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Intensive propagation and utilization of lactic acid bacteria in membrane reactor.

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Intensive propagation and utilization of lactic acid
bacteria in membrane reactor

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SUMMARY

The main object of this project was to investigate the use of membrane cell recycle reactor (MCR) to grow lactic acid bacteria (LAB) to a high cell density and the possibility of using them for industrial application.

Four lactic acid bacteria, *Lactobacillus buchneri*, *L. brevis*, *Oenococcus oeni*, and *Bifidobacterium longum* were investigated in batch, pH-controlled stirred tank reactor (STR), and MCR culture. Nutrient requirements and the physiology of LAB were studied in batch and pH-controlled STR and ultimately in MCR, using optimized media. The simple growth medium contained yeast extract, carbohydrates and mineral salts. During the culture of LAB in batch, pH-controlled STR and MCR, the material balances were studied through the substrate % product stoichiometries. Growth rates and product yields were also studied.

The four LAB were grown in the MCR. The cell mass of the four LAB during the culture in MCR reached a high concentration (13 ~ 23 DCW, g/l) and was typically over 7 times that obtained in batch and pH-controlled STR culture. The cell production rates in the MCR were from 10 times to 33 times greater than that in batch and pH-controlled STR culture, even though growth rates during the culture were lower in MCR than in batch and pH-controlled STR culture. Membrane fouling was significant limitation not only to growth of LAB at high growth rates, but also to increase of the final cell concentration attainable.

High cell density cultures of *L. brevis* and *O. oeni* were then used to study malolactic fermentation of cider in the MCR. Two strains were compared for malolactic activity, organic acid and the volatile compound formation, the specific malolactic activity, K_S for malate uptake, cell concentration, and the pH difference after MLF. The malolactic strains showed a good tolerance in the extreme conditions such as low pH and high alcohol concentration (13%, w/v) of the green cider during the MLF in MCR. Two ciders, General Cider and pure apple juice cider (Scrumpy Jack), showed significant differences when subject to MLF of the LAB. The MLF of Scrumpy Jack by O. *oeni* was inhibited more than that of General cider. The malolactic strains also showed large differences in the transformation of acetaldehyde, alcohols, and esters during MLF of Scrumpy Jack. These are important characteristics when considering the quality of the final product.

Overall, this project shows that MCR can grow LAB to high cell density and that high cell density transformation can be used to overcome significant problems at the traditional MLF processes. This technology is widely applicable to biotransformation of other foods and chemicals.

DECLARATIONS AND STATEMENTS

DECLARATION

This work has not previously accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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CHAPTER 1: INTRODUCTION

1.1. SCOPE OF THE PROJECT.

The lactic acid bacteria (LAB) have been closely tied to human history and development. They have conferred many benefits to humans, helping digestion and in the absorption of nutrition as a symbiosis in human gastro-intestinal tract. Also, they have been related to human life as contributory food stuffs like fermented foods and beverages. Indeed, they are now being manipulated systematically to enhance their utility and further develop new products.

Recently, increasing health concerns have taken lactic acid bacteria into the field of the functional food, pre- and pro-biotic.

The starter cultures for the introduction of lactic acid bacteria into processes are by the use of dense cells to increase the possibility of success against indigenous unfriendly growing bacterial flora. The density of cultures approachable by traditional batch culture techniques is limited by end product inhibition. This inhibition has meant that enhancing culture is not possible with the traditional intensive bacterial culture methods such as continuous or fed-batch cultures. The intensive cell propagation technique chosen here was the use of membrane because of the complex nutritional requirement and end product inhibition. The introduction of a membrane into the cultivation system allows a bioreactor to continuously cultivate the cell at high growth rates because of continuous removal of an inhibitory substance through the membrane as permeate and the introduction of new fresh feed.

Many attempts have been made to obtain high cell concentration of LAB using membrane cell recycling reactor (MCR), however what has been done is on small scale and only empirical. Engineering robustness to make viable and efficient propagation system has not been considered.

This project has been concerned with LAB cultured in MCR. Four strains have been investigated and have a broad range of industrial applications.

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Bifidobacterium longum is significant for pre- and pro-biotic. *Lactobacillus buchneri* is a putative candidate for the field of silage. *Lactobacillus brevis* and *Oenococcus oeni* have been shown to be of importance in cider- and wine-making industry. All these applications range from intensive cell culture to biotransformation.

1.2. LACTIC ACID BACTERIA AND THEIR IMPORTANCE.

The contribution of lactic acid bacteria to mankind has been since prehistoric times. The amount of food that could be found in a day was very important in times of a hunter-gatherer society. The development of agriculture and the ability to produce and to store large amounts of food allowed the efforts of people to be diverted to culture and science. Ultimately this led to development of food preservation in process of the civilization and the birth of cities.

Some of the earliest evidence of food preservation came from the post-glacial era, from B.C 15,000 to 10,000, but the first recovered and systematic use of biological methods was from B.C 6000 to 1000 when fermentation was used to produce beer, bread, wine, vinegar, yoghurt, cheese, and butter [Soomro et al. (2002)]. The bible tells us about vineyards, wine, and the effects of too much alcohol consumption. Hannah, the mother of the prophet Sammuel, was mistakenly admonished by the high priests to "put away thy wine from thee" [The first book of Samuel 1 ver.9 ~ 18]. Also, there were many reports about beer- and wine-making in the ancient Egyptian and Mesopotamian societies. Brewing was a home industry in a privileged post of the society. More certain evidence, the code of law handed down by the Babylonian lawgiver king Hammurabi (reigned 1792 ~ 50 B.C) included a regulation about sale price of beer and its minimum alcohol content [Brewing for Millenium. 2004]. However, the agents in brewing were not determined until the middle of the 19th century.

In 1864, Louis Pasteur suggested the evidence that microorganisms in food were the major cause of food spoilage. With the existence of microorganism, it was recognized that food spoilage is a result of a process called fermentation, which changes the food quality, a procedure proceeding only in the presence of microorganisms. But in a contradiction to the ideas of Louis Pasteur, Eduard Buchner created a theory that even

cell that had been killed by heating can be used for fermentation through experiments with enzymes, juices extracted from yeast cells.

Fermentation of foodstuffs by LAB mainly has been dependent on natural resources. Dairy food, wine, and beer in European culture, and pickles and Asian wines in Oriental culture are very good examples. The bacteria associated with fermentation have been developed by natural selection. Many discovered from the flora are associated with the human body.

Today, these bacteria have been isolated and used in purified culture alone or in mixed cultures. Many kinds of lactic acid bacteria were isolated from foods like dairy products, wine, beer, pickles etc.

Oenococcus oeni was isolated from a wine-making process and *Lactobacillus buchneri* came from ensilage. *Lactobacillus brevis* was a representative bacteria found at spoilage of beer. Some of bacteria like *Bifidobacterium longum* were found at very peculiar environment such as infant's faeces. Due to the specified nutrient requirement, high tolerance to high salt, acidic and anaerobic circumstance, the LAB have been naturally involved in food and beverage production.

With increasing world population, more production of food is needed likewise so as to enhance the shelf life and the safety of food, and make mass- and economical production of food possible. The introduction of the starter cultures into the producing line places these food fermentation processes under control overcoming unpredictability of natural spontaneous processes.

The introduction of *Lactobacillus* or *Pediococcus* into cheese manufacturing process protects cheese from contamination by *Listeria, Clostridium, Staphylococcus*, and *Bacillus sp.* and enhances the shelf life [Soomro et al. (2002)]. The use of the starter culture in wine- and beer-making process has been in trial to improve productivity.

At the end of the 19th century, it was recognized that lactic acid bacteria, which are harmless, might give a benefit to the mankind by reducing putrefaction in the intestinal tract. With the use of lactic acid bacteria as pro-biotic, *Lactobacilli* became the first genus of bacteria suspected to have health benefit, rather than to be agents of

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disease. Furthermore, with the finding that *Lactobacillus acidophilus* has been responsible for the longevity of some eastern European people who have traditionally consumed them, it is now believed that *Lactobacilli* have a positive effect on human health. Nowadays, pro-biotics are becoming very popular all over the world and their market has increased rapidly. The kinds of the strains used for the commercial pro-biotic are very broad and includes the genera of *Lactobacillus, Bifidobacterium, Streptococcus*, and *Enterococcus*. According to recent reports, in developing new brands of pro-biotic a combination of *Lactobacillus* and *Bifidobacterium* can be very good at human health because of the complementary nature.

In the middle of ensilage process, the use of the starter culture for cooperation with hetero- and homo-fermentative LAB enhanced the stability of silage even after exposure to the air.

With the every use in the field of the food preservation and the promotion of the human health, lactic acid bacteria give a contribution to manipulation of livestock feed too. When lactic acid bacteria are used as silage additives, there have been many reports on improved preservation efficiency and enhanced animal growth and performance [Weinberg and Muck. (1996)].

Nowadays, the synthetic chemicals used as preservatives in food are significant product of concerns for consumers. As alternatives, recent use of antimicrobial metabolites in preserving food could provide a solution to this dilemma. Lactic acid bacteria can produce many kinds of organic acids, fatty acids, hydrogen peroxide, diacetyl, and bacteriocins to prevent themselves from other competitor growing in the same place. Bacteriocins in particular produced by lactic acid bacteria are the subject of intense research because of their antimicrobial activities against food-borne pathogens. Of those bacteriocins, Nicin is only LAB bacteriocin currently used as a food additive.

Another use of LAB, the development of LAB as vehicle to transport heterogeneous antigens into the human body and production of high pure lactic acid are considerably drawing a lot of interests.

Lactococcus lactis is a harmless and non-invasive bacterium with history of safe use in the food industry. Reuter et al. [2003] designed *L. lactis* as live vaccine carrier to carry out expression of tetanus toxin fragment C (TTFC) in mice, using genetic engineering. They could elicit antigen-specific IgA and protective level of serum antibodies by administering vaccines through mucus membrane with antigen-expressing *Lactococcus*. The use of genetically engineered *Lactococcus lactis* as live bacterial vaccine is more efficient than the existing use of live attenuated human pathogens in that achieving human immunization [Wells et al.(1995), Pouwels et al. (1998)].

Lactic acid is one of most widely used food preservatives and a very common substrate for chemical synthesis. With recently increasing concerns about environmental pollution by packing materials such as vinyl and plastics, development of the environmentally friendly packing materials with high pure lactate increased the worldwide demand for lactic acid [Jeantet et al. (1996)].

1.3. COMMERCIALLY IMPORTANT PROCESSES MEDIATED BY LAB.

1.3.1. Dairy products.

Cheese has been the representative product, which is most popular and is majority on the world dairy market. The conversion of peptides into free amino acids and their subsequent utilization is a central metabolic activity of LAB in ripening and flavour forming process of cheese.

The LAB have many peptidase and they can metabolize a variety of species of bacterial peptides to amino acids. In manipulation process of cheese, milk casein is separated from lipid or carbohydrate and partially hydrolyzed with acid or pepsin. LAB is added into the slurry of milk casein hydrolyzates as the starter culture. Here, LAB hydrolyzes peptides into free amino acids using their peptidases and they synthesize a variety of flavour components through the catabolism of free amino acids.

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Pro-biotic can be defined as fermented food containing specific live microorganism or a live microbial food or feed supplement, which beneficially affects intestinal microflora of humans or the host animals (Table. 1~4).

In contrast with Japan where freeze-dried microorganism is consumed, in Europe, pro-biotic is only used in certain fermented dairy products like yoghurt [Kalantzopoulos (1997)].

In manipulation process of pro-biotic, the LAB is used as starter culture and added after pasteurization of milk. The LAB uses free amino acids and produces a variety of flavour components.

Usually, the agents are selected through the serial tests. These (i) adhere to epidermic cells in human gut intestinal tract; (ii) exclude or reduce pathogenic adherence; (iii) survive or proliferate; (iv) produce acid, hydrogen peroxide, and bacteriocin antagonistic to pathogen growth; (v) resist vaginal microbicides, including spermicides, and bile salts; (vi) be safe, non-carcinogenic, and non-pathogenic; (vii) co-aggregate and form a normal flora [Ouwehand et al. (2002), Kalantzopoulos. (1997), Soomro et al. (2002), Annuk et al. (2003), Dunne et al. (1999), Perdigon et al. (1995); Saxelin et al. (1999)].

Table.1.1 ~ Table1.3 describe commercial pro-biotic organisms on the European and Japan Market. The commercial pro-biotic organisms for European was analysed and taken as a sample at grocery store shelves of Germany, and Japan organisms were pro-biotic bacteria registered by FOSHU (Food for Specified Health Use (Sept. 1. 1991)) according to their in vitro basis to support utility [Sanders and Huis in't Veld. (1999)].

FOSHU, which was launched on 1991, is the legislation of Japan government to circulate the functional foods through the domestic markets and it contains clinical and nutritional documentation demonstrating the health effect, the safety, the dosage, and the stability of the food or its constituents.

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In addition to organisms listed in Table1.1, Table1.2, and Table1.3, Table1.4 contains many examples of pro-biotic *Lactobacillus* strains which have been clinically studied in vitro and in vivo. As pro-biotic examples with a few basic scientific or peer-reviewed basis, other strains, such as *Lactobacillus plantarum* 299V (product of Probi, Lund, Sweden), *L. johnsonii* LJ1 (marketed as LC1 by Nestle, Lausanne, Switzerland), *L. acidophilus* NCFB 1748, *L. crispatus* CVT05 (product of Gynelogix, Boulder, Colo), and *L. casei* DN114 (Danone, Paris, France) have shown a promise in some human studies or in the clinical trials [Reid. (1999)].

Producers Type of product	Product name	Organism on the label	Organism identified in products	Bacterial No. /ml
Drink type products (adapted from Reuter et al.[1997] and Holzapfel et al. [1998])				
Yakult(NL) pH 3.77	Yakult	*L.casei Shirota*	*L.casei*	9.2×10^8
Südmilch(GE) pH 3.91	Vifit	*L.casei* LGG	*L.rhamnosus* *S.thermophilus* *L.bulgaricus*	3.1×10^7 3.3×10^7 5.1×10^7
Chambourcy(F) pH 4.23	LC1 drink	*L.acidophilus* 1	*L.johnsonii* *S.thermophilus*	2.4×10^4 8.5×10^8
Mona, Campina pH 4.34	Fysig drink	*L.acidophilus*	*S.thermophilus*	5.6×10^8
Melkunie(NL)		Gilliland		
Molkerei pH 4.48	Feine	BB 536	*B.longum*	1.9×10^4
Schöppingen(GE)	Schwedenmilch		*S.thermophilus*	2.2×10^4
Danone(F) pH 4.05	Actimel	*L.casei* Imunitas	*L.casei* *L.bulgaricus*	1.5×10^8 2.8×10^4
Emmi(SW) pH4.03	Akifit plus	*L.casei* LGG *L.acidophilus* *S.thermophilus* *B.bifidum*	*L.rhamnosus*	1.8×10^8
Nestlé(SW) ND	Lc1 Go	*L.helveticus*	ND	ND

Table.1.1 Pro-biotic strains in drink type products sold in European market and their producers. *This data was adapted from Reuter et al. [1997] and Holzapfel et al. [1998]. NL; Netherland, GE; Germany, F; France, SW; Swizerland, L; Lactobacillus,*

B; Bifidobacterium, S; Streptococcus [Sanders and Huis in't Veld. (1999)]

Producers Type of Product	Product name	Organism on the label	Organism identified in products	Bacterial No. /ml
Yoghurt type products				
Sŭdmilch(GE) pH 4.08	Vifit	*L.casei* LGG	*L.rhamnosus* *L.acidophilus* *S.thermophilus* *L.bulgaricus*	1.4×10^7 8.2×10^6 4.2×10^7 1.5×10^7
Toni Lait(SW) pH 4.45	SymBalance	*L.casei* 01 *L.acidophilus* La5 *L.reuteri* *Bifidobacterium*	*L.casei* *L.acidophilus* *L.reuteri* *S.thermophilus* *L.bugaricus*	9.5×10^8 3.6×10^7 1.7×10^5 5.2×10^8 1.7×10^7
Nestlé(SW) pH 4.14	LC1(vanille,	*L.acidophilus* La1	*L.johnsonii*	2.6×10^7
Chambourcy	nature, pur 1)		*S.thermophilus*	2.5×10^8
Mona, Campina pH 4.21	Fysiq yoghurt	*L.acidophilus*	*L.casei*	2.6×10^6
Melkunie (NL)	Gilliland	*L.acidophilus*	*L.acidophilus* *S.thermophilus* *L.bulgaricus*	6.0×10^6 9.6×10^8 7.2×10^6
Danone(F) pH 4.33	Bio au casei Actif	*L.casei*	*L.casei* *L.bulgaricus*	2.0×10^8 6.0×10^5
Molkerei pH 4.20	Bi'Ac	*B.longum* 536	*L.acidophilus*	1.9×10^7
Müller(GE)			*S.thermophilus*	2.3×10^8
Danone(F) pH 4.16	Actimel joghurt	*L.casei* Immunitas	*L.casei* *L.bulgaricus*	1.2×10^8 5.0×10^5

Table.1.2 Pro-biotic strains in yogurt type products sold in European market and their producers. *This data was adapted from Reuter et al [1997] and Holzapfel et al.[1998]. NL; Netherland, GE; Germany, F; France, SW; Swizerland, L; Lactobacillus, B; Bifidobacterium, S; Streptococcus [Sanders and Huis in't Veld. (1999)]*

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Pro-biotic strains	Examples of products	Manufacturer
L.rhamnosus GG Torkyo	ONAKA-HE-GG (Drink yoghurt)	Takanashi milk products,
B.longum BB536 Torkyo	Bifidus plain yoghurt	Morinaga milk industries,
L.bulgaricus & Torkyo	Meiji Bulgaria yoghurt LB81	Meiji milk product,
S.thermophilus	Meiji Bulgaria nomu yoghurt LB81 plain	
L.acidophilus SBT-2062 Torkyo	Yukijirushi Nachure (yoghurt)	Snowbrand milk products,
B.longum SBT-2928 L.casei Shirota	Yakult (fermented milk drink) Joie (fermented milk drink)	Yakult Honsha, Torkyo
B.breve strain Yakult	Mil Mil Mil Mil E	Yakult, Honsha, Torkyo

Table.1.3 Pro-biotic examples in Japan market and their producers. *The strains on Japan market have been marketed with FOSHU (Food for specified Health Use (Sept. 1. 1991)) approval, L; Lactobacillus, B; Bifidobacterium, S; Streptococcus. This data was adapted from Sanders and Huis in't Veld. [1999].*

Strains	Distributor	Site of human application
L. rhamnosus GG	Valio, Helsinki, Finland	
	Campina Melkunie, Zaltbommel, Netherland	Intestine
L.acidophilus NCFM	Rhodia, Madison, Wis.	Intestine
L.casei Shirota	Yakult, Torkyo, Japan	Intestine
L.reuteri MM53	BioGaia, Stockholm, Sweden	Intestine
L.casei CRL431	Chr, Hansen, Milwaukee, Wis.	Intestine
L.rhamnosus GR-1	Urex, London, Canada	Urogenital tract
L.fermentum RC-14	Urex, London, Canada	Urogenital tract

Table 1.4 Pro-biotic *Lactobacillus* strains marketed in basis of the scientific publication or the clinical trials [Sanders and Huis in't Veld, 1999].

1.3.3. Malolactic fermentation (MLF)

Most wine-making process includes two microbiologically mediated steps, alcohol fermentation by yeast and malolactic fermentation by lactic acid bacteria.

Yeast grows better than lactic acid bacteria in high concentrations of sugar and acidic pH typically low in grape must. Alcoholic fermentation starts quickly fermenting most of the sugars to ethyl alcohol. When most of sugars are consumed, growth of yeast declines and then LAB starts growing with an increase MLF activity [Aline. (1999)]. Malolactic fermentation starts sequentially when the population of malolactic bacteria approaches 10^6 cfu / ml [Maicas et al. (2000), Aline. (1999)]. In malolactic fermentation, *Oenococcus oeni* is the dominant, even if other malolactic organisms are present: *Lactobacillus, Pediococcus, Leuconostoc* and *Oenococcus spp.* [Carreté et al. (2002), Katariina et al. (2000), Aline. (1999)]. Nearly all red wines and white wines are obtained with these two fermentation steps. *Oenococcus oeni* is the preferred species for wine-based MLF due to its alcohol and acid tolerance together with the flavour profile produced. De-acidification through MLF in wine is particularly desirable for high- acid wine produced in cool-climate regions, such as Northern France, New Zealand and Canada. In addition to its use in wine process, MLF occurs in other fermented beverages, such as cider and beer [Liu. (2002)]. It is now accepted that the principal role of MLF is de-acidification in which it degrades di-carboxylic L-malic acid into mono-carboxylic lactic acid and CO_2, but there are more flavour changes other than just de-acidifying process. The complexity and diversity of the metabolic activity of lactic acid bacteria implies that MLF may affect wine quality in many ways.

Lactobacillus brevis was also isolated in cider-making process and has been studied for its introduction into maturing process of cider and beer. They are inoculated after alcoholic fermentation and filtered before bottling. They form various flavour components through catabolism of free amino acids and tannin derivative substances during MLF [Sakamoto et al. (2001), (2002)].

1.3.4. Silages.

Silage is the fermentation of vegetable matter to pickle it, so as to preserve it for long term use during the unproductive winter months. Lactic acid bacteria play an important role in producing a number of preservative materials.

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Fermentation of forage in a silo was an uncontrolled process and the practical bacteria responsible for improving the quality of forages was not clear. However, the loss of dry matter (DM) on forages during ensiling has been a significant problem because of generation of CO_2 by yeast or fungus growth as a contaminant. Particularly, after sealing the silo, rapid removal of air is very important to prevent the growth of contaminants in the silage. Since the silage is frequently exposed to the air during feeding out, once exposed, the spoilage by yeast contaminants is common. Once yeast has grown, yeast increases pH by consuming lactic acid. Finally, the increase in pH usually induces the growth of the secondary contaminants like *Enterobacteria* or *Clostridia* further spoiling the biomass. In recent years, the use of the additives to help protect the silage from contaminants has been systematically developed and it resulted in the use of microbial inoculants, enzymes, buffered propionic acid [Ranjit and Kung Jr. (2000), Kung Jr. and Ranjit. (2001), Driehuis et al. (1999), Taylor and Kung, Jr. (2002), Selmer-Olsen. et al. (1999)].

Homofermentative LAB, which produces only lactic acid as product, has been strongly recommended as the microbial inoculants. Homofermentative LAB can produce the large quantity of lactic acid during the growth, it is not only effective in lowering pH, but also excellent in preventing a loss of DM as there is to no generation of CO_2. Furthermore, since the forage fermentation naturally involves the growth of pathogens, the uses of microbial inoculants can also improve the aspect of hygiene. *Lactobacillus plantarum*, *L. acidophilus*, *Pediococcus acidolactici*, *P. pentacaceus*, and *Enterococcus faecium* are the representatives of micro-organisms frequently recommended in recent years [Ranjit and Kung Jr. (2000), Kung Jr. and Ranjit. (2001)].

During the fermentation, the rate and the extent of pH decline is a crucial factor to determine a degree of nutrient loss in the forages. However, frequently the uses of microbial inoculants are not enough to inhibit contaminants due to physiological characteristics and insufficient microbial adaptation of applied inoculants against particularly unfriendly circumstance in the forage. Exposure to air caused by poor management of the processes during the preservation process enable an aerobic spoilage to create adverse conditions.

Consequently, Weinberg et al. [1996] suggested criteria to select the best micro-organism to use as the inoculants; i) using LAB isolates more specific to the target crops, ii) addition of heterofermentative LAB producing volatile fatty acids to inhibit yeast or mould in aerobic exposure, iii) bacteria other than LAB to inhibit pathogenic growth, iv) LAB genetically engineered to inhibit specific contaminant, v) LAB to metabolize carbohydrates in a broad spectrum through natural selection or genetic cloning. They also suggested microbial inoculants should grow vigorously and rapidly to be sufficiently competitive in unfriendly environment of the forage.

Inclusion of other LAB into microbial inoculants is one of the alternatives often recommended. For example, the growth of yeast can be inhibited more by acetic acid and propionic acid than L-lactic acid [Tayor and Kung, Jr. (2002)]. It has been reported that heterofermentative species, *L. buchneri* increases aerobic stability in silage after the exposure to air [Taylor and Kung, Jr. (2002), Driehuis et al. (1999)].

1.3.5. Other products by LAB.

Of products produced by LAB, for example, bacteriocin has been found in fermented food in recent years. Bacteriocins are a group of low molecular weight peptides, which can exert a beneficial effect by inhibiting the growth of pathogens in food and intestinal microflora. As an alternative to traditional food additives added for preservation, bacteriocin is antimicrobial produced by many LAB, including the peptides that inhibit the growth of food-borne pathogens.

Of the bacteriocins discovered so far, Nicin produced by *Lactococcus lactis* is a permitted food additive in more than 50 countries including USA and Europe under trade name Nisaplin [Soomro et al. (2002), Ouwehand et al. (2002), Kalantzopoulos. (1997)].

Lactic acid is one of the most widely used organic acids in the food industry and is a very common substrate for chemical synthesis. Moreover, in recent years, there has been increased attention on production of pure lactate because it can be used as raw material for the production of biodegradable polymers with applications in the medical, pharmaceutical, and food industries.

A variety of *Lactobacilli* like *L. helveticus*, *L. delbrueckii*, and *L. plantarum* have been used in trials to produce pure lactate with fermentation using whey permeate and molasses as carbon source [Aeschlimann and Stockar. (1990), Payot et al. (1999),Yoo et al. (1997), Kwon et al. (2000)].

In recent cutting-edge research, there has been increasing interest in developing delivery vehicles for use as nasal administered vaccine. *Lactobacillus lactis* is more acceptable because it has long history of safe use in food industry. With a number of potential antigens such as *HIV-I* and *Clostridium tetani*, human proteins like interleukin used in immunotherapy have been cloned to be expressed in *L. lactis* by genetic engineering [Fooks. (2003). Reuiter et al. (2003)].

Also, in parallel with steps to develop LAB as live vaccine, considerable advances have been made in the genetic and molecular biology of lactic acid bacteria [Reuter et al. (2003)]. *L. lactis* containing expression vector cloned with a number of allergens is introduced into mucosal surface by nasal administration. *L. lactis* as live vaccine can express allergens by inducible or constitutive expression promoter to induce the production of immunoglobulin through mucosal layer [Pouwels et al. (1998), Wells et al. (1995), Willem. (1999)].

1.4. PHYSIOLOGY OF LACTIC ACID BACTERIA.

1.4.1. General physiology.

Lactic acid bacteria can be divided into two sub-groups by the products produced via the metabolic pathways from glucose. Homofermentors can produce two moles of lactic acid through *Embden-Meyerhof pathway* (EMP) (Fig.1.1) whereas heterofermentors use *the oxidative pentose phosphate pathway* (PPP) to produce others than lactic acid (Fig.1.2) in anaerobic circumstance.

Homofermentor produces 2 mole of lactic acid through homofermentative pathway consuming 1 mole of hexose, but heterofermentor produces 1 mole each of ethanol, lactic acid, and carbon dioxide.

Glucose

NAD$^+$ + ATP

NADH + ADP

Glucose-6-phosphate

Fructose-6-phosphate

ATP

ADP

Fructose-1,6-diphosphate

D-Glyceraldehyde-3-phosphate ⇄ Dihydroxyacetone-phosphate

NAD$^+$ + Pi

NADH

1,3-Diphosphoglycerate (2 mole)

ADP

ATP

3-Phosphoglyceric acid (2 mole)

2-Phosphoglyceric acid (2 mole)

Phosphoenol-pyruvate (2 mole)

ADP

ATP

Pyruvate (2 mole)

NADH (2 mole)

NAD$^+$ (2 mole)

Lactate (2 mole)
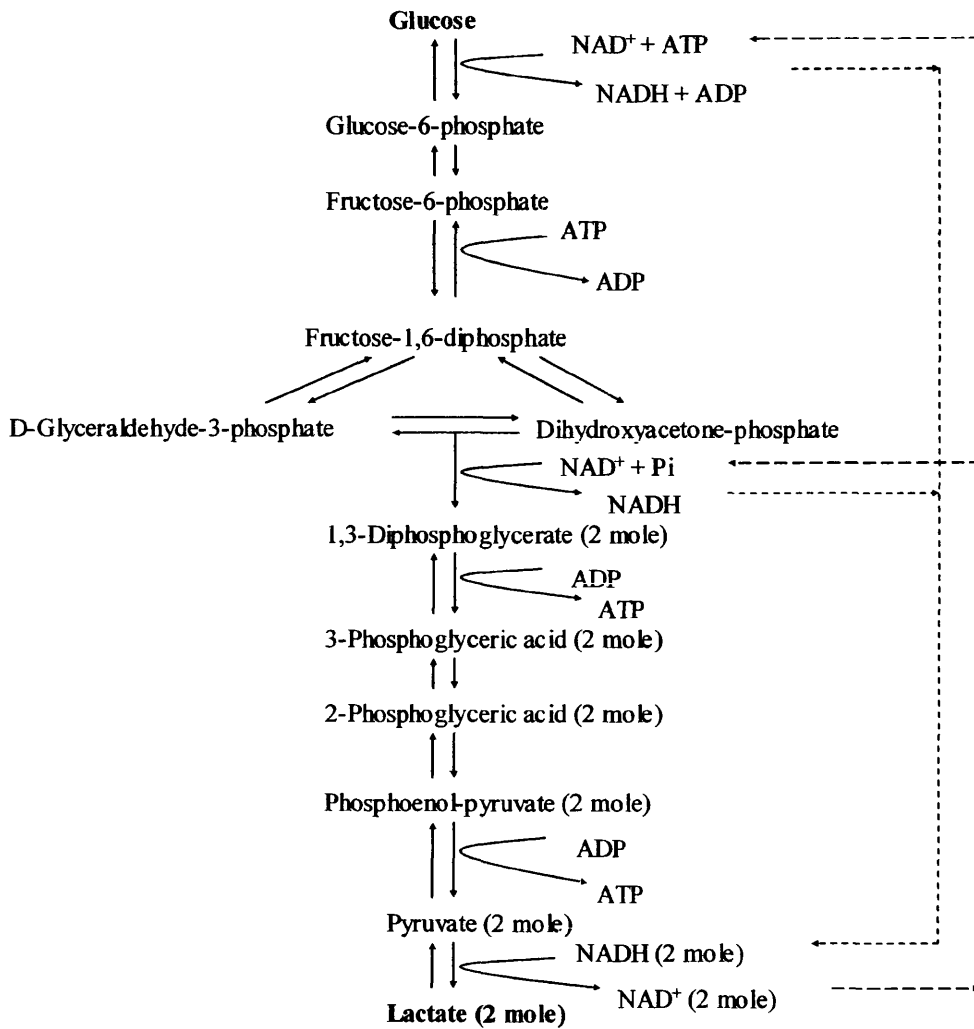
Fig.1.1. The pathway for glucose dissimilation by homofermentative lactic acid bacteria (*Embden-Meyerhof pathway*). *Homofermentor can produce two moles ATP and one mole NADH with two moles lactate [Ref. Stainer et al, The microbial world; Fifth edition.]*

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Glucose

| $\longrightarrow$ ATP
$\downarrow$ $\searrow$ ADP

Glucose-6-phosphate

| $\longleftarrow$ NAD
$\downarrow$ $\searrow$ NADH

6-phosphogluconic acid

$\longleftarrow$ NAD

CO_2 $\longleftarrow$ $\longrightarrow$ Ribulose-5-phosphate

$\searrow$ NADH

Glyceraldehyde-3-phosphate $\longleftarrow$ $\longrightarrow$ acetyl-phosphate

NADH $\longrightarrow$ $\downarrow$ $\downarrow$ $\longleftarrow$ NADH

NAD $\nearrow$ 1,3-diphosphoglyceric acid   acetaldehyde $\searrow$ NAD

$\downarrow$ $\longleftarrow$ ADP   NADH $\longrightarrow$ $\downarrow$

3-phosphoglyceric acid $\searrow$ ATP   NAD $\nearrow$ **ethanol**

$\downarrow$

Phosphoenolpyruvic acid

$\downarrow$ $\longleftarrow$ ADP

Pyruvic acid $\searrow$

NADH $\longrightarrow$ $\downarrow$ ATP
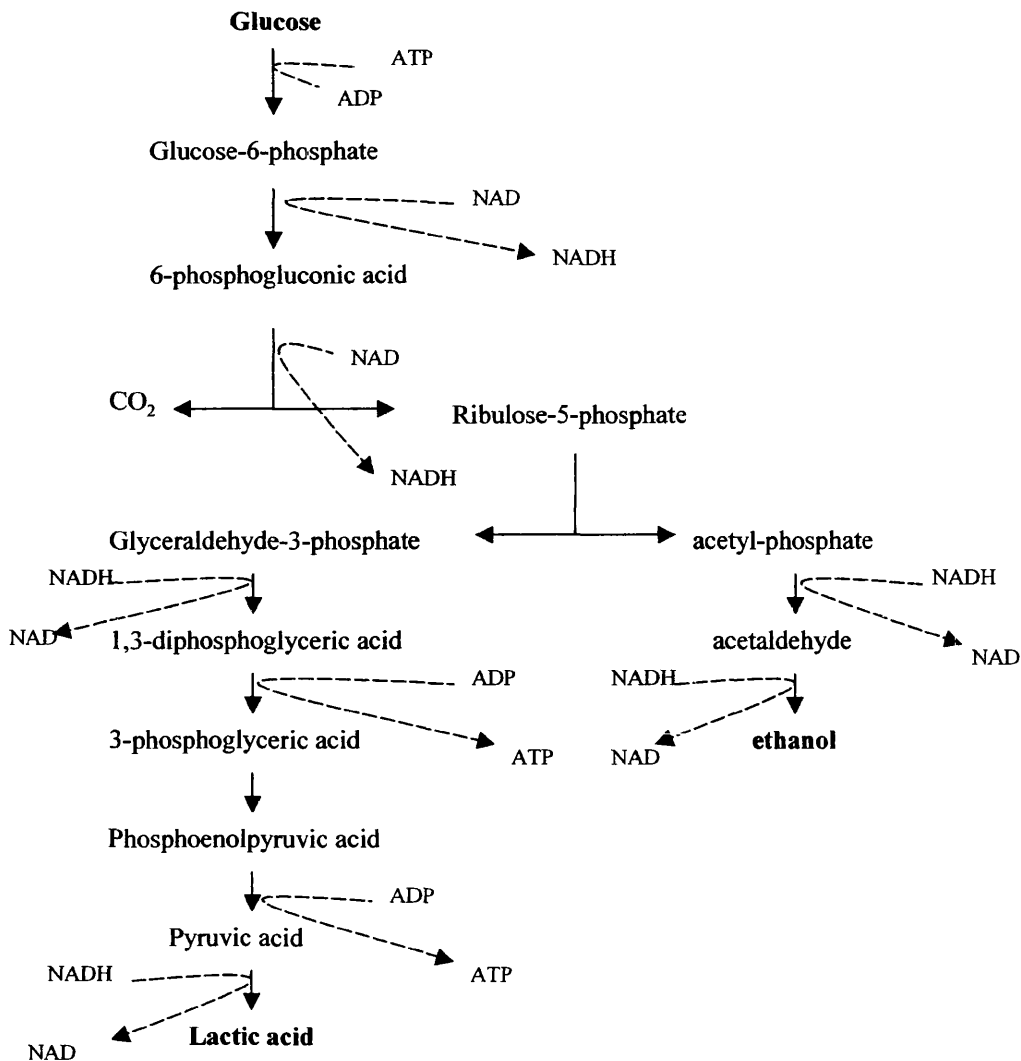
NAD $\nearrow$ **Lactic acid**

Fig.1.2 The pathway of glucose dissimilation by heterofermentative lactic acid bacteria. *The reduction from acetyl-phosphate to ethanol can not occur. The end products are described with bold characters. Heterofermentor can produce one mole ATP and two moles NAD^+ with one mole each lactate, ethanol, and CO_2 per unit glucose consumed [Ref. Stainer et al. the microbial world; Fifth edition]*

In anaerobic circumstances, LAB produce ATP by *substrate level phosphorylation* (SLP). Since ATP production by anaerobes is always less than that by aerobes, product yield to grow anaerobes must be less than that of aerobes. Comparing homofermentative pathway with heterofermentative pathway, the homofermentative pathway can produce 2 moles ATP per mole of glucose, but the heterofermentative pathway produces 1.5 moles ATP. Therefore, homofermentative pathway theoretically produces more cell mass than heterofermentative pathway per

unit substrate consumed.

In anaerobic fermentation, regeneration of NADH is very important process. The failure in supplying of NAD^+ often causes serious problem in anaerobic growth, preventing further oxidation of substrate due to a lack of an NAD^+ pool to accept electrons. Since aerobes can oxidize NADH, producing ATP and NAD^+ through the oxidative phosphorylation, there is no problem to supply again with NAD^+ for another oxidation. In contrast, anaerobes must find alternative route to recycle NADH to allow the fast and intensive growth. Consequently, homofermentors regenerate NAD^+ by producing two moles lactic acid and heterofermentors do it by producing lactic acid, ethanol, and CO_2. In case of heterofermentator with mannitol dehydrogenase, they can also do it reducing fructose to mannitol.

1.4.2. Specified physiology relevant to processes

1.4.2.1. Anaerobic degradation of lactic acid by Lactobacillus bucheri.

L. buchneri can grow using mannitol dehydrogense with fructose instead of O_2. Mannitol dehydrogenase can allow the oxidation of NADH into NAD^+ to allow LAB grow in anaerobic circumstance producing mannitol at the expense of fructose and one mole of NADH.

In recent study, Stefanie et al. [2001] proposed anaerobic degradation of lactic acid into equi-mole of 1,2-propanediol and acetic acid, and trace amounts of ethanol (Fig.1.3). They proposed a novel pathway of regeneration of NAD^+ by the high level of NAD-linked 1,2-propanediol-dependent oxidoreductase.

Taylor and Kung Jr. [2002] investigated that L. buchneri positively influenced on the fermentation and aerobic stability of high moisture corn in silo. Additionally Driehuis et al. [1999] studied anaerobic degradation of lactic acid during ensilage of whole crop maize inoculated with L. buchneri and they found a positive effect in inhibiting yeast growth and improving aerobic stability. As another positive result, Driehuis et al. [1999] used L. buchneri as the additive to the grass silage.
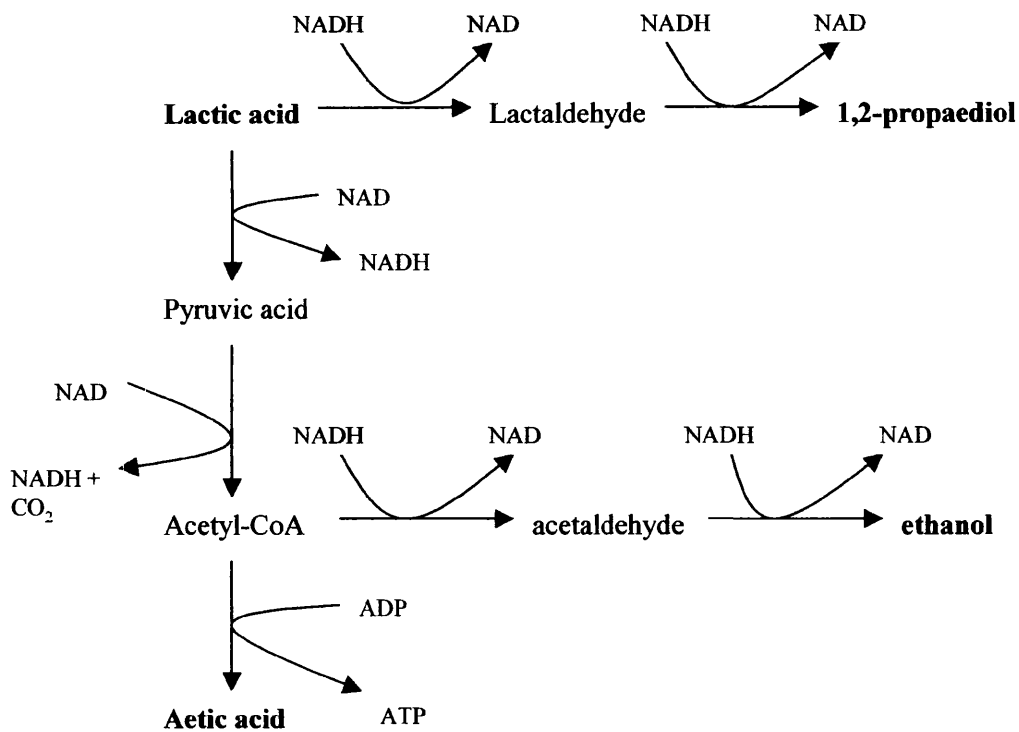
Fig.1.3 Anaerobic degradation pathway of lactic acid into acetic acid. 1,2-propanediol, ethanol, and CO_2 in *Lactobacillus buchneri*. *This novel pathway can be activated in pH under 5.8 and this degradation pathway of lactic acid is to maintain the intracellular redox potential in anaerobic conditions, but the energy produced with acetic acid can not be reused for bacterial growth in general. This energy is to be used as the maintenance energy for the survival in low pH conditions [Stefanie et al. (2001)]. The end products are descried as the bold.*

They made the inoculation of *L. buchneri* as heterofermentative LAB with *L. plantarum* or *Pediococcus pentosaceus* as homofermentative LAB at the same time. Consequently 1,2-propanediol and acetic acid concentration were increased and the aerobic stability of the grass silage was dramatically increased with reduced yeast and mould counts. In fact, the growth of yeast, which generally causes the onset of aerobic silage spoilage, can be inhibited more by acetic acid and propionic acid than L-lactic acid [Tayor and Kung Jr. (2002)].

The minimum limit of the inoculants, which is to become the predominant in the silage, showing the positive effect in ensilage quality was found in a range of $10^5 - 10^6$ viable cells / g of silage, and use of the inoculants up to the minimum limit

dramatically increased the stability and the quality of the silage [Ranjit and Kung Jt. (2000), Kung Jr and Ranjit. (2001), Weinberg and Muck. (1996)].

1.4.2.2. Physiology of Oenococcus oeni for malolactic fermentation.

The main value of malolactic fermentation is the biological de-acidification which results from the degradation of L-malic acid to L-lactic acid. This includes a change in pH, and in wine taste. Decarboxylation of L-malic acid is catalyzed by malolactic enzyme (MLE), which is different from the malic enzyme leading L-malate to pyruvate (Fig.1.4).

The malolactic activities of entire cells are strictly dependent on the integrity of the bacterial membrane, and the basic requirements for the maximum enzyme activity are to be essential for survival and adaptation against the unfriendly environment of wine. In fact, such a difficulty in protecting enzyme system from the inhibitory outside has been a disturbing factor in developing enzyme reactor for MLF.

The genes encoding the MLE proteins, *mles* were identified in *L. mesenteroides*, *L lactics*, and *O. oeni*, and it was functionally expressed in *Saccharomyce cerevisiae* [Denayrolles et al. (1995)]. Even successful integration of *mles* gene into the genome of wine yeasts could not be a technical solution to simplify the wine-making process attempting alcohol fermentation and MLF at the same time. It would not be applicable to all wines since malolactic fermentation includes not only the decarboxylation of malic acid but also a set of numerous reactions mediated by LAB.

Ultimately to overcome the difficulties in malolactic fermentation, winemakers and wine microbiologists have had the idea of inoculation with selected malolactic starter culture. The inoculations with malolactic LAB have not always been successful because of stressed conditions in wine after alcoholic fermentation [Simona et al. (2002), Maréchal et al. (2000), Bourdineaud et al. (2003), Teixeira et al. (2002)]. Inoculations by very high cell numbers that have been improved through re-activation step means that the survival and activity of bacteria newly inoculated to wine has been possible.

The reactivation is an attempt for adaptation through pre-incubation of malolactic LAB in a suitable medium, mainly composed of grape juice or wine, and yeast extract, for one or two days before use.

NAD^+ $CO_2 + NADH + H^+$ $NADH + H^+$ NAD^+

Pyruvic acid ⟶ L-Lactate

Mn

$ATP + CO_2 + H_2O$ ADP

NAD^+ $NADH + H^+$ E-Biotin E-Biotin-CO_2 $NADH + H^+$ NAD^+

L-Malate ⟶ Oxaloacetate ⟶ Pyruvate ⟶ L-Lactate

Mg^{++} Zn^{++}

NADH NAD^+

L-Lactate

Mn
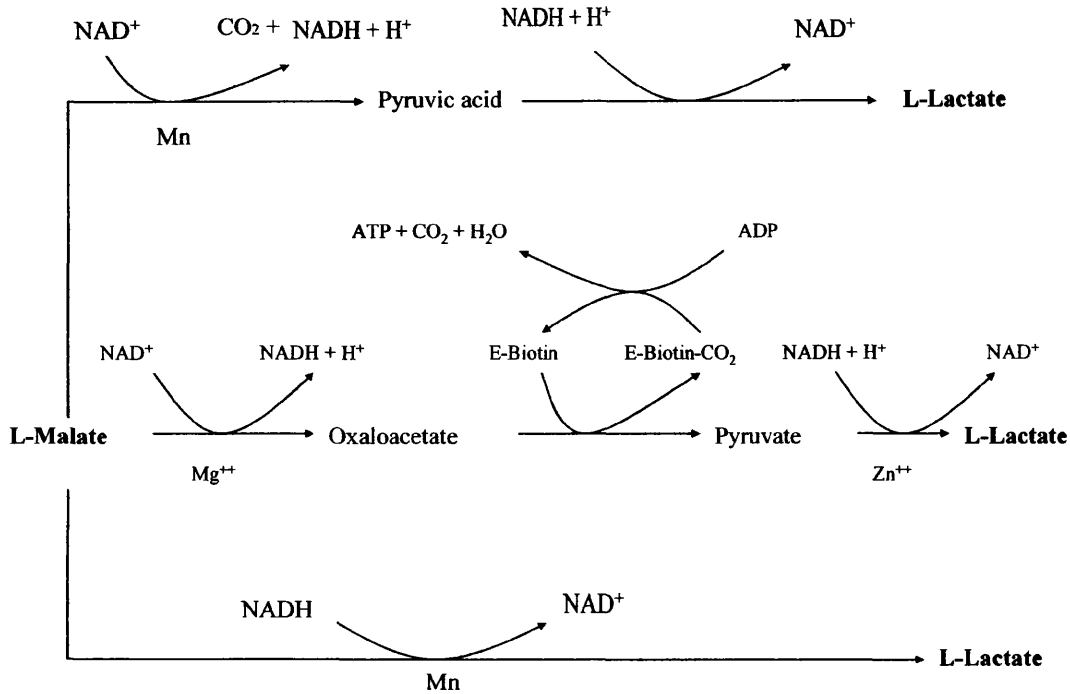
Fig.1.4 Proposed mechanisms of degradation of L-malate to L-lactate and the generation of ATP for growth in *O. oeni* [Tracey and Britz. (1989)].

This incubation not only propagates the population of the bacteria, but also results in adaptation step [Aline. (1999)]. *Oenococcus oeni* of all the malolactic LAB are the preferred starter culture.

Citric acid metabolism (Fig.1.5) by wine lactic acid bacteria is another significant factor to determine wine quality. Diacetyls produced through citric acid metabolism provide a butter flavour to wine and the accumulated amount greatly varies with the malolactic strain. The transformation can also be affected by diacetyl reductase activity in either yeast or lactic acid bacteria [Aline. (1999), Saguir and Manca de Nadra. (2002)].

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The improvement on the sensory quality is another main benefit from malolactic fermentation. Degrading L-malic acid to L-lactic acid, the strong green taste of malic acid is replaced with the less aggressive and softer taste of lactic acid. Furthermore, since most variants of *O. oeni* can metabolize citric acid more slowly than it degrades malic acid, almost all citric acid in grape must be able to be metabolized into acetic acid and C_4 compound. The accumulation of diacetyl, acetoin and acetic acid in wine varies according to the rate of MLF. Usually more acetic acid can be produced from given amount of citric acid during the fast MLF [Aline (1999), Nielsen and Richelieu (1999), Rodriguez et al. (1990), Nedovic et al. (2000), Cogan (1987), Osborne et al. (2000), Liu et al. (1997)].

On the other hand, *Oenococcus oeni* can also be involved in spoilage of wine. Usually malolactic fermentation takes place at the end of alcoholic fermentation, but when it is slow or when it stops, lactic acid bacteria can grow increasing the acidity of wine. Likewise reducing sugars to be fermented to alcohol by yeast tends to be used by lactic acid bacteria with formation of too high acetate and lactate concentration. The best way to establish good reliable wine-making is that alcoholic fermentation is strictly controlled through on-line monitoring, and secondly step must be taken to ensure that by the malolactic fermentation under control. Therefore, use of the starter culture should be another benefit to lead it to success [Aline (1999)]

Two groups of substances identified in wine have been undesirable with respect to the hygienic quality; biogenic amines and ethylcarbamate (EC) known as a possible carcinogen. Many kinds of lactic acid bacteria could transform a variety of amino acids into biogenic amines during malolactic fermentation. *O. oeni*, of lactic acid bacteria releasing biogenic amine, has been defined as histamin-producing strain from histidine, furthermore a novel pathway to produce EC was confirmed in *O. oeni* by cloning and sequencing *arc*A, *arc*B, and *arc*C, which are genes encoding key enzymes in producing EC from arginine [Simona et al. (2002), Aline (1999), Liu (2002)].

To date, the production of these substances can not be stopped with malolactic fermentation, but the accumulated amount can be reduced by preventing undesirable contaminants.

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The third benefit is of the hygienic control of malolactic fermentation to prevent undesirable contaminant growing [Aline (1999), Simona et al. (2002), Arena et al. (1999), Tonon and Aline (2000)].
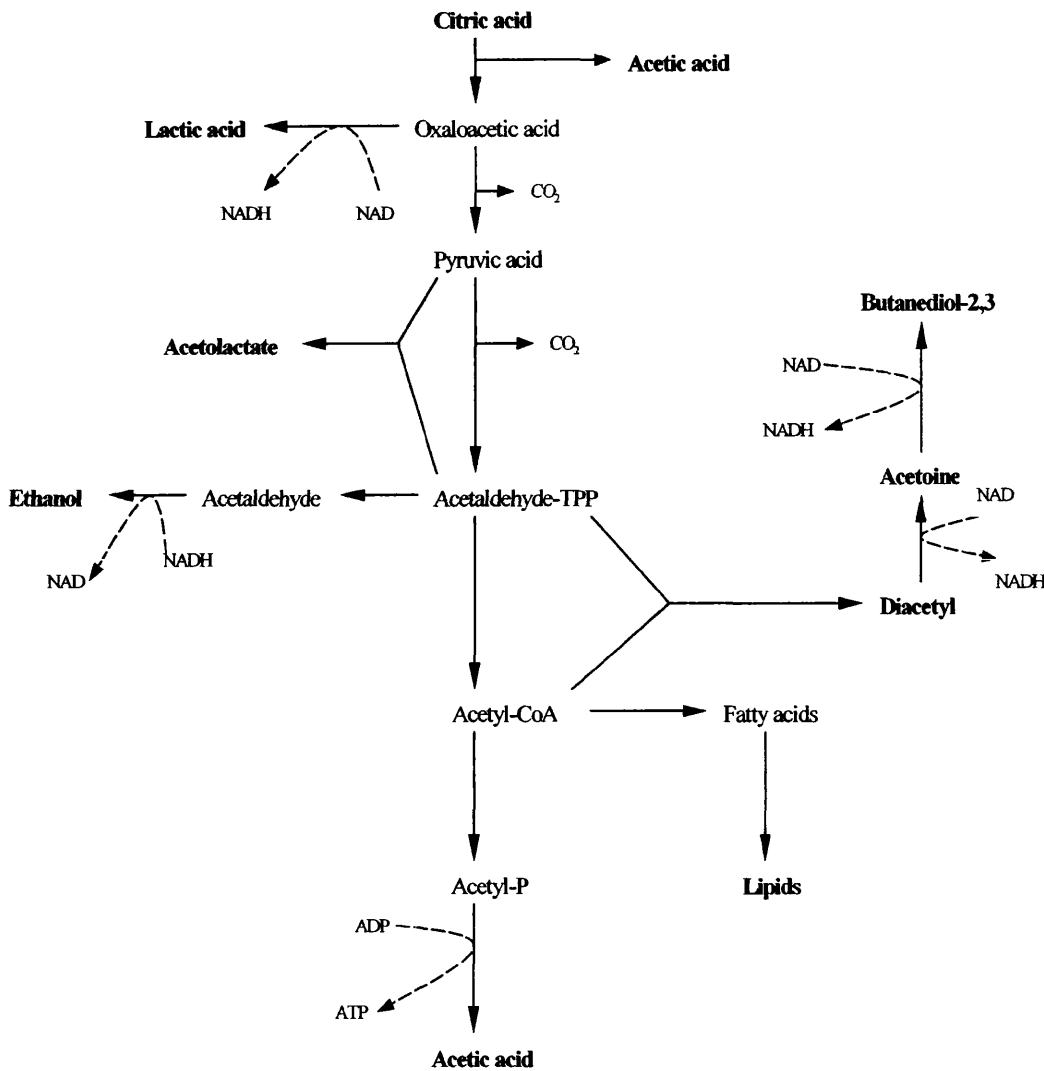
Fig.1.5 Citric acid metabolism by wine lactic acid bacteria [Aline. 1999]. *End products are described with bold characters. Citric acid metabolism can give the wine butter flavor producing diacetyls, acetates, and fatty acids.*

1.4.2.3. Physiology of Bifidobacterium longum for use as pro-biotic.

Bifidobacteria are generally characterized as gram-positive, non-spore-forming, non-motile, catalase-negative anaerobes [Gomes and Malcata (1999)].

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The *Bifidobacteria* are currently used as pro-biotic contains: *B. bifidum, B. longum, B. infantis, B. adolescentis, B. thermophilum, B. animalis.*

Bifodobacterum longum was isolated from human ileum by Dunne et al. [1999]. *Bifidobacteria* are distributed in various ecological niches in the human gastrointestinal tract, which are determined by age and diet. *B. infantis* and *B breve* are replaced by *B. adolescentis* during human growth, but *B. longum* persists throughout human lifetime. *Bifidobacterium spp.* has been prevailing in pro-biotic-containing dietary products because of the major production of L-type lactic acid and β-galactosidase activity in human gastrointestinal tract. Consumers, who have malfunction in digesting milk products, cause problems due to indigestible galactose released from the cleavage of lactose, not by lactose. *Bifidobacterium longum* existing as microbiota in human gastrointestinal tract can help consumers digest galactose using its inherent galactosidase activity [Jiang et al. (1996), Gomes and Malcata (1999)].

B. longum can produce acetic acid and lactic acid in the molar ratio 3:2 without CO_2 production except for the degradation of gluconate. The enzyme fructose-6-phosphate phosphoketolase, the key enzyme of glycolytic pathway, serves for a taxonomic identification of the genus. Furthermore, it can produce ethanol and formate under certain growth condition like glucose-limited [Degnan and Macfarlane (1994)]. In this case, pyruvic acid is reduced to yield lactic acid with the reoxidation of NADH produced through upper stream of glucose degrading pathway or alternatively it can be cleaved by a phosphoroclassic enzyme to formate or acetyl phosphate. Half of acetyl-phosphate must be reduced to ethanol with the reoxidation of NADH and the rest is used to generate more ATP with formation of acetic acid (Fig.1.6). It is more favorable than homolactic fermentation producing 5.5 moles of ATP for every two moles of glucose. *Bifidobacteria* usually exhibit a weak growth in milk and absolutely require anaerobiosis, low redox potential in the early phase growth. Additionally, they can require the growth promoting factors such as vitamin enriched protein hydrolysates to archive a desired growth rate [Ibrahim and Bezkorovainy (1994)].

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The nutrient requirements for growth of *Bifidobacteria* are species-dependent, so any new strain in process needs to be extensively studied. Generally speaking about growth factors, the growth of *B. bifidum* and *B. longum* could be enhanced by *Bifidobacterial* growth-promoting activities of bovine milk casein-hydrolytes digested by trypsin [Poch and Bezkorovainy (1991)], and similar behaviour was observed with yeast extract or hog gastric mucin.

As another *Bifidobacteria* growth-stimulating enhancer, the effects of natural rubber serum powder (NRSP) was indicated by Oiki et al. [1996]. According to his results, ammonium sulphate, one of the major components of NRSP, could assist growth as a nitrogen source.

Bifidobacterium species prefer oligoglucosyl-inositol and fructo-oligosacharides (FOS) [Sato et al. (1991)]. It was found that glucose comprising FOS and sugar might sustain growth and cell proliferation, while fructose might enable the production of the major metabolites, using *B. infantis* ATCC 15697. *B. longum* fermented glucose more rapidly than lactose [Hyun et al. (1995)]. On the other hand, when grown anaerobically at pH values above 5.0 and on a complex medium containing excess lactose, *B. longum* could generate exopolysaccahride comprising glucose, galactose and small amounts of uronic acid and hexosamines [Andaloussi et al. (1995)]. The highest yield could be obtained when the cells were cultivated in a peptone/yeast extract medium of pH 5.8 with NH_4OH.

In spite of the significance of *Bifidobacterium*, its low tolerance to oxygen has been considered as another obstacle in industrial application. Generally, superoxide dismutase (SOD), which is one of the most important factors in oxidative defence systems, has been classified into four types of SOD; Mn-SOD, Fe-SOD, Cu/Zn-SOD, and Ni-SOD, from studies on aerobic organism depending on a feature of cofactor. Each of the SOD requires the indicated metal cofactor for its activation. In several aerotolerant bacteria including *Lactobacillus plantarum*, however, non-enzymatic dismutation system which accumulates high intracellular level of Mn^{++} and stoichiometrically removes superoxide radicals has been evolved.

In case of *B. longum* and *B. infantis*, these apparent dismutation activities might be due to a non-enzymatic dismutation system using divalent metal ions such as Mn^{++} and Fe^{++} [Chang and So (1998)]. Furthermore, oxygen-tolerant anaerobic *B. longum* isolated from human fecal samples showed a number of physiological differences under oxygen [Ahn et al. (2001)].

2 glucose

2 ATP

2 ADP

2 glucose-6-phosphate

fructose-6-phosphate ◄——— fructose-6-phosphate

ADP   ATP

erythrose-4-phosphate ◄———► Acetyl-phosphate ———► **Acetic acid**

Sugar phosphate rearrangements

heptose-phosphate + triose-phosphate

2 acetyl-phosphate ◄———► 2 glyceraldehyde-3-phosphate

2 ADP   2 NAD   4 ADP

4 ATP

2 ATP   2 NADH

2 acetic acid

2 pyruvic acid   **Lactic acid**

2 NAD

2 NADH

Phophoroclastic enzyme

formic acid ◄———

Acetyl-phosphate

0.5 ADP   0.5 NADH

0.5 ATP   0.5 NAD

0.5 acetic acid   **0.5 ethanol**

Fig.1.6 The pathway of glucose fermentation by *Bifidobacterium*; the end products are described in bold characters. *More acetic acid and formic acid can be produced during the exponential period of growth by phosphoroclastic enzyme in Bifidobacterium longum, B. bifidum, and B. aldolescentis [Degnan and Macfarlane. (1994)]*

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1.4.2.4. The effects of Lactobacillus brevis in MLF and its putative use in pro-biotic and fermented food.

Lactobacillus brevis exists in wine and beer as heterolactic fermentative bacteria producing lactic acid, acetic acid and ethanol with CO_2. While this bacterium is surviving in wine or beer after alcoholic fermentation, it has been defined as the agent for the spoilage of beer or biogenic amines produced in the wine [Sakamoto et al. (2001), (2002)]. *L. brevis* has been used in maturing process of cider and have a good facility to degrade malic acid to lactic acid.

The amount of biogenic amines can be controlled in the wine [Christensen et al. (1999), Patrick and Aline (2002)]. Tyrosine decarboxylase was defined in *L. brevis* IOEB 9809. Moreno-Arribas et al. [2000] investigated for the presence of tyramine-producing strains in the wine containing high levels of biogenic amines. They isolated two different *L. brevis* strains (IOEB9809 and IOEB9901) in wine and also detected production of tyramine by *L. brevis* from ATCC. Tyrosine decarboxylase is not just only amino acid assimilating enzyme in *L. brevis*. Yokoyama et al. [2002] used *L. brevis* to produce γ-amino-butyric acid from free glutamic acid in *shochu kasu*, which is Japanese distilled alcoholic beverage produced from rice, barley, buckwheat, sweet potato, and sugar cane in Kyushu and Okinawa. Glutamate decarboxylase in *L. brevis* can convert free glutamic acid into γ-amino-butyric acid through decarboxylation [Christensen et al. (1999)]. *L. brevis* is the hetero-lactic fermentative bacteria using pentose phosphoketolase pathway (PKP) to metabolise glucose [Ye and Saier, Jr. (1995), Saier et al. (1996)]. These bacteria can obtain glucose or lactose through glucose or lactose: H^+ symporter. Sugar uptake can be controlled under allosteric regulation by cooperative binding of glucose and HPr (ser-P). Glucose or lactose permease is inducible during the growth in the presence of glucose or lactose.

L. brevis can catalyse the conversion of fructose to mannitol with mannitol dehydrogenase [Martinez et al. (1963)]. This enzyme can use NADH to covert fructose into mannitol as cofactor and also use NAD^+ for the reversible reaction producing fructose from mannitol depending on the substrate concentration and pH. Generally speaking, *L. brevis* produce two moles mannitol, one mole each lactate and

acetate with CO_2 in the expense of three moles fructose [Peterson and Fred (1920), Martinez et al. (1963)]. However, depending on the substrate and culture conditions, ratio of products can be changed. According to the results from Peterson and Fred [1920], the amount of ethyl alcohol produced during the cultivation varied with the certain amount of fructose in use. As stated above, while fructose is available in the culture medium, *L. brevis* can use fructose as an alternative instead of reducing acetaldehyde in metabolic pathway to regenerate NAD^+ exhausted during the catabolism of glucose. But *L. brevis* should regenerate NAD^+ through a branched pathway toward ethyl alcohol when fructose is limited.

Also, Rönkä et al. [2003] re-evaluated *L. brevis* as source of pro-biotic. Using *Lactobacillus brevis* ATCC 8287 and ATCC 14869, they proved the strains has good applicability at in vitro test for adherence to human Caco-2 and intestine 407 cells, and they were tolerant against low pH, bile acids and pancreatic fluids under in vitro conditions. Furthermore it showed long survival during a cold storage period of 28 days and even if *L. brevis* was not able to produce Yorghurt with milk, it proved a good possibility to use it as the additive in diverse ways.

1.4.2.5. Physiology of Lactobacilli in cheese making process.

Cheese ripening involves biochemical activities such as glycolysis, lipolysis, and proteolysis. Proteolysis of cheese casein and lysis of starter cells incorporated in the cheese curd produce free amino acids that may serve as substrate for secondary microflora including LAB. The catabolism of amino acids by the starters contributes to the cheese ripening process.

A variety of LAB are active to produce flavour from free amino acids. Since dairy LAB have many peptidases, they can digest milk casein to free amino acids. Amino acids can be catabolised by LAB through a number of pathways: de-carboxylation to form amine and CO_2; de-amination to produce ammonia and α-keto acids; transamination to synthesize other amino acids. Amine, α-keto acids, and amino acids are further transformed to aldehydes, alcohols, acids, sulphur-containing compounds, indole, phenol, and cresol. Also, CO_2 produced through decarboxylation by LAB is very important to make texture in cheese [Tavaria et al. (2002), Liu et al. (2003)].

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To provide another example in recent food industry, a role of *L. brevis* in sourdough fermentation has been investigated by Meignen et al. [2001]. Bacterial starters have been developed for various cereal products to improve theirs sensory and technological qualities. To produce more aromas in bread, they found that fermentation of sourdough with *L. brevis* before baker's yeast could help yeast produce aroma from free amino acids.

There are also many reports about its uses in fermented foods such as bread-making, [Meignen et al. (2001)], pickles and African foods-making [Susana et al. (2001), Plessis et al. (2003). Mugula et al. (2003)].

1.4.3. Inhibition by organic acids of LAB growth

The growth of LAB can be inhibited by the certain concentration of lactic acid and acetic acid. Most of organic acids are produced during the bacterial growth and accumulated to a sufficient concentration to give the serious growth inhibition. Indeed, this is the basis of their use in food preservation.

Generally, all LAB produce organic acids such as lactic acid, acetic acid, propionic acid, and formic acid in addition to alcohols such as ethanol, propanol, and butanol. Organic acids and alcohols are the major substances that create unfavorable environment for the growth of other bacteria. The LAB have a variable tolerance toward the end product inhibition.

Giraud et al. [1991] and Gonçalves et al. [1997] found the pH-dependent inhibitory mechanism by lactic acid in *Lactobacillus rhamnosus*. According to their results, in alkali pH the growth rate was lowered by high lactic acid concentration. Since the energy obtained with production of lactic acid is not used for the cell growth in this condition, the energy must be used for cell homeostasis with its function as proton pump.

In contrast with alkali pH, the growth inhibition by lactic acid in acid circumstance attributes to non-dissociated lactic form accumulated within the cytoplasm.

The inhibition by most weak organic acids is intimately related to the solubility of the non-dissociated form within the cytoplasmic membrane and the insolubility of the ionised acid form. This caused acidification of cytoplasm and the collapse of the motive force, preventing the nutrients from transporting (Fig.1.7).

Bifidobacterium spp. is no more tolerant against the acid condition when compared to other lactic acid bacteria. However, the case of *B. longum* survives better than other *Bifidobacterium* in the acid condition and they are able to tolerate a bile concentration as high as 4% [Lankaputhra (1995)].

Oenococcus oeni are highly tolerant of acidic pH being able to grow even at pH 3. Evidence that acid tolerance is related with H^+-ATPase activity at low pH is not sure yet, but Arginine deiminase pathway (ADI) clearly attributes to acid tolerance. This pathway catalyzes the cleavage of one mole arginine into one mole ornitine, two moles ammonia, and CO_2 forming one mole ATP. Ammonia (NH_3) can to be used to neutralise proton and ATP can be used to enable the exclusion of H^+ by F_1F_0–ATPase. An arginine/ornithine anti-porter completes the system and allows the exchange of these two molecules at no energy cost [Bourdineaud et al. (2003), Fortier et al. (2003), Galland et al. (2003)].

As another system for pH homeostasis in *Oencoccus oeni*, the decarboxylation of malic acid into lactic acid across the cellular membrane can be effective to enable to increase the alkalinity of the cytoplasm in low pH. The acidity decreases through malolactic fermentation and produced lactic acid is exported through an electrogenic uniport in which allow the synthesis of ATP at low pH [Ramos et al. (1994), Poolman et al. (1991), Salema et al. 1996; Arena et al. (1999)].

In addition to these protection systems to acid condition, an alteration on the composition of the cell wall plays a role in acid tolerance. A small heat shock protein (smHSP) was found in *Oenococcus oeni* after heat shock and acid stress. These proteins are expressed into the cell membrane after the stress and it induced an alteration on the integrity of the cell wall [Guchte et al. (2002), Guerrini et al. (2002), Maréchal et al. (2000), Teixeira et al. (2002), Tracey and Britz (1989)].
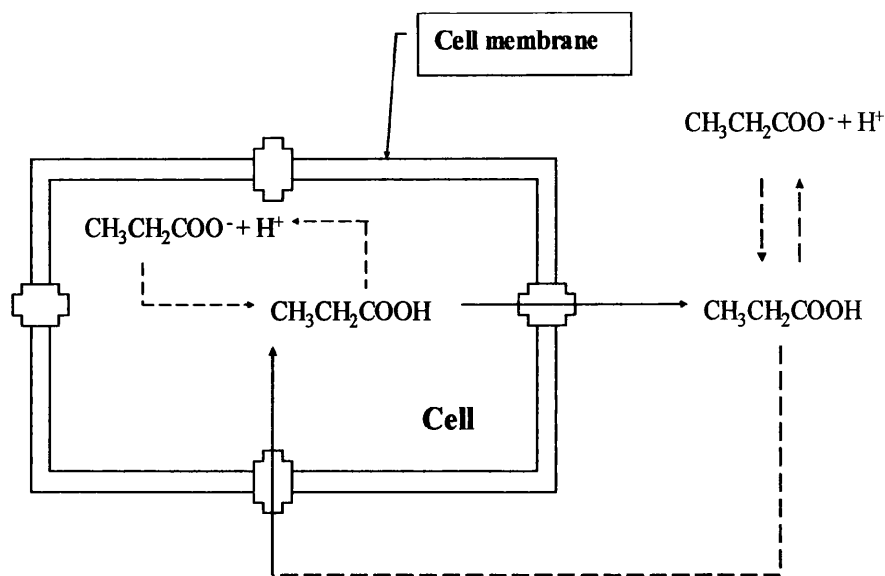
Fig.1.7. pH-dependent inhibitory mechanism by lactic acid in *Lactobacillus rhamnosus*. *Lactate-H and lactate $^-$ H^+ exist in equilibrium state between outside and inside of the cell , and only lactate-H form is transported to outside of cell from inside or to inside of the cell from outside. Free movement of lactate-H establishes the balance of the lactate concentration between the intra and the extra-cell. Through such a transport system, L. rhamnosus can accomplish the transportation of lactate and H^+, and establish the balance between the outside and the inside of the cell.*

1.5. THE DEMAND FOR HIGH CELL DENSITY FERMENTATION.

1.5.1. Launching of high density in LAB

Large quantities of LAB are required for many applications (Section 1.5.3). High density fermentation is, therefore, required to provide efficient use of fermenter. High concentration of cell is also required for biotransformation processes such as maturation and the enzymatic reactions. High concentration of product by high cell density fermentation simplifies the recovery procedure in downstream process later coming up, and affects the quality of the end products [Richter and Nottelmann (2004)]. Eventually, high density fermentation has been turned out as a new technique for innovation in many fields.

However, there are many problems associated with growing LAB to high cell density.

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At first, low productivity of anaerobes related to aerobe has made it look extravagant in terms of media required and the volumes consumed. The low yield on carbon source and the end product inhibition made that cell concentrations above 4 ~ 5 g/L are almost the maximum cell concentration expectable in conventional batch and continuous culture [Chapter 3 and 4].

The second main limitation to high cell density fermentation is the end product concentration that ultimately inhibits growth. Using the best (*Lactobacillus plantarum*) adapted to these conditions that shows very high tolerance to lactic acid (0.5 M), only about 5 g/L dry cell can be produced. If this is comprised to Fed batch *E.coli* or aerobic yeast fermentation, the concentration of over 50 g/L are typical and in some case to over 100 g/L can be obtained.

Another obstacle in obtaining high cell density of LAB in the traditional batch culture is their complex nutrient requirements. *Bifidobacterium* sp and *O. oeni* generally requires the growth factors to grow them at a high growth rate. Most of *Lactobacilli* need a variety of amino acids for the fast growth. But mainly, such ingredients are too expensive to use in large scale.

Overall, it has meant that this is a need to develop innovative culture such as the membrane cell recycling bioreactor (MCR).

As an example, spontaneous processes dependent on either the regional or the natural microflora are very robust, time-consuming, and uncontrolled, it is non-economic. The use of the starter culture has been developed to increase the productivity because of explosively increasing population and multi-culture in the world.

There have been still so many difficulties with the use of the starter culture when initially applied. Typically, stressed conditions in the beginning of fermentation have made so many problems to the bacterial adaptation and growth. Therefore, the large numbers of viable cells have always been required to overcome the inhibitory barriers such as tolerance, toxicity and adaptation.

For successful silage process, the inoculants must be predominantly from the early stage of ensiling process. In case of *Lactobacillus plantarum*, at least over 10^6 cfu (colony forming units) per g of the wet forage are necessary for producing the consistent result according to the report of Kung Jr. and Ranjit [2001] and Ranjit and Kung Jr. [2000].

Maicaset al. [2000] investigated how many number of *O. oeni* must be inoculated to diminish plenty of detrimental factors in the circumstance of MLF with high densities of non-proliferating cell. When dosage of $10^7 \sim 10^8$ cfu/ml was applied, inhibition of MLF was diminished even at low pH of pH 3.5 and bacterial development was not necessary for the degradation of L-malic acid. However, when the bacterial number in inoculation dropped to be 10^6 cfu/ml, the efficiency of MLF was influenced by the species in MLF, low pH, and even by the concentration of L-malic acid.

Also, as $10^9 \sim 10^{10}$ viable microbes per ml of yoghurt has been required to provide health effects as the minimum dose in daily ingestion, the cell should be grown to high density.

Overall, it has meant that this is a need to develop innovative high cell density culture such as the membrane cell recycling bioreactor (MCR).

1.5.2. Cost reduction of LAB fermentation.

Cost reduction in LAB fermentation has been studied in various ways.

Major nitrogen source in the growth medium is yeast extract. Yeast extract is highly effective, but relatively expensive to the market price of lactic acid. According to the economic analysis for the production of lactic acid by Yoo et al. [1997], the price of yeast extract accounts for over 38% of the total cost in the production of lactic acid.

The trials to replace cheap substrates for yeast extract have been studied with Soybean hydrolysates [Yoo et al. (1997), Kwon et al. (2000)] or whey [Aeschlimann and Stockar (1990)].

Another consequent factor in cost is the scope of the reactor and the downstream process required for low density culture.

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The largest problem in obtaining high cell density of LAB in traditional batch culture, continuous culture, and fed-batch culture is the end product inhibition.

Ultimately, the growth of LAB is inhibited by the end product inhibition during the fermentation. Particularly, the cease of the exponential growth period is mainly due to end product inhibition.

MCR is the simplest cell growth equipment designed to continuously remove inhibitory substances while allowing the cell retained in bioreactor with a filtrate. Likewise, the cell can continuously grow in high growth rates without an inhibition simultaneously removing fermented medium containing inhibitor and products. Furthermore, as the growing cells in the bioreactor are continuously supplied with the nutrients, they are not starved.

With recent improvements in membrane filtration studies of MCR, ceramic membrane [Suzuki et al. (1994), Grespo et al. (1992), Boyaval et al. (1996), Kamoshita et al. (1998), Suzuki et al. (1994), Persson et al. (2001)], nanofiltration [Jeantet et al. (1996)], and electro-dialysis (ED) membrane [Nomura et al. (1999)] have been used in MCR. The studies of cell retention culture techniques have dominated in investigations for intensive production of ethanol [Marañón et al. (1997)], lactic acid [Jeantet et al. (1996), Kwon et al. (2001), Kamoshita et al. (1998)], and biomass [Bibal et al. (1991), Suzuki. (1996), Boyaval et al. (1996), Grespo et al. (1992), Persson et al. (2001), Taniguchi et al. (1987)] because of economic and technical reasons. Principally ceramic membrane has been chosen for trials in Lab or industry due to endurance and usability.

Many attempts to obtain high concentration of viable cells have been limited by membrane fouling, which seriously reduces the membrane flux.

Membrane fouling is a complex process caused by a number of related phenomena at the membrane surface. The reducing flux may be the build-up of particles on the membrane surface by concentration polarization. However, concentration polarization has no in-depth relationship with membrane fouling because it is reversible.

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Solute and particle deposition on the membrane surface is likely to occur when the permeation flux is very high compared to the back transport mechanisms [Belfort et al. (1994)]. However, another significant cause is absolutely a change of the feature and character on membrane surface or in membrane pore by the colloidal materials such as proteins, peptides, amino acids, and carbohydrates [Bowen and Gan (1991), Burns and Zydney (1999)]. Usually, the first layer of soluble proteins formed on the membrane surface stimulates formation of another layer and it builds the cake on the membrane surface. The first binding of soluble protein to the membrane surface can take place due to electrostatic or ionic interactions depending on surrounding environmental parameters. Once protein binds successfully to the membrane surface, the second binding between proteins is easier. Ultimately overlapping protein layers forms a thick layer of protein and seriously reduces the flux. Persson et al. [2001] found the membrane fouling was attributed to the physico-chemical variation of the medium resulting in after fermentation. Mainly a change in pH affected the electrostatic interaction on both of bacteria and membrane so that bacteria adhere to membrane surface or to each other. Trials to reduce the fouling during the high density cell culture in the MCR have been studied through the trouble-shooting such as the intermittent back-flushing of using membrane [Kuberkar et al. (1998)], introducing ultra-sonication into membrane module, or using them by in turn the membranes for cleaning others [Marañón et al. (1997)].

All ways considered to improve membrane flux can be roughly classified into two categories depending on where membrane units are placed. In general, fitting membrane unit inside bioreactor [Kamoshita et al. (1998); Suzuki et al. (1994), Suzuki. (1996)] is very difficult and expensive in set-up and scale-up, but it can produce more viable cells due to small variation in growth environment. On the other hand, external units like centrifugation, sedimentation, or membrane filtration can increase the separation rate, but it can cause significant problems to the cultures such as contamination and deactivation of cell due to additional environmental stresses [Bibal et al. (1990); Grespo et al. (1992). Kwon et al. (2000)].

In the case using the MCR to intensify microbial growth, real problems take place in case where the organisms produce extracellular proteins or exopolysaccharides.

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Many kinds of *Lactobacillus* and *Bifidobacterium sp* are involved in production. Surface-layer protein of *Lactobacillus brevis* [Yasui et al. (1995), Mårtensson et al. (2003), Savijoki et al. (1997)] and exopolysaccharides by *Bifidobacterium longum* [Nagaoka et al. (1995), Roberts et al.(1995)] are good examples. Production of these substances must be controlled by many physico-chemical factors.

1.6. MASS BALANCE EQUATIONS FOR THE GROWTH OF LACTIC ACID BACTERIA

Growing lactic acid bacteria by high cell density has many advantages. So as to assess and develop processes for the bacterial cultivation, a good understanding of the bacterial growth kinetics or mass balance can give better results. But in contrast with the growth of many bacteria, LAB fermentation has many factors that can affect the final results. Consequently, the kinetics of end product inhibition must be involved to describe the growth kinetics in LAB fermentation.

1.6.1. Mass balance equations in the batch culture.

The growth rate in the batch culture was estimated like following

$$\frac{dX}{dt} = \mu X \tag{1}$$

In which X is the dry cell weight per unit volume of culture broth, t is time, and the constant of proportionality μ is *the specific growth rate*. where

$$\mu = \frac{\mu_m S}{K_S + S} \tag{2}$$

Upon integration of (1) between the limits of t_2 and t_1, and X_2 and X_1,

$$\int_{X_1}^{X_2} dX = \int_{t_1}^{t_2} \mu dt$$

$$\ln X_2 - \ln X_1 = \mu(t_2 - t_1)$$

$$\mu = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)} \tag{3}$$

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Yield coefficient for biomass can be estimated as a certain amount of biomass produced per a certain amount of glucose consumed in the same time like follows.

$$Y_{X/S} = \frac{(X_2 - X_1)}{(S_1 - S_2)} \tag{4}$$

Also, *the specific production rate of cell mass* is evaluated as the biomass production per unit time per unit culture volume.

$$P_{X/t} = \frac{(X_2 - X_1)}{(t_2 - t_1)} \tag{5}$$

In lactic fermentation inhibitory materials like lactic acid and alcohol are constantly accumulated in duration of fermentation. Therefore it is necessary to incorporate an inhibitory effect by by-products into mass balance equation.

To take an account for product inhibition, a simple generalization of the Monod equation by Levenspiel. [1980]

$$\mu = \mu_m (1 - \frac{C_p}{C_p^*})^n (\frac{S}{K_S + S}) \text{ or } \mu = \mu^* (\frac{S}{K_S + S}) \tag{6}$$

when $\mu^* \neq \mu$

$$\mu^* = \mu_m (1 - \frac{C_p}{C_P^*})^n \tag{7}$$

As stated at equation (7) the growth of lactic acid bacteria would be inhibited by extent of inhibitory factor (n) and concentration (C_p).

To identify *production rate (v) of organic acids* in batch culture by Leuderking and Piret. [1959],

$$\frac{dP}{dt} = \alpha(\frac{dX}{dt}) + \beta X \tag{8}$$

$$\mu = (\frac{1}{X})(\frac{dX}{dt}) \tag{9}$$

$$\frac{1}{X}\frac{dP}{dt} = \alpha\mu + \beta \tag{10}$$

$$\frac{1}{X}\frac{dP}{dt} = \alpha\mu^* \left(\frac{S}{K_S + S}\right) + \beta \qquad \text{here, if} \frac{1}{X}\left(\frac{dP}{dt}\right) = v \text{, the specific production rate}$$

$$v = \alpha\mu^* \left(\frac{S}{K_S + S}\right) + \beta \qquad\qquad\qquad (11)$$

The constants of proportionality α and β can be experimentally obtained by plotting the specific production rate (v) of organic acids to the growth rate (μ). The product concentration (P) changing in time (t) depends on the stoichiometric constant (α) in growth-associated model and the proportionality constant (β) depending on cell concentration (X) existing at time t. The equation (10) can be obtained by incorporating the equations (6), (7), (8) and (9) into (10). Here, the K_s is Monod constant describing nutrient-limiting growth rate and the substrate concentration below the K_s may reduce the growth rate of the cultivation.

Finally, the substrate consumption rate is the sum of the substrate consumption rate for production, cell mass and maintenance

$$\frac{dS}{dt} = -(\frac{X}{Y_{P/S}})v + \left\{-(\frac{X}{Y_{X/S}})\mu^*\right\} - \frac{mX}{Y_{X/S}} \qquad (12)$$

1.6.2. Mass balance equations in MCR.

The mathematical equations in the MCR can be obtained by taking a constant consumption of the ingredients in washing out into the mathematical equations observed in batch culture. Also, the products in the MCR can not be retained in the bioreactor and washed out, but biomass can be retained and accumulated.

Therefore, the production rate of biomass in the MCR can be described by the equation (6). But, the mass balance equations about the dynamic of the specific production rate (15) and the accumulation of substrate (16) in the MCR can be described as following,

$$\frac{dP}{dt} = D(C_0 - C_i) + (\alpha\mu + \beta)X \tag{13}$$

When $C_O \cong 0$, dilution rate $D = \frac{f}{V}$

$$\frac{1}{X}\frac{dP}{dt} = (\alpha\mu + \beta) - \frac{DC_i}{X}$$

$$v = \alpha\mu + \beta - \frac{D}{X}C_i \tag{14}$$

As a result, the specific production rate of the product P in the MCR is

$$v = \alpha\mu^* \left(\frac{S}{K_S + S}\right) - \frac{D}{X}C_i + \beta \tag{15}$$

Here, V and f predict the volume (L) of bioreactor and the flow rate (L/h), respectively.

$$\frac{dS}{dt} = \frac{f}{V}(S_0 - S_i) - \frac{\mu^* X}{Y_{X/S}} - \frac{vX}{Y_{P/S}}$$

$$\frac{dS}{dt} = -\left(\frac{\mu^*}{Y_{X/S}} + \frac{v}{Y_{P/S}}\right)X - \frac{f}{V}(S_i - S_0) \tag{16}$$

As stated above, mass balance equations were constructed with general forms used for growth kinetics in addition to inhibitory forms by lactic acid according to Levenspiel. [1980]. Here, bacterial growth can be inhibited by inhibitor concentration accumulated during the cultivation and its toxic power. Toxicity (n) is different depending on using bacteria, the kind of inhibitory substances, and its environmental conditions, therefore it must be determined by the experiments.

1.7. AIMS AND OBJECTIVES.

The aims of this project are to provide a general method for the production of high cell density and their usability. Also, this technique was used in maturation to bio-transformation of cider.

1.7.1. Choosing bacteria in good industrial use.

The first objective was to choose bacteria that are generally representative of LAB of importance.

Lactobacillus buchneri has been re-evaluated to use in ensilage with homofermentative bacteria. Traditionally, homofermentative bacteria like *Lactobacillus plantarum* has been in use because it quickly drops down pH in ensilage. Quick drop in pH is very useful to protect ensilage from contamination by yeast or *Clostridia*. But due to so often exposure to aerobic circumstance, the contamination was very usually occurred.

L. buchneri can anaerobically convert lactic acid to acetic acid, 1,3-propanediol, and propionic acid, which prevent the growth of contaminants, when it was inoculated together.

Lactobacillus brevis and *Oenococcus oeni* are good subjects to compare for each other in the field of malolactic fermentation. *L. brevis* has been used for MLF of cider-making process and *O. oeni* has been used in wine-making process. Both two bacteria have shown similar malolactic activity, but they are different from each other in that they produce different flavour components and have physico-chemical features.

Finally, *Bifidobacterium longum* has been used in pro-biotic. Principally, *Lactobacilli* was the main subject in the field of pre- and pro-biotic, but in recent studies, the use of *Bifidobacterium sp* with *Lactobacilli* is more effective in that promotes human health. Likewise, *B. longum* was chosen as the example for cell product.

1.7.2. Investigation of growth in the batch cultures.

To investigate growth and growth requirements batch cultures were undertaken in serum vials without pH control. The physico-chemical characteristics of bacteria were checked to use later on the growth in the MCR or on the maturation of cider. The nutrient requirements were selected in basis of the growth rate and total cell mass

produced. Optimized culture media selected through the batch culture were used for large scale growth in the stirred tank reactor (STR) and the MCR.

1.7.3. Investigation of growth in the STR.

Bacteria grew at the STR of 5 L with working volume of 3 L with pH control.

While growing bacteria at the STR, the optimum pH control level and the critical substrate concentration were checked. A range of pH control was selected in basis of the growth rates, product yield, and specific production rates of cells.

The optimum substrate concentration is a range of the substrate concentration to possibly remove lag period and to produce the maximum cell mass at high product yield and high specific production rates.

The pH control level selected through the growth at the STR was used to control pH during the growth at the MCR, and the critical substrate concentration was referred as the least substrate concentration to keep high growth rates during the growth at the MCR.

1.7.4 Investigation of growth in the membrane cell recycle reactor (MCR).

Having investigated the selected organisms and optimized the media in the batch, the next stage was to develop the method for maximizing cell cultivation in the MCR.

1.7.5. The Comparison of each culture methods.

The efficiencies during the growth at each of culture methods will be compared on the basis of the growth kinetics, growth rates, product yield, and the specific production rates. The superiority of the MCR in that obtains high cell mass in the high specific production rates was confirmed through the comparison.

1.7.6. The Maturation of cider by high cell density bio-transformation as a model application (MLF).

The trials to mature wine or cider with proliferating high cell density of LAB have been undertaken in Lab or small scales.

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However, maturation of cider was fulfilled at the practical scale through this investigation. It showed the possibility to use proliferating high cell density of LAB in industrial level.

General cider and Scrumpy Jack will be used to compare through the maturation in this project, commonly using *O. oeni* as malolactic bacterium. Two ciders provide the malolactic strain a different physico-chmical circumstance during MLF. Two ciders are different in the composition and the concentration of nutrients. Such a different circumstance affected the malolactic efficiency.

L. brevis and *O. oeni* will be compared for each other in the malolactic efficiency and in variations of the components during the MLF.

Monitored components are the essential substances to determinately affect to the flavour and acidity of the product. The volatile compounds and organic acids will be monitored for the variation during the MLF, and also the variations in the enzyme activity according to alcohol concentration were compared with two strains.

- Nomenclature -

X : biomass in dry cell weight (g /L). t : time (h). μ: the specific growth rate (h^{-1}). μ_m: the maximum growth rate (h^{-1}). α: the stoichiometric constant in growth-associated model. β: the proportionality constant depending on cell concentration in t time. $Y_{X/S}$: yield constant for biomass (Dimensionless). $Y_{PL/S}$: yield constant for lactic acid (Dimensionless). $Y_{PA/S}$: yield constant for acetic acid (Dimensionless). $Y_{PE/S}$: yield constant for ethyl alcohol (Dimensionless). S: the substrate concentration (mole).S_t: the substrate concentration in output in t time (mole). S_O: the substrate concentration in input in zero time (mole). C_t: a product concentration in output in t time (mole). C_O: A product concentration in input in zero time (mole). C_P :concentration of product giving end product inhibition (mole). C_P^*: the critical concentration of product to stop the cell growth (mole). f: flow rate (L/ h). V: volume in bioreactor (36 L). D: dilution rate (h^{-1}). m: constant for cell maintenance. n: toxic power of organic acids. K_s : the saturation constant of substrate. T_P ; productivity. v ; The specific production rate. $P_{x/t}$: the specific production rate of cell mass.

MATERIALS AND METHODS

CHAPTER.2: MATERIALS AND METHODS

2.1. STRAINS.

Oenococcus oeni NCIMB 11648 and *Bifidobacterium longum* NCIMB 702259 used in this investigation were purchased from NCIMB LTD (23 St. Machar Drive, Aberdeen, U.K). *Lactobacillus buchneri* was gifted from Interprise Ltd in U.K. and *Lactobacillus brevis* was collected from the strains in which it has been used in this Lab. All strains were selected from simple selection in the agar plate with MRS medium (Difco Lab., Detroit, MI, USA), and particularly MRS medium supplemented with tomato juice was used to propagate *Oenococcus oeni* at first, but later MLM-1 medium, which was developed from investigation in the batch, was used for propagation.

2.2. REAGENTS.

Soy-peptone purchased from IDG(U.K) Ltd (Torey House, 52 Wash Lane, Bury, England, BL9 6AU), and yeast extract (salt free) gifted from Interprise Ltd in U.K were used as organic nitrogen sources. Glucose monohydrate and fructose were industry-based carbon resources purchased from Cerestar U.K Ltd (GB-Manchester, M17 1PA, U.K).

And, all others than the reagents mentioned above were purchased from Aldrich & Sigma (The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT) and Fisher Scientifics (Bishop Meadow Road, Loughborough, Leichestershire, LE11 5RG). Sorbitan mono-oleate (Tween-80) purchased from Merck.Ltd (Hunter Boulevard, Magna Park, Lutterworth, LEICS, LE17 4XN) was used for formulation of the medium. Glycerol (Molecular biology grade) used to preserve the strains was purchased from Merck Co. Ltd.

2.3.PREPARATION OF THE STARTER CULTURE.

2.3.1. General.

The starter cultures undertaken prior to the batch culture were to propagate and activate the strains taken out of $-70°C$ ultra-freezer.

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Generally speaking, relatively the small number of cell is preserved through freezing and thawing process.

2.3.2. Preparation of the starter culture medium for activation and propagation.

The media for propagation and activation of the strains were nominated as MLM-1. The composition of MLM-1 (w/v) was yeast extract (2%), soy-peptone (1.7%), KH_2PO_4 (0.39%), Tween-80 (0.05%), $MgSO_4.7H_2O$ (0.05%), and $MnSO_4.4H_2O$ (0.005%). The reason for addition of glucose after autoclaving was to protect the medium from caramelization.

MLM-1 medium used for the starter culture was developed to enhance the growth of LAB according to Ledesma et al. [1977]. They used the synthetic medium supplemented with diverse amino acids, and commercial MRS medium included mineral salts like $Mn(SO_4)$ or $Mg(SO_4)$ necessary for growth of LAB. Consequently, MLM-1 medium was created by combination of MRS medium and the synthetic medium by Ledesma et al. [1977]. To supply amino acids, soy peptone was selected. Yeast extract was used as source for vitamin as well as various amino acids and some sugars.

All media were made up to 880 ml, and pH after autoclaving was in a range from pH 6.2 to pH 6.4. Initial pH was adjusted to pH 5.8 with HCL (5M) in case of *O. oeni*, and to pH 8.3 with NaOH (5M) for *B. longum*. Initial pH was not adjusted for *L. brevis* and *L. buchneri*.

The medium was boiled to remove dissolved oxygen (DO), and distributed into 50 ml serum vials, where the air had been flushed out with nitrogen gas. Each of the serum vials was filled to 8.8 ml with the medium. All serum vials were sealed with rubber stopper and aluminum seal, and then sterilized in the steam autoclave for 10 min.

Anaerobic solutions of glucose and fructose as mentioned above were made at the concentration of 50%, w/v, and separately autoclaved by the same procedure. Sterilized anaerobic stock solutions of glucose and fructose were added into the medium with 0.6 ml and 0.4 ml just before use, respectively.

This made the final concentration to 3%, w/v glucose and 2%, w/v fructose in the starter culture medium. Finally, inoculation was made by adding 0.2 ml cell suspension after thawing the vial.

In the case of *O. oeni*, the starter culture medium of 860 ml instead of 880 ml for a further addition of L-cysteine was made. Cysteine stock solution was made to 0.1%, w/v and autoclaved as described above. L-cysteine stock solution of 0.2 ml was added with glucose and fructose solutions into every serum vials just before use, and then inoculated with the seed of 0.2 ml. This addition is effective to reduce trace amounts of oxygen.

2.3.3. Preservation of the strains.

All strains were propagated in anoxic sterilized pressure tubes with 10 ml MLM-1 medium.

Fig.2.1. Photograph of the cryo-vial for preservation of the strains at - 70°C ultra-freezer.

Glucose stock solution (0.6 ml) and fructose solution (0.4 ml) of 50%, w/v were added. The strains grown in appropriate temperature were harvested and separated by centrifugation at 12,000 rpm for 3 min.

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After washing all impurities out with anaerobic sterilized saline water by three washes, the cell pellets were again suspended in 2.5 ml of sterilized glycerol solution (25%, w/v). Ultimately, the microbial suspensions (0.2 ml) were distributed into each of Cryo-vial (Fig.2.1) and straight preserved in -70°C ultra-freezer (U67856 from New Brunswick Scientifics) after labeling. All strains were safely stored for approximately 6 months and vial stocks of the strains were reproduced in every 6 months.

In order to propagate and activate the strains in the ultra-freezer, Cryo-vials taken out of the ultra-freezer were thawed in 30°C and inoculated into MLM-1 for the propagation. They were grown for 24 h in appropriate temperature. Fully grown cells were then inoculated into the starter culture medium.

2.3.4. Sterilization of the starter culture

The vials for the batch culture were sterilized at steam autoclave for 10 min. Pressure and temperature increased to 121°C and 15 psi and maintained for 10 min. The serum vials were taken out from autoclave after cooling to near room temperature.

In the same way, the starter culture bottles for the stirred tank reactor (STR) and the MCR were autoclaved in the steam autoclave 121°C for 15 min.

2.3.5. Propagation of Lactobacillus buchneri in the starter culture.

After activation of the strains in the pressure tube for 24 h, 0.5 ml the culture suspension was injected into the starter culture medium of 9.5 ml using sterilized disposable syringe (1 ml). The starter culture was grown at the rotary shaker automatically controlled at 30 °C (Rotary shaking incubator with 120 rpm and 3 inch power stroke supplied by New Brunswick Scientific Ltd.) for 19 h. Since LAB can produce variety of organic acids and alcohols, it is very important to inoculate before physiological changes caused by the end product inhibition occur.

The starter culture suspension of 1.5 ml was used to inoculate 30 ml the batch culture.

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2.3.6. Propagation of Lactobacillus brevis in the starter culture.

After activation for 24 h, the culture liquid of 0.5 ml collected from the pressure tube was inoculated into the starter culture medium using sterilized disposable syringe (1 ml). The starter culture of *L. brevis* was grown stagnantly in a water bath automatically controlled at 28°C for 20 ~ 25 h. The starter culture suspension of 1.5 ml was inoculated into the batch culture medium of 28.5 ml.

2.3.7. Propagation of Oenococcus oeni in the starter culture.

After activation for 24 h, 0.5 ml the culture solution collected from the pressure tube was inoculated into the starter culture medium. The starter culture of *Oenococcus oeni* was grown stagnantly at constant temperature room automatically controlled at 28°C. After cultivation of starter culture for 24 h, 3 ml the starter culture was used to inoculate the batch culture medium.

2.3.8. Propagation of Bifidobacterium longum in the starter culture.

After activation of the starter culture for 24 h in the pressure tube, the culture liquid of 0.5 ml collected from the pressure tube was inoculated into the starter culture medium. The starter culture of *Bifidobacterium longum* was grown stagnantly in a water bath automatically controlled at 37°C for 12 h. The starter culture liquid of 1.5 ml was inoculated into the batch culture medium.

2.4. PREPARATION OF THE BATCH CULTURE.

2.4.1. General

All small batch cultures were prepared in serum vials. The physiology and the nutritional requirements of the strains under investigation were studied and optimized in serum vials of batch cultures. Information from these cultures was used to scale-up growth of LAB to the STR and the MCR.

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2.4.2. Preparation of batch culture medium and inoculation.

The serum vials with the growth medium of 30 ml were used in the batch culture (Fig.2.2).
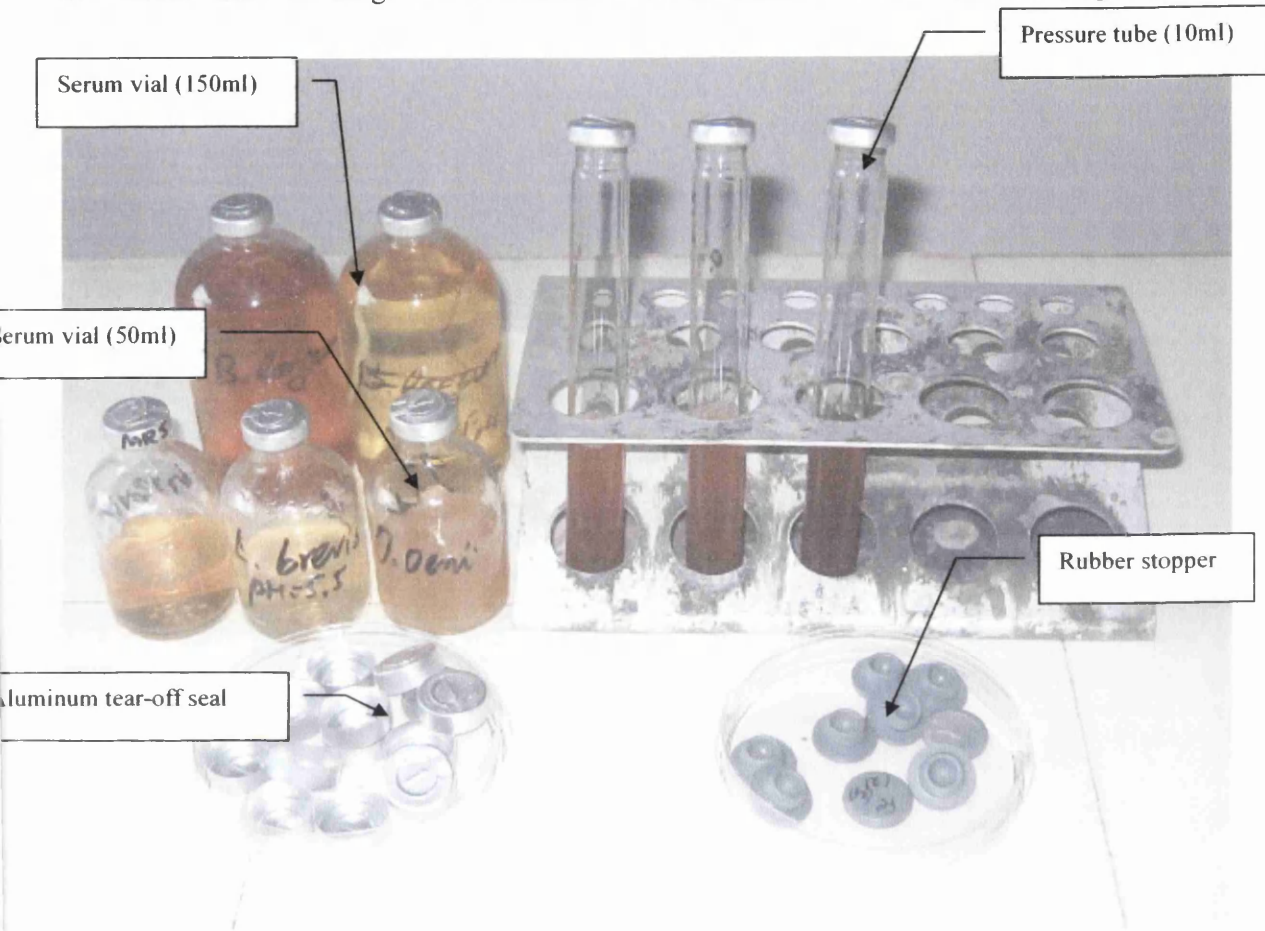
Fig.2.2. Photograph of the Serum vial and the pressure tubes used in the starter and the batch cultures. *Every serum vials were sealed with rubber stopper and aluminium seal after flushing oxygen out with nitrogen gas to switch it over to anoxic. The serum vial of 50ml was used for the microbial growth and the serum vial of 150 ml was used for the stock solution of nutrients. Tear-off aluminium seal and rubber stopper were shown in the front left and the front right, respectively.*

All ingredients to make the growth medium of 1 L were dissolved in tap water of 500 ml with mixing. Initial pH was adjusted with NaOH (5M) or HCL (5M) before autoclaving. This was for the case of investigating the physical conditions such as inoculums size and inoculation timing and the effect of agitation and initial pH.

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When the physiological condition and nutritional requirements were investigated, all ingredients under investigation were added after autoclaving as sterile solution. The initial pH was adjusted depending on the strain and the nutrients under investigation.

The media (25 ml) were distributed to serum vial and sterilized in the steam autoclave for 10 min according to the aforementioned way *(Refer to section 2.3.4)*. All components under investigation were made as a sterile concentrated stock solution, and added just before use.

The growth medium in the serum vial was filled up to 28.5 ml with sterilized distilled water, and inoculated with 1.5 ml the starter culture. The exception was for *O. oeni* where 3 ml inoculation was used.

2.4.2.1. Composition of the growth medium for Lactobacillus buchneri.

The composition (w/v) of the optimized medium for the batch culture was glucose (2%), KH_2PO_4 (0.39%), yeast extract (0.9%), $MgSO_4 \cdot 7H_2O$ (0.74%), $MnSO_4 \cdot 4H_2O$ (0.1%), D,L-aspartic acid (0.67%).

Investigation about the nutritional requirements of *L. buchneri* was begun with MLM-1 medium as the basic medium. All components were tested to look for the critical concentration to support the microbial growth. The basic media without ingredient under investigation were separately sterilized in the steam autoclave, and then concentrated stock solutions of those ingredients were mixed with the basic media just before use. Sterilized glucose solution of 1.2 ml was added to make up to glucose concentration of 2% (w/v) in the growth medium, and then initial pH was adjusted with NaOH (1M) or HCL (1M) just before inoculation. As a result, optimized growth medium for *L. buchneri* was developed from MLM-1 starter culture medium. Finally, initial volume of 28.5 ml was made up with sterilized tap water.

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2.4.2.2. Composition of the optimized growth medium for Lactobacillus brevis.

The composition (w/v) of optimized batch culture medium for *L. brevis* was glucose (1.0%), fructose (2.5%), KH_2PO_4 (0.39%), yeast extract (1.5%), $MnSO_4 \cdot 4H_2O$ (0.018%), Tween-80 (0.1%).

That optimized medium was developed using MLM-1 as the basic medium like the case of *L. buchneri* (Refer to section 2.4.2.1)

2.4.2.3. Composition of the optimized growth medium for Oenococcus oeni.

The composition (w/v) of optimized batch culture medium for *O. oeni* was glucose (2.5%), fructose (1.0%), KH_2PO_4 (0.39%), yeast extract (1.5%), $MgSO_4 \cdot 7H_2O$ (0.05%), $MnSO_4$ (0.005%), $(NH_4)_2SO_4$ (0.05%), Na-citrate (0.88%), L-Cys (0.1%).

The optimized medium was developed using MLM-1 as the basic medium and the initial pH was adjusted to pH 5.8. The initial volume in serum vial was filled up to 27 ml as the inoculation volume was 3 ml.

2.4.2.4. Composition of the growth medium for Bifidobacterium longum.

The composition (w/v) of optimized batch culture medium for *B. longum* was glucose (1.0%), fructose (0.9%), KH_2PO_4 (0.39%), yeast extract (3.0%), $MgSO_4 \cdot 7H_2O$ (0.14%), $(NH_4)_2SO_4$ (1.0%).

That optimized medium was developed using MLM-1 as the basic medium. Initial pH was adjusted to pH 6.0 with NaOH (1M) according to the same procedures described above.

2.4.3. Microbial growth in the batch culture and sampling.

Microbial growth in the batch culture was carried out in the stagnant incubator with temperature control. The pH was not controlled during the batch culture, and cultivation was terminated at the point where the microbial growth had stopped.

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Temperature of the incubator was set to 28°C for *L. buchneri* and *O. oeni*, to 37°C for *B. longum*, and to 30°C for *L. brevis*.

Samples were taken in every 3 or 6 h during the incubation, and the cell mass, the growth rates, and pH were measured.

2.5. PREPARATION OF LAB GROWTH IN STIRRED TANK REACTOR (STR).

2.5.1. General.

The stirred tank reactor (STR) for the microbial growth (Fig.2.3) was designed in this Lab.
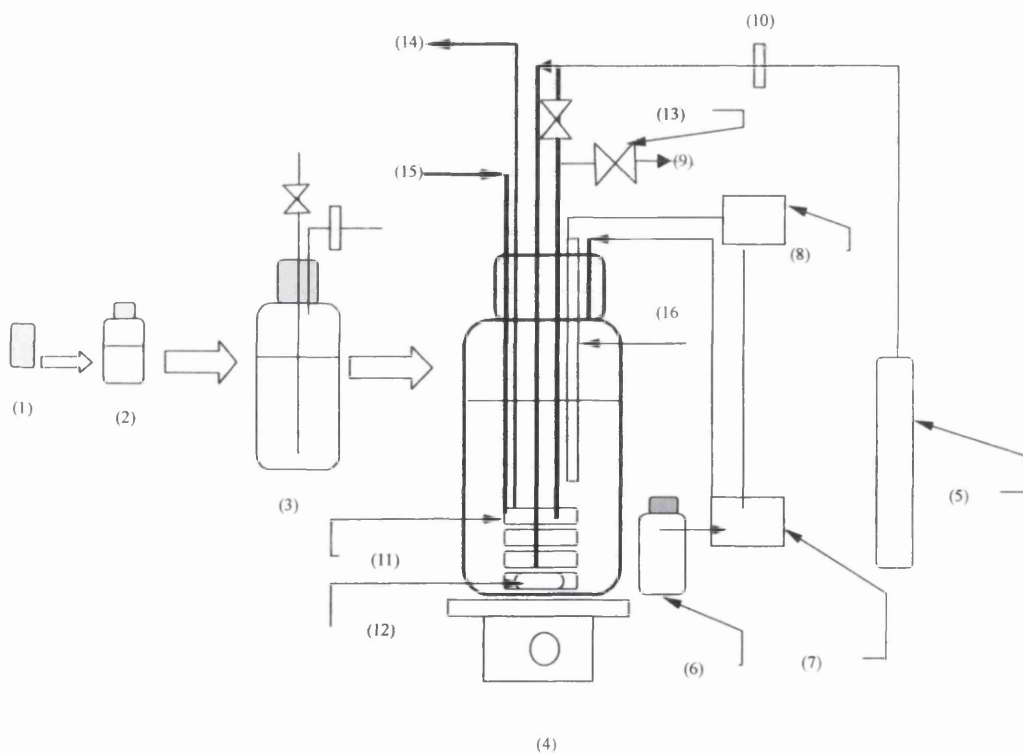
Fig.2.3 Diagram of the stirred tank reactor (STR) for the batch culture of lactic acid bacteria. *(L) bacterial stock (0.2 ml), (2) the activation (10 ml) of the bacterial stock in the serum vial (50 ml), (3) the pre-culture (150 ml) in anoxic starter culture bottle (500 ml), (4) the main culture (3.0 L) in the glass vessel (5.0 L), (5) oxygen free nitrogen gas cylinder, (6) the glass bottle with NaOH (10M), (7) the peristaltic pump, (8) pH meter, (9) the sample port, (10) air filter, (11) heat exchanger, (12) magnetic bar, (13) on-off valve, (14) cooling water out, (15) cooling water in, (16) pH electrode.*

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The bioreactor had total 5.0 L operating volume incorporated with pH electrode, sampling port, sparger for continuous supply of nitrogen gas. The heat exchanger was used to maintain temperature, and that bioreactor was stirred with the magnetic bar coupled to magnetic stirrer unit.

The microbial growth in 5.0 L the batch culture showed quite different growth patterns from that in the bath cultures because of pH control. Faster growth rates and more cell mass are obtained. Moreover, all kinetics obtained during microbial growth in the STR is more realistic because growth is not influenced by changing pH at the end of the batch culture. All kinetics parameters such as the growth rate, product yield, end products and specific production rate and the stoichiometry were investigated through the cell cultivation in the pH-controlled STR.

2.5.2. Preparation of the starter culture in the STR.

The propagation and activation of the strain under investigation was carried out with MLM-1 medium *(Refer to section 2.3)*. The culture suspension (7.5 ml) collected from the starter culture was again inoculated into MLM-1 medium of 142.5 ml, and then grown as described above.

2.5.2.1. The growth of LAB and preparation of the starter culture medium.

The growth medium for the starter culture was prepared with tap water of 127.5 ml at the bottle of total 500 ml. All ingredients to make 150 ml of the growth medium were dissolved in 127.5 ml of tap water excluding carbohydrates. The growth medium of 127.5 ml was loaded into the starter culture bottle in anoxic with boiling.

The starter culture bottle was sterilized in the steam autoclave for 15 min.

Glucose solution (9 ml) and fructose solution (6 ml) of 50% (w/v) were mixed with MLM-1 of 127.5 ml just before inoculation. Initial pH was adjusted with NaOH (5M) or HCL (5M) just before autoclaving. The culture suspension (7.5 ml) activated in 10 ml serum vial were injected into 142.5 ml of the starter culture medium with sterilized disposable syringe.

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The starter cultures were incubated according to the conditions which the strains require as described above *(Refer section to 2.3)*.

2.5.2.2. Preparation of the starter culture bottle.

Aspirator bottle (500 ml) was constructed to grow LAB and make inoculation into the STR easy (Fig.2.4).

Fig.2.4. Starter culture bottles for inoculation into the STR. *One bottle was used for the growth of the starter culture and for inoculation, and another was used for the feed of concentrated carbohydrate solution into STR just before inoculation. All tubes were made of rubber to prevent the culture system from exposing to the air. Long stainless steel tube reaches the bottom of the bottle and it was connected with inoculating line of STR. Short stainless steel tube was connected with high pressure nitrogen gas cylinder via the air filter and the manometer. Nitrogen gas from gas cylinder pressurized the starter culture bottle and pushed the culture liquid into STR through long tubing. All LAB were grown at this bottle for the starter culture, and both lines were blocked with clips to protect the culture from the contamination for cultivation.*

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As shown at Fig.2.4, the bottles have two stainless tubes, the long one to reach the base of the bottle for inoculation and another short one entering the headspace. Short one was fitted with the air filter. This can be connected with nitrogen gas cylinder via the manometer in case of inoculation where the pressurized headspace will push the culture out through the long tube into 5.0 L the batch culture.

2.5.2.3. Inoculation into STR.

When inoculation was required, long silicone line of the starter culture bottle was connected with inoculating port on top of the STR, and short silicone line was connected with nitrogen gas cylinder via the manometer and the air filter. During the cultivation, all lines were closed with clips to protect it from contamination.

The starter culture bottle was pressurized with nitrogen gas via the short line, and that positive pressure pushed the culture liquid into the reactor via long silicone line and inoculating port. Gas pressure was controlled with the manometer for the safety.

2.5.3. Preparations for the microbial growth in the STR

In this section, all preparations essential to grow the microbes in the STR will be deserved, including calibration of pH electrode, preparation of the growth medium in the STR, how to sterilize the STR in the steam autoclave, and how to operate the STR during the cultivation. However, the composition of the growth medium will not be discussed because it was the same composition as that used in the batch culture *(Refer to section 2.4)*.

2.5.3.1. Construction and operation of STR.

The microbial growth was carried out in the reactor of total 5.0 L with the working volume of 3.0 L. That reactor was incorporated with sampling line, inoculating line, sparger line to continuously supply with nitrogen gas, the heat exchanger to control temperature, and alkaline feeding line for pH control. All connections were constructed with silicone lining except for sparger line connected with nitrogen gas cylinder because silicone lining can be exposed to the outside oxygen.

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In this section, equipment fitted in the reactor are discussed in detail, including the equipment to control pH, to supply with nitrogen gas, to take samples, and how to operate them in this system.

● *pH control and its operation.*

The equipment for pH control during the cultivation includes pH electrode, pH controller, digital balance, and NaOH (5M). The pH electrode is wired to pH controller via a connector, and pH controller has two peristaltic pumps, which pump with alkali and acid solutions. Silicone tubing is used to connect between alkaline tank and the reactor. In these cultures only the alkaline was used and solution was fed via the peristaltic pump.

● *Continuously supply with nitrogen gas to maintain anoxic condition.*

All tubes to supply with nitrogen gas were constructed with butyl tube. Silicone tube was not used because the risk of the exposure to the outside oxygen. A sparger was inserted into the reactor and the reactor was connected with high pressure nitrogen gas cylinder via the pressure valve and the air filter. The air filter continuously filtered the nitrogen gas and the pressure valve was used to control the pressure of nitrogen gas. Nitrogen gas was fed in the rates of 0.167 vvm / L during the microbial growth.

● *The equipment for sampling.*

The butyl tube carrying with nitrogen gas was split into two streams, which comprise of sampling line and sparger line. Nitrogen gas was supplied through the route toward sparger via the air filter from nitrogen gas cylinder. Whenever sampling is required, the flow of nitrogen gas was switched toward sampling line to push out the culture liquid in sampling line for a while. After making sure all liquid is out of sampling line, a sample was taken with 10 ml syringe. Once the sample was taken, the nitrogen gas was switched back to the sparger line (Fig.2.3).

Fig.2.5. Photograph of the stirred tank reactor (STR) used to grow LAB. *The bioreactor of total volume 5 L was agitated with magnetic bar on hotplate, and agitation speed was controlled by hotplate. Alkaline solution (5M NaOH) for pH control was reserved in the plastic bottle and its weight was measured with digital balance. Alkaline consumption rate was measured with reducing rate of the weight. Alkaline solution was fed with peristaltic pump controlled by pH controller. Temperature was controlled with the heat exchanger connected with the water bath. Another thermometer was fitted into the culture liquid was used to measure real temperature of the culture liquid.*

• *The heat exchanger to control temperature.*

The fermentation requires the heat during the cultivation. Incubation temperature was above the ambient air. Heat was therefore needed and then was supplied via a heat exchanger. The heat exchanger was made of spiral types of glass tube. Both ends of glass tube are connected

Chapter.2 Materials and Methods

with the water bath, and the spiral part was inserted into the reactor with sealing from the outside. That water bath can forcibly circulate the water between the water bath and the reactor. Since temperature of water bath can be automatically controlled with thermo-indicator & controller (TIC), temperature in the reactor can be controlled at constant level as temperature of the water bath (Fig.2.3 & 2.5).

● *The equipment for agitation.*

In general, the growth of anaerobes does not need much agitation because of the risk of aeration. In spite of aeration, gaseous end product inhibition was possible with CO_2 and hydrogen. Therefore, it was important to disperse gaseous inhibitory substances. The culture liquid was also stirred to prevent the growing cells from settling down and to allow heat transfer. The reactor was set with the magnetic bar on magnetic stirrer unit.

2.5.3.2. Calibration of pH electrode.

The pH electrode was purchased from Fisher Scientific Ltd.. When pH electrode was purchased, it was calibrated with two factors of the slope adjustment (pH 4.0) and the zero point adjustment (pH 7.0), but once it was autoclaved, it was calibrated with only zero point adjustment just before autoclaving.

At first, the pH electrode was put into pH 4 standard buffer solution (Fisher Scientific Ltd.), and difference between displayed digital and pH 4 standard buffer solution was adjusted with the slope adjustment. In the next, after washing with distilled water, the pH electrode was put into pH 7 standard buffer to readjust the zero point. After adjusting slope and zero point with pH 4 and pH 7 standard buffer solutions, the pH electrode was then fitted into the STR.

2.5.3.3. Preparation of the growth medium in STR.

All ingredients to make 3.0 L growth medium were dissolved with tap water of 2.55 L with magnetically coupled stirring, excluding carbohydrate addition. Initial pH was adjusted with NaOH (5M). The dissolved medium was added into the reactor, and pH electrode was fitted

on top. Sampling line, sparger line, and alkali line except for the vent line were sealed with clips to prevent the culture liquid from overflowing during autoclaving. The vent line was loosely sealed with cotton wool, and then all lines were wrapped with aluminum foil.

The reactor with the growth medium of 2.55 L was sterilized in the steam autoclave for 15 min *(Refer section to 2.3.4)*. After sterilization, the reactor was taken out of the autoclave, and connected straight away to nitrogen gas cylinder via the air filter at low pressure (2 psi) and allowed to cool.

After cooling, the reactor was connected with the water bath to control temperature and stirred with the magnetic bar. The pH electrode was connected with pH controller after cooling the reactor down to the culture temperature.

2.5.3.4. Inoculation into the STR.

When inoculation was required, the long tube of the starter culture bottle (Fig.2.4) was connected with inoculating port on top of the STR (Fig.2.3), and short tube was connected with nitrogen gas cylinder via the manometer and the air filter. During the cultivation, all lines were closed with clips to protect it from contamination.

The starter culture bottle was pressurized with nitrogen gas via the short line, and that positive pressure pushed the culture liquid into the reactor via long silicone line and inoculating port. Gas pressure was controlled with the manometer for the safety.

2.6. PREPARATION OF THE STARTER CULTURES AND MLF IN MEMBRANE CELL RECYCLE REACTOR (MCR).

The MCR was designed to grow lactic acid bacteria to high cell densities (Fig.2.6).

All information and knowledge obtained through the batch culture and the microbial growth in the STR were referred to grow the microbes in the MCR. End product inhibition detected in the batch and the STR culture can be released during growing lactic acid bacteria in the MCR even though not perfect.

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Below one outlined in the following, in this section, construction and design of the MCR for the growth of lactic acid bacteria and MLF of the cider; preparation of the growth medium and the cider; sampling and operation of the MCR; preparation of inoculums to be served into the MCR and inoculation; cleaning of the membrane and the bioreactor; and how to control the feeding rates of the growth medium.

2.6.1. Construction and design of MCR (Fig.2.6).

The MCR can be divided into three sections according to their function, which are the equipment associated with feed section, the reactor section, and the product separation and cell recycling section. Construction and design of the MCR will be separately discussed in this section.

2.6.1.1. The feed section.

The feed is supplied to the reactor as a membrane sterilized medium. The whole part to feed the growth medium and the cider into the bioreactor was constructed with the medium feed tank, the cider feed tank, the centrifugal pump, the diaphragm valve, and the pressure gauge at analogue type (Fig.2.6).

• *The medium feed tank (Fig.2.6-No.1).*

Feed tank of 100 L was made of Stainless Steel SUS 316 and connected with the feed membrane through the butterflyl valve C (Fig.2.7). The growth medium was drown from the bottom of the tank through the centrifugal pump and recycled back to the tank via the feed membrane. The feed loop and diaphragm valve incorporated with the heat exchanger and the heat exchanger was connected with the water bath automatically controlled in constant temperature. It comprises of a reservoir tank and membrane system to continuously sterilize the feeds, as growth medium or cider is fed to the bioreactor section.
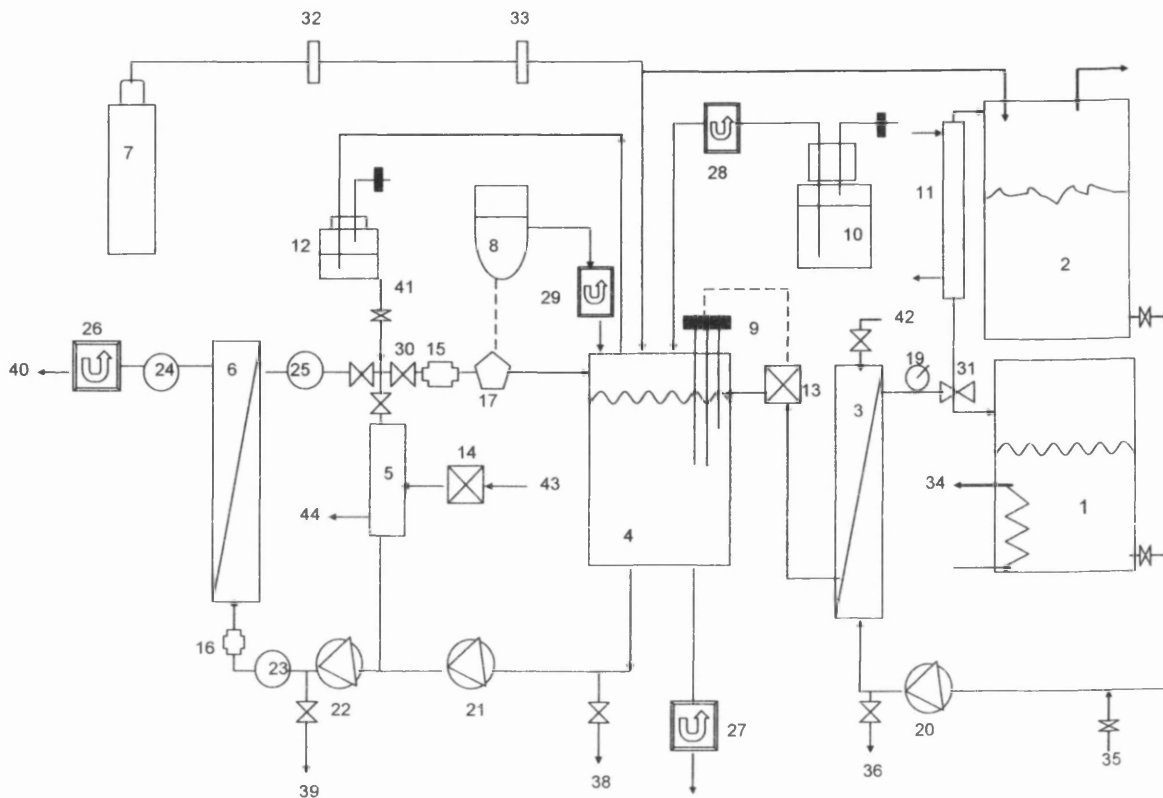
Fig.2.6 The diagram of the membrane cell-recycle bioreactor: *(1) Feed tank (100 L), (2) feed cider tank (400 L), (3) two feed membranes (total 0.4 m^2), (4) bioreactor (26 L), (5) heat exchanger, (6) product membrane (1 m^2), (7) nitrogen gas cylinder, (8) alkaline tank (6 L), (9) level indicator, (10) starter culture (20 L), (11) heat exchanger, (12) bubbling trap, (13) pinch valve, (14) solenoid valve, (15) flow meter for reactor/transmitter, (16) flow meter for recycling/transmitter, (17) load cell/transmitter, (18) pH indicator/transmitter, (19) pressure gauge, (20) centrifugal pump/PLC control, (21) magnetic pump for reactor fluid/PLC control, (22) magnetic pump for recycling fluid/PLC control , (23) pressure indicator 1/transmitter, (24) pressure indicator 3/transmitter, (25) pressure indicator 2 / transmitter, (26) production pump/PLC control, (27) bleeding and sampling pump/PLC control, (28) inoculating pump, (29) alkaline pump/PLC control , (30) diaphragm v/v to control the reactor flow, (31) diaphragm valve, (32) manometer, (33) air filter, (34) heat exchanger (35) original cider in, (36) drain out, (37) bleeding and sampling, (38) drain out of bioreactor, (39) drain out of production membrane, (40) production out, (41,42) steam in (43,44) cooling water in & out. Total volume of the fluid to circulate through the system was 36 L including the volumes of bioreactor and pipe-lines.*

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• *Connection with the cider feed tank (Fig.2.6-No.2).*

The cider feed tank was made of Stainless Steel SUS 316 and has the maximum capacity of 400 L. It has total four ports, which are three ports for the gas- in and -out, and recycling-in of the fluid on top, and one port for recycling-out of the fluid in bottom. The cider tank was connected with the feed membrane through valve A as shown at Fig.2.7.
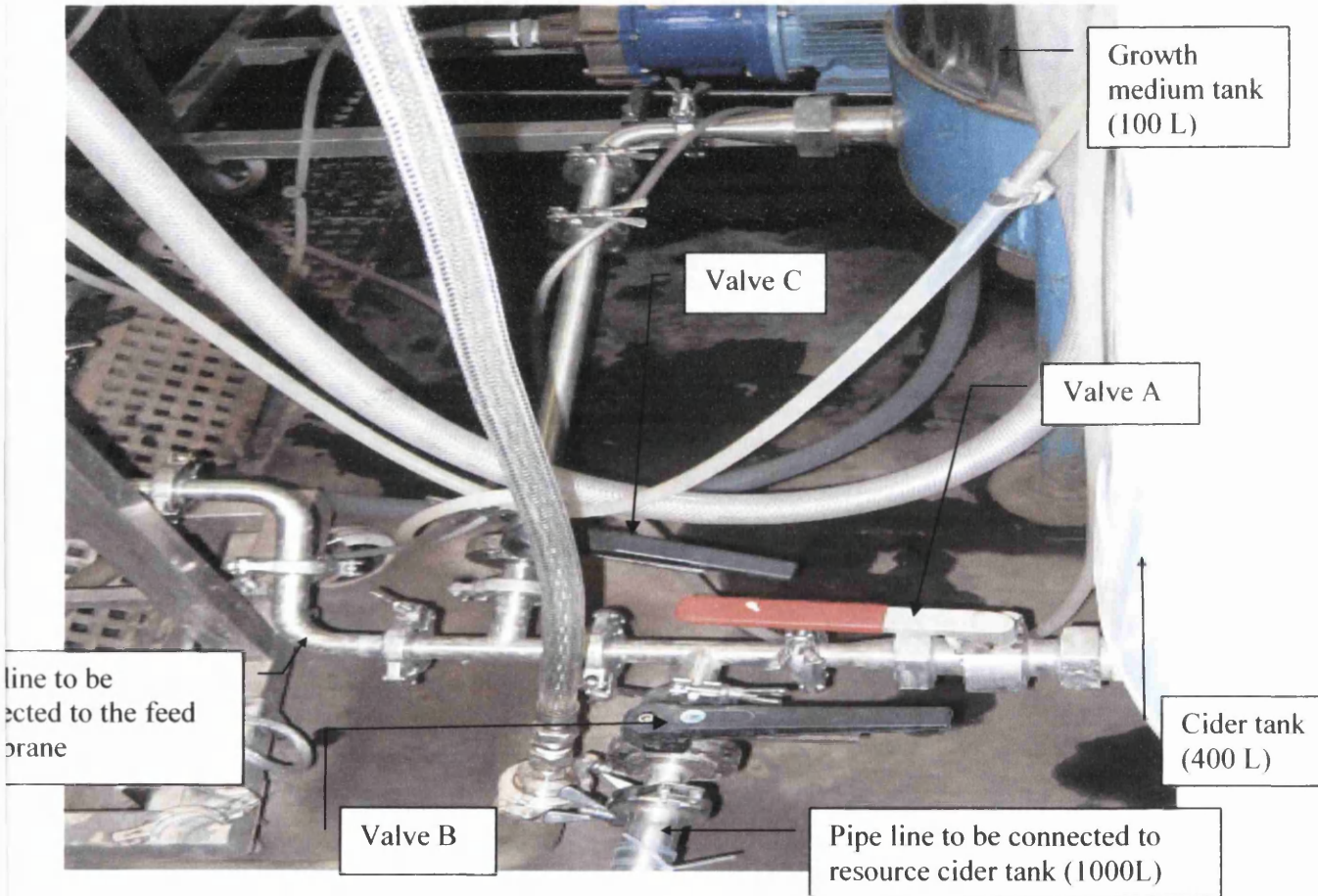
Fig.2.7. Photograph to display the arrangement and the joints of each of the feed tanks.
- *Valve A was fitted in the front of the feed cider tank and used to control recycling fluid of the cider.*
- *Valve B was fitted in the front of the resource cider tank, and used to control supply of the resource cider.*
- *Valve C was fitted in the front of the feed tank, and used to control recycling the growth medium or to formulate the feed cider.*

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• *Connection with the resource cider tank (Fig.2.6.-No.35).*

The original cider in the plastic reservoir (1 m^3) was supplied from Bulmers Ltd (Scotish Newcastle) in Hereford, England. The plastic reservoir was connected with the feed membrane through the valve B as shown at Fig.2.7. The connection was tightly jointed with the clamp and the O-ring, and Tygon tubing of 38 mm diameter. Cider could be transferred via this way from the reservoir to the feed tank.

• *Connection with the heat exchanger (Fig.2.6.-No.11).*

Temperature of the feed cider increased while circulating the feed cider at feed section. Since increase of temperature can affect the cider flavour, the heat exchanger should be fitted to cool the circulating cider down. The heat exchanger was fitted on the recycling stream after the feed membrane, and the cooling water was separately circulated through the water jacket of the heat exchanger. Therefore, temperature of the cider in the feed cider tank could be maintained below 18 °C during the circulation of the cider.

• *The feed membrane (Fig.2.6-No.3).*

Two feed membrane were made of the two ceramic modules with total filtering area of 0.4 m^2. The pore size was 0.2 μm in average diameter. The membranes were in two stainless Steel SUS 316 modules. The pipes, clamps and seals were supplied by PCI-Memtech Ltd. in UK. Two permeates from the two membrane modules were combined using Y-shape plastic tube and fed into the bioreactor via pinch valve.

• *The centrifugal pump (Fig.2.6.-No.20).*

Centrifugal pump in the feed section (Stuart Turner pump, Italy) was controlled with main control panel. It was fitted in the bottom of the feed membrane and used for recycling the fluid in the feed sector.

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- *The flow control valve (Fig.2.6.-No.31).*

The flow control valve (Diaphragm valve) was fitted between the feed tank and the pressure gauge, the recycling stream from the feed membrane to the feed tank, and used to control the flow rate and the pressure of the feed membrane.

- *The butterfly valves (Fig.2.6.-☒) for feeding medium or cider.*

Total five butterfly valves were fitted in the feed section.

Valve 1 (Fig.2.6.-No.42) was fitted in the steam inlet of the feed part to be connected with the steam line, and used to turn on / off the steam in just case of the sterilization.

Valve 2 (Fig.2.6.-No.36) was fitted in the drain line, and used to drain out the growth medium in the medium feed tank in just case of cleaning.

Valve 3 (Fig.2.6.-No. 35) was fitted in the inlet of the original cider and used for supply of the cider from the cider tank brought down from Bulmers Ltd.

Valve 4 was fitted in the bottom of the medium feed tank, and used as the gate for recycling –out of the medium from the medium feed tank to the feed membrane.

Valve 5 was fitted in the bottom of the cider feed tank, and used as the gate for recycling-out of the diluted cider from the cider feed tank to the feed membrane.

- *Pinch valve (Fig.2.6.-No.13).*

The pinch valve was fitted on the sterile permeate of the feed membrane to the inlet of the bioreactor and was used to control the feeding rate. The pinch valve was under control of the level detector in the bioreactor.

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• *Pressure gauge (Fig.2.6.-No.19).*

It was the gauge of the oil filled type displayed in a range from 0 bar to 6 bar. The pressure of the fluid flowing through the pipe was transported by oil and displayed on the gauge.

• *Piping components (Fig.2.8).*

All pipes were constructed with Stainless Steel SUS 316. The connection between the units was sealed with autoclavable O-ring, and tightly pressed for each other with the clamps for sealing.

• *Feed connection to the bioreactor.*

It was constructed with silicone tube (Dia; 8 mm). The silicone tube was connected with the inlet of the bioreactor via pinch valve controlled by level indicator.

Fig.2.8. Photograph of all stainless steel SUS 316 clamps
and pipes, and O-rings used to construct the MCR.

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2.6.1.2. The bioreactor section and inoculation procedure.

The bioreactor section were constructed 210 mm stainless steel pipe with top and bottom plate clamp, ports, the top and base plates, level detector, centrifugal pumps, two peristaltic pumps, PIC (pH indicator / controller), alkaline feed tank fitted with a load cell, TIC (Thermo-indicator/ controller), solenoid valve, diaphragm valve, four butterfly valves, and the flow sensor and heat exchanger.

A port of inoculation was constructed in the top plate and was manually controlled using a peristaltic pump, silicone tube and the plastic connectors. These sensors and control systems are via a PLC within the control panel.

• *The bioreactor (Fig.2.6.-No.4, Fig.2.9).*

The bioreactor was made of Stainless Steel SUS 316, and the capacity was 25 L. One sight glass was fitted in its side to check the fluid level. The bioreactor has five ports in the inlet, which are gas-in, feeding, recycling-in, inoculating, and level indicator, and four ports, which are gas-out, recycling-out, breeding and sampling, and draining. The ports for draining and recycling-out were constructed in the bottom of the bioreactor, and the rest of them were constructed in top of the bioreactor.

• *The level indicator (Fig.2.6.-No.9).*

The level indicator was fitted in top plate of the bioreactor. It senses the level of the fluid by conductivity. The electric signals from level indicator were transmitted to PLC to control pinch valve in the feeding line.

• *The magnetically coupled pump (Fig.2.6.-No.21).*

The main fluid circuit was driven by two magnetically coupled centrifugal pumps. They were Q-Max series pumps (Caster Pumps, Italy). They were used, via control converters, to regulate the fluid loop for membrane loop and recycle from the bottom of the bioreactor. This means that there are no seals within the pumps so avoiding possible contamination.

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The reactor sector allows for mixing and control of feed entry, gas exchange, environmental control (alkaline addition), sampling, and inoculation etc.

• *Peristaltic pumps and tubing (Fig.2.6.-No.27 &29).*

Two peristaltic pumps (type 323u) were fitted to feed alkaline solution, and bleed the culture liquid out when required. They were supplied by Watson Marlow Ltd..
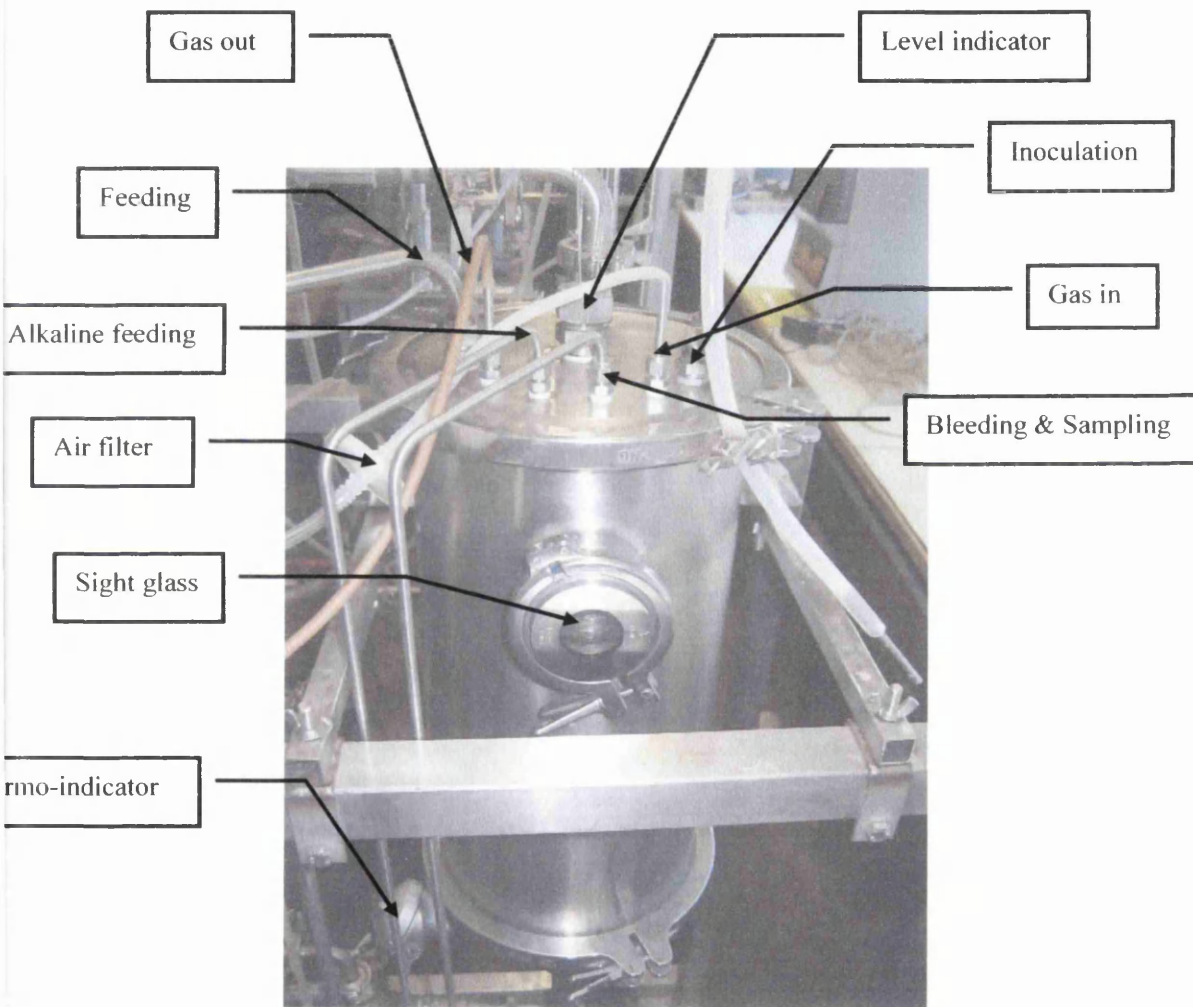
Fig.2.9. Photograph of the bioreactor and top plate. *The reactor was made of stainless steel SUS 316, and the capacity was 25 L. It was fitted with one sight glass in its side to check the fluid level. It has five ports in the inlet, which are gas-in, feeding, recycling-in, inoculating, and level indicator, and other five ports, which are gas-out, recycling-out, bleeding, sampling, and draining.*

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The peristaltic pump connected with PLC to control the feed of alkaline solution was fitted with silicone tubing between alkaline tank and the bioreactor, and another was fitted in the bottom of the bioreactor for cell bleeding and sampling.

Silicone tubing (Dia; 8 mm) was used to transport alkaline solution and the fluids in the bleeding and the sampling.

● *PLC / transmitter (Fig.2.6.-No.18).*

The pH electrode was purchased from Fisher Scientific Ltd in U.K, and the length of the pH electrode was 20 cm to be fitted in the recycling stream to the bioreactor from the production membrane or the heat exchanger. It was autoclavable. The controller was purchased from AWE Instruments and the model number was 3630.

The controller was fitted in PLC (Fig.2.10). The signals detected by the pH electrode were transmitted to the controller in PLC and displayed. It was used to control on/off of the peristaltic pump for feeding of alkaline solution.

● *TIC / transmitter (Fig.2.6.-No.45).*

Thermo-sensor fitted in the bottom of the bioreactor was used to sense temperature of the culture fluid. The signals detected by thermo-sensor were transmitted to the controller in PLC and displayed. The controller (DR 500C) was supplied by Piodem (Fig.2.10).

The signal was used to control on/off of solenoid valve (Fig.2.6.-No.14), which regulates the cooling water to pass through the water jacket of the heat exchanger (Fig.2.6.-No.5).

● *Alkaline tank and a load cell / transmitter (Fig.2.6.-No.8 &17).*

Alkaline tank of total volume 6 l was made of the plastic, and hung up to the load cell fitted in the frame of the MCR. The weight of the alkaline tank was transmitted to PLC and displayed.

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• *Solenoid valve for heat exchanger (Fig.2.6.-No.14).*

The solenoid valve (type 6213 A) was supplied from Burkert in Germany. It was fitted in the water jacket of the heat exchanger. Solenoid valve regulated the flow of the cooling water, which passes through the water jacket. Solenoid valve was controlled by the thermo-sensor via thermo-controller in PLC.

Data logger (not used)

PLC

PIC

Motor inverter controller

TIC

On/off switch
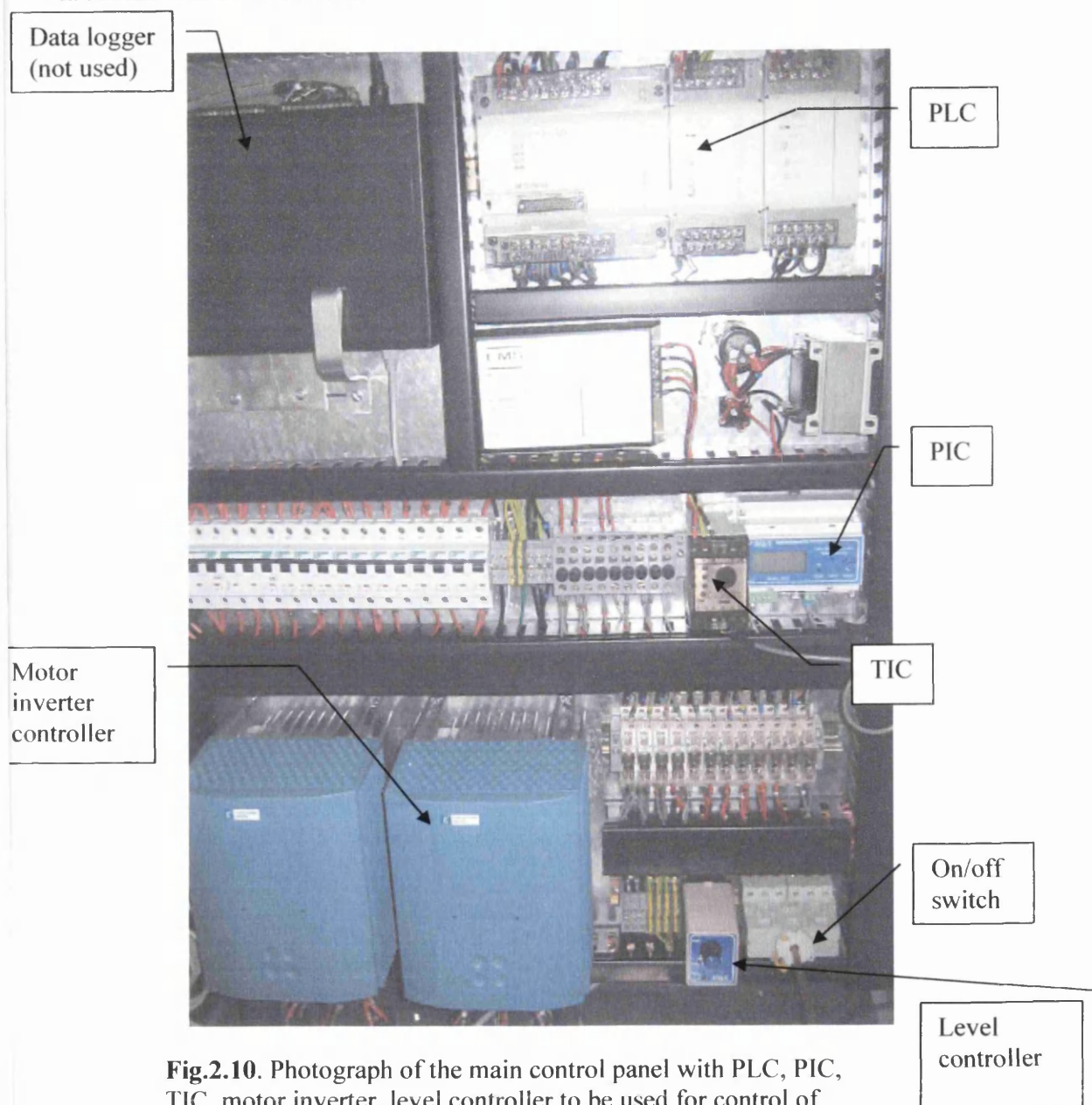
Level controller

Fig.2.10. Photograph of the main control panel with PLC, PIC, TIC, motor inverter, level controller to be used for control of MCR.

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- *Diaphragm valve (Fig.2.6.-No.30).*

The diaphragm valve was the same as described in the feed section of the system. *(Refer section to 2.6.1.1).*

   The valve was fitted between the flow meter and the heat exchanger, and it regulated the flow of the recycled culture fluid and pressure to the bioreactor from the membrane loop.

- *Butterfly valve (Fig.2.6.-No.38) for the bioreactor.*

There is one butterfly valve in the bioreactor sector. The butterfly valve is for draining out of the culture fluid in the bioreactor. It was fitted in the bottom of the bioreactor and used to drain the fluid out for cleaning.

- *The flow sensor / transmitter (Fig.2.6.-No.15).*

The flow sensor (Model No. 8030) was supplied by Burkert, Germany. It was fitted between the diaphragm valve and the bioreactor, and used to measure the flow rate of recycling stream to the bioreactor from the product membrane loop and the heat exchanger. It transmitted a signal to PLC for display.

- *Inoculation port (Fig.2.6.-No.10 & 28).*

The bioreactor was connected with the starter cultures (3 X 20 L) through silicone tubing (Dia; 8 mm) to inoculation port. The flow rate was manually controlled by the same peristaltic pump (Waston Malow Model No 505u). It was disconnected during the cultivation.

2.6.1.3. The product and separation section.

The separation loop were constructed with the heat exchanger, the production membrane, centrifugal pump, three pressure sensor, a flow sensor, the five butterfly valves, and a peristaltic pump. The product permeate flow from the membrane is controlled by a peristaltic

pump. The valve allows the control of steam flow during sterilization. The permeate flow sets the feed rate via the level control in the reactor section.

• *The heat exchanger (Fig.2.6.-No.5).*

The heat exchanger made of Stainless Steel SUS 316 is a concentric tube water jacket and was fitted in the separation loop. The inner part of heat exchanger was completely isolated with the outer part, which is the water jacket. Therefore, the culture fluid passes through the inner part and the cooling water passed through the outer part at the same time. The flow of cooling water was controlled by a solenoid valve so controlling the temperature.

• *The membrane modules (Fig.2.6.-No.6).*

The product membrane supplied by Memtech Ltd. in U.K was a Membrolox membrane module and was fitted in the production loop. The membrane was made of the ceramic and it was covered with the housing made of Stainless Steel SUS 316. The pore size was 0.2 µm in average diameter and total filtering area was 1 m^2. The module was fitted with 3 ceramic filters with 3 mm channels in each.

• *The magnetically coupled pump (Fig.2.6.-No.22).*

The same type of magnetically coupled pump as mentioned in the bioreactor section *(Refer to 2.6.1.2)* was fitted in the production section and used to control the flow of the culture fluid heading to the membrane, which was called the recycling flow.

• *Three pressure sensors / transmitters (Fig.2.6.-No.23, 24, & 25).*

Three pressure sensors were fitted in the production membrane to check the degree of the fouling on the membrane. Pressure sensor 1 (Fig.2.6-No.23) was fitted in the inward flow heading to the production membrane and pressure sensor 3 (Fig.2.6.-No.24) was fitted in permeate coming out from the production membrane. Therefore, pressure sensor 3 was to check the pressure in the production line and the P1 – P3 gives ΔP across the membrane.

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P1 – P2 gives the $\triangle P$ within the membrane. Pressure sensor 2 (Fig.2.6-No.25) was fitted in the outward flow heading to the bioreactor from the production membrane. Differences between P1 and P3 give some idea about the extent of the fouling on the production membrane.

• *The flow sensor (Fig.2.6.-No.16).*

The same flow meter as mentioned in the bioreactor part *(Refer to 2-6-1-2)* was fitted in the production part to check the flow rate in the recycling stream. The signal created in this sensor was transmitted to PLC and displayed in the control panel.

• *The five butterfly valves for production and separation.*

There were five butterfly valves.

Valve 1 was fitted in the next to the flow meter. It stayed in open during the cultivation, but it was used to give the bioreactor or the heat exchanger more steam in only the case of the sterilization.

Valve 2 (Fig.2.6.-No.39) was placed at the bottom for draining out of the fluid in the production membrane during cleaning.

Valve 3 was fitted in the production line to control the flow rate in the production line. It was often closed to regenerate the production membrane in case of a serious fouling.

Valve 4 was fitted in the next to the heat exchanger, and used to give the production membrane more steam during the sterilization. It stayed in open during the cultivation.

Valve 5 (Fig.2.6.-No.41) was fitted in the inlet of the steam, and used to be connected with the steam line.

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At first, the frozen (-70 C°) microbial stock vial, which contains the microbe suspension (>10 ^{12} cfu/ml) was thawed, and used to inoculate into the pressure tube (10 ml). After incubation for 24 h, the starter culture was inoculated into 100 ml the growth medium in 150 ml the serum vial.

This inoculum was used to inoculate into 1.0 L the starter culture bottle. After a further 24 h this was used to inoculate 10 L growth medium. Finally after a further 24 h 10 L starter culture was divided into three inoculums of 2.0 L and these were inoculated into 20 L three starter cultures.

The starter cultures smaller than 10 L were incubated in the water bath controlled in constant temperature, and the starter cultures of 10 L and 20 L were incubated in the constant temperature room depending on the culture condition of the strains *(Refer to 2.3)*. All cultures were stirred with the magnetic bar on stirrer during the cultivation.

2.6.2.2. Preparation of the growth medium and dilution of the original cider.

• *Preparation of the growth medium.*

All media used for the growth of LAB were the same as mentioned at section 2.4.

All ingredients to make 50 L were dissolved in 10 L of tap water, and then pH was adjusted with NaOH (10M) or HCl (10M) to optimum pH. Concentrated growth medium of 10 L was diluted to 50 L with tap water in the feed tank.

The centrifugal pump was used to circulate the fluid through the membrane for mixing. The growth medium was sterilized with the membrane filtration during the operation of the MCR.

• *Dilution of the original cider.*

The original liquid of 1000 L in the plastic tank was brought from Bulmer Ltd in Hereford, England, U.K. The original cider contained alcohol of almost 13% (w/v) and was diluted with tap water in the feed cider tank to 9% (w/v) in the most cases.

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The following procedure was adopted for dilution. At first, the Valve A, B, and C were closed, and tap water of a certain volume was loaded in the feed tank of 100 L (Fig.2.7). Only Valve C was opened, and then tap water in the feed tank was transported to the feed cider tank through the feed membrane by the centrifugal pump. Next, Valve C was closed and Valve B was then opened. The original cider in the resource cider tank was transported to the feed cider tank through the feed membrane by the centrifugal pump. The diluted cider in the feed cider tank was recycled through the feed membrane to mix for a few minutes.

The alcohol concentration was adjusted in a range from 9% (w/v) to 13% (w/v), and the original cider was used without any dilution in case to investigate the malolactic efficiency in the alcohol concentration of 13% (w/v).

2.6.2.3. Feed of the growth medium and the diluted cider into MCR from the feed reservoirs.

• Feeding of the growth medium.

The growth medium formulated with tap water of 50 L in the feed tank was sterilized by filtering through the feed membrane instead. Valve A & B were closed and the only Valve C was opened (Fig.2.7). The growth medium was forcibly recycled from the bottom of the medium feed tank via Valve C, the centrifugal pump, and the feed membrane to the top of the medium feed tank again. Permeate was fed from the feed parts into the bioreactor via pinch valve and the filtrate was recycled to the feed tank.

The feed rate was controlled by the filtration rate of the product membrane. Using a level sensor, a pinch valve regulated the feed flow into the reactor. Therefore flow through the reactor was normally controlled by the product filtration pump (Fig 2.6).

The carbohydrate concentration in the reactor was checked with HPLC in every 3 h to keep up the least glucose concentration during the cultivation. If the level dropped significantly the product feed was increased to allow the growth medium feed to the reactor.

The pressure in the feed membrane was indicated in the pressure gauge and that pressure was regulated by the diaphragm valve (Fig.2.6.-No.31).

The pressure was controlled at about 1 bar during feeding the growth medium. Since the medium feed tank was not sealed, the contamination in the tank was a considerable problem.

This was avoided by frequently changing with the fresh medium, and temperature in the feed tank was shifted up and down from 15 °C to 60 °C with the heat exchanger controlled by the water bath. That was to reduce the chance that the contaminants can grow in the medium feed tank and decrease the membrane flux of the feed allowing faster filtration.

● *Feeding of the diluted cider.*

The diluted cider was recycled through the feed membrane by the centrifugal pump. Valve B & C were closed and only Valve A was opened (Fig.2.7). The pressure was controlled at 0.5 bar to keep membrane flux high. The feed rate was automatically controlled by pinch valve under control of the level sensor in the bioreactor.

The cider feed rate was controlled at the retention time of 12 h so as to allow a steady increase of the alcohol concentration giving a period of adaptation of the strains against high alcohol concentration..

Usually, the cell mass declined for the first period but then stabilized and increased. When the optical density did not increase further, the feed rate was stepwise increased in terms of the retention time of 12 h, 9 h, 6 h, and 3 h.

2.6.2.4. Inoculation of the starter culture into the MCR.

The starter culture of total 60 L was prepared in three 20 L bottles (Fig.2.11), and inoculated into the bioreactor using manually controlled the peristaltic pump. Once inoculation of the first starter culture of 20 L was completed, the fluid was recycled through whole system with two magnetic pumps (Fig.2.6.-No.21, & 22).

The pH was controlled by PIC and temperature was controlled by TIC during inoculation.

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NaOH solution of 2M was fed from the alkaline tank through peristaltic pump under PIC control into the bioreactor whenever pH dropped down under control level. Production through the production membrane was commenced controlling the speed with peristaltic pump (Fig.2.6.-No.26) under PLC control.

This peristaltic pump connected with the product membrane was handled with care because it could lead to serious fouling. The manipulation of the membrane pressure was controlled using the main control panel. The pressures for P1 and P2 were controlled at about 30 psi and at about 15 psi, respectively.

The starter culture fluid was fed in the rate of 300 ml/min and production rate was controlled to prevent the culture liquid from overflowing. When the feed of the starter culture fluid was completed, it was changed with the fresh medium of 36 L, which is the volume of the MCR. It was to reduce the concentration of inhibitory substances and to supply with more nutrients to the MCR at the beginning of the growth.

2.6.2.5. The growth of LAB in the MCR.

• *Control of the flow rate and the pressure.*

The culture liquid was recycled in the whole system. Recycling speed was controlled with two magnetic coupled centrifugal pumps. Pump 21 controlled the flow rate of the fluid from the bioreactor to the production membrane and the heat exchanger, and Pump 22 controlled the flow rate of the fluid heading to the production membrane from Pump21. Therefore, when Pump 21 and 22 were speeded up, the production membrane was pressurized and the filtration rate increased (Fig.2.6).

Diaphragm valve 30 was fitted in the next to the production membrane, and used to control the flow rate of the culture fluid recycled to the bioreactor and simultaneously the pressure loaded on the production membrane. Therefore, when diaphragm valve 30 was closed, the flow rate of the fluid in the bioreactor part reduced and the pressure (sensor 23 and 25) increased.

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The flow rate in the product line was controlled with the peristaltic pump (Fig.2.6.-No.26) and this sets the liquid recycling time in the whole system.

- *Saturation of the bioreactor and the cider feed tank with nitrogen gas.*

The bioreactor and the cider feed tank were purged with nitrogen gas from a nitrogen gas cylinder via the gas filter (Fig.2.6-No.33) and a manometer (Fig.2.6-No.32). The gas flow rate was measured with the manometer. Excess gases from the bioreactor and the cider feed tank were naturally ventilated through the bubbling trap (Fig.2.6-No.12), unit was fitted at the end of ventilating line to visually check the smooth flow of nitrogen gas.

- *Control of pH.*

Alkaline solution (2M NaOH) was fed into the bioreactor whenever pH dropped below the control level. The feed rate of alkaline solution was regulated by the peristaltic pump (Fig.2.6-No.29). The pumping rate of alkaline solution increased at high cell density as the reactor become more active. The consumption rate of alkaline was measured by load cell and monitored. This gave an excellent increase of malolactic activity to the culture.

- *Control of temperature.*

A great deal of heat was generated with the reactor, mainly from the pumps and the actively growing culture. Temperature was measured by thermo-sensor fitted in the bioreactor, which was displayed on the control panel via PLC and the signals were used to control a solenoid valve fitted to the water feed jacket of the heat exchanger.

- *Control of the fluid level in the bioreactor.*

The feed rate of the growth medium was controlled with the level sensor and pinch valve (Fig.2.6). The level sensor was based on conductivity using three wires. Once the circuit is closed, the feed was resumed via valve activation.

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• *Sampling, bleeding, and draining.*

The sampling and the bleeding were taken through the peristaltic pump (Fig.2.6.-No.27) under PLC control and the cell mass was measured with spectrophotometer.

Also, the culture fluid was drained out through butterfly valves located in the bottom of the bioreactor (Fig.2.6.-No.38 & 39) for cleaning. Usually, drain valve was used to drain the bioreactor out or to drain the condensed water out during the sterilization.

2.6.2.6. Control of the retention time.

The retention time is a very important variable either in the microbial growth or the cider maturation.

The continuous feed of the growth medium for the microbial growth means either to continuously supply with a new nutrients to the growing cells or to continuously remove the inhibitory substances. Even if there is no drawback like washing out shown in high dilution rate of the conventional continuous culture, the overfeeding of the nutrients can be wasteful and unreasonable on economic. Therefore, the dilution rate should be controlled depending on the consumption rate of carbohydrates. The carbohydrate concentration was monitored in every three hours with HPLC to control carbohydrate at the least concentration during the cultivation. The feed rate of the cider during the cider maturation plays a crucial role to determine the flavour and acidity of the cider after maturation. Therefore, the retention time was significant objective to investigate in either the microbial growth or the cider maturation. What increases the speed of permeate coming out of the production membrane resulted in increase on the feed rate of the fluid served from the feed part to the bioreactor.

Generally, the maximum feed rate was 15 L/h., which means the bioreactor is fully changed in every 2.4 h. Therefore, the microbial growth at the beginning of the cultivation was controlled with 4.8 L/h. of the feed rate, and it was increased step by step depending on the consumption rate of the carbohydrate to the maximum feed rate.

2.6.2.7. Sterilization of the MCR.

The MCR was separately sterilized with two parts, which are the feed part, and the bioreactor and the production part, because two parts was mechanically separated.

- _The sterilization of the feed section._

The feed preparation mainly consisted of the medium feed tank, the cider feed tank, and the feed membrane. The sterilization of the feed section was mainly performed through all pipes and the feed membrane. The steam was supplied in a pressure range from 3 bar to 5 bar by the main boiler and was controlled under 0.5 bar for sterilization. The steam was supplied into the line through ball valve (Fig.2.6.-No.42), and then split out into two ways, which are one toward bottom of the feed tanks via the feed membrane and the centrifugal pump, and another toward top of the feed tank via the pressure gauge and the diaphragm valve.

The steam was vented through the silicon tube permeate of the feed membrane and through the draining valve (Fig.2.6.-No.36) at bottom of the feed tank. In the next, the silicon tube permeate of the feed membrane was loosely closed to give more steam to the feed tank. The condensed water in the line was removed through the drain valve. After increasing temperature by 100 °C, temperature was maintained at 100 °C for 20 min, passing the steam through whole lines of the feed part. The butterfly valves (Fig.2.6.-No.42, No.36) and the silicon tube were firmly closed and then the steam line was disconnected from the feed parts.

- _The sterilization of the bioreactor and production parts._

The steam was supplied through the butterfly valve (Fig.2.6.-No.41) into the whole system. At first, the way toward the heat exchanger and the diaphragm valve (Fig.2.6-No.30) were closed to give the production membrane more steam. At this point, the steam reached the magnetic pump (Fig.2.6-No.22) via the production membrane and some portion of the steam came out of the production membrane through permeate (Fig.2.6.-No.40). When the steam was full in the production membrane, it was confirmed to come out with the condensed water through the drain valve (Fig.2.6.-No.39).

At this point, the valve toward the heat exchanger was opened to pass the steam to another magnetic pump (Fig.2.6.-No.21) via the heat exchanger. When both lines through the production line and the heat exchanger were full of the steam, the steam was pushed toward the temperature sensor fitted in the bottom of the bioreactor.

When the temperature increased to over 100 °C, the diaphragm valve was opened to pass the steam to the bioreactor. At this point, the temperature instantly declined a bit due to the split of the steam. When the temperature increased again by 100 °C, all lines for the alkaline feed, the sampling and bleeding, gas-in and –out, and inoculation were opened to allow steaming out, convincing the steam vented through them for 15 min at least. After 15 min, all lines were closed. The temperature increased again by 100 °C, and was maintained at over 100 °C for 20 min at least.

After completion of the sterilization with the steam, all drain valves fitted in the bioreactor were closed, and supplying the steam was stopped. The nitrogen gas was quickly supplied to prevent the pressure of the whole system from dropping down. At last, the steam line was disconnected from the system and temperature controller was switched on. Finally, the ventilation of nitrogen gas and the positive pressure in the whole system was checked with bubbling in the bubble trap.

2.6.2.8. The membrane fouling.

The degree of membrane fouling was displayed by $\triangle P$ between P3 and P1. Since P1 indicates the pressure of the fluid in inlet of the product membrane and P3 is for the pressure of permeate of the product membrane, difference ($\triangle P$) between P3 and P1 indicates the degree of membrane fouling of the product membrane. First of all, the membrane fouling is affected by how to control each of valves and how to control the flow rate of the fluid passing through the whole system. Closing the diaphragm valve (Fig.2.6-No.30) increased the pressure and effectively increased the fouling in the production membrane. Speeding up the flow rate in permeate of the production membrane also increased the risk of the membrane fouling in the production membrane.

Two magnetically coupled pumps to control the flow rate of the culture fluid are the main causes of the membrane fouling. The flux through the production membrane slowly declined with increasing cell density indicating build-up of the cake on the membrane inner-surface. Consequently, the production membrane must be regenerated to increase again the flux. It is very important to maximize the efficiency of the MCR that keeping high dilution rate is deeply related to keeping high growth rate in high cell mass.

In order to regenerate the production membrane, the butterfly valve in permeate of the production membrane was firmly closed, and the recycling flow was speeded-up to the maximum to create the back pressure on the production membrane until the pressure of P3 increases to P1. When the pressure of P3 was equal to P1, the recycling flow was back to the normal speed. The butterfly valve in permeate of the production membrane was opened again to restart production with care. Once the fouling on the production membrane occurred, it was very hard to regenerate, apart from taking it off-line and cleaning.

2.6.2.9. Cleaning.

When the growth of LAB was completed, the culture fluid was drained out of the MCR through two drain valves and washed out divided into two parts, which are the feed part, and the bioreactor and the production part.

• *Cleaning of the feed sector.*

The growth medium in the feed tank and the lines were drained out through the butterfly valve (Fig.2.6.-No.36). The feed tank was washed out with tap water, and the feed membrane was washed with tap water, detergent, NaOH (0.1M), and citric acid (0.1M).

At first, the feed membrane was washed circulating hot water (50 ~ 60 °C) through the whole feed part. Impurities in the feed membrane came out with hot water and the rinsing water with impurities was drained out through the butterfly valve (Fig.2.6-No.36). Rinsing with tap water was repeatedly continued until rinsing water was clean.

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NaOH (0.1M) was made of hot water of 20 L in the feed tank. That alkaline solution was circulated through the feed membrane for 1 h. Both of permeate and filtrate were recycled to the medium feed tank. While circulating for 30 min, the colour of alkaline solution changed to the dark brown. Alkaline solution was drained out of the medium feed tank, and the feed membrane was repeatedly rinsed with hot water until pH of rinsed water dropped to the neutral pH.

When pH reached neutral, 20 L of a hot citric acid solution (0.1M) in the feed tank was circulated through the feed membrane for 1 h again. The colour of citric acid solution was changed to the dark brown again. After 1 h., citric acid solution was drained out from the medium feed tank, and the feed membrane was repeatedly rinsed with hot water until the pH increased up to neutral.

• *Washing of the bioreactor and the production sector.*

After completion of the culture, the broth was drained out through the two drain valves (Fig.2.6-No.38 and 39). The bioreactor and the production part were washed with hot water, NaOH (0.1M), and citric acid (0.1M). The bioreactor was reloaded with hot water and then circulated at the whole system without temperature control. The rinsing water was drained out again through the two drain valves. The rinsing was repeated until the colour of the rinsed water was clean.

NaOH solution (10M) of 360 ml was added into the bioreactor and straight way the bioreactor was reloaded with hot water diluting to 0.1M NaOH solution in the bioreactor. NaOH solution was circulated through the whole system for 1 h without temperature control. The colour of NaOH solution was changed to the dark brown and temperature increased to a range between 70 °C and 80°C. NaOH solution was drained out and rinsing of the whole system was repeated with tap water until pH of the rinsing water dropped to neutral.

Citric acid of 690g was dissolved with hot water of 36 L in the bioreactor and then the whole system was washed out with circulation for 1 h and rinsed according to the same procedure as the case of NaOH.

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After washing the whole system, the production membrane and the bioreactor were reserved in the clean water until the next use.

2.7. ANALYSES.

2.7.1. Measurement of cell growth.

The cell growth was monitored by the optical density in the wave length of 660 nm with the spectrophotometer (type Unicam 330) supplied by Unicam Co. Ltd..

During the measurement the cuvette with 1 cm path was fitted into spectrophotometer in the measurement cell while the reference was air. Optical density measured in spectrophotometer was converted to dry cell weight (g/L) using a calibration.

The samples were diluted with distilled water by 10 times in the case of batch culture and the microbial growth in the STR to give O.D below 2. The volumetric flask of 100 ml was usually used for the dilution. At first, the sample of 10 ml was loaded into the volumetric flask to volume up. After rigorously mixing, the diluted sample of 2 ml was loaded into the cuvette, and the optical density was read at the wave length of 660nm.

The samples were diluted by 100 times in case of the microbial growth in the MCR or the maturation of the cider in the MCR. The cultured sample of 5 ml was loaded into the volumetric flask of 500 ml and diluted with tap water.

• *The measurement of dry cell weight (DCW).*

The filter paper (Whatman No.2, Dia 5cm) was numbered and dried in the oven controlled at 80 °C for over night. Each of dried filter paper was weighed. And then 10 ml of cell suspension was taken and filtered through the filter paper. After filtering, the filter papers including cells were dried in the oven for over night again. In the next day dried filter papers and cells were weighed again. Difference in the weights of the former and the latter was recognized as DCW (g/L).

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All optical densities (O.D) obtained during cultivating were converted into DCW in basis of the standard curve established the relationship between DCW and O.D.

Therefore, equations to convert O.D into DCW concerning with species is shown in Table.2.1.

Strains	The relationship between optical density (660nm) and DCW ,g/L	Reference
L. buchneri	Y = 0.26 X – 0.01	Appen.2
L. brevis	Y = 0.4X + 0.13	Appen.4
B. longum	Y = 0.37X + 0.21	Appen.1
O. oeni	Y = 0.41X + 0.06	Appen.3

Table.2.1: The relationship between optical density and dry cell mass (g/L). *Optical densities obtained during the cultivation were converted into DCW (glL) by these equations.*

2.7.2. HPLC equipment for analyses of the organic acids and carbohydrates.

The LAB produced organic acids like lactic acid and acetic acid through the metabolism of carbohydrates during the growth. These organic acids and carbohydrates were analyzed with HPLC. In this section, how to analyze organic acids using HPLC, and how to prepare the samples for HPLC analysis will be discussed. Finally, the organic acid or carbohydrate concentration was estimated using the equations shown at Table.2.2.

2.7.2.1. Organic cid analysis.

Organic acids were measured by conductivity detector using HPLC fitted with Ion-exchange column.

● *The column for analyses of organic acids.*

This Varian HPLC system was fitted Ion Pac $^{®}$ ATC-1 trap column(9 X 2.4mm) located between the pump and the injection valve. Since this system used the gradient elution of NaOH, ATC-1 trap column was used to strip anionic contaminants such as carbonate in the hydroxide eluents.

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These contaminants often interfere with the precision of the analysis in trace level analyses [ATC-1 manual; Doc No. 032697-06].

IonPac ® ATC-1 (4 X 50mm) as the guard column was placed between the injection valve and the main column. This is to remove the insoluble in the sample and protect the main column from contamination.

Ion-Pac AS11 HC anion exchange column (4 X 250mm) as the main column was fitted in the heating chamber (Waters, Millipore) under control of Waters temperature control module and controlled in constant temperature. All columns were purchased from Dionex Corporation in USA.

• *Detector for HPLC analysis of organic acids*

ED 40 electrochemical detector was used wired to analysis cell DS3 Detector stabilizer (Model: DS3-1 from Dionex Corporation). This instrument was used to remove from chromatogram the noisy detectable in case using ED 40 electrochemical detector. ASRS-Ultra 4mm (P/N 53946), which is the pressure controller (Dionex Corporation in USA) was placed in the next to DS-3 Detector stabilizer, and used to protect the analysis cell from fracturing by the pressure transported from the HPLC pump. The output in case analyzing organic acids by ED 40 electrochemical detector was displayed by the conductivity in the mvoltage-based.

• *The injector.*

The samples were injected through the injection loop of 20 μl fitted in the injector with the HPLC syringe of 1 ml. That injector was purchased from Waters & Millipore Corporation in USA.

• *The solvent delivery system.*

The solvent delivery system of this HPLC system was constructed with two pumps purchased at Varian Pro-Star Corporation in Canada.

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The gradient module of NaOH was produced using two pumps in case to analyze organic acids, the isocratic module was produced using only one pump in case to analyze carbohydrates.

• *The tubing.*

The connections among all equipments were constructed with PEEK tubing, which is a polymer ideal for HPLC. That is high pressure rating and used as substitutes for the conventional stainless steel. The stainless tubing was not suitable for use with ED 40 electrochemical detector, because an ion affecting the interpretation of HPLC results can be released into the mobile phase from the stainless steel. The diameter of PEEK tubing was ID 0.25 mm.

• *Temperature*

Temperature of Ion-Pac AS11 HC anion exchange column the main column for organic acid analysis was controlled in 35 °C with the heating chamber purchased from Waters & Millipore Corporation. Temperature of Ion Pac $^®$ ATC-1 trap column and IonPac $^®$ ATC-1 (4 X 50mm) the guard column were not controlled.

• *Mobile phase of HPLC analysis for Organic acids.*

The HPLC analysis of organic acids was performed in the gradient module using NaOH solution from 0.5 mM to 50 mM. That gradient module was begun with NaOH solution of 0.5 mM and maintained at the same concentration for 5 min. At the next, NaOH concentration was increased from 0.5mM to 5.5mM for 10 min and then increased again from 5.5 mM to 50.5mM for 5 min. At the last, NaOH solution was kept up at 50.5 mM for 5 min and then dropped down to 0.5 mM for 3 min. Therefore, it took 28 min for one cycle of organic acids analysis.

The gradient module was established with two Varian HPLC pumps. The Pump A was used for the delivery of NaOH solution of 0.5 mM, and the Pump B was used for delivery of NaOH

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solution of 100 mM. The Pump B was opened for longer in higher concentration than in lower concentration to increase NaOH concentration under control of the program module proposed by Varian Corporation. The flow rate was controlled at 1.0 ml/min and the pressure was controlled in a range from 1500 psi to 1850 psi..

Lactic acid peak was appeared at the retention time between 13 min and 15 min just before acetic acid peak, and malic acid peak was appeared at the retention time between 23 min and 24 min.

● *Washing the columns for organic acid analysis.*

The assay columns were regularly washed once in a week.

Mainly, since the main columns were protected from contamination by the guard columns, the guard columns were washed.

When guard column was contaminated by insoluble in the sample, the operating pressure increased. Therefore, when the operating pressure increased over 1900 psi, the guard column was regenerated.

IonPac $^®$ ATC-1 guard column was separated from the main column, and washed in the opposite direction with NaOH solution 0.5 mM. The operating pressure declined during the washing, and when the operating pressure did not declined any more and was stabilized, the washing stopped and the guard column was fixed in the normal direction. The flow rate was 4 ml/min during the washing.

When Ion-Pac AS11 HC anion exchange column the main column was contaminated, the noisy on the chromatogram increased and disturbed the analysis of the chromatogram.

The main column was regenerated with NaOH of 50 mM until the conductivity dropping down under 8 mVolts. The flow rate during the washing of the main column was 1 ml/min.

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2.7.2.2. Carbohydrate analysis.

●. *HPLC columns for carbohydrate analysis.*

The trap column was not used for analysis of carbohydrates, and CarboPac ™ MA-1(4 X 50mm) as the guard column and CarboPac ™ MA-1 (4 X 250mm) as the main column were used. All columns were purchased from Dionex Corporation in USA.

● *Detector for HPLC analysis of carbohydrates.*

The analysis cell to measure INT Amperometry was wired to ED40 electrochemical detector, and the output was displayed in the mVolts-based.

● *Temperature*

Temperature of CarboPac ™ MA1 (4X50mm) the guard column and CarboPac ™ MA-1 (4 X 250mm) the main column for carbohydrate analysis was not controlled.

● *Mobile phase of HPLC analysis for carbohydrates.*

NaOH solution of 480 mM was used in isocratic mode for carbohydrate analysis. NaOH solution was delivered with only the pump A. The flow rate was controlled at 0.4 ml/min and the pressure was controlled in a range from 1500 psi to 1800 psi.

Ethyl alcohol was measured with gas chromatography (CP-3800 from Varian). All data obtained from HPLC and G.C was analyzed with pro-star workstation data analysis file supplied from Varian Co. in Canada. Standard curves were defined in a limited range showing a linear relationship between the concentration and indicator. The concentrations of all substances tried in this investigation were estimated through the first order equation obtained through standard curve.

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• *Washing the columns for carbohydrates analysis.*

When CarboPac ™ MA-1 (4 X 50mm) the guard column was contaminated, the operating pressure increased over 1900 psi. The guard column was washed with de-ionized water by the same procedure as the washing of IonPac ® ATC-1 guard column until the pressure drops down and stabilizes. The flow rate was maintained 4 ml/min during the washing. When CarboPac™ MA-1 (4 X 250mm) the main column was contaminated, the column was washed with de-ionized water. The flow rate was maintained at 0.3 ml/min for over night.

2.7.2.3. Analysis program.

All data obtained from HPLC was analyzed with pro-star workstation data analysis file supplied from Varian Co. in Canada. Generally, the concentration of the target substance was automatically estimated by pro-star workstation data analysis file in the basis of the main peak area appeared on the chromatogram.

2.7.3. Preparation of the samples and the standard curves.

• *Preparation of the standard curves.*

All standard curves were established in certain concentration range showing a linear relationship between the organic acid or carbohydrates concentration and the peak area on the HPLC chromatogram (Table.2.2). All references were serially diluted with distilled water as shown in table and injected. Here, τ means the average accurate as the standard deviation.

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Organic acids or Carbohydrates	The linear relationship between the substance concentration (mM) and the HPLC peak area.	Reference
Lactate	$Y = 314005 X + 6566.08$ ($\tau = 0.996$)	Appen.8
Acetate	$Y = 94949.64 X + 3573.26$ ($\tau = 0.999$)	Appen.7
Glucose	$Y = 93485.46 X + 5388.07$ ($\tau = 0.999$)	Appen.6
Fructose	$Y = 29998.77 X - 1099.45$ ($\tau = 0.998$)	Appen.5
Manitol	$Y = 160609 X + 26.95$ ($\tau = 0.999$)	Appen.9
Malate	$Y = 314269X - 1401.91$ ($\tau = 0.999$)	Appen.10

Table.2.2. The linear relationship between organic acids or carbohydrates concentrations and the peak area on HPLC chromatogram shown in certain range of the concentration.

• *Preparation of the samples for organic acid analysis.*

The samples taken during the microbial growth in STR were diluted with distilled water before injection into HPLC. Usually, the samples taken before the exponential growth period were diluted by 50 times, and the samples taken in the exponential growth period were diluted by 250 times. Finally, the samples taken after the exponential growth period were diluted by 500 times with distilled water *(Appendix 22, 24, 27, 29)*.

When the samples were injected in high concentration, the peaks for lactate and acetate were not separated. Therefore, the samples should be diluted to appropriate concentration for fine resolution.

The samples taken during the microbial growth in MCRB and during the cider maturation were diluted with distilled water by 400 times from the beginning of the cultivation to the end *(Appendix 30, 31, 32, 33, 34, 35, 36, 37, 38)*.

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• *Preparation of the samples for carbohydrate analysis.*

The samples for carbohydrate analysis were diluted by 20 times in all cases *(Appendix 20, 23, 25, 28)*.

2.7.4. Equipment and operation for the volatile compound analyses in Gas chromatography (G.C)

Ethyl alcohol produced during the microbial growth and the volatile compounds produced during MLF of the cider were analysed with G.C. All compounds were quantitatively checked in basis of the standard curves at Table.2.3. The methods will be discussed in this section.

2.7.4.1. Operation of Gas chromatography.

• *The stationary phase and equipment*

ChromPac Capillary Column (WCOT fused SILICA, 25M X 0.32 mm ID, Coating CO-WAX 57CB, DF = 0.2) from Varian in Canada was used for all volatile compound analyses.

• *The mobile phase.*

Oxygen free nitrogen gas and air was supplied for analysis, and all gases during the operation were supplied under control at 4.0 bar from gas cylinder. The flow / pressure of all gases in the capillary column were controlled at 10.0 psi.

• *Detector.*

FID was used as detector in all cases. Rear FID was controlled at 220°C for analysis of ethyl alcohol, and at 230°C for analysis of the volatile compounds.

• *Injector.*

This G.C. has two injectors, which are the front and the rear injector. The front injector was used for injection, and flamed with H_2 gas. H_2 gas was generated by Hydrogen gas generator

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purchased from Domnick hunter (Model: UHP-20H). The pressure was controlled at 6.5 psi. Temperature of the front injector was controlled at 200 °C.

● *Control of column temperature.*

- *Control of column temperature for ethyl alcohol analysis.*

Column oven temperature was controlled at 30°C for the first 2.0 min, and the column temperature was increased in the rate of 40 °C/min to 100 °C and held at 100°C for 0.5 min.

The column temperature was increased again in the rate of 80 °C/min to 200 °C and held at 200 °C for 1 min. Therefore, it took 6.50 min for one cycle of analysis. The column temperature was settled down to 30 °C for 6.50 min to get ready for the next analysis.

- *Control of the column temperature for the volatile compounds analysis.*

The column temperature was controlled at 30°C for the first 2.0 min, and increased in the rate of 2.0°C/min to 40°C and held at 40°C for 10 min. The column temperature was increased again in the rate of 4.0°C/min to 80°C and held at 80°C for 10 min.

The column temperature was increased again in the rate of 8.0°C/min to 200°C and held at 200°C for 15 min. Therefore, it took 67 min for one cycle of analysis.

- *Control of the column temperature for cleaning.*

The column temperature was kept up to 200°C for 60 min without any injection.

Cleaning of the column was conducted to settle down the noisy on the chromatogram just before the start of analysis.

Chapter.2 Materials and Methods

2.7.4.2. Preparation of the samples for gas chromatography.

● *Preparation of the standard samples.*

The standard samples of ethyl alcohol and the volatile compounds were prepared in the serum vial (50 ml) according to the head space method.

The distilled water of 10 ml was added into the serum vial, and the water of 1 ml was removed again from the serum vial. Ethyl alcohol of 1g was accurately added into the serum vial on the digital balance establishing the ethyl alcohol concentration of 10% (w/v) in the serum vial. That serum vial was sealed with the rubber stopper and the tear-off aluminium seal to prevent ventilation of the alcohol. That standard ethyl alcohol solution of 10% (w/v) was serially diluted in the same serum vials with the distilled water of 10 ml, and all serum vials were sealed.

Each of the serum vials was warmed for 10 min in the water bath controlled at 50°C just before the injection. Gas of 100 μl was taken off from the head space of the serum vial using the syringe specified to use in G.C., and injected through the front injection port into G.C column.

Each of the standard volatile compound solutions was prepared in the serum vials according to the same procedure. These standard solutions were serially diluted in the serum vials to the certain concentration range, and warmed in the water bath controlled at 50°C for 5 min just before injection. The sample of 20 μl was accurately taken off the head space of the serum vial, and injected through the front injection port into the G.C. column.

Ethyl alcohol and the volatile compounds showed a linear relationship between the ethyl alcohol or the volatile compound concentration in the certain range and the peak area on G.C. chromatogram (Table.2.3).

Volatile compounds	The linear relationship between the volatile compound concentration (mM) and the peak area on G.C. chromatogram.	Reference
Ethyl alcohol	$Y = 656.69X + 656$ ($\tau = 0.999$)	Appen.11
Acetaldehyde	$Y = 63283.75X - 2402.862$ ($\tau = 0.999$)	Appen.12
Ethylacetate	$Y = 478669.38\ X + 4395.61$ ($\tau = 0.999$)	Appen.13
Isoamyl alcohol	$Y = 94901.79\ X - 1691.488$ ($\tau = 0.999$)	Appen.14
Isoamyl acetate	$Y = 45701189.03\ X - 14310.51$ ($\tau = 0.999$)	Appen.15

Table.2.3. The linear relationship between the volatile compound concentration and the peak area on G.C. chromatogram. τ was the standard deviation and means the accuracy of analysis.

• *Preparation of the samples for ethyl alcohol analysis (Appendix 21, 26).*

The samples taken from the microbial growth in STR were used after the centrifugation and the samples of MCR were directly used without the centrifugation because the samples were permeate coming out of the production membrane.

The sample of 1 ml was mixed with the distilled water of 9 ml in the serum vial, and then warmed in the water bath of 50°C for 10 min. Gas of 100 μl was taken off from the head space of the serum vial using the syringe specified to use in G.C. analysis and injected through the front injection port into the G.C. column.

• *Preparation of the sample for the volatile compound analysis (Appendix39, 40, 41, 42, 43, 44).*

The volatile compound analysis was conducted with only the samples taken during MLF of the cider. Therefore, the centrifugation was not necessary because the samples were filtered through the production membrane.

Chapter.2 Materials and Methods

The samples were added into the serum vial without dilution and sealed. That sample was warmed in the water bath of 50°C for 5 min and the sample of 20 µl was taken off from the head space of the serum vial using the syringe specified to use in G.C. analysis. That sample was injected through the front injection port into G.C. column.

2.7.4.3. Analysis program for G.C.

The chromatograms were analyzed with analysis program CP-3800 purchased from Varian Co. in Canada.

3 GROWTH OF LACTIC ACID BACTERIA AND OPTIMIZATION OF THE BATCH

CHAPTER 3: GROWTH OF LACTIC ACID BACTERIA AND OPTIMIZATION OF BATCH CULTURE.

3.1. INTRODUCTION.

The lactic acid bacteria that are predominant in the human intestine, have diverse nutritional requirements and as a consequence they have been found in diverse ecological niches. Lactic acid bacteria are Gram-positive and microaerophilic catalase-negative bacteria which grow under microaerophilic to strictly anaerobic conditions. They are non-spore forming and heterogeneous group that ferment hexose to lactic acid [Klein et al. (1998), Adamberg et al. (2003)].

Many workers have studied the physiology and nutritional requirements of lactic acid bacteria using synthetic media, but none of media used can support the growth of all groups of *Lactobacilli*. Therefore, the complex substance or mixtures are necessary to support the growth of LAB in a wide range of species. Moreover, considering industrial application of lactic acid bacteria, the economics in growth media formulation should be considered in the development of processes.

Consequently, as the starting point, the investigations about the physiology and the nutritional requirements of four strains, *L. buchneri*, *L. brevis*, *B. longum*, and *O. oeni* are the subjects of this chapter

The aims were to produce high yields of cell mass in a cheap medium and ultimately propagate it to high cell concentration in the MCR. Carefully considering the economics, the investigations were carried out in a basis of the growth rate and total cell mass yields as the main optimized parameter necessary to save the time and the production cost.

The preserved strains taken out of -70°C ultra-freezer are very unstable to straight use for inoculation. Therefore, the strains must be activated and propagated before inoculation into the main culture medium.

For the consistency of data and reliable propagation of the strains taken out of -70 °C ultra-freezer, a starter culture was introduced into the seeding procedure before the main culture.

All manipulations were undertaken at the 50 ml serum vial in anoxic conditions. The pH was not controlled during the batch cultures.

3.2. PHYSIOLOGY AND THE NUTRITIONAL REQUIREMENTS OF LACTOBACILLUS BUCHNERI.

3.2.1. The starter culture of Lactobacillus buchneri

The starter culture of *Lactobacillus buchneri* was performed in MLM-1 medium. Even if the first propagation of the strain gifted from Interprise company was performed in commercial MRS medium, the growth in MRS medium was very poor. As a result, MLM-1 medium was developed on basis of the composition of the synthetic medium by Ledesma et al. [1977]. However the media components such as trace elements and amino acids were amounted. Complex media components like yeast extract or soy peptone were used to support the growth of all strains. The growth of *L. buchneri* in MLM-1 medium was compared with that in MRS medium.

MLM-1 medium was prepared according to the procedure described in section 2.3.2, and MRS was purchased at Lab M. Co in U.K. The experiment was carried out according the methods described in section 2.3.5 and the samples were periodically taken to determine pH and growth, optical density. These data were converted to dry weight by means of a standard curve *(Refer to Appendix -2)*.

Figure 3.1 compares the growth of *L. buchneri* in MLM-1 medium with that in MRS medium. According to Fig.3.1, when *L. buchneri* was grown in MRS medium, the growth rate was 0.23 h $^{-1}$, but the growth rate in MLM-1 medium increased by 13% as 0.26 h $^{-1}$. Ultimately total cell mass of 3.38 DCW, g/L obtained at MLM-1 medium during the cultivation for 35 h. It increased by 40% when compared with 2.45 DCW, g/L in MRS medium.

Chapter.3 Growth of lactic acid bacteria and optimization of batch culture

Also, when MLM-1 medium is used as the medium for the start culture, the exponential period was extended by 15 ~ 19 h. Therefore, appropriate timing for inoculation during the starter culture was identified to be in a range between 15 h and 19 h.
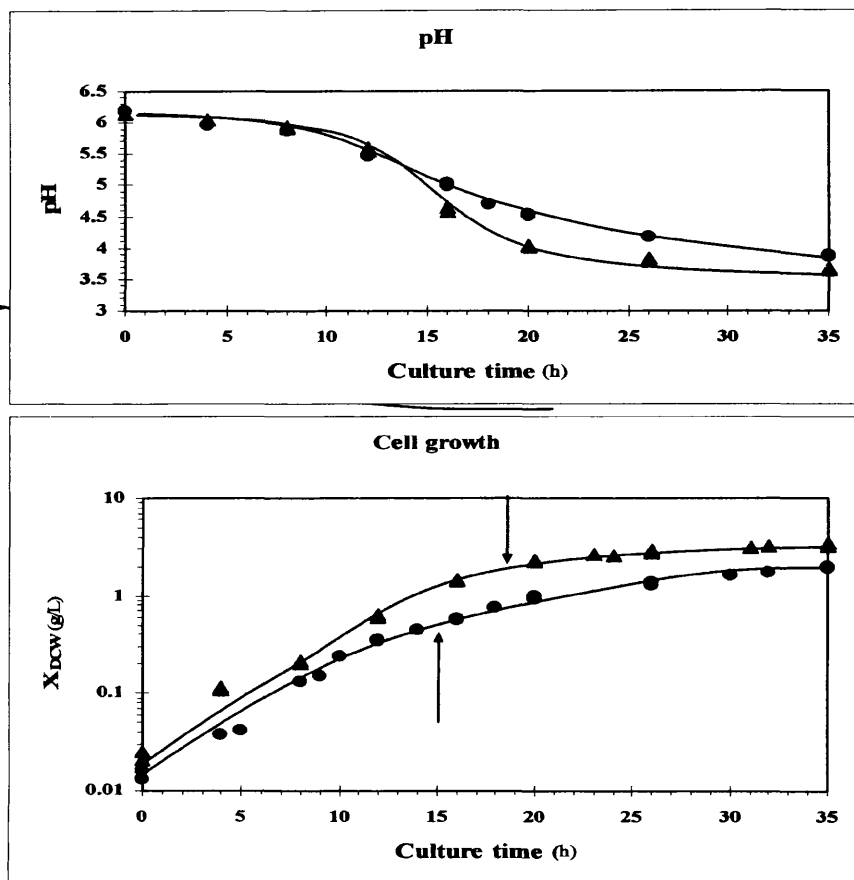
Fig.3.1 A Comparison of MRS medium with MLM-1 medium in the growth of

L. buchneri. These starter cultures were prepared according to the procedure described in chapter 2 (Section 2.3.5), and cultivated in the incubator of 30 °C. Two arrows marked the end of the exponential growth period.

The growth in MRS medium : ●, the growth in MLM-1 medium : ▲

3.2.2. Optimization of the physical conditions for growth of L. buchneri.

In order to optimize the physical conditions for growth of *L. buchneri* all tests were carried out using the starter culture medium (MLM-1) in batch culture *(Refer to 2.3.5)*.

3.2.2.1. The effects of shaking in the starter culture.

Generally, anaerobic culture is usually carried out without agitation to reduce possible aeration of the medium. But, when the growth was completely sealed in an anoxic environment, agitation should not affect the growth. The end products (mainly lactic acid) accumulated in the vicinity of growing bacteria might cause the growth inhibition during the stagnant culture. Therefore, agitation can improve growth by removing diffusion barriers allowing inhibitory substances to be diluted. The influence of shaking on the growth of *L. buchneri* was investigated. The experimental conditions were described at above section and after inoculation the cultures were compared for optical density, thus data were then converted to dry cell weight.
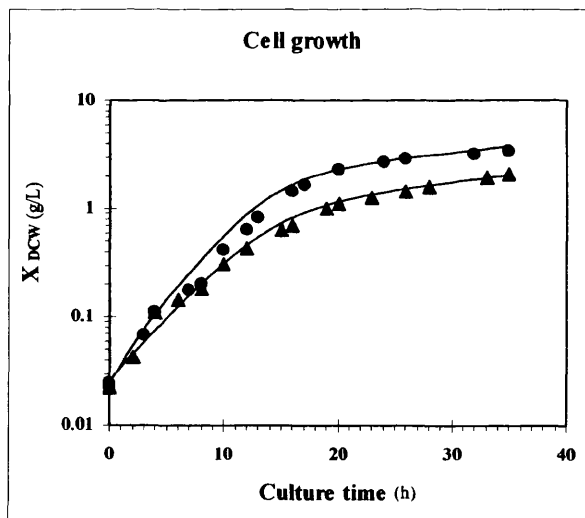
Fig.3.2 The effect of agitation in the starter culture of *Lactobacillus buchneri*. *MLM-1 medium was used to grow L. buchneri. All experiments were duplicated. Shaking incubator was purchased from New Brunswick Scientific Ltd (120 rpm and 3-inch power stroke.) Shake culture: ●, Stagnant culture : ▲*

Chapter.3 Growth of lactic acid bacteria and optimization of batch culture

Figure 3.2 shows that *L. buchneri* had better growth in shaking than in stagnant conditions. Even if the growth in stagnant culture initially showed higher growth than that in shaking, the growth in shaking conditions was higher than that in stagnant conditions from the middle of the cultivation (6 h). Furthermore, growth in shaking condition showed a large extension in the exponential growth phase. Ultimately, the cultivation in the shaking condition also produce more total cell mass. The introduction of a strong starter into the microbial cultivation was very important to reduce the error range and increase reliability of the growth.

3.2.2.2. The effect of inoculation volume

In this section, the investigation was undertaken to decide an appropriate inoculum size.

The inoculation must be undertaken with an appropriate volume. Using too much volume of the starter culture for the inoculation, the growth inhibition can be induced by the inhibitory substances at the early stage of the cultivation. On the other hand, if too small volume is used, the lag phase is longer.

A broad range of the starter culture volumes from 1% (v/v) to 20% (v/v) were investigated. The cultures were inoculated and sampled during the growth (Fig.3.3). The growth rate decreased as the inoculation volume increased. Generally, the inoculation volume made no difference in the final cell mass. However a large inoculum affected the growth rate and the length of the exponential growth phase.

When the volume of 1% (v/v) was inoculated, a short lag phase was detected in the early stage of the cultivation. Moreover, when the inoculation volume over 10% (v/v) was used, a reduction of the growth rate during the exponential growth phase was noted. The inoculation volume between 5% (v/v) and 10% (v/v) was chosen as an appropriate volume for *L. buchneri*. According to the result of Fig.3.3, final cell mass produced was the same in all cases, but the exponential growth phase reduced with the increasing inoculation volume. Only in the cases started under 5% (v/v) the exponential growth phase could maintain for over 10 h.
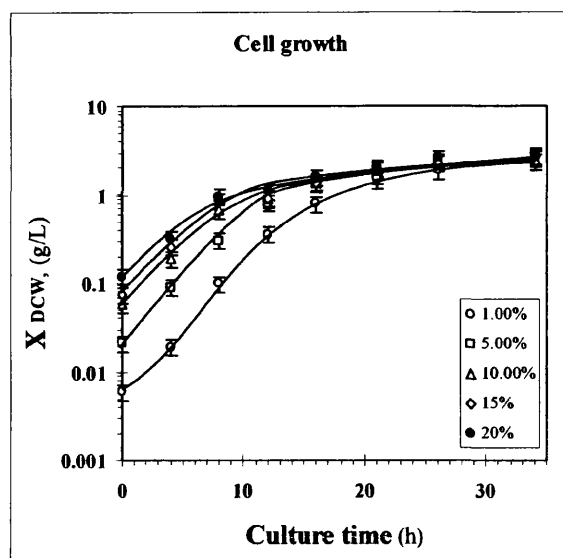
Fig.3.3 The effect of the starter volume for inoculation on the batch culture of *Lactobacillus buchneri. MLM-1 medium was used to grow L. buchneri. The starter culture volumes of inoculation in this investigation were 1% (v/v), 5% (v/v), 10% (v/v), 15% (v/v), and 20% (v/v) of the main culture volume. All experiments were undertaken in duplicate, and all data were in an error range of ± 5%.*
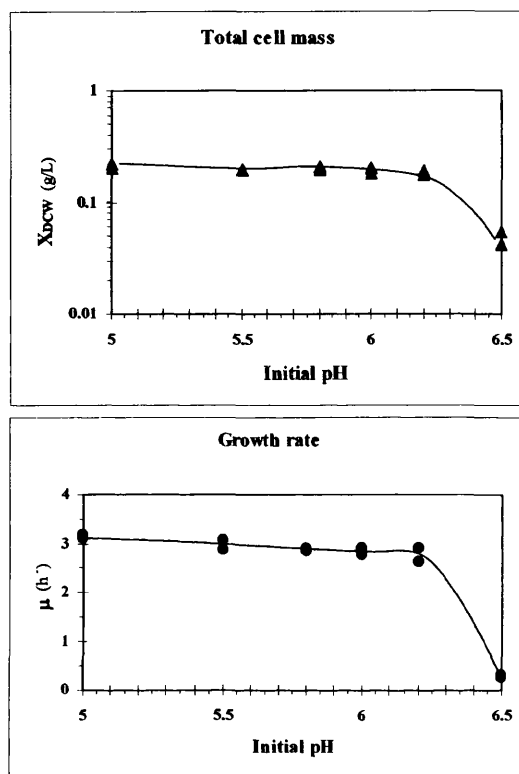
3.2.2.3. The effect of initial pH.

The growth of LAB can be significantly affected by initial pH of the batch culture. Appropriate initial pH is very effective in enhancing the bacterial growth to produce more total cell mass as the pH drops during the growth caused by organic acids. Therefore, the best initial pH is typically slightly higher than the optimum pH for growth.

The growth of LAB was therefore investigated in a pH range between pH 5.0 and pH 6.5. MLM-1 medium was used for the growth of *L. buchneri*, and the cultivation was carried out as described in the previous chapter *(Section 2.3.5)*. Samples were taken and the dry weight was estimated and the specific growth rate was determined. Figure 3.4 shows the effect of the

initial pH on the growth rate and the total cell mass during the cultivation of *L. buchneri*. The growth rate and total cell mass of *L. buchneri* showed a sharp reduction when the cultivation started at the higher than pH 6.25, and the growth almost stopped at the initial pH of pH 6.5.

Fig.3.4 The effect of initial pH on the growth rate and the cell mass in the batch culture of *Lactobacillus buchneri*. *L. buchneri was grown at MLM-1 medium and the growth condition as described at the previous chapter (Section 2.3.2 and 2.3.5). All experiments were duplicated, and all results were in an error range of ± 5%.*

3.2.3. The investigation of the nutrient requirements for growth of L. buchneri.

All ingredients were selected through the tests in the batch culture in basis of MLM-1 medium *(Section 2.3.2 and 2.3.5)*, and optimization on the medium composition was conducted by removing and adding the ingredients of MLM-1 medium and then observing their effects on the growth. Ultimately, optimum composition for growth of *L. buchneri* was developed *(Section 2.4.2.1)*.

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Chapter.3 Growth of lactic acid bacteria and optimization of batch culture

3.2.3.1. The effect of glucose concentration on growth.

In this section, an appropriate glucose concentration to use in the growth medium was investigated.
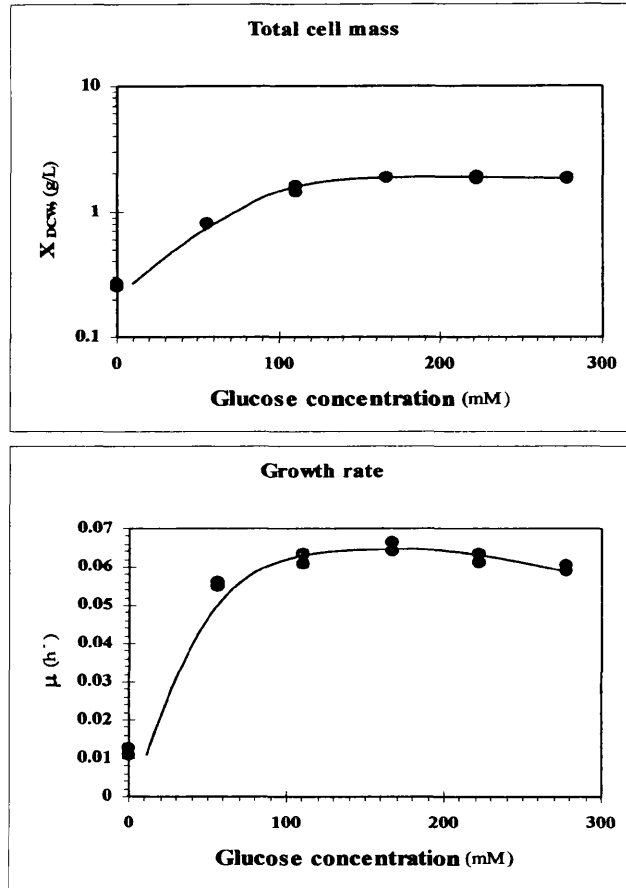
Fig.3.5 The effect of glucose concentration on the growth rate and the total cell mass in the batch cultures of *Lactobacillus buchneri*. *L. buchneri was grown with the MLM-1 as the basic medium, and only glucose concentration was changed in a concentration range from nil to 300 mM.*

If too much glucose is added to the medium, it must produce organic acids like lactic acid or acetic acid, which cause the end product inhibition during the cultivation. Therefore, the amount of glucose added to the growth medium should be controlled. Appropriate glucose concentration is also important to get the maximum cell mass during the batch culture. The effect of carbon source was tested in a wide concentration range from nil to over 250 mM using glucose.

The effect of the addition was then determined by means of the specific growth rate and growth yield.

The growth rate was the highest at the glucose concentration of 50 mM. Total cell mass reached the maximum when glucose of about 80 mM was used in the medium and did not increase any more in higher glucose concentration. Consequently, it was confirmed that *L. buchneri* can grow as well even at low concentrations of glucose (Fig.3.5).

3.2.3.2. The effect of nitrogen sources on growth.

Yeast extract contains diverse nutrients like carbohydrates, amino acids, peptides, growth factors, vitamins, and minerals. Yeast extract has been used to support a wide range of the bacterial groups very well. Soy peptone contains carbohydrate, peptides and amino acids but is not sufficient in the growth factors like vitamins or minerals. Therefore, soy peptone was used as alternative of yeast extract on production of lactic acid by *Lactobacillus rhamnosus* by Yoo et al. [1997] and Kwon et al. [2000]. Likewise, appropriate yeast extract concentration in the growth medium can enhance the growth, and it has been very economic alternative of amino acids in industrial use.

In this section, yeast extract (Fig.3.6) was investigated together with soy peptone (Fig.3.7) in a range from 0% (w/v) to 2% (w/v) for the growth of *L. buchneri*. The tests were undertaken with MLM-1 medium as the basic medium, and glucose concentration used in this investigation was 167 mM. *L. buchneri* was grown at the same growth condition as described at the previous chapter *(Section 2.3.5)*. The growth rate and growth yield were determined.

At first, yeast extract did influence in the growth rate and total cell mass in a concentration range from 0 to 1.2% (w/v) (Fig. 3.6). Soy peptone did not affect the growth rate but it showed a slight but steady non-proportional increase on total cell mass (Fig.3.7).
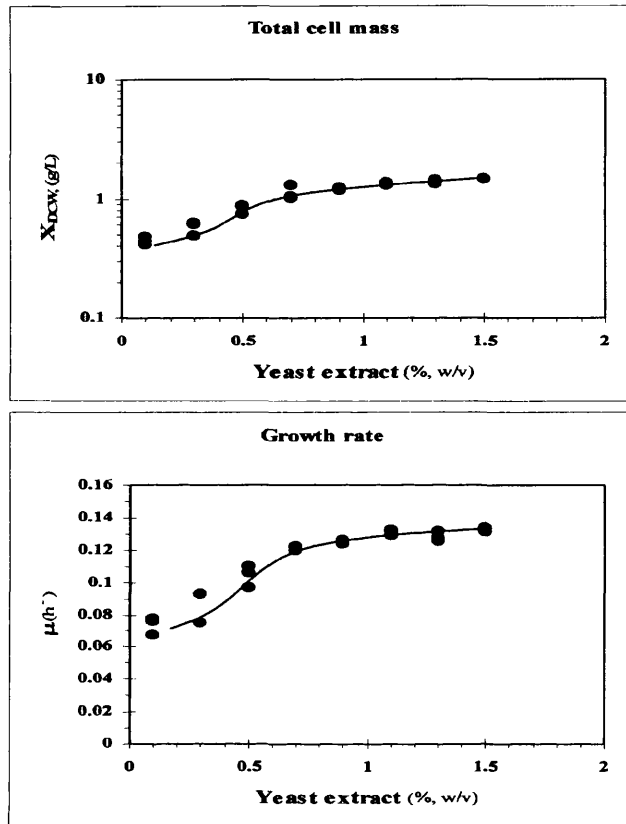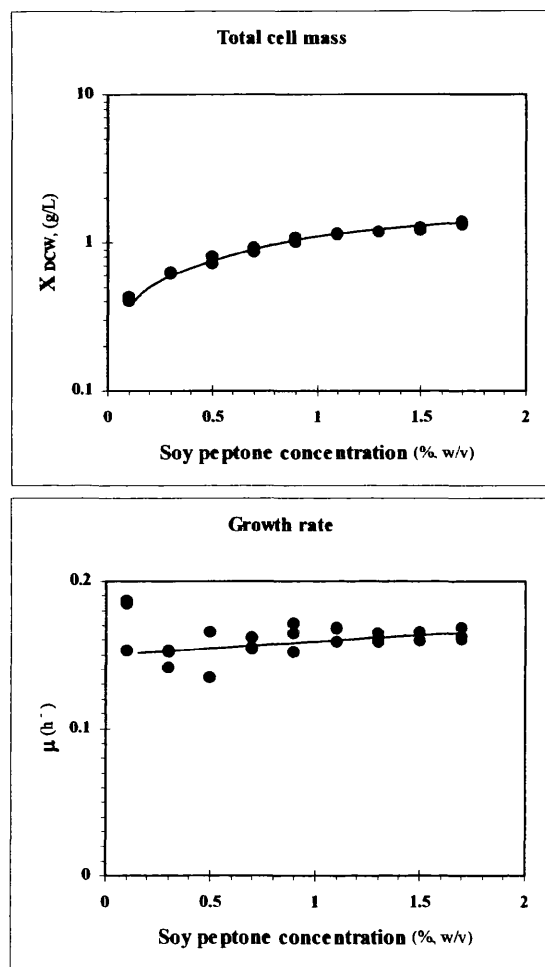
Fig.3.6 The effect of yeast extract concentration on the growth rate and total cell mass in the batch culture of *Lactobacillus buchneri*. *L. buchneri was grown with MLM-1 medium as the basic medium changing only yeast extract concentration in a range from nil to 1.5% (w/v).*

Consequently, yeast extract was selected as nitrogen source because it positively affected both of the growth rate and total cell mass. When yeast extract was used in concentrations less than 0.7% (w/v), the growth rate and total cell mass were reduced dramatically.

Yeast extract concentration of 0.9% (w/v) or 1.0% (w/v) was appropriate to use in the growth medium of *L. buchneri*.

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Fig.3.7 The effect of soy peptone concentration on the growth rate and the cell mass in the batch culture of *Lactobacillus buchneri*. *L. buchneri was grown with MLM-1 medium as the basic medium changing only soy peptone concentration in a range from nil to 1.7% (w/v).*

3.2.3.3. The standard growth in the batch culture

The growth of *L. buchneri* was tested with a variety of chemicals in MLM-1 medium containing yeast extract of 0.9% (w/v) and glucose of 128 mM.

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L. buchneri has the nutritional requirements of niacin, pantothenate, and riboflavin as vitamin and glutamic acid, valine, and diverse amino acids depending on the strain. If except for L-aspatic acid, all chemicals did not show any improvement on the growth of *Lactobacillus buchneri* with the yeast extract-based complex medium. It was very different from the results with the synthetic medium showing a good growth effect at diverse amino acids [Ledesma et al. (1977)]. Moreover, the effect of L-Asp could be due to buffering capacity of L-Asp around pH 4.7 because *L. buchneri* could show the fast growth in that pH (Fig.3.4). Consequently, when L-Asp was added, the growth rate at the beginning of the cultivation was lower than the growth without L-Asp, but the cell mass at the end of the cultivation increased because pH was maintained around pH 4.5 during the final period of cultivation. Likewise, D,L-Asp was used to maintain pH in only the vial culture due to the unreasonable cost to use in bulk base indeed.

The growth rate in the balanced growth decreased from 0.097 h^{-1} to 0.093 h^{-1}, but the yield ($Y_{x/s}$) increased from 0.048 h^{-1} to 0.06 h^{-1} with use of L-Asp. Cultivation with glucose of 127.8 mM could produce the cell mass of 1.12 DCW, g/L bringing pH down from pH 4.71 to pH 2.58 in 53 h (Fig.3.8).

Mg^{++}, Mn^{++}, and Fe^{++} can improve the growth of LAB [Shin and Park. (1997), Ahn et al. (2001), Shimamura et al. (1992), Chang and So. (1998)]. These elements can play a role as key catalyst or stabilizer of tertiary structure on the metalo-enzymes. It is very important for use of Mn^{++} as cofactor for superoxide dismutase (SOD) to neutralize the toxicity by oxygen for anaerobic survival as many as reported use of Mg^{++}. Adequate levels of two materials were added into the medium.
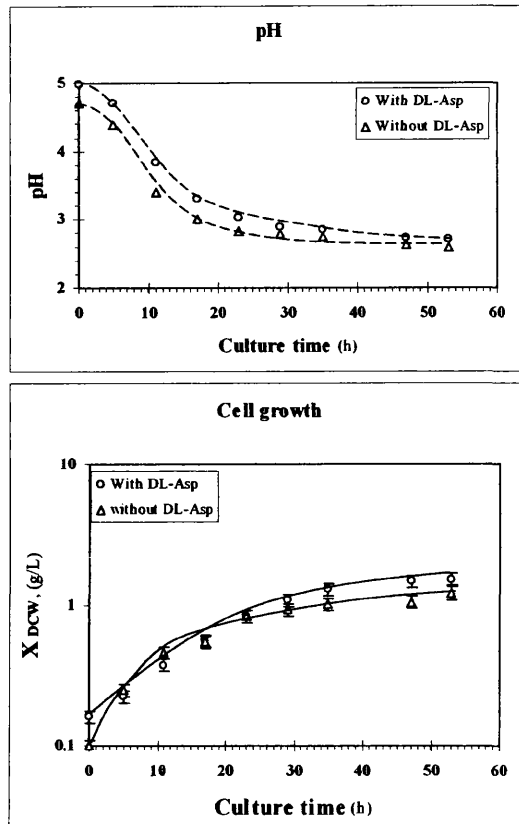
Fig.3.8 The standard growth pattern of *Lactobacillus buchneri* at the optimized medium composition, and the effect of D,L-Asp on the growth of *L. buchneri*. *L. buchneri was grown with the optimized growth medium improved from MLM-1 medium and the growth condition as described at the previous chapter (Section 2.4.2 and 2.4.2.1). DL-Asp of 50 mM was added into the growth medium of the same composition for comparison. Glucose was used in the concentration of 127.8 mM in the both cases. All experiments were undertaken five times, and all data were in a range of ± 5%.*

3.2.3.4. The inhibition by organic acids.

The influence of organic acids on growth was investigated to gauge the tolerance of *L. buchneri* to these substances. The growths of LAB are typically inhibited up to a certain concentration and type of organic acids.

Chapter.3 Growth of lactic acid bacteria and optimization of batch culture

Since such a growth inhibition by lactic acid has been a major problem in obtaining the high cell mass, the simulation and kinetics of the end product inhibition by lactic acid has been enormously considered by many workers [Dutta et al. (1996), Levenspiel. (1980)] in LAB research.

Likewise, the inhibitory effect by the end products was evaluated during the growth of LAB in the batch culture. The organic acids, lactic acid or acetic acid were added at the beginning and then the growths were measured during the early stages of the cultivation. Figure 3.9 shows that lactic acid is stronger inhibitor than acetic acid. According to Fig.3.9, the critical concentration of lactic acid to give a complete inhibition in the growth of *L. buchneri* was 525 mM, and in case of acetic acid it was theoretically 2.2 M.
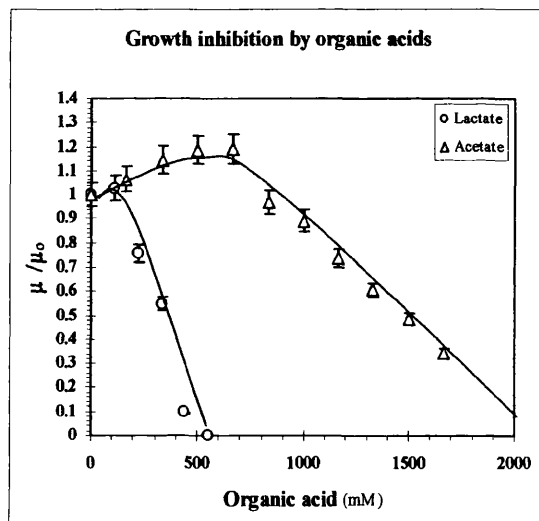
Fig.3.9 The growth inhibition by lactic acid and acetic acid during the cultivation of *Lactobacillus buchneri*. *The growth rates were checked in 12h after inoculation, and the inhibition was described as the percentage to the control (μ_o) incubated without addition of organic acid. The medium used to check the growth inhibition of organic acids was the optimized medium without D,L-Asp, and the cultivation was performed by the same condition (Section 2.4.2 and 2.4.2.1). All experiments were duplicated, and all data were in error range of $\pm 5\%$.*

3.3. PHYSIOLOGY AND THE NUTRITIONAL REQUIREMENTS OF BIFIDOBACTERIUM LONGUM.

3.3.1. The starter culture of B. longum.

The starter cultures of *Bifidobacterium longum* were grown at MLM-1 medium *(Section 2.3.2 and 2.3.8)*.
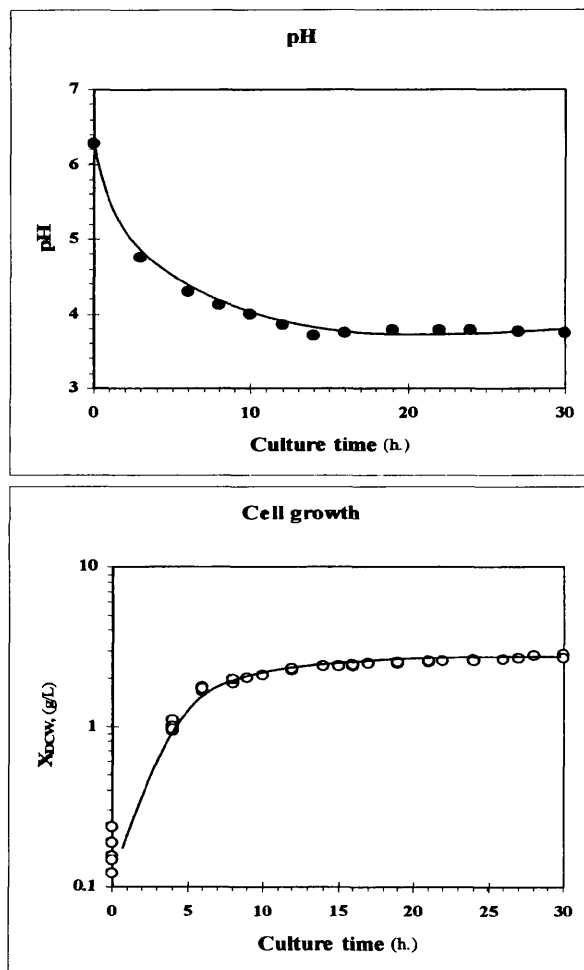
Fig.3.10 The standard growth pattern in the starter culture of *Bifidobacterium longum*. *The medium used to grow the strain was MLM-1 including glucose of 111 mM. B. longum was grown at the same condition as described at the previous chapter (Section 2.3.2 and 2.3.8). The growths were undertaken five times, and all data were in error range of ± 5%.*

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Chapter.3 Growth of lactic acid bacteria and optimization of batch culture

As described in Fig.3.10, the exponential growth phase was less than 6 h with the growth rate of 0.39 h^{-1}, and then an inhibited growth with a growth rate of 0.007 h^{-1}. During 30 h of cultivation, the starter culture produced the cell mass of 2.53 DCW, g/L.

3.3.2. Optimization of the physical growth condition for growth of B. longum.

In order to investigate the optimum physical conditions, *B. longum was* grown in MLM-1 medium *(Section 2.3.2)*.

3.3.2.1. The effect of inoculation timing from the starter culture.

Using inoculum prepared as above, the inoculation timing was investigated. Generally speaking according to Fig.3.10, inoculation timing from the starter culture was between 9 h and 12 h. Therefore, inoculation was taken in terms of every 6 h from 0 to 24 h, and Figure 3.11 shows the growth curves obtained from the experiment.
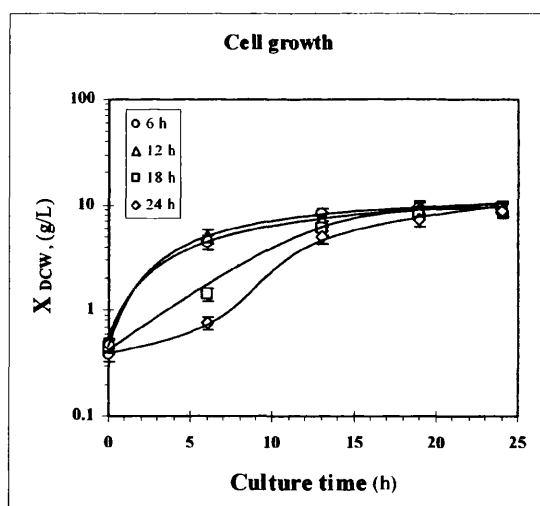
Fig.3.11 The effect of inoculation timing in the batch culture of *Bifidobacterium longum. Inocula were collected in 6 h, 12 h, 18 h, and 24 h of the starter culture. B. longum was grown at MLM-1 medium containing glucose of 111 mM, and the same condition as described at the previous chapter (Section 2.3.2 and 2.3.8). All experiments were duplicated, and all data were in error range of ± 5%.*

Chapter.3 Growth of lactic acid bacteria and optimization of batch culture

The growth rate was respectively 0.42 h $^{-1}$ in the batch culture with inoculum in 6 h $^{-1}$ of the starter culture and 0.41h $^{-1}$ in 12 h, but it dropped to 0.21 h $^{-1}$ at 18 h and 0.27 h $^{-1}$ in 24 h. As shown in Fig.3.10, the physiological condition of the bacteria in the starter culture after 12 h did not look very well due to the multiple stresses in an environment worsen during the batch culture.

3.3.2.2. The effect of initial pH on growth.

The growth of *B. longum* was tested in a range of initial pH from pH 3.0 to pH 6.5. This experiment was carried out as described in Section 2.3.8 and dry cell weight was estimated according to a standard curve to convert optical density to dry cell weight as described in Appendix-1.
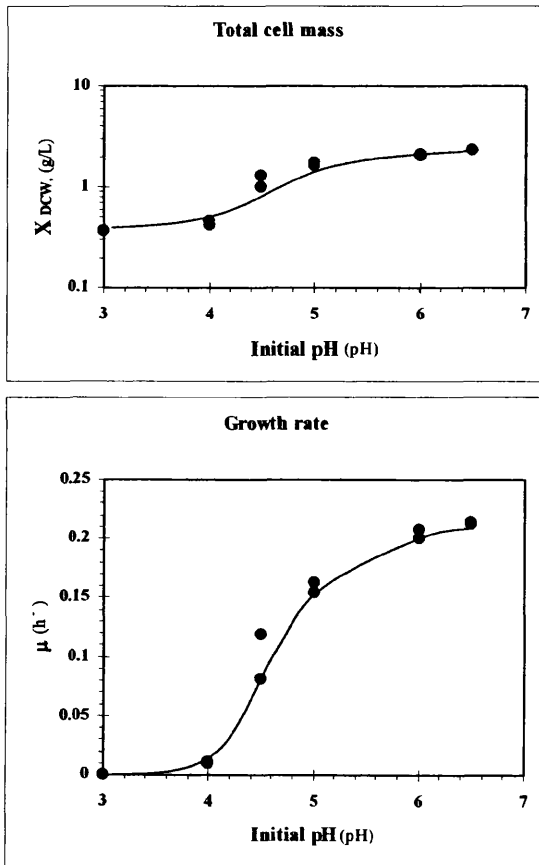
Fig.3.12 The effect of initial pH in the batch culture of *Bifidobacterium longum*. *The bacterial growth was begun in initial pH of 3.0, 4.0, 4.5, 5.0, 6.0, and 6.5. The medium used for the bacterial growth was MLM-1 including glucose of 111 mM, and B. longum was grown in the same growth condition as described at the previous chapter (Section 2.3.2 and 2.3.8). All experiments were duplicated, and all data were in error range of ± 5%.*

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The growth rate showed a declining trend when initial pH lower than pH 6.0 was tried, and was completely inhibited at pH 4.0. Above pH 6.0 the growth rate increased at higher initial pH than pH 6.5. These data suggested that *B. longum* would grow at neutral pH (Fig.3.12).

During the cultivation of *B. longum*, total cell mass increased as initial pH increased from pH 4.0 to pH 5.5, but showed no further to pH 6.5.

3.3.3. Investigation of the nutrient requirements for growth of B.longum.

In order to determine the nutrient requirements and optimum physiological conditions, *B. longum* was grown in the batch culture using MLM-1 medium as the basic medium *(Section 2.3.2 and 2.3.8)*. All ingredients necessary for growth were added into or removed from the medium composition of MLM-1. Ultimately, optimum medium composition for *B. longum* was developed *(Section 2.4.2.4)*

3.3.3.1. The effect of carbon sources on growth.

The MLM-1 medium was used as the basic medium and glucose was compared with fructose in the same concentration 111 mM for the growth of *B. longum* (Fig.3.13).
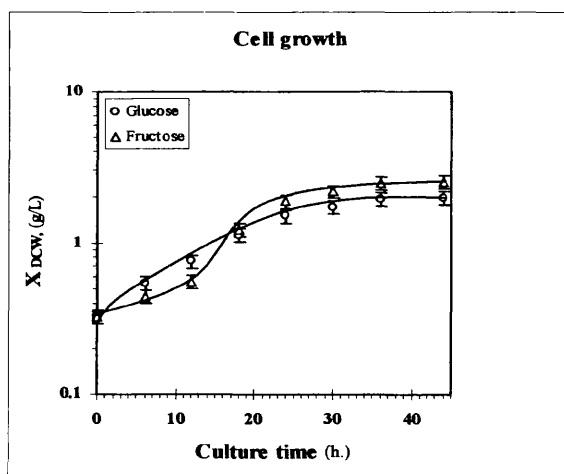
Fig.3.13 Comparing glucose with fructose on the growth of *Bifidobacterium longum* as single carbon source. *B. longum was grown at MLM-1 medium containing carbohydrates of 111mM and the same growth condition as described at the previous chapter (Section 2.3.2 and 2.3.8). All experiments were undertaken three times, and all data were in error range of ± 5%.*

B. longum was found to be able to grow at both carbohydrates, but it produced 30% more cell mass with fructose. But, the growth on glucose was faster than that on fructose at the early stage of the cultivation. As shown at Fig.3.13, when initiating the fermentation with only fructose, a lag phase was detected.

In the next experiment, the effect of the carbohydrate concentration was investigated on MLM-1 medium with the carbohydrate concentration from nil to 280 mM.
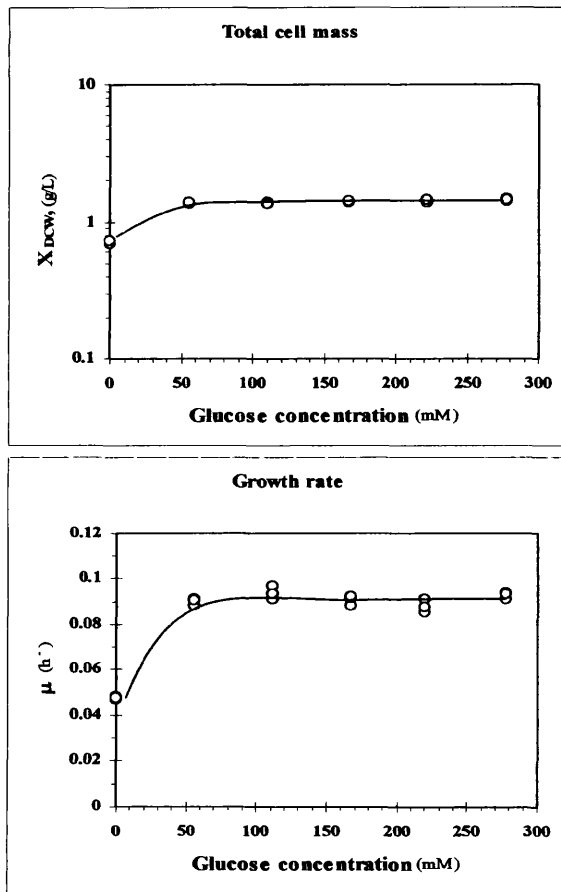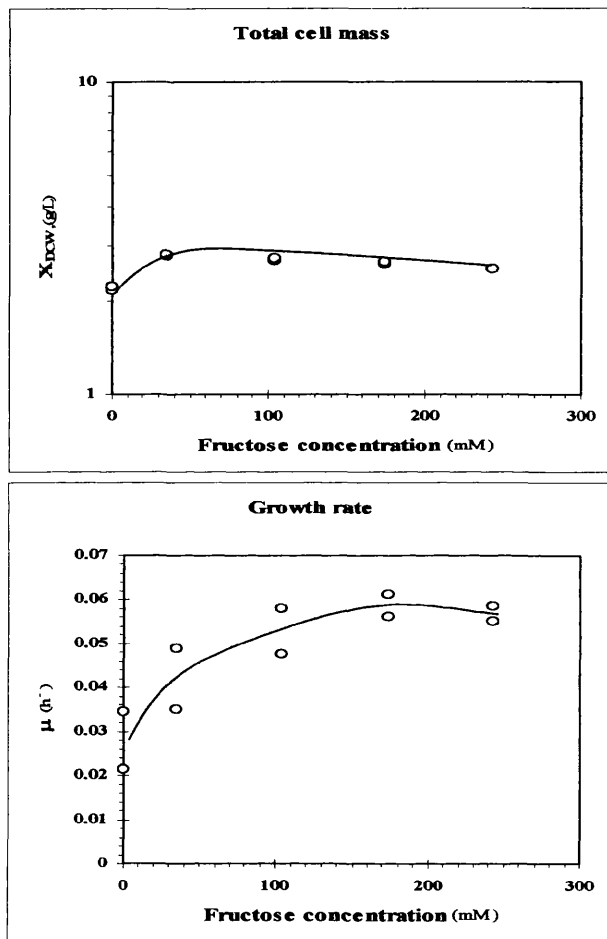

Fig.3.14 The effect of glucose concentration in the batch culture of _Bifidobacterium longum_.

B. longum was grown at MLM-1 medium containing a serial glucose concentration from nil to 280 mM and at the same growth condition. All experiments were undertaken three times. As a result about usability of the carbohydrate, 56 mM of glucose and 168 mM of fructose were chosen for the growth of B. longum in the batch culture.

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Figure 3.14 shows the effect of glucose concentration as single carbon source on the growth rate and total cell mass. When the glucose concentration over 56 mM was used, the growth rate and the total cell mass did not increase further. In the other hand, the use of fructose would be able to produce the steady and upward growth rate as shown in Fig.3.15. The total cell mass, however, did not decrease significantly over the range of fructose added.

Fig.3.15 The effect of fructose concentration in the batch culture of *Bifidobacterium longum. B. longum was grown at MLM-1 medium containing a serial fructose concentration from nil to 280 mM, and the same growth. All experiments were undertaken three times, and all data were in error range of ± 5%.*

3.3.3.2. The effect of nitrogen sources on growth of B. longum.

The effect of yeast extract and soy peptone on growth of *B. longum* was investigated. *B. longum* has complex nutritional requirements for organic nitrogen and trypsin digests of casein or yeast extract has been used for the growth of *Bifidobacterium* [Poch and Bezkorovainy. (1991), Oiki et al. (1996)].

The effect of yeast extract, soy peptone, and ammonium sulfate on growth was investigated. The results of these experiments are shown in Fig 3.16. When yeast extract was used with ammonium sulphate, the growth rate of *B. longum* was higher than the growth on other nitrogen sources (Fig.3.16).
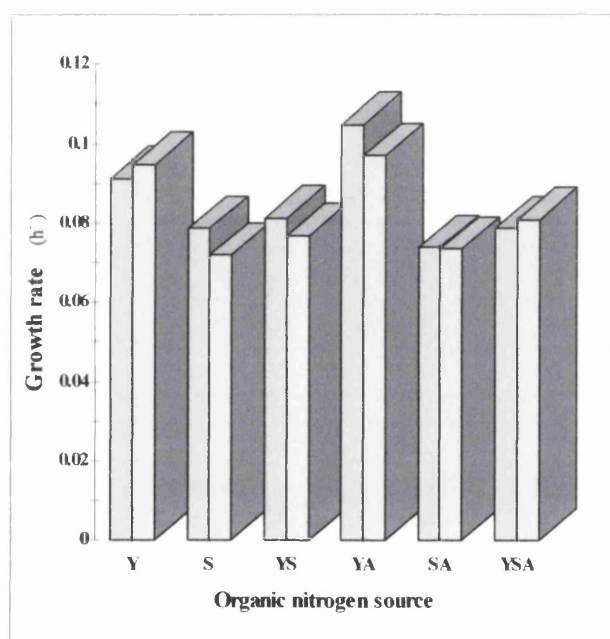
Fig.3.16. The effect of each nitrogen sources on the growth rate during the batch culture of *Bifidobacterium longum*. *The growth rates of B .longum were compared among each others with yeast extract, soy peptone, and ammonium sulphate as nitrogen source at MLM-1 medium. Glucose of 111 mM and fructose of 139 mM were used as the carbon sources. All experiments were duplicated, and all data were in error range of ± 5%.*
Y; yeast extract (1%,w/v), S; soy peptone (1%,w/v), YS; mixture of yeast extract (0.5%,w/v) and soy peptone (0.5%,w/v), A; ammonium sulphate (10 mM).

Figure 3.17 also shows the effect of yeast extract concentration on the growth rate and the total cell mass. The growth rate and the total cell mass shows a steady increase in a range from nil to 5% (w/v) of yeast extract. The use of $(NH_4)_2SO_4$ with yeast extract also lead to an improvement of the growth rate (Fig.3.18) as investigated by Oiki et al. [1996]. They found that natural rubber serum has a stimulating effect on the growth of B. *longum*. Furthermore they investigated the alternatives facilitating the growth of B. *longum*, and they found that a combination of yeast extract and ammonium sulphate could produce the same effect. The growth rate showed an extra increase by 10% in a range from 10 mM to 30mM of ammonium sulphate.
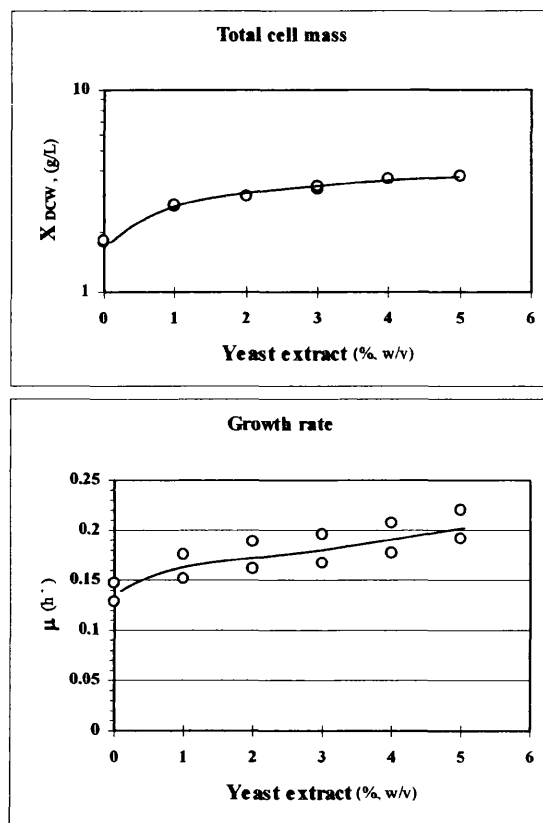
Fig.3.17 The effect of the yeast extract concentration on the growth rate and the total cell mass during the cultivation of Bifidobacterium longum. B. longum was grown at MLM-1 medium changing only yeast extract concentration from nil to 5% (w/v). All experiments were duplicated, and all data were in error range of ± 5%.

Total cell mass

Growth rate
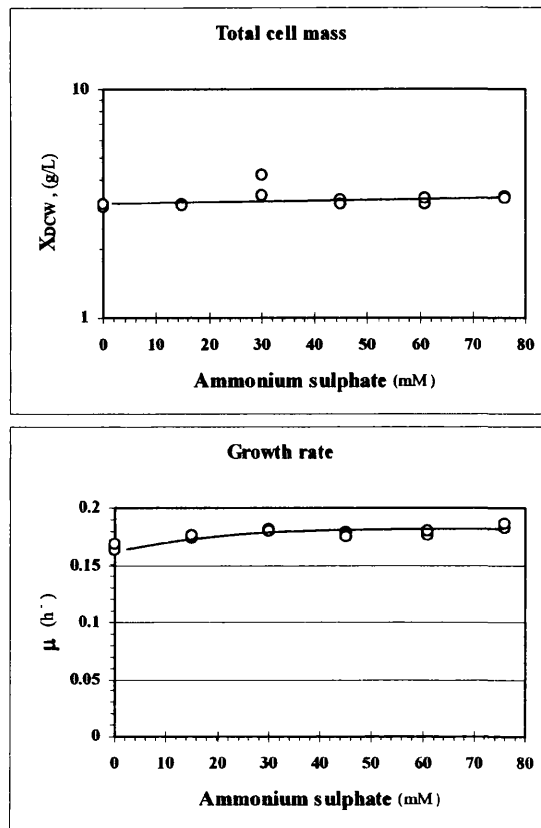
Fig.3.18 The effect of ammonium sulphate in the presence of yeast extract. *Bifidobacterium longum was grown in MLM-1 medium containing yeast extract of 3% (w/v). Ammonium sulphate was used in a range from nil to 78 mM. All experiments were duplicated, and all data were in error range of ±5%.*
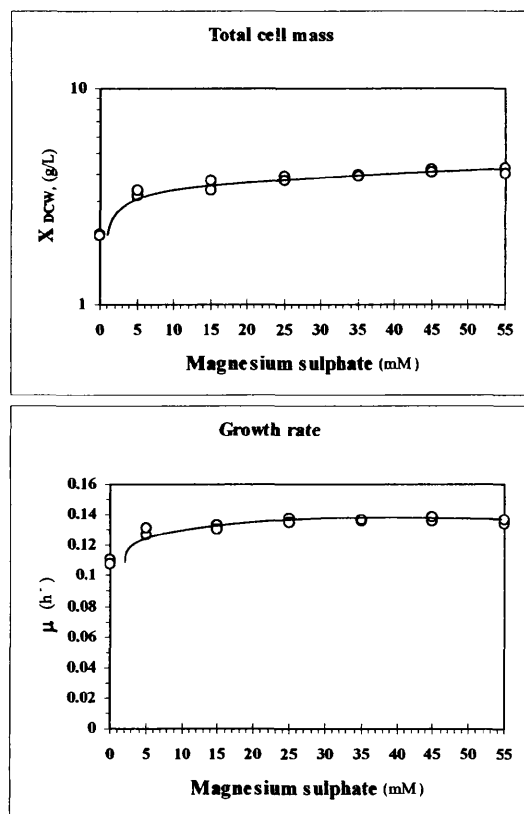
3.3.3.3. The effect of magnesium sulphate on growth.

B. longum is known as microaerophilic anaerobes and its oxidative defense system comprising NADH-oxidase and NADH-peroxidase can accumulate divalent metal ions like Mn^{++}, Mg^{++} or Fe^{++} for an intracellular establishment of non-enzymatic dismutation different from superoxide dismutase (SOD). The combination of both enzymes can remove the toxicity of oxygen, producing water with 2 mole of NAD^+. [Shin and Park. (1997), Ahn et al. (2001), Shimamura et al. (1992), Chang and So. (1998)].

B. longum was grown at optimized medium containing glucose (111 mM), fructose (139 mM), yeast extract (3%,w/v), and ammonium sulphate (10 mM) and the effect of Mg(SO₄) was investigated by addition of nil to 55 mM Mg $^{++}$.

Figure 3.19 shows the growth rate increases by addition of magnesium sulphate 10 mM, but the growth rate did not increase further with further additions. However, the total cell mass was strongly related to growth at concentrations lower than 10 mM.

Therefore, in the presence of 10 mM of Mg $^{++}$ the growth rate reached 0.13 h $^{-1}$ after 7 h of cultivation and total cell mass of 3.56 g/L was obtained in 24 h. The pH dropped from pH 7.04 to pH 3.81.

Fig.3.19 The effect of magnesium sulphate concentration on the growth rate and the total cell mass during the cultivation of *Bifidobacterium longum*. *B. longum was grown at optimized medium changing magnesium sulphate concentration from nil to 55 mM. Mg(SO₄) 7H₂O was used as magnesium source. All experiments were duplicated, and all data were in error range of ± 5.*

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3.3.3.4. The growth of B. longum on standard medium in the batch culture.

The optimized composition of the batch culture medium as developed above is shown at section *2.4.2.4. B. longum* was grown at this medium for 24 h, and produced the cell mass of 3.2 DCW, g/L. The growth rate was 0.23 h^{-1} during the exponential growth phase from the start to 7 h (Fig.3.20).
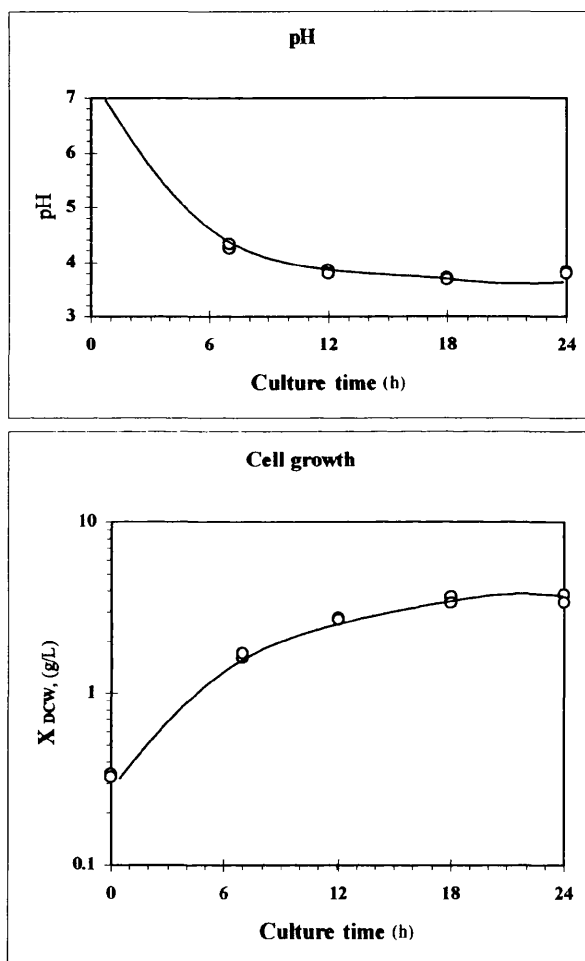
Fig.3.20 The standard growth pattern in the batch culture of *Bifidobacterium longum*. *B. longum was grown at the optimized medium(w/v) including glucose (1.0%), fructose (0.9%), KH$_2$PO$_4$ (0.39%), yeast extract (3.0%), MgSO$_4$·7H$_2$O (0.14%), (NH$_4$)$_2$SO$_4$ (1.0%) (Section 2.4.2.4) for 24h. The experiments were duplicated, and all data were in error range of ± 5%.*

*3.3.3.5. The inhibition by organic acids*

Bifidobacterium spp. is heterolactic fermentative bacteria using a different pathway to assimilate carbon source. They have a relatively economic pathway that can produce 5 mole of ATP with lactic acid and acetic acid in the ratio of 2:3 from 2 mole of glucose (See Fig.1.6).

To a great extent, the unique nutrient requirements of *Bifidobacterium* and such a low acid tolerance have been obstacles in producing the cell mass of *Bifidobacteria*. *Bifidobacterium spp.* has very low acid tolerance when compared to other lactic acid bacteria. In fact, the growth of *B. longum* was inhibited by a relatively low concentration of organic acids when compared with the cases of *Lactobacillus* or *Leuconostoc spp.* But, *B. longum* is the most tolerant of the *Bifidobacteria sp.* against organic acids, even in acidic condition and at the bile concentration as high as 4% [Lankaputhra. (1995)].

The effect of organic acids was investigated using the standard medium and additions of organic acids. Figure 3.21 shows that acetic acid is a stronger than lactic acid in inhibition. According to Fig.3.21, 150 mM lactic acid and acetic acid was serious enough to inhibit the growth of *B. longum*. Either organic acid completely stopped the bacterial growth in 250 mM. These records are similar to the results of Taniguchi et al. [1987].

Growth inhibition by organic acids

Legend: ● Lactate, ▲ Acetate

Y-axis: μ / μ_o
X-axis: Organic acids (mM)
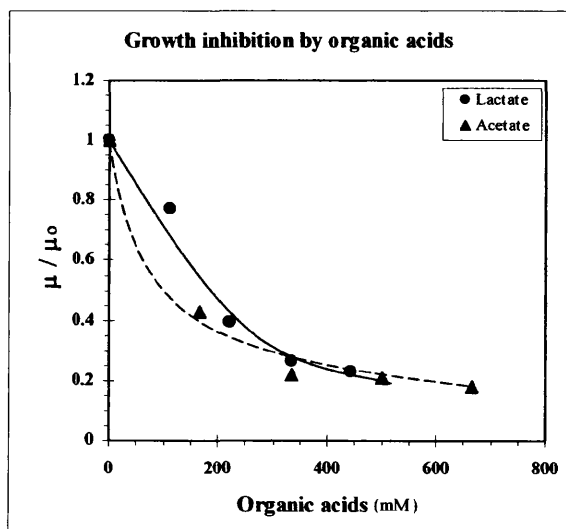
Fig.3.21 Growth inhibition by lactic acid and acetic acid during the cultivation of *Bifidobacterium longum. B. longum was grown in the optimized growth medium as described before (Section 2.4.2), and the growth condition as described at the previous chapter (Section 2.4.2.4). The growth rates were checked in 12h after inoculation, and the inhibition was described as the percentage to the control (μ_o) incubated without addition of organic acid.*

3.4. PHYSIOLOGY AND THE NUTRITIONAL REQUIREMENTS OF OENOCOCCUS OENI.

3.4.1. The starter culture of Oenococcus oeni.

The original strains of O. oeni used tomato juice as the essential part of the medium required. However, tomato juice was not used in this investigation using MLM medium.

Figure 3.22 shows the growth pattern of O. oeni in the starter culture performed at MLM-1 containing glucose (56 mM) and fructose (56 mM). The starter culture was carried out as described above (Section 2.3.7).
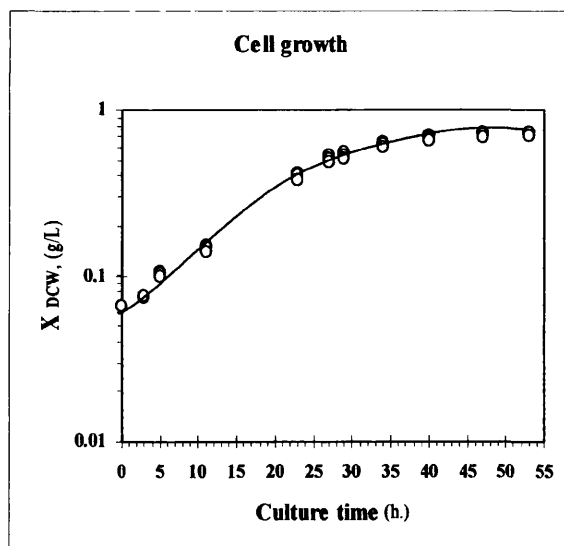
Fig.3.22 The growth pattern of *Oenococcus oeni* in the starter culture performed at MLM-1 medium containing glucose (56mM) and fructose (56mM). *O. oeni was grown at the same condition as described at the previous chapter (Section 2.3.7) for 53 h. All cultures were undertaken three times, and data out of error range of ±5% were not collected for this figure.*

O. oeni grew at the growth rate of 0.15 h^{-1} during the exponential growth phase from 3 h to 24 h. Cultivation of O. oeni produced total cell mass of 0.614 DCW, g/L in 53 h. Therefore, appropriate collection for inoculation was performed from the starter culture in a range between 24 h and 30 h.

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The cultivation with inoculation taken in 35 h of the starter culture grew at the lower growth rates.

3.4.2. Optimization of the physical growth condition for growth of O. oeni.

In order to determine the optimum inoculation volume and initial pH, *O. oeni* was grown at MLM-1 medium *(Section 2.3.2)* and the same growth condition *(Section 2.3.7)*. The initial pH and inoculation volume were adjusted before inoculation at the MLM-1 medium.

3.4.2.1. The effect of initial pH on growth.

O. oeni has been known as an acidophilic microorganism. Therefore, the effect of initial pH to grow *O. oeni* was investigated.
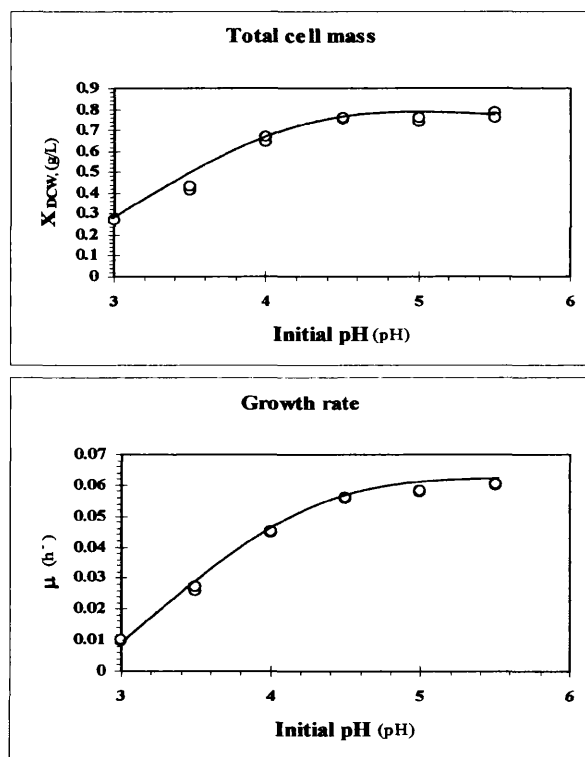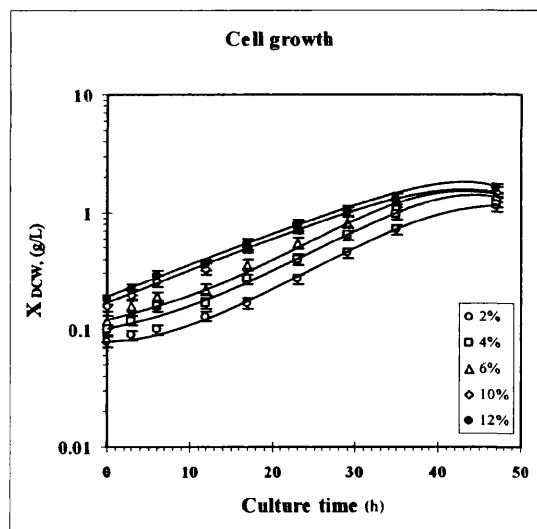

Fig.3.23 The effect of initial pH on the growth rate and the cell mass during the cultivation of *Oenococcus oeni*. *O .oeni was grown at MLM-1 medium (Section 2.3.2) and the growth condition described at the previous chapter (Section 2.3.7). All experiments were duplicated, and all data were in error range of ± 5%. .*

Figure 3.23 shows the effect of initial pH on the growth rate and the total cell mass during the cultivation of *O. oeni*. According to Fig.3.23, the growth rates and the cell mass dramatically increase from pH 3.0 to pH 4.5, and above pH 4.5 initial pH has no effect on these parameters. The highest cell mass was 0.783 g/L. The growth rate was 0.061 h^{-1}.

3.4.2.2. The effect of inoculation volume on growth.

As the growth of *O. oeni* was very slow, a strong and large inoculation volume was required to avoid a long lag at the early stage of the cultivation and the risk of the contamination.

Fig.3.24 The effect of the inoculation volume on the growth rate and the cell mass during the cultivation of *Oenococcus oeni*. *The batch culture was inoculated with the inoculation volume of 2% (v/v), 4% (v/v), 6% (v/v), 10% (v/v), and 12% (v/v) collected in 24 h of the starter culture. O. oeni was grown at MLM-1 medium (Section 2.3.2) and the growth condition described at the previous chapter (Section 2.3.7). All experiments were duplicated, and all data were in error range of ± 5%.*

Therefore, the effect of inoculation volume was checked with MLM-1 medium using a volume range from 2% (v/v) to 12% (v/v). Figure 3.24 shows a variation on the growth pattern of *Oenococcus oeni* with respect of inoculation volume. Inoculations performed at the volume below 6% (v/v) led to the lag phases.

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When inoculation volume of 10% (v/v) was used at least, the lag phase disappeared. Consequently, at least an inoculum size of 10% (v/v) should be used.

### 3.4.3. Investigation of nutrient requirement for growth of O. oeni.

Nutritional requirements for growth of *O. oeni* was investigated at MLM-1 medium as the basic medium, and all ingredients necessary for growth of *O. oeni* were added or removed in MLM-1 medium. Ultimately, optimum composition of the medium was developed *(Section 2.4.2.3)*.

3.4.3.1. The effect of carbon sources on growth.

O. oeni prefers fructose to glucose, but the cultivation produced more cell mass with the consumption of glucose than fructose in the vial culture. Most of fructose can be converted to mannitol regenerating NAD^+ recycled in the catabolic pathway from glucose to lactic acid and acetic acid. Maicas et al. [2002] reported up to 80% of consumed fructose was transformed into mannitol with regeneration of NAD^+ and 20% can be metabolized through heterolactic fermentative pathway to generate the cellular metabolites. Likewise, it is essential to use a mixture of glucose and fructose for a high cell mass and high growth rate [Richter et al. (2003), Maicas et al. (2002), Maicas et al. (1999)].

During the growth of *O. oeni* in appropriate combination of fructose and glucose, the growth rate increased from 0.049 h $^{-1}$ in the case using only fructose (139 mM) to 0.058 h $^{-1}$, and total cell mass increased 1.37 times more than the case using only glucose (139 mM), producing total cell mass of 0.85 g/L (Fig.3.25). Therefore, a low growth rate was found at glucose (139 mM) as single carbon source and low total cell mass was obtained at fructose (139 mM) as a single carbon source. There was a significant improvement when a carbohydrate mixture of glucose (56 mM) and fructose (83 mM) was used.

The effect of the carbohydrates on the growth rate was investigated using glucose or fructose as single carbon source.
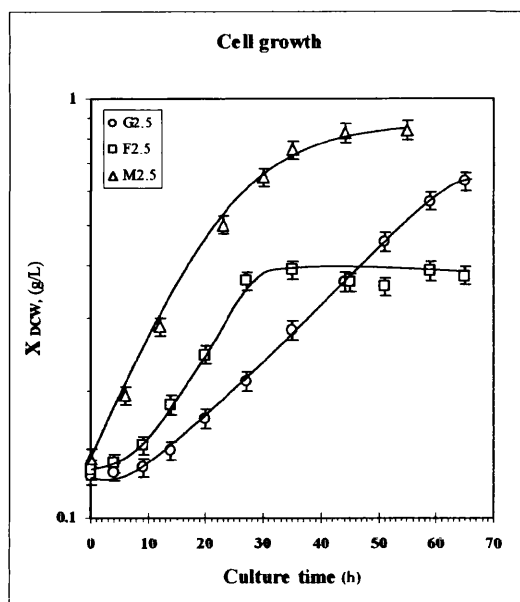
Fig.3.25 The effect of glucose, fructose and a mixture of glucose and fructose on the growth pattern of *Oenococcus oeni* by carbon sources. *The carbon sources were glucose (139 mM), fructose (139 mM), and the mixture of glucose (56 mM) and fructose (83.3 mM). O. oeni was grown at MLM-1 medium to check the effect of carbon sources. All experiments were duplicated, and all data were in error range of ± 5%.*

O. oeni was grown at MLM-1 medium containing glucose or fructose in a concentration range up to 300mM. *O. oeni* showed the higher growth rate in whole range of investigation when fructose was used as single carbon source (Fig 3.26). From the results of these investigations (Fig.3.25 and Fig.3.26), glucose can be used as the main carbon source for the growth while fructose high enough to control the balance between NAD^+ and NADH existing in the growth medium.
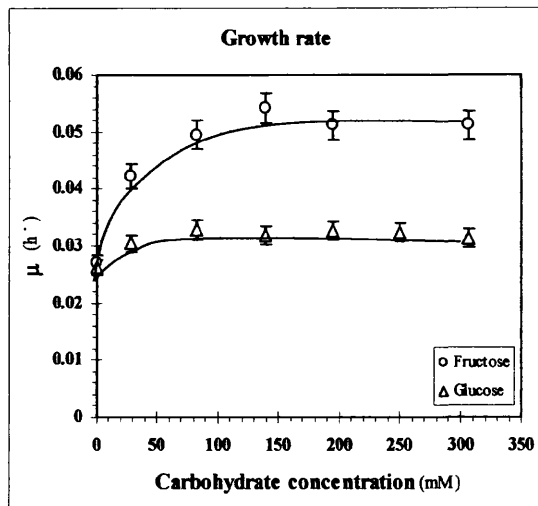
Fig.3.26. The effect of the carbohydrate concentration on the growth rate during the cultivation of *Oenococcus oeni*. *O. oeni was grown at MLM-1 medium changing glucose or fructose concentration from nil to 300 mM as a single carbon source. All experiments were duplicated, and all data were in error range of ± 5%.*

3.4.3.2. The effect of nitrogen sources on growth.

Nitrogen sources were investigated and yeast extract was compared with soy peptone to investigate their influence on the cultivation of *O. oeni* (Fig.3.27). The investigation was performed at MLM-1 medium as the basic medium, replacing for only nitrogen source yeast extract (1%, w/v) and soy peptone (1%, w/v). *Oenococcus oeni* was grown better in yeast extract than in soy peptone of the same concentration.
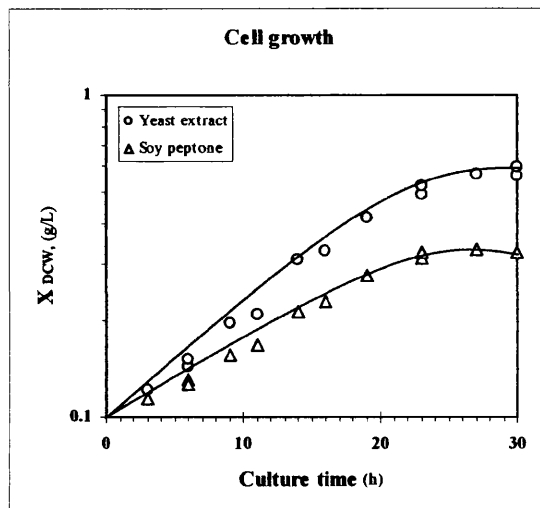
Fig.3.27 The effect of the nitrogen sources on the growth of *Oenococcus oeni*. *Yeast extract was compared with soy peptone in the concentration of 1.0% (w/v). O. oeni was grown at MLM-1 medium to check the effect of the nitrogen source, and the carbohydrate mixture of glucose (139 mM) and fructose (56 mM) was used.*

With selection of yeast extract as nitrogen source, the effect of the yeast extract concentration was investigated to assess the growth rate and total cell mass (Fig 3.28). The growth rate and total cell mass dramatically increased when yeast extract was added, and yeast extract concentration above 1.0% (w/v) had no further effect.

The growth of *O. oeni* did not show any difference on the growth rate and on total cell mass when magnesium sulphate, ammonium sulphate, and manganese sulphate were tried in a concentration range from 0% (w/v) to 0.9% (w/v).
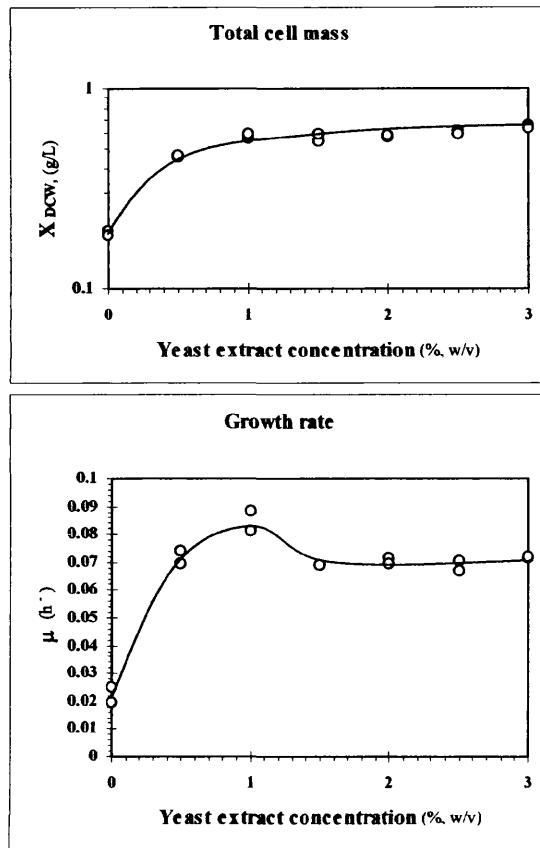
Fig.3.28 The effect of yeast extract concentration on the growth rate and the total cell mass during the cultivation of *Oenococcus oeni*. *O. oeni was grown at MLM-1 medium containing the carbohydrate mixture of glucose (139 mM) and fructose (56 mM). Yeast extract concentration was changed from nil to 3% (w/v) as a single nitrogen source. All experiments were duplicated, and all data were in error range of ± 5%.*

3.4.3.3. The effect of organic acids on growth.

The effect of organic acids was investigated. Addition of citric acid stimulated the growth of *O. oeni* (Fig.3.29). At acidic pH, *O. oeni* can produce diacetyl, acetoin, and 2,3-butylene glycol through co-metabolism of glucose and citric acid (See Fig.1.5). Citric acid is metabolized to produce acetate with more ATP. According to Cogan. [1987], co-metabolism of glucose and citric acid in *O. oeni* led to an activation of acetate kinase with production of more acetate than an amount of acetate shown at the consumption of citric acid.

Furthermore, he reported the growth is stimulated with co-metabolism of glucose and citric acid inhibiting production of ethanol.

O. oeni was grown at MLM-1 medium containing yeast extract (1.5%,w/v) and the carbohydrate mixture of glucose (56 mM) and fructose (83.3 mM) to investigate the effect of citric acid and acetic acid in a concentration range up to 90 mM. Figure 3.29 shows the effect of citric acid on growth. Citric acid promoted the growth rate and total cell mass proportionally to up to 30 mM. But at higher citric acid concentration, the growth rate declined (Fig.3.29).
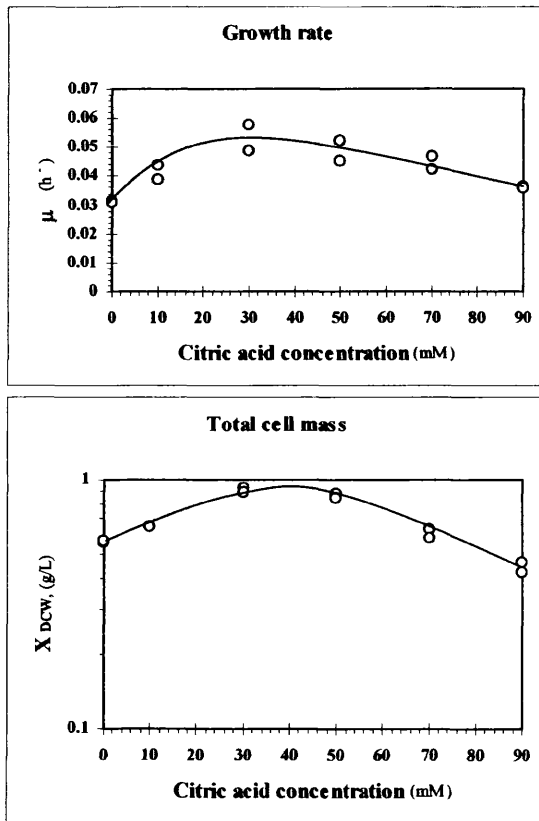
Fig.3.29 The effect of glucose and citric acid co-metabolism on the growth rate and the cell mass during the cultivation of *Oenococcus oeni*. *The effect of citric acid was investigated at MLM-1 medium containing citric acid in a range from nil to 90 mM with the carbohydrate mixture of glucose (139 mM) and fructose (56 mM). All experiments were duplicated, and all data were in error range of ± 5%.*

Acetic acid did not stimulate the growth of *O. oeni*. When acetic acid was added, the growth rate and the total cell mass showed slightly increasing trends by 50 mM, but in the concentration over 50 mM more improvement was not detected (Fig.3.30).
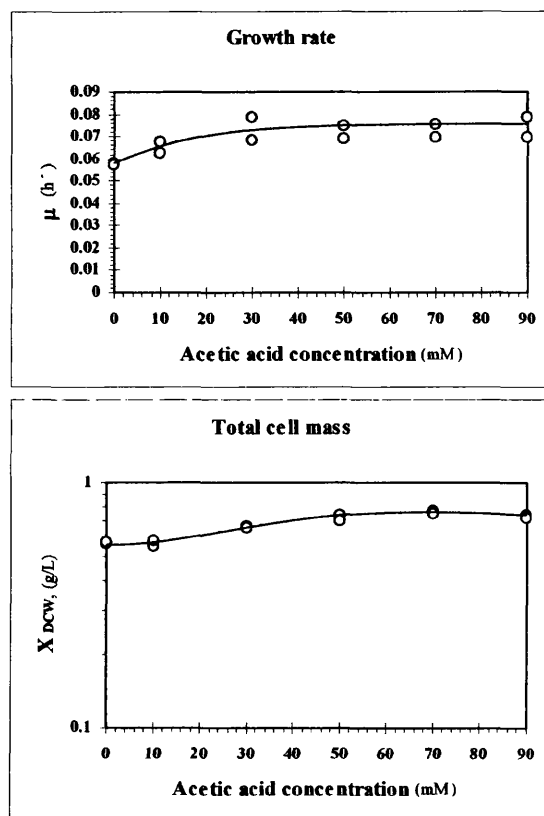
Fig.3.30 The effect of acetic acid concentration on the growth rate and the total cell mass during the cultivation of *Oenococcus oeni*. *The effect of acetic acid was investigated at MLM-1 medium containing acetic acid in a range from nil to 90 mM with the carbohydrate mixture of glucose (139 mM) and fructose (56 mM). All experiments were duplicated, and all data were in error range of ± 5%.*

3.4.3.4. The standard growth pattern in batch culture of O. oeni.

O. oeni was grown at optimized growth medium for 48 h *(Section 2.4.2.3)*. *O. oeni* was grown at the growth rate of 0.081 h^{-1}, and reached the cell mass of 0.92 g/L (Fig.3.31).

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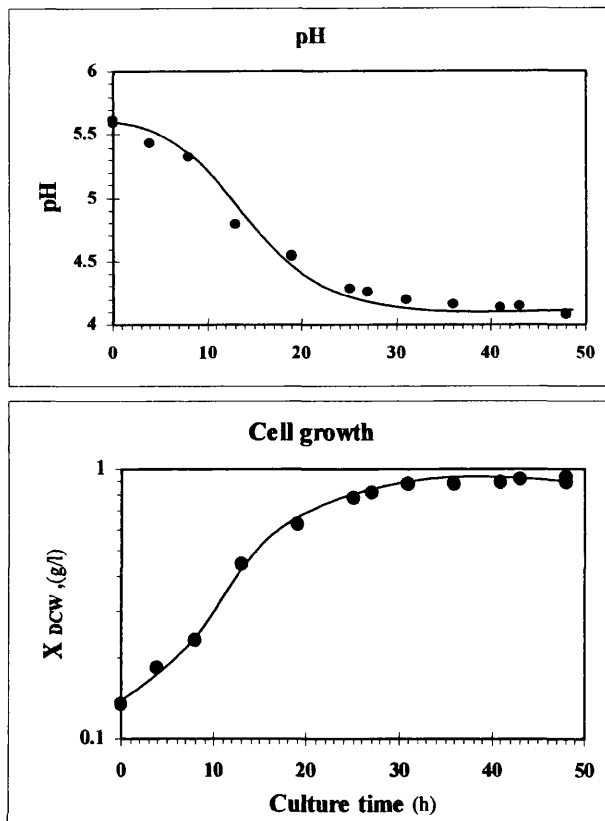
Fig.3.31 The standard growth pattern of *Oenococcus oeni* in optimized growth medium and the growth condition *(Section 2.4.2 and 2.4.2.3)*. *O. oeni was grown at the growth rate of 0.081 h $^{-1}$ during the exponential growth phase, and total cell mass reached 0.92 DCW, g/L during 48 h. All experiments were duplicated, and all data were in error range of ± 5%.*

3.4.3.5. The growth inhibition by organic acids

In cultivation of *O. oeni*, acetic acid and lactic acid are the main products of carbohydrate metabolism. The expected stoichiometry is 1 mole lactic acid and ½ mole acetic acid and ½ mole ethanol per mole of consumed glucose, but these proportions are more or less specific [Cogan. (1987)]. According to the reports so far, *O. oeni* is the most tolerant on acidic conditions of all lactic acid bacteria. *O. oeni* could be grown in a pH range from pH 3.5 to pH 5.5, however *O. oeni* was seriously inhibited even in a low concentration of organic acids.
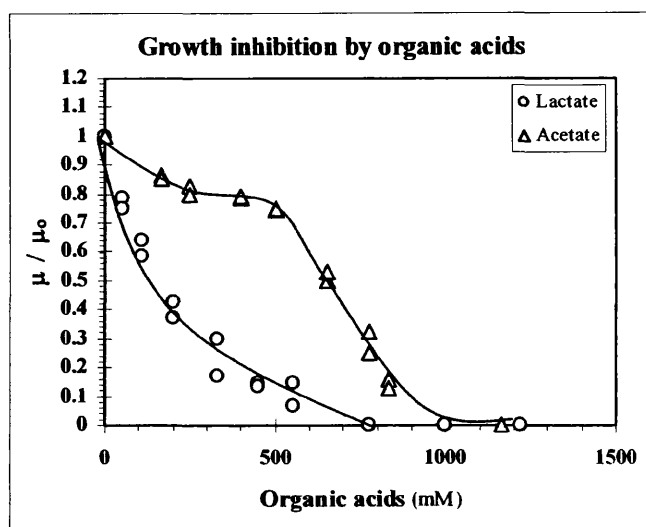
Fig.3.32 The effect of lactic acid and acetic acid concentration on growth of *Oenococcus*

oeni. O. oeni was grown at optimized medium and the growth condition to check the inhibitory effect of organic acids (Section 2.4.2 and 2.4.2.3). Each of organic acid was added in the beginning of the culture, and the growth rates were measured in 12 h of incubation, and the inhibition was described as the percentage to the control (μ_o) incubated without addition of organic acid. All experiments were duplicated, and all data were in error range of ± 5%.

Lactic acid of 700 mM was high enough to stop the growth of *O. oeni*. In case of acetic acid, the growth rate slowed down by 30% at the acetic acid concentration below 600 mM, but acetic acid gave a very serious inhibition in the concentration over 600 mM (Fig.3.32).

3.5. PHYSIOLOGY AND THE NUTRITIONAL REQUIREMENTS OF LACTOBACILLUS BREVIS.

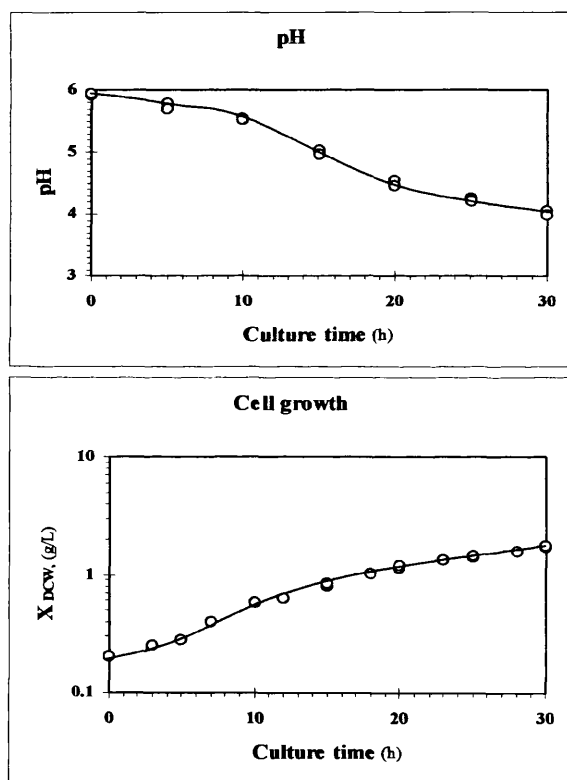### 3.5.1. The starter culture of Lactobacillus brevis.

The starter culture of *Lactobacillus brevis* was undertaken at MLM-1 medium including glucose 56 mM and fructose 56 mM *(Section 2.3.2)*. During the starter culture of *L. brevis*, a long lag was detected at the early phase of the cultivation, but *L. brevis* was grown at the growth rate of 0.11 h $^{-1}$ during the exponential growth phase (from 5 h to 15 h).

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This was progressed by a steady increase to the end of the stationary phase (Fig. 3.33). The starter culture of *L. brevis* produced the cell mass of 1.52 DCW, g/L in 30 h. Although not shown here, the growth rate of *L. brevis* in the batch culture was not affected even if the inoculation after 25 h of the starter culture was tried.

3.5.2. Optimization of the physical condition for growth of L. brevis.

In order to investigate optimum physical condition for growth of *L. brevis*, *L. brevis* was grown at MLM-1 medium changing initial pH and inoculation timing *(Section 2.3.2 and 2.3.6)*.
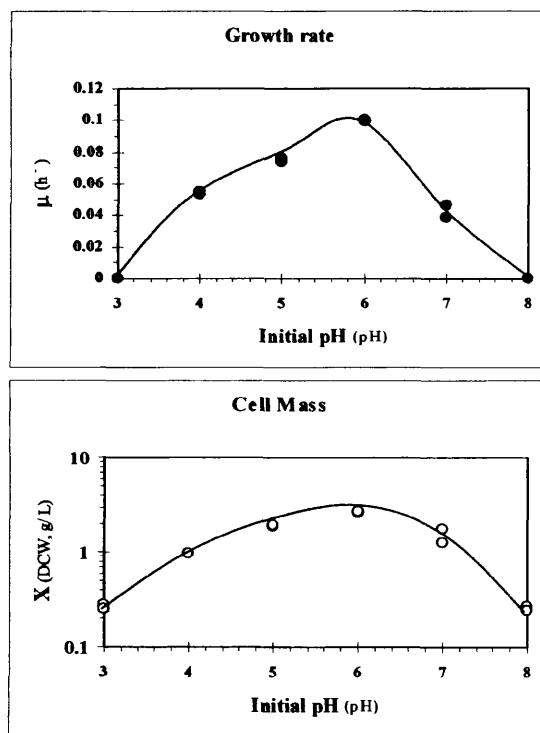

Fig.3.33 The growth of *Lactobacillus brevis* on MLM-1 medium containing glucose (56 mM) and fructose (56 mM) during the starter culture. *L. brevis was grown at the same growth medium (Section 2.3.2) and condition (Section 2.3.6) as described at the previous chapter. All experiments were undertaken three times, and all data out of error range of ± 5% were removed.*

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3.5.2.1. The effect of initial pH on growth.

The effect of initial pH on the growth rate and total cell mass changing the only initial pH from pH 3.0 to pH 8.0 is shown in Fig 3.34. The growth rate and the total cell mass of *L. brevis* were the maximum when the initial pH was between pH 5.5 and pH 6.0, and the growth rate was 0.18 h $^{-1}$. The growth of *L. brevis* stopped below pH 3.0 and above pH 8.0. The growth rate dropped by 20% when the cultivation was started at the initial pH lower than pH 5.0, and when the cultivation was started at the neutral pH, the growth rate dropped by 45%. In the other hand, the total cell mass relatively kept up at pH range between pH 4 and pH 7.

Fig.3.34 The effect of initial pH on the growth rate and the cell mass during the starter culture of *Lactobacillus brevis*. *L. brevis was grown at MLM-1 medium containing glucose (56 mM) and fructose (56 mM), and initial pH was adjusted with NaOH or HCl (1M) just after inoculation in a range from pH 3.0 to pH 8.0.*

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3.5.3. Investigation of nutrient requirements for growth of L.brevis.

The nutritional requirements for growth of *L. brevis* were investigated at MLM-1 as the basic medium. All ingredients necessary for growth of *L. brevis* were added or removed from MLM-1 medium during the investigation through this batch culture *(Section 2.3.2 and 2.3.6)*. Ultimately, optimum composition of the growth medium was developed (Section *2.4.2.2)*.

3.5.3.1. The effect of carbon source on growth.

L. brevis was grown at MLM-1 with glucose and fructose to observe their effect on growth. First of all, glucose and fructose were separately investigated in the concentration up to 390 mM. Figure 3.35 and Fig 3.36 show the effect of glucose and fructose on growth of *L. brevis*.
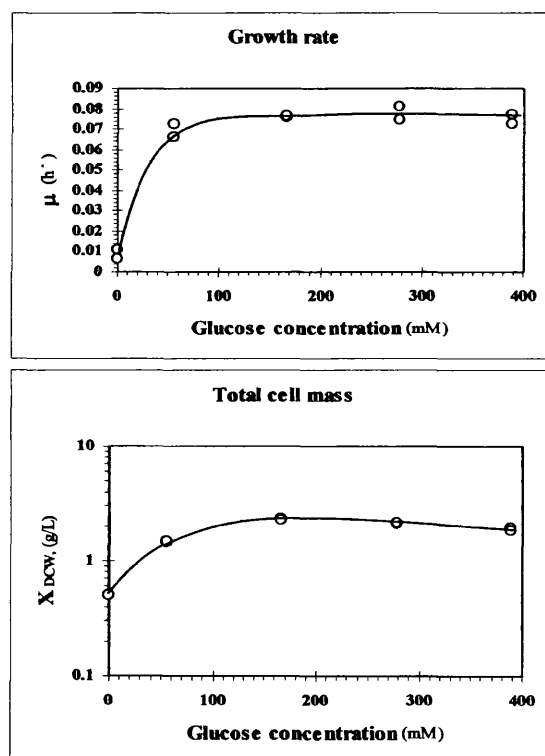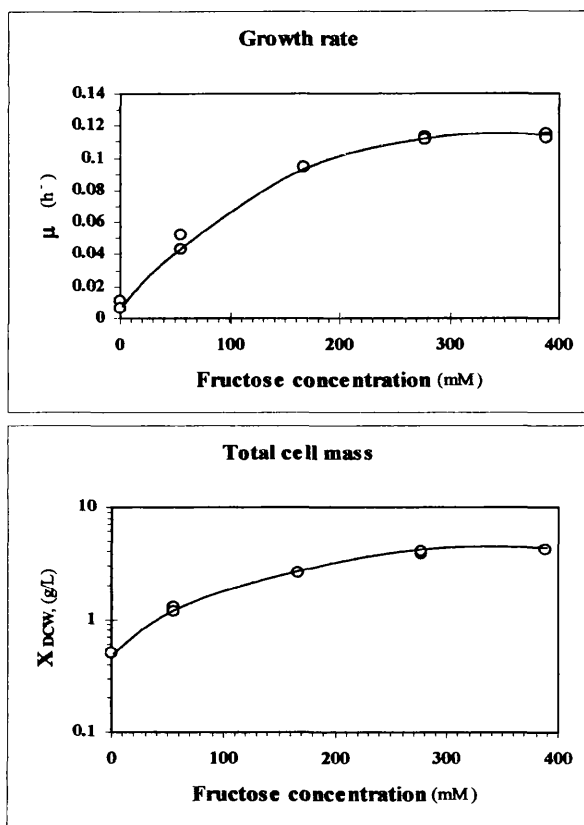

Fig.3.35 The effect of glucose concentration on the growth rate and the total cell mass during the cultivation of *Lactobacillus brevis*. *L. brevis was grown at MLM-1 changing only the glucose concentration from nil to 400 mM as a single carbon source. All experiments were duplicated, and all data were in error range of ±5%.*

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The change on glucose concentration did not affect the growth rate, but the growth was significantly affected by fructose concentration. The growth rate and total cell mass of *L. brevis* at glucose concentration over 56 mM did not increase (Fig.3.35), but the growth rate and total cell mass of *L. brevis* increased as the fructose concentration increased (Fig.3.36).

The growth rates and total cell mass in the batch cultures using fructose as carbon source were always higher than them in the cases using glucose.

Fig.3.36 The effect of fructose concentration on the growth rate and the total cell mass during the cultivation of *Lactobacillus brevis*. *L. brevis was grown at MLM-1 changing the only fructose concentration from nil to 400 mM as a single carbon source. All experiments were duplicated, and all data were in error range of ± 5%.*

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The regeneration of NAD^+ is necessary for growth of *L. brevis*. Fructose can be used not only for production of the energy, but also for regeneration of NAD^+. Ultimately, using the mixture of glucose and fructose produced better results in both of the cell product yield and the growth rate than using glucose or fructose as carbon source.

3.5.3.2. The effect of nitrogen sources on growth.

L. brevis was grown at MLM-1 with yeast extract or soy peptone to investigate the effect of nitrogen source on growth of *Lactobacillus brevis*. Yeast extract was compared with soy-peptone in terms of the growth rate and total cell mass.

Glucose (56 mM) and fructose (139 mM) as carbon source were used together in MLM-I. When yeast extract was investigated (Fig.3.37), the growth rate increased as the yeast extract concentration was increased to up to 1.0%, w/v.
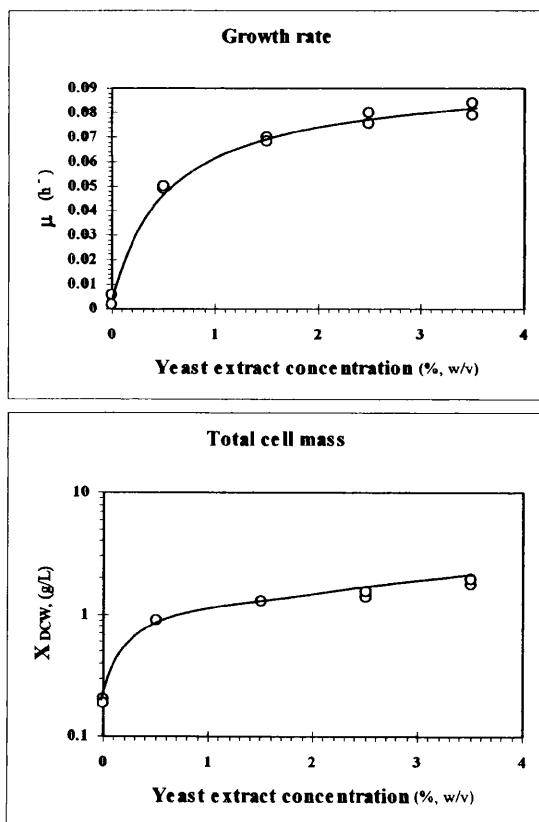
Fig.3.37 The effect of yeast extract concentration on the growth rate and the cell mass during the cultivation of *Lactobacillus brevis*. *L. brevis was grown at MLM-1 changing only the yeast extract concentration from nil to 3.5 % (w/v). The carbohydrate mixture of glucose (56 mM) and fructose (139 mM) was used as the carbon source. All experiments were duplicated, and all data were in error range of ± 5%.*

In the case of soy peptone (Fig.3.38) the growth rate increased as the soy peptone concentration increased but total cell mass did not show a big difference when compared with the case of yeast extract. Consequently, Yeast extract was selected as the nitrogen source because of its influence on the growth rate.

Growth rate

Total cell mass
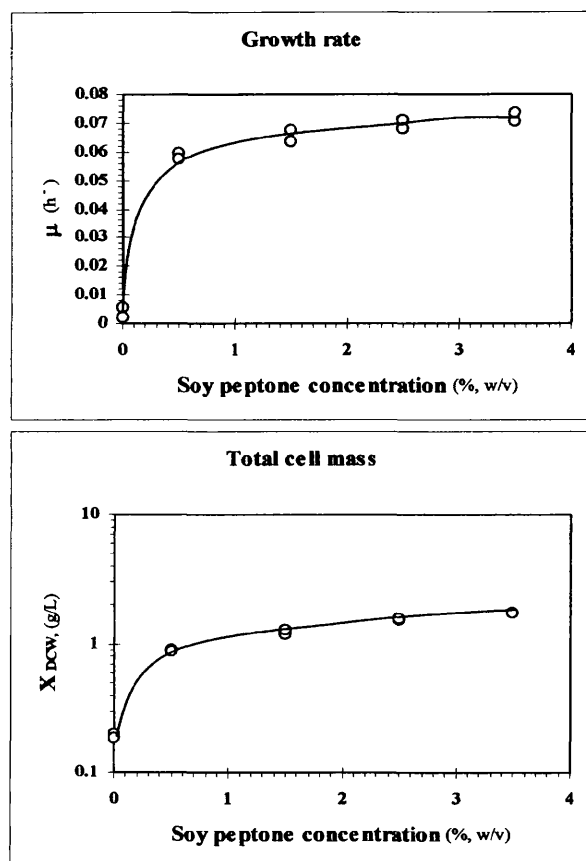
Fig.3.38 The effect of soy peptone concentration on the growth rate and the cell mass during the cultivation of *Lactobacillus brevis*. *L. breis was grown at MLM-1 changing only the soy peptone concentration from nil to 3.5 %(w/v). The carbohydrate mixture of glucose (56 mM) and fructose (139 mM) was used as the carbon source. All experiments were duplicated, and all data were in error range of ± 5%.*

Also, organic acids like citric acid and acetic acid, and these elements of two positive valences such as Mn^{++} and Mg^{++} were investigated about their effects on the growth of *Lactobacillus brevis*, but these elements showed no effect during the growth in the research range between 0 and 25 mM, and between 0 and 9 mM, respectively.

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3.5.3.3. The standard growth of L. brevis in the batch culture

L. brevis was grown at optimized growth medium and the growth conditions *(Section 2.4.2 and 2.4.2.2)*. The growth had a lag period for 6 h at the early stage of the cultivation, and *L. brevis* was grown at the growth rate of 0.2 h^{-1} during the exponential growth phase. The exponential growth phase was followed with the stationary period after 12 h (Fig.3.39).

The growth rate was inhibited to 0.047 h^{-1} during the stationary phase. This culture produced cell mass of 2.2 DCW, g/L in 26 h. The cell product yield ($Y_{X/S}$) was 0.06.
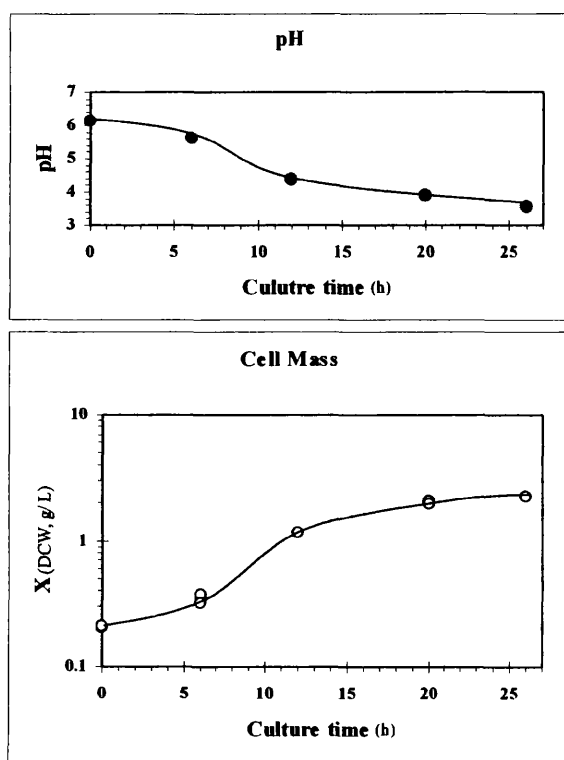
Fig.3.39 The standard growth of *L. brevis* in the batch culture. *L. brevis was grown at optimized medium (w/v) including glucose (1.0%), fructose (2.5%), KH_2PO_4 (0.39%), yeast extract (1.5%), $MnSO_4 \cdot 4H_2O$ (0.018%), Tween-80 (0.1%) and the growth conditions (Section 2.4.2). All experiments were performed in three times, and all data were in error range of ± 5%.*

3.5.3.4. The inhibition by organic acids.

Inhibition by organic acids on growth of *Lactobacillus brevis* was investigated at the optimized medium *(Section 2.4.2.2)* and the growth condition *(Section 2.4.2)*. Acetic acid and lactic acid were initially added into the growth medium of *L. brevis*, and then the growth rate was measured in 12 h. Figure 3.40 shows the results of the investigations. *L. brevis* was more resistant against organic acids than *L. buchneri*. However lactic acid was still strong growth inhibitor. The growth rate of *L. brevis* was inhibited by 50% with lactate concentration of 441 mM or with acetate concentration of 894 mM.
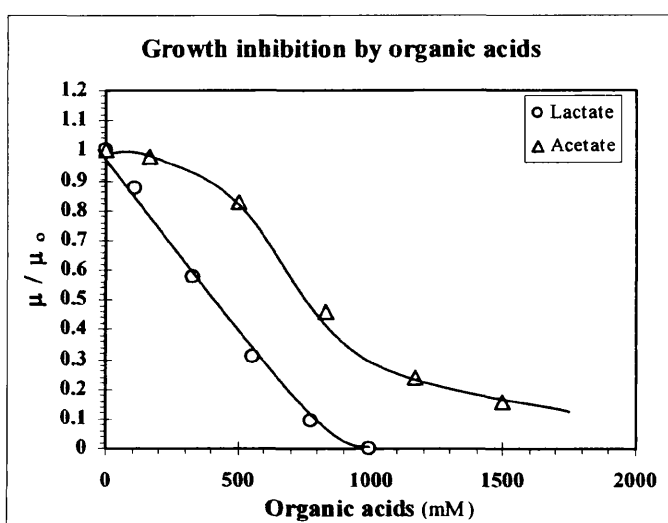
Fig.3.40 The effect of lactic acid and acetic acid on the initial growth rate of *Lactobacillus brevis*. *L. brevis was grown at optimized medium and the growth condition (Section 2.4.2 and 2.4.2.2) to gauge the inhibitory effect of organic acids. Each of organic acids was added into the growth medium before starting of the cultivation, and the growth rates were measured in 12h. The inhibition was described as the percentage to the control (μ_o) incubated without addition of organic acid. All experiments were duplicated, and all data were in error range of ±5%.*

3.6.Discussion.

3.6.1. Comparison by the growth kinetics (Table.3.1).

Lactobacillus buchneri was grown at the growth rate of 0.093 h^{-1} at the growth medium

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optimized through a series of the batch cultures. The growth medium contained 128 mM glucose, and produced a cell mass of 0.06 DCW g per g of consumed glucose, which is product yield. Ultimately, it produced the total cell mass of 1.12 DCW, g/L in 53 h.

Lactobacillus brevis was grown at the growth rate of 0.2 h^{-1} in the optimized growth medium including 56 mM glucose and 139 mM fructose. When compared to growth of *L. buchneri*, *L. brevis* kept growing after the exponential growth phase even if it grew at a low growth rate. Ultimately, the cultivation could produce the total cell mass of 2.2 DCW, g/L in 26 h. The cell product yield was 0.086.

Cunha and Foster. [1992] investigated the effect of glycerol-sugar co-fermentation on growth of heterofermentative lactic acid bacteria such as *L. buchneri and L. brevis*. They obtained 0.093 h^{-1} the growth rate in the case where grow *L. buchneri* in glucose and glycerol and 0.035 h^{-1} the growth rate in only the case where glucose was used as a single carbon source. The growth rate increased by 2.66 times than that in investigation by Cunha and Foster [1992] if comparing the case where glucose was used as a single carbon source. Furthermore considering the case of *L. brevis*, the growth rate was higher 7.41 times using fructose than that using glycerol.

Bifidobacterium longum was grown at the growth rate of 0.232 h^{-1} in the optimized medium including glucose of 56 mM and fructose of 50 mM during 24 h of the cultivation, and ultimately it could produce the total cell mass of 3.2 DCW, g/L. Product yield was 0.17.

The growth of *B. longum* was investigated by Hyun et al. [1995]. He obtained 0.042 h^{-1} the growth rate while growing *B. longum* in the growth medium containing lactose as a single carbon source and peptone and yeast extract as nitrogen source and 0.118 h^{-1} the growth rate in the growth medium containing glucose as single carbon source. Consequently, using a mixture of glucose and fructose, the growth rate of *B. longum* increased by 1.97 times than case where using glucose as a single carbon source.

Finally, *Oenococcus oeni* was grown at the growth rate of 0.081 h^{-1} in the optimized growth medium including 139 mM glucose and 56 mM fructose.

Chapter.3 Growth of lactic acid bacteria and optimization of batch culture

The cultivation could produce the total cell mass of 0.91 DCW, g/L in 48 h. Product yield was 0.026.

Richter et al. [2001, 2003] investigated the growth of *O. oeni* in the growth medium containing lactose or glucose. In the pH uncontrolled STR culture, he obtained 0.034 h^{-1} the growth rate in the growth medium containing glucose and 0.065 h^{-1} with lactose. Saguir and Manca de Nadra [2002] investigated the effect of L-malic acid and citric acid with amino acids on growth of *O. oeni*. In the basic medium containing a sufficient ingredient necessary for the growth, he obtained the growth rate 0.094 h^{-1}.

Strains	μ (h^{-1})	$Y_{x/s}$ (g/g)	X (DCW, g/L)	Time (h)	$P_{x/t}$ (DCW,g/L,h)	Growth inhibition (50%) by Organic acid	
						L(mM)	A(mM)
L. buchneri	0.093	0.06	1.120	53	0.021	332	1490
L. brevis	0.20	0.06	2.224	26	0.086	441	894
B. longum	0.23	0.17	3.230	24	0.135	182	131
O. oeni	0.08	0.03	0.907	48	0.019	217	779

Table.3.1. Comparing the growth kinetics and the growth inhibition by organic acids during the batch culture. *Tolerance was presented with the organic acid concentration to reduce the growth rate by 50%. Total cell mass was determined at the end of the cultivation. μ; the specific growth rate, $Y_{x/s}$; product yield, $P_{x/s}$; specific production rate. L; lactate, A; acetate*

The inhibitory effect of organic acids on growth of lactic acid bacteria was presented as the organic acid concentration required for reduction on the growth rate by 50%. In Table.3.1, tolerance of *B. longum* against organic acids was relatively low to other bacteria. In general, it has been known that *Bifidobacterium* species against organic acids is the most weak tolerant to the organic acids.

In contrast *L. buchneri* was resistant against acetic acid, but it was weaker tolerant than *L. brevis* against lactate. *O. oeni* showed low tolerance against lactate.

Table 3.1 shows the growth kinetics and the inhibition of organic acids on growth of four different types of lactic acid bacteria as described above.

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3.6.2. Comparison by nutritional requirements or physiological features.

Lactobacillus buchneri was grown at the shaking culture of 30°C. *L buchneri* was grown at this medium without any other special nutrient requirements. This is contrast to the result obtained with the synthetic medium by Ledesma et al. [1977]. However it seems to grow by higher cell mass when $MgSO_4$ was at high concentration. Yeast extract, which stimulates both of the growth rate and the cell yield, was used at the concentration of 0.9% (w/v) after comparison with soy peptone. For optimum cultivation initial pH from 5.8 to 6.0 gave the highest growth rate and product yield.

Lactobacillus brevis was grown at the shaking culture of 28 °C. The cultivation of *L. brevis* reached the maximum growth rate at 1.5% (w/v) the higher yeast extract concentration than that of *L. buchneri*. It required fructose for high growth rates. Mannitol accumulated can be consumed through an anaerobically induced Phosphoenolpyruvate-dependent fructose-specific Phosphotransferase system after conversion to fructose at the final stage because the enzymatic reaction by mannitol dehydrogease in *L. brevis* is reversible [Martinez et al. (1963), Saier et al. (1996)].

They proved that fructose can be metabolized by fructose-1-phosphate kinase and fructose-1,6-bisphosphate aldolase induced in the presence of fructose under anaerobic conditions.

The requirement for the minerals except for $MnSO_4$ was not detected, furthermore when it was given to the growth medium, growth was inhibited. When the cultivation was begun in a pH range from 5.5 to 6.0, high growth rate and cell mass were obtained.

Bifidobacterium longum was grown at the shaking culture of 37°C. The cultivation of *B. longum* was undertaken with 3% (w/v) yeast extract, and carbon sources were used in low concentration of 56 mM glucose and 50 mM fructose so as to reduce the production of polysaccharides. *B. longum* particularly required ammonium sulphate with yeast extract as nitrogen source. Only when yeast extract was used with ammonium sulphate, the mixture acts as growth stimulant.

The growth of *B. longum* was also stimulated when it was begun at the neutral pH. When the growth medium was formulated, the pH was in a pH range from pH 6.0 to pH 6.5.

Oenococcus oeni was grown at the growth medium including the mixture comprising glucose and fructose and growth temperature was 28 °C. Fructose stimulated the growth rate of *O. oeni* with glucose. Fructose enables *O. oeni* to metabolize glucose regenerating NAD^+ like the case of *L. brevis*. Also, as the growth stimulant, citric acid was used to be co-metabolized. Even if citric acid increases the production of acetic acid, it is very helpful for the growth of *O. oeni* because of more production of ATP and NAD^+.

4

GROWTH OF LACTIC ACID BACTERIA IN STIRRED TANK

CHAPTER 4: GROWTH OF LACTIC ACID BACTERIA IN A STIRRED TANK REACTOR.

4.1. INTRODUCTION.

To gain further understanding of growth kinetics these four different strains was grown in a stirred tank reactor (STR). The working volume in the STR was 3 L, which is 10 times larger than that batch culture. The STR creates a more realistic production system than batch culture. The bacterial physiology such as the concentration of the carbohydrate and pH control level can be more precisely investigated.

The optimum level for pH control was first studied as this would be required in the operation of membrane cell recycling reactor. The critical concentration of carbon sources, which should be maintained during the growth of LAB in membrane cell recycling reactor, was also investigated.

All optimized growth conditions should be as economic as possible to produce a high cell concentration. The judgements of these investigations can be made in basis of the cell product yield and the growth rate as in the previous chapter (Chapter 3).

4.2. THE GROWTH OF LACTOBACILLUS BUCHNERI IN THE STR.

4.2.1. The standard growth of Lactobacillus buchneri in the STR.

Lactobacillus buchneri was grown in the 5 L reactor with working volume of 3 L during the cultivation of 30 h. Initial pH was adjusted to pH 5.8, and then pH dropped to pH 5.5 with the inoculation due to acidic pH of the starter culture.

Figure 4.1 shows typical pH controlled cultivation. During incubation in 30 °C, the pH dropped quickly, and controlled at a pH 5.2 with 5 M NaOH until the end of the cultivation. The composition of the growth medium was used as in the batch culture *(Section 2.4.2.1)*.

The cultivation showed a dramatic increase in the growth rate from 0.097 h^{-1} in the batch culture to 0.2 h^{-1} during the exponential growth phase in the pH-controlled STR.
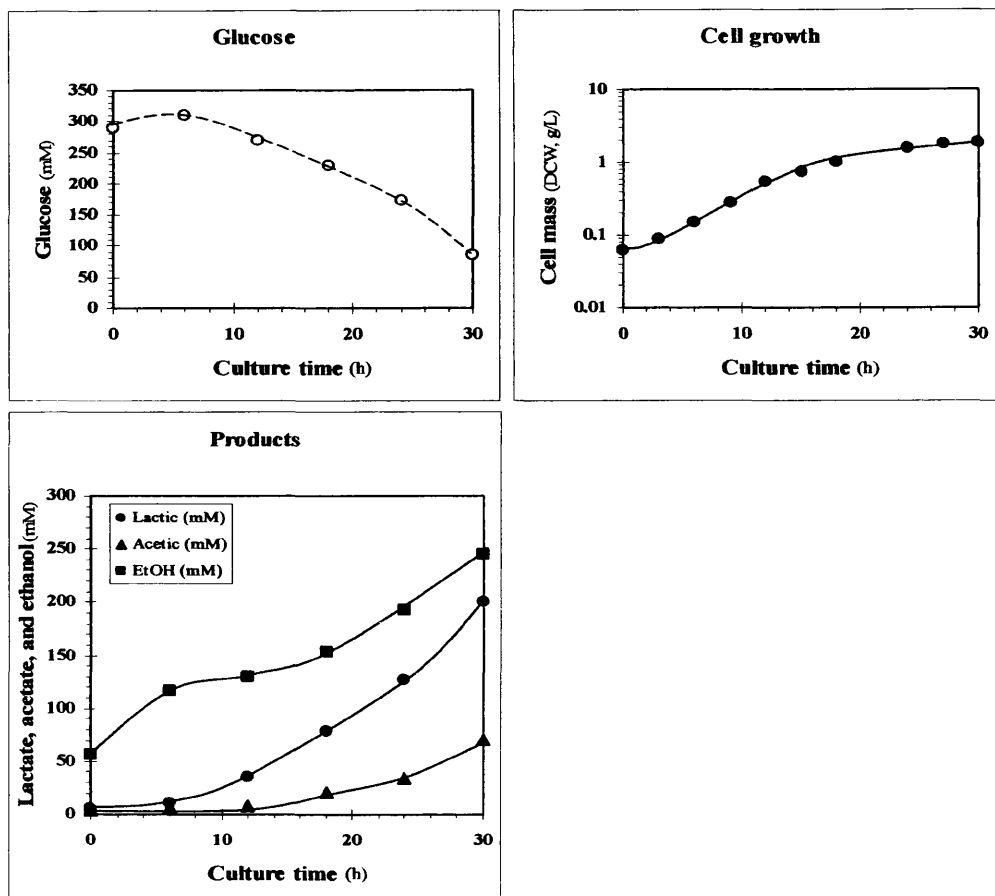
Fig.4.1. The growth of *Lactobacillus buchneri* during the cultivation in the pH-controlled STR. *The cultivation was controlled at pH 5.2 with 5M NaOH, and initial pH was set at pH 5.8. The composition of the growth medium was the same as that in the batch culture (Section 2.4.2.1) containing glucose of 310 mM. The cell mass was grown to 1.83g/L in 30 h. The production of lactic acid, acetic acid, and ethanol were monitored with the glucose consumption during the culture.*

Ultimately the culture produced total biomass of 1.83 DCW, g/L in 30 h. The cell product yield, $Y_{x/s}$ was 0.068 higher than 0.057 observed in the batch culture. After this period, the growth rate declined to 0.06 h^{-1} for the stationary phase from 15 h to the end of the cultivation due to the end product inhibition even if there was still carbon source enough to use. In chapter.3, when inhibition by organic acid was investigated, *L. buchneri* showed good tolerance against acetate, but its growth was inhibited by 50% at 332 mM of lactate *(Section 3.2.3.4)*.

Chapter.4 Growth of Lactic Acid Bacteria in a Stirred Tank Reactor.

Figure 4.1 shows the production rate of all organic acids also increased rapidly from 15 h the start of the stationary phase, and consequently the growth rate dropped after 20 h.

The stoichiometry study was carried out with 278 mM glucose as single carbon source (Fig.4.1) and was as follows:

Glucose (1M) $\rightarrow$ Lactate (0.8M) + Acetate (0.3M) + Ethanol (0.85M) + CO_2 (1.15M)

The carbon recovery was 95%. *L. buchneri* can degrade approximately one M of lactic acid to 0.5 M acetic acid, 0.5 M 1,2-propanediol with 0.5 M CO_2 producing ATP. This novel pathway of *L. buchneri* for anaerobic lactic acid degradation, which is activated at acidic pH lower than pH 4.5, is entirely pH dependent [Cunha and Foster. (1992), Stefanie et al. (2001)]. Since glucose was not limiting at the end of the cultivation and pH was maintained at over pH4.5, more lactate was produced during the cultivation. But, there may be a possibility for the presence of 1,2-propanediol or 1,3-propnediol even though this was not investigated, but from the carbon balance it says convectively that significant diols can be produced.

4.2.2. The effect of glucose concentration.

The growth of *L. buchneri* was investigated in a range from 3% (w/v) to 7% (w/v) of glucose with proportionately increasing yeast extract concentration from 0.9% (w/v) to 2.1% (w/v) at the same time. The growth conditions and the concentrations of other ingredients excluding glucose and yeast extract in the growth medium were the same as them in the batch culture *(Section 2.4.2.1)*. The results of this study are shown below at Fig 4.2.

The maximum growth rate increased in proportion to the glucose concentration, but the lag phase was extended at the glucose concentration up to 278 mM, implying substrate inhibition to certain extent.
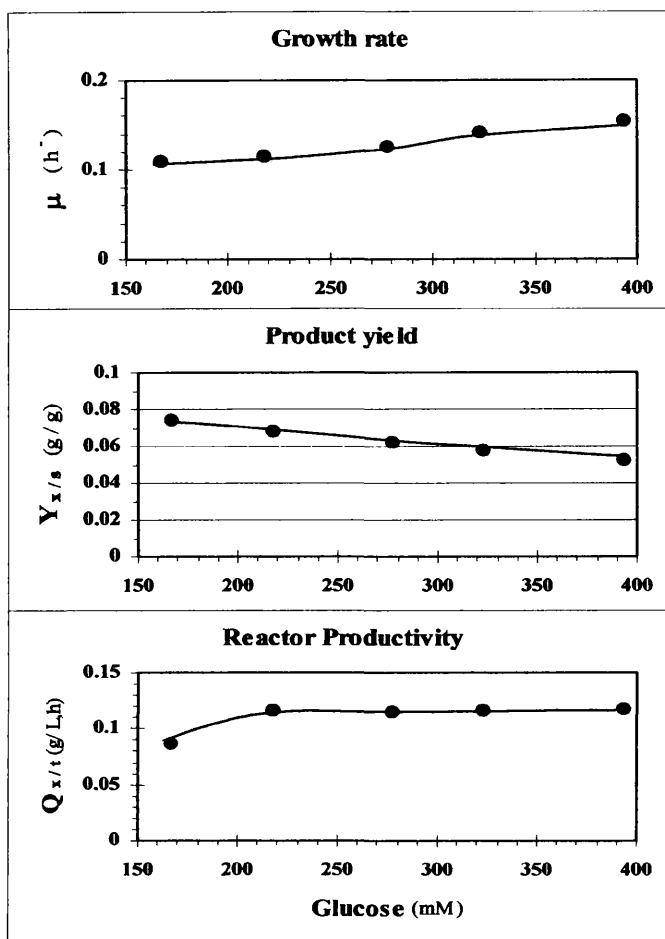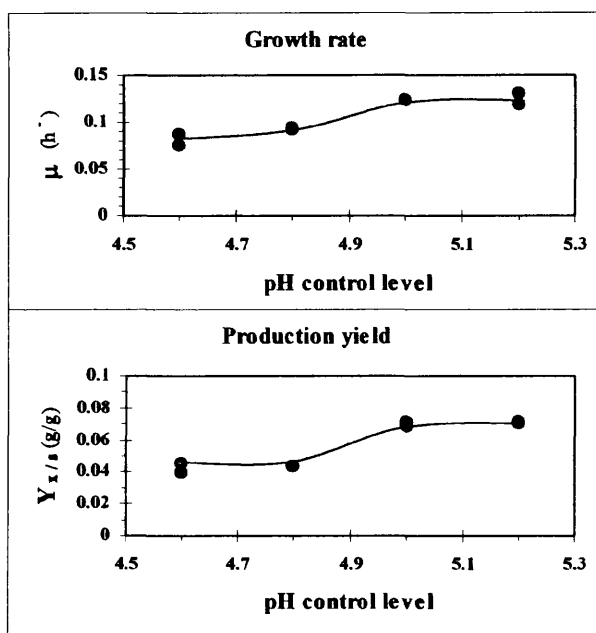
Fig.4.2. The effect of glucose concentration on the growth rate, the volumetric productivity, and the product yield during the cultivation of *Lactobacillus buchneri*. *The glucose concentration was increased from 167 mM to 38 9mM while yeast extract (w/v) being increased from 0.9 to 2.1%, respectively. The product yield (g/g) was displayed in g of produced biomass per g of consumed glucose. The production rate was displayed as g/L of biomass produced for a unit time (h).*

The product yield was decreasing in basis of the glucose consumption with increasing glucose concentration, but the growth rate increased. The productivity (g of produced biomass/L. h) increased up to 200 mM of glucose, but showed a plateau without more increase in the glucose concentration above this (Fig.4.2) and reflects the existence of the critical glucose concentration for the fast growth of LAB. Glucose over the critical concentration was not necessary for more cell growth leading to the extension of total culture time with longer period of the stationary growth phase.

4.2.3. The effect of pH control level.

L. buchneri was grown at the optimized medium containing glucose 167 mM and growth conditions previously used for the batch culture (Section 2.4.2.1). The cell growth was initiated at pH 5.8, and pH quickly dropped to pH 5.2 the control level. The pH control was begun with feeding of alkaline solution (5 M NaOH). The highest growth rate was found when pH control level rose from pH 5.0 to pH 5.2 (Fig.4.3).

Fig.4.3. The effect of pH control level on the growth rate and the product yield during the cultivation of Lactobacillus buchneri. The same growth medium as that in the batch culture was used with glucose concentration 167 mM. The initial pH was set at pH 5.8 and the culture was allowed to drop to pH control level before being controlled. The product yield was displayed as g of produced biomass per g of consumed glucose.

The growth rate and product yield were reduced when pH control was at lower pH than pH 5.0. Growing LAB at low pH diverts more portion of the growth energy into the maintenance energy to survive LAB in low pH circumstance so reducing the overall yield.

Chapter.4 Growth of Lactic Acid Bacteria in a Stirred Tank Reactor.

4.3. THE GROWTH OF LACTOBACILLUS BREVIS IN THE STR.

4.3.1. The standard growth of Lactobacillus brevis in the STR

Lactobacillus brevis was grown at the growth medium and the growth condition previously used for the batch culture containing 138.9 mM glucose and 56 mM fructose for 36 h. Initial pH was set in a range from pH 6.0 to pH 6.4, and pH quickly dropped with the growth of *L. brevis*. Temperature was controlled at 28 °C *(Section 2.4.2.2)*. With 195 mM carbohydrate consumed, the culture produced a total biomass of 2.33 DCW, g/L in 36h. Likewise the cell product yield (Yx/s, DCW, g of biomass/g of consumed carbohydrate) was 0.066. *L. brevis* was grown at the growth rate of 0.162 h^{-1} during the exponential growth phase.

The results of the experiments were shown at Fig 4.4. Figure 4.4 shows that *L. brevis* was grown up with a short lag phase and grown rapidly during the exponential phase until 12 h and then a slower log phase (μ = 0.034 h^{-1}) until the end of the culture.

A study of the stoichiometry was established during the growth of *L. brevis* in the STR. The end products were lactic acid, acetic acid, ethyl alcohol, and mannitol with CO_2 consuming glucose (139 mM) and fructose (56 mM) and gave the following overall reaction with a carbon recovery of 97%.

Hexose (1M)→Lactate(0.8M)+Acetate(0.25M)+Ethanol(0.87M)+Mannitol(0.03M)+CO$_2$(1.0M)
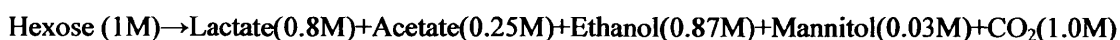
From the stoichiometry, a part (29.5 mM) of used fructose (56 mM) was converted into mannitol for the regeneration of NAD$^+$ in the middle of the culture and, after the shortage of fructose in the medium, mannitol (5.7 mM) slowly disappeared by the end of the cultivation. However, after fructose was limited and the growth rate reduced, a second growth corresponds to the consumption of the residual glucose and mannitol [Peterson and Fred. (1920)].

From the material balance, 3% carbon, 0.83% oxygen, and 4.7% hydrogen were not accounted for the cultivation. The unrecoverable materials may be small amounts of glycerol or 1,2 -propanediol [Taylor and Kung, Jr. (2002), Driehuis et al. (1999)].

Chapter.4 Growth of Lactic Acid Bacteria in a Stirred Tank Reactor.
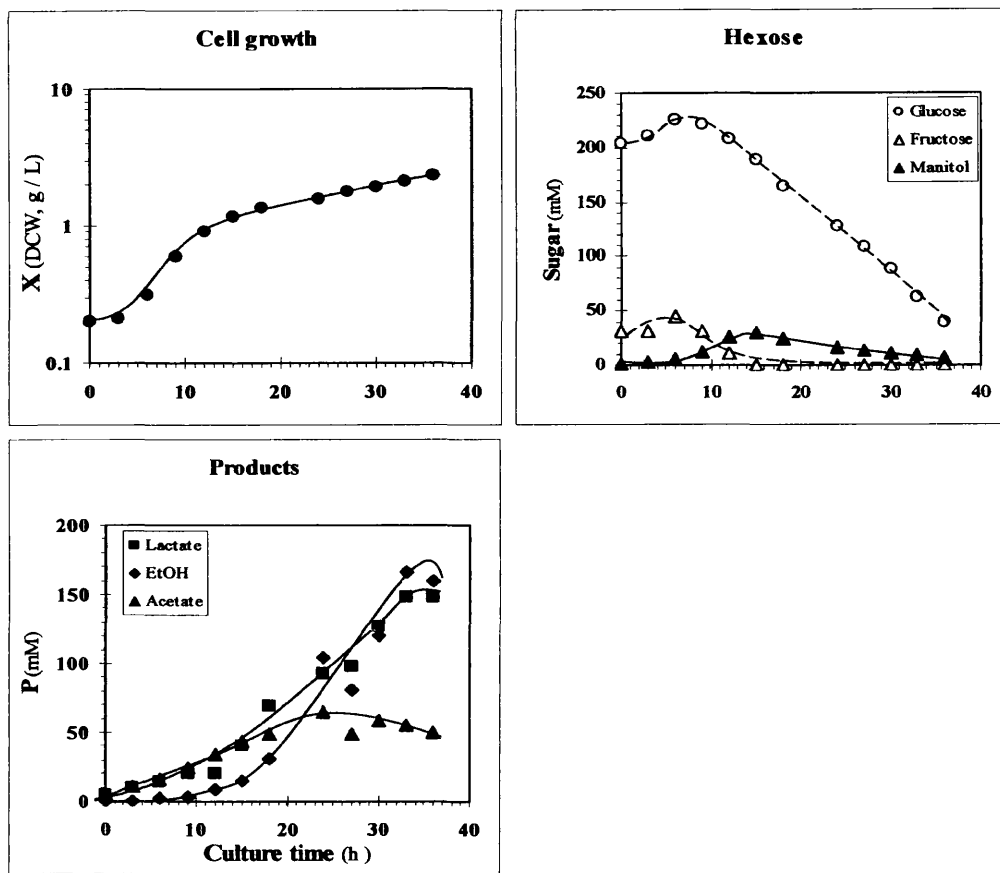
Fig.4.4. The growth of *Lactobacillus brevis* on the pH-controlled batch culture (pH 6.0). *L. brevis was grown at the growth medium as in the batch culture containing glucose 139 mM and fructose 56 mM for 36 h. Initial pH was set in a range from pH 6.0 to pH 6.4, and pH quickly dropped with the growth of L. brevis. Temperature was controlled at 28 °C. Lactic acid of 144 mM, acetic acid of 44 mM, ethanol of 159 mM, mannitol of 5.7 mM were produced with the cell mass of 2.33 g/L. Particularly, mannitol was accumulated by 29.5 mM in the middle of the fermentation, but it slowly disappeared when fructose was limited.*

4.3.2. The effect of the carbohydrate concentration on growth.

To investigate an effect of glucose concentration on the growth of *L. brevis*, the concentration of glucose with the yeast extract concentration is increased in proportion while the fructose concentration was fixed at 56 mM (Fig.4.5). When the glucose concentration increased from 138 mM to 243 mM, the yeast extract concentration (w/v) was proportionately increased from 1.5% to 2.63%. The composition of the growth medium except for the carbohydrate and yeast extract was the same as that in the batch culture.

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Initial pH was pH 6.0, and pH quickly dropped down to pH control level pH 5.5 with the growth of *L. brevis (Section 2.4.2.2)*.

The growth rate showed a peak at the glucose concentration of 170 mM, but the cell product yield decreased at the glucose concentration of 179 mM. The cell product yield did not change significantly over the range of glucose used. End product inhibition seams to reduce the growth rate and product yield in a high sugar concentration.
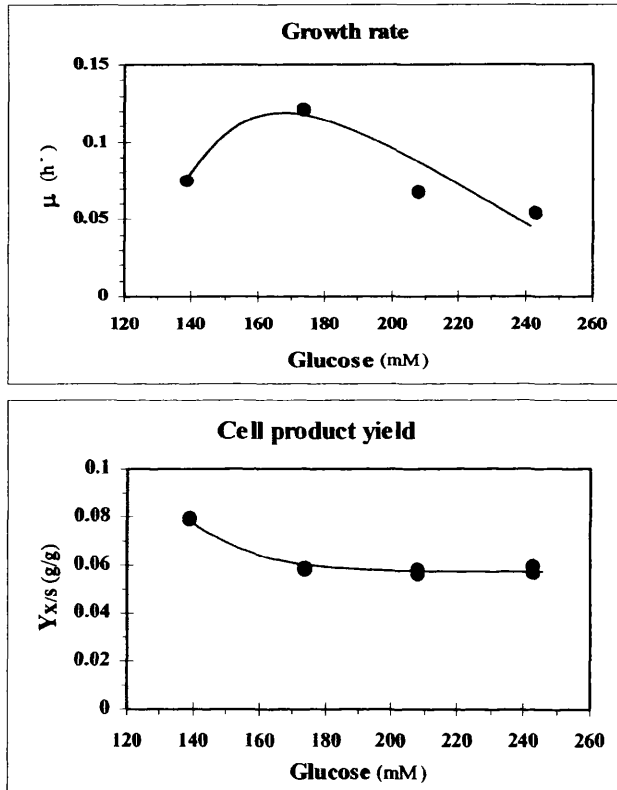
Fig.4.5. The effect of the glucose and yeast extract concentration on the growth rate and the cell product yield during the cultivation of *Lactobacillus brevis*. *The glucose concentration was increased from 139 to 243 mM while yeast extract concentration (w/v) being increased from 1.5% to 2.63%. The concentration of fructose was fixed at 56 mM. Initial pH was set at pH in certain range between pH 6.0 and pH 6.4, and pH was controlled at pH 5.5.*

Fructose plays an important role in growth significantly stimulating cell product yield and the growth rate.

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It was thought that the switch from mannitol to ethanol significantly slows down regeneration rate of NAD^+ (Fig 4.4). Consequently, fructose (56 mM) can be used for consuming 170 mM glucose at least. At high glucose concentration, the majority of glucose was consumed to produce ethyl alcohol for regeneration of NAD^+ rather than acetate and a high cell mass resulting in the lower cell product yield (Fig.4.5).

4.3.3. Investigating pH control level.

To search for the optimum pH control level, an investigation was performed over a pH range between pH 5.2 and pH 6.2. Initial pH of the cultivation was set in a pH range between pH 6.0 and pH 6.4. The composition of the growth medium was used as in the batch culture, and the cultivation was started with the mixture containing 138.9 mM glucose and 56 mM fructose *(Section 2.4.2.2)*. The growths are presented in Fig 4.6. The growth rate did not change significantly over the range investigated.
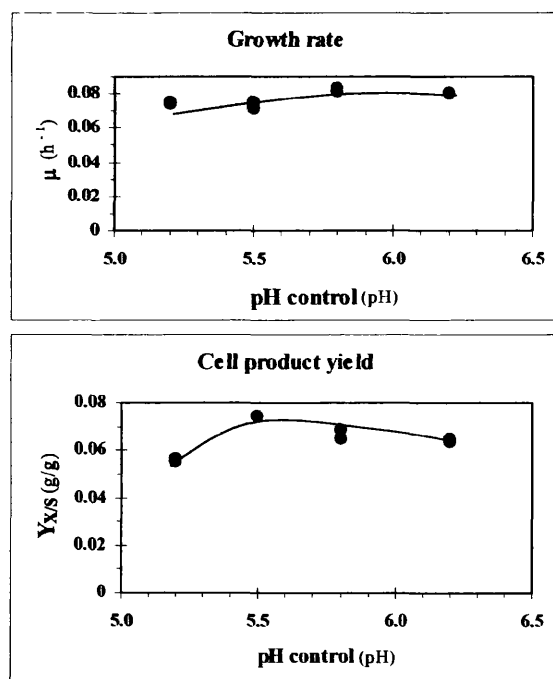
Fig.4.6. The effect of pH on the growth rate and cell product yield during the pH-controlled batch growth of *Lactobacillus brevis*. *The cultivation was performed with the same medium as that in the batch culture containing the mixture of 140 mM glucose and 56 mM fructose as carbon source. Initial pH was set in a certain range between pH 6.0 and pH 6.4. When pH reached pH 5.5, pH control was started with 5M NaOH solution.*

The maximum cell product yield increased in a range between pH 5.5 and pH 5.8. Consequently, the optimum pH control level was found between pH 5.5 and pH 5.8.

4.4. THE GROWTH OF BIFIDOBACTERIUM LONGUM IN THE STR.

4.4.1. The standard growth pattern of Bifidobacterium longum in pH controlled STR.

Figure 4.7 shows the standard growth pattern of *Bifidobacterium longum* in the STR. The cultivation of *B. longum* in the STR was performed with the same medium as the batch culture containing carbohydrate mixture of glucose 54.4 mM and fructose 38.4 mM. Initial pH was set in a range between pH 6.0 and pH 6.4. When pH dropped to pH 5.8, pH control was achieved by the addition of 5M NaOH solution *(Section 2.4.2.4)*.

The major end product was acetic acid and it was produced on the growth phase with glucose and fructose consumed in the same rate. The growth rate reached 0.28 h^{-1} during the exponential growth phase and the biomass (DCW) of 4.04 g/L was obtained in 11 h (Fig.4.7). The following stoichiometry was established.

Hexose (2M) → Lactate(1.2M)+Acetate(3.4M)+Formate(0.8M)+ETOH(0.4M)

According to the stoichiometry, 2 M glucose generated 1.2 M lactic acid, 3.4 M acetic acid with 0.8 M formate and 0.4 M ethanol. Generally speaking, 2 M glucose can produce lactic acid and acetic acid in proportion of 2:3 as usual, but *B. longum* can produce more acetic acid with a little of ethanol, and formate during the glucose-limited growth [Degnan and Macfarlane. (1994), Lauer and Kandler. (1976)]. The amount of formate produced was theoretically assumed from the amount of acetate and ethanol empirically detected. According to the stoichiometry mentioned above, lactic acid 0.8 M missed from the normal cultivation must be divided into 0.4 M acetic acid and 0.4 M ethanol. The amount of formate must be the total (0.8 M) of the increasing part on acetic acid and ethanol empirically detected.

Phosphoroclastic enzymes in *B. longum* can divert 1 M pyruvate into 0.5 M acetate and 0.5 M ethanol with 1 M formate. Consuming pyruvate, *B. longum* can produce more acetate, ethanol, and formate with more ATP in the glucose-limited condition.

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Chapter.4 Growth of Lactic Acid Bacteria in a Stirred Tank Reactor.

According to Lauer and Kandler [1976], the extent of phosphoroclastic splitting of pyruvate varies with strains even among the same species.
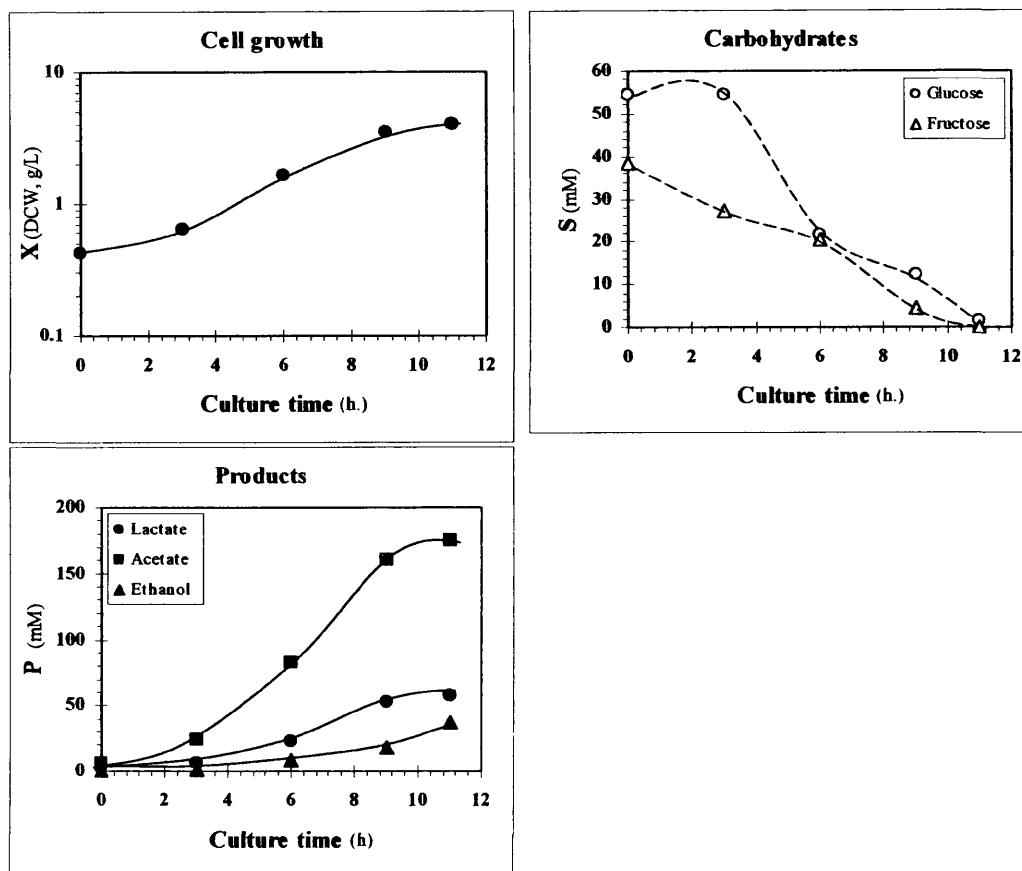
Fig.4.7. The growth of *Bifidobacterium longum* in the pH-controlled batch culture. *B. longum was cultivated in the growth medium used for the batch culture containing carbohydrate mixture of 55 mM glucose and 38 mM fructose (Section 2.4.2.4). Initial pH was set in a range between pH 6.0 and pH 6.4. When pH dropped to pH 5.8, pH control was started with 5M NaOH solution. The cultivation produced the cell mass 4.04 DCW, g/L, 55 mM lactate, and 169 mM acetate with 37 mM ethanol in 11 h.*

4.4.2. The effect of the carbohydrate concentration on growth of B. longum.

The glucose concentration was increased from 56 mM to 167 mM using the growth medium and the growth conditions used for the batch culture *(Section 2.4.2.4)*, while the concentration of fructose being fixed at 56 mM (Fig.4.8).

Increasing the glucose concentration proportionately increase the total organic acids formed with reduction in the cell yield and little or no improvement in the growth rate.

Chapter.4 Growth of Lactic Acid Bacteria in a Stirred Tank Reactor.

Also at the high glucose concentration over 167 mM, an extension in the lag phase was observed at the early stage of the culture. Such a phenomenon was also detected when fructose concentration was increased with the fixed glucose concentration of 56 mM (Fig.4.8). Increase of the fructose concentration can activate phophoroclastic pathway producing more acetate and more cell mass apart from the growth rate. That is, total carbohydrate concentration in the growth medium must be controlled below 150 mM to avoid the substrate inhibition while controlling the cultivation with carbon source. Figure 4.9 shows increase of the acetate concentration in the production when fructose concentration was increased in the growth medium.
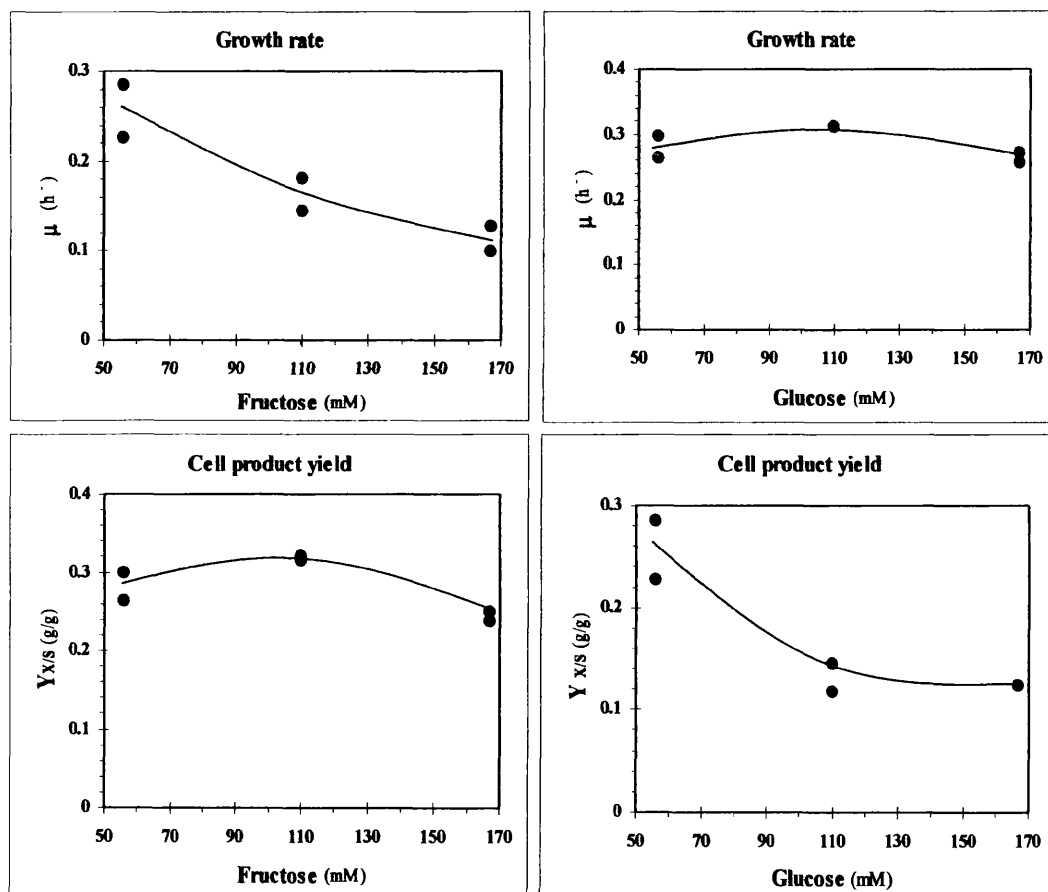
Fig.4.8. The effect of the carbohydrate concentration on the growth rate and the cell product yield during the cultivation of *Bifidobacterium longum*. *Glucose and fructose were used as carbon source. The glucose concentration increased from 56 mM to 167 mM while fructose concentration being fixed at 56 mM. When the fructose concentration increased in the same range, the glucose concentration was fixed at 56mM. The composition of the growth medium and the growth condition were the same as them in the batch culture.*

According to Fig.4.9, the lactate concentration does not show an increase, but the acetate concentration does increase. That is, when the fructose concentration was increased in the growth medium, the productivity of acetate increases with the production of energy through phosphoroclastic enzyme route in anaerobic condition. *B. longum* can produce more ATP producing acetate instead of producing lactate from pyruvate [Degnan and Macfarlane. (1994), Lauer and Kandler. (1976)]. Therefore, that energy could be used for more production of the cell mass or the cell maintenance.

High C:N ratio in the growth medium and use of lactose can lead to production of exopolysaccharides ensuring increase in viscosity of the culture fluid, and it may lead to very serious malfunction in the MCR by the membrane fouling [Roberts et al. (1995), Andaloussi et al. (1995)].
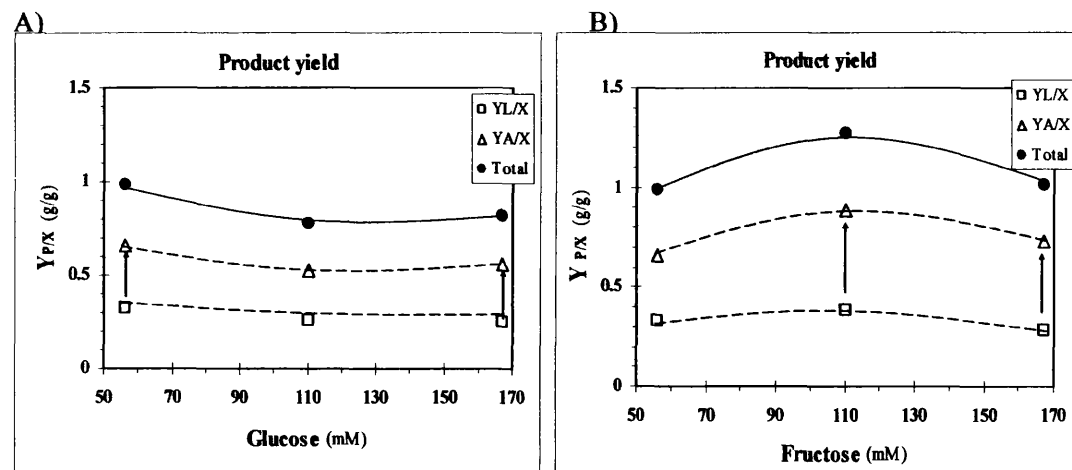
A)

B)

Fig.4.9. The effect of the carbohydrate concentration on the production of organic acids during the cultivation of *Bifidobacterium longum*. *Glucose and fructose were used as carbon source. A) The glucose concentration increased from 56 mM to 167 mM while fructose concentration being fixed at 56 mM. B) When the fructose concentration increased in the same range, the glucose concentration was fixed in 56 mM. The composition of the growth medium and the growth condition were the same as them in the batch culture. When fructose concentration in the growth medium was increased, the productivity of acetate increased whereas the productivity of lactate did not increase*

4.4.3. Investigating pH level to be controlled during the cultivation.

For the investigation of optimum pH controlled level, the cultivation was performed with the same medium and the same growth condition as in the batch culture *(Section 2.4.2.4)* containing the carbohydrate mixture of glucose (110 mM) and fructose (56 mM). Figure 4.10 shows the result of the investigation.
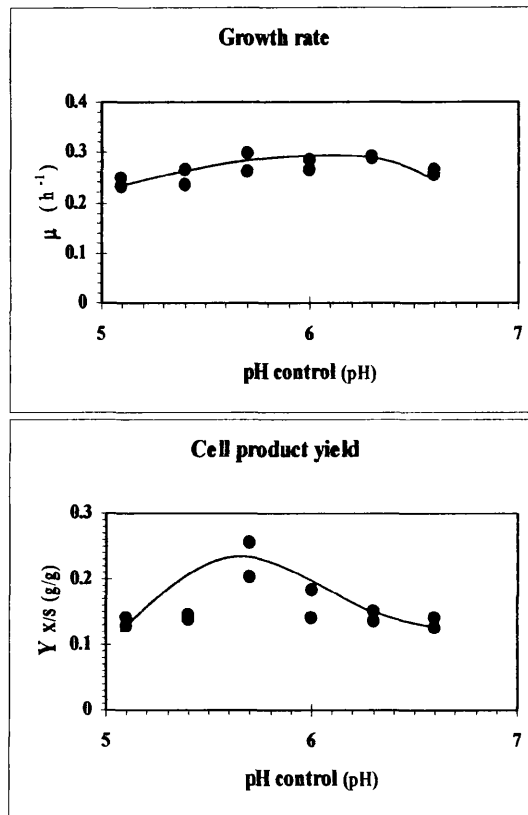
Fig.4.10. The effect of pH control level on the growth rate and the product yield during the cultivation of *Bifidobacterium longum. Initial pH was set in a range between pH 6.0 and pH 6.4. When pH reached the control level, pH control was started with 5M NaOH solution. The cultivation was carried out with the growth medium and the growth condition used in the batch culture containing the carbohydrate mixture of glucose (110 mM) and fructose (56 mM).*

The growth rate of *B. longum* in the STR showed a steady increase as the pH control level rose from pH 5.1 to pH 6.6. The cell product yield also showed a peak in pH 5.7 or pH 5.8. But, since the change in the growth rate was small, the pH was controlled at pH 5.8.

4.5. THE GROWTH OF OENOCOCCUS OENI IN THE STR.

4.5.1. The standard growth of Oenococcus oeni in pH controlled STR

The cultivation was carried out with the growth medium and the growth condition used as described in the batch culture *(Section 2.4.2.3)* containing the carbohydrate mixture of glucose (56 mM) and fructose (167 mM). Initial pH was set in a range between pH 5.5 and pH 5.8, and pH quickly dropped to the control level. At pH 4.8, the pH was controlled by addition of 5M NaOH.

Figure 4.11 shows the results of the growth experiment. The growth rate was 0.108 h^{-1} at the exponential growth phase for about 20 h. Glucose and fructose were simultaneously consumed with the production of mannitol. The major end products were acetate and lactate with little or no ethanol.

According to Cogan [1987], *O. oeni* can produce lactate and ethanol with CO_2 without fructose in the growth medium. However, in this investigation ethanol was not produced because of addition of fructose. Instead, acetate produced was associated with improved yield. Fructose was used to regenerate NAD^+ producing mannitol instead of producing ethanol from pyruvate. The mannitol produced was not consumed again and accumulated in the end of the cultivation.

That is, fructose of 56 mM was reduced into mannitol to regenerate NAD^+ while glucose of 150 mM was consumed for the production of end products and the cell growth. The ideal ratio of glucose to fructose in the mixture was 3:1.

In another report, Richter et al. [2001] explained over production of acetate during the limitation of pantothenate in glucose fermentation. According to their report, HSCo-A and acetyl-CoA are coenzymes of phosphotransacetylase and acetaldehyde dehydrogenase, and pantothenic acid is the structural component of coenzyme A. Considering their function in the metabolic pathway to produce ethanol, if there is a limitation of pantothenate in fermentation of *O. oeni*, more metabolites might be shifted to produce erythritol, acetate and glycerol.

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Cell growth

Hexose

Products
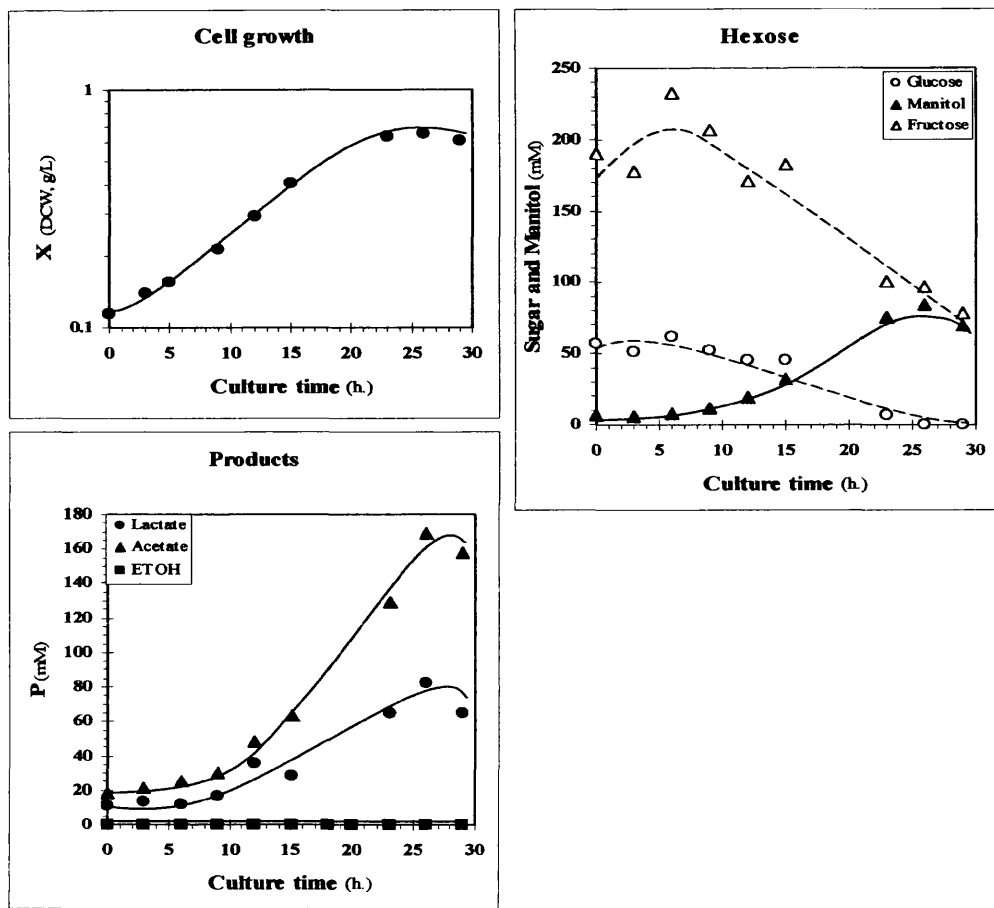
Fig.4.11. The growth of *Oenococcus oeni* in the pH-controlled batch culture. *O. oeni was cultivated with the same medium as that in the batch culture containing the carbohydrate mixture of glucose (56 mM) and fructose (167 mM). Initial pH was set in a range between pH5.5. When pH reached pH4.8, pH control was started with 5M NaOH solution. Mannitol, lactate, acetate, and ethanol were produced with the cell mass of 0.542 DCW, g/l in 30 h.*

The stoichiometry for *Oenococcus oeni* from the end product analyses was determined and was as follows.

Hexose (1M) → Lactate(0.4M) + Acetate(0.8M) + CO_2(0.59M) + Mannitol(0.42M)
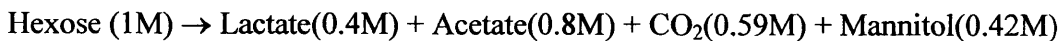
According to the stoichiometry, the cultivation of *O. oeni* could produce 0.4 M lactic acid, 0.8 M acetic acid, 0.42 M mannitol, and 0.59 M CO_2 with the cell mass of 0.542 DCW, g/L.

From the material balance, carbon 1.5%, hydrogen 4.3% was not recovered and more oxygen 8.3% was detected.

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4.5.2. The effect of glucose: fructose ratio on growth.

The effect of carbohydrate concentration was investigated using the same medium and the same condition as described in the batch culture *(Section 2.4.2.3)* and increasing only the proportion of glucose in 190mM total carbohydrate. The glucose proportion of carbohydrates was increased from nil to 2.5 times fructose concentration in the growth medium. Initial pH was set in a range between pH 5.5 and pH 5.8, and pH quickly dropped to pH 4.8 where it was controlled by addition of 5M NaOH. Figure 4.12 shows the results of the study.
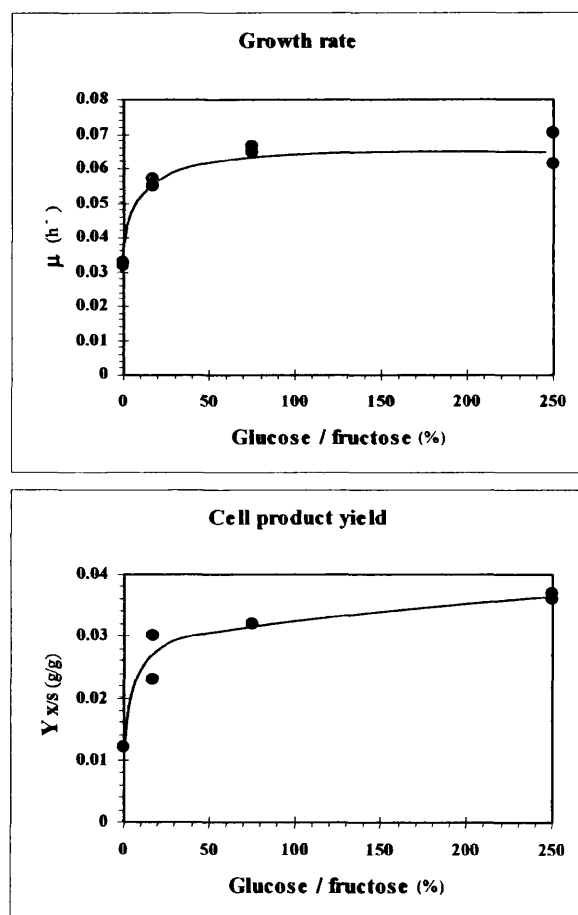
Fig.4.12. The effect of more glucose fraction of carbohydrates (glucose / fructose) on the growth rate and the cell product yield during the cultivation of *Oenococcus oeni.* *The total concentration of carbohydrates was 194 mM, and only the ratio of glucose and fructose was altered within fixed total carbohydrate concentration. All growth conditions were the same as that of the batch culture.*

Increasing up to 75% the glucose fraction of total carbohydrates in the growth medium increases the growth rate, however further addition did not affect the growth rate. The cell product yield also increased with small additions of glucose, but then showed a slow increase as the proportions of glucose increased further.

This means that fructose contributed to control the redox balance associated with regeneration of NAD^+, and glucose is metabolised to produce the cell mass. Some part of fructose can be assimilated through fructose-6-phosphate [Richter et al. (2003), Maicas et al. (2002), (1999)].

4.5.3. The effect of pH control level on growth.

The effect of pH on growth was investigated. The cultivations were carried out with the growth medium and the growth condition used in the batch culture containing the carbohydrate mixture of glucose (56 mM) and fructose (167 mM). Initial pH was set in a range between pH 5.5 and pH 5.8. At pH 4.8, pH was controlled by additions of 5M NaOH solution *(Section 2.4.2.3)*.

The pH level for control was investigated with the STR in a range between pH 4.0 and pH 5.0. Figure 4.13 shows the results that the growth rate and product yield show the peak around pH 4.8.
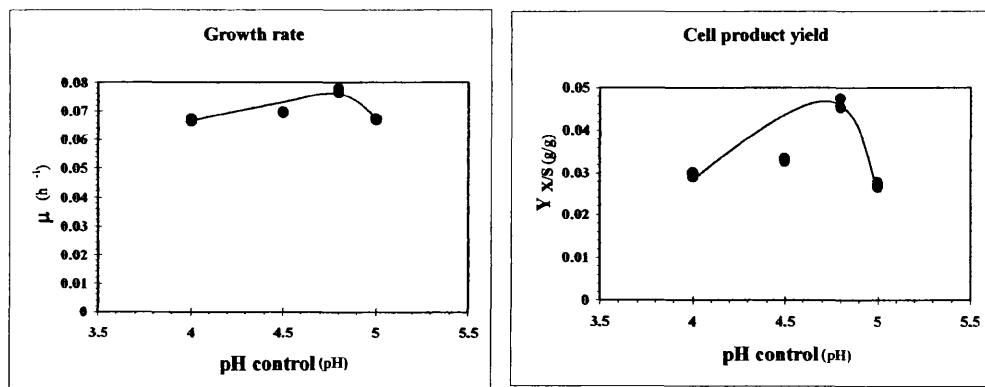
Fig.4.13. The effect of pH control level on the growth rate and the cell product yield during the cultivation of *Oenococcus oeni* in the pH-controlled STR. *The cultivations were carried out with the growth medium containing the carbohydrate mixture of glucose (56 mM) and fructose (167 mM) and the growth condition used for the batch culture. Initial pH was set in a range between pH 5.5 and pH 5.8, and pH control was started with 5M NaOH solution at pH 4.8.*

The growth rate and the cell product yield slowly increased until pH 4.8, but both showed sharp drops around pH 5.0. That means that *O. oeni* is very sensitive to alkaline conditions. Other studies have shown this organism to be highly tolerant of acidic pH and to survive at pH 3.2.

4.6. DISCUSSIONS.

In this section comparisons of the results are made. The results are summarized at Table 4.1.

4.6.1. Comparison of growth of four strains in the STR (Table 4.1).

L. buchneri was grown at the growth rate of 0.202 h^{-1} during the exponential growth phase in the pH-controlled STR and reached the cell mass of 1.832 DCW, g/L in 30 h. The growth medium contained 310 mM glucose and the product yield was 0.068.

L. brevis was grown at the growth rate of 0.162 h^{-1} during the exponential growth phase in the STR, and reached the cell mass of 2.329 DCW, g/L in 36 h. The cell production yield was 0.067.

In the pH-uncontrolled batch culture *(Section 3.6.1)*, the growth rates of *L. buchneri* and *L. brevis* were higher than them observed by the researchers. The growth rate in the pH controlled batch culture was improved as shown at Table 4.1 and Table 3.1.

B. longum was grown at the growth rate of 0.278 h^{-1} for the exponential growth phase in the STR, and reached the cell mass of 4.04 DCW, g/L in 11 h. The growth of *B. longum* was the fastest of four strains in this investigation. The cell mass of 0.213, DCW, g/L was produced for consumed glucose of 1g.

Taniguchi et al. [1987] investigated the growth of *B. longum* in the pH-controlled batch culture. They used lactose as a single carbon source. The growth rate was 0.208 h^{-1}, but the maximum growth rate reached 0.37h^{-1}.

O. oeni was grown at the growth rate of 0.108 h^{-1} for the exponential growth phase in the pH controlled STR, and reached the cell mass of 0.542, DCW, g/L in 29h.

Chapter.4 Growth of Lactic Acid Bacteria in a Stirred Tank Reactor.

The growth of *O. oeni* was the slowest of four strains in this investigation. The cell mass of 0.022, DCW, g/L was produced for 1g glucose consumed.

Cogan [1987] investigated the growth of *O. oeni* in the growth medium containing glucose and citric acid. The growth rate was varied from 0.075 h^{-1} to 0.109 h^{-1} depending on the variants used in the cultivation. Even if the mixture of fructose and glucose was used with citric acid as the carbon source in this investigation, the growth rate did not improve further.

4.6.2. Comparing the product yields (Table4.1).

Strains	μ (h^{-1})	X_{DCW} (g/L)	T (h)	Product yield (g/g)					$P_{X/t}$ (g/L,h)
				$Y_{X/S}$	$Y_{L/S}$	$Y_{A/S}$	$Y_{E/S}$	$Y_{M/S}$	
L.buchneri	0.202	1.832	30	0.068	0.44	0.1	0.22	N.D	0.061
L.brevis	0.162	2.329	36	0.067	0.4	0.08	0.22	0.03	0.065
B.longum	0.278	4.038	11	0.213	0.3	0.57	0.05	N.D	0.367
O.oeni	0.108	0.542	29	0.022	0.2	0.27	N.D	0.43	0.019

Table. 4.1. Summary of the parameters obtained during the cultivation of four strains in STR. μ : *the growth rate in the exponential growth period, X_{DCW}: the cell mass, T: the culture time, $Y_{X/S}$: the cell product yield, $Y_{L/S}$: the lactate product yield, $Y_{A/S}$: the acetate product yield, $Y_{E/S}$: the ethanol product yield, $Y_{M/S}$: the mannitol product yield, $P_{X/t}$: the specific cell production rate.*

The major product during the cultivation of *L. buchneri* was lactate. Lactate 0.44 g, ethanol 0.22g, and acetate 0,1g were produced for 1g glucose consumed.

According to the report by Cunha and Foster [1992], lactate 0.5g, acetate 0.4g, and ethanol 0.166g were produced for 1g glucose consumed. When glycerol was added, productivity of acetate was increased with reduction in productivity of lactate and the growth rate was improved. Products accumulated were more than equimolar with sugar used in this fermentation.

Products during the cultivation of *L. brevis* were similar to them of *L. buchneri*, but mannitol was produced because of fructose in the growth medium. Produced mannitol was consumed again when fructose was dried up in the growth medium (Fig 4.4)

The major product during the cultivation of *B. longum* was acetate 0.57 g produced for 1g carbohydrate consumed. Lactate the second product was produced in the product yield 0.3g per 1g carbohydrate consumed. Ethanol of 0.05g was produced for 1g carbohydrate consumed.

According to Taniguchi et al. [1987], *B. longum* produces 2 M lactate and 3 M acetate per 1 M glucose. Desjardine et al. [1990] reported 1.60 in production ratio of acetate: lactate and a little of ethanol. The results by Desjardine et al. [1990] were the same as the results obtained in this investigation.

The most of fructose consumed during the cultivation of *O. oeni* was converted into mannitol to regenerate NAD^+. Produced mannitol was not consumed again and accumulated showing a different result from *L. brevis* (Fig 4.11). Ethanol was not produced while fructose being available in the growth medium. Lactate 0.2g and acetate 0.27g were produced for 1g carbohydrate consumed.

According to the report by Richter et al. [2003], about 30% of fructose consumed is fermented via the phosphoketolase pathway and the rest is used for the electron acceptor for NAD(P)H reoxidation. However, in this investigation all fructose was converted to mannitol, and lactate 0.6 M and acetate 1.14 M were produced per glucose M consumed. *O. oeni* was grown at the high growth rate. Acetate is produced more than lactate in the fast growth to produce more ATP from the enzyme step acetyl-phosphate kinase and more mannitol is produced from fructose with the reduction of ethanol production. Likewise, pyruvate electron acceptor is not used for lactate production but used for acetate production.

4.6.3. Problems anticipated at the growth of LAB in the MCR.

L. brevis and *O. oeni*, which are the main strains for MLF of the cider, showed some different patterns in their growth to the carbohydrates. *L. brevis* could be slowly grown during the limitation of fructose in the growth medium whereas the growth of

O. oeni completely stopped. Therefore, *O. oeni* in the MCR should be supplied for the fast growth with sufficient and balanced carbon sources such as glucose and fructose.

O. oeni showed very low cell product yield because of a relatively low growth rate. Therefore, the growth of *O. oeni* in the MCR might be non-economical due to consuming more medium. Therefore, it can be very important to determine the minimum amounts of the ingredients wasted through product line.

L. brevis and *B. longum* produce the extracellular polymers. Generally speaking, *L. brevis* produces the extracelluar proteins so called as Surface-layer protein, and *B. longum* produces exopolysaccharides in the case where too many carbohydrates to the nitrogen sources exist in the growth circumstance. Therefore, severe fouling on the product membrane is anticipated during the growth in the MCR.

L. buchneri and *L. brevis* produce large quantities of CO_2 during the cultivation. The gas in the system affect to the fluid stream leading to bad operation of pumps. The fluctuation on the fluid consequently affects the feed rate of the growth medium and the flow rate of the fluid in the reactor.

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GROWTH OF LACTIC ACID BACTERIA IN MEMBRANE CELL RECYCLE REACTOR (MCR)

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CHAPTER 5: GROWTH OF LACTIC ACID BACTERIA IN A
MEMBRANE CELL RECYCLE REACTOR.

5.1 INTRODUCTION.

Lactic acid bacteria can produce a variety of metabolites during the cultivation. The growth
can be inhibited by them and one of the most important obstacles in approaches for high cell
density, even if LAB is very resistant, is end product inhibition.

The microbial cultivation in the membrane cell-recycle reactor (MCR) allows the
continuous removal of inhibitory materials produced during the incubation and continuous
feeding of the fresh medium to the microbes. Growing the microbes in high cell density
provides many economical benefits. High concentration of the product in the downstream
processing can cut down the production time and the cost, and improve the quality of the
final product [Senthuran et al. (1997), Kwon et al. (2001)]. In lactic acid production, high
product concentration is important to make the downstream process economic. Generally,
lactate yield increases with increase cell mass, but the volumetric productivity of lactate was
lower in the MCR than in the batch culture. Senthuran et al. [1997] and Kwon et al. [2001]
studied to improve the lactate productivity using diverse membranes.

The basic principle of the MCR is that the liquid perfuses through system while the cells are
retained by the use of membrane. In materials and methods section, the MCR has been
described. The key features of the system are the use of ceramic membrane (1 m^2) connected
to the 25 L reactor. Two pumps are used to give the most effective filtration while a third
pump is one to cause the flow through the reactor. The feed rate is controlled by a level
controller so as to control the working volume of the system. The pH and temperature are
also carefully controlled. Therefore, the MCR can maintain the fast growth through
continuous removal of the harmful metabolites during the cultivation. Furthermore,
distinctive from the conventional continuous culture, the MCR can retain the growing
microbes, using the membrane as a part of the reactor to protect growing microbes from
washing out. On the other hand, the microbial cultivation in the MCR has its problems
because of membrane fouling.

Chapter.5 Growth of Lactic Acid Bacteria in Membrane Cell Recycle reactor.

At high cell density, the substrate consumption rate grows in parallel with an increase cell concentration and the production rate. Consequently the membrane flux must also be increased to maintain the high growth rate and to control the concentration of inhibitory substances below a critical concentration so as to prevent the microbial growth from significant inhibition.

This chapter investigates the growth of four LAB species with an effective system for LAB production and the possible causes of limitation.

5.2. FACTORS EFFECTIVE FOR THE MEMBRANE FLUX.

Key feature of the membrane cell recycle bioreactor (MCR) is a special bioreactor incorporating the membrane filter so as to continuously allow removal of substances causing end product inhibition with the aim to keep LAB growing in the maximum growth rate without inhibition. In recent studies, ceramic membranes have been often used in the MCR [Kamoshita et al. (1998), Persson et al. (2001), Suzuki (1996), Suzuki et al. (1994)]. Since ceramic membranes are capable of withstanding extreme conditions such as high pressure, high temperature, and acidic or alkaline condition.

There are, however, many problems in the use of the membrane at the MCR. The main problem is the membrane fouling which ultimately limits productivity. Control of membrane fouling is dependent on the operating conditions of the membrane, the properties of the fluid circulating through the membrane and the physical properties of the membrane. Therefore, discovering the properties of the membrane is very important to effectively apply them in the MCR because ultimately it will limit the dilution rate of the medium within the reactor. Therefore, the effects of pH, yeast extract concentration, and temperature on the membrane flux were investigated.

Investigations of the variables to the membrane flux were performed using the small membrane unit with filtering area of $0.2m^2$. Figure 5.1 shows the membrane flux of yeast extract solution with time. The flux was dramatically reduced in 30 min, and then a slow reduction on the flux.

Chapter.5 Growth of Lactic Acid Bacteria in Membrane Cell Recycle reactor.

Therefore, all data obtained during the investigation about the membrane flux were checked in 60 min.
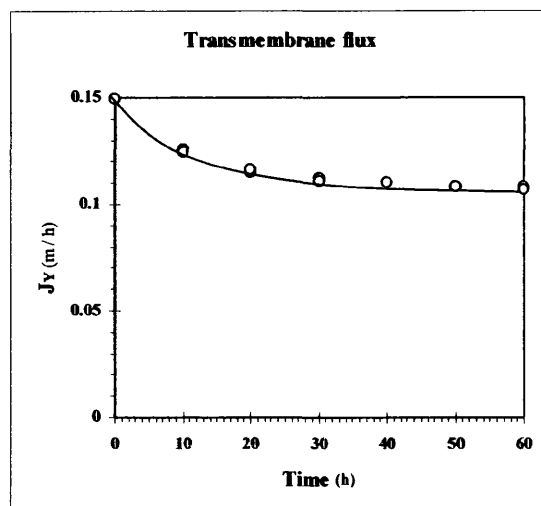
Fig.5.1 Variation on the flux of yeast extract solution (0.9%, w/v) through the ceramic membrane shown as a function of the time. *The filtration was recycled during the investigation. This investigation was undertaken at the room temperature, one bar, and neutral pH. The filtering area used in this investigation was 0.2 m^2.*

5.2.1. The effect of temperature

Temperature is one of the important parameters that affect the membrane flux. The temperature can change the fluid properties such as viscosity and surface tension, and ultimately influence on the membrane flux. Increasing the operating temperature will not only to decrease viscosity of the fluid, but also to change the conformation of proteins and the rates interaction by which fouling takes place, while reducing in temperature causes the flux decline.

Generally speaking, an increase in 1°C should increase the flux of pure water by 2% and this is directly related to the changing viscosity. Figure.5.2 shows the effect of the operating temperature on the membrane flux (J_W =0.00579X + 0.1107).

Chapter.5 Growth of Lactic Acid Bacteria in Membrane Cell Recycle reactor.

In this investigation, the effect of the operating temperatures were investigated comparing with the membrane fluxes of water (J_w), growth medium ($J_m = 0.004586X + 0.0173534$), and growth medium including Tween-80 as a detergent ($J_{md} = 0.004339X + 0.02445$). The composition (w/v) of the used growth medium was glucose (4%), yeast extract (0.9%), $MgSO_4.7H_2O$ (0.25%), $MnSO_4.4H_2O$ (0.025%), and KH_2PO_4 (0.2%), and additionally the detergent was added into the growth medium in the concentration of 0.05%.

Higher temperature increased the flux with increasing rate of 0.0057 m/h per every increase of 1°C in the case of water, but in the case of the medium it increased with lower increasing rate of 0.0049 m/h. Furthermore, the addition of Tween-80 showed a lower increasing rate (Fig.5.2).
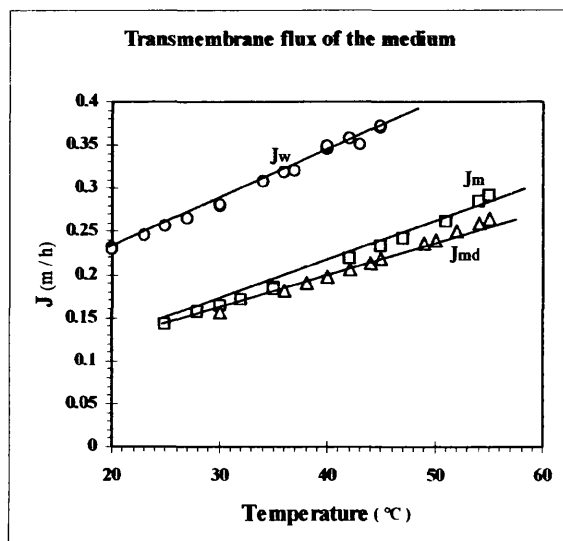
Fig.5.2 The effect of the operating temperature on the membrane flux filtering of growth media through the ceramic membrane. *The membrane flux was measured using the growth medium (Jm) containing glucose (4%,w/v), yeast extract (0.9%,w/v), MgSO$_4$.7H$_2$O (0.25%,w/v), MnSO$_4$.4H$_2$O (0.025%,w/v), and KH$_2$PO$_4$ (0.2%,w/v), water (Jw) and the growth medium (Jmd) including Tween-80 of 0.05% (w/v) as a detergent. Temperature was changed from 20 ℃ to 60 ℃. Permeates were recycled to the reservoir tank, and the measurements were undertaken in 60 min of operation.*
$J_W = 0.00579X + 0.1107$ (r = 0.99803), $Jm = 0.004586X + 0.0173534$ (r = 0.99739)
$J_{md} = 0.004339X + 0.02445$ (r = 0.999368)

Chapter.5 Growth of Lactic Acid Bacteria in Membrane Cell Recycle reactor.

According to Fig.5.2, while the temperature rising from 35°C to 60°C, the membrane flux of the growth medium increased by 61% (from 0.18 m/h to 0.29 m/h). The temperature could be used to change the transmembrane flux while operating the MCR. The increase in temperature gave a linear increase in the flux which is contrary to the general viscosity rate above.

5.2.2. The effect of the Yeast extract concentration

Yeast extract is not only the major nitrogen source in the growth medium but one of the major factors to reduce the membrane flux. It is well known that changing organic components will reduce the flux. The effect on the flux by yeast extract concentration was therefore investigated. Figure 5.3 shows the results of the study. The membrane still maintained the membrane flux over 0.11 m/h with yeast extract concentration of 0.9% (w/v) at 20°C, but it dropped rapidly when yeast extract was added further and showed a downward trend with increase yeast extract concentration.

Generally, yeast extract not only caused membrane fouling, but also contributed to the production of by-products. Consequently, since yeast extract concentration affects bacterial metabolism, this determination of the critical yeast extract concentration for the fast growth and for reduction of the side effects is essential for an efficient operation of the MCR.
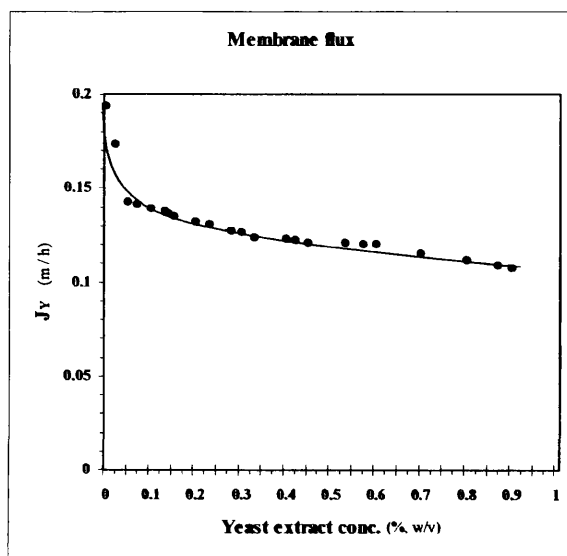

Fig.5.3. The effect of yeast extract concentration on the flux through the ceramic membrane. *The membrane flux was checked with yeast extract solution in a concentration range from 0 to 0.9% (w/v) without any other ingredients. The measurements were performed in 60 min of operation. This investigation was performed in 20 °C and pH 6.4.*

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5.2.3. The effect of pH

The pH changes the properties of the process fluids and the membrane surface. The pH is increased from pH 3 to pH 7 in the both cases of water J_W and growth medium Jm, but the flux of the medium (Jm) dramatically increased at alkaline pH between pH 9 and pH 11.0.

The flux of water and growth medium as a function of pH was investigated at 20°C. The results of this investigation are shown in Fig 5.4.
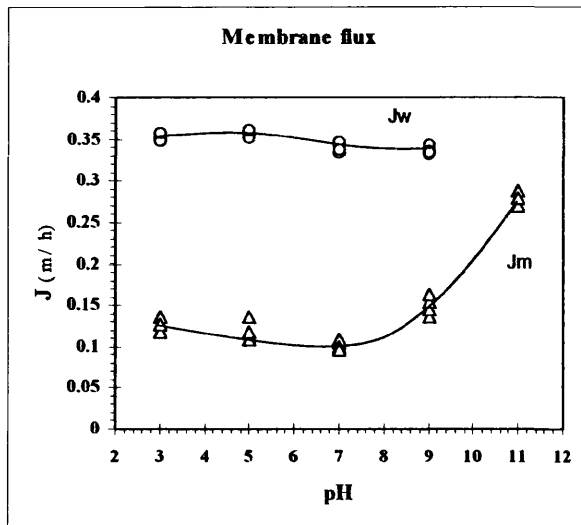
Fig.5.4. The effect of pH on the flux of the ceramic membrane. *The membrane fluxes were measured with water (J_w) and the growth medium (J_m) containing glucose (2%,w/v), yeast extract (0.7%,w/v), KH_2PO_4 (0.39%,w/v), $MSO_4.7H_2O$ (0.74%,w/v), $MnSO_4.4H_2O$ (0.06%,w/v), and Twen-80 (1ml/L). This investigation was performed in 20 °C, and at pH 6.4. Permeates were recycled to the reservoir tank. The measurements were made in 60 min.*

As the pH changes between pH 3 to pH 7, the change on the ionic distribution between the water and the membrane, on the electrostatic interactions between organics (amino acids, peptides, and proteins), and on the electrochemical potential of the materials in the system affect the membrane flux and the conformation of soluble proteins [Burns and Zydney. (1999), Persson et al. (2001)].

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The charged organics could effectively change volume and charged membrane surface could effectively shrink the pore size. The electric surface double layer is changed and such volumetric changes result in a significant change on the membrane flux.

At a high pH over pH 9, denatured proteins and membrane charge induce an increase on the membrane flux (Fig.5.4) reducing the medium/surface interactions. Therefore, the flux of J_m approached to J_w in alkaline conditions over pH 11.0. A slightly reduced flux of J_w in a range from pH 4 to pH 9 can be due to change in the pore size caused by changes in the charged membrane surface.

5.3 INVESTIGATION OF GROWTH OF LACTOBACILLUS BUCHNERI IN MCR.

The fast growth was found on the growth of *L. buchneri*. The medium and the culture conditions used and optimized in the previous work *(Chapter 3, 4)* were used for the preparation of inoculum to be used in this section. The methods for operation and for inoculation of the MCR were described in Chapter 2.

As a basic strategy to control the growth of *Lactobacillus buchneri* in the MCR, glucose was present at 70 mM for growth. The feed medium including glucose of 166.7 mM *(Section 2.4.2.1)* was supplied at a sufficient rate to maintain the glucose concentration. The critical concentration of glucose to support the maximum growth rate of *L. buchneri* was 100 mM, and the growth of *L. buchneri* could be restricted in the case to control the glucose in the concentration below 100 mM. Figure 5.5 shows the effect of glucose concentration on the growth rate of *L. buchneri*.

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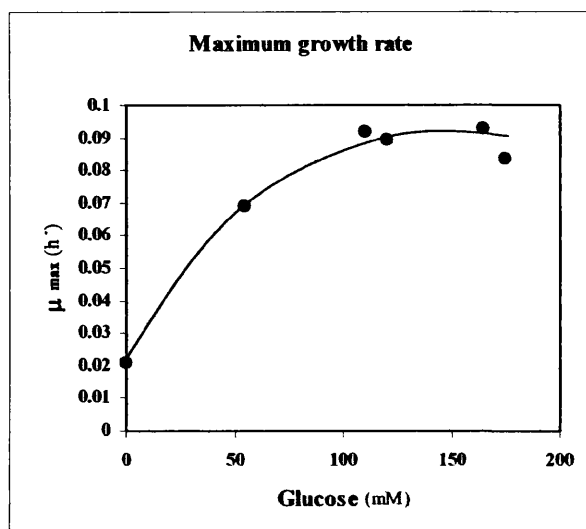
Fig.5.5 The effect of glucose concentration (mM) on the growth rate of *Lactobacillus buchneri*. *L. buchneri was grown at the same medium and the same growth conditions as mentioned at previous chapter 2 and the measurements of the growth rate were made in 12 h after start of the cultivation.*

Figure 5.6 shows the results for the growth of *L. buchneri* in the MCR with three graphs about dilution rate, cell growth, and products and substrates. The pH was controlled at pH 5.2. The fermentation was terminated at 21 h. Recycling pumps did not work appropriately due to CO_2 gas generated in high cell mass and the reduction of the membrane flux in product membrane led to reduction of the feed rate of the growth medium. The cell mass was measured in every 3 h and when the cell mass did not increase further, the culture was terminated.

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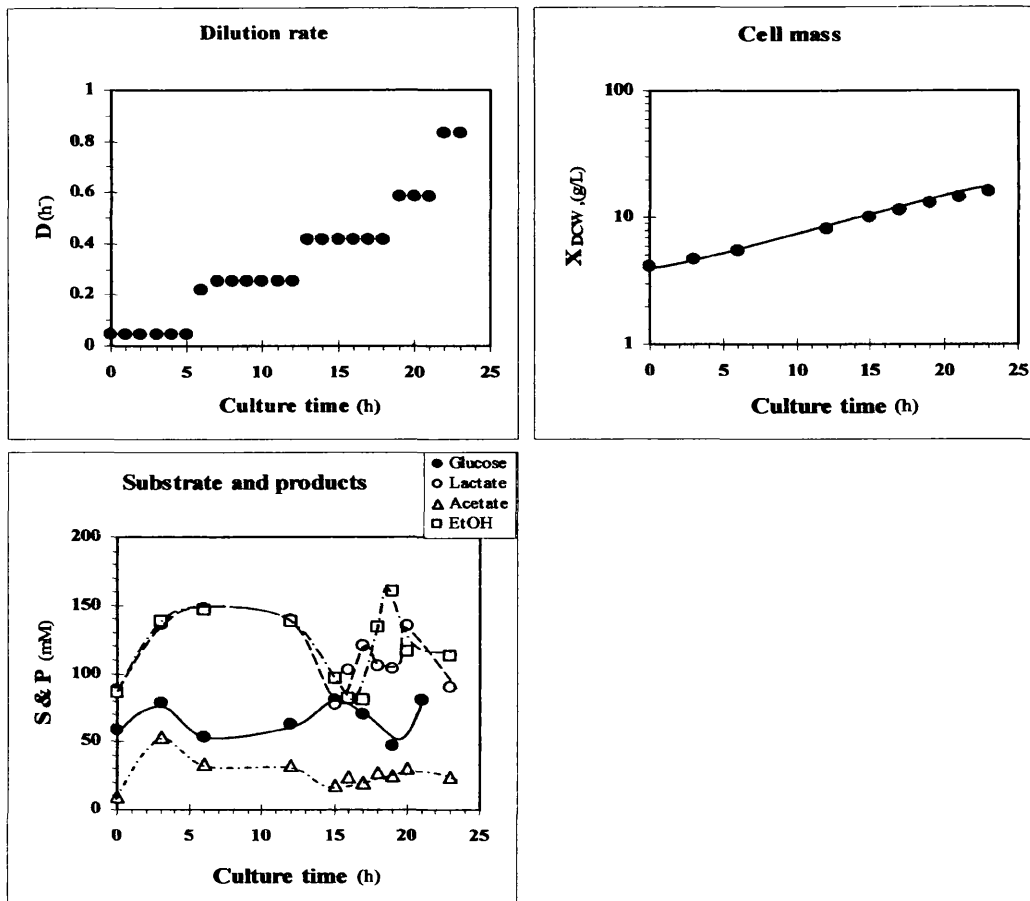
Fig.5.6. The growth of *Lactobacillus buchneri* during the cultivation in the membrane cell recycle reactor (MCR). *The cultivation produced the cell mass 16.2 DCW, g/L with by-products like lactic acid, acetic acid, and ethanol with the feed medium of total 320 L, including glucose of 221 mM. Dilution rate was increased step by step from 0.047 h⁻¹ to 0.83 h⁻¹. The composition of growth medium was the same as that in the batch culture of L. buchneri (Section 2.4.2.1).The pH was controlled at pH 5.2, and temperature was 30 °C*

The feed rate was increased along an increase growth. The dilution rate was 0.8 h^{-1} at the end of the fermentation (20 h). During the culture in the MCR the cell growth was in an exponential phase. Biomass increased on the maximum growth rate 0.064 h^{-1} from 15 h to 21 h, and ultimately it produced biomass of 16.2 DCW. g/L. Furthermore, the stoichiometry indicated a decrease on lactic acid production with an increase on ethanol production but that is similar to that formed in the batch culture.

Hexose (1M) $\rightarrow$ Lactate (0.75M) + Acetate (0.28M) + Ethanol (0.95M) + CO_2 (1.23M)

As shown at Fig.5.6, the growth rate during the cultivation in the MCR did not drop at the end of the cultivation, but the observed growth rate was lower than that in the batch culture. The glucose concentration during the cultivation was controlled in a range from 60 mM to 80 mM, the concentration of ethanol and lactic acid could not be controlled below the critical concentration. During the cultivation, 1% carbon and 6% hydrogen were underestimated and 3.7% oxygen was overestimated in the material balance.

The concentrations of acetate and lactate were at the concentration range from 80 mM to 170 mM respectively during the cultivation in the MCR. The growth of *L. buchneri* in the MCR was inhibited even at continuous removal of inhibitory substances such as ethanol and lactic acid. On the other hand, the product yields $Y_{x/s}$ was 0.076 higher than the cases of either the batch culture or the vial culture. Even if the dilution rate was increased stepwise from 0.047 h^{-1} to 0.83 h^{-1} and the concentration of inhibitory substances could not increase to up to the critical concentration (Table 3.1), but slow growth might be due to the complex effect of the end products and glucose concentration lower than the critical concentration to be controlled. The growth rate was 0.057 h^{-1} in the early stage from 1 h to 12 h and continuously increased 0.064 h^{-1} at the final stage.

5.4. INVESTIGATION OF GROWTH OF LACTOBACILLUS BREVIS IN MCR.

The medium and the culture conditions were optimized in the previous chapters *(Chapter 3,4)*. Optimized medium and the culture conditions were used to grow *Lactobacillus brevis* in the MCR and the methods for operation and inoculation of the MCR were described at the previous chapter *(Chapter 2)*. *L. brevis*, from previous investigations *(Section 3.5.3)*, needs both of glucose and fructose for high yield and the fast growth, even if it can grow in only glucose or in only fructose. The feed medium including glucose (139 mM) and fructose (56 mM) was supplied through the ceramic membrane with 0.2 m^2 of filtering area to avoid of the shortage of carbohydrate during the cultivation in the MCR *(Section 2.4.2.2)*.

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Figure 5.6 shows the results of this experiment.
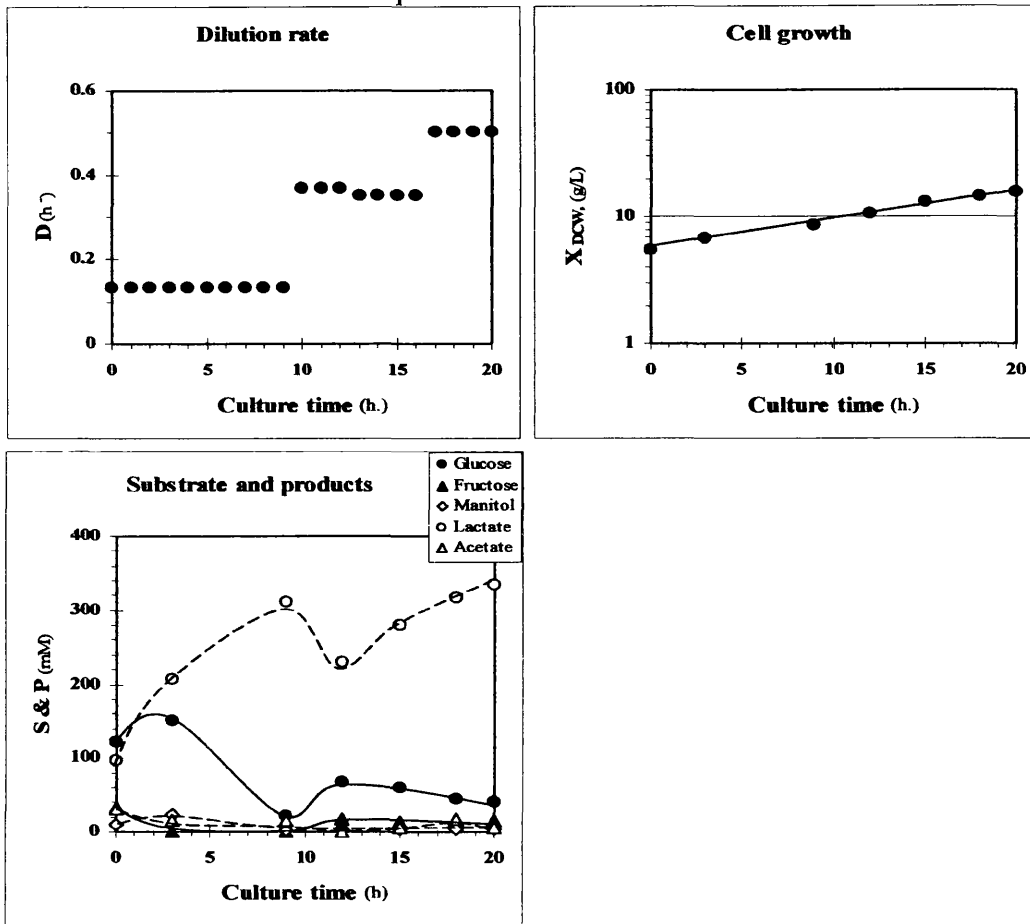
Fig.5.7. The growth of *Lactobacillus brevis* during the cultivation in the membrane cell-recycle reactor (MCR). *The cultivation produced lactic acid, acetic acid, and mannitol with 15.48 DCW, g/L of the cell mass, continuously feeding with the medium containing glucose (139 mM) and fructose (56 mM) for 20 h. During the cultivation, the dilution rate was increased from 0.13 h^{-1} to 0.483 h^{-1} at the end. Total volume of bioreactor was 36 l. The pH was controlled at pH 5.5, and temperature was 28 °C.*

The strategy for growth was to increase the dilution rate along an increase cell mass because the consumption rate of the medium will increase. The dilution rate was increased from 0.13 h^{-1} to 0.48 h^{-1}. The culture was maintained during the exponential growth phase of 20 h with a low growth rate. The fermentation was terminated in 20 h because severe membrane fouling on product membrane resulted in a limitation on the dilution rate.

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Glucose concentration was present in a range from 100 mM to 140 mM, but at the end glucose concentration dropped beneath 40 mM because of increase substrate consumption rate by a high cell mass. By the way, fructose concentration was maintained in an extremely low concentration (Fig. 5.7).

The growth rate decreased from 0.064 h^{-1} in the beginning (from 0 h to 10 h) to 0.04 h^{-1} at the end (from 10 h to 20 h) of the cultivation and ultimately the cell mass reached 15.5 DCW, g/L in 20 h. When compared to the batch culture, the growth rate was lower in the MCR than in the batch culture (0.162 h^{-1}). During the cultivation, lactic acid and only a trace of acetic acid were detected without production of ethyl alcohol.

Mannitol-forming bacteria like *L. brevis* have two metabolic pathways for the growth [Cunha and Foster. (1992), Richter et al. (2003)]. In high growth rate, this bacterium uses fructose for regeneration of NAD^+ and production of ATP, and produces mannitol and acetic acid as products. However, in low growth rate this bacterium uses fructose as energy source like glucose and produces equimolar lactic acid to fructose used. *L. brevis* in the MCR grew as if homofermentative lactic acid bacteria grow producing lactic acid as the main product with a low growth rate.

Looking at the stoichiometry on the batch culture (Fig 4.4), acetic acid and ethanol were the main products when fructose and glucose was sufficient in the medium, but lactic acid rather than acetic acid was the main product in the end of the batch culture where fructose was completely consumed. Furthermore the growth rate when fructose was sufficient was a maximum 0.16 h^{-1}, but the growth rate was decreased to 0.03 h^{-1} after the exponential phase where fructose was not detected. The stoichiometry was studied on the MCR with the following mass balance.

Hexose(1M) $\rightarrow$ Manitol (0.005M) + Lactate (1.8M) + Acetate (0.05M) + CO_2 (0.05M)

Mannitol produced during the cultivation could be partially consumed or partially washed out through the membrane without the accumulation.

The products have not been precisely recovered as per the amount of carbohydrate consumed during the cultivation according to the stoichiometry. Most of carbohydrates consumed during the cultivation were metabolized into lactic acid ($Y_{L/S} = 0.9$), and only a trace amount of carbohydrate was used to produce acetic acid ($Y_{A/S} = 0.017$). The cell product yield $Y_{X/S}$ was 0.075

In the MCR, fructose was continuously supplied and fructose was reduced to mannitol without production of ethanol, but the growth rate was obviously decreased in low fructose concentration. Mannitol produced can be consumed by *L. brevis* again (Fig.4.4). In high cell mass almost all carbohydrates could be metabolized to lactic acid maintaining a low growth rate and only trace quantities of ethanol and acetic acid were produced.

There were about 6% unrecoverable on carbon balance. The fermentation of mannitol-forming bacteria can produce succinic acid, glycerol, and erythritol in a trace amount too. However they were not measured in this investigation.

According to Peterson and Fred [1920], it was reported that the amount of carbon dioxide produced during the cultivation is almost equal to the amount of ethyl alcohol and acetic acid because almost all of carbon dioxide was taken off from pyruvate during decarboxylation of pyruvate to either acetate or ethanol.

5.5. INVESTIGATION OF GROWTH OF BIFIDOBACTERIUM LONGUM IN MCR.
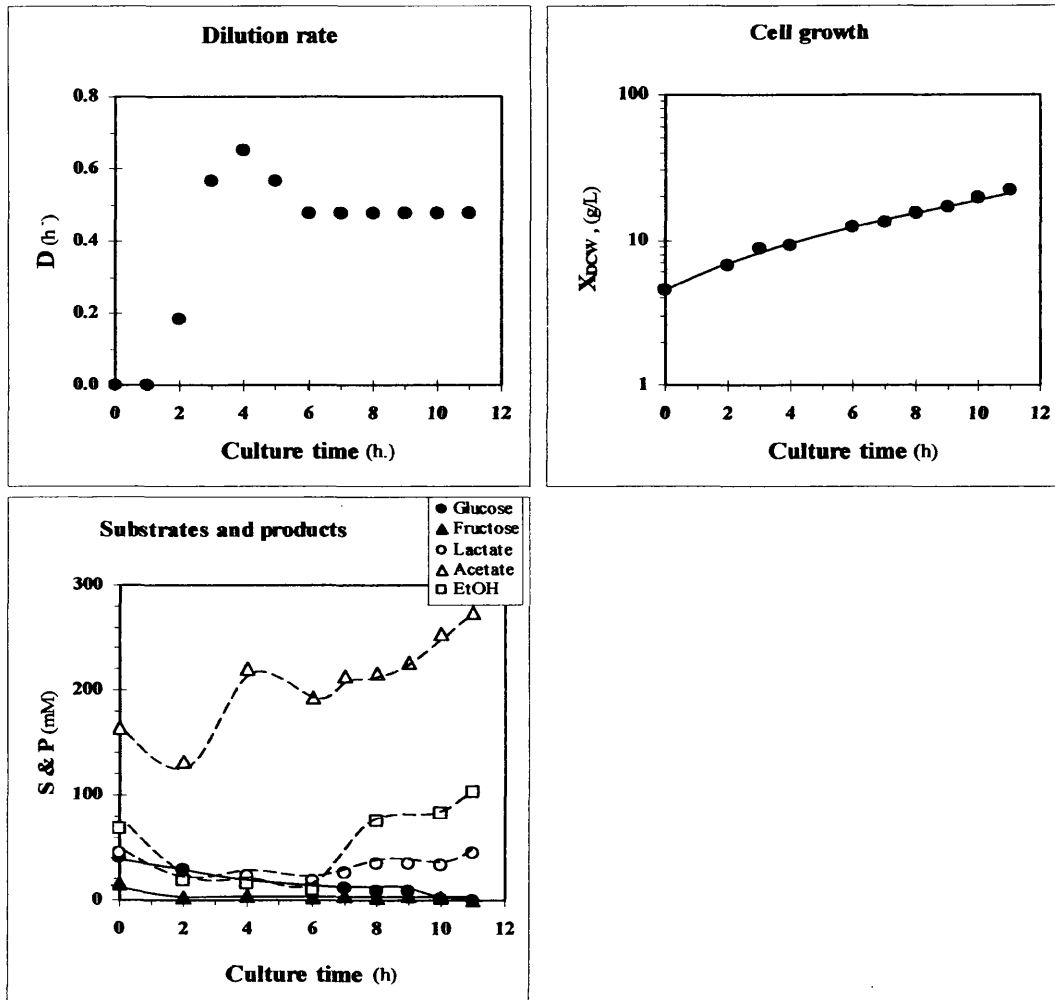
The growth of *Bifidobacterium longum* in the batch culture and nutritional requirements were shown at the previous chapter *(See Chapter 3,4)*. The optimized growth medium and conditions determined at Chapter 3 and 4 were used in this investigation to grow *B. longum* in the MCR. The methods for operation and inoculation were also described at Chapter 2.

As a basic strategy to control the growth of *B. longum* in the MCR, glucose concentration was maintained in a relatively low concentration during the cultivation with the feed medium including carbohydrates of total 106 mM in the mixture, which is containing glucose of 56 mM and fructose of 50 mM *(Refer to 2-4-2-4)*.

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As *Bifidobacterium spp* can produce exopolysaccharides in a high carbohydrate concentration, which is fatal to the membrane fouling, the carbohydrate concentration should be controlled at the least concentration. Figure 5.8 shows the results of the investigation about growth of *B. longum* in the MCR.


Fig.5.8. The growth of *Bifidobacterium longum* during the cultivation in the MCR. *The cultivation produced lactic acid, acetic acid, and ethanol with the cell mass 22.2 DCW, g/L, consuming total the feed medium 151 L containing the carbohydrate mixture of glucose (56 mM) and fructose (50 mM) in 11 h. Dilution rate was controlled at 0.183 h⁻¹ for 2 h in the beginning of the cultivation, and gradually increased to 0.567 h⁻¹ at the end. Total volume of bioreactor was 36 L. The pH was controlled at pH 5.8, and temperature was 37 °C. B. longum was grown at the same medium as that in the batch culture (Refer to 2-4-2-4).*

B. longum during the exponential growth phase increased at the growth rate of $0.13\ h^{-1}$, and ultimately it produced the cell mass 22.2 DCW g/L. The following product stoichiometry was established:

Hexose (2M) $\rightarrow$ Lactate (0.5M) + Acetate (3.75M) + Formate (1.5M) + Ethanol (0.75M)
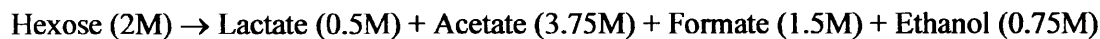
The stoichiometry on the MCR indicated a decrease production of lactic acid with an increase other products reflecting a partial glucose-limited growth. The concentrations of lactic acid and acetic acid were maintained at a concentration range of 20 mM to 100 mM and of 120 mM to 260 mM, respectively. These concentrations were enough to inhibit the growth of *B. longum*. Dilution rate beginning at $0.18\ h^{-1}$ was raised to $0.57\ h^{-1}$ at the end of fermentation consuming 4.2 times that to total bioreactor volume but quickly dropped again to $0.48\ h^{-1}$ due to a serious membrane fouling on product membrane.

According to the stoichiometry, carbons forming lactic acid were diverted to other products. lactic acid 1.2 M produced during the batch culture dropped into 0.5 M during the growth in the MCR. The rest carbon to form lactic acid was reused into 0.35 M of acetic acid and 0.35 M of ethyl alcohol with 0.7 M of formic acid.

Generally speaking, in a high concentration of the cell mass, the fluid in the fermentation can not be a homogeneous and a partial substrate limitation can be caused, creating a more extreme condition than the batch culture. The content of acetate and alcohol in the batch culture increased more than that of lactate at the end of the culture where glucose was limited.
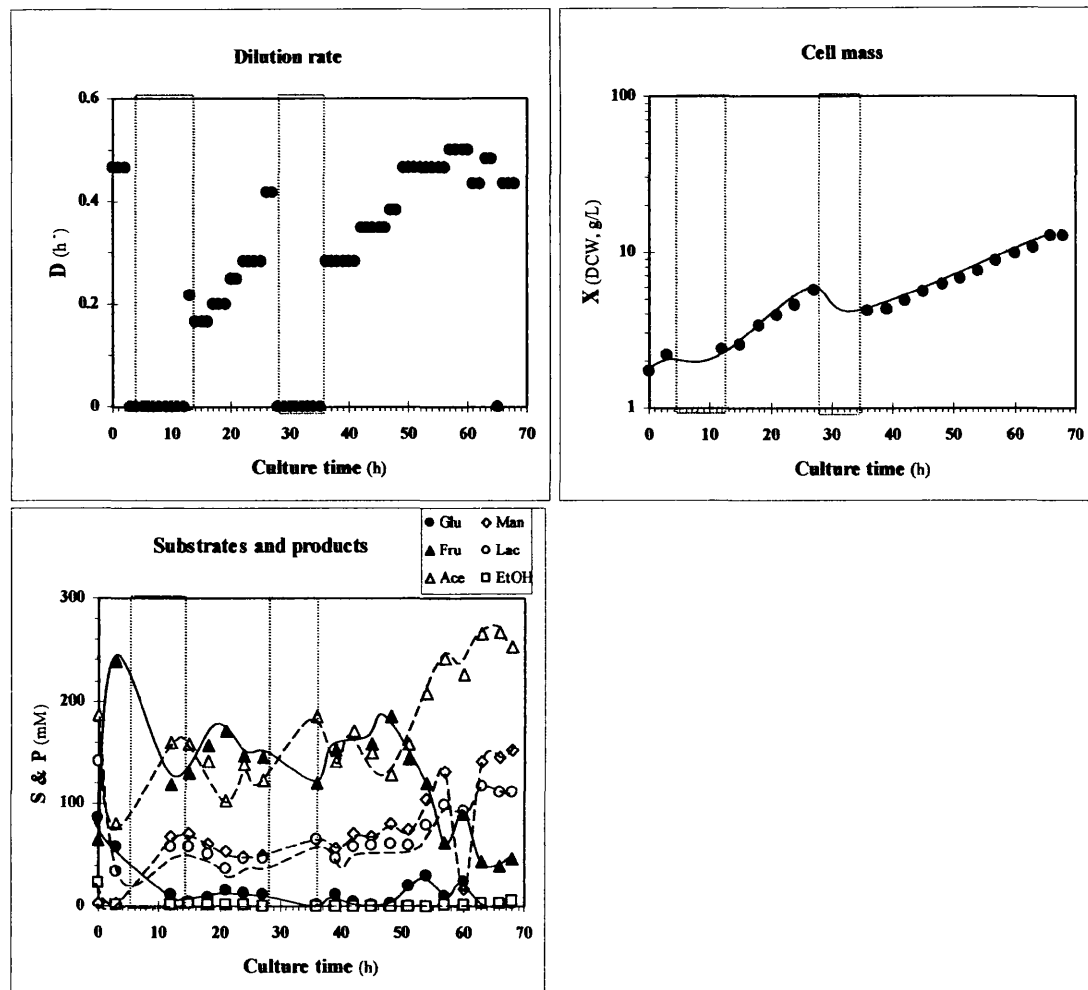
5.6. THE GROWTH OF OENOCOCCUS OENI IN MCR.

The growth of *Oenococcus oeni* and nutritional requirements were shown at the previous chapters *(See Chapter 3,4)*, which investigated at the batch culture with and without pH control. Optimized growth medium and conditions were used in this investigation to grow *B. longum* in the MCR. The methods for operation and inoculation were described at Chapter 2.

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. Figure 5.9 shows the results obtained during the growth of *O. oeni* in the MCR.

Fig.5.9. The growth of *Oenococcus oeni* during the cultivation in the membrane cell recycle reactor. *This cultivation produced lactate, acetate, mannitol and ethanol with cell mass of 11.1 DCW, g/L, consuming total 626 L of the feed medium containing glucose (50 mM) and fructose (194 mM). Dilution rate was stepwise increased from 0 h^{-1} to 0.5 h^{-1}. The growth rate after 35 h was lower than that of 3 h to 12 h because of a lower increase dilution rate. Dilution rate increased from 0.167 h^{-1} to 0.42 h^{-1} for 13 h from 13 h to 26 h and from 0.283 h^{-1} to 0.5 h^{-1} for 21 h from 36 h to 57 h. The cell growth was slowed and instantaneously dropped while stopping the feeding from 3 h to 12 h and from 28 h to 35 h. The growth was restarted with the feeding of the growth medium. The pH was controlled at pH 4.8, and temperature was 25 °C (Section 2.4.2.3).*

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The growth of *Oenococcus oeni* was supplied with the feed medium including the carbohydrate mixture of total 244 mM, which contains 50 mM glucose and 194 mM fructose *(Section 2.4.2.3)* during the cultivation in the MCR. *O. oeni* was grown at the growth rate of 0.067 h^{-1} and ultimately reached the optical density (OD) 31 at the wavelength of 660 nm, which is 12.8 DCW, g/L in 68 h. The growth simultaneously stopped when stopped the feeding of the growth medium for two periods of 3 h to 12 h and 28 h to 35 h and the growth restarted with the beginning of feeding. However, the growth rate was lower between 36 h to 57 h than that between 13 h to 26 h because an increase in the feeding rate was slower. At the end of the cultivation the consumption rate of fructose increased and productivity of acetate increased more than that of lactate.

The stoichiometry on the MCR indicated a production of lactic acid, acetic acid, and mannitol with CO_2 and small quantity of ethanol. There was a lower production of mannitol in the MCR than in the batch culture and production of ethanol can reflect a carbohydrate limited growth condition. The stoichiometry was studied as the following mass balance.

Hexose(1M)→ Lactate(0.35M)+Acetate(0.85M)+Ethanol(0.1M)+Mannitol(0.39M)+CO_2(0.6M)
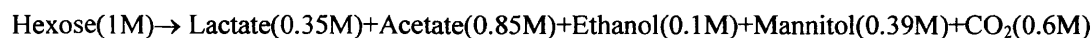
During the cultivation in the MCR, lactate decreased to 0.35 M from 0.4 M in the batch culture, and productivity of acetate and ethanol increased. The productivity of mannitol and carbon dioxide in the MCR was similar to that in the batch culture. The productivity of acetate increased at the end of the cultivation with an increase consumption rate of fructose. Acetate, erythrytol, and mannitol can be produced via an alternative pathway with fructose instead of glucose. Fructose can be used for production of ATP and regeneration of $NAD(P)^+$ producing erythritol, acetate, and mannitol [Richter et al. (2003)].

5.7. COMPARISON AND ANALYSIS OF THE CULTIVATION OF LAB IN MCR.

The summary data for comparison is shown in Table 5.1.

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5.7.1. Comparison of the cell growth (Table.5.1).

L. buchneri was grown to the total cell mass 16.2 DCW, g/L at the growth rate of 0.065 h^{-1} in 23 h. Glucose (210 g/L) as the single carbon source was consumed during the whole fermentation time. Therefore, total cell product yield (DCW) was 0.077. This result shows slightly higher product yield ($Y_{X/S}$) in the MCR than in either the batch cultures with pH control (0.068) or without pH control (0.06). The growth rate was lower in the MCR than in either the batch culture with pH control or without pH control.

In the pH-controlled STR culture lactate and acetate were maintained at lower concentration than 50 mM and only the concentration of ethanol was at 130 mM during the exponential growth phase at which the growth rate increased to 0.2 h^{-1}, but during the stationary phase at which lactate concentration was over 100 mM, the growth rate dropped to 0.06 h^{-1} (Fig 4.1). Therefore, the growth rate of *L. buchneri* in the MCR could be affected by the end products in high concentrations enough to inhibit growth. The concentrations of lactate and ethanol were maintained in a range of 100 mM to 150 mM.

The growth of *L. brevis* was started at the growth rate of 0.064 h^{-1}, but the growth rate decreased 0.04 h^{-1} in 10 h. The fermentation produced the total cell mass 15.5 DCW, g/L consuming a carbohydrate mixture 207 g/L of glucose and fructose. The total cell product yield was 0.075. Total cell product yield of *L. brevis* was higher in the MCR than in either the batch cultures with pH control ($Y_{X/S} = 0.067$) or without pH control ($Y_{X/S} = 0.06$). In the investigation about the effect of the glucose and the fructose to the growth rate (See Fig 3.35 and Fig 3.36), the growth rate increased with an increase fructose concentration, but glucose did not affect the growth rate. The growth rate in the batch culture was high in the presence of fructose until 10 h, but the growth rate after 10 h decreased to 0.034 h^{-1} with the shortage of fructose in the growth medium (See Fig 4.4). The fructose concentration was very low during the cultivation in the MCR. As shown at Fig 5.6, the dilution rate was not increased to over 0.48 h^{-1} because of a serious membrane fouling in product membrane. A limitation in feeding created carbohydrate limitation during the cultivation. Therefore, the growth rate was lower in the MCR than in the batch culture.

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B. longum was grown at the growth rate of 0.128 h^{-1} and the cell mass reached DCW 22.2 g/L in 11 h. The carbohydrate of 126 g/L in a mixture of glucose and fructose was consumed during the culture. Therefore, total cell product yield was 0.176. Total cell product yield was lower in the MCR than in the batch culture ($Y_{X/S}$ = 0.213) with pH control, but it was the same as the batch culture ($Y_{X/S}$ = 0.17) without pH control. The growth rate in the MCR was lower in the MCR than in either the pH controlled STR culture (μ = 0.27 h^{-1}) or the batch culture (μ = 0.23 h^{-1}). In growth of *B. longum* shown at Fig.4.7 the growth rate decreased in 9 h when the acetate concentration was in a range of 150 mM to 200 mM. In Fig 5.7 the growth rate of *B. longum* was high during the early stage of 0 h to 4 h when acetate concentration was in a range of 150 mM to 200 mM and the growth rate decreased when acetate concentration increased to over 200 mM in 4 h. The dilution rate did not increase more than 0.57 h^{-1} because of a serious membrane fouling in product membrane. The growth rate of *B. longum* was affected by acetate accumulation. This result is very similar to them obtained by Desjardine et al. [1990].

O. oeni was grown at the growth rate of 0.067 h^{-1} and the cell mass reached DCW 12.8 g/L in 68 h consuming carbohydrate of 610 g/L in a mixture of glucose and fructose. The total cell product yield was 0.021. The total cell product yield was the same in all cultures. The growth rate was lower in the MCR than in either the pH controlled STR culture (μ = 0.108 h^{-1}) or the batch culture (μ = 0.08 h^{-1}). In investigation for the effect of carbohydrates on the batch culture (Fig. 3.25) *O. oeni* had a long lag during the early stage and was slowly grown when glucose or fructose was limited. There is no lag phase at the early stage in the presence of both glucose and fructose. In Fig.3.26 glucose did not effectively improve the growth rate. In glucose-limited growth conditions the growth rate decreased and acetate production increased more than lactate production (Fig 4.11). Figure 4.11 showed that the growth rate decreased in the glucose concentration under 20 mM and *O. oeni* stopped growing further when glucose was limited, but the acetate concentration still increased with the consumption of fructose. The growth of *O. oeni* in the MCR as shown at Fig 5.8 could be progressed with only fructose in a glucose-limited condition. The growth rate decreased and the productivity of acetate increased consuming fructose as the main carbon source.

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5.7.2. Comparison of the product yields (Table 5.1).

Strains	X$_{DCW}$ (g/L)	T (h)	μ (h^{-1})	Product yields (g/g)					P$_{X/T}$ (g/L,h)
				Y$_{x/s}$	Y$_{L/S}$	Y$_{A/S}$	Y$_{E/S}$	Y$_{M/S}$	
L. buchneri	16.2	23	0.065	0.077	0.375	0.093	0.243	N.D	0.704
L. brevis	15.5	20	0.064	0.075	0.9	0.017	N.D	0.005	0.774
B. longum	22.2	11	0.128	0.176	0.25	1.251	0.192	N.D	2.018
O.oeni	12.8	68	0.067	0.021	0.175	0.284	0.033	0.394	0.188

Table.5.1. Comparison of all parameters obtained during the cultivation of LAB in the MCR.

The cell mass (X) in DCW, g lL, the culture time(T) in hour, the growth rate (μ) in h^{-1}, the cell production yield (Y$_{X/S}$) in the cell mass (glL) produced per the carbohydrate (glL) consumed, the lactic yield (Y$_{L/S}$) in lactic acid (g/L) produced per the carbohydrates (g/L) consumed, acetic yield (Y$_{A/S}$) in acetic acid (g/L) produced per the carbohydrates (g/L) consumed, ethyl alcohol yield (Y$_{E/S}$) in ethyl alcohol (glL) produced per the carbohydrates (g/L) consumed, mannitol yield (Y$_{M/S}$) in mannitol (g/L) produced per the carbohydrates (g/L) consumed, and the specific cell production rate (P$_{X/T}$) in the cell mass (glL) produced per hour were shown at this table.

The major organic acid produced during the cultivation of *L. buchneri,* the product yield of lactate was 0.375. The second major product, the product yield of ethyl alcohol was 0.243. Mannitol was not produced during the fermentation of *L. buchneri.* All product yields in the batch culture were the similar to them in the MCR. Mannitol was not produced because fructose was not used.

L. brevis in the MCR was grown like homofermentative lactic acid bacteria mainly producing lactate. The product yield of lactate increased from 0.4 during the batch culture to 0.9 during the cultivation in the MCR and other product yields decreased.

In general, *L. buchneri* and *L. brevis* have two metabolic pathways deeply related to the growth rate. *L. brevis* uses fructose like glucose producing lactate in a low growth rate, but fructose is used for regeneration of NAD^{+} in a high growth rate.

As shown at Fig 5.6, the dilution rate did not increase more than 0.48 h^{-1} because of a serious membrane fouling in product membrane.

A limitation in feeding created carbohydrate limitation during the cultivation. The growth rate of *L. brevis* because of fructose limitation was lower in the MCR than in the batch culture and lactate was produced as the major product. The production of ethanol is inhibited by the presence of fructose, but the productivity is increased in high growth rate. The reason why ethanol was produced in the batch culture is because the growth was controlled in a high growth rate and ethanol was produced from the point when fructose was limiting in the growth medium.

The cultivation of *B. longum* was affected by the acetate concentration the major product at the end of fermentation. The product yield of lactate decreased from 0.3 in the batch culture to 0.25 during the cultivation in the MCR and the product yield of acetate and ethanol increased to 1.5 times them in the batch culture. In Fig 5.7 the feeding of the growth medium was not increased further because of a serious membrane fouling in product membrane. Therefore glucose and fructose was completely consumed in 6 h. In such a glucose-limited condition *B. longum* can produce acetate, ethanol, and formate rather than lactate [Degnan and Macfarlane. (1994)].

While *O. oeni* was being grown in a glucose-limited condition of the MCR, the product yield of lactate decreased to 87% that in the batch culture and the product yield of acetate and ethanol showed a slight increase 5%. *O. oeni* produces lactate, acetate, ethanol, and erythritol via phosphoketolase pathway from fructose in low growth rate and mannitol in a high growth rate [Richter et al. (2003)]. Mannitol was produced in a ratio of one third to fructose used, but 9% more mannitol was produced during the cultivation in the batch culture. Dilution rate did not increase more than 0.5 h^{-1} because of a serious membrane fouling in feed membrane.

5.7.3. The limits in growing LAB to high cell density.

A severe fouling on the membrane produced the limitation also in increase dilution rate of the growth medium during the cultivation of *L. brevis* and *B. longum*.

Chapter.5 Growth of Lactic Acid Bacteria in Membrane Cell Recycle reactor.

In general, S-layer proteins produced by *L. brevis* and exopolysaccharides produced by *B. longum* were the main clues of fouling in product membrane. At the end of cultivation where cell density is high, a serious fouling in product membrane made a limitation on feeding the growth medium

In the case of *O. oeni*, a membrane fouling was not in product membrane, but a fouling in feed membrane made a problem because of a long culture period. by a low growth rate making a limitation on feeding rate. The feed ultimately caused this problem. The membrane fouling limited the dilution rate and the carbon is completely consumed causing an accumulation of end products which are at high enough concentration to inhibit the growth. The filtering area should be increased and the flux must be improved by an alternative use of membrane or intermittent back-flushing of membrane *(Section 1.5.2)*.

Another problem for the growth of *O. oeni* in the MCR was the amount of the growth medium consumed during the cultivation because *O. oeni* consumed three times that consumed during the culture of other LAB because of low growth rate resulting in non-economic.

When the cell reached high cell density, there was a severe foaming in the culture fluid. Foaming in the culture fluid was another problem. Foams led to malfunction of the level controller contaminating level indicator, and consequently affected the feed rate of the growth medium. Foams also disturbed the function of the magnetic centrifugal pumps resulting in disturbing the flow of the culture fluid.

According to the results obtained during the cultivation of four lactic acid bacteria, the membrane bioreactor for the cell retention culture can be strategically designed for a high product yield and high production rate. In order to give bacteria the best growth the concentration of medium must be lowered to reduce the risk of the end product inhibition. High substrate concentration produces high end product concentration and it can provide the clue of end product inhibition. Therefore, the substrate concentration must be controlled in the critical range of certain concentration not to inhibit the growth. Consequently, in $D = Jm = \mu_{max}$, the efficiency of the MCR will be the maximum.

Chapter.5 Growth of Lactic Acid Bacteria in Membrane Cell Recycle reactor.

When the substrate concentration is controlled in the critical concentration, the substrate can be limited in the middle of the fermentation. Therefore, the feeding rate of the growth medium must be controlled in a high rate so that the growth can not be inhibited by the substrate limitation. High feeding rate needs a large filtering area and membrane fouling must be prevented. However, unfortunately, the cost of membrane is a burden in design of membrane reactor.

CHAPTER 6: MALOLACTIC FERMENTATION OF CIDER by L. BREVIS AND O. OENI.

6.1. INTRODUCTION

Malolactic fermentation (MLF) is the microbiologically mediated de-acidifying process typically associated with alcoholic beverages such as wine, cider, and beer. Mainly, during the alcoholic fermentation by yeast, wine and cider have a strong sour flavour by alcohols, aldehydes, and esters together with citric acid and malic acid derived from the fruit source. These astringent tastes in wine and cider can be quantitatively reduced through MLF.

There are a wide range of bacterial groups isolated in wine-making processes. *Oenococcus oeni* and *Lactobacillus brevis* have been most predominant in MLF of wine-making and cider-making process, respectively. Both species are very resistant against low pH and high alcohol concentration, and very effective in transforming malic acid into lactic acid with a resultant de-acidification.

O. oeni has been known as the main species to control the fragrance of wine forming ester compounds like ethyl acetate and fatty ethyl esters by the esterification between aldehydes and alcohols or fatty acids. Butter flavours, diacetyl, acetoin, and 2,3-butanediol, can also be generated through the metabolism of citric acid (Fig.1.5) [Aline (1999), Saguir and Manca de Nadra (2002)].

In general, acetaldehyde is the major component to affect wine aging, color, and stability, and is mainly produced by yeast during alcoholic fermentation. Also, Liu et al. [1997] and Ott et al. [2000] noted the production of acetaldehyde by *Lactobacilli*. In terms of catabolism of acetaldehyde, Osborne et al. [2000] mentioned that acetaldehyde can be consumed to produce the growth energy during MLF by *O. oeni*, generating acetic acid or ethanol with ATP.

L. brevis has been known as the common spoilage contaminant of beer for a long time because of its strong resistance to the bitter flavour of hops, and it has been tried in

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maturation process of cider for recent years [Sakamoto et al. (2001), (2002)].

Conventional MLF depends on the spontaneous and natural selection of malolactic species, but there have been a couple of trials to carry out MLF in the bioreactor with high density malolactic species [Ref. HP. Bulmer] The first trials were to add starter culture into the system. Although MLF improved in reliability, they have not significantly speeded the process up. Such trials can be problematic because cider conditions during the alcoholic fermentation are unfriendly to growth of LAB. These systems typically have not been well adapted for such MLF and it is still possible to get a failure.

This chapter describes the investigation into the use of *L. brevis* and *O. oeni* in the rapid maturation of cider. In this situation, the MCR was used to propagate the cells as previously described, and then followed by a prolonged biotransformation phase of the MLF. *L. brevis* and *O. oeni* were compared with their efficiency and differences in carrying out MLF of the cider. The effects of the retention time and the type of cider on MLF were investigated.

6.2. THE ASSESSMENT OR THE MEMBRANE FLUX OF THE GREEN CIDER.

In the maturation process, the green cider is filtered to remove all bacteria, yeast and other particles. Therefore, it was very important to measure the membrane flux of the substrate the green cider through the ceramic membrane as this determines the feed rate. In general, the efficiency of MLF is strongly influenced by the feed rate of the green cider in the feed stream. The green cider after alcoholic fermentation contains low concentration of soluble proteins, but the presence of yeast and tannin must be significantly considered due to the membrane fouling that the compounds were.

The control of pH or temperature to improve the membrane flux could not be utilized during the cider maturation. Therefore, the operating pressure was considered as the only parameter that could be used to control the membrane flux of the green cider in feeding. Figure 6.1 shows the result of filtering the green cider at the different pressures.

According to the result shown in Fig.6.1, the membrane flux dramatically drops in 20 min, and then showed a steady decline over the rest of the experiment. Fluxes did not change over a large range of pressures 0.5 ~ 1.0 bar, but significantly slower rates were observed at 0.3 bar.
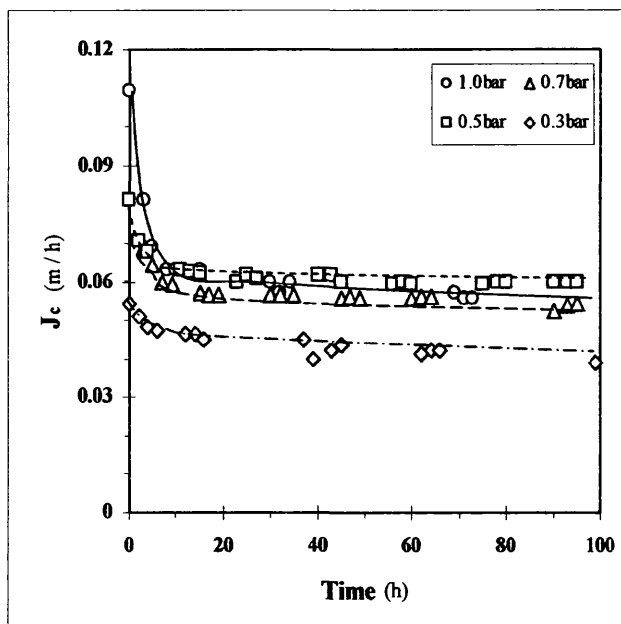
Fig.6.1 The effect of operating pressure on the membrane fluxes of the green cider. *The filtering area of the ceramic membrane was 0.2 m² and operating pressure was given in the interval of 0.3, 0.5, 0.7, and 1.0 bar. Permeate and filtrate recycled at the system. Cider used in this investigation was diluted to 9% (w/v) in terms of the alcohol concentration. Temperature was not controlled and pH of the recycling cider was in a range between pH 3.5 and pH 3.7.*

6.3. MALOLACTIC FERMENTATION OF CIDER BY OENOCOCCUS OENI.

6.3.1. Malolactic fermentation in different types of the green ciders.

Two types of the green ciders, which are General cider and Scrumpy Jack, were compared with the effect by the types of cider on malolactic fermentation. General cider has low content of malic acid and alcohol when compared with Scrumpy Jack. Also, the soluble proteins or the residual carbohydrates concentration might be different as General cider contains less apple juice.

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The green cider is a resource cider to make General cider or Scrumpy Jack as a cider just after alcoholic fermentation of the apple juice. The green cider can become General cider or Scrumpy Jack after MLF by lactic acid bacteria. The experiments were carried out with the green cider of General cider and Scrumpy Jack.

6.3.1.1. Malolactic fermentation of General cider by O. oeni.

In general, the strains take up and metabolise nutrients in General cider for survival and propagation. The cider residence time has a large effect on the concentrations of components in the product stream during MLF.

Concentrations of organic components in the product stream after MLF could be controlled by the reaction rate, which is the cider residence time in the reactor. Cells are growing very slowly and the cider residence time, the reaction rate (V_{max}) and the K_S for substrate uptake are the main factors to control concentrations of organic components so affecting the flavour of the alcoholic beverage.

The reactor was efficiently run in propagation mode so that *L. brevis* or *O. oeni* was grown to high concentration. This was done using the growth methods and the media outcome previously in the Materials and Methods (Chapter 2). Once sufficient cells were present, the fluid flow was switched to the cider reservoir tank and General cider was passed through the MCR after washing with General cider by 5 times volume of the MCR. This also served as a flow adaptation period. The MLF of General cider was performed in a certain range of the retention time from 3 h to 10 h. The samplings were made in every 12 h and analyses were carried out as soon as possible after the sampling. Ethanol concentration was controlled at 9% (w/v). Operation of the MCR was followed to the methods described in Chapter.2 *(Refer to 2-6-2)*.

The results of this cider maturation are summarised at Fig 6.2, Fig. 6.3, and Fig.6.4. Figure 6.2 shows the effect of the retention time on the cell mass and pH between the feed and the product, and Figure6.3 shows the effect of the retention time on organic acids during MLF of General cider by *O. oeni.*

Using this data a double reciprocal plot of malate concentration and the reaction rate could give the K_S for malate uptake in General cider. The K_S for malate uptake in General cider by the biocatalyst LAB was determined by difference between malate concentrations in the feed and the product stream after MLF and was shown at Fig.6.4.

• *The effect of the cider retention time on pH and the cell mass during MLF of General cider by O. oeni.*

Figure 6.2 shows the effect of the retention time on pH and the cell concentration during the MLF of General cider.
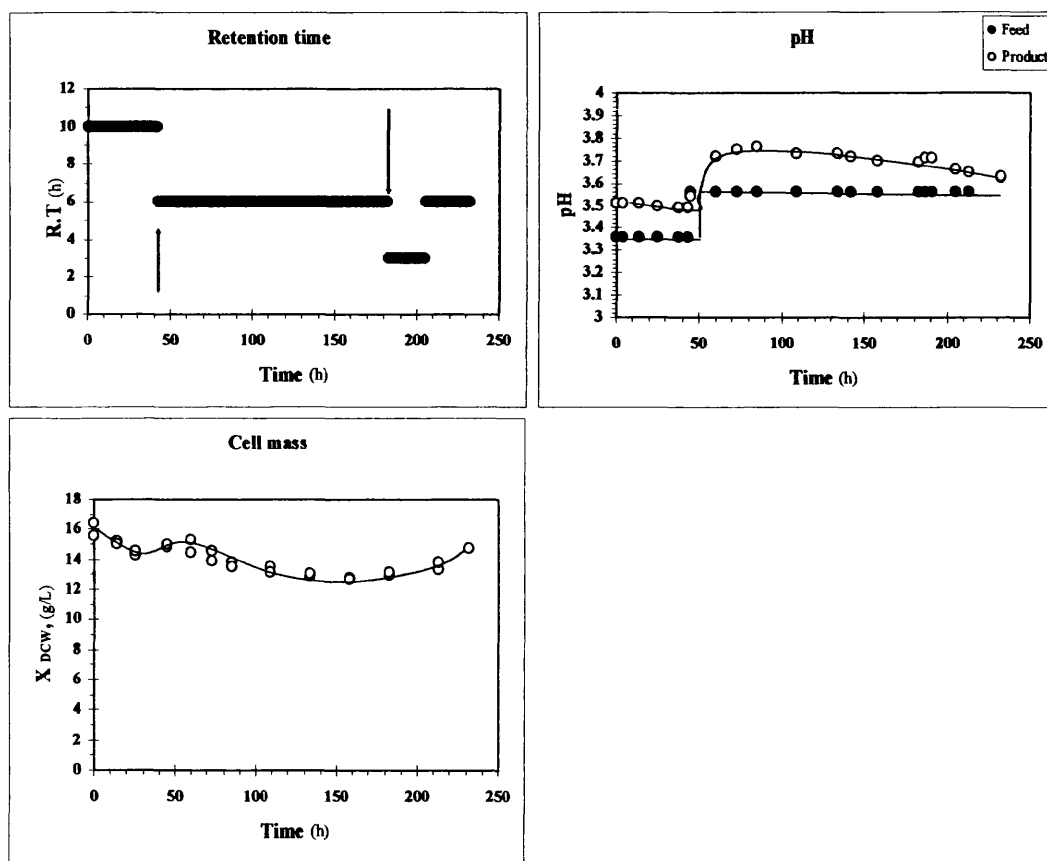
Fig.6.2. The effect of cider retention time on the cell mass, and pH during MLF of General cider by *Oenococcus oeni*. *The feeding of the green cider was carried out in terms of 10 h, 6 h, and 3 h. Alcohol content was 9% (w/v). The pH and cell mass were monitored in a function of the cider retention time. The arrows mark an instant stop of feeding during the change of the cider batch in the cider feed tank. Temperature was 28 °C.*

According to Fig. 6.2, the cell mass declined from 15.15 DCW, g/L while running the MCR in the retention time of 10 h to 13.75 DCW, g/L in the retention time of 3 h. The pH difference between the feed and the product stream reduced with the shorter retention time.

• *The effect of the cider retention time on organic acid during MLF of General cider by O. oeni.*

During MLF of General cider, the consumption rate of malate was strongly affected by the retention time. The majority, 4.3 mole of total malate 6.8 mole in the feed, was consumed for 42 h of the retention time 10 h and total malate 31.4 mole in the feed was reduced to total 17.3 mole in the product for 165 h of the retention time 6 h. Lower consumption rates of malate are detected with the shorter retention time. A total malate 12.1 mole in the feed was reduced to 7.4 mole in the product for 22 h of the retention time 3 h (Fig.6.3, Table 6.1). General cider 151.2 L was consumed during MLF in the retention time 10 h and General cider 990 l was consumed during MLF in the retention time of 6 h. During MLF in the retention time of 3 h General cider 264 L was consumed.

The malate concentration in the product during MLF reduced to below 20 mM lower concentration than K_s for malate uptake in the retention times of 10 h and 6 h, but increased to 22 mM again in the retention time of 3 h.

Lactate was produced with the consumption of malate. Lactate (1 M) could be produced through de-carboxylation reaction from malic acid (1M) with CO_2 (1 M). Production of lactate decreased with the longer retention time whereas production of acetate increased (Table.6.1).

R.T (h)	Time (h)	T-Vol. (L)	Feed (mole)			Product(mole)			Variation (mole)		
			M	L	A	M	L	A	M	L	A
10	42	151	6.8	4.3	1.3	2.5	7.3	4.0	-4.3	3.0	2.7
6	165	990	31.4	15.4	5.9	17.3	33.7	14.7	-14.1	18.3	8.8
3	22	264	12.1	3.3	1.5	7.4	9.4	2.4	-4.7	6.1	0.9
Total	229	1405	50.3	23	8.7	27.2	50.4	21.1	-23.1	27.4	12.4

Table.6.1 The effect of the retention time in organic acids during MLF of General cider by *O. oeni*. *Variations were described with the real quantities (mole) of reduction or increase to the feed after MLF. Therefore, the negative shows a reduction and the positive shows an increase. Malate (M), Lactate (L), and acetate (A) were described in mole. T-vol. means total volume displayed in litter.*

Figure 6.3 shows the composition of the components in the feed and the product stream during MLF of General cider. Malate 23 mole was transformed to lactate 27.4 mole. Acetate 12.4 mole was thought to be produced by the dissimilation of the residual carbohydrate and citric acid in the green cider after alcoholic fermentation. Malate can also be transformed to acetate via pyruvate from malate by intracellular malic enzymes.
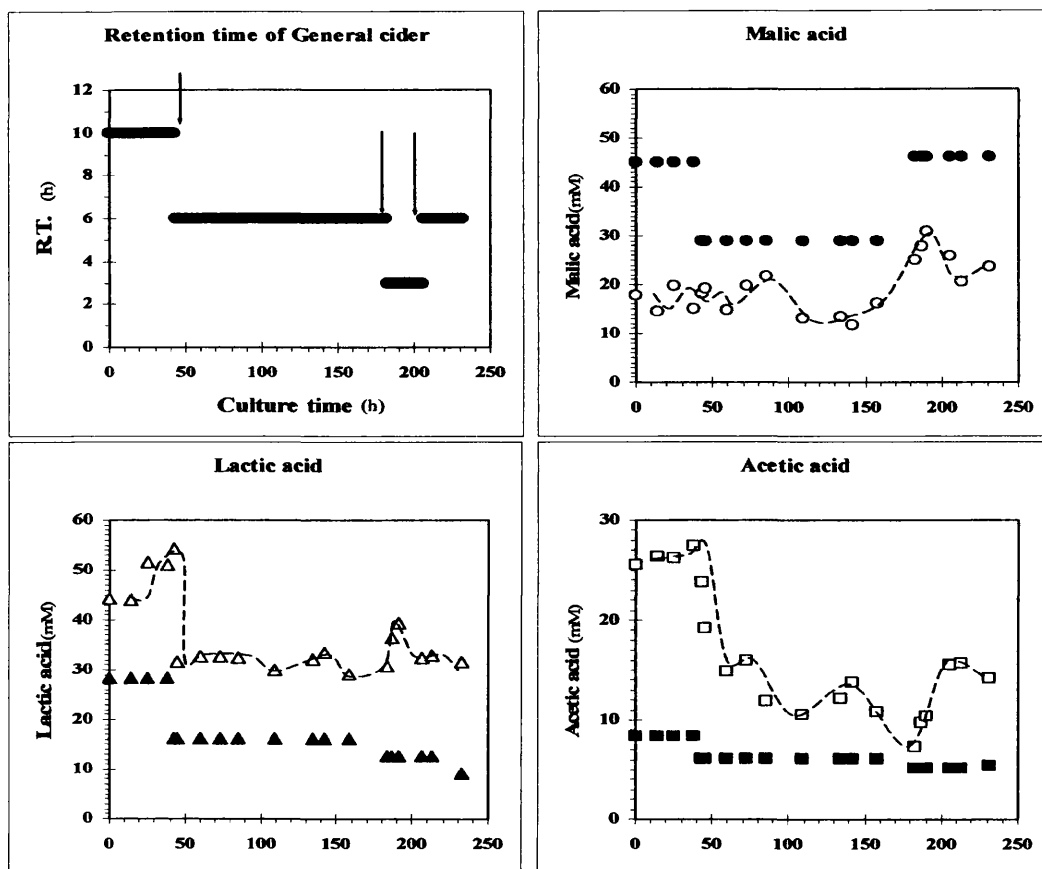
Fig.6.3. The effect of the retention time on organic acids during MLF of General cider by proliferating high density *Oenococcus oeni*. *Organic acids in the feed stream (the closed) and the product stream (the opened) were described. Each of organic acid was monitored as a function of the retention time, and the arrows mark an instant stop of feeding during the change of the cider batch in the feed cider tank. General cider was fed in the residence times of 10 h, 6h, and 3h. Alcohol content was 9% (w/v). Temperature was 28 °C*

Figure 6.4 shows the effect of the retention time on the transformation activity and the specific transformation activity of organic acids during MLF of General cider by *O. oeni*.
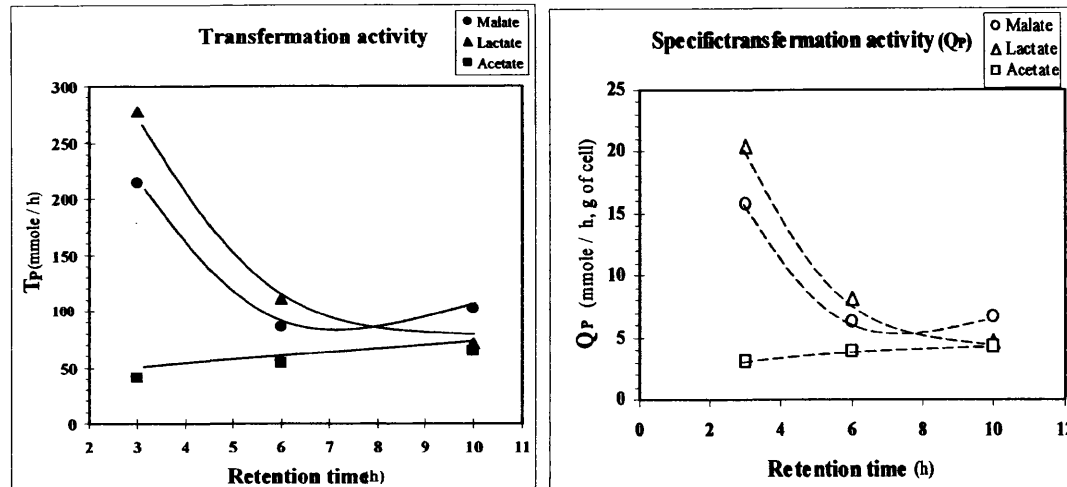
Fig 6.4. The effect of the retention time on the transformation activity and the specific transformation activity of organic acids during MLF of General cider by *O. oeni*. *The feed rate was controlled in terms of the retention time 3, 6 and 10 h. The alcohol concentration was 9% (w/v)*

The specific transformation activity of organic acids did not show a great difference between the retention times of 6 h and 10 h, but when the feeding rate increased to the retention time of 3 h, the specific transformation activity of malate and lactate increased.

However, the productivity and the specific productivity of acetate show a slow increase with increase retention time.

• *The K_s for malate uptake and transformation of malate by O. oeni in General cider.*

Figure.6.5 shows the apparent *Ks* for malate uptake in biotransformation of malate by *O. oeni* in General cider. As shown at Fig.6.5, the *Ks* for malate uptake was 31.4 mM with General cider.
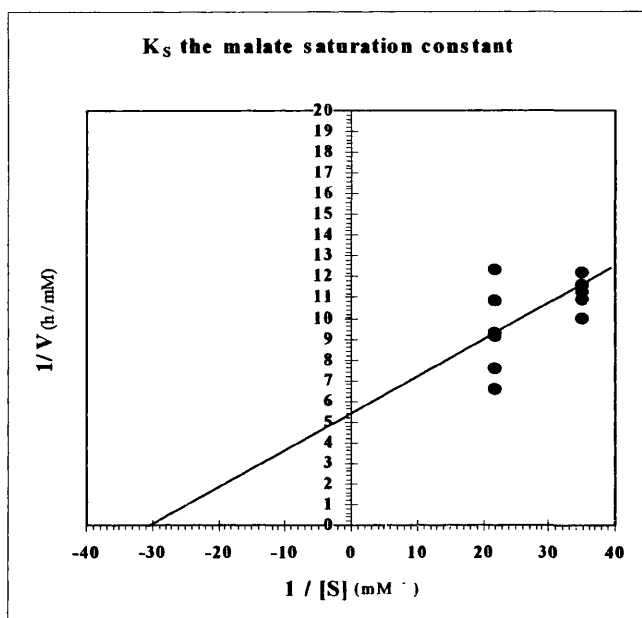
Fig.6.5. Determination of the K_S for malate uptake in biotransformation of malate by *O. oeni* in General cider. *The K_S was determined by Lineweaver-Burk equation a double reciprocal plot of the transformation rate to the malate concentration (M). The equation of line was designated as Y = Ks/Vm X 1/[S] + 1/Vm = 0.1739X + 5.5356, and consequently, Ks = 0.0314 M.*

6.3.1.2. Malolactic fermentation of Scrumpy Jack by O. oeni.

Scrumpy Jack is the cider containing more tannin, higher ethyl alcohol concentration, higher malate concentration than General cider as comparison of pure cider apples rather than the blends. The residual carbohydrates, tannin, and ethyl alcohol can affect the transformation rate and the K_S for malate uptake.

The experiment was performed along the same method as the previous section. Before starting MLF in the MCR the growth medium after the culture of LAB was changed with a sufficient amount of Scrumpy Jack (about 5 volumes) to stabilize the cider flow through the MCR. MLF of Scrumpy Jack was carried out among the retention times from 3 h to 12 h.

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The samplings were made in every 12 h and analyses were carried out as soon as possible after the sampling. The ethanol concentration was controlled at 9% (w/v) during MLF.

The results are summarised at Fig 6.6, Fig.6.7, Fig.6.8 and Fig 6.9. Figure 6.6 shows the effect of the retention time on the cell mass and pH between the feed and the product stream. Figure 6.7 shows the effect of the retention time in the composition of organic acids during MLF of Scrumpy Jack by *O. oeni*. Figure 6.8 shows the effect of the retention time on the transformation activity and the specific transformation activity of organic acids during MLF of Scrumpy Jack. The determination of the *Ks* for malate uptake by *O. oeni* in Scrumpy Jack is shown at Fig.6.9.

- *The effect of the cider retention time on pH and the cell mass during MLF of Scrumpy Jack.*

Figure 6.6 shows the effect of the retention time on pH and the cell mass during MLF of Scrumpy Jack by *O. oeni*. According to Fig 6.6, the cell mass increased without an influence by the retention time during MLF of Scrumpy Jack.

The pH difference between the feed and the product increased with increase retention time. The pH of product was maintained at pH 0.05 higher than pH of the feed during MLF of Scrumpy Jack at the retention time of 6 h, but pH difference decreased to pH 0.03 during the retention time of 3 h. The pH difference increased again to pH 0.1 during the retention time of 12 h

- *The effect of the cider retention time on the composition of organic acids during MLF of Scrumpy Jack by O. oeni.*
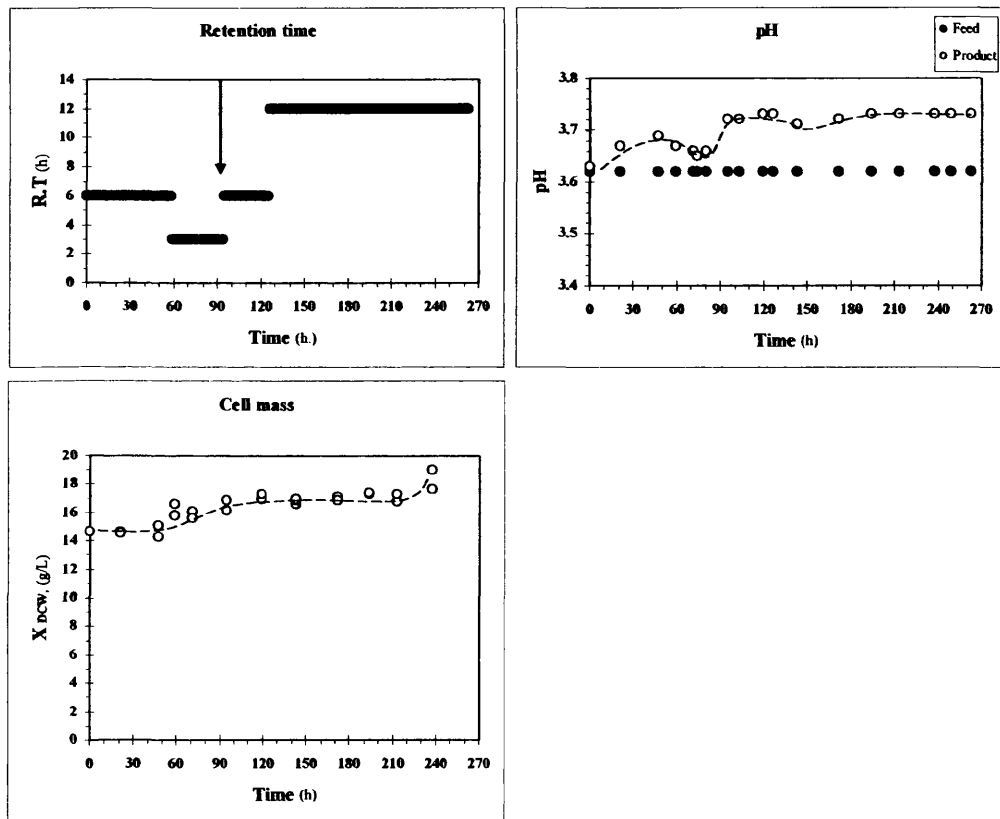
Figure 6.7 shows the effect of the retention time in the composition of organic acids during MLF of Scrumpy Jack. Total malate 27.3 mole in the feed reduced to total 14.5 mole in the product consuming Scrumpy Jack 560 L for 88 h of the retention time 6 h and total malate 17.5 mole reduced to 14.4 mole in the product consuming Scrumpy Jack 420 L for 35 h of the retention time 3 h.

Malate, 10 mole of total 17.1 mole in the feed stream, was transformed consuming Scrumpy Jack 411 L for 137 h of the retention time 12 h.

Transformation rate of malate did not vary for the retention times of 6 h and 12 h. The transformation rate was thought to be entirely governed by the K_S for malate uptake.

Fig.6.6. The effect of the retention time on the cell mass, and pH during MLF of Scrumpy Jack by *Oenococcus oeni.* The pH of the feed stream (the closed) and the product stream (the opened) were described. The retention time was controlled in terms of 12 h, 6 h, and 3 h. Ethyl alcohol concentration during MLF was 9% (w/v). The pH and cell mass were monitored on a function of the retention time, and the arrow marks an instant stop of feeding for the change of the cider batch. Temperature was 28 °C

The transformation activity of *O. oeni* during MLF of Scrumpy Jack was inhibited to a certain extent. Figure 6.7 shows the composition of organic acids during MLF of Scrumpy Jack by *O. oeni.*
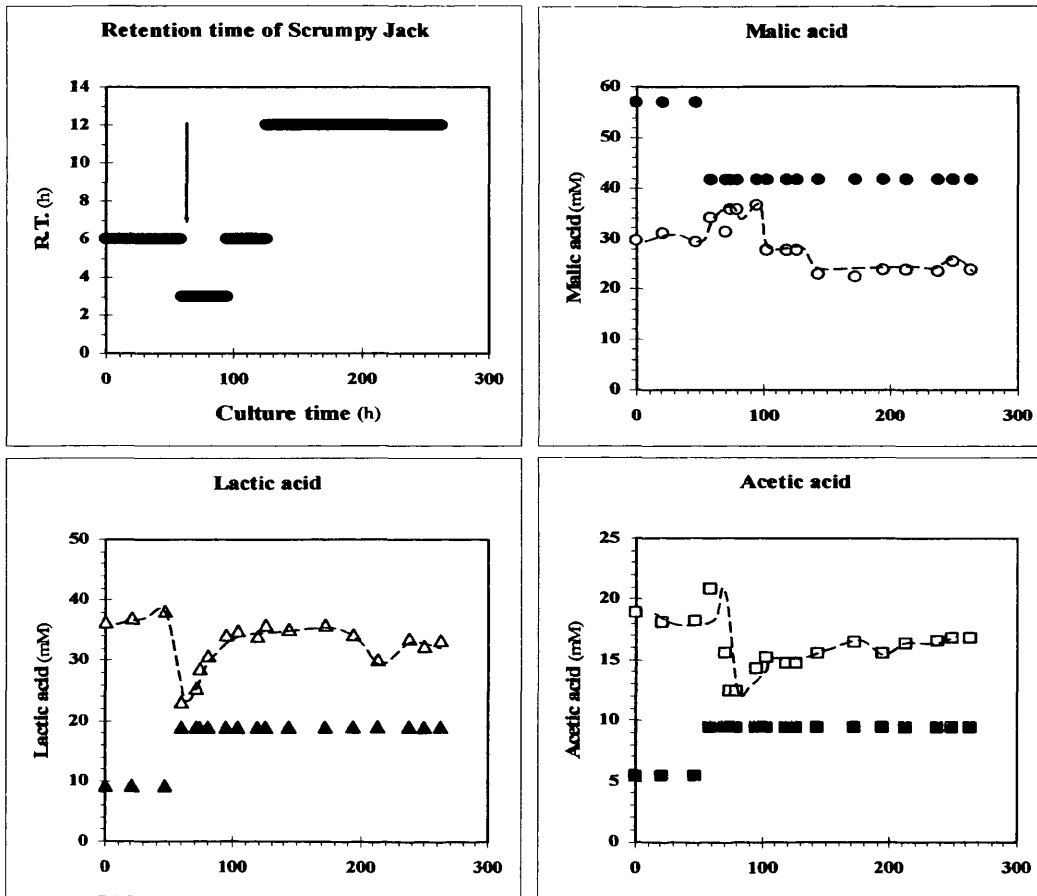
Fig.6.7. The composition of organic acids during MLF of Scrumpy Jack by high density of proliferating *Oenococcus oeni*. *Organic acids in the feed stream (the closed) and the product stream (the opened) were described. The MLF was carried out the retention time of 12 h, 6 h, and 3 h. The ethanol concentration during MLF was 9% (w/v). The composition of organic acids was monitored as a function of the retention time, and the arrow marks an instant stop of feeding for the change of the cider batch. Temperature was 28 °C.*

The malate in the product was over 20 mM even at the retention time of 12 h. Although the cell mass was in a higher concentration during MLF of Scrumpy Jack than during MLF of General cider, the malate concentration in the product was higher 34.2 mM for the retention time of 3 h and 28.2 mM for the retention time of 6 h in Scrumpy Jack than in General cider.

The malate concentration in the product decreased to 24 mM for the retention time of 12 h.

R.T (h)	Time (h)	T-Vol. (L)	Feed (mole)			Product(mole)			Variation (mole)		
			M	L	A	M	L	A	M	L	A
12	137	411	17.1	7.9	3.9	10	13.9	6.0	-7.1	6.0	2.1
6	88	560	27.3	6.6	8.3	14.5	19.5	9.1	-12.8	12.9	0.8
3	35	420	17.5	8.0	4.0	14.4	11.4	6.4	-3.1	3.4	2.4
Total	260	1391	61.9	22.5	16.2	38.9	44.8	21.5	-23	22.3	4.5

Table.6.2 The effect of the retention time in the composition of organic acids during MLF of Scrumpy Jack by *O. oeni*. *Variations were described with real quantities of reduction or increase of organic acids in the product after MLF. Therefore, the negative marks a reduction and the positive marks an increase. Malate (M), Lactate (L), and acetate (A) were described in mole.*

According to Table 6.2, malate in the product stream was controlled in the lower concentration (26 mM) than the K_S (57 mM) for the malate uptake (Fig.6.9) by *O. oeni* in Scrumpy Jack for the retention time of 12 h and 6 h. When the feed rate of Scrumpy Jack increased to the retention time of 3 h, the malate concentration in the product increased to 34.3 mM. This is still lower than the K_S for malate uptake by *O. oeni* in Scrumpy Jack.

The lactate concentration increased to 33.8 mM in the product from 20.2 mM in the feed for the retention time of 12 h and to 34.8 mM in the product from 11.3 mM in the feed for the retention time of 6 h. For the retention time of 3 h the lactate concentration was increased from 19.1 mM in the feed to 27.1 mM in the product.

The acetate concentration in the product increased from 9.5 mM in the feed to 14.6 mM with the retention time of 12 h. For 6 h retention time it increased from 14.8 mM in the feed to 16.3 mM, and it increased from 9.5 mM in the feed to 15.2 mM with the retention time of 3 h.
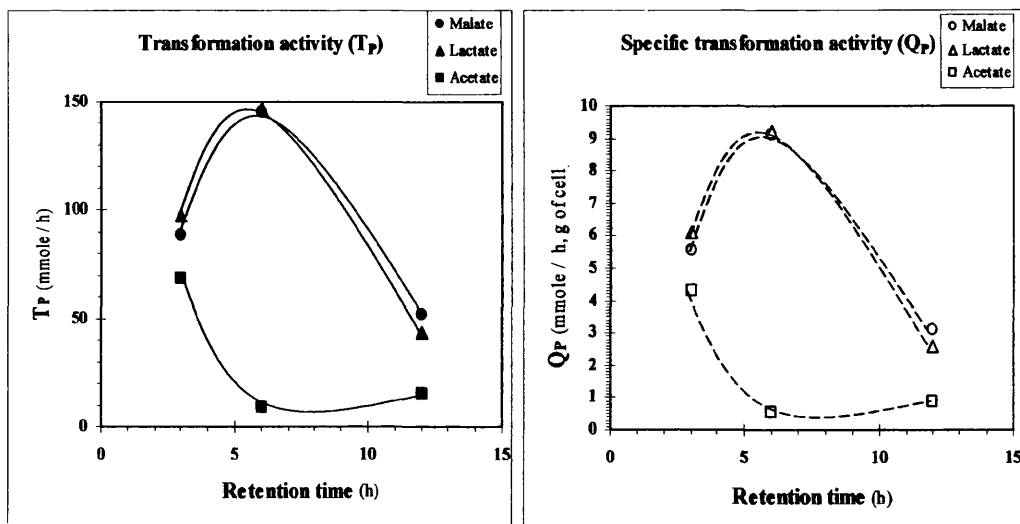
Fig. 6.8 The effect of the retention time on the transformation activity and the specific transformation activity of organic acids during MLF of Scrumpy Jack by *O. oeni. The feed rate was controlled in terms of the retention time 3, 6 and 12 h. The alcohol concentration was 9% (w/v).*

The transformation activity and the specific transformation activity of organic acids increased with increase feed rate as shown in Fig 6.8. The transformation activity and the specific transformation activity of malate and lactate dramatically increased with increase retention time in a certain range from 12 h to 6 h except for the case of acetate. The productivity and the specific productivity of acetate did not show an increase in the retention time from 6 h to 12 h, but when the retention time reduced to 3 h the productivity of acetate increased.

• *Determination of the K_s for malate uptake by O. oeni in Scrumpy Jack.*

Figure 6.9 shows the apparent K_s for malate uptake by *O. oeni* during MLF of Scrumpy Jack. The K_S for malate uptake is inhibited by the presence of unknown inhibitory components in Scrumpy Jack. This conclusion was made by comparing Fig.6.5 with Fig 6.9, the K_s value increased from 31.4 mM during MLF of General cider to 57.6 mM during MLF of Scrumpy Jack.
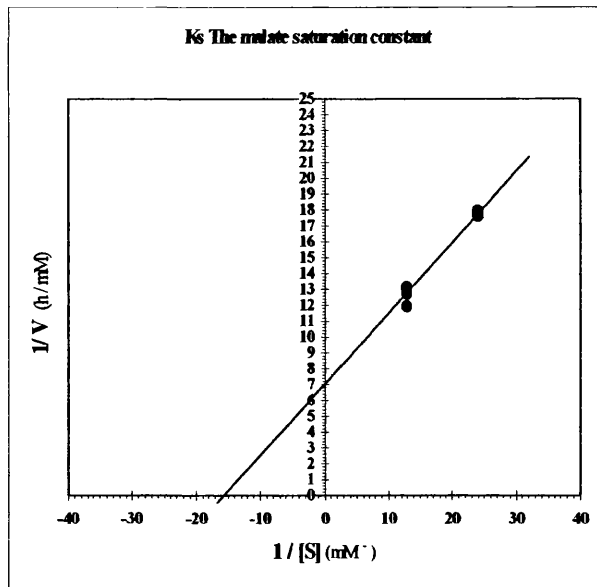
Fig.6.9. Determination of the *Ks* for malate uptake by Lineweaver-Burk equation during MLF of Scrumpy Jack by *Oenococcus oeni*. *The equation of line was designated as* $Y = Ks/Vm \times 1/[S] + 1/Vm = 0.4249X + 7.3733$, *and consequently*, $Ks = 0.0576$ *M.*
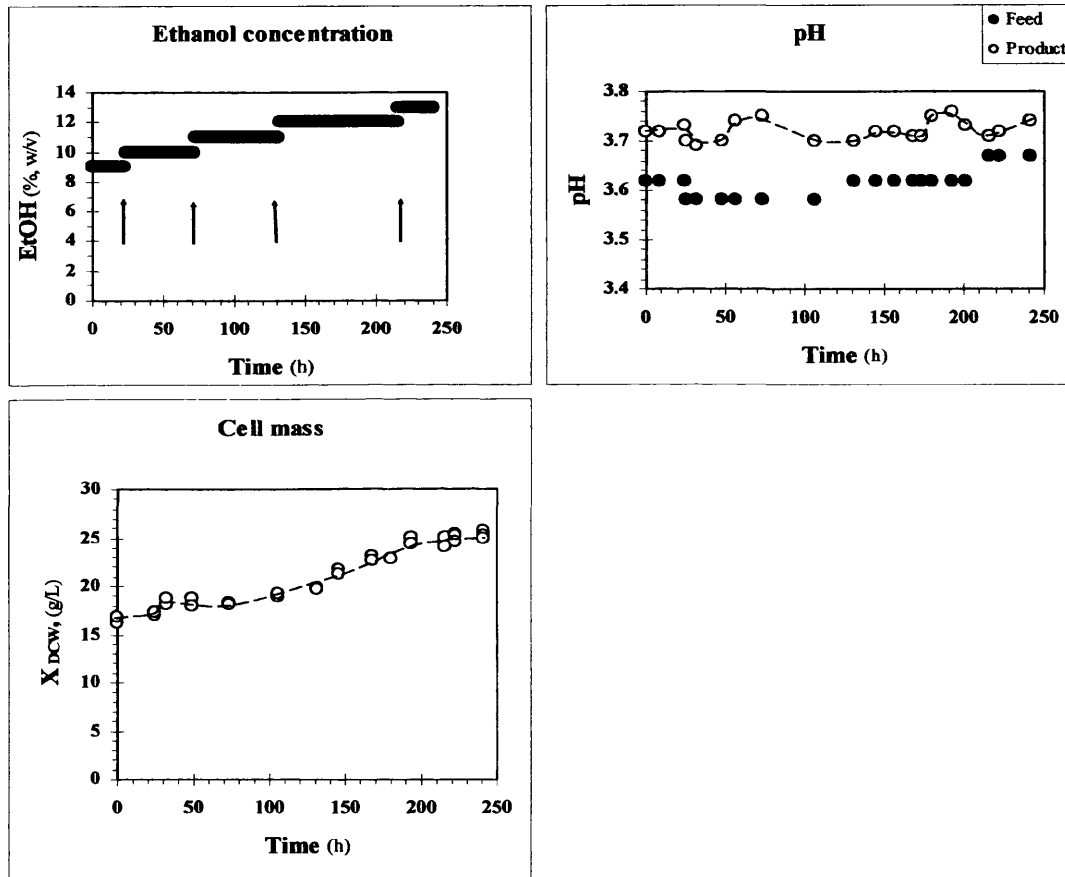
6.3.2. The effect of the alcohol concentration during MLF of Scrumpy Jack by Oenococcus oeni.

The Scrumpy Jack was supplied as a high alcohol concentration (13%,w/v) and was diluted with water to investigate the effect of the feed strength *(Refer to 2-6-2-2)*. The feeding rate of the cider was at the retention time of 6 h through the experiment. The membrane fouling was not significant at this feeding rate. The viability of the cell or the enzyme activity could be influenced by the alcohol concentration in the bioreactor. The critical alcohol concentration effective for malolactic activity and the effect of alcohol concentration on the components in the product were investigated.

6.3.2.1. The effect of the alcohol concentration on the cell mass and pH during MLF of Scrumpy Jack by O. oeni..

The cell mass of _O. oeni_ increased while the alcohol concentration in the feed was increasing. The average cell mass at the start of MLF was 16.74 DCW, g/L, but the cell was grown to 25.18 DCW, g/L even at high alcohol concentration of 13% (w/v). The cell mass increased with the average rate 0.002 h^{-1} (Fig.6.10).

Fig.6.10. The effect of alcohol concentration on the cell mass, and pH during MLF of Scrumpy Jack by proliferating high density _Oenococcus oeni_. The _pH and cell mass were monitored as a function of the alcohol concentration. The pH in the feed (the closed) and the product (the opened) were described. The feeding rate was controlled at the retention time of 6 h. The ethyl alcohol concentration in the feed was changed from 9% (w/v) to 13% (w/v). The arrows are an instant stop of feeding for changing the cider batch in the feed cider tank. Temperature was controlled at 28 °C._

The pH difference between the feed and the product become larger when the alcohol concentration increased from 9% (w/v) to 10% (w/v), but the pH difference reduced at the alcohol concentration over 10% (w/v). That means the malolactic activity being inhibited by alcohol concentration higher than 10% (w/v). *O. oeni* could grow using malate in cider [Tracey and Britz (1989)]. The growth of *O. oeni* is influenced by the temperature. When MLF by *O. oeni* was carried out at 25°C the growth of *O. oeni* was not affected by the alcohol concentration 10% (w/v). In the experiment a temperature 28°C supported the growth of *O. oeni* even at the alcohol concentration 13% (w/v).

6.3.2.2. The effect of the alcohol concentration on the composition of organic acids during MLF of Scrumpy Jack by O. oeni.

Figure 6.11 and Table 6.3 shows a variation in the concentration of organic acids during MLF. The malate concentration in the product dropped to average 28 mM in over the range from the alcohol concentration 9% (w/v) to 11% (w/v). The malate concentration in the product was at lower concentration than the Ks (57 mM) for malate uptake in Scrumpy Jack.

Alc (%,w/v)	Time (h)	T-Vol (L)	Feed (mole)			Product (mole)			Variations (mole)		
			M	L	A	M	L	A	M	L	A
9	23	138	5.8	3.0	1.3	3.8	4.5	2.5	-2.0	1.5	1.2
10	49	294	13.7	3.0	3.8	8.7	6.1	7.2	-5.0	3.1	3.4
11	59	354	18.8	4.4	1.7	9.4	14.7	8.3	-9.4	10.3	6.6
12	84	504	31.7	8.2	3.1	17.6	22.9	8.2	-14.1	14.7	5.1
13	26	156	11.3	4.0	1.1	6.4	8.2	3.5	-4.9	4.2	2.4
Total	241	1446	81.3	22.6	11	45.9	56.4	29.7	-35.4	33.8	18.7

Table.6.3 The effect of alcohol concentration in organic acid during MLF of Scrumpy Jack by *O.oeni*. *Variations were described with the real quantities of each component in the feed and the product stream after MLF. Therefore, the negative was reduction and the positive was increase. Malate (M), Lactate (L), and acetate (A) were described in mole.*

The malate concentration in the product increased to 35 mM at the alcohol concentration of 12% (w/v) and to 42.5 mM at the alcohol concentration of 13% (w/v). The malate concentration increased in the product must be due to the inhibition by the high alcohol concentration of the feed.
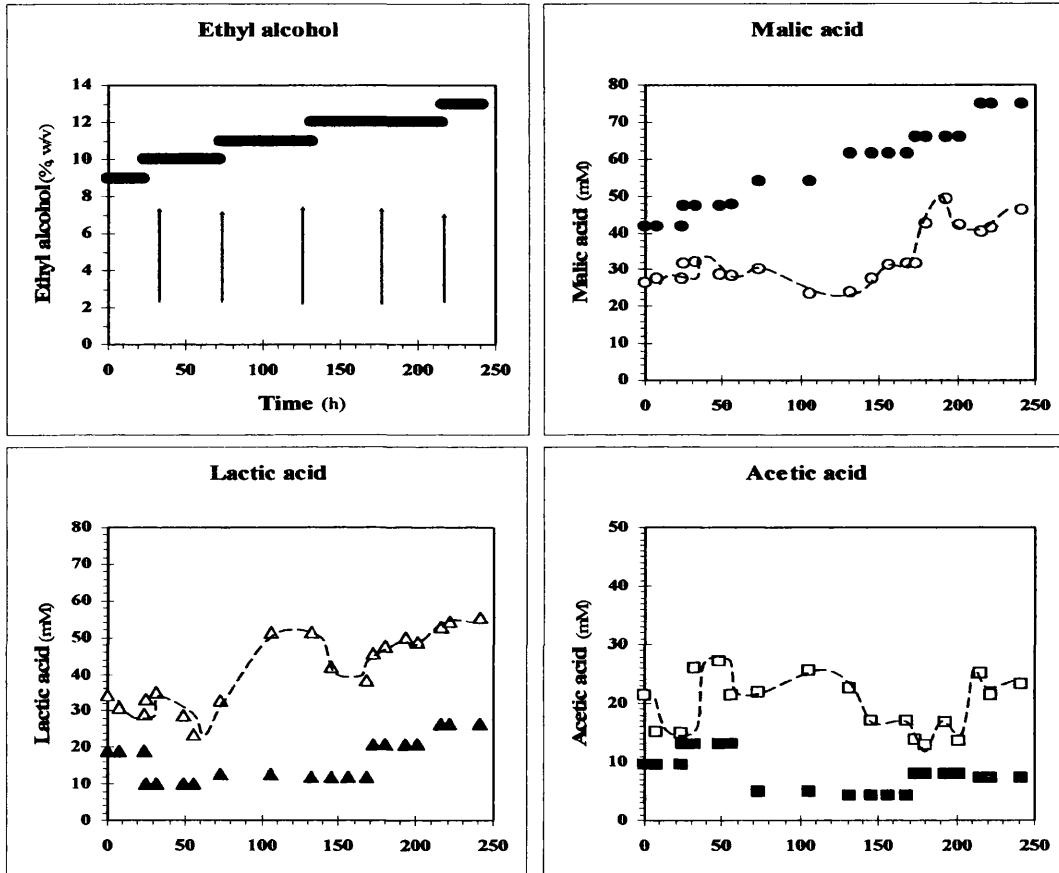
Fig.6.11. The effect of alcohol concentration on organic acids during MLF of Scrumpy Jack with proliferating high density *Oenococcus oeni*. *Organic acids in the feed (the closed) and the product (the opened) were described. Each of organic acid was monitored as a function of the alcohol concentration. The feeding rate was controlled at the retention time of 6 h for all the time. The arrows mark and instant stop of feeding for changing Scrumpy Jack batch in the cider feed tank. Temperature was 28 °C*

The malate concentration was reduced to 27.5 mM in the product from 42 mM in the feed at the alcohol concentration 9% (w/v) and to 29.6 mM in the product from 46.6 mM in the feed at the alcohol concentration of 10% (w/v). The malate concentration was reduced to 26.6 mM in the product from 53.1 mM in the feed at the alcohol

concentration of 11% (w/v), to 34.9 mM in the product from 62.9 mM in the feed at the alcohol concentration 12% (w/v) and to 41 mM in the product from 72.4 mM in the feed at the alcohol concentration 13% (w/v).

The malolactic activity of malate reduced from 86.9 mmole / h to 188 mmole / h when the alcohol concentration increased from 9% (w/v) to 13% (w/v), and the specific malolactic activity of malate was inhibited in the alcohol concentration above 12% (w/v). The productivity of lactate was also influenced in the alcohol concentration above 12% (w/v) and the specific productivity was inhibited in the alcohol concentration above 12% (w/v). The productivity and the specific productivity of acetate reduced in the alcohol concentration above 12% (w/v) (Fig.6.12).
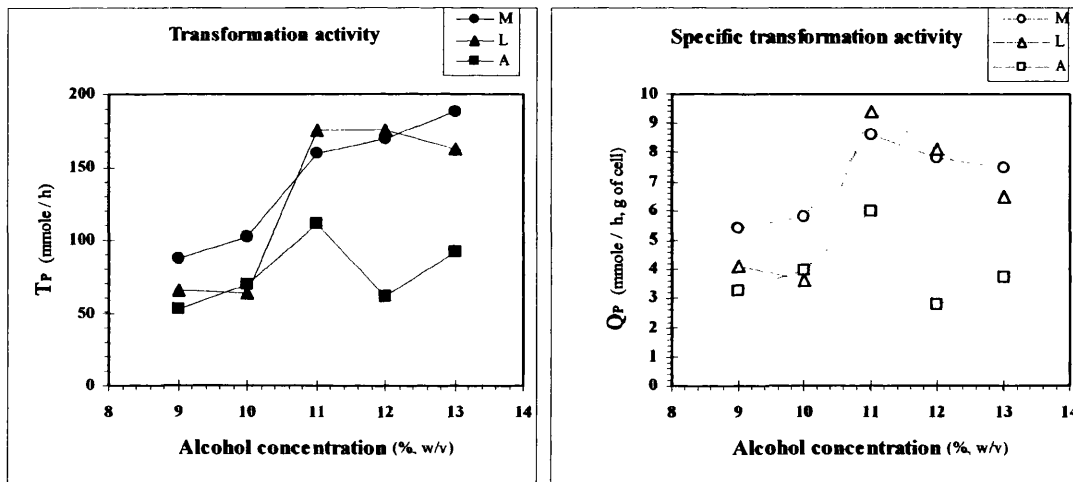
Fig.6.12. The effect of the alcohol concentration on the transformation activity (T_P) and the specific transformation activity (Q_P) of organic acids during MLF of Scrumpy Jack by *O. oeni. M, L and A mark malate, lactate and acetate, respectively.*

6.3.2.3. Variation on the volatile compounds during MLF of Scrumpy Jack by
        Oenococcus oeni.

Several volatile compounds were also monitored during the malolactic fermentation. The variation on acetaldehyde during MLF by *O. oeni* showed a large difference from the case of MLF by *L. brevis,* implying two strains have very different mechanism for the metabolism of acetaldehyde.

In the case of MLF by *L. brevis*, acetaldehyde was produced, but in the case by *O. oeni* it was consumed. A similar result was observed by Osborne et al. [2000], the majority of acetaldehyde in the feed was consumed during MLF and the reduction rate of acetaldehyde declined in the alcohol concentration above 11% (w/v), and it was thought that some portion of consumed acetaldehyde was used to produce ethylacetate (Fig.6.13) with ethyl alcohol and iso-amylacetate with iso-amylalcohol (Fig.6.14).

- *The effect of the alcohol concentration on acetaldehyde and ethylacetate during MLF of Scrumpy Jack by O. oeni.*

The reduction rate of acetaldehyde was influenced by the alcohol concentration of cider during MLF (Fig.6.13).
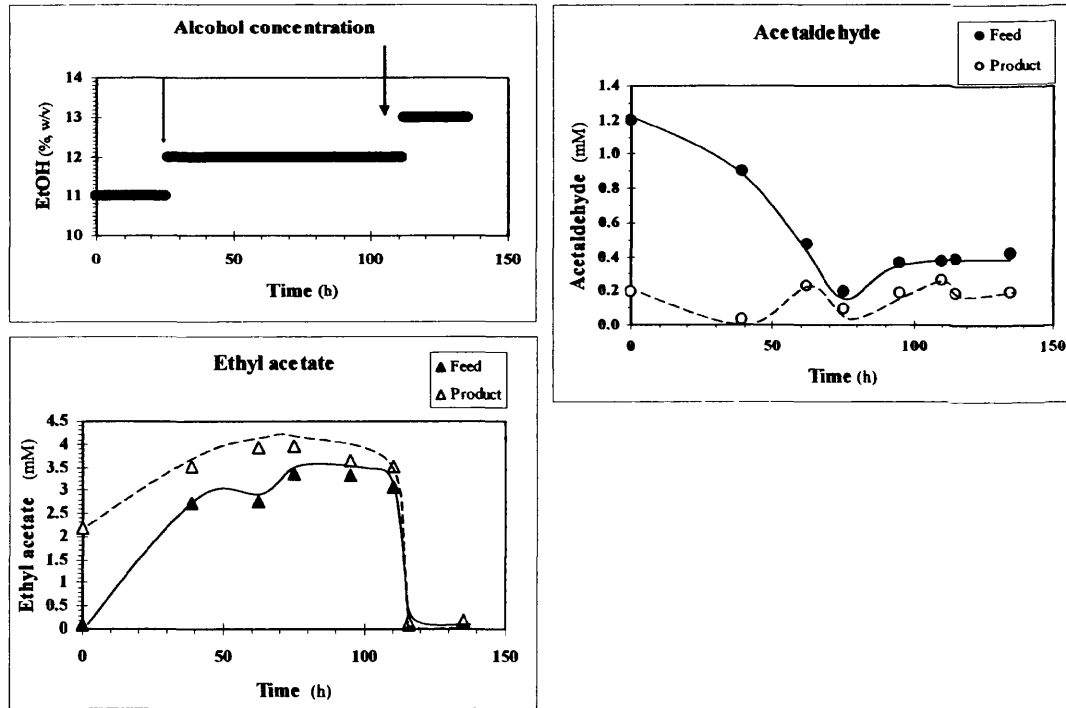
Fig.6.13. The effect of the alcohol concentration on production of ethylacetate during MLF of Scrumpy Jack by proliferating high density *Oenococcus oeni*. The alcohol concentration was controlled at 11, 12 and 13% (w/v) during MLF. The feed rate was controlled at the retention time of 6 h. Acetaldehyde concentration was monitored as a function of the alcohol concentration in feed. Temperature was 28 °C. The arrows mark an instant stop of feeding for changing the cider feed batch.

Most esterification was inhibited by the higher alcohol concentration than 12% (w/v). Consumed acetaldehyde is converted either to ethanol or acetic acid to produce the energy for growth or to ethylacetate with ethyl alcohol.

The acetaldehyde concentration was reduced from 1.15 mM to 0.19 mM in the product during MLF at the alcohol concentration 11% (w/v), from 0.46 mM to 0.16 mM in the product at the alcohol concentration 12% (w/v) and from 2.44 mM to 1.03 mM in the product at the alcohol concentration 13% (w/v).

Conversely, the ethyl acetate concentration increased during MLF. The ethyl acetate concentration increased from 0.1 mM to 2.1 mM in the product at the alcohol concentration 11% (w/v), from 3 mM to 3.7 mM in the product at the alcohol concentration 12% (w/v) and from 0.12 mM to 0.17 mM in the product at the alcohol concentration 13% (w/v) (Table 6.4).

- *The effect of the alcohol concentration on iso-amylacetate and iso-amylalcohol concentration during MLF of Scrumpy Jack by O. oeni. .*

The concentration of iso-amylacetate increased a very little but significant amount during MLF (Fig.6.14, Table 6.4). Iso-amylalcohol concentration reduced from 0.85 mM to 0.41 mM in the product during MLF at the alcohol concentration of 11% (w/v), from 1.1 mM to 0.85 mM in the product during MLF at the alcohol concentration 12% (w/v) and from 1.04 mM to 0.98 mM in the product during MLF at the alcohol concentration 13% (w/v). However, iso-amylacetate concentration increased during MLF. Figure 6.15 shows the effect of the alcohol concentration on the transformation activity and the specific transformation activity of acetaldehyde, ethyl-acetate, iso-amylalcohol and iso-amylacetate during MLF of Scrumpy Jack by *O. oeni*. The transformation activity was negatively affected at the alcohol concentration higher than 11% (w/v).
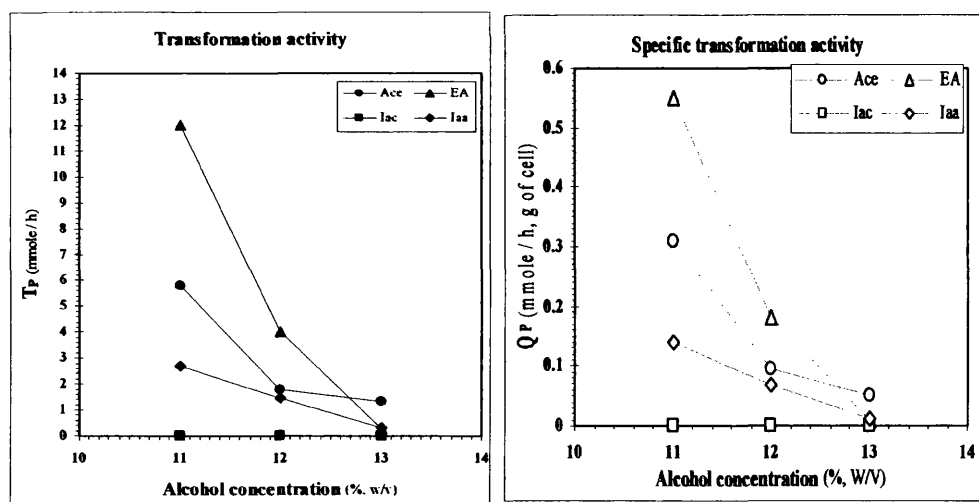
Ethyl alcohol concentration

Isoamyl-alcohol ● Feed ○ Product

Isoamyl-acetate ▲ Feed △ Product
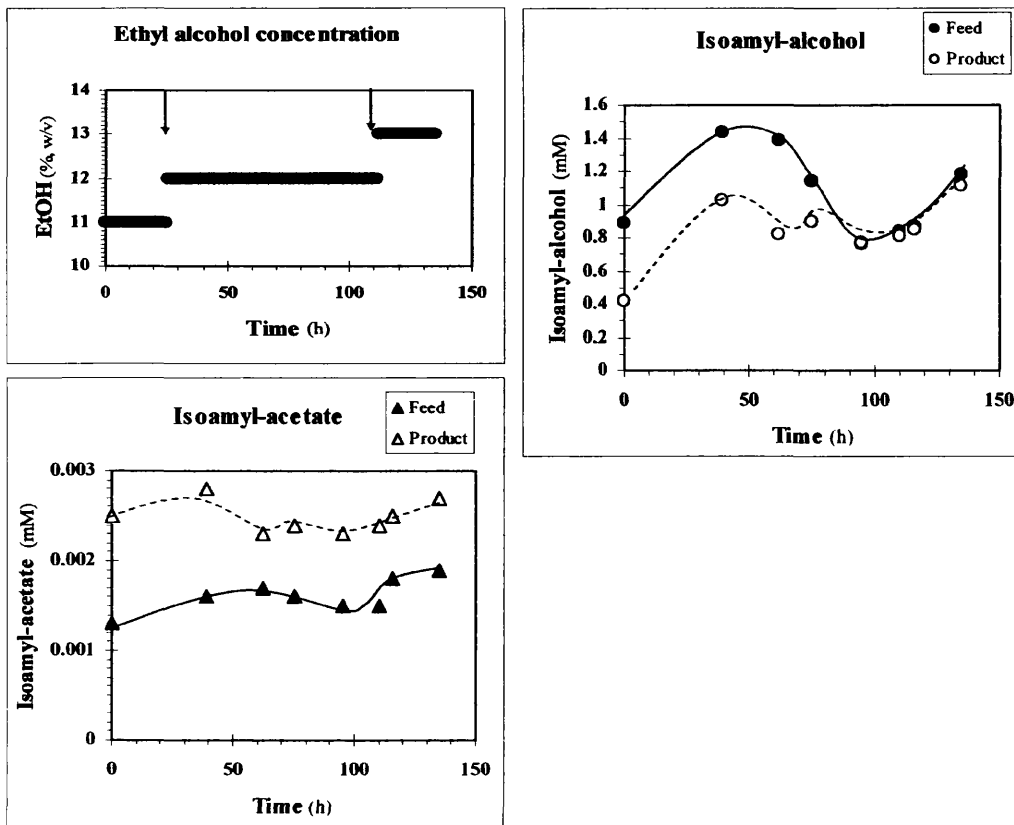
Fig.6.14. The effect of the alcohol concentration on production of iso-amylacetate during MLF of Scrumpy Jack by proliferating high density *Oenococcus oeni*. *Iso-amylalcohol and iso-amylacetate concentration were monitored as a function of the alcohol concentration. The feeding rate was controlled at the retention time of 6 h for all the time. Temperature was 28 °C. The arrows mark an instant stop of feeding for changing the cider feed batch.*

Alc (%,w/v)	T (h)	T-Vol. (L)	Feed (mmole)				Product (mmole)				Variations (mmole)			
			Ace	EA	Iac	Iaa	Ace	EA	Iac	Iaa	Ace	EA	Iac	Iaa
11	26	156	180	15	0.2	133	29	327	0.4	64	-151	312	0.2	-69
12	86	516	236	1553	0.8	567	83	1896	1.2	441	-153	343	0.4	-126
13	23	138	56	17	0.3	142	27	23	0.4	135	-29	6	0.1	-7
Total	135	810	472	1585	1.3	842	139	2246	2.0	640	-333	661	0.7	-202

Table.6.4 The effect of alcohol concentration in volatile compounds during MLF of Scrumpy Jack by *O.oeni*. *Variations were described with the real quantities of components in the feed and the product stream after MLF. The negative presents reduction and the positive presents an increase. Acetaldehyde (Ace), Ethyl-acetate (EA), Iso-amylacetate (Iac), and Iso-amylalcohol (Iaa) were described in mmole.*

Fig.6.15 The effect of the alcohol concentration on the transformation activity and the specific transformation activity of acetaldehyde, ethylacetate, iso-amylalcohol and iso-amylacetate during MLF of Scrumpy Jack by *O. oeni*. *The alcohol concentration was controlled at 11, 12 and 13% (w/v). The feeding rate of Scrumpy Jack was at the retention time 6 h. Ace, EA, Iac and Iaa mark acetaldehyde, ethyl-acetate, iso-amylacetate and iso-amylalcohol, respectively.*

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6.4. MLF OF SCRUMPY JACK BY LACTOBACILLUS BREVIS.

6.4.1 The effect of cider retention time during MLF of Scrumpy Jack by L. brevis.

The MLF of Scrumpy Jack by *L. brevis* was initially investigated on a function of the retention time. Many components in the product are thought to be changed as a function of cider retention time during MLF.

The retention time was changed from 4 h to 12 h. Organic acids and volatile compounds in the feed and the product stream after MLF were measured with HPLC and GC as soon as possible after sampling. The alcohol concentration in the feed was controlled at about 9% (w/v). Figure 6.16 shows the effect of the retention time on pH and the cell mass during MLF of Scrumpy Jack by *L. brevis*, and its effect on organic acids are shown in Fig.6.17. Figure 6.18 shows the effect of the retention time on malolactic activity and specific malolactic activity. The *Ks* for malate uptake by *L. brevis* in Scrumpy Jack was obtained in basis of difference between malate concentration in the feed and the product stream after MLF, and is shown in Fig.6.19.

6.4.1.1. The effect of the cider retention time on the cell mass and pH during MLF of Scrumpy Jack by L. brevis.

The cell mass declined with reduction of cider retention time. The cell mass was at 8.94 DCW, g/L for the high retention time of 12 h, but declined to 7.99 DCW, g/L for the retention time of 6 h and to 7.57 DCW, g/L for the retention time of 4 h (Fig 6.16).

The pH difference between the feed and the product during MLF of Scrumpy Jack by *L. brevis* was also influenced by the shorter retention time than 6 h. The pH of the product dropped to pH 3.82 during the retention time of 6 h or 3 h from pH 3.88 during the retention time of 12 h or 9 h. The reason of reduction in pH difference is due to reduction of cell mass with increasing the feeding rate.
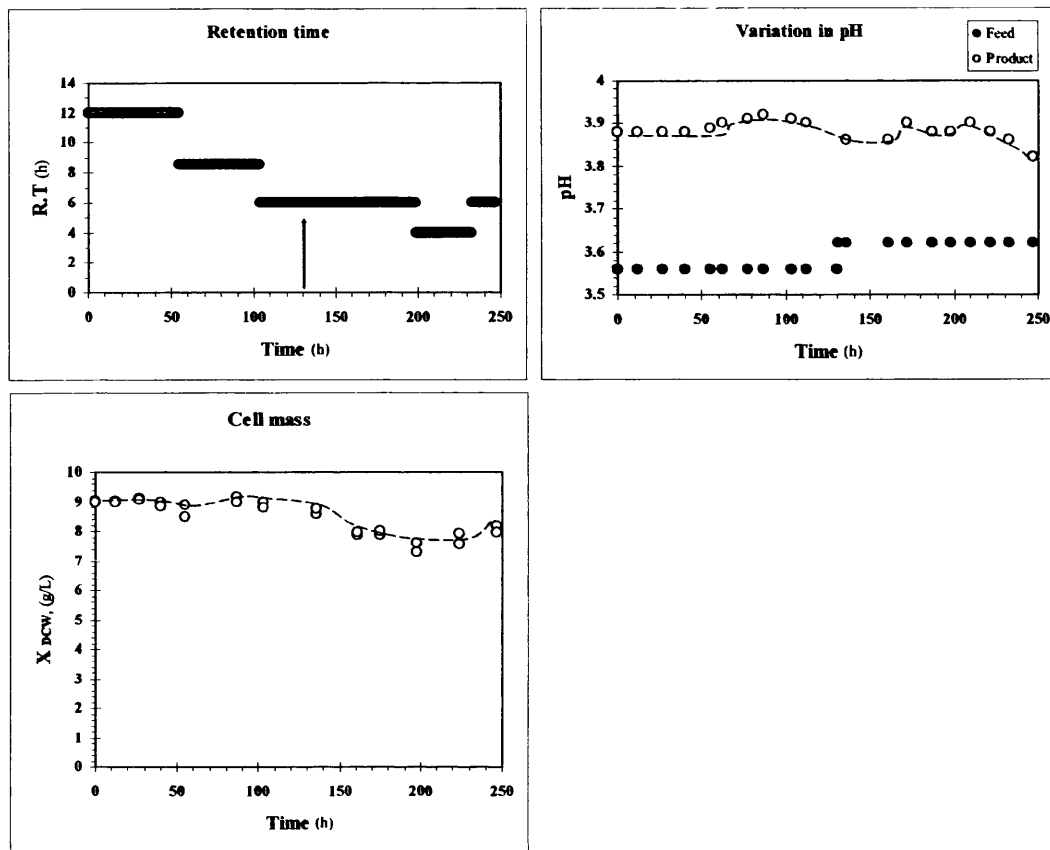
Fig.6.16. The effect of the cider retention time on the cell mass, and pH during MLF of Scrumpy Jack by proliferating high cell dense _Lactobacillus brevis._ Variations on pH and the cell mass were monitored as a function of the retention time. Retention time was controlled in terms of 12 h, 9 h, 6 h, and 4 h. The alcohol concentration in the feed was controlled at 9% (w/v) for all the time. The arrow marks an instant stop of feeding during the change of the cider batch. Temperature was 28 °C.

6.4.1.2. The effect of the cider retention time on organic acids during MLF of Scrumpy Jack by L. brevis.

The malate concentration in the feed was reduced during MLF by _L. brevis_ and the reduction rate proportionately increased when the feeding rate was increased. Malate (45.5 mM) in the feed was reduced to 23 mM in the product for the retention time of 12 h, and the malate concentration reduced from 46.8 mM to 23 mM for the retention time of 8.6 h, from 53.2 mM to 34.4 mM for the retention time of 6 h and from 54.3 mM to 33 mM for the retention time of 4 h (Fig.6.17, Table.6.5).
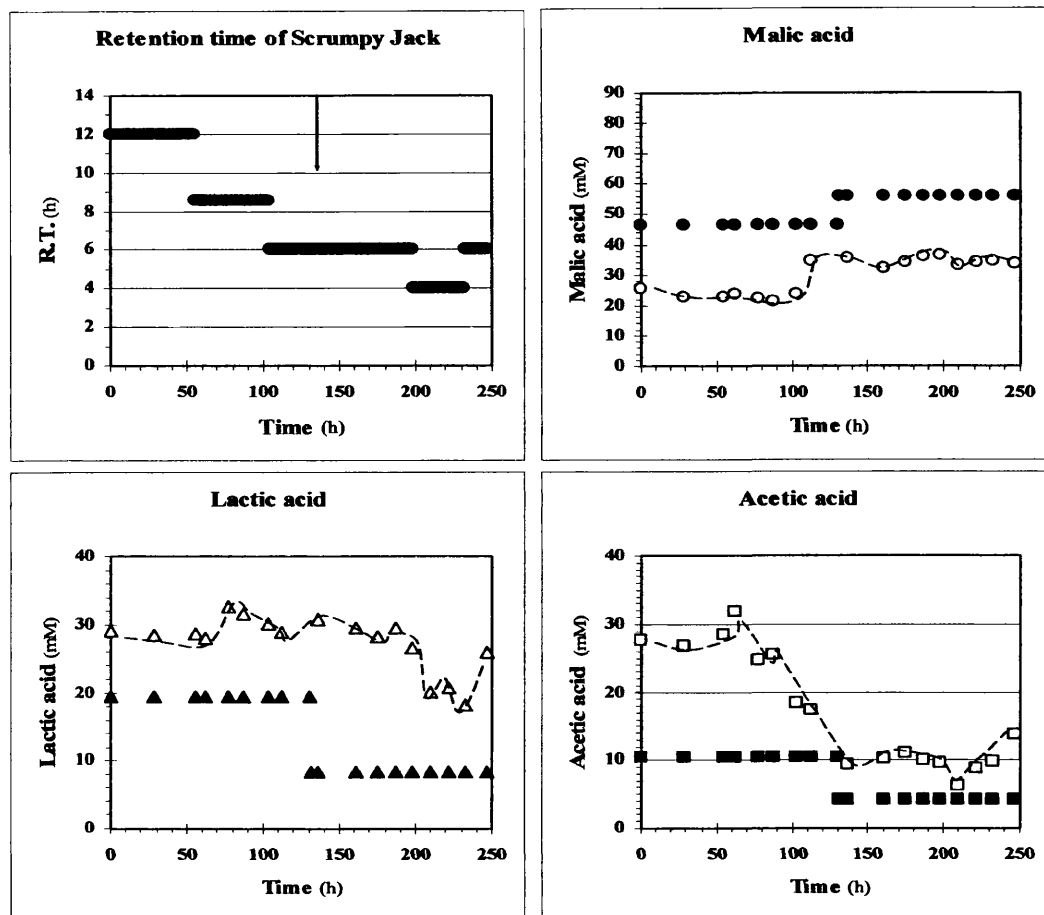
Fig.6.17. The effect of the cider retention time on organic acids during MLF of Scrumpy Jack by proliferating high dense *Lactobacillus brevis. Organic acids in the feed (the closed) and the product (the opened) were described. Each of organic acid was monitored as a function of the retention time. The arrow marks an instant stop of feeding during the change of Scrumpy Jack batch in the cider feed tank. The retention time was controlled in terms of 12 h, 8 h, 6 h, and 4 h. Temperature was 28 °C.*

The malate concentration in the product increased at the retention time shorter than 6 h. The malate concentrations in the product were 23 mM for the retention times of 12 h and 8.56 h, but it increased to 34.5 mM for the retention time of 6 h and 4 h

The lactate and the acetate concentrations increased during MLF by *L. brevis* in contrast to decrease malate concentration in the product.

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The lactate concentration increased from 19.4 mM in the feed to 28.5 mM in the product for the retention time of 12 h, from 19.4 mM to 30.3 mM in the product for the retention time of 8.6 h, from 10.9 mM to 27.8 mM in the product for the retention time of 6 h and from 8.2 mM to 19.3 mM in the product for the retention time of 4 h.

The acetate concentration increased from 10.3 mM in the feed to 27.3 mM in the product for the retention time of 12 h, from 10.5 mM in the feed to 25.9 mM in the product for the retention time of 8.6 h. The acetate concentration did not increase further for the retention time shorter than 6 h.

R.T (h)	Time (h)	T-Vol (L)	Feed (mole)			Product (mole)			Variations (mole)		
			M	L	A	M	L	A	M	L	A
12	55	165	7.5	3.2	1.7	3.8	4.7	4.5	-3.7	1.5	2.8
8.6	48	201	9.4	3.9	˙2.1	4.6	6.1	5.2	-4.8	2.2	3.1
6	109	654	34.8	7.1	3.7	22.5	18.2	7.9	-12.3	11.1	0.8
4	34	306	16.6	2.5	1.2	10.1	5.9	2.5	-6.5	3.4	1.3
Total	246	1326	68.3	16.7	8.7	41	34.9	20.1	-27.3	16	8

Table.6.5. The effect of cider retention time on organic acids during MLF of Scrumpy Jack by proliferating high dense *Lactobacillus brevis*. *Variations are described with the real quantities (mole) of each component in the feed and the product after MLF. The negative marks the reduction of organic acids. Malate (M), lactate (L), and acetate (A) were described in mole.*

Figure 6.18 shows the effect of the retention time on organic acid during MLF of Scrumpy Jack by *L. brevis*. The transformation rate and the specific transformation rate of malate and lactate increased with an increase feed rate, but the case of acetate reduced with an increase feed rate (Fig 6.18).

L. brevis can convert lactate to acetate, 1,2-propanediol and ethanol with more production of ATP [Stefanie et al. (2001); Cunha and Foster (1992)].

This conversion can be carried out in acidic condition below pH 5.8 and is pH dependent. MLF was carried at pH 3.6 or pH 3.4. The fast flow of the fluids facilitated MLF, but inhibited the anoxic degradation of lactate.

For the MLF of Scrumpy Jack by *L. brevis*, the malate concentration in the product was 34 mM for the retention time of 6 h, but the malate concentration in the product increased to 33 mM for the retention time of 3 h in the case of *O. oeni.*
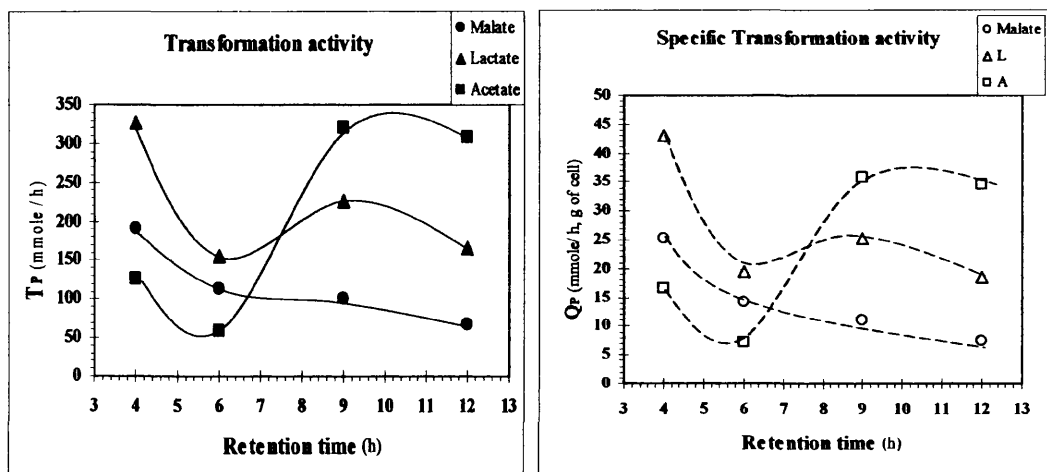

Fig 6.18 The effect of the retention time on the transformation activity and the specific transformation activity of organic acids during MLF of Scrumpy Jack by *L. brevis*. *The retention time the residence time was controlled in terms of 4 h, 6 h, 9 h, 12 h.*

6.4.1.3. The K_s for malate uptake by L. brevis in Scrumpy Jack.

Figure.6.19 shows the determination of the Ks for malate uptake of *L. brevis* in Scrumpy Jack through a double reciprocal plot of Lineweaver-Burk. According to Fig 6.19, the Ks was 81.3 mM. As compared to Ks (57.6 mM) in *O. oeni* (Fig. 6.9), therefore, the malate concentration in the product during MLF of Scrumpy Jack by *L. brevis* should not be lower than them by *O. oeni,* even in a high retention time, and the specific malolactic activity of *L. brevis* in Scrumpy Jack can be higher than that by *O. oeni* (Fig 6.8).

That means that more reaction time is required to maintain the malate concentration low in the product stream during MLF of Scrumpy Jack by *L. brevis*. The specific malolactic activity of *L. brevis* showed the higher reaction rate than that of *O. oeni* even though the K_S (81.3 mM) of *L. brevis* was higher than that of *O. oeni* (56.7 mM). It was because the cell mass (from 15 to 18 DCW, g/L) of *O. oeni* during MLF was higher than that (from 8 to 9 DCW, g/L) of *L. brevis*. The growth of *L. brevis* could be influenced more by the retention time during MLF. As mentioned at the previous section, the malate concentration in the product increased to 34 mM in the case of *L. brevis* at the retention time 6 h, while the malate concentration in the product increased to 34 mM in the case of *O. oeni* at the retention time 3 h.
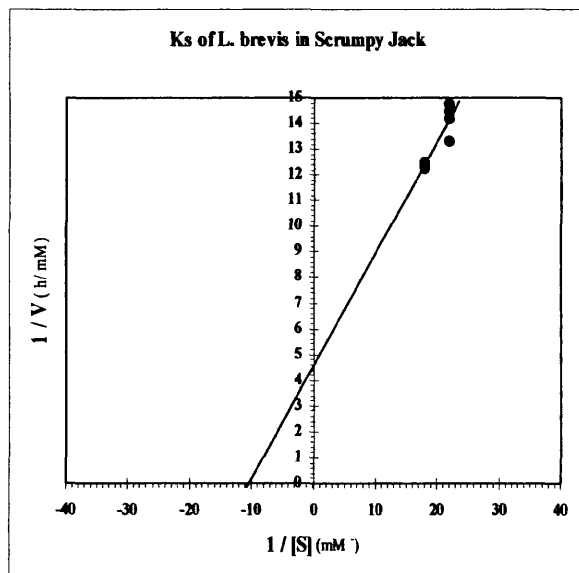
Fig.6.19. Determination of the *Ks* for malate uptake by *Lineweaver-Burk* equation during MLF of Scrumpy Jack by *Lactobacillus brevis*. *The K_S was determined by a double reciprocal plot (Lineweaver-Burk equation) to the reaction rate (V) and the substrate concentration (mM). The equation of line was designated as Y = Ks/Vm X 1/[S] + 1/Vm =0.410859X + 5.0554, and consequently, Ks = 0.0813M.*

6.4.2. The effect of the alcohol concentration during MLF of Scrumpy Jack by L. brevis.

The alcohol concentration in the feed was raised from 9% (w/v) to 13% (w/v) during MLF of Scrumpy Jack by *L. brevis*. The alcohol concentration of Scrumpy Jack after alcohol fermentation by yeast was at the concentration over 13% (w/v).

During the investigation, Scrumpy Jack was diluted with water to each of alcohol concentrations in the feed, and ultimately, the highest alcohol concentration of 13% (w/v) in the feed was used without any dilution of the original Scrumpy Jack.

The feed rate was controlled at the retention time of 7.5 h. However, when the retention time decreased to 6 h, the permeate flux through the product membrane in the MCR was seriously influenced by the high cell density of *L. brevis* because of membrane fouling.

Figure 6.20 shows a variation on the cell mass, pH and the alcohol concentration during MLF and Fig 6.21 with Table 6.6 shows the effect of the alcohol concentration on organic acids concentration during the investigation. Figure 6.22 shows a variation on the malolactic activity and the specific malolactic activity during the investigation.

6.4.2.1. The effect of the alcohol concentration on the cell mass and pH during MLF of Scrumpy Jack by L. brevis..

Figure 6.20 shows the effect of the alcohol concentration on pH and the cell mass during MLF of Scrumpy Jack by *Lactobacillus brevis*.

As shown at Fig.6.20, the cell mass did not increase and was in a range between 7.0 and 9.0 DCW, g/L while investigating the effect of the alcohol concentration on the cell mass. The cell mass was at 8.0 DCW, g/L at the alcohol concentration of 9% (w/v) and slightly dropped to 7.46 DCW, g/L at the alcohol concentration of 11% (w/v), but increased again to 8.26 DCW, g/L at the alcohol concentration of 13% (w/v).

The pH of the cider in the product was always at higher pH from 0.25 to 0.33 than pH in the feed stream. Sudden and instantaneous drops of pH in the product were detected while exchanging the feeding cider in 90 h and 150 h, but quickly recovered to the previous levels.
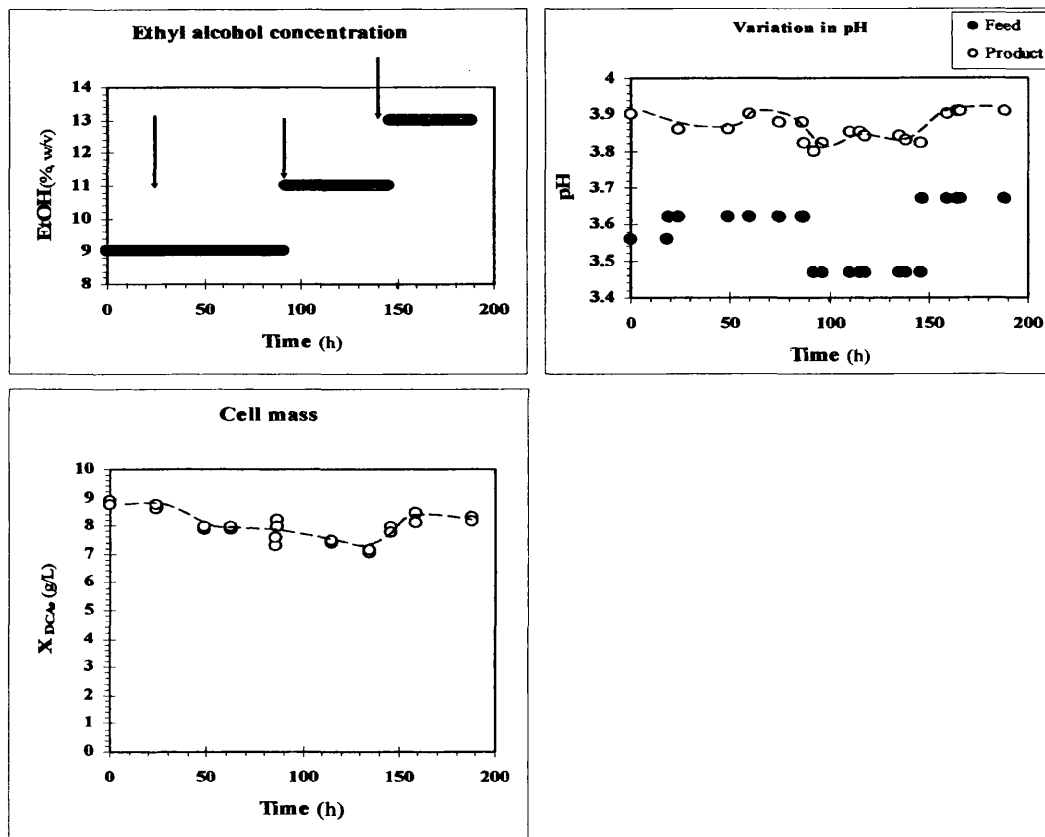
Fig.6.20. The effect of the alcohol concentration on the cell mass, and pH during MLF of Scrumpy Jack by proliferating high dense _Lactobacillus brevis_. _The pH and the cell mass were monitored as a function of the alcohol concentration. The feed rate was controlled at the retention time of 7.5 h. The alcohol concentration was controlled in terms of 9% (w/v), 11% (w/v), and 13% (w/v). Temperature was 28 °C. The arrows mark an instant stop of feeding during change of the cider feed batch._

6.4.2.2. The effect of the alcohol concentration on organic acids during MLF of Scrumpy Jack by L. brevis.

The degradation rate of malate was influenced at higher alcohol concentration than 12% (w/v) during MLF of Scrumpy Jack by _L. brevis_ (Fig.6.21, Table.6.6).

ALC (%,w/v)	T (h)	T-vol. (L)	Feed (mole)			Product(mole)			Variations(mole)		
			M	**L**	**A**	**M**	**L**	**A**	**M**	**L**	**A**
9	92	442	25.9	4.6	2.6	15.2	13.6	5.8	-10.7	1.2	3.2
11	54	260	18.9	4.5	2.2	9.3	11.7	4.0	-9.6	7.2	1.8
13	43	206	13.0	5.7	2.6	9.1	8.4	3.1	-3.9	2.7	0.5
Total	189	908	57.8	14.8	7.4	33.6	33.7	12.9	-24.2	11.1	5.5

Table.6.6. The effect of the alcohol concentration on organic acids during MLF of Scrumpy Jack by a high density *Lactobacillus brevis*. *Variations were described with the real quantities of components in the feed and the product stream after MLF. Therefore, the negative marks reduction and the positive marks increase. Malate (M), lactate (L), and acetate (A) were described in mole.*

Malate (58.6 mM) in the feed was degraded to 34.4 mM in the product during MLF of Scrumpy Jack by *L. brevis* at the alcohol concentration of 9% (w/v), and the malate concentration was reduced from 72.7 mM in the feed to 35.8mM in the product during MLF in the alcohol concentration 11% (w/v) and from 63.1 mM in the feed to 44.2 mM in the product during MLF in the alcohol concentration of 13% (w/v). The malate concentration in the product increased with increase alcohol concentration.

The lactate and the acetate concentrations were higher in the product than in the feed. The lactate concentration increased to 30.8 mM in the product from the lactate concentration 10.4 mM in the feed at the alcohol concentration of 9% (w/v), to 45 mM in the product from 17.3 mM in the feed at the alcohol concentration of 11% (w/v) and to 40.8 mM in the product from 27.7 mM in the feed when the alcohol concentration in the feed was at 13% (w/v).

The acetate concentration in the product also increased to 13.1 mM from 5.9 mM in the feed in the alcohol concentration of 9% (w/v), to 15.4 mM from 8.5 mM in the feed at the alcohol concentration of 11% (w/v) and to 15.1 mM from 12.6 mM in the feed at the alcohol concentration of 13% (w/v).
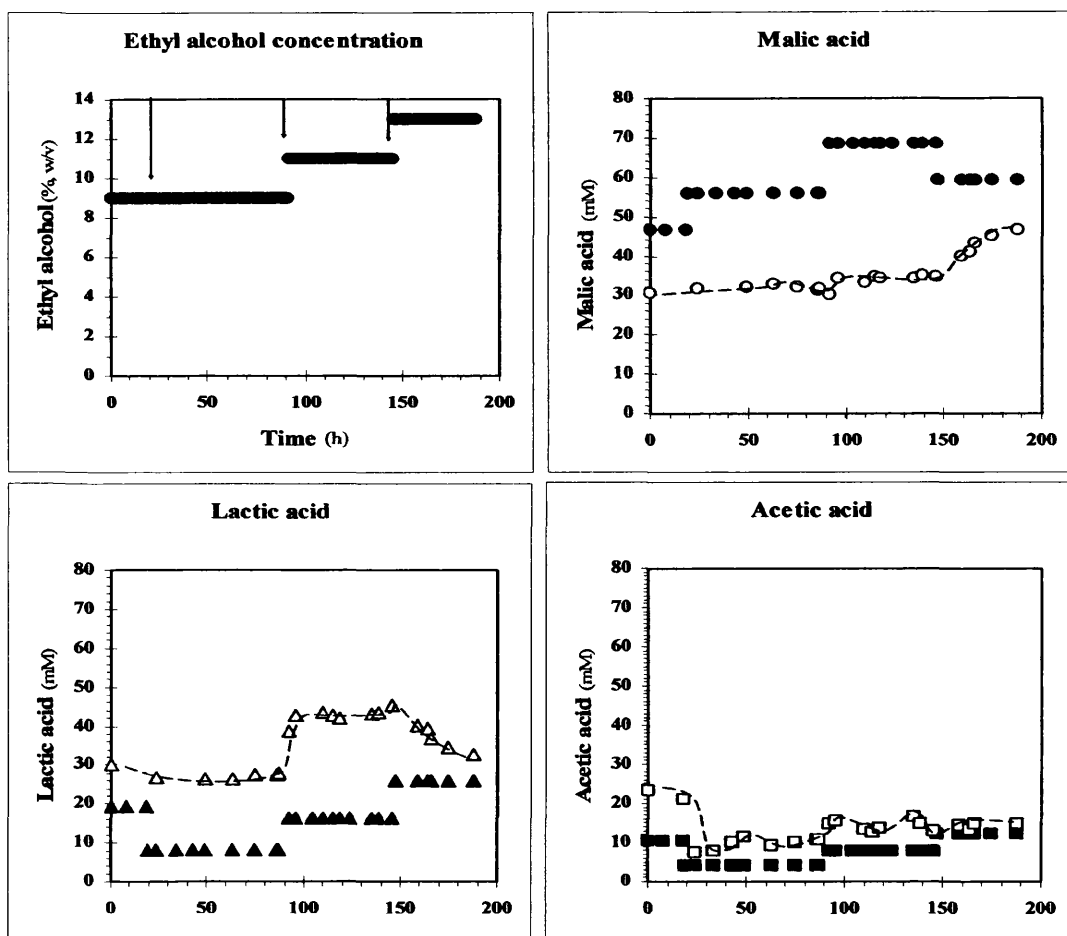
Fig.6.21. The effect of the alcohol concentration on organic acids during MLF of Scrumpy Jack by proliferating high dense *Lactobacillus brevis*. *The organic acid concentrations in the feed (the closed) and the product (the opened) were described. Each of organic acids was monitored as a function of the alcohol concentration. The arrows mark the change of Scrumpy Jack batch. The feeding rate was controlled at the retention time of 7.5 h. The alcohol concentration was controlled in terms of 9% (w/v), 11% (w/v), and 13% (w/v). Temperature was 28 °C. The arrows mark an instant stop of feeding during the change of the*

Figure 6.22 shows a variation in the malolactic activity and the specific malolactic activity of organic acids during MLF. The malolactic activity increased when the alcohol concentration increased from 9% (w/v) to 11% (w/v), but the activity decreased in the alcohol concentration of 13% (w/v).
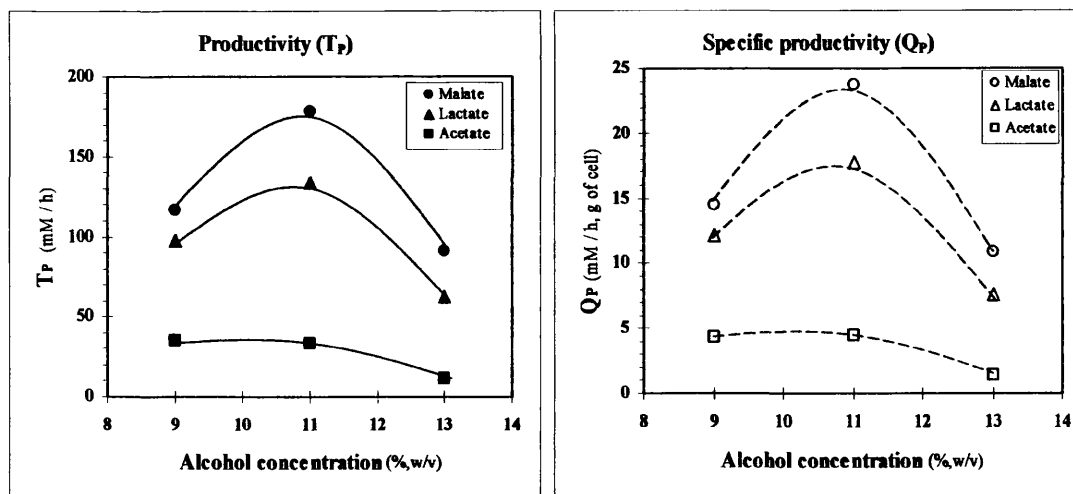
Fig. 6.22 The effect of the alcohol concentration on the transformation activity and the specific transformation activity of organic acids during MLF of Scrumpy Jack by *Lactobacillus brevis*. *The feed rate of the Scrumpy Jack was controlled at the retention time of 7.5 h. The alcohol concentration (%,w/v) in the feed was controlled in terms of 9, 11 and 13% during MLF.*

6.4.3. The effect of the alcohol concentration on the volatile compounds during MLF of Scrumpy Jack by Lactobacillus brevis.

The contribution of the volatile compounds to the quality of the alcoholic beverage is a great. Diols, diacetryls, and esters have been discussed as considerable. Diacetyls and diols can be produced through the dissimilation pathway of citric acid (Fig.1.5), and esters can be produced through esterification among aldehydes, alcohols and fatty acids.

Figure 6.23 and Fig 6.24 with Table 6.7 show a variation on the concentrations of acetaldehyde, ethylacetate, iso-amylalcohol and iso-amylacetate during MLF of Scrumpy Jack by *L. brevis*. Figure 6.25 shows the effect of the alcohol concentration on the transformation activity and the specific transformation activity of volatile compounds during the investigation.

6.4.3.1. The effect of the alcohol concentration on the acetaldehyde and ethyl acetate during MLF of Scrumpy Jack by Lactobacillus brevis.

Acetaldehyde is mainly produced by yeast during the alcoholic fermentation, and the

concentration can be raised by *Lactobacilli* during MLF. Figure 6.23 shows an increase on acetaldehyde concentration during MLF of Scrumpy Jack by *L. brevis*.
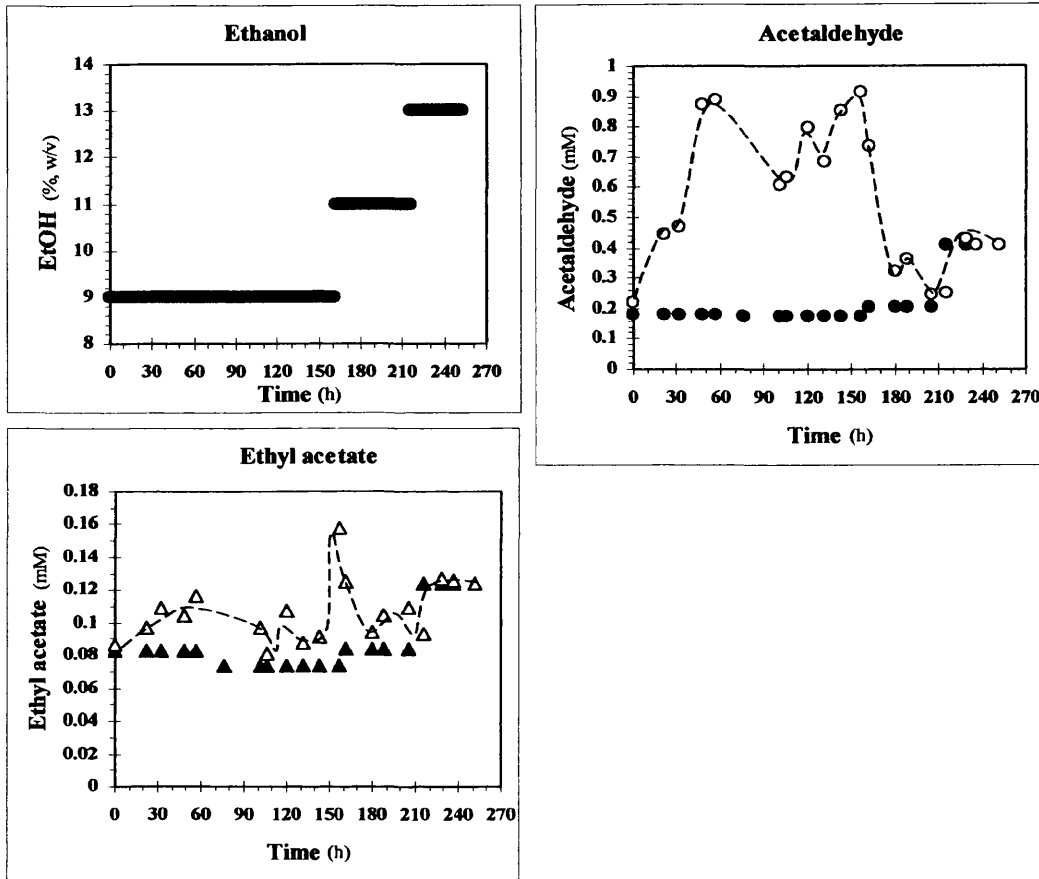
Fig 6.23. The effect of the ethyl alcohol on acetaldehyde and ethyl alcohol during MLF of Scrumpy Jack by proliferating high density *Lactobacillus brevis*. *Acetaldehyde and ethylacetate were monitored as a function of the alcoholic concentration. The feeding rate was controlled at the retention time of 7.5 h. Ethanol concentration was controlled in terms of 9% (w/v), 11% (w/v), and 13% (w/v). Temperature was 28 °C.*

Acetaldehyde concentration 0.22 mM in the feed increased to 0.83 mM in the product during MLF in the alcohol concentration of 9% (w/v) and the acetaldehyde concentration increased from 0.26 mM in the feed to 0.52 mM in the product during MLF in the alcohol concentration of 11% (w/v). But acetaldehyde concentration did not increase during MLF in the alcohol concentration of 13% (w/v).

The ethylacetate concentration increased during MLF in the alcohol concentration of

9% (w/v) and 11% (w/v).

However, the increase amount was a relatively little when compared with the case of acetaldehyde. (Table 6.7 and Fig 6.23). The concentration of ethylacetate during MLF of Scrumpy Jack by *L. brevis* increased in the product, but it was a relatively little when compared with the production of ethyl acetate by *O. oeni*.

6.4.3.2. The effect of the alcohol concentration on iso-amylalcohol and iso-amyl acetate during MLF of Scrumpy Jack by Lactobacillus brevis.

The iso-amylacetate and the iso-amylalcohol concentrations were not changed much when compared with the case by *O. oeni* (Fig. 6.24 and Table 6.7).

The production of iso-amylacetate during MLF of the cider by *O. oeni* has been reported by Nedovic et al. [2000]. They mentioned the production of iso-amylacetate was accomplished by yeast when they designed MLF of the cider by co-immobilized yeast and *O. oeni*. It was very hard to define the exact reaction to produce iso-amylacetate. In fact, as *Lactobacilli* and malolactic *Oenococci* have esterase activity, it might be accomplished by these bacteria. Here, a little production of iso-amylacetate during MLF was detected.

Figure 6.24 shows the variations in iso-amylacetate and iso-amylalcohol during MLF of Scrumpy Jack by *L. brevis*. The iso-amylacetate concentration did not increase during MLF, but the iso-amylalcohol concentration increased from 1.24 mM in the feed to 1.39 mM in the product during MLF in the alcohol concentration of 9% (w/v) and from 1.3 mM in the feed to 1.53 mM in the product during MLF in the alcohol concentration of 11% (w/v). Increases in both volatile compounds were not found during MLF in the alcohol concentration of 13% (w/v).
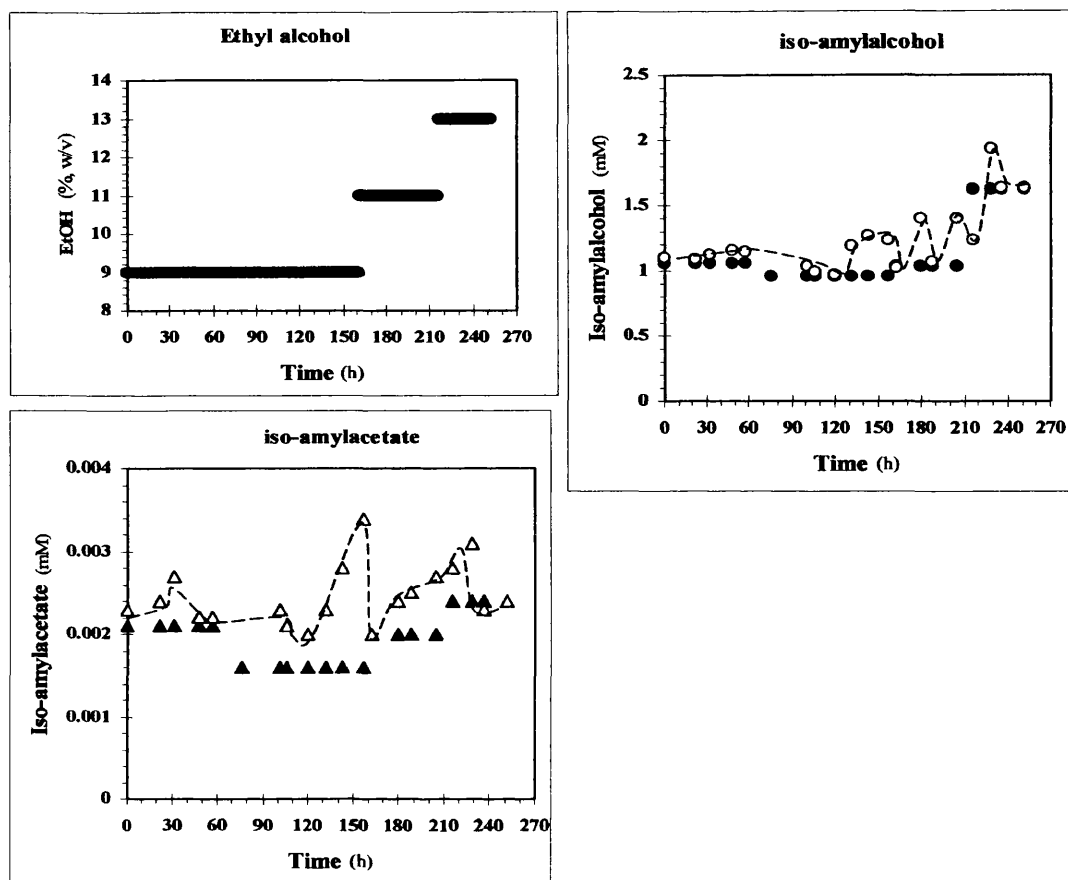
Fig.6.24. The effect of the ethyl alcohol concentration on iso-amylacetate and iso-amylalcohol during MLF of Scrumpy Jack by proliferating high density *Lactobacillus brevis*. *Iso-amylacetate and iso-amylalcohol were monitored as a function of the ethanol. The volatile compound concentrations in the feed (the closed) and the product (the opened) were described. The feeding rate was controlled at the retention time of 7.5 h. Ethanol concentration was controlled in terms of 9% (w/v), 11% (w/v), and 13% (w/v). Temperature was 28 °C.*

The variations in the transformation activity and the specific transformation activity of the volatile compounds during MLF of Scrumpy Jack were shown in Fig 6.25. The transformation activity and the specific transformation activity of acetaldehyde reduced with a increase alcohol concentration and the transformation activity of acetaldehyde reduced to 1.26 mmole/h in the alcohol concentration 11%(w/v) when compared with 2.93 mmole/h in the alcohol concentration 9% (w/v). At an alcohol concentration of 13% (w/v), the activity was not found at all.
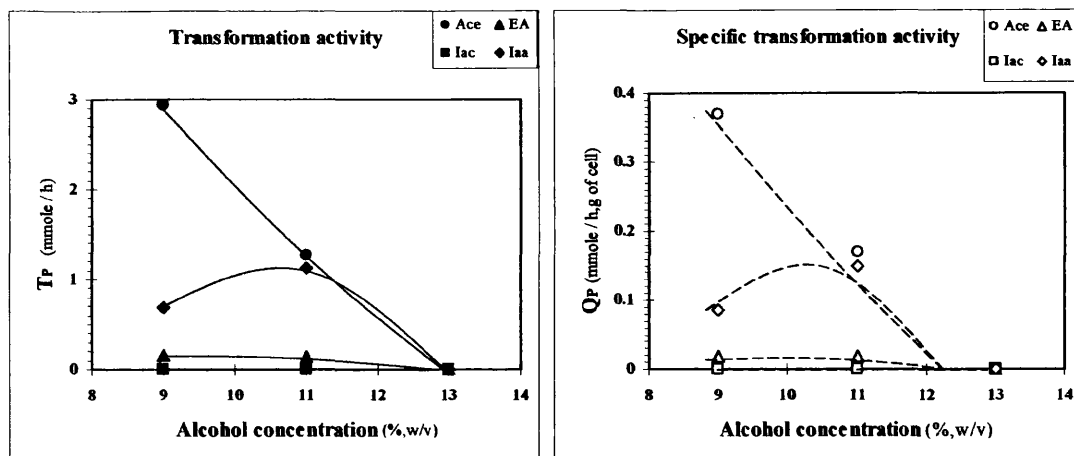
Fig 6.25 The effect of the alcohol concentration on the transformation activity and the specific transformation activity of volatile compounds during MLF of Scrumpy Jack by *Lactobacillus brevis*. *The alcohol concentration was controlled at 9% (w/v), 11% (w/v) and 13% (w/v). The feed rate was at the retention time of 7.5 h.*

Alc (%)	T (h)	T-vol (L)	Feed (mmole)				Product (mmole)				Variations (mmole)			
			Ace	EA	Iac	Iaa	Ace	EA	Iac	Iaa	Ace	EA	Iac	Iaa
9	162	778	171	76	1.8	968	645	99.7	2.3	1079	474	23.7	0.5	111
11	53	254	65	27	0.7	329	132	34.4	0.8	389	67	7.4	0.1	60
13	37	178	88	27	0.5	351	81	25.4	0.6	347	-7	-1.6	0.1	-4
Total	252	1210	324	130	3	1648	858	125	3.7	1464	474	29.5	0.7	167

Table.6.7 The effect of alcohol concentration in volatile compounds during MLF of Scrumpy Jack by *L. brevis*. *Variations were described with the real quantities of each component in the feed and the product stream after MLF. Therefore, the negative marks reduction and the positive marks increase. Acetaldehyde (Ace), Ethyl-acetate (EA), Iso-amylacetate (Iac), and Iso-amylalcohol (Iaa) were described in mmole.*

6.5. Conclusions by analysis and comparison during MLF of the cider by L. brevis and O. oeni.

L. brevis and *O. oeni* were compared with effectiveness during MLF of the cider. General cider and Scrumpy Jack were used to compare with the effect on the maturation by the types of cider during MLF.

6.5.1. The effect of the types of the ciders during MLF by O. oeni.

The types of the ciders affected the results of MLF in ways including the cell concentration produced and maintained, the *Ks* for malate uptake, and the concentrations of organic acids and the volatile compounds in product.

The cell mass declined with an increase feed rate of General cider (Fig.6.2), but was not strongly influenced by the retention time during MLF of Scrumpy Jack. The cell mass increased with the time during MLF of Scrumpy Jack (Fig 6.6). This is most significant due to the higher level of adaptation to unfriendly culture environment in the cider after yeast alcohol fermentation.

The pH difference between the feed and the product decreased in the both cases where the feed rate increased.

The consumption rate of malate in the feed was strongly influenced by the retention time during MLF of General cider. During MLF of General cider, the consumption rates of malate and lactate did not show a large difference in the retention times of 6 h and 10 h, but the malolactic activities of malate and lactate dramatically increased in the retention time of 3 h. The transformation activity and the specific transformation activity of acetate show a different pattern from malate and lactate. The transformation activity and the specific transformation activity of acetate slowly increased with an increase retention time (Fig 6.4)

Figure 6.4 and Fig 6.8 show that the specific malolactic activities of organic acids are higher in General cider than in Scrumpy Jack.

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The *Ks* for malate uptake by *O. oeni* in General cider was 31.4 mM and was 57.6 mM in Scrumpy Jack. The maximum reaction rate (V_m) for malate was 12.9 mmole/h, g of cells in General cider, and 6.5 mmole/h, g of cells in the case of Scrumpy Jack (Fig 6.5, Fig 6.9).

Consequently, the malate in the product declined to the lower concentration in General cider than in Scrumpy Jack. The malate concentration in the product during MLF of General cider dropped to 16.6 mM and 17.5 mM in the retention times of 10 h and 6 h, but the malate concentrations in the product of Scrumpy Jack were at 24.3 mM and 25.9 mM in the retention times of 12 h and 6 h, respectively. Furthermore, the malate concentration increases to 34.2 mM in Scrumpy Jack and 28 mM in General cider when the feed rate increased to the retention time of 3 h (Table 6.1, Table 6.2).

6.5.2. The effect of the malolactic strains on MLF of Scrumpy Jack.

- *Variation on the cell mass and pH between the feed and the product during MLF of Scrumpy Jack.*

During MLF of Scrumpy Jack by *L. brevis*, the cell mass decreased with an increase feed rate of Scrumpy Jack. The cell mass was maintained at 8.94 DCW, g/L in the alcohol concentration of 9% (w/v), but decreased to 7.99 g/L in the alcohol concentration of 11% (w/v) and to 7.57 DCW, g/L in the alcohol concentration of 13% (w/v) (Fig. 6.16).

This was very different from that of *O. oeni*. *O. oeni* was not influenced by the retention time, and the cell mass increased from 16.74 DCW, g/L to 25.18DCW, g/L with an increase alcohol concentration by 13% (w/v). The alcohol tolerance of *O. oeni* was higher than *L. brevis* and *O. oeni* can use citric acid or residual sugars in the cider as energy source for growth (Fig 6.6). The pH difference during the maturation was larger in *L. brevis* than in *O. oeni*. The pH difference between the feed and the product in *O. oeni* was from pH 0.12 to pH 0.04 depending on the retention time (Fig 6.6) or the alcohol concentration (Fig 6.10). The pH difference increased to from pH 0.25 to pH 0.33 in *L. brevis* (Fig 6.16, Fig 6.20).

Chapter.6 Malolactic Fermentation of Cider by *L. brevis* and *O.oeni*.

• *The effect of malolactic strains on organic acids during MLF of Scrumpy Jack.*

The malolactic activities of organic acids were different between two malolactic strains according to the retention time and the alcohol concentration during MLF of Scrumpy Jack.

Considering about the retention time, the malolactic activity of *L. brevis* was higher than that of *O. oeni* in general. The specific malolactic activity of *O. oeni* was 9 mmole/h, g of cell and the specific malolactic activity of *L. brevis* was 15 mmole/h, g of cell in the same retention time 6 h. According to the retention time, the malolactic activity of both strains increased with an increase feed rate (Fig. 6.8, Fig 6.18). The malolactic activity of *O. oeni* shows an increase with retention times between 6 h and 12 h while *L. brevis* shows an increase with retention times between 6 h and 3 h. However, in the case of acetate, the transformation activity and the specific transformation activity increased in an increase retention time with *L. brevis*, while decreased in an increase retention time with *O. oeni*. *L. brevis* can convert lactate to acetate in anoxic and acidic circumstance, but there has been no report about degradation of lactate to acetate by *O. oeni* in anoxic condition.

The cell mass of *L. brevis* was maintained around 8.5 DCW, g/L and the cell mass of *O. oeni* was maintained around 16.5 DCW, g/L during the investigation (Fig 6.6, Fig 6.16). According to Fig 6.9 and Fig 6.19, the K_S for malate uptake on *L. brevis* was 81.3 mM and the K_S on *O. oeni* was 57.6 mM using Scrumpy Jack as the substrate. Also Figure 6.9 and Fig 6.19 showed the maximum malate degradation rate of 23.2 mmole/h, g of cells in the case of *L. brevis* and of 8.2 mmole/h, g of cells in the case of *O. oeni*.

Consequently, *O. oeni* showed high malolactic activity because of the high cell concentration used during MLF of Scrumpy Jack. Moreover, *O. oeni* was better than *L. brevis* at adapting to the extreme growth condition of the green cider after yeast fermentation. *O. oeni* is better than *L. brevis* in maintaining a good flux during MLF because *L. brevis* produces extracellular proteins.

The filtering area of the membrane must be increased to use high density *L. brevis* or the problem of the membrane fouling must be solved.

The malolactic activity of both strains to malate in Scrumpy Jack showed similar patterns in an environment with increase alcohol concentration. The malolactic activity of malate and lactate for both strains increased to the maximum in 11% (w/v) alcohol concentration and then the malolactic activity was inhibited in higher alcohol concentration than 11% (w/v). However, the formation of acetate showed a different pattern from malate and lactate. In the case of *O. oeni*, the specific activity was in a range between 3 mmole/h, g of cells and 6 mmole/h, g of cells, but the case of *L. brevis* showed a pattern slowly declining with increase alcohol concentration (Fig 6.12, Fig 6.22). The malate concentration in the product was at 35 mM during MLF in the alcohol concentration from 9% (w/v) to 11% (w/v), but it increased to 44.2 mM when the alcohol concentration increased to 13% (w/v) in *L. brevis*. In the case of *O. oeni*, the malate concentration in the product increased to 34.9 mM in the alcohol concentration 12% (w/v) and to 41 mM in the alcohol concentration 13% (w/v) from 27.9 mM in certain range of the alcohol concentration from 9% (w/v) to 11% (w/v) (Table 6.6, Table 6.9).

• *The K_S for malate uptake and the maximum reaction rate of two malolactic strains.*

The K_S for malate uptake and the maximum reaction rate (V_m) were determined through a double reciprocal plot between the reaction rate (V) and the substrate concentration (S) by Lineweaver-Burk

The K_S for malate uptake by *L. brevis* in Scrumpy Jack was 81.3 mM, and the maximum reaction rate was 23.2 mmole/h, g of cell. The K_S of *O. oeni* in Scrumpy Jack was 57.6 mM, and the maximum reaction rate was 8.2 mmole/h, g of cell, while the K_S of *O. oeni* in General cider was 31.4 mM. The maximum reaction rate (V_m) in General cider was 12.9 mmole/h, g of cell. Typically, Scrumpy Jack contains higher concentrations of inhibitory materials such as tannin and malate than General cider.

Chapter.6 Malolactic Fermentation of Cider by *L. brevis* and *O.oeni.*

- *The effect of malolactic strains on the volatile compounds during MLF of Scrumpy Jack.*

Aldehydes are very important to determine the sensory and colour property of cider and wine. Acetaldehyde mainly produced by yeast fermentation affects wine aging and colour stability. In usual process to remove the excess acetaldehyde in wine SO_2 is added into wine after yeast fermentation. However because addition of SO_2 can affect the wine sensory properties, MLF by lactic acid bacteria can be preferred to addition of SO_2 so as to remove acetaldehyde.

The consumption of acetaldehyde by *O. oeni* was thought to be due to the esterification between acetaldehyde and alcohols and the metabolic conversion of acetaldehyde into ethanol and acetate [Osbourne et al. (2000), Liu et al. (2002); Nevodic et al. (2000)]. Ethylacetate and iso-amylacetate were produced during MLF by *O. oeni*. The investigation of Nevodic et al. [2000] using co-immobilized yeast and *O. oeni* for continuous cider fermentation reported high production of ethylacetate and diacetyls. The iso-amylalcohol concentration was high in the batch process, but was low in the continuous process. The acetaldehyde concentration was very low after the fermentation using co-immobilized yeast and *O. oeni*.

A significant difference between two malolactic strains during MLF was in acetaldehyde. *L. brevis* produced acetaldehyde and *O. oeni* consumed acetaldehyde (Table 6.4, Table 6.7). *L. brevis* produced acetaldehyde in the rate of 1.3 mmole/h during MLF in alcohol concentration 11% (w/v) and in the rate of 2.93 mmole/h during MLF in alcohol concentration of 9% (w/v). *O. oeni* consumed acetaldehyde in the rate of 5.8 mmole/h during MLF in alcohol concentration 11% (w/v) and in the rate of 1.8 mmole/h during MLF in alcohol concentration 12% (w/v). Both strains showed a trend of declining rate with an increase alcohol concentration (Fig 6.15, Fig 6.25).

The production of ethylacetate was detected in both strains. The transformation activity and the specific transformation activity of ethylacetate reduced in a range from alcohol concentration 9% (w/v) to 13% (w/v) in *L. brevis* and from alcohol concentration 11% (w/v) to 13% (w/v) in *O. oeni.*

The transformation activities of iso-amylacetate and iso-amylalcohol were detected during MLF in the cases of both malolactic strains. The transformation activity and the specific transformation activity of two volatile compounds reduced with increase alcohol concentration.

CHAPTER 7: CONCLUSIONS.

7.1 INTRODUCTION

This project was set out to investigate two main applications of the MCR, namely cell propagation and biotransformation using LAB so as to intensify process productivity. The result of this work has shown that generally it is possible to produce high cell concentrations of LAB with very high productivity as compared with the traditional anaerobic culture systems. Once the cells were produced, a specific transformation involving LAB was investigated, using the maturation processes with these LAB. It was possible to run maturation process up to two months with high biotransformation activity at high cell density.

7.2 GROWTH OF LAB IN SIMPLE BATCH CULTURE, pH-CONTROLLED STR CULTURE AND MCR

The work and its significance can be discussed on the basis of economics and the possibility to apply the system. All media were formulated with yeast extract, a combination of carbohydrates and minerals. Although LAB were grown at the high growth rate during the batch culture and the STR culture, the final yield and the growth rate were limited by end product inhibition even in the MCR, which was also a factor in the MCR.

The capacity of the membrane in the MCR, as pointed out, was of critical importance. The extent of the membrane fouling shown at operation of the MCR with *L. brevis* and *B. longum* was very serious and significantly reduced the productivity of the MCR. Therefore, the membrane to be used in the MCR must be investigated in advance.

LAB concentration in the MCR reached the densities from 7 times to 10 times than that approached in the batch culture or in the STR, without a large difference in the cell product yield ($Y_{X/S}$). That high cell density obtained in the MCR was high enough to allow direct use them without further concentration as the starter cultures in ensilage, pro-biotics, and malolactic fermentation.

Chapter.7 Conclusions

7.2.1. Growth of Lactobacillus buchneri.

In the simple batch culture *Lactobacillus buchneri* was grown at shaking incubator of 30°C without any other special nutrient requirements. Yeast extract and glucose were used to formulate the growth medium with minerals. The starter culture medium and the main culture medium were optimized in basis of the synthetic medium by Ledesma et al. [1977]. Yeast extract effective to stimulate both of the growth rate and the cell yield was used at the concentration of 0.9% (w/v) after comparison with soy peptone. The initial pH for the optimum culture was given in a pH range from pH 5.8 to pH 6.0 to produce the highest growth rate and product yield.

Table 7.1 shows a difference at the growth kinetics obtained during scaling up the culture of *L. buchneri* from the simple batch culture to the MCR.

Kinetics / Culture	μ (h⁻)	X (DCW, g/L)	Time (h)	Product yields (g/g)					$P_{X/t}$ (g/L, h)
				$Y_{X/S}$	$Y_{L/S}$	$Y_{A/S}$	$Y_{E/S}$	$Y_{M/S}$	
Batch	0.093	1.12	53	0.06	N.D	N.D	N.D	N.D	0.021
STR	0.202	1.83	30	0.068	0.44	0.1	0.22	N.D	0.061
MCR	0.065	16.2	23	0.077	0.375	0.093	0.243	N.D	0.704

Table 7.1 The growth kinetics obtained during the culture of *L. buchneri* in the batch, the STR and the MCR. μ; the growth rate, X; cell mass, $Y_{X/S}$; cell product yield, $Y_{L/S}$; Lactate product yield, $Y_{A/S}$; Acetate product yields, $Y_{E/S}$; ethanol product yields, $Y_{M/S}$; mannitol product yields, $P_{X/t}$; the specific cell production rate, N.D; not determined

Lactobacillus buchneri was grown at a growth rate of 0.093 h⁻¹ at the growth medium optimized through a series of the batch cultures. The growth rate in the STR culture increased to 0.202 h⁻¹ because of the growth in optimum pH level and again decreased to 0.065 h⁻¹ in the MCR because of end product inhibition. In the growth medium of *L. buchneri*, the carbon source was glucose. *L. buchneri* can reproduce NAD^+ necessary for glycolysis by producing ethanol.

Chapter.7 Conclusions

Growth showed large improvement at total cell mass (X $_{DCW}$), cell product yield (Y$_{X/S}$), and the specific cell production rate (P$_{x/t}$). The cell concentration improved 8.9 times more in the MCR than in the STR culture and the total cell production rate was 11.5 times higher in the MCR than in the STR culture. Even more cell mass could be obtained during the prolonged culture in the MCR.

The product yield of lactate was 0.375. The product yield of ethanol was 0.243. Mannitol was not produced during the fermentation of *L. buchneri*. All product yields in the batch culture were the similar to that in the MCR.

7.2.2 Growth of Lactobacillus brevis.

Lactobacillus brevis was grown at the shaking incubator of 28 °C with 1.5% (w/v) yeast extract concentration.

High growth rate and cell mass were obtained at the initial pH range from pH 5.5 to pH 6.0. Table 7.2 shows the growth kinetics obtained during the growth of *L. brevis* in the simple batch culture, the STR and the MCR.

Lactobacillus brevis was grown at a growth rate 0.2 h^{-1} in the optimized growth medium including 56 mM glucose and 139 mM fructose.

Kinetics Culture	μ (h⁻)	X (DCW, g/L)	Time (h)	Product Yields (g/g)					P$_{X/t}$ (g/L, h)
				Y$_{X/S}$	Y$_{L/S}$	Y$_{A/S}$	Y$_{E/S}$	Y$_{M/S}$	
Batch	0.2	2.23	26	0.058	N.D	N.D	N.D	N.D	0.086
STR	0.162	2.33	36	0.067	0.4	0.08	0.22	0.03	0.065
MCR	0.052	15.5	20	0.075	0.9	0.02	N.D	0.01	2.018

Table 7.2 The growth kinetics obtained during the culture of *L. brevis* in the batch, the STR and the MCR. *μ; the growth rate, X; cell mass, Y$_{X/S}$; cell product yield, Y$_{L/S}$; Lactate product yield, Y$_{A/S}$; Acetate product yields, Y$_{E/S}$; ethanol product yields, Y$_{M/S}$; mannitol product yields, P$_{X/t}$; the specific cell production rate, N.D; not determined*

Chapter.7 Conclusions

L. brevis kept growing, even if it was grown at a low growth rate, after the exponential growth phase. Ultimately, the cultivation could produce the total cell mass of 2.2 DCW, g/L in 26 h. The cell product yield was 0.075. The growth rate in the batch culture was higher 7.41 times using fructose than the result obtained with glycerol by Cunha and Foster [1992] and the growth rate decreased stepwise from the batch culture to the MCR culture. The cell mass obtained with the MCR culture was 7 times higher than that obtained in the batch culture and the specific cell production rate increased 23.5 times. The cell product yields increased from 0.058 in the batch culture to 0.075 in the MCR.

One of the most striking differences was the fact that *L. brevis* in the MCR grew like homofermentative LAB mainly producing lactate. The product yield of lactate increased from 0.4 in the batch culture to 0.9 in the MCR and other product yields decreased. *L. brevis* have two metabolic pathways depending on the growth rate. *L. brevis* can use fructose like glucose producing lactate in a low growth rate by end-product inhibition or in glucose-limited condition, but at high growth rate, fructose can also be reduced to regenerate NAD^+ using glucose as a main carbon source. As shown at Fig 5.7, the dilution rate did not increase more than 0.48 h^{-1} because of a serious membrane fouling. The growth rate of *L. brevis* in fructose-limited condition was lower in the MCR.

7.2.3. Growth of Bifidobacterium longum.

Bifidobacterium longum was grown in shaking incubator of 37°C with 3%(w/v) yeast extract, and carbon sources in low concentration of 56 mM glucose and 50 mM fructose so as to reduce the production of polysaccharides. *B. longum* particularly required ammonium sulphate with yeast extract as nitrogen source. Only when both of yeast extract and ammonium sulphate are present, *B. longum* grew fast. The growth of *B. longum* was the best in initial pH from pH 6.0 to pH 6.5.

Table 7.3 shows the growth kinetics obtained during the culture of *B. longum* in the batch, the STR and the MCR. The cell mass in the MCR was 7 times that obtainable in the batch culture. The specific cell production rate in the MCR was 13.5 times greater than that in the batch culture.

Chapter.7 Conclusions

The growth rate of *B. longum* showed a slight increase between the batch culture and the STR culture, but it decreased to 0.13 h $^{-1}$ in the MCR culture from 0.28 h $^{-1}$. The cultivation of *B. longum* is thought to be affected by end product inhibition. The product yield of lactate decreased from 0.3 in the batch culture to 0.25 during the MCR culture and the product yield of acetate and ethanol increased to 1.5 times that of the batch culture.

In Fig 5.7 the feeding of the growth medium could not be increased further because of a serious membrane fouling.

Therefore, glucose and fructose were limited in 6 h. In such a glucose-limited conditions *B. longum* can produce acetate, ethanol, and formate rather than lactate [Degnan and Macfarlane. (1994)].

Kinetics / Culture	μ (h $^{-1}$)	X (DCW, g/L)	Time (h)	Product Yields (g/g)					$P_{X/t}$ (g/L, h)
				$Y_{X/S}$	$Y_{L/S}$	$Y_{A/S}$	$Y_{E/S}$	$Y_{M/S}$	
Batch	0.23	3.23	24	0.17	N.D	N.D	N.D	N.D	0.135
STR	0.28	4.04	11	0.21	0.3	0.57	0.05	N.D	0.367
MCR	0.13	22.2	11	0.18	0.25	1.25	0.19	N.D	2.018

Table 7.3 The growth kinetics during the culture of *B. longum* in the batch, the STR and the MCR. *μ; the growth rate, X; cell mass, $Y_{X/S}$; cell product yield, $Y_{L/S}$; Lactate product yield, $Y_{A/S}$; Acetate product yields, $Y_{E/S}$; ethanol product yields, $Y_{M/S}$; mannitol product yields, $P_{X/t}$; the specific cell production rate, N.D; not determined*

7.2.4 Growth of Oenococcus oeni.

Growth of *Oenococcus oeni* was the best in the growth medium, which includes glucose and fructose with growth temperature of 28 °C. Fructose stimulated the growth rate of *O. oeni* when used together with glucose.

Fructose enables *O. oeni* regenerate NAD^+ necessary for metabolizing glucose as in the culture of *L. brevis*. Also, as the growth stimulant, citric acid can be co-metabolized with more production of ATP and NAD^+.

Table 7.4 shows the growth kinetics obtained during the culture of *O. oeni* in the batch, the STR and the MCR. Total cell mass of *O. oeni* in the MCR improved by 14 times that in the batch culture, but the growth rate did not improve. Low growth rate of *O. oeni* make the culture unreasonable due to increasing medium cost by long culture time.

O. oeni could not consume mannitol accumulated in the middle of the culture as compared with the culture of *L. brevis*. Most of mannitol converted from fructose was wasted through permeate in the MCR culture.

Kinetics / Culture	μ (h^{-1})	X (DCW,g/L)	Time (h)	Product Yields (g/g)					$P_{X/t}$ (g/L, h)
				$Y_{X/S}$	$Y_{L/S}$	$Y_{A/S}$	$Y_{E/S}$	$Y_{M/S}$	
Batch	0.08	0.91	48	0.026	N.D	N.D	N.D	N.D	0.019
STR	0.11	0.54	29	0.022	0.2	0.27	N.D	0.43	0.019
MCR	0.07	12.8	68	0.021	0.18	0.29	0.03	0.4	0.188

Table 7.4 The growth kinetics obtained during the culture of *O. oeni* in the batch, the STR and the MCR. μ; the growth rate, X; cell mass, $Y_{X/S}$; cell product yield, $Y_{L/S}$; Lactate product yield, $Y_{A/S}$; Acetate product yields, $Y_{E/S}$; ethanol product yields, $Y_{M/S}$; mannitol product yields, $P_{X/t}$; the specific cell production rate, N.D; not determined

The total cell production rate in the MCR was increased by 10 times than in the batch and the STR. The membrane in the MCR was not significantly fouled by *O. oeni*, so high cell density in the MCR could be used for MLF during the prolonged running of the MCR.

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7.3 LIMITATIONS TO MCR CULTURE OF LAB.

From the comparison of data shown above, there are a couple of limitations in using the MCR. Severe fouling on product membrane produced a limitation where dilution rate could not be increased as in the case of *L. brevis* and *B. longum*. In general, S-layer proteins produced by *L. brevis* and exopolysaccharides produced by *B. longum* were thought to be the main cause of the fouling in product membrane. Such membrane fouling limits the removal of end product from the MCR and the feed of new growth medium. In the case of *O. oeni*, membrane fouling was not in product membrane, but in feed membrane because a long incubation period by a low growth rate. That is, the membrane was fouled with medium components.

As another problem for the growth of *O. oeni* in the MCR, the cultivation of *O. oeni* required three times more growth medium when compared with others because of low growth rate and yield. When either glucose or fructose was limiting under the critical concentration, the growth rate and cell yield reduced. It is important to keep both fructose and glucose non-limiting so as to keep the growth rate high.

When the cell concentration reached high density in the MCR, there was severe foaming. Foams led to malfunction of the level controller contaminating of level indicator, and consequently affected the feed rate of the growth medium. Foams also disturbed the function of the magnetic centrifugal pumps fluctuating the flow of the culture fluid.

To reduce foam in the reactor, detergents such a polypropylene glycol must be used, but it must be careful because use of detergents can be another cause of membrane fouling.

According to the results obtained during the cultivation of four lactic acid bacteria, the membrane bioreactor for the cell retention culture is designed with strategies for a high product yield and production rate. In order to give bacteria the best growth the concentration of medium must be controlled to reduce end product inhibition. High substrate concentration produces high end product concentration so producing end product inhibition.

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Therefore, the substrate concentration must be controlled in a range between these two extremes. The feed in a low concentration must be supplied in a high rate so increasing the membrane flux of the feed stream. In the condition of $D = Jm = \mu_{max}$, the efficiency of the membrane reactor will be the maximum.

7.4 BIOTRANSFORMATION AND MCR.

L. brevis and *O. oeni* are involved in intensive maturation process of cider, the represent approach for another industrial use. Two strains were compared with the efficiency during MLF of cider. The K_S for substrate malate uptake was affected by the types of cider and malolactic species. Two types of the green ciders in comparison were General cider and Scrumpy Jack. Even if the quality of the matured cider was affected by the cider types and the malolactic strains, the quality could be controlled by the cider retention time in the MCR

7.4.1. Comparison of two malolactic strains in MLF of Scrumpy Jack.

L. brevis and *O. oeni* were compared from the point of view of organic acid, volatile compound and cell maintenance during the MLF of Scrumpy Jack. Organic acids, volatile compounds and cell mass were affected by the retention time and the alcohol concentration during MLF. Therefore, the MCR represents an important tool for biotransformation of chemicals and other food related products.

7.4.1.1. Comparison of two malolactic strains in cell maintenance during MLF of Scrumpy Jack.

Two malolactic strains of *L. brevis* and *O. oeni* were compared with their efficiency at malolactic fermentation of the green cider. *L. brevis* has been predominant at MLF of cider and *O. eni* has been predominant at MLF of wine.

Both strains showed a resistance against acidic and alcoholic condition after alcoholic fermentation by yeast. *L. brevis* and *O. oeni* showed a difference in cell maintenance (cell yield) during MLF of cider.

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L. brevis showed a decline trend different from *O. oeni* on an increase during MLF of cider. As shown at Table.3.1, *L. brevis* was stronger than *O. oeni* against organic acids, but *L. brevis* could not be grown in the acidic condition and the alcoholic condition of the post-alcoholic fermented cider because of poor ethanol and acid tolerance.

O. oeni showed the growth during MLF of cider. There is no evidence that *O. oeni* could produce the growth energy through the degradation from malic acid to lactic acid or acetic acid [Tracey and Britz. (1989)] and from acetaldehyde to acetic acid or ethanol [Osborne et al. (2000), Liu et al. (1997)], but *O. oeni* could grow during MLF of cider using residual sugars and amino acids.

When the alcohol concentration in the feed increased, the cell mass of *L. brevis* showed a steady decrease from 9% (w/v) to 13% (w/v) alcohol concentration, but the cell mass of *O. oeni* increased even in 12% (w/v) alcohol concentration and *O. oeni* was more tolerant.

7.4.1.2. Comparison of two malolactic strains in the K_S for malate uptake and organic acid during MLF of Scrumpy Jack .

The K_S for malate uptake was affected by the components in the substrate such as alcohol, organic acid and tannin. When the K_S of two malolactic strains was compared using Scrumpy Jack as a substrate, two strains showed a large difference. This clearly represents a key factor for selection of strain to be used in MLF.

Due to the high K_S value, the malate concentration in the product stream was controlled by the cider retention time. The malate concentration in the product stream was 23 mM in the retention time longer than 6 h, but it increased to above 34.4 mM when the retention time reduced to below 6 h during MLF of Scrumpy Jack by *L. brevis*. The malate concentration in the product stream was 24.3 mM at the retention time longer than 3 h and the malate concentration increased to 26 mM in the retention time of 6 h and increased further to 34.3 mM in the retention time of 3 h during MLF by *O. oeni*.

The reduction rate of malate during MLF of Scrumpy Jack by *L. brevis* and *O. oeni* was affected by the alcohol concentration higher than 11% (w/v).

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The reduction rate of malate in *L. brevis* increased from 15 mmole/h g of cell to 23 mmole/h g of cell when the alcohol concentration increased from 9% (w/v) to 11% (w/v). When the alcohol concentration increased further to 13% (w/v), the reduction rate of malate decreased to 11 mmole/ h g of cell. The reduction rates of malate in MLF by *O. oeni* was increased from 5.4 mmole/h, g of cell to 8.6 mmole/h g of cell when the alcohol concentration increased from 9% (w/v) to 11% (w/v). The reduction rate of malate decreased again to 7.5 mmole/ h g of cell when the alcohol concentration increased further to 13%(w/v).

The specific malolactic activity of *L. brevis* was higher than that of *O. oeni* when using Scrumpy Jack as the substrate. The reason why the specific malolactic activity of *L. brevis* was higher than that of *O. oeni* even if the K_S of *L brevis* was higher than that of *O. oeni* is because the cell concentration of *L. brevis* was low when compared with that of *O. oeni* in the reaction. *L. brevis* should be used in lower cell concentration than *O. oeni* because of the membrane fouling by extracellular proteins in high cell density.

7.4.1.3. Comparison of two malolactic strains in the transformation of the volatile compounds during MLF of Scrumpy Jack.

O. oeni consumed acetaldehyde in cider during MLF as a result by Osborne et al. [2000] and Liu et al [1997]. Ethyl alcohol and iso-amylalcohol in cider partially reduced with production of ethyl acetate and iso-amylacetate, respectively, and some part of acetaldehyde in cider could be used to produce ethyl alcohol and ethyl acetate in matured cider. Therefore, the use of *O.oeni* in MLF of cider was better than *L. brevis* for flavour development to enrich aroma. The reduction rate of acetaldehyde was affected by cider retention time and the alcohol concentration as shown at the section about organic acid during MLF of Scrumpy Jack.

The reduction rate of acetaldehyde during MLF by *O. oeni* was inhibited by 11%(w/v) alcohol concentration and the production rate of acetaldehyde during the MLF by *L. brevis* was inhibited even by 9% (w/v) alcohol concentration.

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7.4.2. Comparison of two types of ciders during the MLF by O. oeni.

General cider and Scrumpy Jack were compared from the point of view of organic acid and cell maintenance during MLF, using *O. oeni* as malolactic strains. The K_S for malate uptake and cell maintenance could be affected by the types of ciders in test.

Scrumpy Jack contains more nutrients such as tannin, organic acids and the residual sugars after the alcoholic fermentation than General cider. This promotes growth, but may inhibit biotransformation.

7.4.2.1. The effect of types of cider on the cell maintenance during MLF.

During the MLF by *O. oeni* as malolactic strain, two ciders were compared by changing the retention time of feeding cider from the point of view of the cell maintenance and pH. The cell mass was not affected by the retention time whatever types of cider was used.

While General cider was used, the cell mass was in a range from 12 to 16 DCW, g/L, and the cell mass was in a range from 16 to 18 DCW, g/L when Scrumpy Jack was used.

7.4.2.2. The effect of types of cider on organic acid and the K_S for malate uptake.

The K_S for malate uptake by *O. oeni* was affected by the types of feeding cider. The K_S in General cider was 31.4 mM and the K_S in Scrumpy Jack was 57.6 mM. The malate in the feeding cider was reduced in higher rate during MLF of General cider than during MLF of Scrumpy Jack.

Unknown chemicals or high concentration of ingredients in Scrumpy Jack could give an inhibition during MLF. Likewise, malate in the product stream was at lower concentration during MLF of General cider than during MLF of Scrumpy Jack.

7.5. LIMITATION ON MCR FOR BIOTRANSFORMATION.

Biotransformation is about the technology using biological catalysts to produce the products. When compared to the existing chemical syntheses, biotransformation is more

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environment-friendly and is more specific than the chemical syntheses in selectively producing optical isomers such as chiral compounds.

As a biotransformation, the MLF process shows how more rapid and controllable than the traditional maturation process. However, there are still limitations in application of biotransformation.

From the cider transformation studied above, considerable enhancements of biotransformation in general are possible. The cider biotransformation illustrates the key advantages. Advantages and disadvantages of the MCR system can be listed as follows

● Advantages

- Operation at relative high cell concentration, but with good mass-transfer
- Operation in slow growth or non-growing cultures, inhibited cultures, full cell retention and no-washout.
- End products continuously removed.
- Propagation and biotransformation system can be combined.
- Control of the reaction rate by the residence time.
- Intensive processes with relatively small footprint.
- Generic form of immobilization.
- Economic use of catalysts by recycling in process.
- Well suited to anaerobic culture.
- Well suited to stable biocatalyst.

● Disadvantages.

- Relatively expensive to construct and operate.
- Membrane fouling limits operational range
- Not well suited to aerobic culture.
- Not useful with short-lived biocatalysts

7.6. INDUSTRIAL APPLICATIONS OF THE MCR TECHNOLOGY.

The MCR has been used to grow bacteria to high cell density and to use it as biocatalysts for biotransformation. Using high cell density as inoculants can be one of methods to

stimulate the bacterial adaptation to poor environment of wine- and cider-making processes, silage, and pro-biotics. Secondly, the shelf life of LAB in dairy foods like pro-biotics can be longer.

In this section efficiency in use of the MCR will be shown in terms of high cell density culture and biotransformation concerning about commercialization.

7.6.1. Application of high cell density culture for commercialization.

Using the MCR for bacterial growth can present the chance to grow microorganism to the maximum. As shown at this investigation, all LAB in use were grown to more or 7 time cell concentration approachable at the traditional incubation methods.

Usually, high cell concentration has been required to stimulate the initial growth of microorganisms at poor environment. However, such a high cell density could not be obtained in the traditional culture methods because of end product inhibition or substrate limitation when the culture is in high cell density.

The MCR can be more often applied for alcohol fermentations and lactic fermentations at which end product inhibition has been a serious problem. At lactic acid fermentation it is very economic because water the main contaminant in pure lactate production can be easily removed in the high lactic acid concentration produced by the high cell mass.

Single cell proteins or single cell oils can reach higher product concentration when the cell mass is as high as possible because products are intracellular. New growth medium should be continuously supplied without the risk of washing-out in the high feeding rate. However, in the traditional continuous culture when the feeding rate exceeds the growth rate, the cell concentration is reduced by washing-out. The cells can be accumulated in the reactor by the retaining membrane during the MCR culture while continuously feeding new growth medium into the reactor.

The number of living LAB in manufacturing pro-biotics is very important. Most of LAB is very vulnerable to extremely acidic pH in the human gastric tracts.

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A few LAB survive in the acidic pH and pass to the human intestine. Consequently, more number of Lab in pro-biotics provide more possibility to pass LAB in pro-biotics via the gastric tracts to the human intestinal mucous membrane and to maximize the function of pro-biotics.

7.6.2. Application of biotransformation for commercialization.

At the rate of biotransformation using biocatalysts can be described as following,

$$V = V_S a = -\frac{dS}{dt} = \frac{V_{max} S_1}{[K_m + S_1]}$$ (1)

V : reaction rate at biocatalysts surface. V_S : reaction rate per unit surface area of biocatalysts. a : unit area for reaction per surface area of biocatalysts. S_1 : substrate concentration in biocatalysts surface.

As shown at above the rate equation (1), the reaction rate (V) is in a proportion to the surface area (a) of biocatalysts and the substrate concentration (S_1) in a boundary layer along biocatalysts. Consequently, increase biocatalysts concentration is only parameter which can positively affects the transformation rate if the substrate concentration S_1 is constant. The use of the MCR to grow bacteria biocatalyst to high concentration is necessary so as to increase the productivity in biotransformation and well maintain high mass transfer.

Biocatalysts have been involved to manufacture products in diverse area of industry. Biotransformation has been used to selectively produce optically specific substances such as chiral compounds or isomers even if chemical syntheses have been traditionally used to produce them. Production of chiral compounds by biotransformation is more efficient in the point of view which can produce with better productivity than the chemical syntheses and which can make downstream process easier than the chemical syntheses because of optical specificity on biotransformation.

However, use of biocatalysts is very rare in producing the bulk chemicals because the price of biocatalysts is too expensive when compared to the retail price of chemicals in

the market. They have been traded in higher price than the bulk chemicals. Even if the MCR can not be used to produce the bulk chemicals because of expensive membrane and biocatalysts, and financial barrier demanding a huge investment, the MCR can be used to produce chiral compounds or intermediates for chemical syntheses.

Usually the membrane filtration or centrifugation is used to separate the products from biocatalysts in downstream process after transformation. The separation is necessary. However, using the MCR without an extra process for separation, the products can be harvested right way after transformation.

The MLF in wine- or cider-making is one example of biotransformation. Carrying out MLF with high cell density in the MCR aims to improve the traditional biotransformation by intensifying and controlling the processes.

All strategies such as genetic modification and use of immobilized enzyme catalysts for improvement in biotransformation have limitations because of difficulties in development and production in the high cost. Using the MCR to grow the cells biocatalysts to high concentration and to use it to intensify biotransformation shows another way to overcome a number of limitations in biotransformation.

The existing industries have depended on the large capacity of the plants to increase the productivity and to stabilize the investment. The existing cider-making or wine-making industries have built the huge plants to improve the productivity and to cope with increase consumption. However, such a large capacity is becoming a large barrier in enhancing their agility and flexibility to efficiently cope with unexpectedly changeable market. Therefore, the past producers depending on huge capacity have been expelled in recent market. Quickly fluctuating market is requiring the structural change of the world leading producers.

Recent producers are facing to decision-making which has to choose between cutting-edge technologies and the traditional huge capacity. Therefore, even if the MCR technology is a good for improving and intensifying the traditional industry, novel

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strategies need to present advantages enough to overcome the risks coming from huge investment to the producers so as to let them invest to new technologies. The existing producers have to try new technologies for survival and for improvement of the existing production.

The future challenges of the MCR for growth and biotransformation lie in the efficiency of reactor system and in enhancing productivity to overcome the expensive membrane price.

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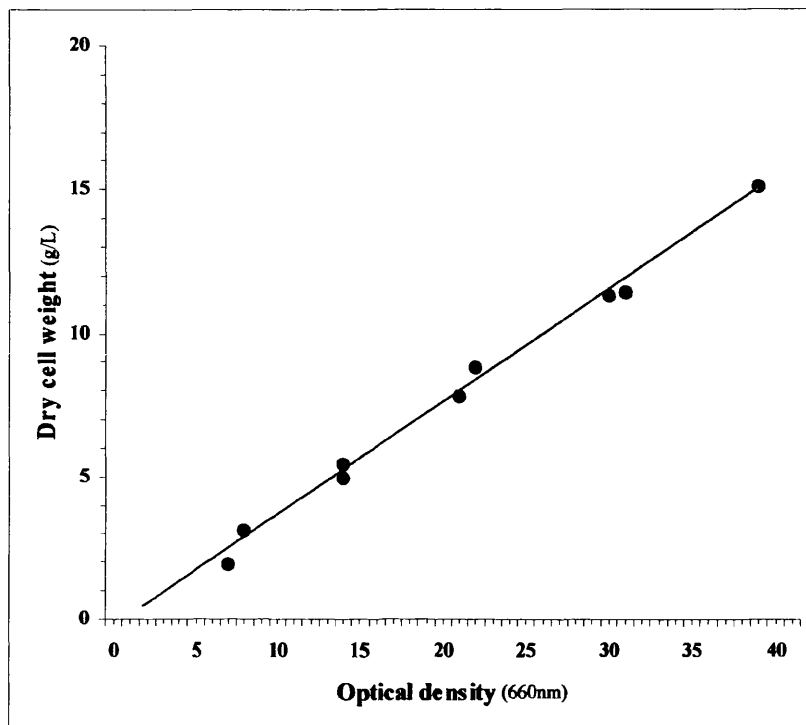
Appendix-1

Standard curve to convert optical densities into dry cell weight of *Bifidobacterium longum*. Equation for conversion was defined as following (Refer to 2-7-1-2).

$Y = 0.37X + 0.21$

Here, Y is DCW and X is O.D (660nm)
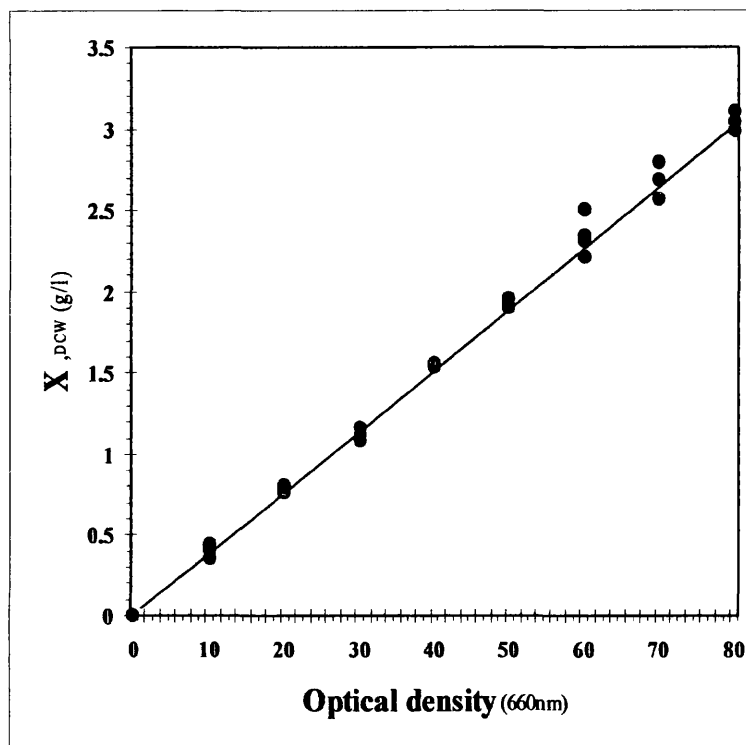
Appendix-2.

Standard curve to convert optical densities into dry cell weight of *Lactobacillus buchneri*. *Equation for conversion was defined as following (Refer to 2-7-1-2).*

$Y = 0.26 X - 0.01$

Here, Y is of DCW and X is of O.D (660nm).

265
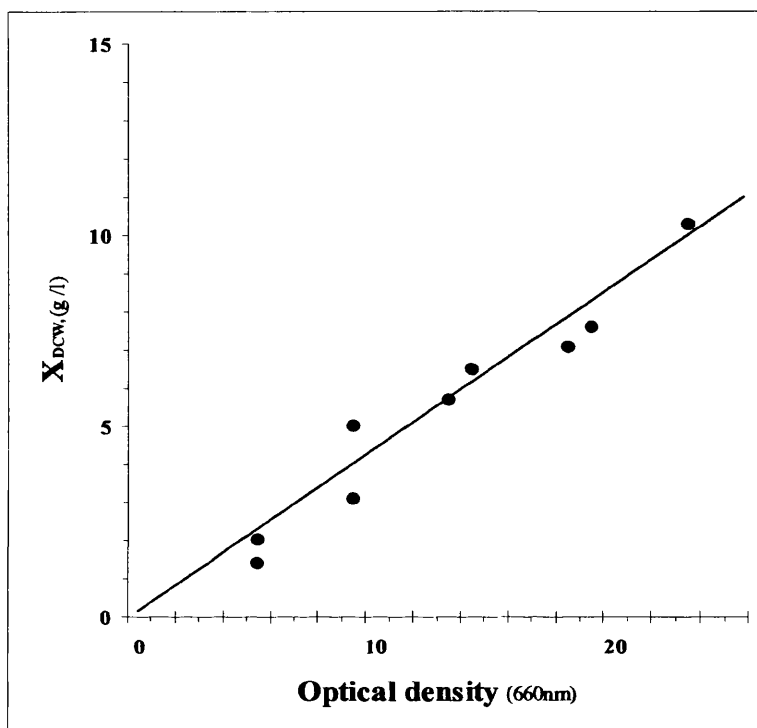
Appendix-3

Standard curve to convert optical densities into dry cell weight of

Oenococcus oeni. Equation for conversion was defined as following (Refer to 2-7-1-2).

$Y = 0.41X + 0.06$

Here, Y is of DCW and X is of O.D (660nm).
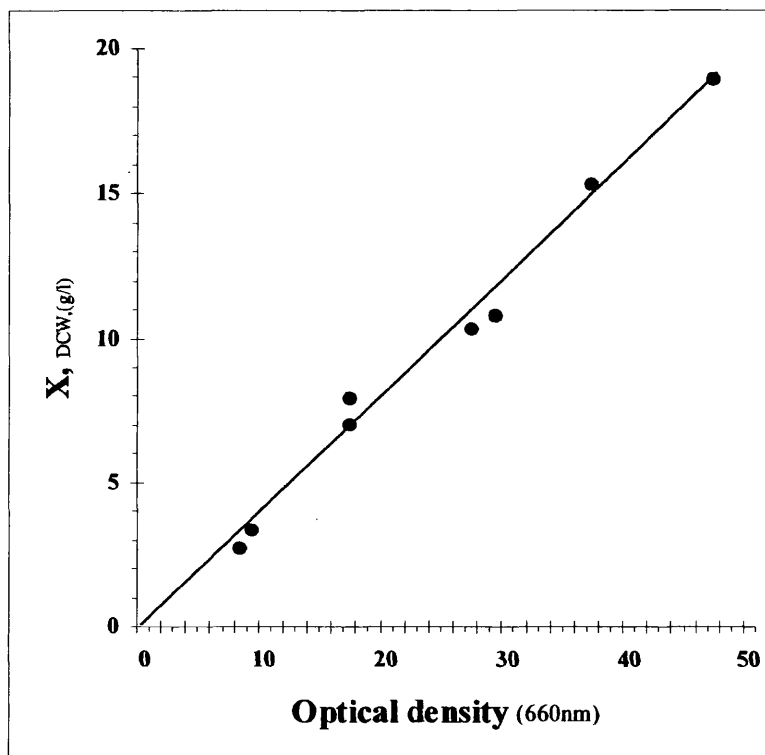
Appendix-4

Standard curve to convert optical densities into dry cell weight of *Lactobacillus brevis*. *Equation for conversion was defined as following (Refer to 2-7-1-2).*

$Y = 0.4X + 0.13$

Here, Y is of DCW and X is of O.D (660nm).
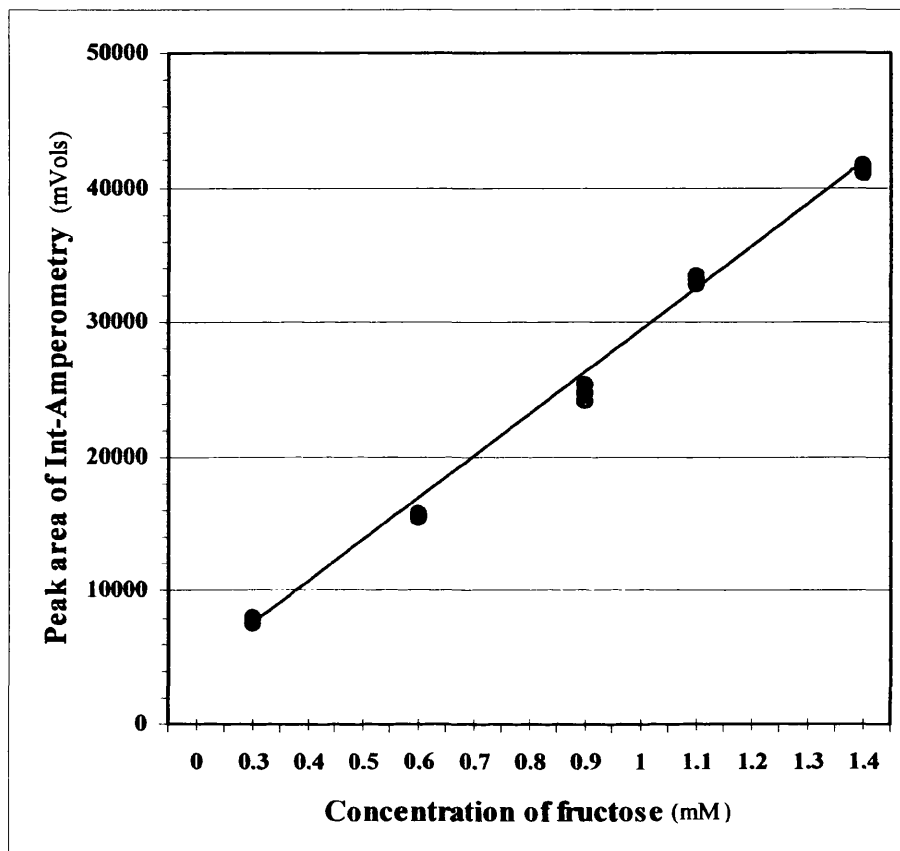
Appendix-5

Standard curve for determination of fructose concentration (mM). This curve was plotted using HPLC incorporating ED. *Equation to determine fructose concentration was defined as following (Refer to 2-7-3-3).*

Y = 29998.76X – 1099.45

An error range of equation was r = 0.998.

X described the concentration (mM) of fructose and Y is for the area of the main peak.
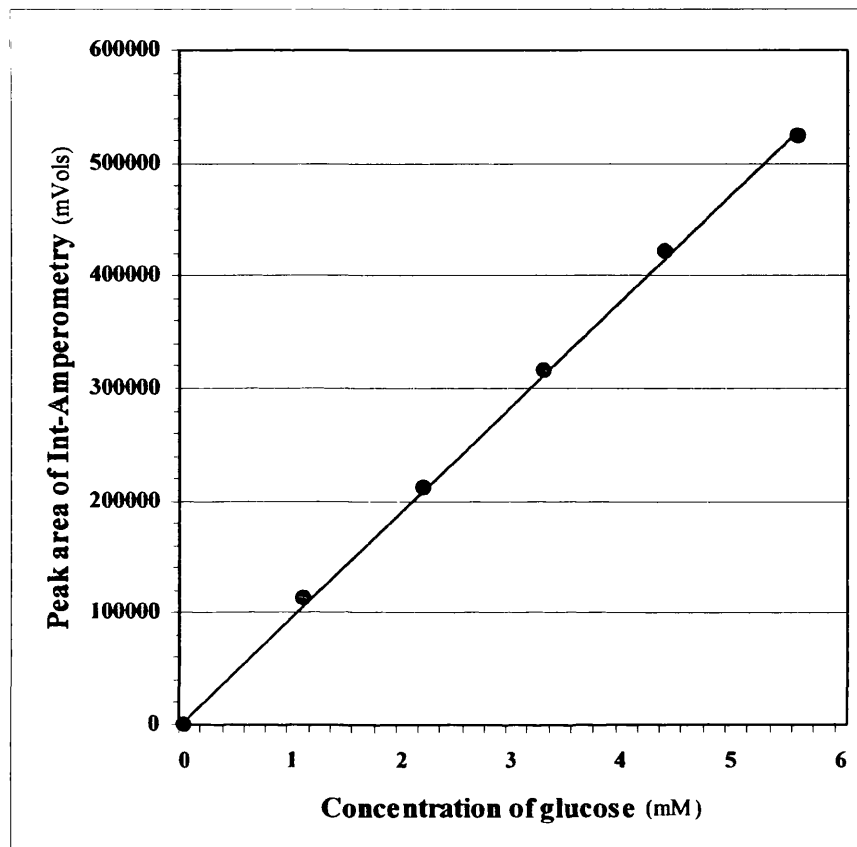
Appendix-6.

Standard curve for determination of glucose concentration (mM). *This curve was plotted using HPLC incorporating ED. Equation to determine glucose concentration was defined as following (Refer to 2-7-3-3).*

$Y = 93485.46X + 5388.065$

An error range of equation was r = 0.999.

Y described the area of the main peak and X is for the glucose concentration (mM).
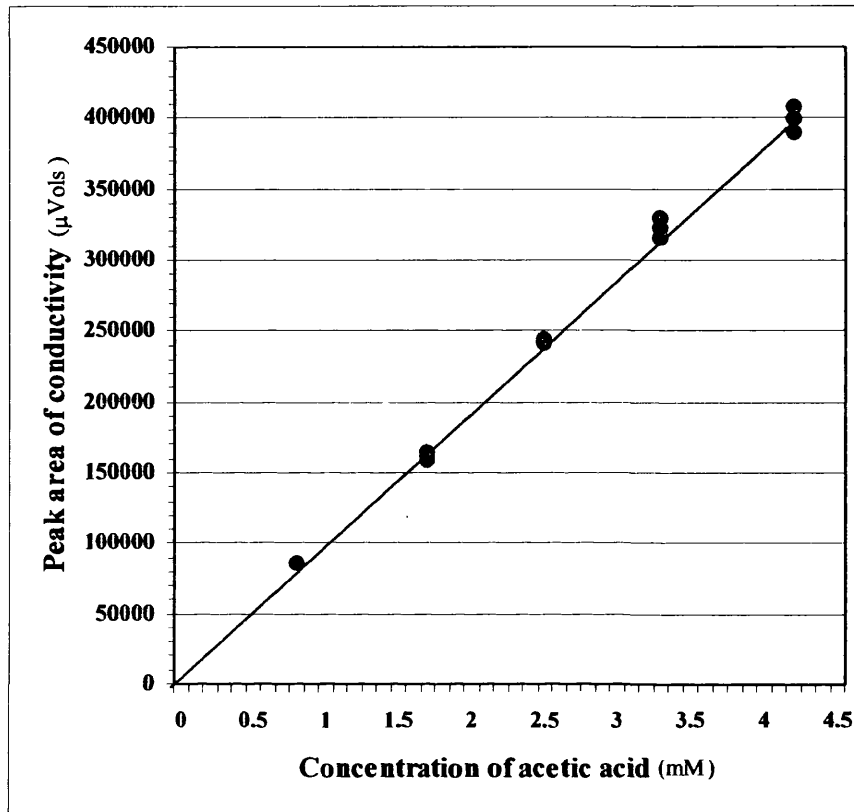
Appendix-7.

Standard curve for determination of acetic acid concentration (mM). *This curve was plotted using HPLC incorporating ED. Equation to determine the concentration of acetic acid was defined as following (Refer to 2-7-3-3).*

$Y = 94949.64X + 3573.26$

An error range of equation was r = 0.999.

Y described the area of the main peak and X is for the acetate concentration (mM).
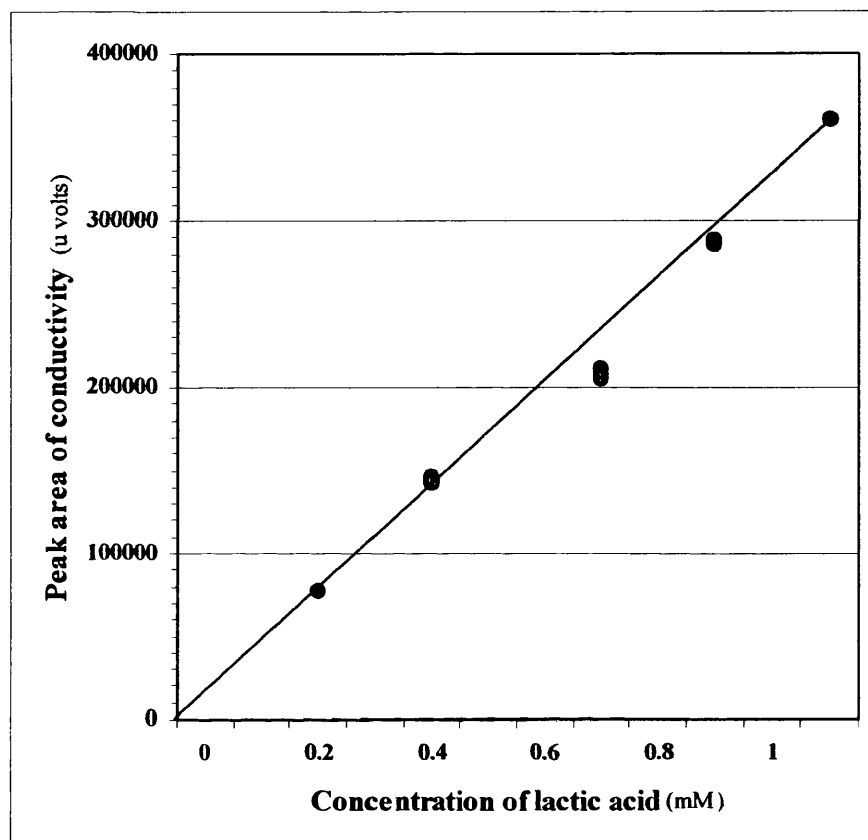
Appendix-8.

Standard curve for determination of lactic acid concentration. *This curve was plotted using HPLC incorporating ED. Equation to determine the concentration of lactic acid was defined as following (Refer to 2-7-3-3).*

$Y = 314005.92X + 6566.08$

An error range of equation was r= 0.996.

Y described the area of the main peak and X is for the lactate concentration (mM).
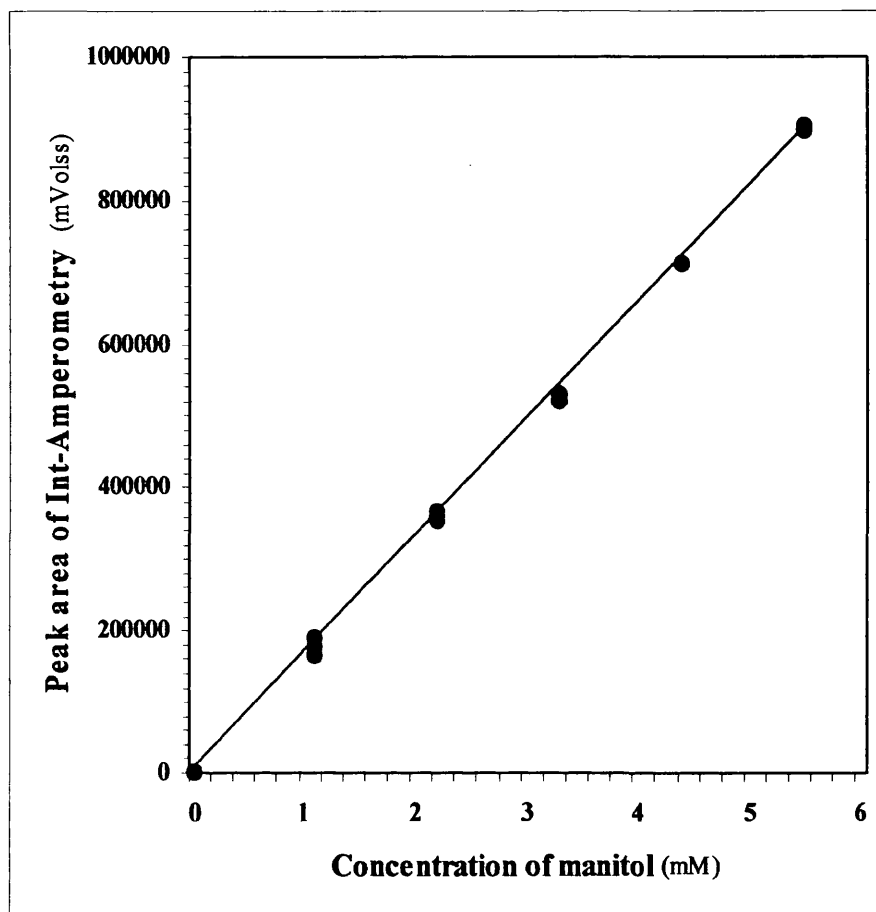
Appendix-9.

Standard curve for determination of mannitol concentration (mM). *This curve was plotted using HPLC incorporating Int-amperometry of ED. Equation to determine mannitol concentration was defined as following (Refer to 2-7-3-3).*

$Y = 160609X + 26.95.$

An error range of equation was r = 0.999.

Y described the area of the main peak and X is for the mannitol concentration (mM).
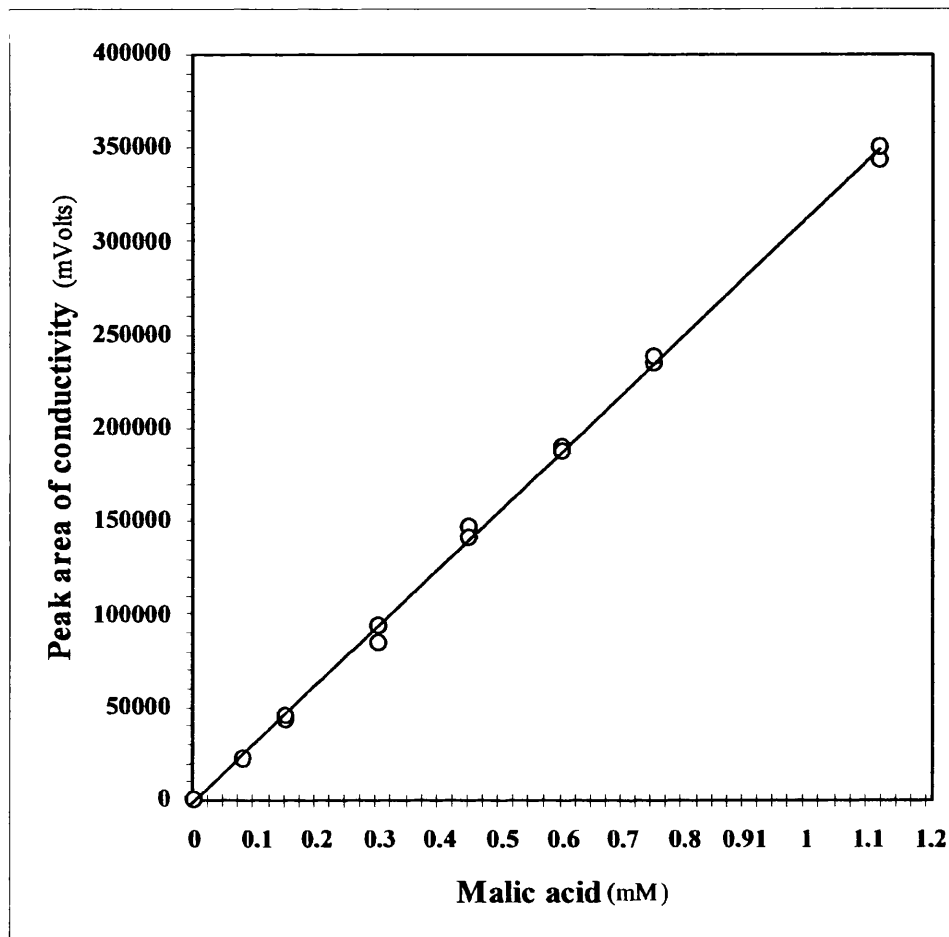
Appendix-10.

Standard curve for determination of malic acid concentration. *This curve was plotted using HPLC incorporating conductivity of ED. Equation to determine the concentration of malic acid was defined as following (Refer to 2-7-3-3).*

$Y = 314269X - 1401.91$

An error range of equation was r = 0.999

Y described the area of the main peak and X is for the malate concentration (mM).
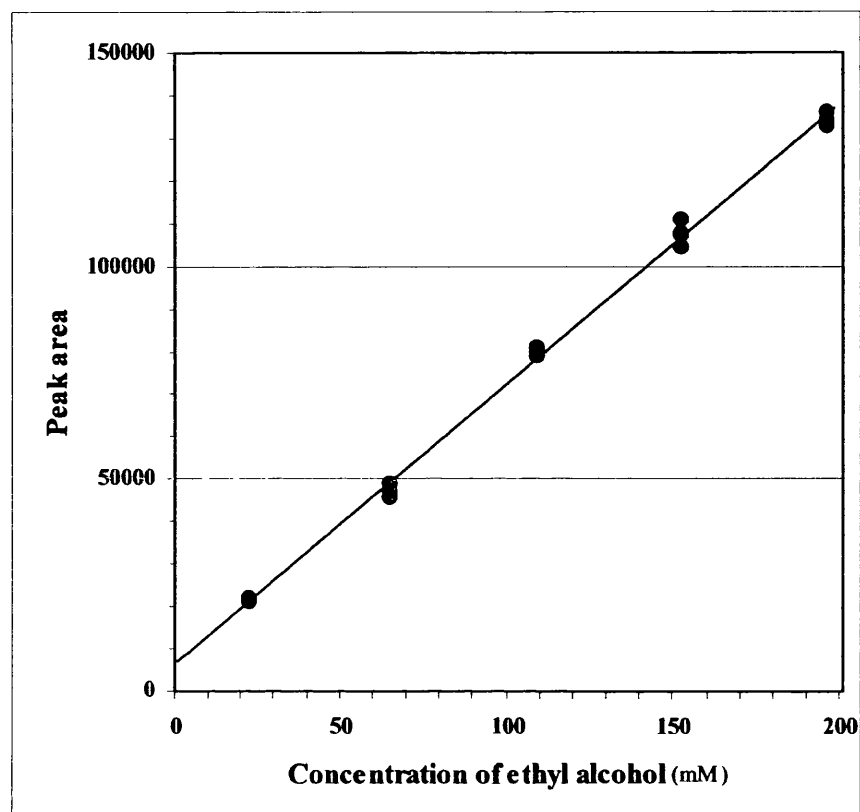
Appendix-11.

Standard curve for determination of ethyl alcohol concentration (mM). *This curve was plotted using G.C. Equation to determine fructose concentration was defined as following (Refer to 2-7-4-6).*

$Y = 656.69X + 66$

An error range of equation was $r = 0.999$

Y described the area of the main peak and X is for the ethanol concentration (mM).
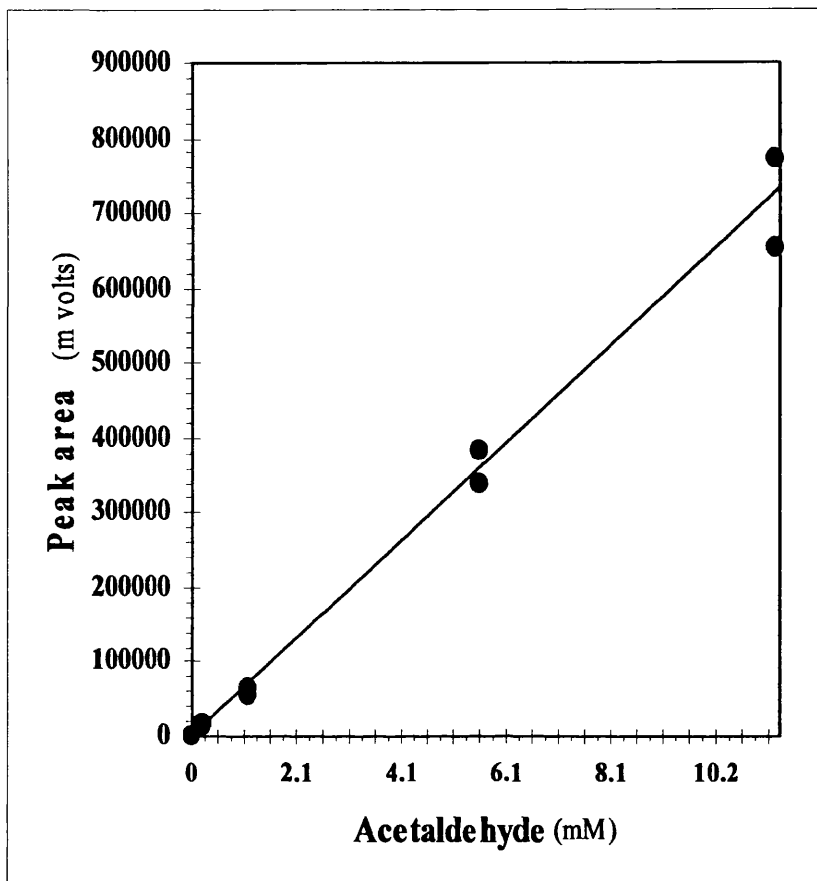
Appendix-12.

Standard curve for determination of acetaldehyde concentration (mM). *This curve was plotted using G.C. Equation to determine acetaldehyde concentration was defined as following (Refer to 2-7-4-6).*

$Y = 63283.75X - 2402.86.$

An error range of equation was r = 0.999.

Y described the area of the main peak and X is for the acetaldehyde concentration (mM).
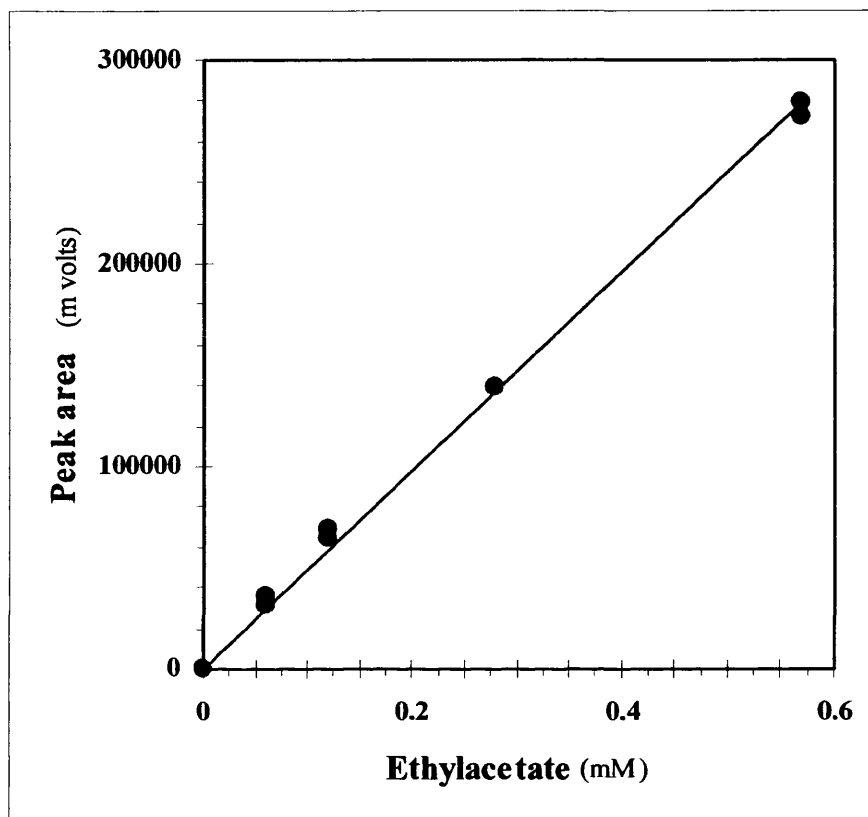
Appendix-13.

Standard curve for determination of ethyl acetate concentration (mM). *This curve was plotted using G.C. Equation to determine ethyl acetate concentration was defined as following (Refer to 2-7-4-6).*

$Y = 478669.38X + 4395.61.$

An error range of equation was r = 0.999.

Y described the area of the main peak and X is for the ethylacetate concentration (mM).

Peak area (m volts) vs iso-amylalcohol (mM)
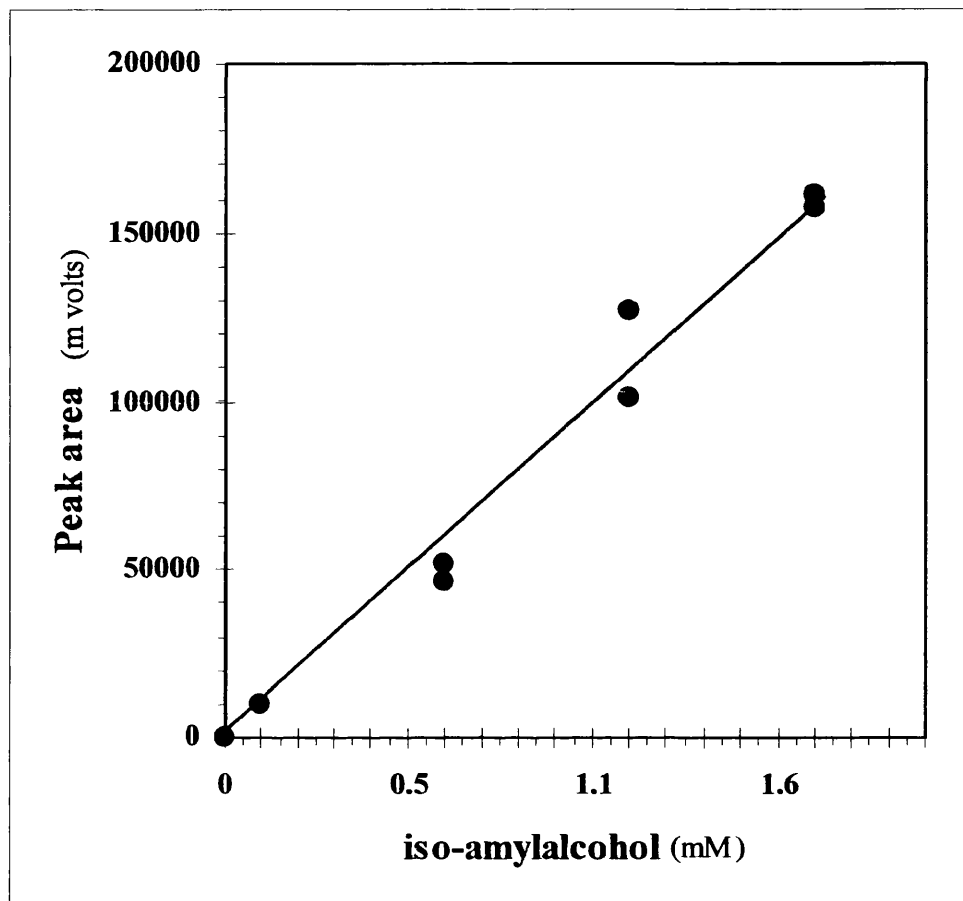
Appendix-14.

Standard curve for determination of iso-amylalcohol concentration (mM). *This curve was plotted using G.C. Equation to determine iso-amyalcohol concentration was defined as following (Refer to 2-7-4-6).*

$Y = 94901.79X - 1691.49.$

An error range of equation was r = 0.999.

Y described the area of the main peak and X is for the iso-amylalcohol concentration (mM).
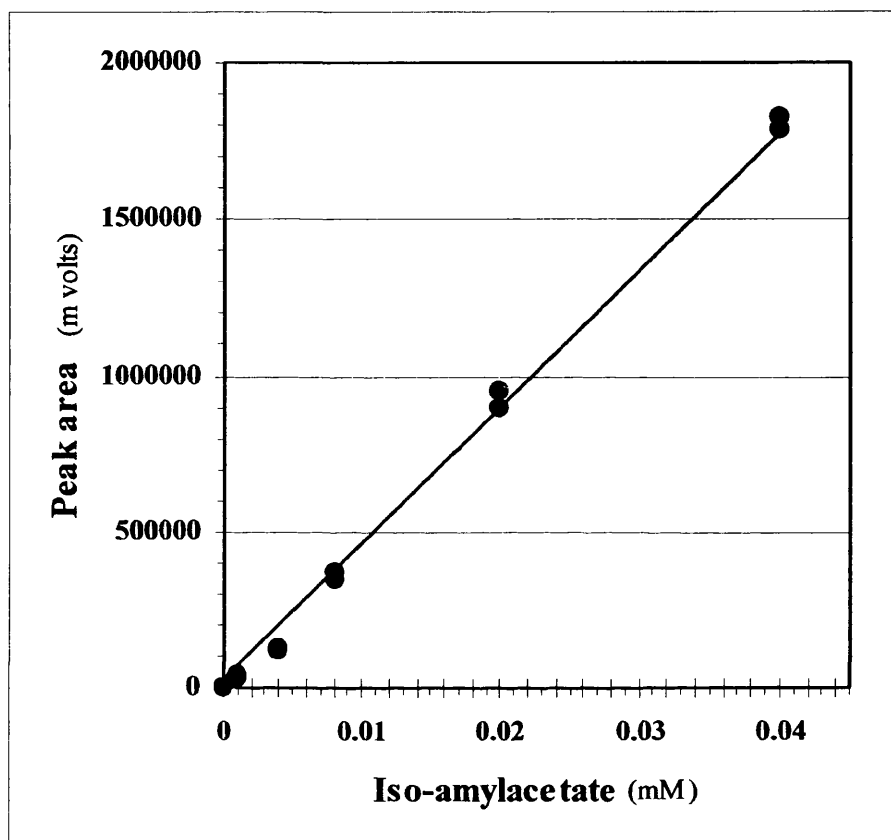
Appendix-14.

Standard curve for determination of iso-amylacetate concentration (mM).

This curve was plotted using G.C. Equation to determine iso-amylacetate concentration was defined as following (Refer to 2-7-4-6).

Y =45701189.03X – 14310.5.

An error range of equation was r = 0.999.

Y described the area of the main peak and X is for the iso-amylacetate concentration (mM) .

```
Title         : Reference - Acetaldehyde (0.1%)
Run File      : C:\Star\ilseon\GCstd\Std-Acetaldehyde(1g-10ml X 100).run
Method File   : c:\star\gc-cider.mth
Sample ID     : Manual Sample

Injection Date: 8/23/04 6:40 PM     Calculation Date: 11/17/04 1:17 PM

Operator    : bob                   Detector Type: 3800 (10 Volts)
Workstation:                        Bus Address  : 44
Instrument : 3800 gc                Sample Rate  : 10.00 Hz
Channel    : Rear = FID             Run Time     : 10.063 min

** Star Chromatography Workstation Version 5.51 ** 01086-7620-243-0714 **

Chart Speed =    5.97 cm/min   Attenuation = 9              Zero Offset = 12%
Start Time  =    0.000   min    End Time    = 3.500   min   Min / Tick  = 1.00
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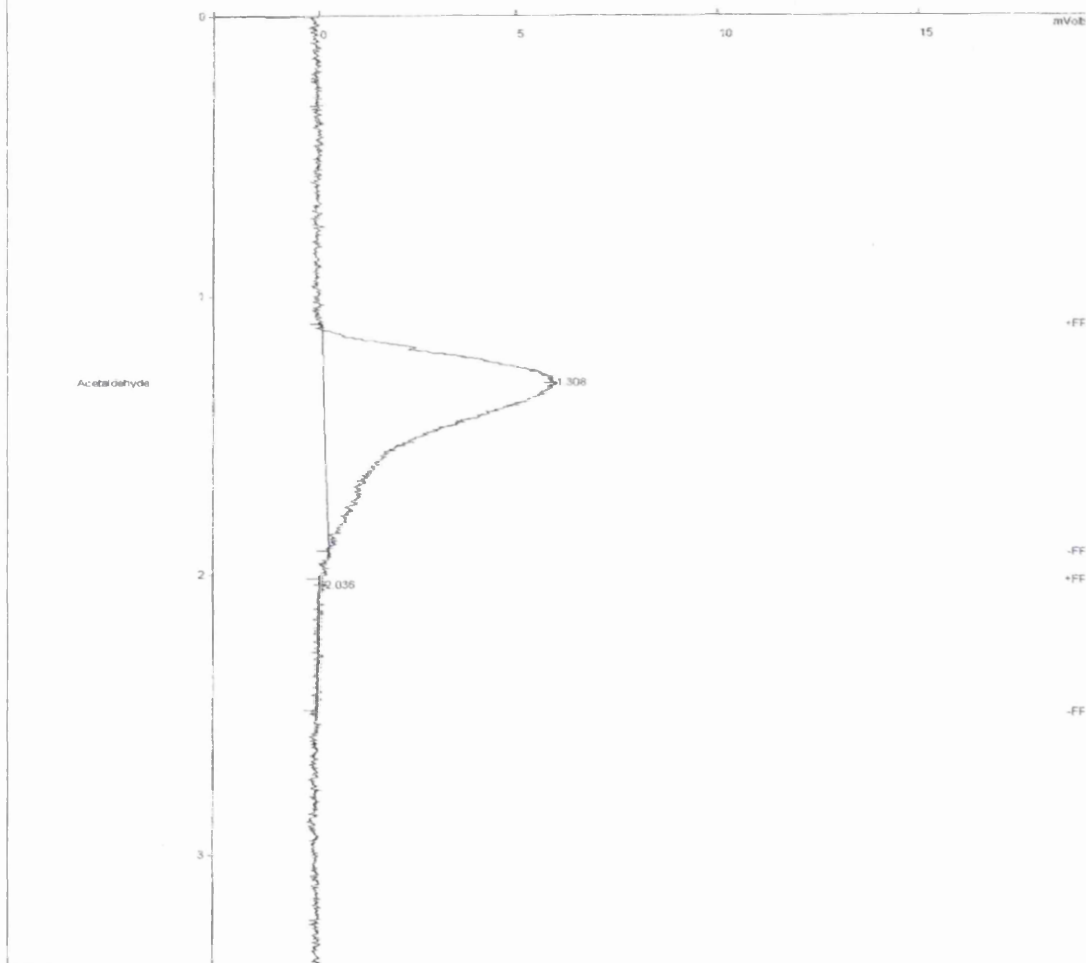

Appendix-15.
Standard GC chromatography of acetaldehyde (0.1%) *(Refer to 2-7-4-6).*
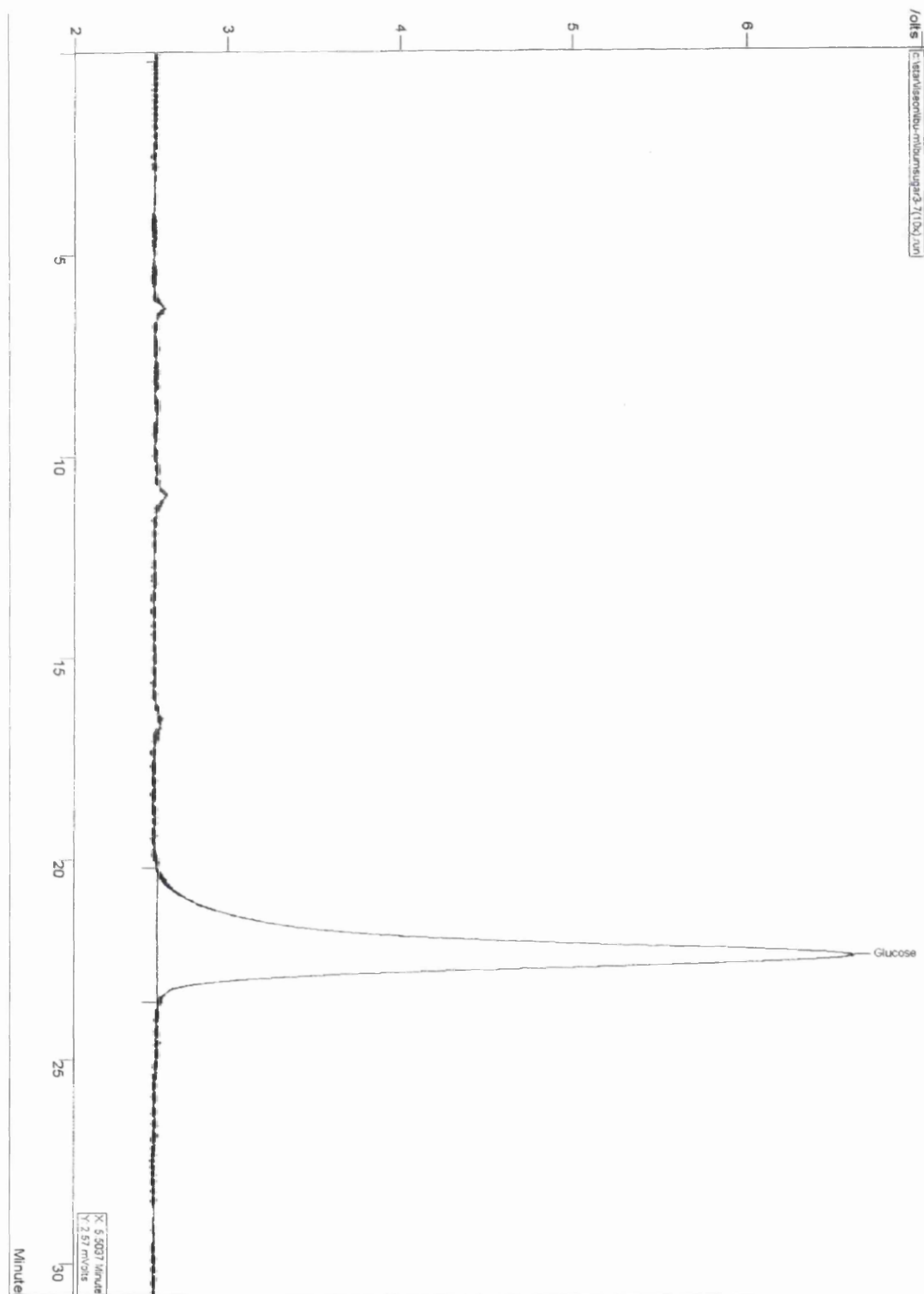
Appendix-16.

Standard GC chromatography of ethyl alcohol (0.3%) *(Refer to 2-7-4-6).*
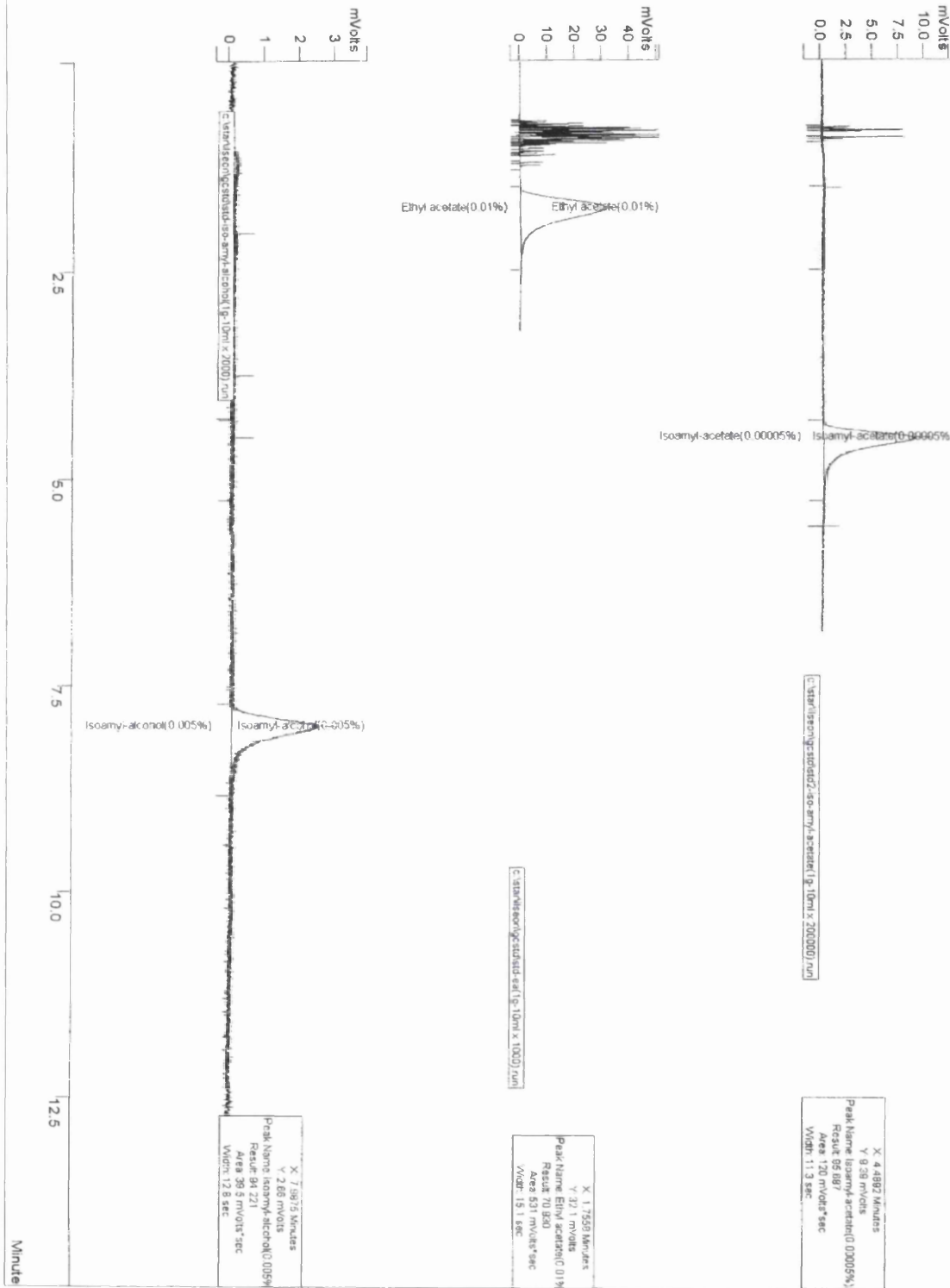
Appendix-17.

Standard GC chromatography of the volatile compounds; iso-amylacetate (0.00005%), ethyl acetate (0.01%), and iso-amylalcohol (0.005%) *(Refer to 2-7-4-6)*.

APPENDIX: Standard curves for determination of dry cell weight, organic acids and carbohydrate concentration, GC and HPLC chromatograpy of cider and nomenclature.
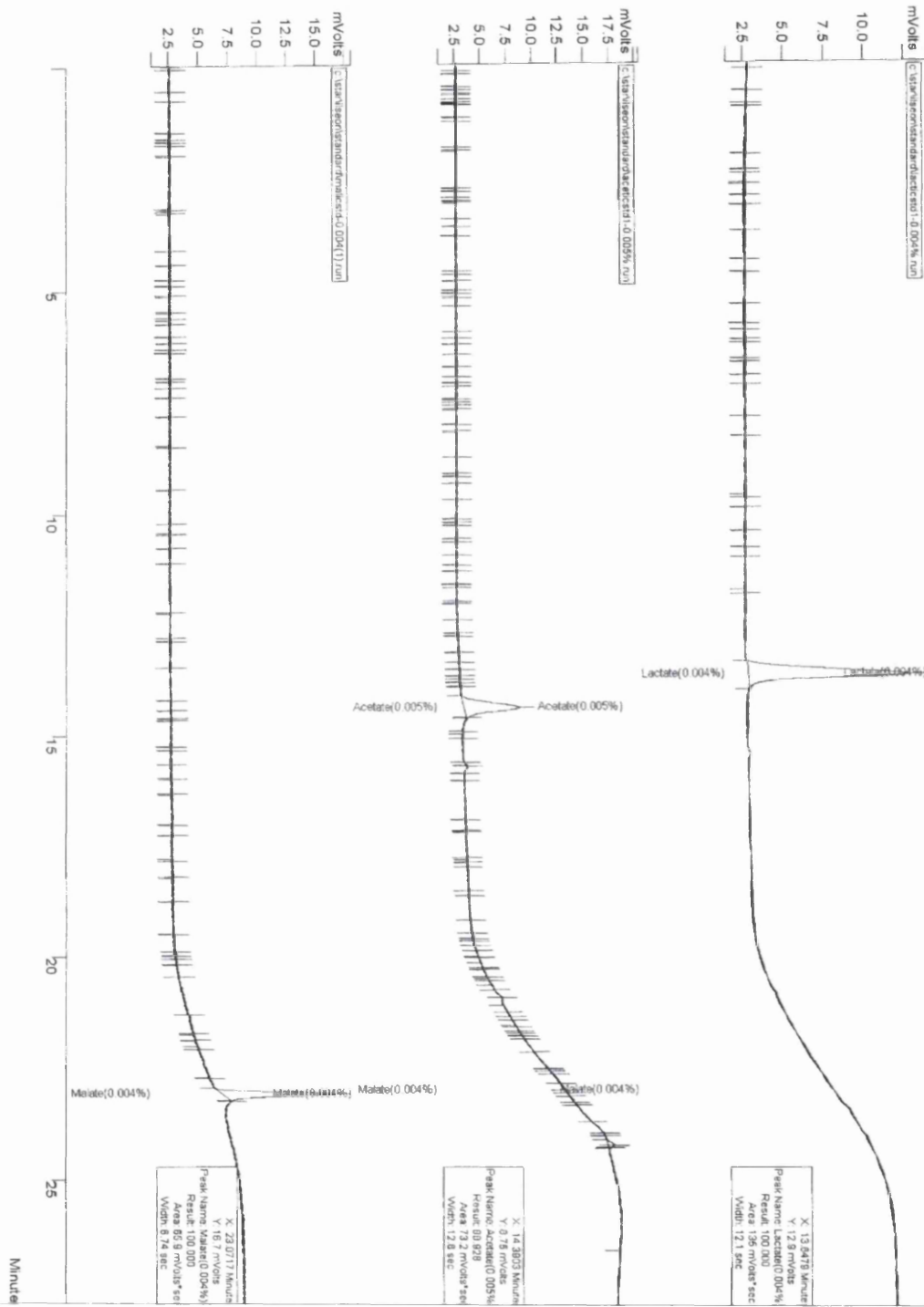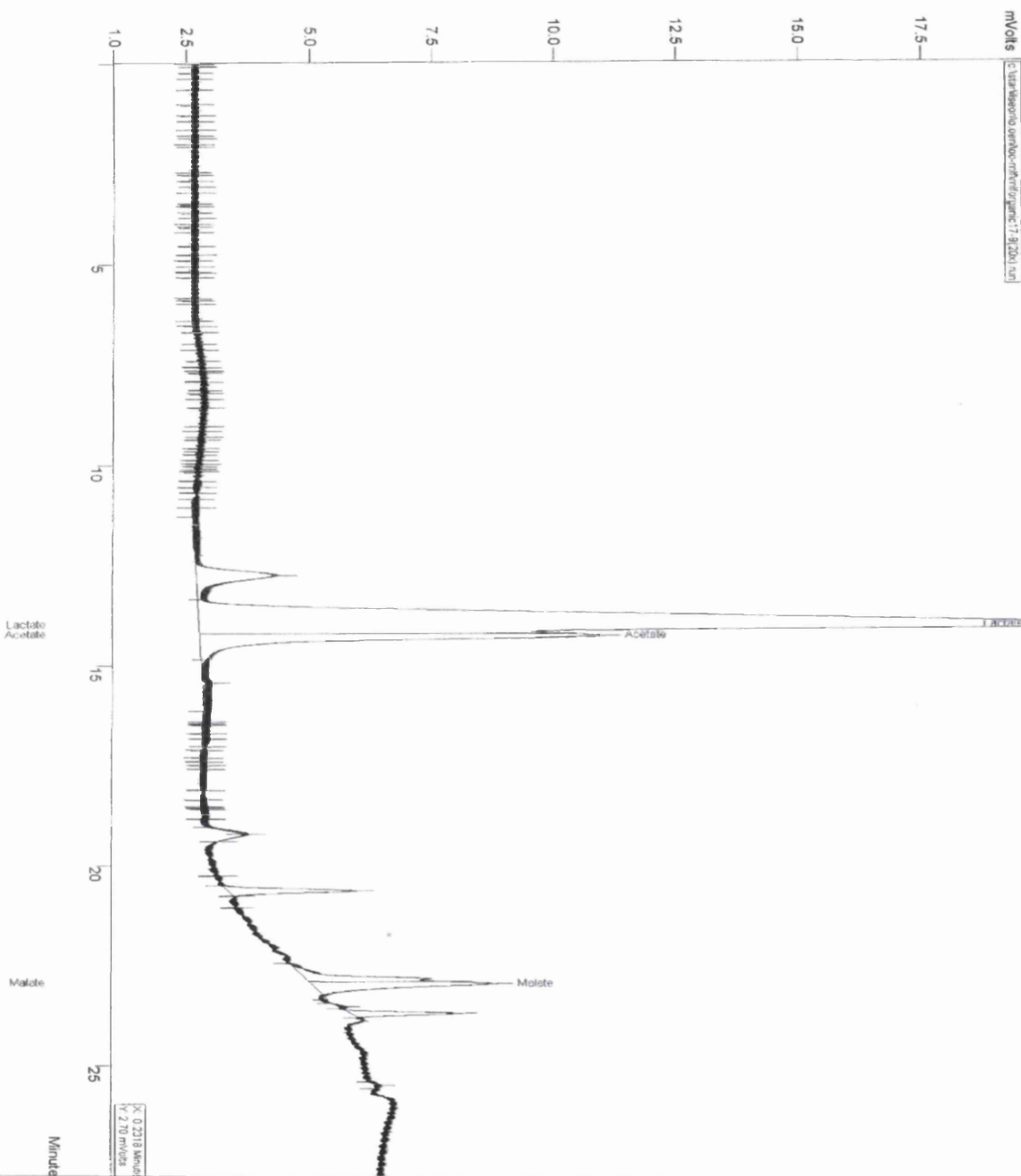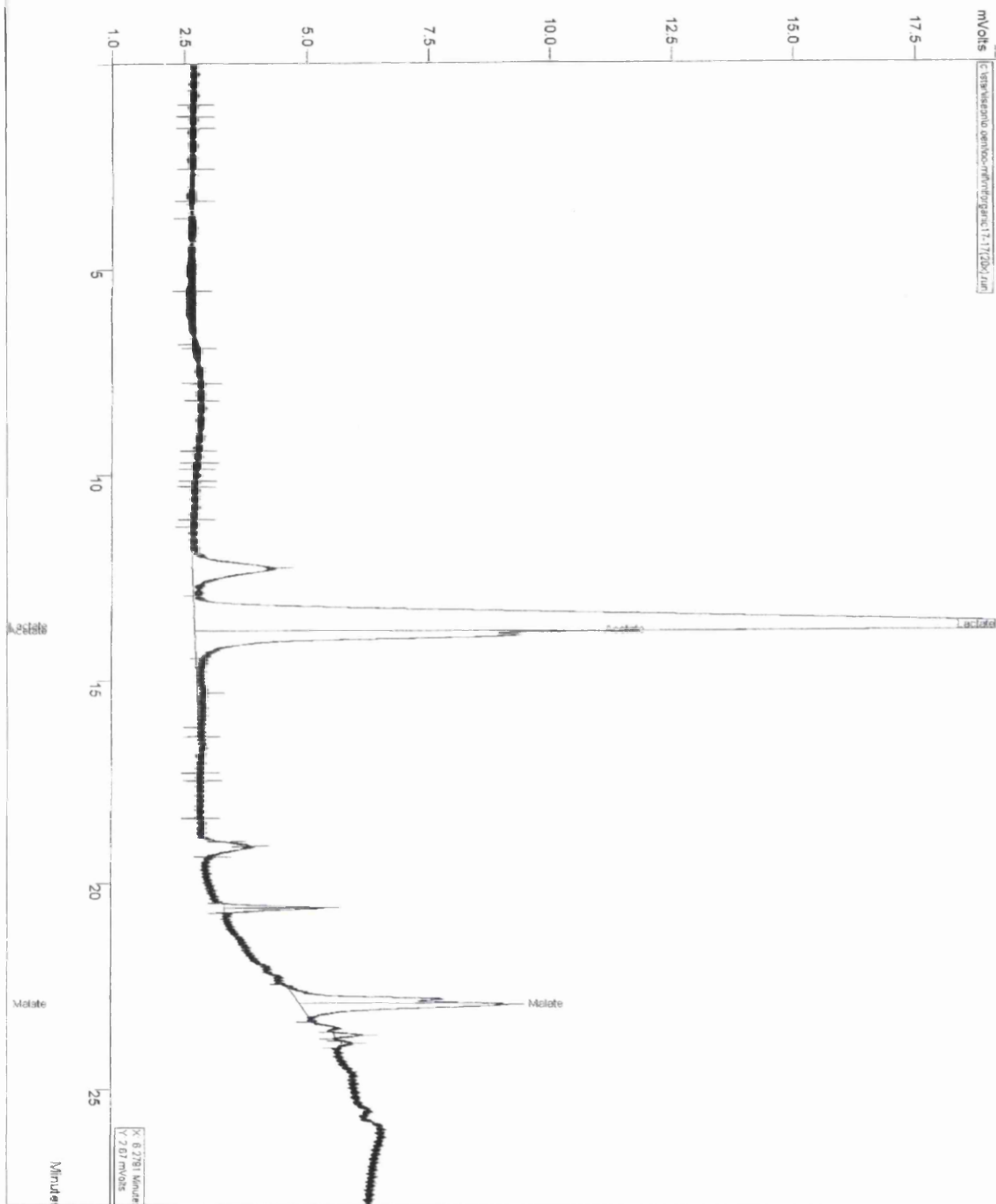

Appendix-18.

Standard HPLC chromatography of organic acids; lactate (0.004%), acetate (0.005%), and malate (0.004%) *(Refer to 2-7-4-6).*
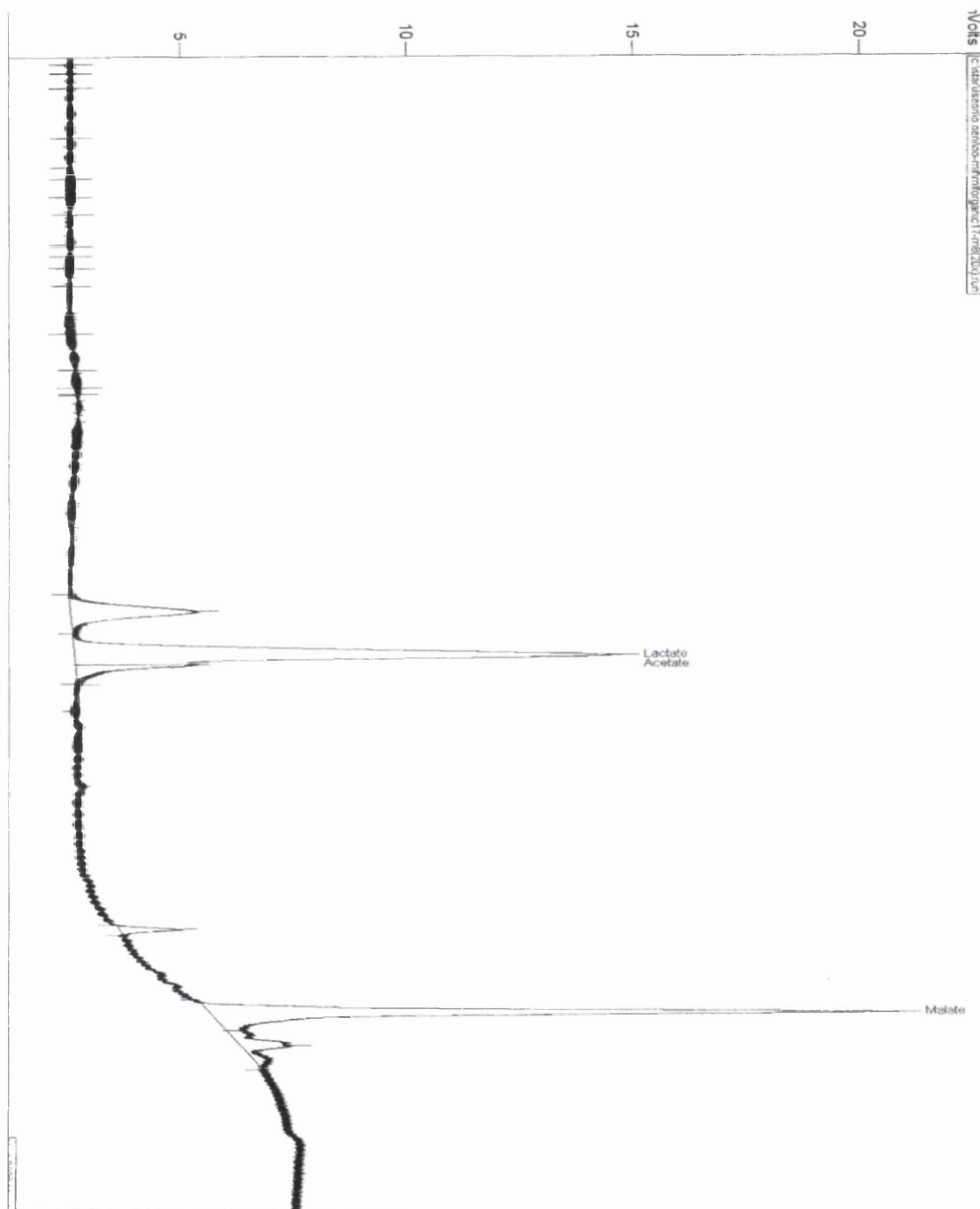
282

APPENDIX: Standard curves for determination of dry cell weight, organic acids and carbohydrate concentration, GC and HPLC chromatograpy of cider and nomenclature.
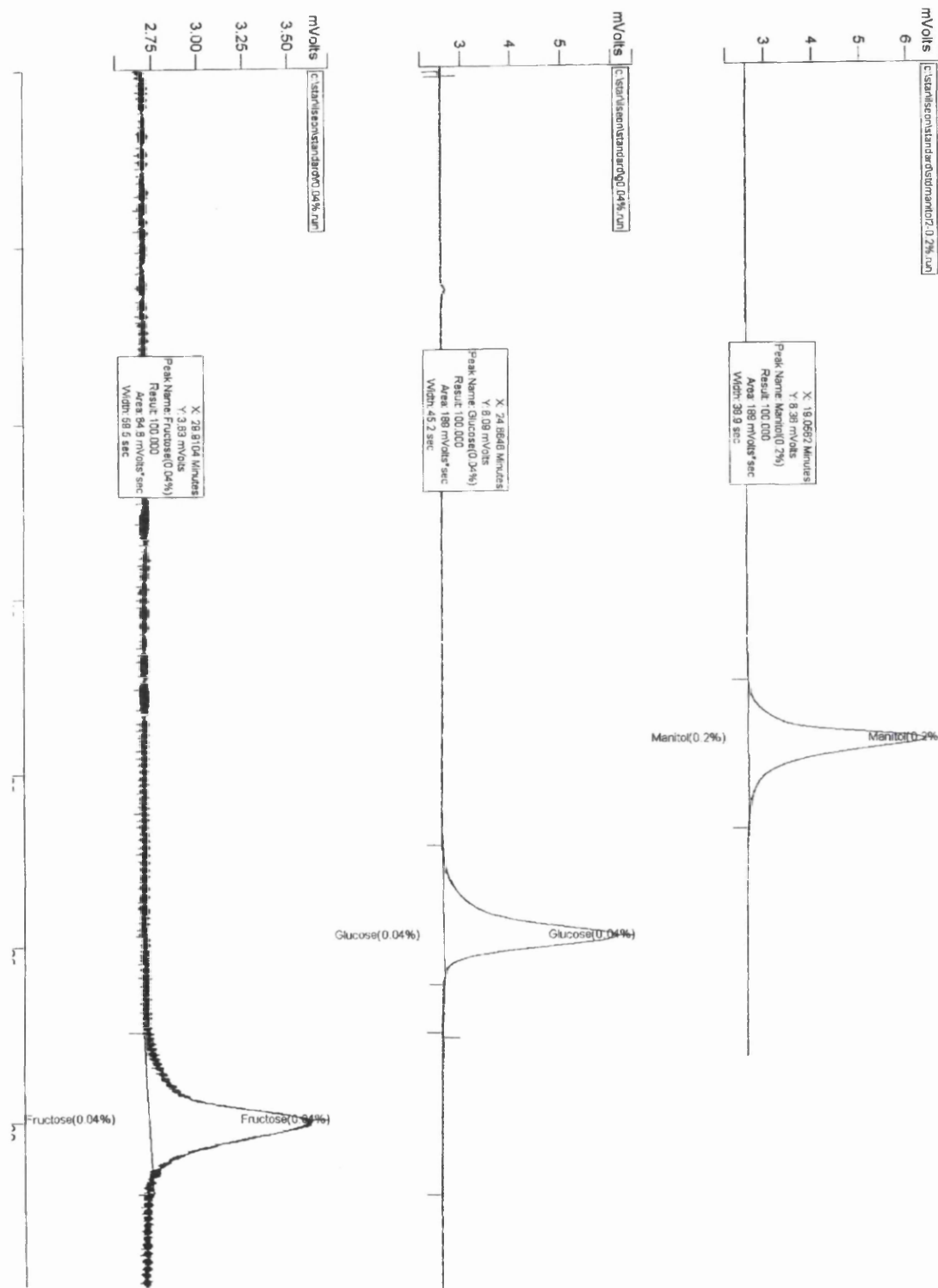
Appendix-19.

Standard HPLC chromatography of carbohydrates; mannitol (0.2%), glucose (0.04%), and fructose (0.04%) *(Refer to 2-7-3-3).*
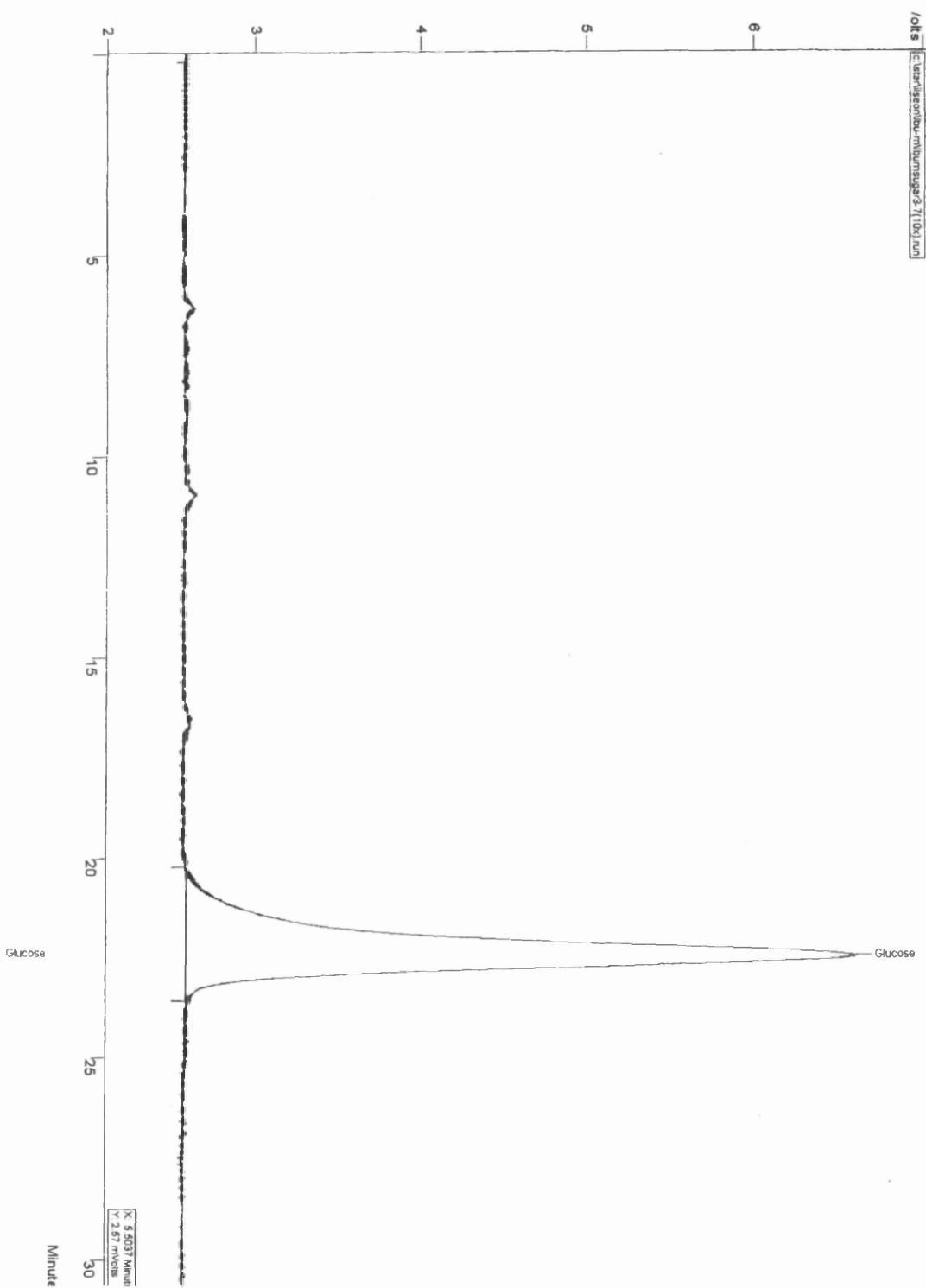
Appendix-20.

HPLC chromatography of glucose during the culture of *L. buchneri* in MCR *(Refer to 2-7-3-3).*
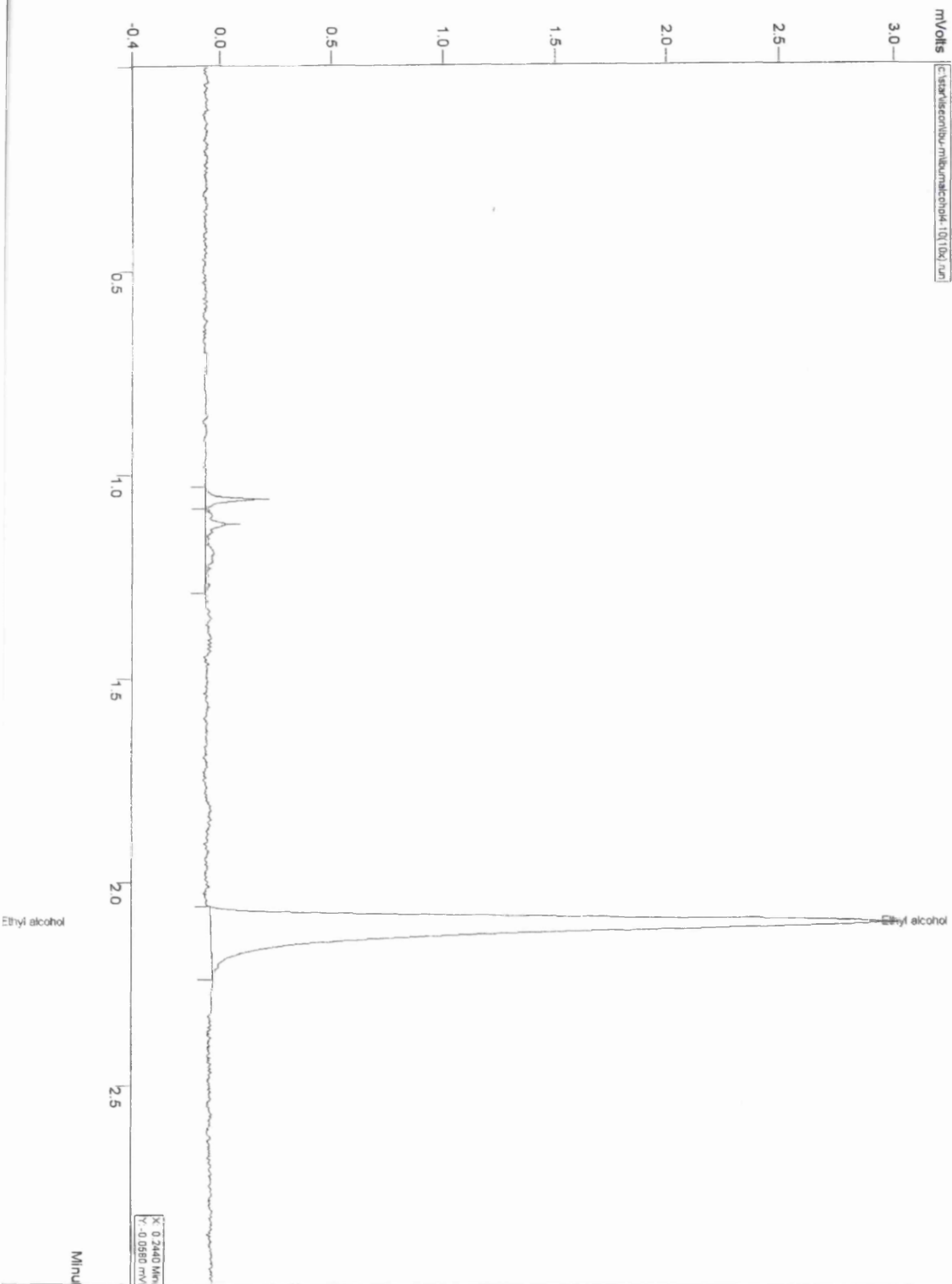
Appendix-21.

G.C chromatography of ethyl alcohol (10X) during the culture of *L. buchneri* in MCR.

(Refer to 2-7-4-6)

APPENDIX: Standard curves for determination of dry cell weight, organic acids
and carbohydrate concentration, GC and HPLC chromatograpy of cider and
nomenclature.
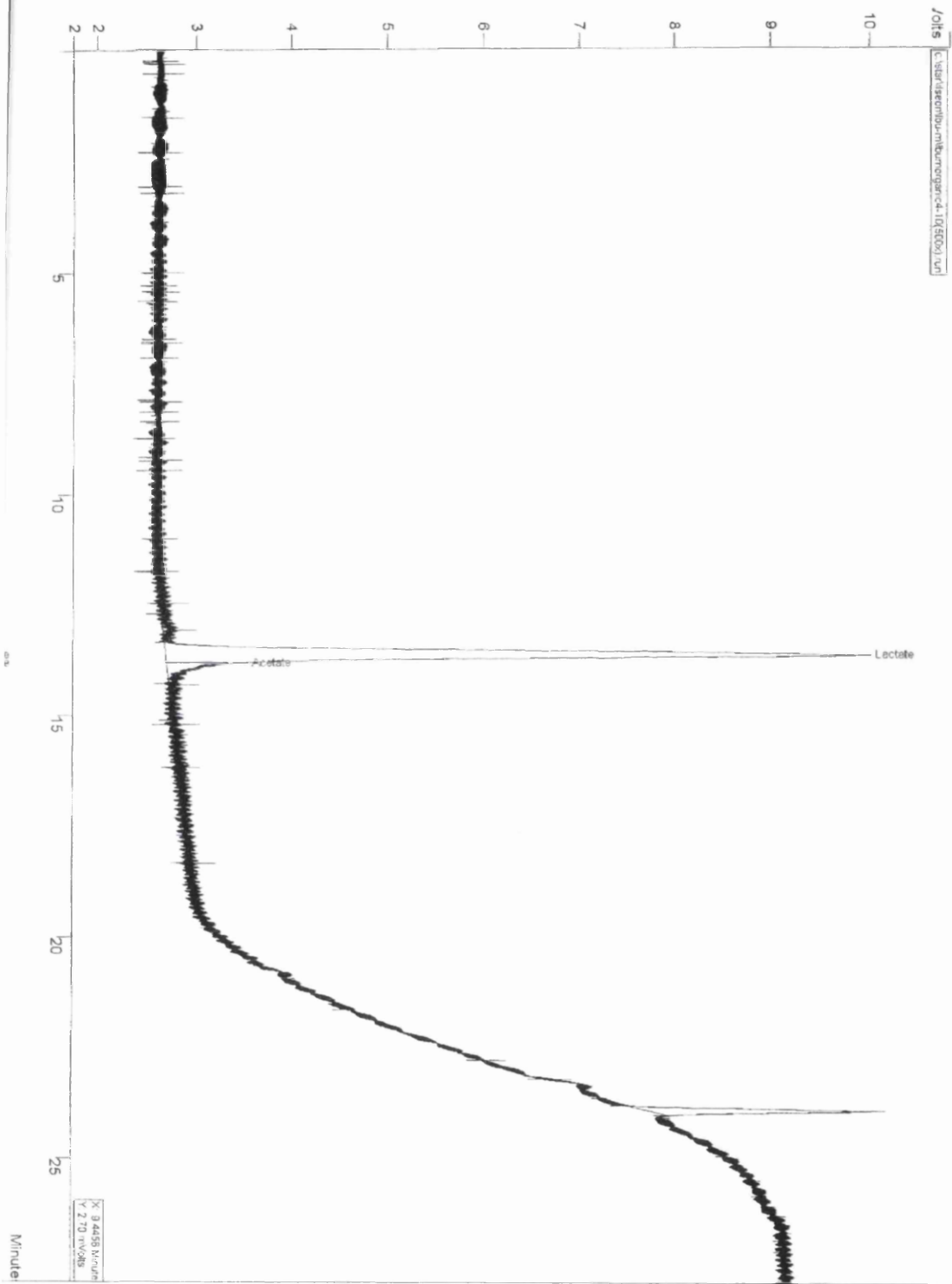
ɔendix-22.

LC chromatography of organic acids (500X) during the culture of *L. buchneri* in MCR
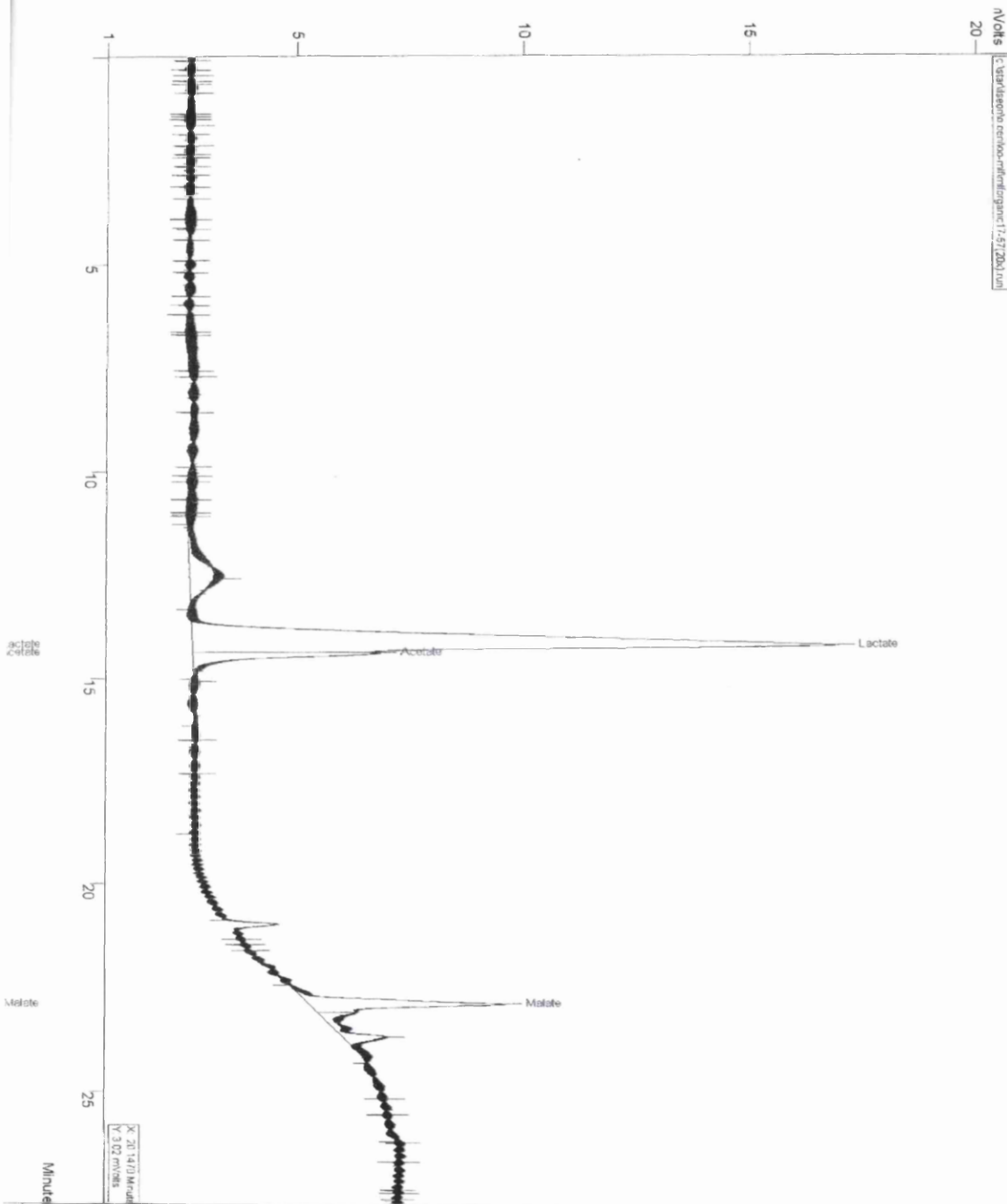
ɩer to 2-7-3-3).

APPENDIX: Standard curves for determination of dry cell weight, organic acids and carbohydrate concentration, GC and HPLC chromatograpy of cider and nomenclature.
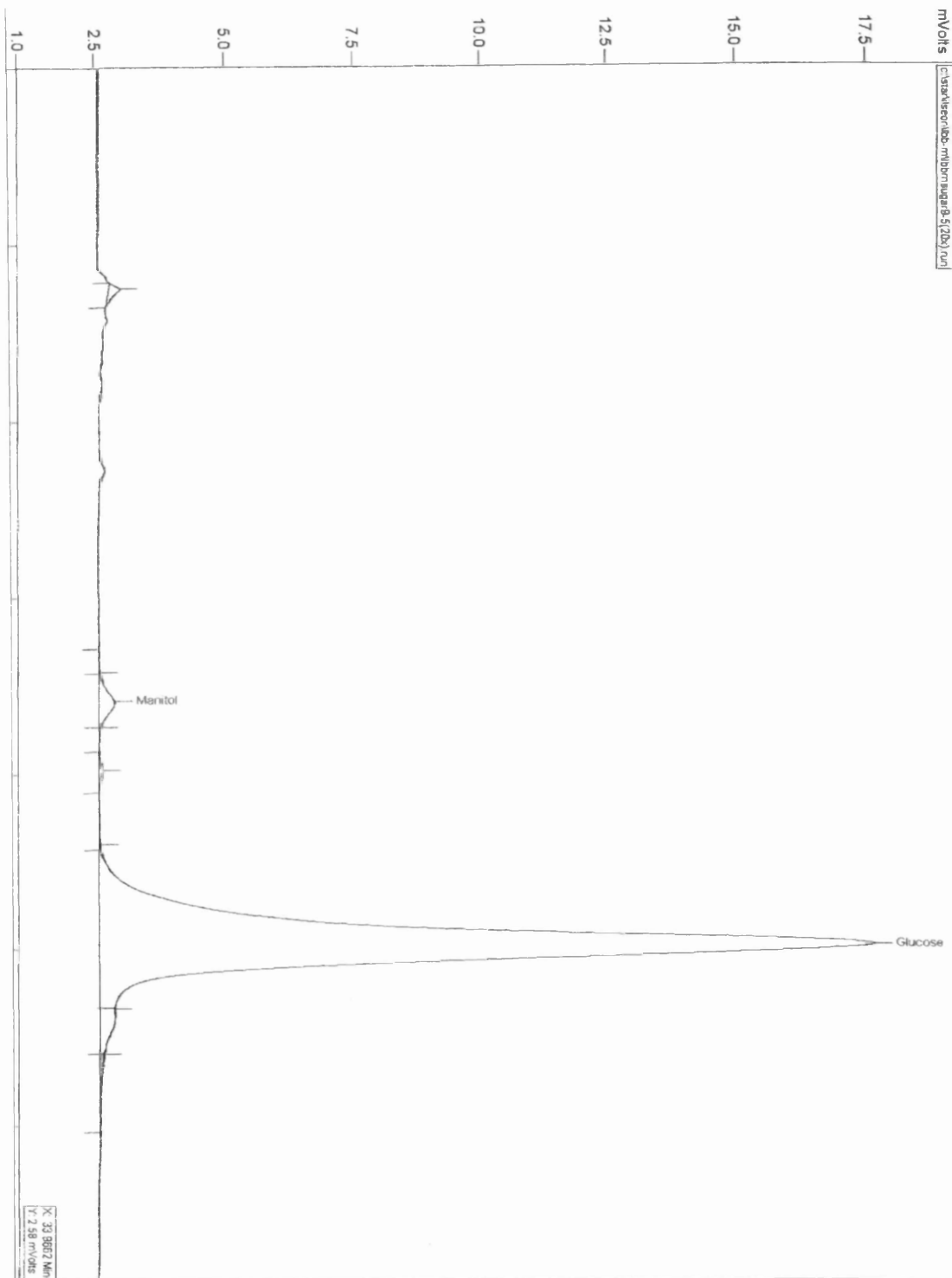
Appendix-23.

HPLC chromatography of carbohydrates (20X) during the culture of *L. brevis* in MCR *(Refer to 2-7-3-3)*.
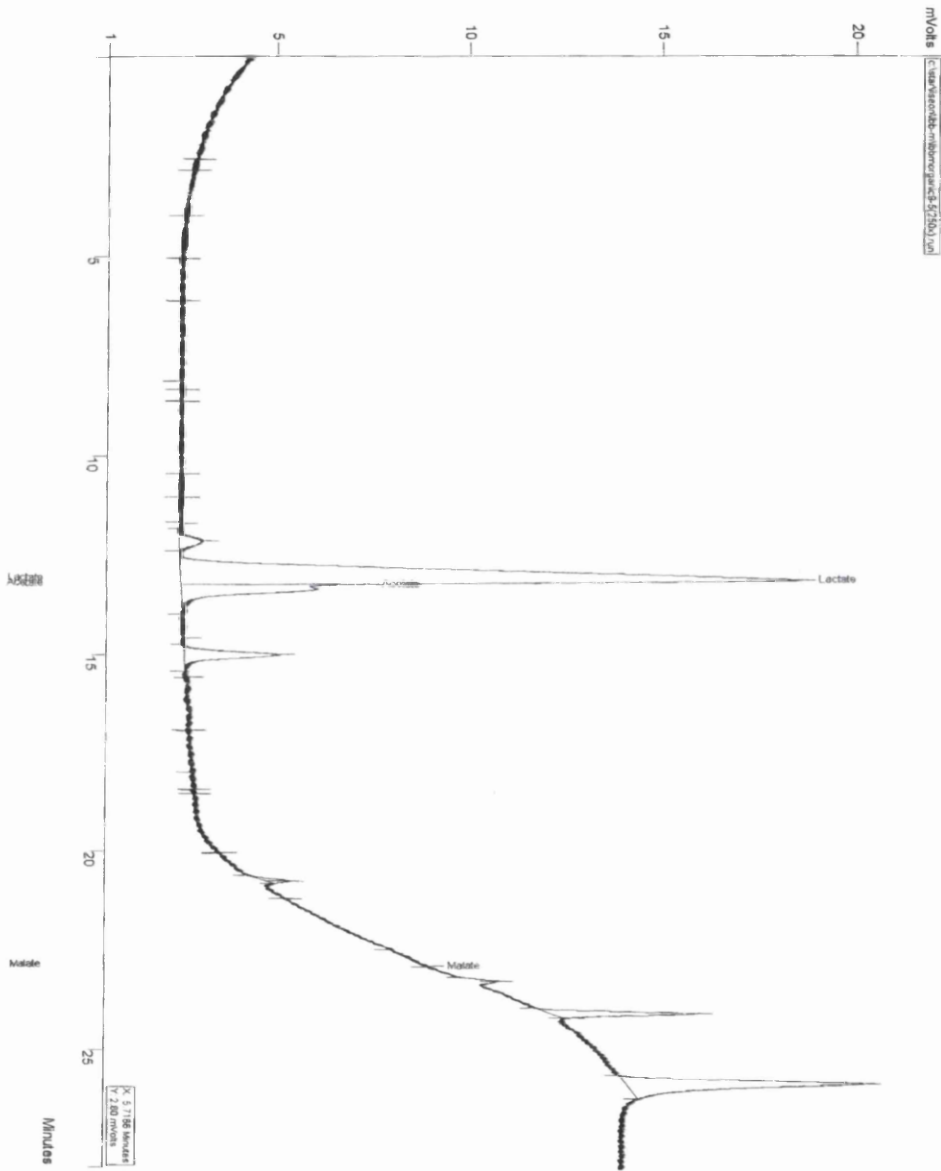
Appendix-24.

HPLC chromatography of organic acids (250X) during the culture of *L. brevis* in MCR *(Refer to 2-7-3-3).*
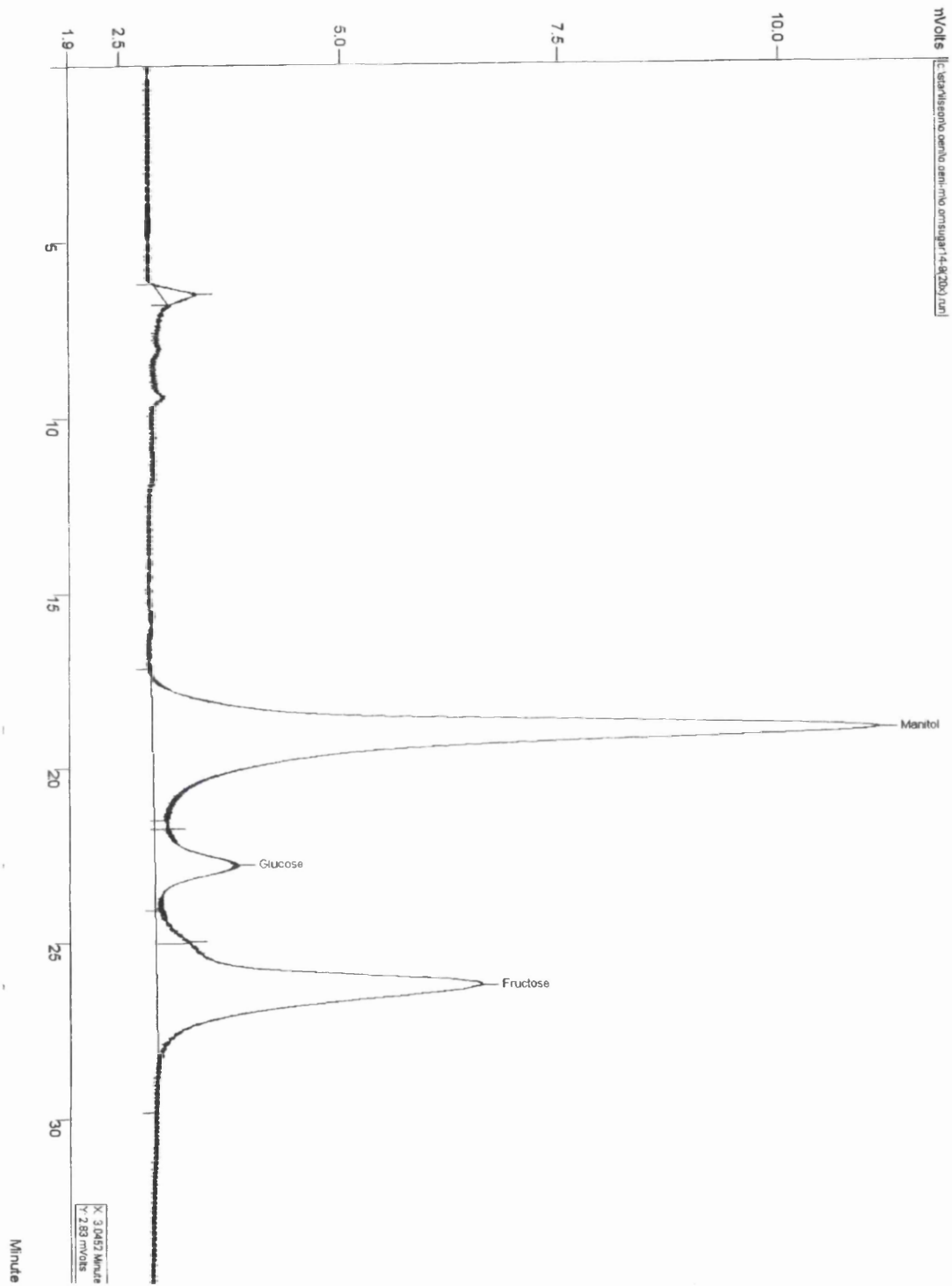
Appendix-25.

HPLC chromatography of carbohydrates (20X) during the culture of *O. oeni* in MCR.
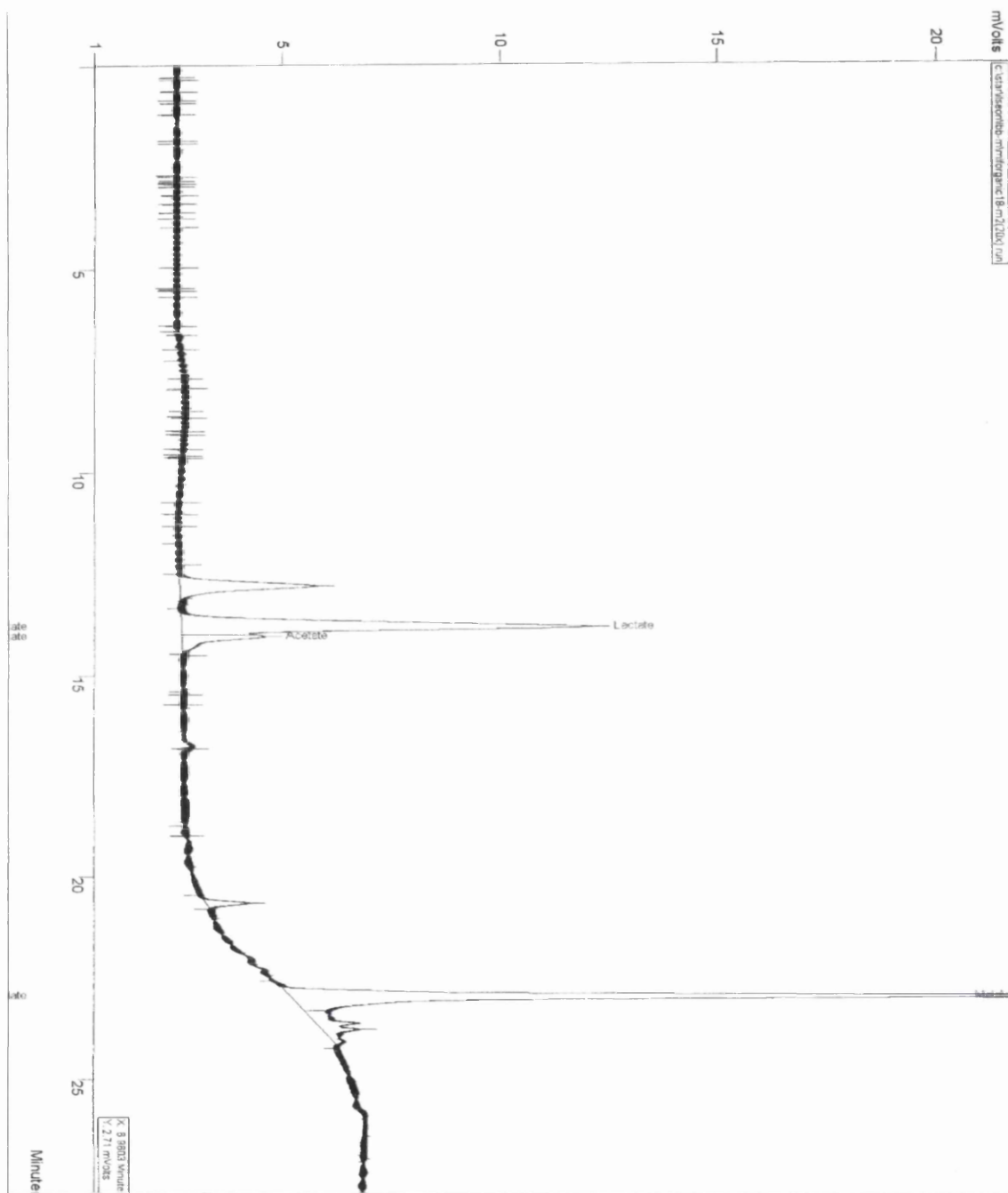*(Refer to 2-7-3-3).*

APPENDIX: Standard curves for determination of dry cell weight, organic acids and carbohydrate concentration, GC and HPLC chromatograpy of cider and nomenclature.

Appendix-26.

G.C chromatography of ethyl alcohol (10X) during the culture of *O. oeni* in MCR *(Refer to 2-7-4-6).*
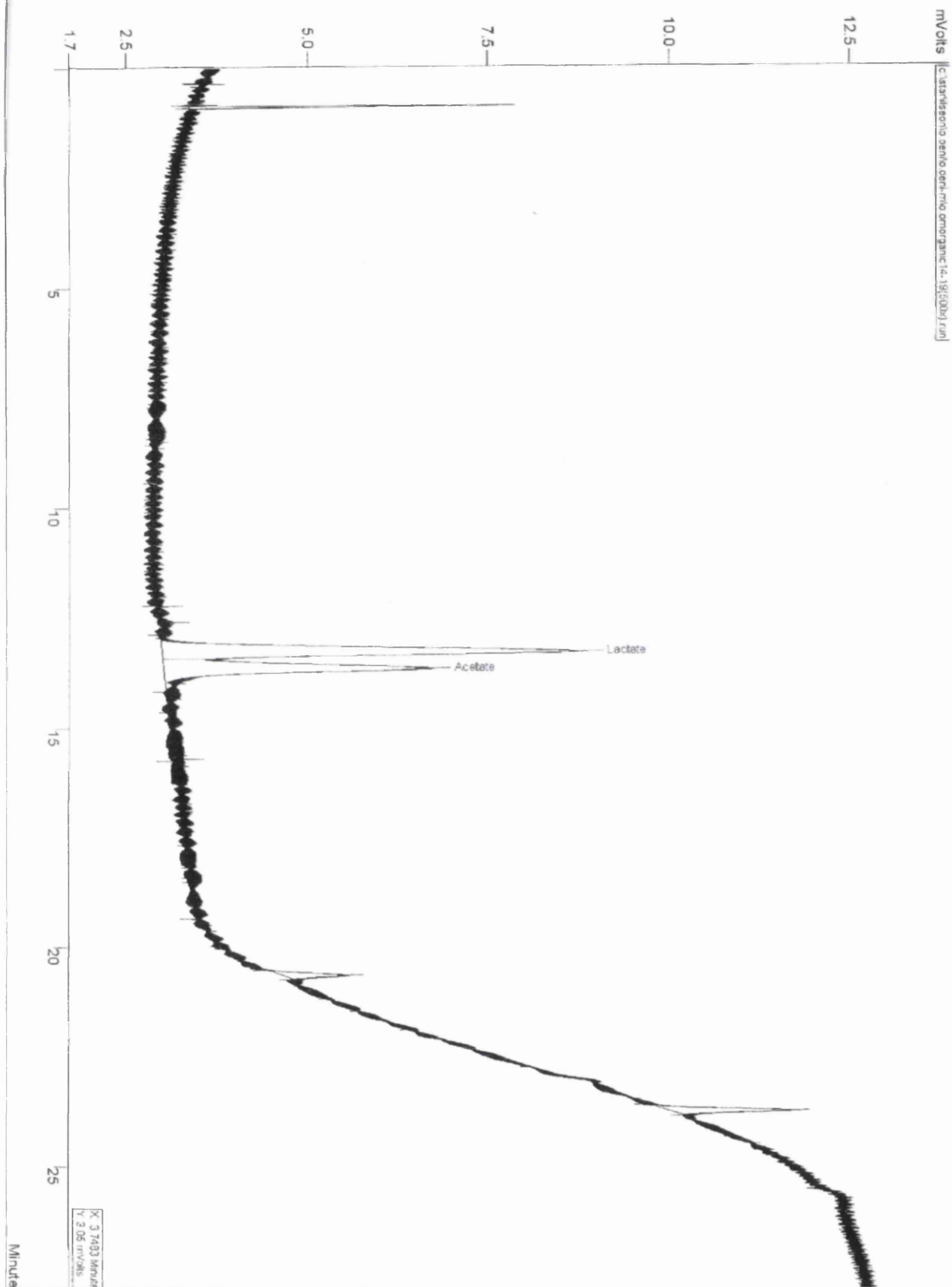
Appendix-27.

HPLC chromatography of organic acids (500X) during the culture of *O. oeni* in MCR *(Refer to 2-7-3-3).*
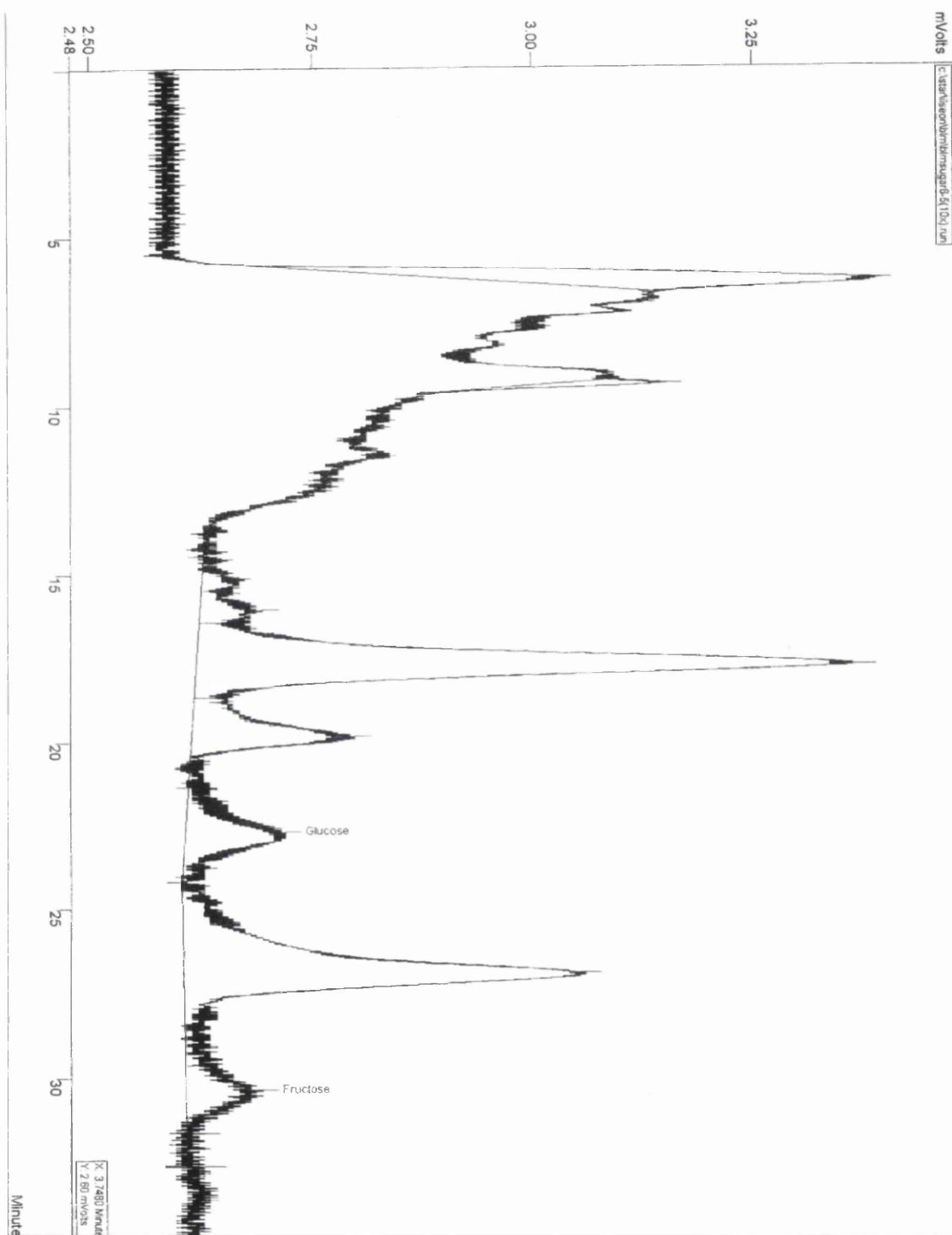
Appendix-28.

HPLC chromatography of carbohydrates (10X) during the culture of *B. longum* in MCR *(Refer to 2-7-3-3).*
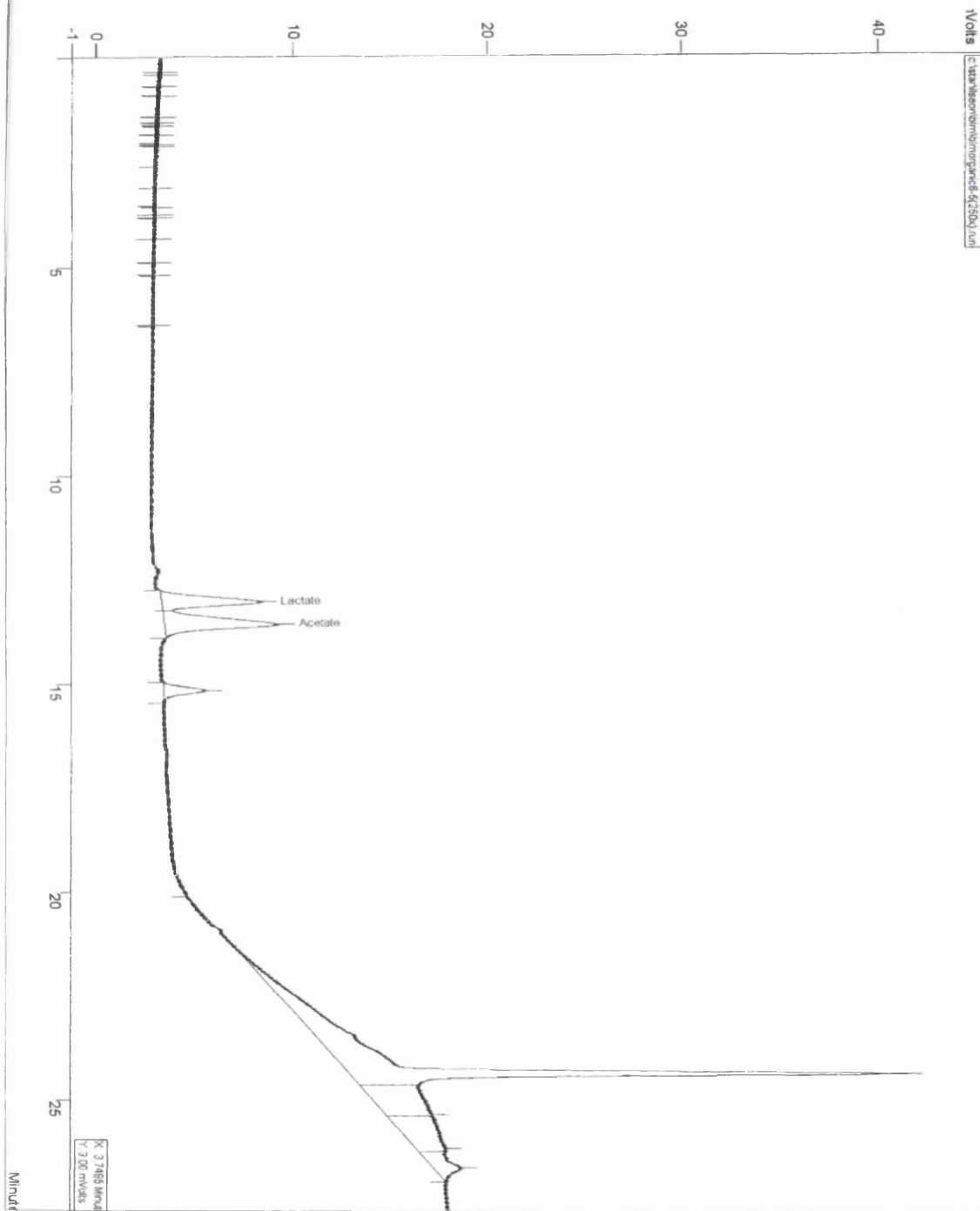
Appendix-29.

HPLC chromatography of organic acids (250X) during the culture of *B. longum* in MCR *(Refer to 2-7-3-3).*
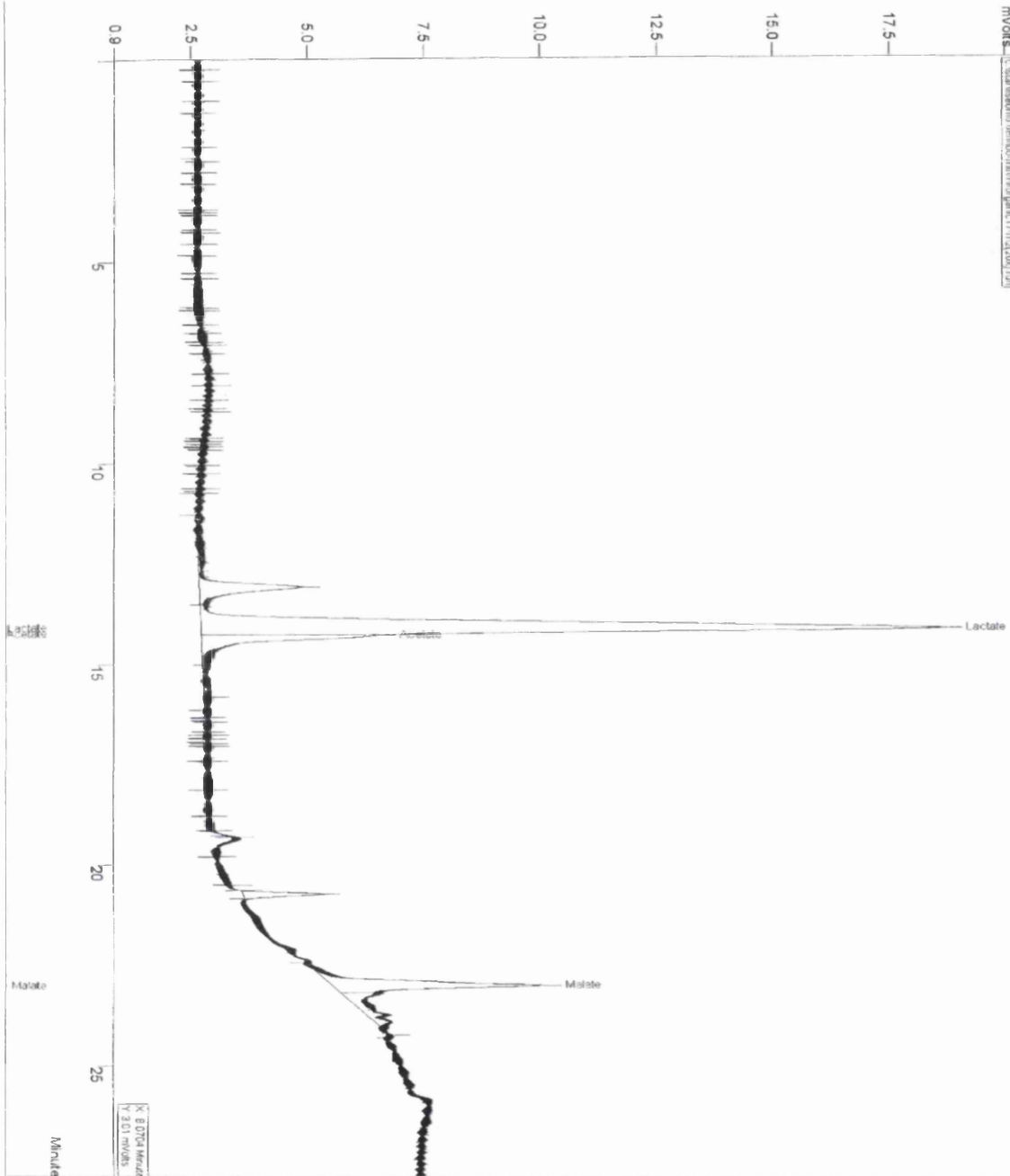
Appendix-30.

HPLC chromatography of organic acids (20X) before MLF of General cider by *O. oeni* in MCR *(Refer to 2-7-3-3)*. *This picture shows the chromatography of lactate, acetate, and malate in General cider. HPLC chromatography of organic acids after MLF of General cider by O. oeni was shown at the following Appendix-31 and Appendix-32. Difference of Appendix 31 and Appendix-32 were in sampling time*

Appendix-31.

HPLC chromatography of organic acids (20X) after MLF of General cider by *O. oeni* in MCR *(Refer to 2-7-3-3). This picture shows the chromatography of lactate, acetate, and malate in product stream after MLF of General cider. The sample was taken in 21h of MLF of General cider. The feeding rate was controlled at the retention time of 8.57h, and the alcohol concentration in feed stream was 9% (w/v). This was product when General cider of Appendix-30 was used as feed.*
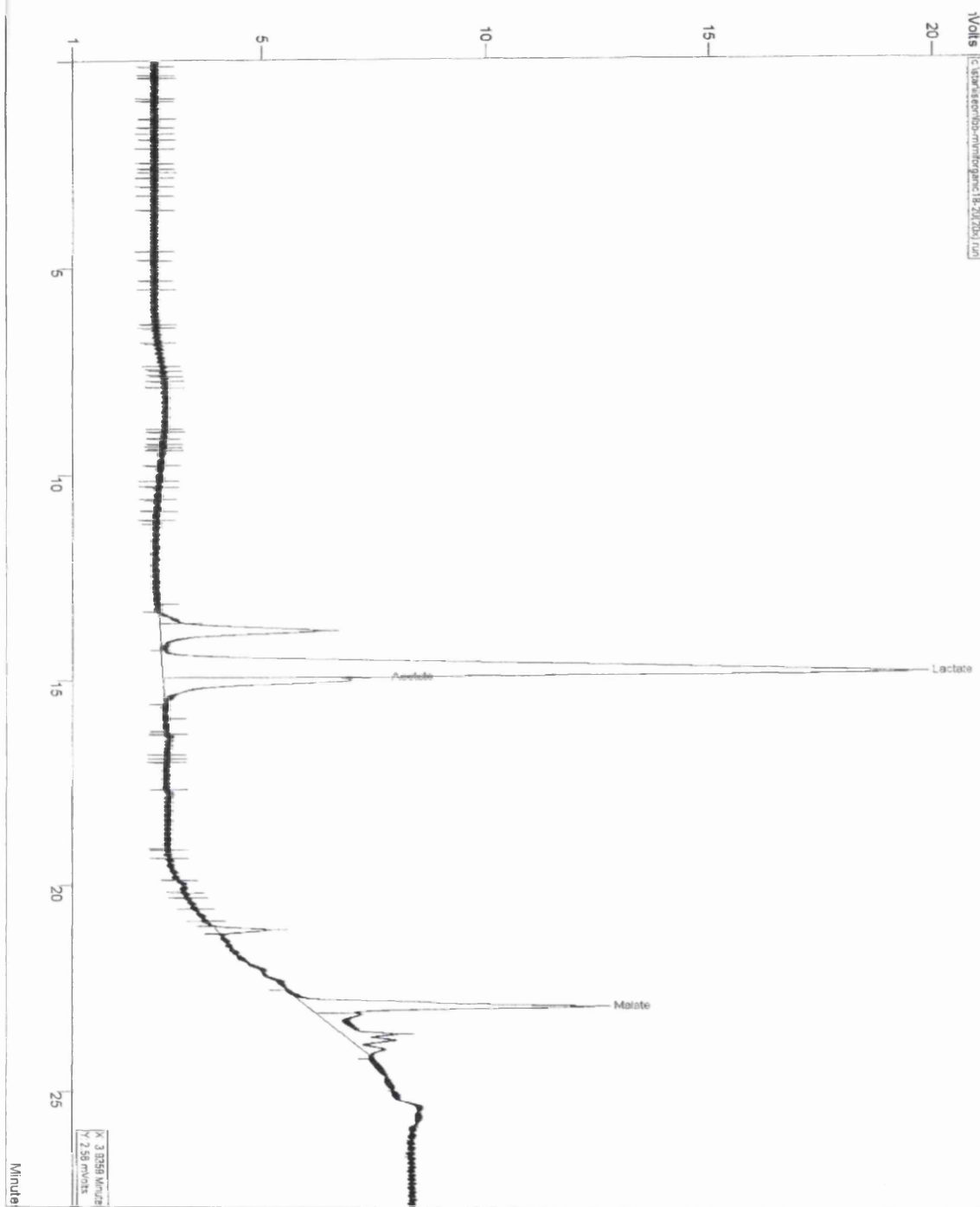
Appendix-32.

HPLC chromatography of organic acids (20X) after MLF of General cider by *O. oeni* in MCR *(Refer to 2-7-3-3)*. *This picture showed the chromatography of lactate, acetate, and malate in product stream after MLF of General cider. The sample was taken in 123h of MLF of General cider. The feeding rate was controlled at the retention time of 8.57h, and the alcohol concentration in the feed stream was 9% (w/v) This was product when General cider of Appendix-30 was used as feed.*

Appendix-33.

HPLC chromatography of organic acids (20X) before MLF of Scrumpy Jack by *O. oeni* in MCR *(Refer to 2-7-3-3). This picture showed the chromatography of lactate, acetate, and malate in feed stream of Scrumpy Jack. The alcohol concentration was 9% (w/v). HPLC chromatography of organic acids after MLF of Scrumpy Jack by O. oeni was shown at Appendix-34.*

Appendix-34.

HPLC chromatography of organic acids (20X) after MLF of Scrumpy Jack by *O. oeni* in MCR *(Refer to 2-7-3-3)*. *This picture shows the chromatography of lactate, acetate, and malate in product stream after MLF of Scrumpy Jack. The sample was taken in 294 h of MLF. The feeding rate was controlled at the retention time of 6 h, and the alcohol concentration in feed stream was 9% (w/v). This was product when Scrumpy Jack of Appendix-33 was used as feed.*
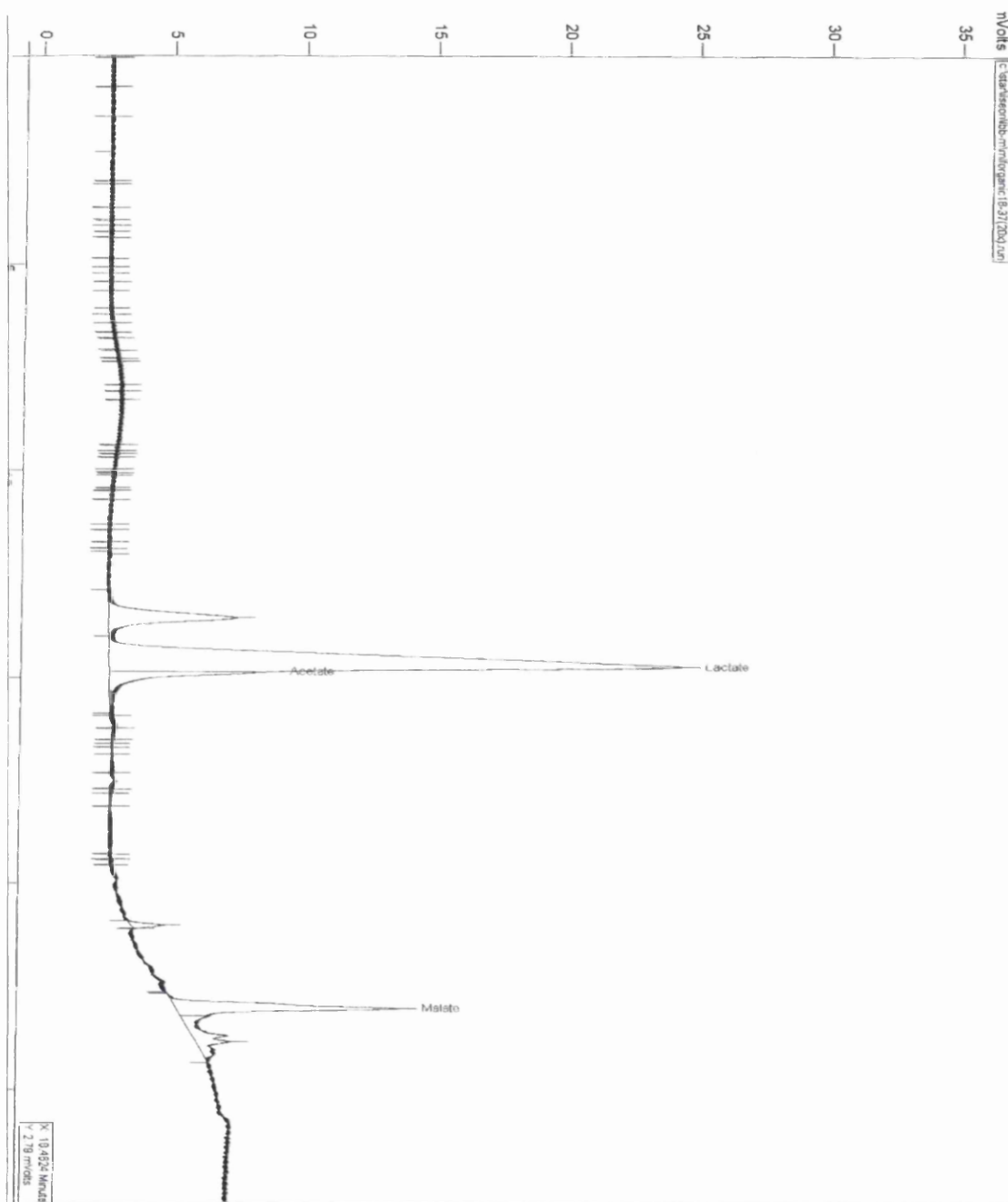
APPENDIX: Standard curves for determination of dry cell weight, organic acids and carbohydrate concentration, GC and HPLC chromatograpy of cider and nomenclature.
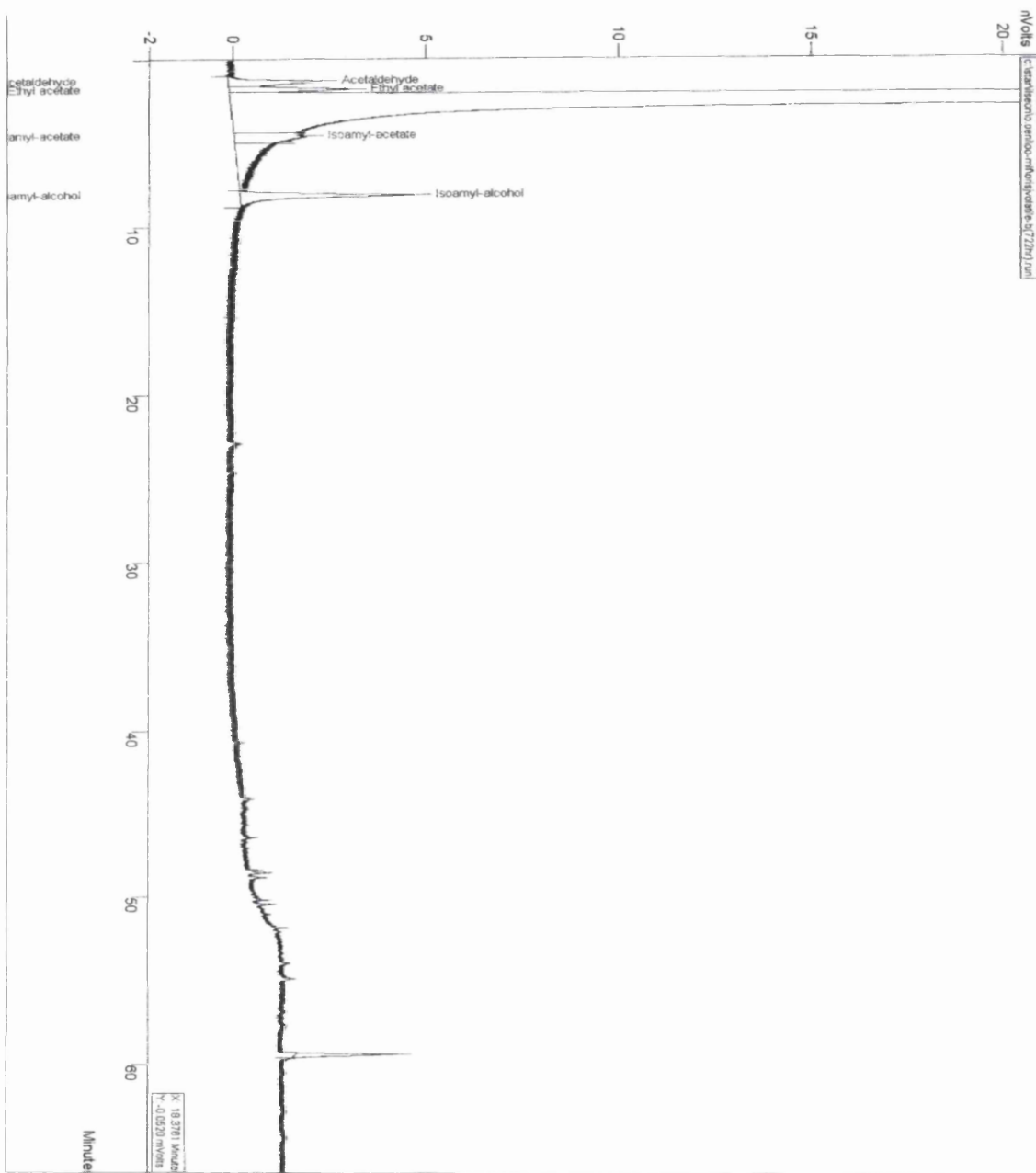

Appendix-35.

HPLC chromatography of organic acids (20X) before MLF of Scrumpy Jack by *L. brevis* in MCR *(Refer to 2-7-3-3). This picture shows the chromatography of lactate, acetate, and malate in feed stream of Scrumpy Jack. The alcohol concentration was 9% (w/v). HPLC chromatography of organic acids after MLF of Scrumpy Jack by L. brevis was shown at Appendix-36.*

Appendix-36.

HPLC chromatography of organic acids (20X) after MLF of Scrumpy Jack by *L.brevis* in MCR *(Refer to 2-7-3-3)*. *This picture shows the chromatography of lactate, acetate, and malate in product stream after MLF of Scrumpy Jack. The sample was taken in 339 h of MLF. The feeding rate was controlled at the retention time of 6 h, and the alcohol concentration in feed stream was 9% (w/v). This is product when Scrumpy Jack of Appendix-35 was used as feed.*
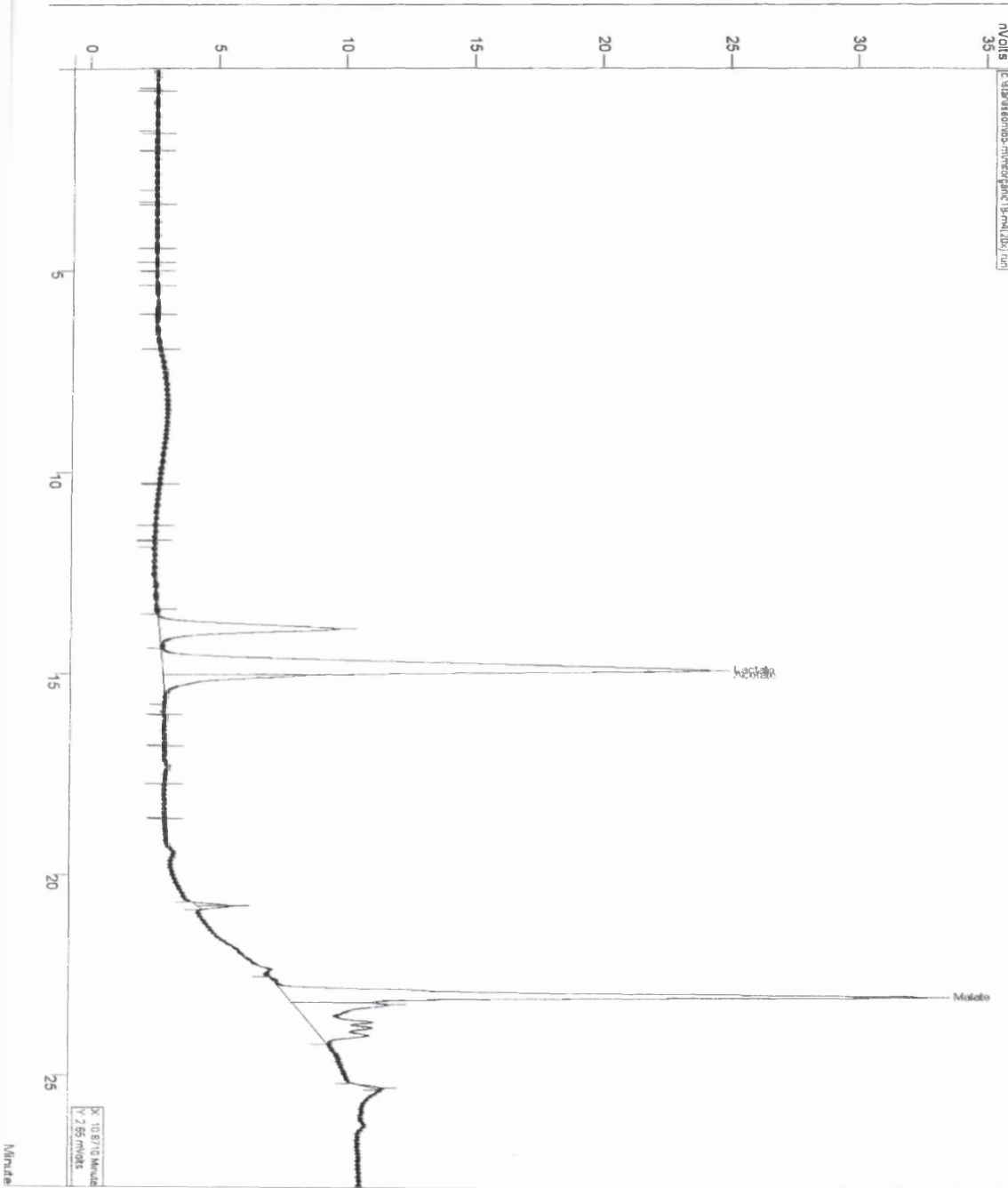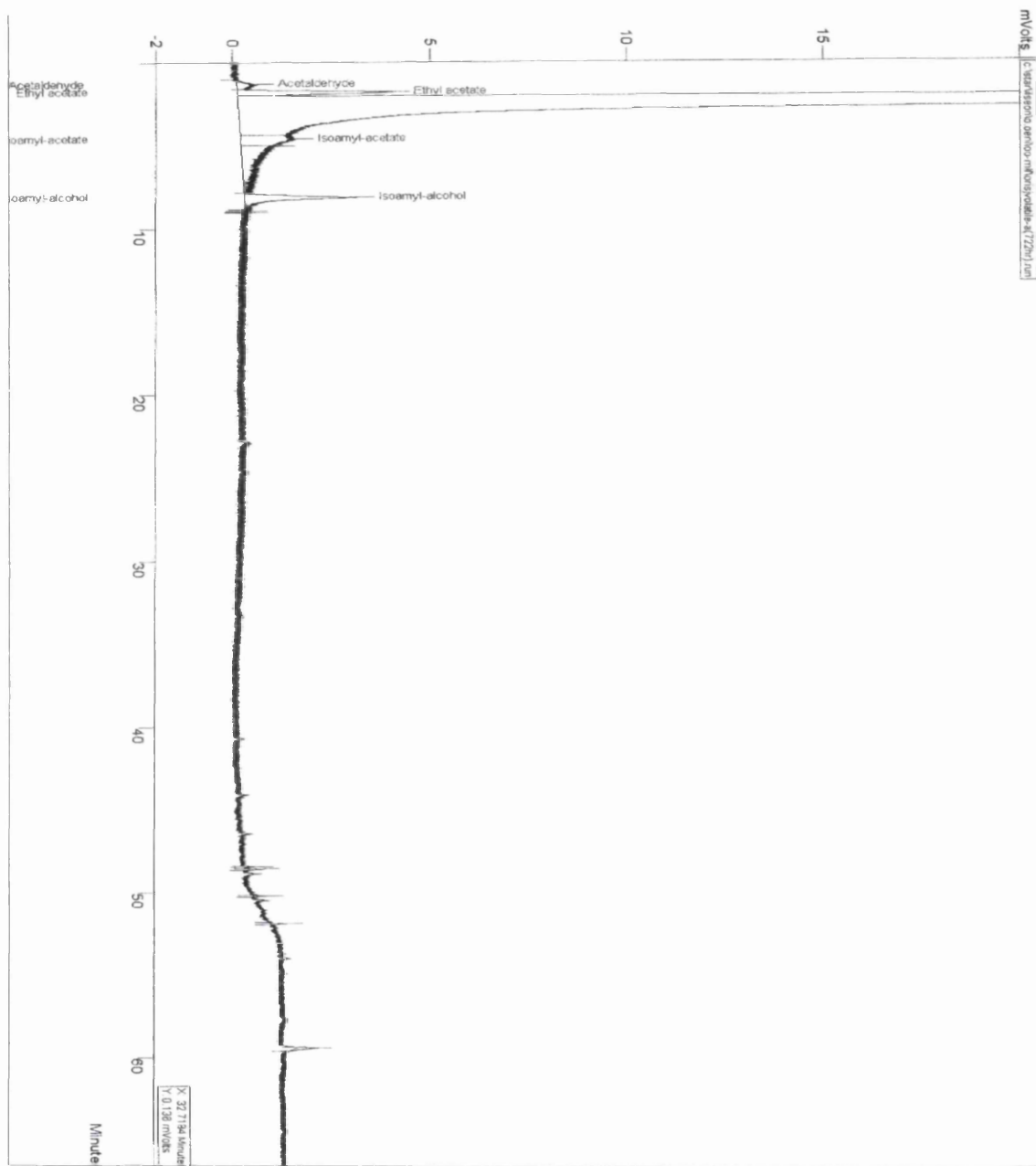
Appendix-37.

HPLC chromatography of organic acids (20X) before MLF of Scrumpy Jack by *L. brevis* in MCR *(Refer to 2-7-3-3). This picture shows the chromatography of lactate, acetate, and malate in feed stream of Scrumpy Jack. The alcohol concentration was 9% (w/v). HPLC chromatography of organic acids after MLF of Scrumpy Jack by L. brevis is shown at Appendix-38.*

Appendix-38.

HPLC chromatography of organic acids (20X) after MLF of Scrumpy Jack by *L. brevis* in MCR *(Refer to 2-7-3-3). This picture shows the chromatography of lactate, acetate, and malate in product stream after MLF of Scrumpy Jack. The sample was taken in 339 h of MLF. The feeding rate was controlled at the retention time of 6 h, and the alcohol concentration in feed stream was 9% (w/v). This is product when Scrumpy Jack of Appendix-37 was used as feed.*

Appendix-39.

G.C chromatography of the volatile compounds (1X) before MLF of Scrumpy Jack by *O. oeni* in MCR *(Refer to 2-7-4-6). This picture showed the chromatography of acetaldehyde, ethyl-acetate, iso-amy-alcohol, and iso-amylacetate in product stream before MLF of Scrumpy Jack. The alcohol concentration was 11% (w/v). GC chromatography of the volatile compounds after MLF of this Scrumpy Jack by O. oeni is shown at Appendix-40.*

Appendix-40.

G.C chromatography of the volatile compounds (1X) after MLF of Scrumpy Jack by *O.oeni* in MCR. *(Refer to 2-7-4-6). This picture shows the chromatography of acetaldehyde, ethyl-acetate, iso-amylalcohol, and iso-amylacetate in product stream after MLF of Scrumpy Jack. When the sample was taken in 722 h of MLF, the alcohol concentration in feed stream was 11% (w/v), and the feeding rate was controlled at the retention time of 6 h. This is product when Scrumpy Jack of Appendix-39 was used as feed.*
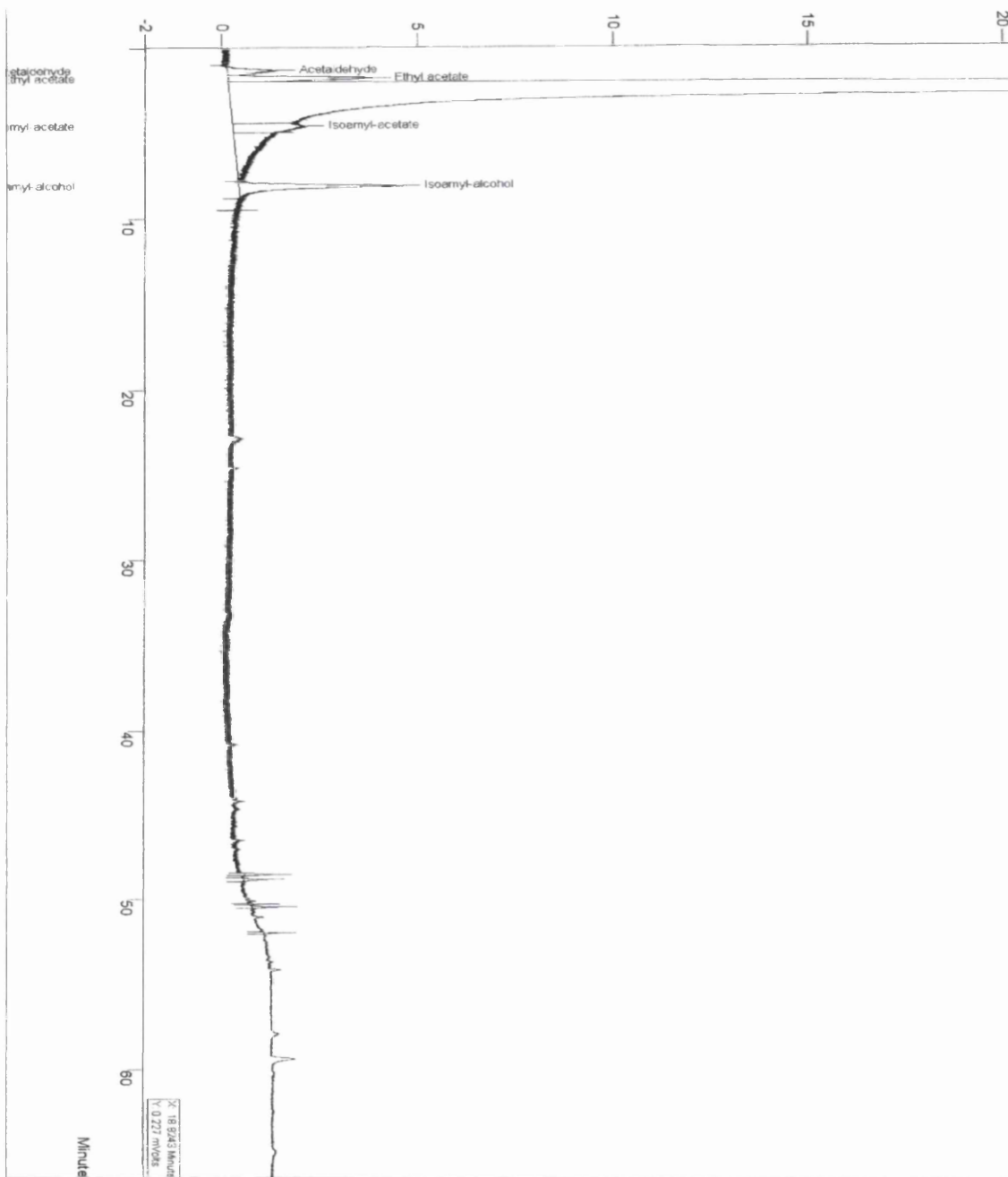
Appendix-41.

G.C chromatography of the volatile compounds (1X) before MLF of Scrumpy Jack by *O. oeni* in MCR. *(Refer to 2-7-4-6). This picture shows the chromatography of acetaldehyde, ethyl-acetate, iso-amylalcohol, and iso-amylacetate in product stream before MLF of Scrumpy Jack. The alcohol concentration was 12% (w/v). GC chromatography of the volatile compounds after MLF of this Scrumpy Jack by O. oeni is shown at Appendix-42.*
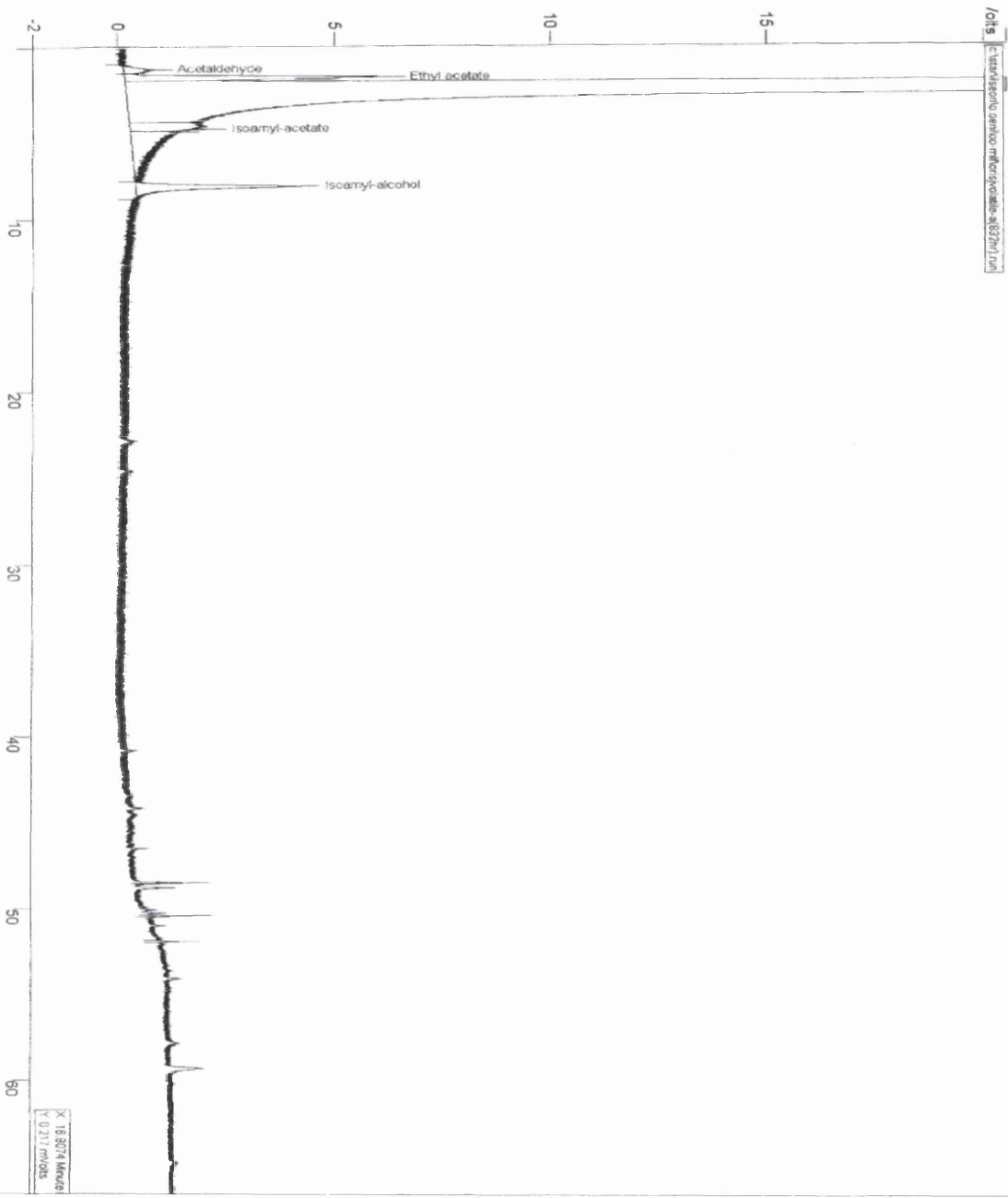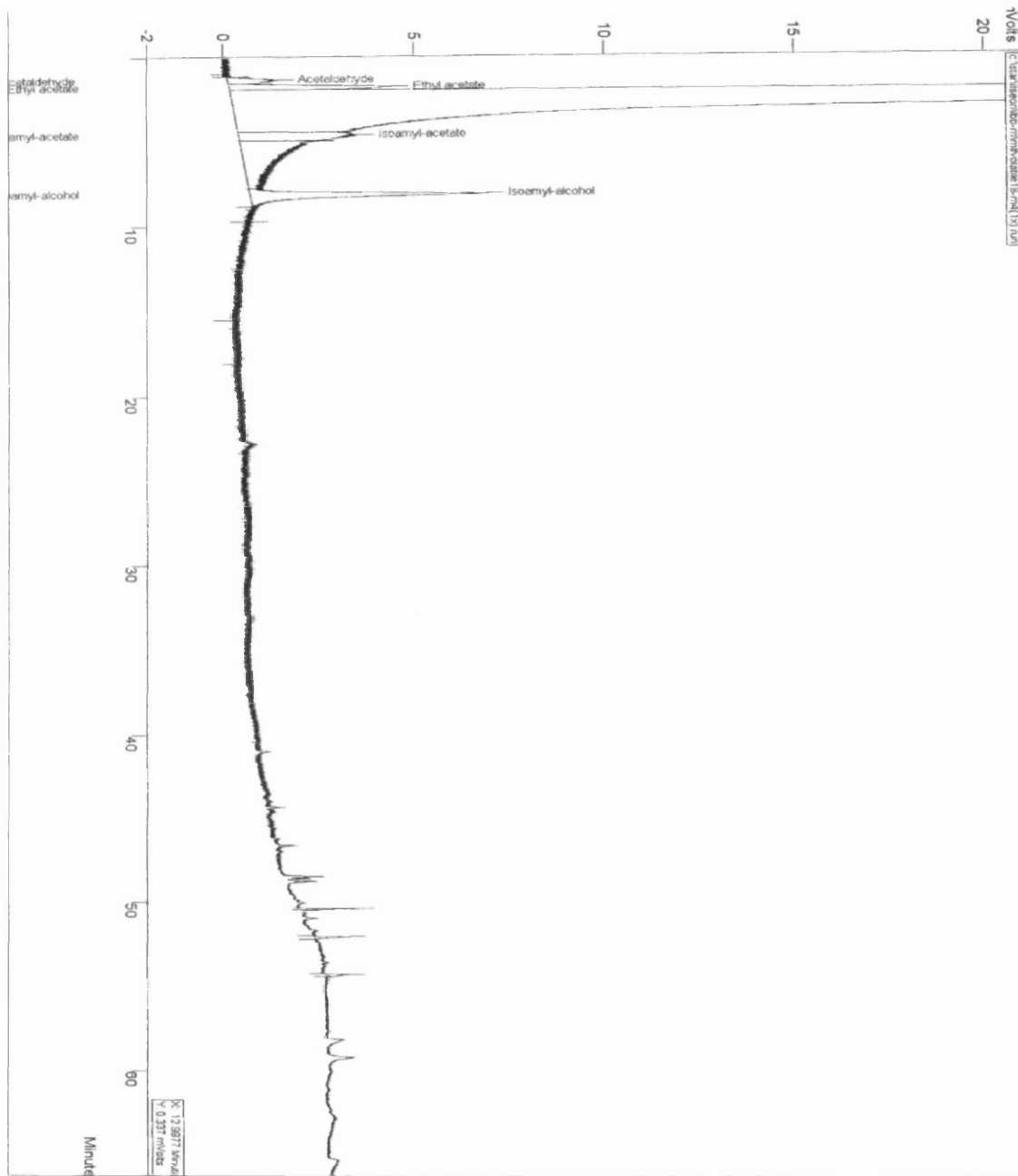
Appendix-42.

G.C chromatography of the volatile compounds (1X) after MLF of Scrumpy Jack by *O. oeni* in MCR. *(Refer to 2-7-4-6). This picture shows the chromatography of acetaldehyde, ethyl-acetate, iso-amylalcohol, and iso-amylacetate in product stream after MLF of Scrumpy Jack. When the sample was taken in 832 h of MLF, the alcohol concentration in feed stream was 12% (w/v), and the feeding rate was controlled at the retention time of 6 h. This is product when Scrumpy Jack of Appendix-41 was used as feed.*
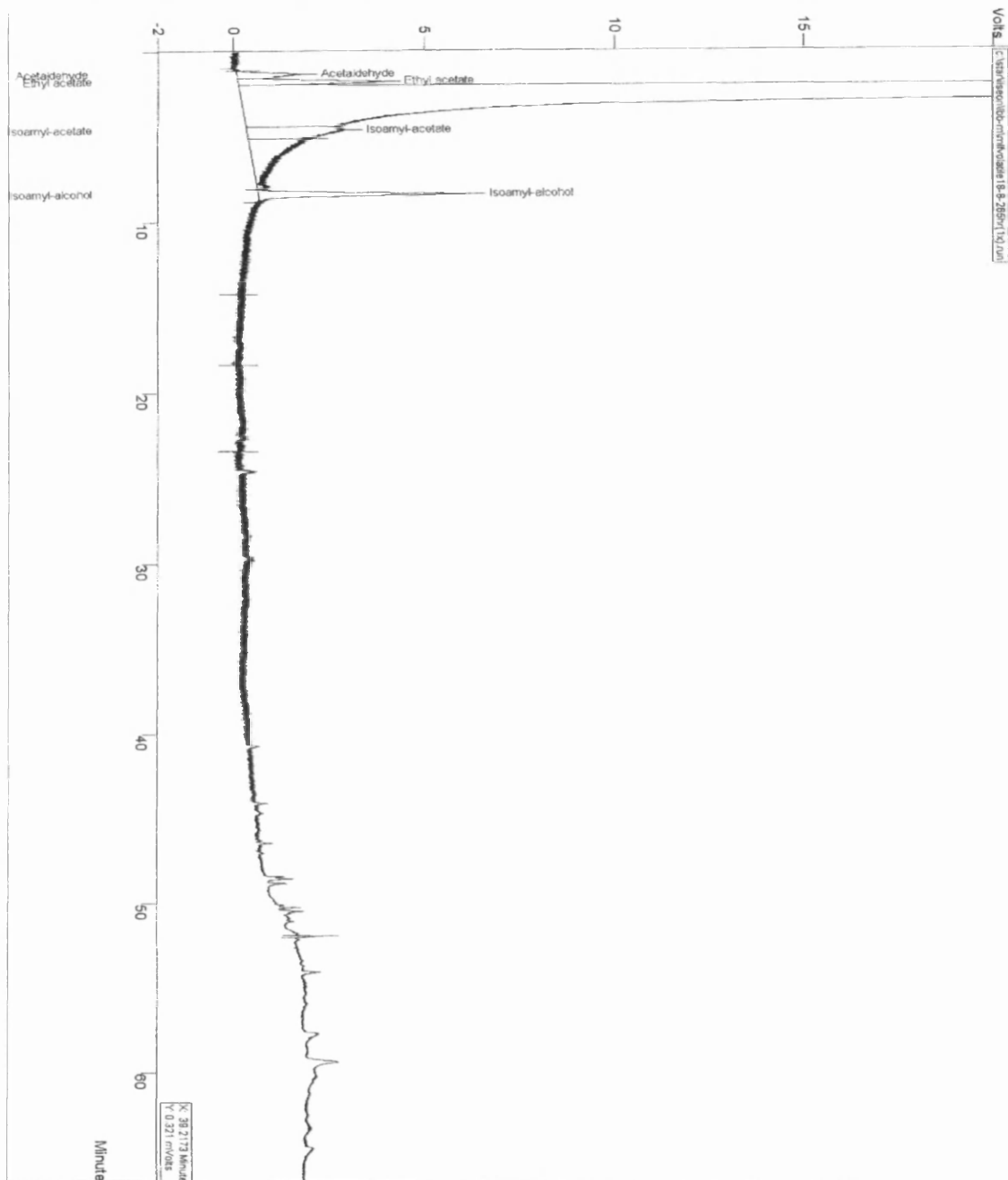
Appendix-43.

G.C chromatography of the volatile compounds (1X) before MLF of Scrumpy Jack by *L.brevis* in MCR. *(Refer to 2-7-4-6). This picture shows the chromatography of acetaldehyde, ethyl-acetate, iso-amylalcohol, and iso-amylacetate in product stream before MLF of Scrumpy Jack. The alcohol concentration was 9% (w/v). GC chromatography of the volatile compounds after MLF of Scrumpy Jack by L. brevis is shown at Appendix-44.*

Appendix-44.

G.C chromatography of the volatile compounds (1X) after MLF of Scrumpy Jack by *L. brevis* in MCR. *(Refer to 2-7-4-6). This picture shows the chromatography of acetaldehyde, ethyl-acetate, iso-amylalcohol, and iso-amylacetate in product stream after MLF of Scrumpy Jack. When the sample was taken in 265 h of MLF, the alcohol concentration in feed stream was 9% (w/v), and the feeding rate was controlled at the retention time of 8.57 h. This is product when Scrumpy Jack of Appendix-43 was used as feed.*

APPENDIX: Standard curves for determination of dry cell weight, organic acids and carbohydrate concentration, GC and HPLC chromatograpy of cider and nomenclature.

Nomenclature

X : biomass in dry cell weight (g /L)

t : time (h)

μ: The specific growth rate (h^{-1})

μ_m: The maximum growth rate (h^{-1})

α: The stoichiometric constant in growth-associated model

β: The proportionality constant depending on cell concentration in t time

$Y_{X/S}$: Yield constant for biomass (Dimensionless)

$Y_{PL/S}$: Yield constant for lactic acid (Dimensionless)

$Y_{PA/S}$: Yield constant for acetic acid (Dimensionless)

$Y_{PE/S}$: Yield constant for ethyl alcohol (Dimensionless)

S: The substrate concentration (mole)

S_t: The substrate concentration in output in t time (mole)

S_O: The substrate concentration in input in zero time (mole)

C_t: A product concentration in output in t time (mole)

C_O: A product concentration in input in zero time (mole)

C_P :Concentration of product giving end product inhibition (mole)

C_P^* : The critical concentration of product to stop the cell growth (mole)

f: flow rate (L/ h)

V: Volume in bioreactor (36 L)

D:Dilution rate (h^{-1})

m: Constant for cell maintenance.

n: Toxic power of organic acids

K_s : The saturation constant of substrate

T_P ; Productivity

v ; The specific production rate

$P_{x/t}$: the specific production rate of cell mass

• *Peristaltic pump and tubing (Fig.2.6.-No.26).*

The peristaltic pump was fitted in the production line, and used to control the flow rate of the fluid in the production line. The peristaltic pump (Model No 505u) was supplied by Waston Malow. Silicone tubing (Dia; 18 mm) was used for the production line.
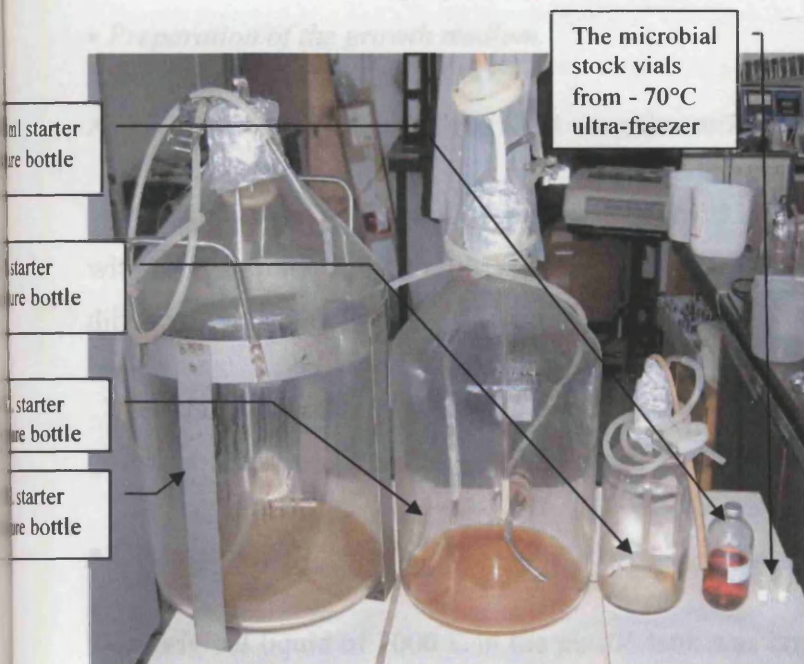
2.6.2. Operation of the MCR for the growth of LAB and the cider maturation.

The operation of the MCR will be discussed. Three parts according to their functions, those are the sterilization, inoculation, and cleaning.

The preparation for the starter cultures, for the growth medium or the feed cider, the feeding of the growth medium or cider, the microbial growth in the bioreactor and the control of production rate will be described in this section.

2.6.2.1. Preparation of the starter culture for the microbial growth in the MCR.

The starter cultures were prepared through a series of the batch cultures (Fig.2.11).

The microbial stock vials from - 70°C ultra-freezer

ml starter
re bottle

starter
re bottle

starter
re bottle

starter
re bottle

Fig.2.11. Photograph of the starter culture bottles used to prepare the inoculum for the further cultivation in the MCR. *The starter culture was progressed from the vial stock suspension of 0.2 µ l to three starter culture of 20 L via 100 ml, 1.0 L, and 10 L. At last, the starter culture of total 60 L was prepared to be inoculated into MCR of total 36 L.*