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PROCESS DEVELOPMENT FOR BIOMASS PRODUCTION OF *ENTEROCOCCUS FAECIUM* FOR PROBIOTICS USE

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for the degree of Master of Science (Biotechnology)
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Sukunya Oaew

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KEY WORDS :PROBIOTICS/ *ENTEROCOCCUS FAECIUM*/ HIGH CELL DENSITY/ LACTIC ACID BACTERIA/ FISH SOLUBLES/ WHEY PROTEIN/ FED-BATCH CULTIVATION/ GROWTH INHIBITION/ BROWNING REACTION PRODUCT

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In order to develop high cell density cultivation of *Enterococcus faecium* for probiotics use, the growth inhibitory effect of lactic acid should be reduced. With the aim of controlling lactic acid formation, the effects of protein as a C-source and aeration conditions on cell growth and lactic acid production were investigated. It was found that protein supplementation as a C-source, coupled with aeration promoted good cell growth and efficiently reduced lactic acid production. Furthermore, it was observed that protein digestion improved the cell growth yield. For industrial scale cell production, medium containing digested fish solubles and whey was successfully developed to replace costly meat extract, tryptone and yeast extract that were present in the original MRS medium used. However, the utilization of protein as the sole C-source was less efficient than when it was combined with sugar supplementation. Although the cell growth yield could be improved by sugar utilization, lactic acid formation due to excess sugar supply had to be avoided. To achieve this, a fed-batch technique was applied to limit the protein and sugar supply and thus control lactic acid formation. The maximum cell density obtained in the fed-batch cultivation was 4.3×10^{10} cfu/ml, which was 7 times higher than that obtained by batch cultivation. However, a leveling off of cell viability was observed when the fed-batch culture reached a high cell concentration. The results also suggested that Browning reaction products formed during the sterilization of high protein media may have a major inhibitory effect on cell growth. In addition, cell viability was much improved in the presence of low quantities of Browning reaction products.

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สุกัญญา แซ่เอี๊ยว : การพัฒนากระบวนการผลิตชีวมวลจุลินทรีย์แลคติคสายพันธุ์ *Enterococcus faecium* สำหรับประยุกต์ใช้เป็นโปรไบโอติค (PROCESS DEVELOPMENT FOR BIOMASS PRODUCTION OF *ENTEROCOCCUS FAECIUM* FOR PROBIOTICS USE) คณะกรรมการควบคุมวิทยานิพนธ์ : สมชาย เชื้อวัชรินทร์, Ph.D., อมเรศ ภูมิรัตน, Ph.D., อภิญญา อัศวนิก, Ph.D. 123 หน้า. ISBN 974-663-802-5

ในการพัฒนากระบวนการผลิตเชื้อจุลินทรีย์ *Enterococcus faecium* ที่ความเข้มข้นสูงสำหรับใช้เป็นสารเสริมชีวนะ (probiotics) จำเป็นที่จะต้องลดอิทธิพลของกรดแลคติคซึ่งมีผลยับยั้งการเจริญเติบโตของเชื้อ ในการศึกษานี้ได้มีการทดสอบผลของการใช้โปรตีนเป็นแหล่งอาหารคาร์บอนและการเติมอากาศต่อการเจริญเติบโตและการสร้างกรดแลคติคของเชื้อ และพบว่าการใช้โปรตีนเป็นแหล่งอาหารคาร์บอนควบคู่ไปกับการให้อากาศ ทำให้เชื้อเจริญเติบโตได้ดีและสามารถลดการสร้างกรดแลคติคได้อย่างมีประสิทธิภาพ นอกจากนี้ยังพบว่าโปรตีนที่ผ่านการย่อยแล้วสามารถเพิ่มการเจริญเติบโตของเชื้อได้ สำหรับการพัฒนาสูตรอาหารเพื่อการเลี้ยงเชื้อในระดับอุตสาหกรรม พบว่าอาหารที่มีส่วนผสมของ digested fish soluble และ whey สามารถทดแทนแหล่งโปรตีนที่มีราคาสูง เช่น meat extract, tryptone และ yeast extract ที่ใช้ในสูตรอาหาร MRS ได้เป็นอย่างดี อย่างไรก็ตามผลการศึกษาต่อมาชี้ให้เห็นว่า การใช้แหล่งโปรตีนเป็นแหล่งคาร์บอนเพียงอย่างเดียวในการเลี้ยง เชื้อจะมีการนำโปรตีนไปใช้ได้เพียงส่วนน้อยเท่านั้น แต่จะใช้ได้มีประสิทธิภาพมากยิ่งขึ้นถ้ามีการใช้น้ำตาลควบคู่ไปด้วย อย่างไรก็ตามแม้ว่าการให้น้ำตาลจะช่วยเพิ่มการเจริญเติบโตของเชื้อ แต่การให้น้ำตาลที่มากเกินไปจะมีผลในการเพิ่มการสร้างกรดแลคติคของเชื้อได้ ดังนั้นจึงมีการประยุกต์ใช้เทคนิคการเลี้ยงเชื้อแบบ fed-batch ในการควบคุมการให้โปรตีนและน้ำตาลเพื่อควบคุมการสร้างกรดแลคติคของเชื้อ ซึ่งพบว่าโดยเทคนิคนี้สามารถเลี้ยงเชื้อที่มีชีวิตได้ความเข้มข้นสูงถึง 4.3×10^{10} cfu/ml ซึ่งมากกว่าการเลี้ยงแบบ batch ถึง 7 เท่า อย่างไรก็ตามในการเลี้ยงเชื้อที่ความเข้มข้นสูงระดับหนึ่ง พบว่าเซลล์ที่มีชีวิตจะไม่สามารถเพิ่มมากขึ้นได้ ทั้งนี้ผลการศึกษาชี้ให้เห็นว่าอาจเนื่องมาจากผลิตภัณฑ์จากปฏิกิริยา Browning ที่เกิดในระหว่างการเตรียมอาหารโปรตีนความเข้มข้นสูงที่อุณหภูมิสูง ซึ่งจากผลการลดปริมาณผลิตภัณฑ์จากปฏิกิริยา Browning พบว่าสามารถช่วยเพิ่มเซลล์ที่มีชีวิตให้สูงยิ่งขึ้นได้

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LIST OF ABBREBIATIONS

AN	Amino nitrogen
Approx.	Approximately
cm	Centimeter
°C	Degree of Celsius
DFS	Digested fish soluble
et al.	Et alii (Latin), and others
etc.	Et cetera (Latin), other things
e.g.	Example gratia (Latin), for example
Fig.	Figure
g	Gram
hr	Hour
i.e.	Id est. (Latin), that is
l, L	Liter
m	Meter
µg	Microgram
mg	Milligram
ml	Milliliter
min	Minute
M	Molar
ME	Meat extract
MRS medium	de Man, Rogosa and Sharpe medium

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nm	Nanometer
N	Normal
%	Percent
rpm	Revolution per minute
TN	Total nitrogen
TR	Tryptone
vol.	Volume
v/v	Volume by volume
w/v	Weight by volume
YE	Yeast extract

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CHAPTER I

INTRODUCTION

Lactic acid bacteria (LAB) have been widely used in manufacturing of foods for centuries. Hence they are categorized as "generally regarded as safe (GRAS)" (1). Several species of lactic acid bacteria are used commercially for the production of fermented dairy and meat products and other foods. These products include yogurt, sour milk, buttermilk, salami-type sausages, and pickled fruits and vegetables. The LAB have been considered to be beneficial to their consumers due to several functions. These organisms are particularly suitable as antagonistic microorganisms in food because they are capable of inhibiting other food-borne pathogens by a variety of means including production of antimicrobial agents such as organic acids, hydrogen peroxide or bacteriocins. Furthermore, they can supply some of the B-vitamins including niacin (B3), pyridoxine (B6), folic acid and biotin and some of certain enzymes essential for promoting the metabolism of consumer's animals.

In animal farming a wide variety of antibiotics have been used to prevent bacterial intestinal disease and increase the feed conversion . The use of antibiotics with farm animals, however, may resulted in tissue residues of the antibiotics and imbalance of normal intestinal flora as well as a reduction in beneficial intestinal microbial populations and the generation of antibiotic-resistant bacteria. In order to overcome the above-mentioned problems, the utility and development of probiotics has

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been recently introduced in veterinary. The term "Probiotics" is defined as "a viable monoculture or mixed culture of microorganisms that, when applied to animals or man, beneficially effects the host by improving the balance of the intestinal microflora" (2), are strongly recommended as an alternative for antibiotics for industrial animals such as pigs, poultry, sheep, goat, and cattle. Among bacteria used as probiotics, (Table 1) lactic acid bacteria (LAB) have been the most commonly used group. As part of the natural flora, they can exert beneficial effects through the production of antibacterial compounds, increasing antibody levels, increasing macrophage activity, organic acid production, lowering of pH and competition for adhesion sites. By these prominent characteristics they are used to manage intestinal disorders such as lactose tolerance, acute gastroenteritis due to rotavirus and other enteric pathogens, adverse effects of pelvic radiotherapy, constipation, inflammatory bowel disease, and food allergy. (3). Because of these attractive properties of LAB, many researches related with probiotics have been conducted around the world not only in Europe and America, but also including Asia. It is now widely used in both men and farm animals.

In Thailand, it was reported that import of probiotics steadily increased from 42.1 tons in 1991 to 100.5 tons in 1995 (4). Hence, it is the most preferable to develop our own technology for producing probiotics in the country to reduce import of both antibiotics and probiotics. According to the literature surveying, a few reports mentioned about the techniques for high cell density cultivation of LAB. Thus basic research to develop novel technique to cultivate the lactic acid bacteria at high cell concentration is important and this could be applied in industrial scale production of

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probiotics as well as ordinary lactic acid bacteria starter culture in fermented food process.

To develop high cell density process for lactic acid bacteria, previous works related with lactic acid and cell production have been focused firstly. In process development of lactic acid production by lactic acid bacteria, there is growth inhibitory effect caused by the produced lactic acid so the high cell density and lactic acid production is difficult to achieve. To cope with the inhibitory effect of lactic acid, several techniques have been introduced to continuously remove lactic acid from the cell culture environment, for examples, electrodialysis (5-10), resin adsorption (11-13) and solvent extraction (14-16). According to these means, continuous lactic acid production and also cell growth could be obtained. Meanwhile, for extending cell growth another approach to decrease lactic acid production by physiological control of lactic acid bacteria could also be considered. In this study, studies on the effect of several parameters such as medium components, culture conditions and cultivation techniques on cell physiology were emphasized in order to control the metabolism of lactic acid production and support cell growth at high cell density. Firstly, the effect of aeration and medium component *i.e.* utilization of several proteineous sources such as yeast extract, soy bean meal, and fish soluble as carbon source on the growth and lactic acid production were investigated for their potential to be used as culture condition and medium. Secondly, studies of the modification of protein sources by acid and enzyme digestion to improve protein utilization efficiency were conducted. Thirdly, fed-batch cultivation technique with medium feeding control was applied to lower

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lactic acid production and extend cell growth. Finally, overall process optimization for high cell density cultivation was developed.

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CHAPTER II

BACKGROUND

1. Probiotics

1.1. Why probiotics?

Due to the presence of numerous types of antibiotics since the 1950s resulted in their widespread use as therapeutic agents and growth stimulants for farm animals. However, there has been a growing concern that the use of antibiotics as growth promoters was resulting in the development of drug-resistance in bacteria which made subsequent increase of antibiotics dosage for therapy necessary. Furthermore, there is also a reaction against the use of antibiotics as therapeutic agents because of the intestinal upsets. The growing concern on the use of antibiotics as growth stimulants for farm animals and about their side-effects when use as therapeutic agents prompted both consumers and manufacturers to look for alternatives. Probiotics are being considered to fill this role and some farmers already use them in preference to antibiotics (2).

1.2 Definition and characteristics

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The term "probiotics" originated from two Greek words meaning "for life". It was first used by Parker (17) to describe "organisms and substances which contribute

to intestinal microbial balance". However, this definition was revised by Fuller (2) as " A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Probiotics can be presented to the animal in various ways. The type of preparation will depend on the intended use. They can be either included in the pelleted feed or produced in the form of capsules, paste, powder or granules. The target species are cattle, sheep, goats, pigs, poultry, horses and domestic pets. Probiotic preparations may consists of single strains or may contain any number up to eight strains. The species currently being used in probiotic preparations are listed in Table 1. However, among the strains, *Lactobacillus* and *Streptococci* are the most commonly used groups in the production of probiotics.

Table 1. Examples of microorganisms used in probiotics products

Products for humans	**Product for farm animals**
Lactobacillus acidophilus	*Lactobacillus acidophilus*
Lactobacillus casei Shirota strain	*Lactobacillus casei*
Lactobacillus delbrueckii supspp.	*Lactobacillus delbrueckii subspp.*
bulgaricus	*bulgaricus*
Lactobacillus johnsonii	*Lactobacillus helviticus*
Lactobacillus reuteri	*Lactobacillus lactis*
Lactobacillus rhamnosus	*Lactobacillus plantarum*
Bifidobacterium adolescentis	*Lactobacillus reutei*
Bifidobacterium bifidum	*Lactobacillus salivarius*
Bifidobacterium breve	*Bifidibacterium bifidum*

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Table 1. (continued)

Products for humans	Product for farm animals
Bifidobacterium longum	*Bacillus subtilis*
Bifidobacterium infantis	*Enterococcus faecalis*
Streptococcus thermophilus	*Enterococcus faecium*
Saccharomyces boulardii	*Pediococcus pentosaceus*
	Streptococcus thermophilus
	Saccharomyces cerevisiae
	Aspergillus oryzae
	Torulopsis spp.

Adapted from Tannock 1997 (1)

1.3 Modes of action

The beneficial effects of probiotics may be mediated by a direct antagonistic effect against specific groups of organisms, resulting in a decrease in numbers or by an effect on their metabolism or by stimulation of animal immunity.(1, 18-27). A summary of possible modes of action of probiotics is shown in Table 2.

For all of these mechanisms there was some supported by experimental data (25). The bacterial numbers could be suppressed by production of antibacterial substances. Primary metabolites, such as organic acids (lactic acid, acetic acid), carbon dioxide, diacetyl, acetaldehyde and hydrogen peroxide, are known to be effective *in vitro*. However, the evidence for the involvement of organic acids in the control of gut bacteria is equivocal. Another mechanism for preventing colonization of pathogens is

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Table 2. Possible modes of action of probiotics

1. Suppression of viable count by:
   (a) production of antibacterial compounds
   (b) competition for nutrients
   (c) competition for adhesion sites
2. Alteration of microbial metabolism
   (a) increased enzyme activity
   (b) decreased enzyme activity
3. Stimulation of immunity
   (a) increased antibody levels
   (b) increased macrophage activity

(from Fuller 1989) (1)

competition for adhesion sites on the gut epithelia surface or competitive exclusion (2, 26-27). Probiotics may also promote their host animal health by increasing the activity of useful enzymes, *e.g.* β-galactosidase in the alleviation of lactose tolerance or by suppressing the activity of β-glucuronidase, nitroreductase and azoreductase which are involved in procarcinogen activation and reduced excretion of mutagens in feces and urine. The animal with a complete gut flora which was supplemented with probiotics was observed for increasing in phagocytic activity and immunoglobulin levels (2-3, 23). Several reports have focused on the ability of LAB to modulate the production of cytokines, the mitogen- and antigen-driven lymphocyte proliferation, natural killer (NK) cell cytoxicity, antibody production and some metabolic pathways of monocytes-

macrophages (2-3, 23). There is also evidence that LAB may be involved in the pathogenesis of some models of autoimmunity in experimental animals and, possibly, in humans. However, the investigation of the immunomodulating properties of probiotics, particularly LAB, may considered as a hard issue because of the complexity of the systems involved, the gut microflora and the cytokines as well as cell networks activated during both mucosal and systemic immune responses.

1.4 Characteristics of good probiotics

For farm animals the most important reason for using a probiotic product is the claim that their presence as the indigenous microflora resulting in the following effects. (22, 28)

- growth promotion of the animals in combination with a better feed conversion;
- health control, especially control of intestinal disturbances in young animals by stimulation of the development of the indigenous microflora and the colonization resistance in the gut;
- stimulation of the (non-) specific immune response;
- synthesis of vitamins
- predigestion of antinutritional factors (ANFs) such as phytic acid, glucosinolates, trypsin inhibitors, lectins and non-starch polysaccharides (dietary fiber).

For study of probiotics effects, although many positive results could be obtained experimentally in laboratory, the results obtained in field trails have been variable. The other problems which has occurred with some of the commercial preparation is poor quality control. Features of good probiotics should be (2):

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- a strain which is capable of exerting a beneficial effect on the host animal, *e.g.* increased growth of resistance to disease
- non-pathogenic and non-toxic
- present as viable cells, preferably in large numbers, although the minimum effective dose was not known
- capable of surviving and metabolizing in the gut environment, e.g. resistant to low pH and organic acids
- stable and capable of maintaining viability for long periods under storage and field conditions.

2. Lactic acid bacteria

The term " lactic acid bacteria (LAB)" (29-31) is used to describe a broad group of Gram-positive, non-spore forming rods and cocci, usually non-motile, that utilize carbohydrates fermentatively and form lactic acid as the major end-product. For practical purposes they are divided into, and referred to as, homo- and hetero-fermentative, according to the metabolic routes they use (Embden-Meyerhof-Parnas or phosphoketolase pathway) and the resulting end-products. Lactic acid bacteria are generally catalase-negative and usually lack cytochromes, although they are produced by some species when grown in media containing blood. Lactic acid bacteria are generally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins. They are generally acidoduric or acidophilic, different species having adapted to grow under widely different environmental conditions. They are widespread in nature, their distribution related to wherever high concentrations of

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soluble carbohydrate, protein breakdown products, vitamins and a low oxygen tension occur. Consequently they are common in milk and dairy products, other fermented foods (meats, vegetables, breads), intact and rotting vegetable material, silage, and intestinal tract and mucous membranes of man and animals. (30-31).

Lactic acid bacteria, which are generally recognized as safe (GRAS) constitute a bacterial group that is beneficial to mankind; several of its members are responsible, wholly or in part, for the production of organoleptic characteristics and/or the preservation of a wide range of food products. For many years there has also been widespread interest in the use of lactic acid bacteria in the biological preservation of foods. These organisms are particularly suitable as antagonistic microorganisms in foods because they are capable of inhibiting other food-borne bacteria by a variety of means including production of organic acids, hydrogen peroxide or bacteriocins. Similarly their use as probiotics, *i.e.* dietary and therapeutic adjuncts, for man and animals is highly recognized. Their favorable effect on growth and health is thought to be due to the modulation of other bacterial growth through one or more of antagonistic factors.

2.1 *Enterococcus faecium*

The genus *Enterococcus* was not included *in Bergey's Manual of Systematic Bacteriology*, Vol 2, 1986, where some of its species were included in the genus *Streptococcus*. A proposal was made by Schleifer and Kilpper-Balz to classify streptococci that had long been informally referred to enterococci as a separate genus called *Enterococcus*. In their publication, *Streptococcus faecalis* and *S. faecium* were

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transferred to a new genus as *Enterococcus faecalis* (type species) and *E. faecium*, respectively. Subsequently *Streptococcus avium* and *S. gallinarum* were transferred by Collins *et al.*, and new species of *Enterococcus* were established for other earlier, streptococcal species and subspecies (*i.e.*, *E. durans, E. casseliflavus* and *E. malodoratus*). Then, a number of newly described species have been added on (32).

E. faecium (32-33). are gram-positive spherical or ovoid cells, 0.6-2.0 x 0.6-2.5 μm, occurring in pairs or short chains in liquid media. Elongated cells may be formed. Endospores are not formed and generally non-motile. Motile strains differ in esterase and protease patterns on polyacrylamide gel. Colonies on solid media are entirely smooth and white. They are facultative anaerobes, chemoorganotrophes with fermentative metabolism; a wide range of carbohydrates are fermented with the production of mainly L (+)-lactic acid but no gas and a final pH of 4.0-4.4. Alteration of glucose metabolism with vigorous aeration is identical to that of *E. faecalis*. Nutritional requirements are complex. They are catalase negative and usually can grow at temperature between 10° and 45°C (optimum at 37 °C) and can tolerate in conditions, pH up to 9.6, 6.5% NaCl, and 40% bile. *E. faecium* are predominantly inhabitants of the gastrointestinal tract of man and animals and also commonly occur in vegetables and plant material.

Strains of enterococci are mainly used in pig and poultry nutrition. However, there are pharmaceutical products which contain enterococi as probiotic cultures for human clinical therapy. Only two subspecies of the genus *Enterococus* are of importance as probiotics: *E. faecium* is mainly used as an animal probiotic but also for human application, whilst *E. faecalis* is primarily used as a human probiotic. These two

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species can easily be differentiated from each other by their ability of fermentation reactions (arabinose, sorbitol) and different growth temperatures (50°C, for *E. faecium*) (31).

Numerous researches (34-39) have been investigated in the bacteriocin production of the genus *Enterococcus*. Bacteriocin-producing enterococci are widespread in nature and they have been isolated from dairy products, fermented sausages, vegetables, fish silage, and mammalian gastrointestinal tract. A large number of bacteriocins have been isolated and characterized to date such as enterocin A (34), enterocin CRI35 (35), and enterocin 226NWC (36). It was also reported that the new pediocin-like bacteriocin, enterocin P, produced by *E. faecium* P13 isolated from a Spanish dry fermented sausage, showed a broad antimicrobial spectrum which includes not only *Listeria monocytogenes* but also a wide range of spoilage and food-borne gram-positive pathogenic bacteria such as *Staphylococcus aureus, Clostridium perfingens* and *Clostridium botulinum* (40).

According to the facts mentioned above, *E. faecium* was used as model probiotics strain in this work.

3. High cell density cultivation of lactic acid bacteria

For high productivity in microbial processes a high cell density cultivation is desired. However, some microorganisms produce growth inhibitory substances which are excreted to the medium and in such cases it may be difficult to achieve the high cell density. In lactic acid bacteria cultivation, lactic acid which is the by-product from the cultivation causes the inhibitory effect on microbial growth (41-44). So by normal

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batch cultivation the high cell density is difficult to be obtained. To cope with this problem, several techniques have been introduced to remove lactic acid from the cell environment, e.g. electrodialysis (5-10), resin adsorption (11-13), solvent extraction (14-16), cross-flow filtration (45-46) and cell recycle (47-50). These techniques could be applied during high cell density cultivation of lactic acid bacteria. Nevertheless, these techniques were difficult to be applied with, costly process and may cause drawback effects to microorganism as well as the process. Another possible approach to avoid the inhibitory effect of lactic acid is to regulate cell metabolism for reducing lactic acid production. Study in metabolism control approach could be done by investigation of several factors, which may influence cell physiology and metabolism.

3.1. The effect of environmental condition on the cell metabolism

Physical and environmental factors such as pH, temperature, and dissolved oxygen can play an important role in cell growth and metabolism (51-61). In some lactic acid bacterial strains, oxygen has a pronounced effect on the growth and metabolism. Many lactic acid bacteria grow well aerobically and the growth rate in aerobic condition may even be higher than that in anaerobic condition. The presence of oxygen may also increase the yield of biomass. In aerobic culture, the formation of acetic acid in *Streptococcus agalactiae* is stimulated and as a result lactic acid production was lower(62). It has also been reported that a heterofermentative *Leuconostoc mesenteroides* grew more efficiently in the presence rather than in the absence of air (53, 55-56, 60-61). This was obvious from shorter lag periods, higher growth rates and greater biomass yields. This is because of the specific activity of

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acetate kinase, which normally converts acetyl phosphate to acetate and yield ATP, is high and as a consequence an increased biomass level was obtained. In addition, the specific activity of NADH oxidase, which regenerats NAD^+ from NADH, is high and as a result, NAD^+ can then enter another round of glycolysis and generate ATP which result in higher growth yield of the cells (53, 58, 60-61). Moreover, the increase in NAD^+-level would result in the reverse conversion of lactate to pyruvate via the coupling of NAD^+, resulting in a decreased in lactic acid formation.

3.2. The effect of nutritional factors on cell metabolism

Lactic acid bacteria are widespread in nature, their distribution is related with the presence of high concentrations of soluble carbohydrate, protein breakdown products, and vitamins. These organisms, in general, are highly saccharolytic and they lack most anabolic pathways. So their habitats would be very complex environments such as plant materials, milk, and the intestinal tract of animals. Normally, they gain energy from breakdown of hexose via glycolysis or the Embden-Meyerhof-Parnas pathway. The enzymes in glycolysis catalyze the splitting of hexose, a six-carbon sugar, into two three-carbon sugars. These sugars is then oxidized, releasing energy, and their atoms are rearranged to form two molecules of pyruvic acid. During glycolysis, NAD^+ is reduced to NADH, and ATP is produced from substrate-level phosphorylation. In lactic acid bacteria, pyruvate is then reduced by NADH to form lactic acid for homolactic or other organic acids for heterolactic characteristic, depending on microbial strains. In this case NAD^+ is regenerated and can enter another

round of glycolysis. As a consequence, glycolysis can continue and ATP is continually generated.

Strictly fermentative lactic acid bacteria lack a complete electron transport systems that functions as a proton pump. In these bacteria, the ATPase functions in the direction of hydrolysis, and the H^+ excretion required to generate PMF (proton motive force) causes less ATP to be available for biosynthetic purposes.(63). Lactic acid bacteria lack various biosynthetic pathways so that they require nutrients, especially amino acids and vitamins. As a consequence the occurrence of these lactic acid bacteria is restricted to habitats that are rich in nutrients and are growing well in complex medium. It has been well-established that many lactic acid bacteria, isolated from milk products, are multiple amino acids auxotropic microorganism. The amount of amino acids requirement for growth is strain dependent and can vary from 4 up to 14 different amino acids. Furthermore the proteolytic system of lactic acid bacteria are varied from strains to strains.(64-67).

As mentioned above, using complex protein sources which contain amino acids, vitamins, growth factors, and total carbohydrate together with aeration for cultivation of lactic acid bacteria might be beneficial for controlling of lactic acid production. In these cases, free amino acids might be applied directly to preserve energy for amino acids synthesis by the cells. Furthermore, some amino acids can by-pass glycolysis pathway and convert directly to pyruvate. In addition, some consumable carbohydrate from complex protein source can be used as energy source, for ATP production. Moreover, in addition to ATP generation by glycolysis, NADH could be efficiently recovered by NADH oxidase under aerobic conditions. In

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comparison with that of anaerobic condition this would result in higher growth rates and yields while lactic acid accumulation would be less.

3.3. Application of fed-batch technique in cell cultivation

Yoshida et al. (68) introduced the term fed-batch culture to describe batch cultures which are fed continuously, or sequentially, with medium, without the removal of culture fluid. A fed-batch culture is established initially in batch mode and is then fed according to one of the following feed strategies: (i) the same medium used to establish the batch culture is added, (ii) a solution of the limiting substrate at the same concentration as that in the initial medium is added, (iii) a concentrated solution of the limiting substrate is added at a rate less than that of (i) and (ii), or (iv) a very concentrated solution of the limiting substrate is added at a rate less than that of (i), (ii) and (iii). Thus, the volume of the culture and total biomass will decrease with time.

The basic characteristic of fed-batch microbial process is that the concentrated of nutrients fed into the culture liquid of the bioreactor can be controlled efficiently by changing the feed rate. In a batch cultivation, nutrient concentrations are not controlled and are progressively used up. Fed-batch is superior to conventional batch operation especially when the level of a nutrient (or nutrients) concentration affect the yield or productivity of the desirable metabolite. There are also several other minor advantages of fed-batch operation, which can be summarized as follows (69-70):

1. Reduce the effect of substrate inhibition. Nutrients such as methanol, ethanol and acetic acid inhibit the growth of microorganisms even at relatively low

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concentration. By adding such substrates properly, the lag-time can be shortened and the inhibition of cell growth is markedly reduced.

2. To achieve very high cell concentrations, *e.g.*, 100 g of dry cells per L. In a batch culture a high concentration of nutrients in the medium is needed. At such high concentrations nutrients become inhibitory, even though they have no such effect at the normal concentrations used in batch cultures.
3. In the production of baker's yeast from malt wort or molasses, ethanol is produced even in the presence of sufficient dissolved oxygen (DO) if excess of sugar is present in the culture liquid (Crab Tree Effect). The loss of carbon in the form of ethanol is the main cause of low cell yields. To prevent alcoholic fermentation, a fed-batch process is generally employed for baker's yeast production.
4. To avoid catabolite repression. When a microorganism is provided with a rapidly metabolizable carbon-energy source such as glucose, the resulting of the intracellular concentration of ATP lead to the repression of enzyme biosynthesis, thus causing a slower metabolization of the energy source. This phenomena is known as catabolite repression. A powerful method of overcoming catabolite repression in enzyme biosynthesis is a fed-batch culture in which the glucose concentration in the culture liquid is kept low, where growth is restricted, and enzyme biosynthesis is depressed.
5. Extension of operation time. In a non growth-associated microbial process, such as antibiotics production, microorganism initially rapidly utilize the carbon-energy source for growth and then synthesize the desired secondary metabolite in the subsequent declining phase and early stationary phase. In conventional batch

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process, this production phase is short, due to the depletion of the carbon-energy source; the subsequent cell autolysis is rapid and severe. It is important to maintain a concentration of the carbon-energy source where the microorganisms are semi-starved but where enzyme activity of synthesis is highest. To prolong and maintain high synthesis of semi-starved microorganisms, the carbon-energy source and/or precursors are fed into the bioreactor at a proper controlled rate.

6. Decreasing viscosity of broth. In microbial biopolymer production of, *e.g.*, dextran, pullulan, or xanthan gum, broth viscosity can be kept low by continuous feeding of nutrients. Otherwise, the markedly increased viscosity of the broth will raise the agitation power consumption and lower oxygen transfer efficiency.

In fed-batch cultivation, controlling of carbon feeding can result in changing of metabolic flux in the metabolic pathway of the cells. It was reported that during fed-batch cultivation of *Escherichia coli* (71-74) the formation of acetic acid as well as its inhibitory activity have been successfully avoided by controlling of sugar concentration in the culture broth at a very low level by exponential feeding strategy. It has also been reported that under carbon source limitation, not only is the formation of acetic acid suppressed but the cells also assimilated acetic acid and convert it into cell mass, resulting in a low acetic acid concentration (75).

In this study fed-batch technique with limiting of carbon-source feeding will be employed to reduce the metabolic flux of glucose to lactic acid and to improve cell yield. Fed-batch cultivation with glucose and protein as feeding medium would result in much improvement in cell density as well as cell viability. However, lactic acid production by the cells should be considered. Thus, a proper feeding strategy is

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necessary. In this work, constant and exponential feeding strategies were investigated for their efficiency in controlling of lactic acid production and promoting cell growth. As a result, optimal feeding strategy for high cell cultivation of *E. faecium* was developed.

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CHAPTER III

MATERIALS AND METHODS

1. Microorganism and storage conditions

E. faecium was isolated from a commercial probiotics sample donated by Alltech Inc., USA. The identification was confirmed by 16S rDNA homology sequence analysis. The bacterium was cultivated in MRS medium (Merck, Germany) at 37°C for 10 h (till the late log phase) and then preserved in 30% glycerol and used as stock culture. The strain was stored at -80°C.

2. Chemicals and reagents

Agar, boric acid, copper sulfate, di-ammonium hydrogen citrate, di-potassium hydrogen phosphate, glucose monohydrate, hydrochloric acid, magnesium sulfate, manganese sulfate, meat extract, MRS medium, skim milk, sodium acetate, sodium carbonate, sodium hydroxide, sulfuric acid were purchased from Merck (Germany). Casein acid hydrolysate (casamino acid), Murashige and Skoog vitamins complex, Biotin, Cacium panthotinate, nicotinic acid, inositol, thiamine HCl, pyridoxine HCl, para-aminobenzoic acid and tryptophan were purchased from Sigma Chemical Company (USA). Kjeldahl catalyst (0.35g copper sulfate plus 3.5g potassium sulfate) was purchased from Gerhardt (Germany). All chemicals and reagents used were

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analytical grade. Yeast extract and tryptone were obtained from Difco Laboratories (USA). Sodium hydroxide commercial grade (50%) was supplied by Aseanpolytrade (Thailand). Fish soluble was purchased from a tuna canning factory, TC Union, Bangkok, Thailand. Soy bean meal hydrolysate was supplied by Thaiwichit soy sauce factory, Samutprakarn, Thailand. Kojizyme and thermostable-α-amylase (Termamyl) of Novo Nordick Industries (Denmark) were donated by East Asiatics Thailand Ltd. Whey was achieved from cheese manufacturing plant of Chitrada Royal Project, Thailand. Whey powder (Murray Goulburn Co-operative, Australia) was donated by MilkCoa Trading, Bangkok, Thailand.

3. Culture media

The composition of MRS medium (g/L) (76) was prepared as follows: glucose, 20; tryptone, 10; meat extract, 8; yeast extract, 4; sodium acetate, 5; di-ammonium hydrogen citrate, 2; tween 80, 1; di-potassium hydrogen phosphate, 2; magnesium sulfate, 0.2; manganese sulfate, 0.04.

4. Preparation of digested fish soluble (DFS) protein

A 150-g aliquot of fish soluble protein with total solid of about 59% w/w (measured by moisture analyzer, Sartorius MA30, Germany) was mixed with 150 ml 5N HCl solution and then heated on a hot plate magnetic stirrer (Snijders Inc., The Netherlands) for 30 minutes. The digestion mixture was continuously stirred by a magnetic stirrer to maintain homogeneity. After a certain time the mixture was allowed to cool down and was neutralized to pH 7 by 10 N NaOH. Then, it was subjected to

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centrifugation to remove the solids at 10,000 rpm, 15 min (Sorvall MC12V, Dupont, USA). The supernatant obtained was used as a protein source for cultivation of *E. faecium*..

5. Preparation of soy bean meal hydrolysis

A 200g soy bean meal sample was mixed with 700 ml distilled water. Before 18 ml of Termamyl enzyme was added, the pH of the mixture was adjusted to 6.5. It was then incubated at 100°C for 3 hr to digest starch in soy bean meal into free sugars. The mixture was then centrifuged at 10,000 rpm for 15 min. The liquid portion which contains sugar was discarded. The solid portion was washed twice with distilled water and then centrifuged to remove the remaining sugar. The washed soybean meal was dried in hot air oven (Memmert, USA) at 50°C overnight. A 50g of dried soy bean meal sample was mixed with 175 ml of 0.17M HCl. The mixture was then refluxed for 3, 5 or 9 hr. It was then cooled and neutralized with 0.34N NaOH. It was further centrifuged at 10,000 rpm, 15 min. The supernatant obtained was used as a protein source for cultivation of the cells.

6. Meat extract digestion with enzyme

Meat extract solution was prepared by dissolving 5g of meat in 60 ml distilled water. It was then autoclaved (SS-320, Tomy autoclave, Tokyo, Japan) at 110°C for 10 min. After cooling down, 0.1g of Kojizyme was added and incubated at 55°C for 17 or 35 h. The enzyme digested meat extract was used as a protein source for cultivation

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of the cells in comparison with non-enzyme digested meat extract, based on equal total nitrogen to 2% yeast extract.

7. Analyses

7.1 Cell density

The culture sample was centrifuged (UNIVERSAL 16R, Hettich, Germany) at 6000 rpm for 15 min. Cells were washed with distilled water and resuspended in distilled water. Cell density was monitored by measurement of the optical density at a wavelength of 660 nm (Novaspec II, Pharmacia, LKB Biochrom, England). Distilled water was used as blank and the absorbance value was measured in the range between 0.2 and 0.6.

7.2 Viable cell count

Viable cell count was determined by total plate count. After serial dilution with sterile distilled water, 0.1 ml of cell suspension was transferred and spread onto MRS agar plates. It was then incubated at 37°C for 2-3 days. The colony count is expressed as cfu/ml.

7.3 Organic acid measurement.

Organic acids such as lactic acid, and acetic acid were analyzed by gas chromatography (GC) (GC-17A, Shimadzu, Kyoto, Japan) with Flame Ionization Detector (FID), using a 2m column of 80/120 CARBOPACK™ B-DA*/4% CARBOWAX 20M, diameter 2 mm. Temperature in column chamber, injection port

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and detector were controlled at 190°C, 210°C and 210°C, respectively. Nitrogen gas (Ultra High Purity Grade) was used as carrier gas. Lactic acid and other organic acids concentrations were calculated according to a standard curve using diluted butyric acid as internal standard.

7.4 Sugar measurement

Total sugar was determined according to the Phenol Sulfuric method.(77). Glucose was analyzed by glucose assay kit using the Trinder method. This kit was purchased from Wako Pure Chemicals Co. Ltd., Osaka, Japan. The reducing sugar was also determined by DNS method (78). See appendix A for all details in sugar measurement methods.

7.5 Total nitrogen (TN) and Amino nitrogen (AN) measurement

Total nitrogen was determined by Kjeldahl method using automated nitrogen analyzer (1206 Kjeldahl®System, Tecator AB, Sweden) according to the instructions of the manufacturer. Amino nitrogen was analyzed by formal titration method according to Melnick *et al.* (79), (see Appendix A).

8. Flask cultivation of *E. faecium*

In laboratory scale, experiments were carried out in baffled flasks (Bellco, USA) containing either 100 ml of MRS or modified MRS broth. One ml of frozen stock culture was thawed and transferred into 100 ml of MRS broth in a baffled flask. The pre-culture was incubated at 37°C with shaking at 150 rpm using a rotary type

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shaker (Revco Scientific Inc, USA). The samples were taken at appropriate time intervals during cultivation and then centrifuged at 6,000 rpm for 12 min. Cell pellets were collected for cell growth measurement and the supernatant was collected and stored at -20°C for further analysis of acids, sugars, total nitrogen and amino nitrogen.

9. Investigation of growth promoting factors in protein sources

9.1 Identification of growth promoting factors in protein sources

A flask experiment was performed to check the effect of growth promoting factors in protein sources on the cell growth. In each flask, protein sources were used as energy source and were prepared as the following: DFS, DFS+tryptone, DFS+ meat extract, DFS+meat extract+tryptone, DFS+casamino acid+tryptone, DFS+casamino acid and DFS+tryptophan, based on total nitrogen equal to 0.46 % w/v. In the flask experiment with the mixture of DFS and other protein sources, DFS was prepared based on 2% w/v of yeast extract (TN=0.22% w/v) and the other protein sources were prepared to total nitrogen of 0.24% w/v.

9.2 Effect of vitamins as growth promoting factors

Flask experiment was conducted to investigate the effect of vitamins on promotion of the cell growth. The medium was prepared by using DFS together with vitamins and the amount of vitamins used in 100 ml broth was as the following: 0.1 g biotin, 0.01 g nicotinate, 0.01 g panthothenate, 0.05 g pyridoxine and 0.01 g riboflavin. Total nitrogen in all experiment was prepared at total nitrogen of 2% w/v yeast extract (TN=0.22% w/v).

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9.3 Investigation of alternative protein sources for growth promoting factors.

E. faecium were cultivated in shake flask by using various sources of milk based protein. The protein content of medium in all experiment was adjusted to total nitrogen of 0.46 % w/v and was prepared as follows: skim milk, DFS+skim milk, whey, DFS+whey, DFS+skim milk+whey and DFS+ME+TR. In the flask experiment with the mixture of DFS and other protein sources, DFS was prepared based on 2% w/v of yeast extract (TN=0.22) and the other protein sources were supplemented to yield a final total nitrogen concentration at 0.46% w/v. $CaCO_3$ was added to medium in order to maintained neutral pH during cultivation.

9.4 The optimal ratio of DFS to meat extract and to tryptone for optimal growth of *E. faecium*

The experiment was conducted in shake flask culture. 2% w/v of glucose was supplemented for C-source. Concentrations of meat extract and tryptone were fixed according to ratio in standard MRS medium content while varying the amount of DFS. DFS was prepared in equivalent to total nitrogen of 0.4, 0.8, 1.2, 1.6 and 2.0 g/100 ml of yeast extract, respectively.

10. Investigation of the effect of Browning reaction products on cell growth

10.1 Growth inhibition of *E. faecium* due to Browning reaction products

Shake flask cultivation with standard MRS medium was performed to study the

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effect of Browning reaction products on the cell growth. The medium was sterilized at 110°C, 15 min or 121°C, 15 min and used for the cell culture.

10.2. The effect of phosphate and citrate concentration on formation of Browning reaction products.

Shake flask experiment was performed to investigate the effect of phosphate and citrate concentration on formation of Browning reaction products. The medium used was modified from MRS medium. Concentrations of meat extract and tryptone were calculated from total amount of those utilized in fed-batch experiment (section 10.3), 22.2 and 17.8 g/100 ml, respectively. Di-potassium hydrogen phosphate and di-ammonium hydrogen citrate concentration were varied as follows: 0, 1, 5 and 10 g/100 ml. The medium was sterilized at 121°C for 15 min. Browning reaction products were determined spectrophotometrically at OD_{420}. The sterilized medium was then used to cultivate the cells to study growth inhibition effect of Browning reaction products.

11. Cultivation of *E. faecium* in the fermentor

11.1 Inoculum preparation

A 500-ml baffled flask containing 100 ml MRS medium was inoculated with 1 ml of melted stock culture. The culture was incubated at 37°C at 150 rpm in a rotary shaker for 10 h when most of the cells were considered to be in the exponential growth phase.

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11.2 Cultivation conditions

The fermentation was performed in a 5-L stirred tank bioreactor (model Maestro, BLSL, Biolafitte, France). Temperature was maintained at 37°C. 6M NaOH solution was automatically added into bioreactor to maintain pH at 6.5 (monitored by pH probe, METTLER TOLEDO, Switzerland). Dissolved oxygen in the medium was measured by dissolved oxygen probe (METTLER TOLEDO, Switzerland) and maintained at concentrations above 10% saturation by regulating the stirrer speed and aeration at 300-900 rpm and 0.5-2.0 vvm, respectively.

11.3 Batch cultivation

A 100 ml of pre-culture (section 10.1) of *E. faecium* was transferred to 5-L fermentor with working volume of 2 L. The media used in the fermentor were MRS or modified MRS medium. For modified MRS medium, digested fish soluble (DFS) was used instead of yeast extract, on the basis of an equal total nitrogen. The cultivation conditions were controlled as mentioned above (section 10.2). During cultivation, culture broth sample was taken every 1 h for measurement of cell density, total sugars, and acids. Cell cultivation was allowed to proceed until the cell growth (determined by OD_{660}) stopped.

11.4 Fed-Batch cultivation

Cell cultivation was performed in modified MRS medium. The composition of medium used for batch part prior to feeding was as follows (g/L): tryptone, 15; meat extract, 12; glucose, 5; di-potassium hydrogen phosphate, 2; tween 80, 2; di-

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ammonium hydrogen citrate, 5; sodium acetate, 2; $MgSO_4.7H_2O$, 0.3; $MnSO_4.H_2O$, ;0.06 and DFS was prepared to equal total nitrogen of 4 g yeast extract. Feeding was started after the glucose in the medium was depleted (about 4 h after cultivation) and also as indication, at that time the concentration of dissolved oxygen as well as pH value started to increase. The feeding medium was fed according to constant feeding or exponential feeding strategy. For exponential feeding strategy, glucose and protein source were fed according to following feeding equation:

$$F = V_0/S_0 * 1/Y_{x/s} * X_0 * \mu * e^{\mu t} \quad (69)$$

where F is the feed rate of glucose or protein source (L/h), V_0 is the initial volume (L), and S_0 is the substrate concentration in the feeding medium (g/L), $Y_{x/s}$ is the growth yield for sugar or protein calculating from experiments (*e.g.* OD_{660}/utilized glucose), X_0 is the initial cell density (OD_{660}), μ is the set point of specific growth rate (h^{-1}) used for manipulating the feed rate, and t is the culture time (h). Actual specific growth rate (μ) was also determined every one hour using cell density (OD660). The feeding rate was controlled by manual adjustment using two peristaltic pumps for feeding of glucose and protein (pump model 101 U/R, Watson-Marlow Inc., England and pump model SJ 1211, Atto Corperation, Japan) which covered the full range of feed rates required. The pump feed rates were calibrated with water using electrical weighing (model PT3100, Sartorius, Germany), before use.

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12. Sterilization

The media in flask and fermentor experiments were sterilized at 121°C for 15 min, with the exception for the experiments that investigated the effect of Browning reaction, the medium was autoclaved at 110°C for 15 and 10 min respectively, and glucose and protein source were autoclaved separately.

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CHAPTER IV

RESULTS

1. Flask cultivation

1.1 Effect of aeration on growth and lactic acid production of *E. faecium* grown in different C-sources.

In the literature review it is reported that some lactic acid bacteria can grow under aerobic conditions and yield higher cell mass (53, 80). So in this experiment we investigate the effects of aeration on growth and lactic acid production *of E. faecium*. In this case cells were cultivated in shaken and non-shaken flasks for comparison and glucose as well as yeast extract were used as carbon source.

It was found that in case of using glucose as carbon source, cell growth and lactic acid production in non-shaken culture were not significantly different from shaken culture (Fig. 1a, 1b). Lactic acid was continually produced along with the cell growth. In contrast, in case of using yeast extract as complex carbon source (Fig. 1a, 1b), aeration by shaking result in increasing of the cell growth yield, while lactic acid production was lower. Furthermore, it was also found that under aeration condition, lactic acid that was produced at the beginning of the cultivation was utilized again after a period of cultivation (5 h) and produced minimum concentration of lactic acid at the

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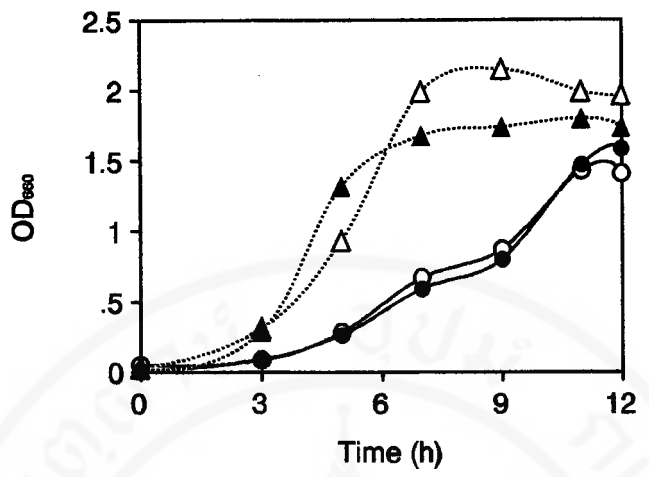

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Fig.1a Cell growth in shaken and non-shaken flask cultivation using glucose and yeast extract. Symbols: —○— 2% glucose, shaken; —●— 2% glucose, non-shaken; ···△··· 2% yeast extract, shaken; ···▲··· 2% yeast extract, non-shaken.

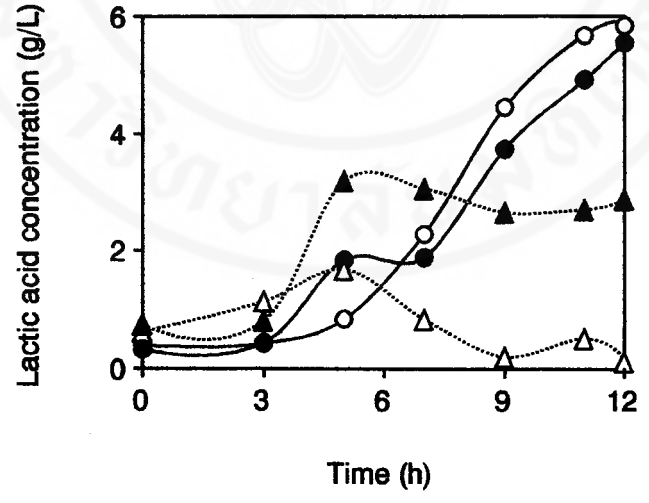

Fig.1b Lactic acid production in shaken and non-shaken flask cultivation using glucose and yeast extract. Symbols: —○— 2% glucose, shaken; —●— 2% glucose, non-shaken; ···△··· 2% yeast extract, shaken; ···▲··· 2 % yeast extract, non-shaken.

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final (Fig 2b, 2% yeast extract with shaken). On the other hand, in case of non-shaken condition the produced lactic acid was retained at a certain level without re-utilization. A further experiment with a higher yeast extract concentration was performed to ascertain this phenomenon. Cells were cultivated in shaken and non-shaken flasks. The results was confirmed that better cell growth was observed in shaken flask than that of non-shaken flasks and lactic acid was re-utilized only in case of shaken flasks (Fig. 2a, 2b).

1.2 An alternative C-source for cultivation of *E. faecium*

Several carbon sources were investigated for cultivation of *E. faecium* (Table 3). It was found that yeast extract was the best complex carbon source and a suitable carbon source. It gave the maximum viable cell count while the minimum lactic acid production. In case of using glucose and molasses as carbon source, lower viable cell count was achieved compared with that of using yeast extract. It is possible that the lower viable count would be due to the inhibitory effect of produced lactic acid. Molasses showed slightly lower viable count compared to that of glucose. This might result from inhibitors present in the molasses, such as heavy metals. On the other hand, glycerol showed the lowest viable count and lactic acid formation of all which imply the difficulty of glycerol assimilation by *E. faecium*.

Fig. 3 demonstrates that cell density as well as viability could be improved by increasing concentration of yeast extract. The results also implied that cell density could be further increased by supplying higher amount of yeast extract. In addition, lactic acid concentration in the culture broth was lower than 0.5 g/L in all cases (data

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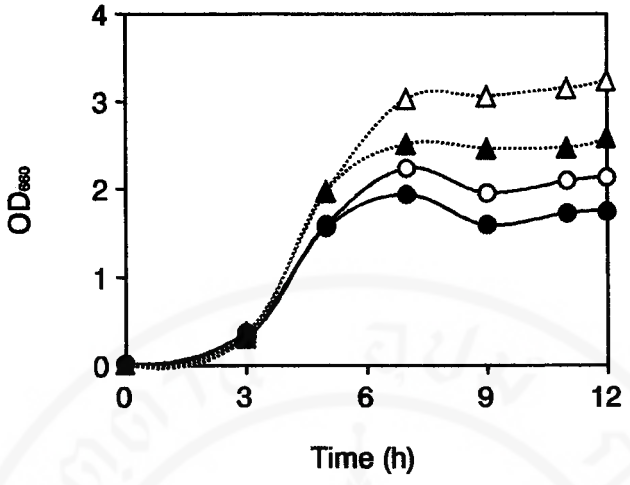

Fig. 2a Cell growth in shaken and non-shaken flask cultivation using various concentrations of yeast extract. Symbols: —○— 2% yeast extract, shaken; —●— 2% yeast extract, non-shaken; ···△··· 4% yeast extract, shaken; ···▲··· 4% yeast extract, non-shaken.

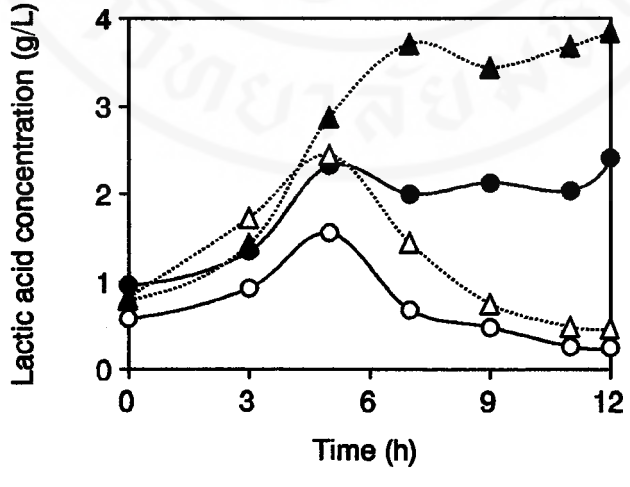

Fig.2b Lactic acid production in shaken and non-shaken flask cultivation using various concentrations of yeast extract. Symbols: —○— 2% yeast extract, shaken; —●— 2% yeast extract, non-shaken; ···△··· 4% yeast extract, shaken; ···▲··· 4% yeast extract non-shaken.

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Table 3 Cultivation of *Enterococcus faecium* using various carbon sources

Carbon source[1] (2% w/v)	Total viable cells count (cfu/ml)±SD[2]	Lactic acid (g/L)
Glycerol	$4.8\pm0.2\times10^{8}$	ND[4]
Molasses[3]	$7.2\pm0.2\times10^{8}$	5.5
Glucose	$1.2\pm0.2\times10^{9}$	5.8
Yeast Extract[5]	$2.3\pm0.1\times10^{9}$	0.5

[1] Modified MRS medium by substituting the glucose with mentioned carbon sources.

[2] Standard deviation of duplicated experiments

[3] prepared according to a basis of equal total sugar with 2% glucose

[4] ND is not determined

[5] prepared by using 2% w/v of yeast extract

not shown) even though a high amount of yeast extract was introduced. Moreover, by using 12% yeast extract, the viable cell (1.58×10^{10} cfu/ml) was about ten times higher than that of 2% glucose (1.24×10^{9} cfu/ml).

Though, yeast extract was proved to be the most suitable protein source and was utilized as carbon and nitrogen sources for high cell density cultivation of *E. faecium*, it still was not economically for lactic acid bacteria production in large industrial scale where cost of raw materials is an important consideration. Another source of carbon which has similar characteristics to yeast extract but is cheap and available in the country would be more preferential.

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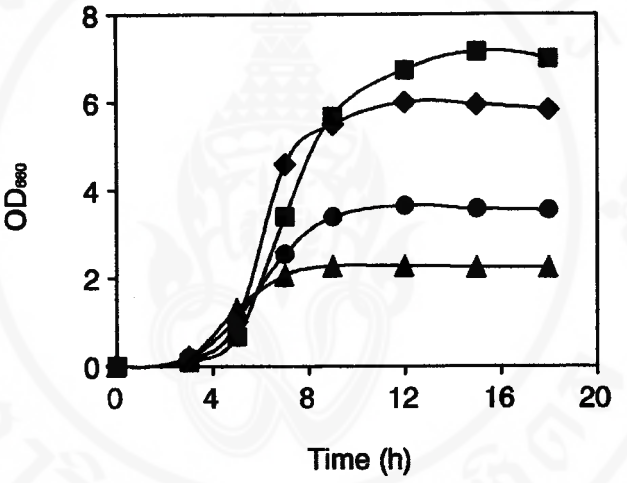

Fig.3 Growth of *Enterococcus faecium* in shaken flask cultivation using various concentrations of yeast extract. Symbols: -▲- 2%w/v yeast extract; -●- 4% w/v yeast extract; -◆- 8% yeast extract; -■- 12% w/v yeast extract.

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1.3 Replacement of other protein sources for yeast extract in cultivation of *E. faecium*

1.3.1 Using soy bean meal hydrolysate (SBMH) as C-source

Soy bean meal acid hydrolysate, derived from soy sauce factory was used for cultivation of *E. faecium*. The major components this hydrolysate are amino acids. During soy bean meal hydrolysis, concentrated hydrochloric acid was which resulted in the formation of NaCl in the neutralization step. This causes the high salt content in SBMH, about 10-15 g/L, hence, electrodialysis (MICRO ACILYZER, Asahi chemical, Kawazaki, Japan) was applied for removal of the salt. In this case low salt content soy bean meal hydrolysate, or electrodialysed soy bean meal hydrolysate (ED-SBMH) was obtained and was used for cultivation of the cells.

The experiment was performed by using 2% w/v yeast extract as control, compared with 5% w/v of electrodialysed and non-electrodialysed soy bean meal hydrolysate (NED-SBMH) which has an equal total nitrogen to 2% w/v yeast extract. The result is shown in Fig 4. It was found that SBMH could be used as carbon source for cultivation of the cells. However, growth rate was low in comparison with yeast extract. This might result from lack of some essentail growth promoting factors which are necessary for growth or the present of some inhibitors in SBMH. A further experiment was performed by increasing concentration of ED-SBMH from 5 to 10 % to study the effect of inhibition and 0.4 % of yeast extract was added as growth supplement. The results (Fig. 4) show that cell density of 10% ED-SBMH was higher than that of 5% ED-SBMH. Furthermore, in case of 10% ED-SBMH supplemented with yeast extract, higher cell growth was observed than that without yeast extract

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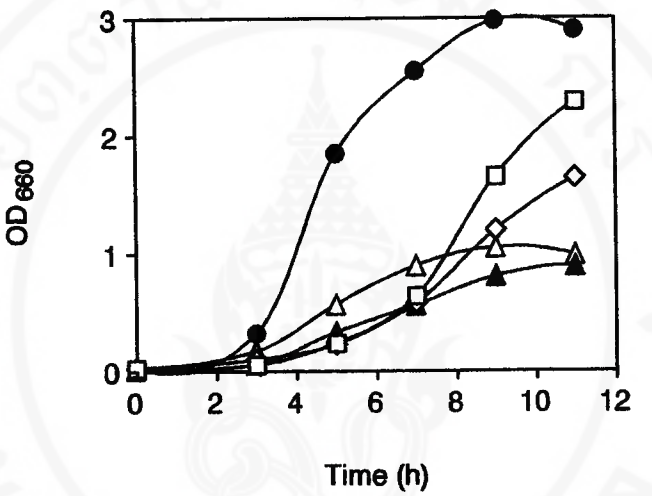

Fig.4 Flask cultivation of *E. faecium* using soy bean meal hydrolysate (SBMH) as C-source. The total protein content in medium was prepared according to equivalent total nitrogen of 2% yeast extract. Symbols: -△- 5% electrodialyzed SBMH; -▲- 5% non-electrodialyzed SBMH; -◇- 10% electrodialyzed SBMH; -●- 2% yeast extract; -□- 10% electrodialyzed SBMH + 0.4 g yeast extract.

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supplement. This showed that cell growth was limited by the carbon source supplementation and some essential growth promoters.

It was also found that when using ED-SBMH as carbon source, cell growth was higher than that of NED-SBMH. This result showed the significant effect of salt inhibition on the cell growth. An additional experiment was performed to study the effect of salt concentration on the cell growth. Various concentrations of NaCl were added to MRS medium and the result of cell growth is shown in Fig 5. The higher the salt concentration, the more cell inhibition was observed. In addition, cell inhibition appeared when salt concentration was equal to 2%. This result set the limited amount of salt in the preparation of protein source used for cell growth.

1.3.2 Process modification for preparation of SBMH

In this study 0.17 M HCl was used in soy bean meal hydrolysis to reduce salt content from the neutralization step. The digestion times were 3 and 9 h, respectively. Cell cultivation was performed by using soy bean meal digested at 3 and 9 h, (SBMH 3 h and SBMH 9 h respectively) and compared with commercial yeast extract SBMH obtained from factory. Total nitrogen was adjusted to be equal to 2% yeast extract in all cases. Fig. 6 indicates that yeast extract was still the best carbon source for cultivation of the cells. However, better cell growth could be observed from SBMH (salt 0.4%) than that of SBMH which contains a higher salt content (approx. 1.5%). Furthermore, soy bean meal digested at 9h could promote higher cell growth than that obtained from 3h digestion. This might be resulted from a higher AN/TN ratio (amino nitrogen to total nitrogen ratio which may indicate the high content of free amino acid

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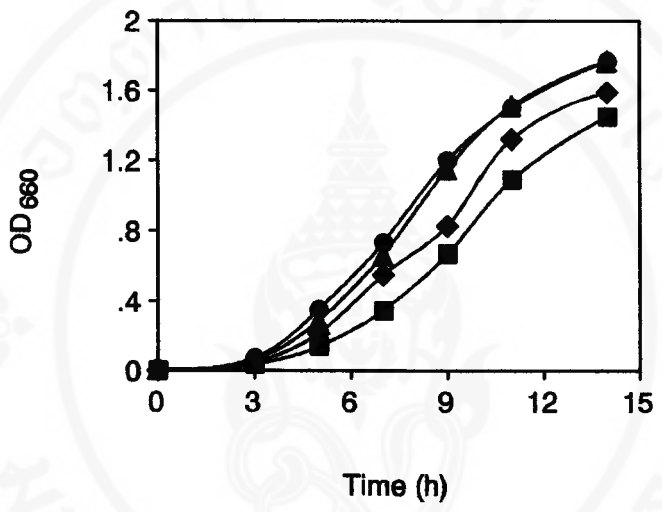

Fig.5 Effect of salt concentration on growth of *E.faecium.* MRS medium was used as a basal medium component which was supplemented with salt at different concentration. Symbols: ●— 0% NaCl; ▲— 1% NaCl ; ◆— 2% NaCl; ■— 3% NaCl.

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and short peptides) in soy bean meal digested at 9 h than at 3 h digestion.

1.3.3 Effect of protein digestion on cell growth

Further experiment was carried out to confirm the effect of protein digestion on cell growth. The enzyme digested meat extract was used for cultivation of the cells compared with non-digested meat extract based on equal total nitrogen. Fig. 7 showed significant cell growth in digested meat extract compared with non-digested meat extract. This result may imply that cells can use carbon source in form of free amino acids and shorter peptides better than in form of long peptides.

1.3.4 Fish soluble as alternative protein source for *E. faecium* cultivation

Apart from soy bean meal which is cheap and widely available in the country, fish soluble extract also has this advantages. Fish soluble extract, a slurry form, is the by-product derived from tuna canning industry. It contains percent solid (%w/w) of 58.9 and low sugar content which benefit for lowering lactic acid production by the cells. From AN/TN analysis it was found that the AN/TN of fish soluble extract was low which indicated that this protein source contains long peptide chains and might not suitable for cultivation of the cells. The digestion process was introduced to increase the fraction of shorter peptides and amino acids. Acid digestion was preferred to enzyme digestion because of its effective and low cost, even though some salt will be formed. Hence, digested fish soluble (DFS) was tested for its applicability in cell cultivation and compared with non-digested fish soluble and yeast extract, based on equal total nitrogen.

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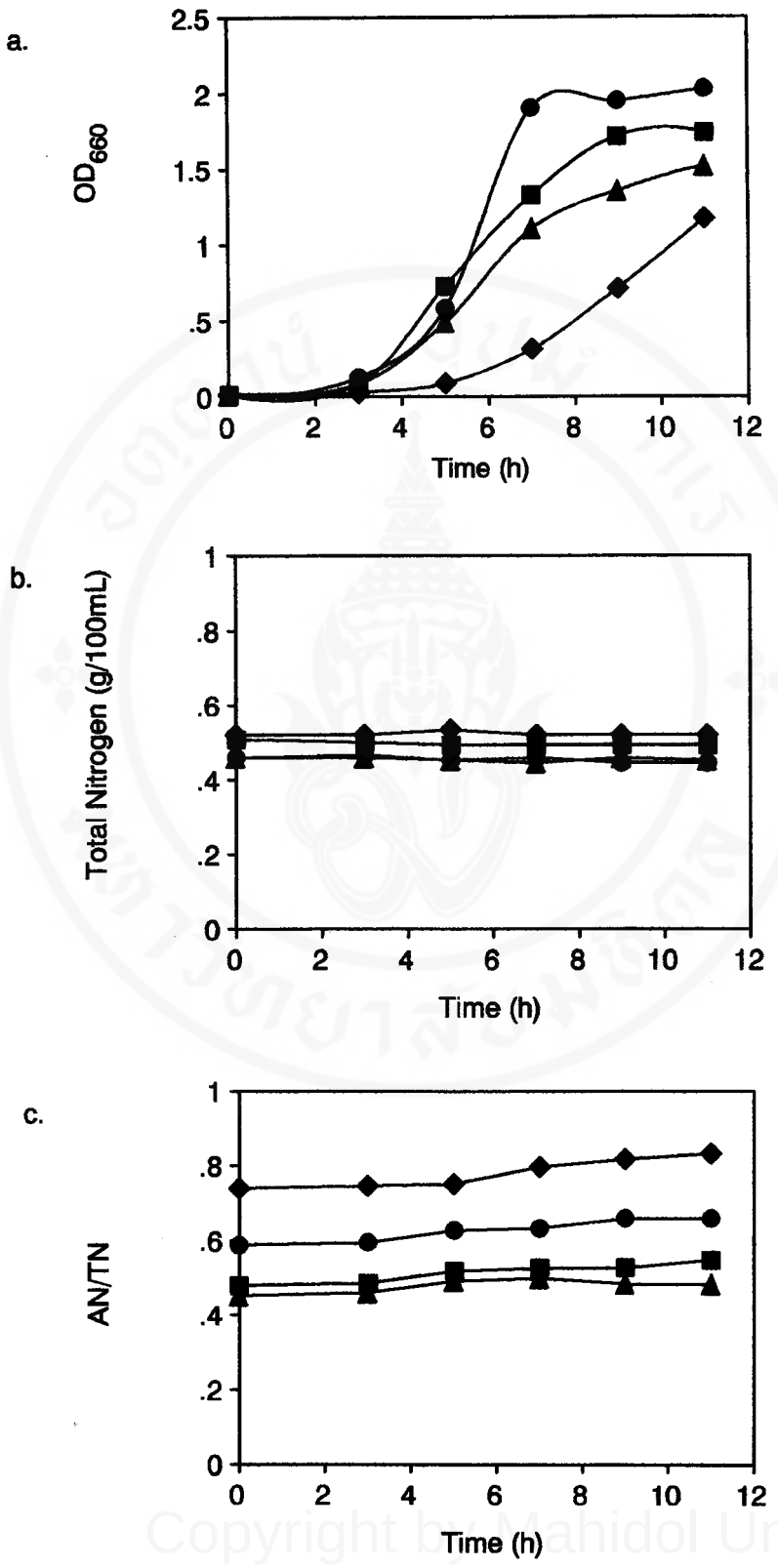

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Fig.6 Cultivation of *E. faecium* using acid hydrolysed soy bean meal in comparison with yeast extract. The medium was prepared on the basis of equivalent total nitrogen in 2% yeast extract. Symbols: ▲ 3h hydrolysed soy bean meal; ■ 9h hydrolysed soy bean meal; ● yeast extract; ◆ soy sauce.

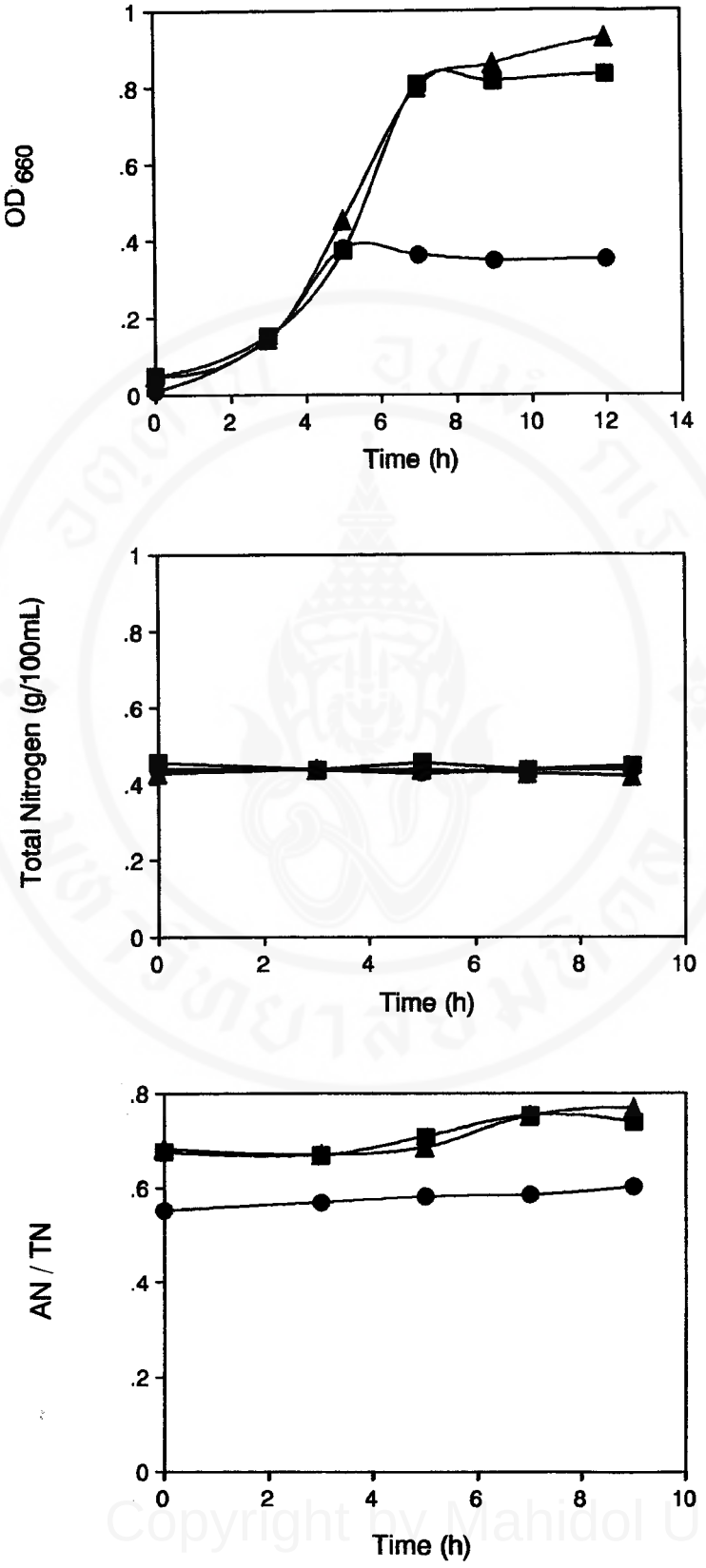

Fig. 7 Cultivation of *E. faecium* using enzymed digested meat extract as C-source. The medium were prepared on the basis of equivalent total nitrogen to 2% yeast extract. Symbols: ●— Meat extract; ▲— 17h Enzyme digested meat extract; ■— 35h enzyme digested meat extract.

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It was found that fish soluble could be used for cultivation of the cells (Fig. 8). Digested fish soluble could provide even higher cell yield than that of yeast extract at equivalent of total nitrogen. Moreover, digested fish soluble at total nitrogen equivalent to 2 and 4% yeast extract provided about two times higher cell growth than those of non-digested fish soluble at equal TN content. However, in case of digested fish soluble at TN equivalent to 8% yeast extract (Fig. 8c), lower cell growth could be observed. This was considered as inhibition effect which might result from the high salt content (4.65%) in culture broth. Since the acid digestion process was investigated, a lot of salt (ca. 30% w/v) was obtained in the neutralization step. Nonetheless, at lower digested fish soluble content, cell growth could be improved by increasing amount of digested fish soluble.

The growth yield obtained when various protein sources were utilized as carbon source, when compare based on equal total nitrogen to 2% yeast extract, were summarized in Table 4. For the growth yield (expressed as the ratio of OD_{660}/total nitrogen), acid digested fish soluble protein gave the highest value, about 11.72, followed by yeast extract, 9.38 and acid digested soy bean meal, 7.9. This indicated that acid digested fish soluble was the most suitable carbon source for cultivation of *E. faecium*. However, it should be noted that the AN/TN-ratio could be considered as rough estimation parameter to determine protein quality in digestion, especially only from the same protein source. Furthermore, optimal AN/TN-ratio for growth utilization would be depended on type and basic composition of the protein source.

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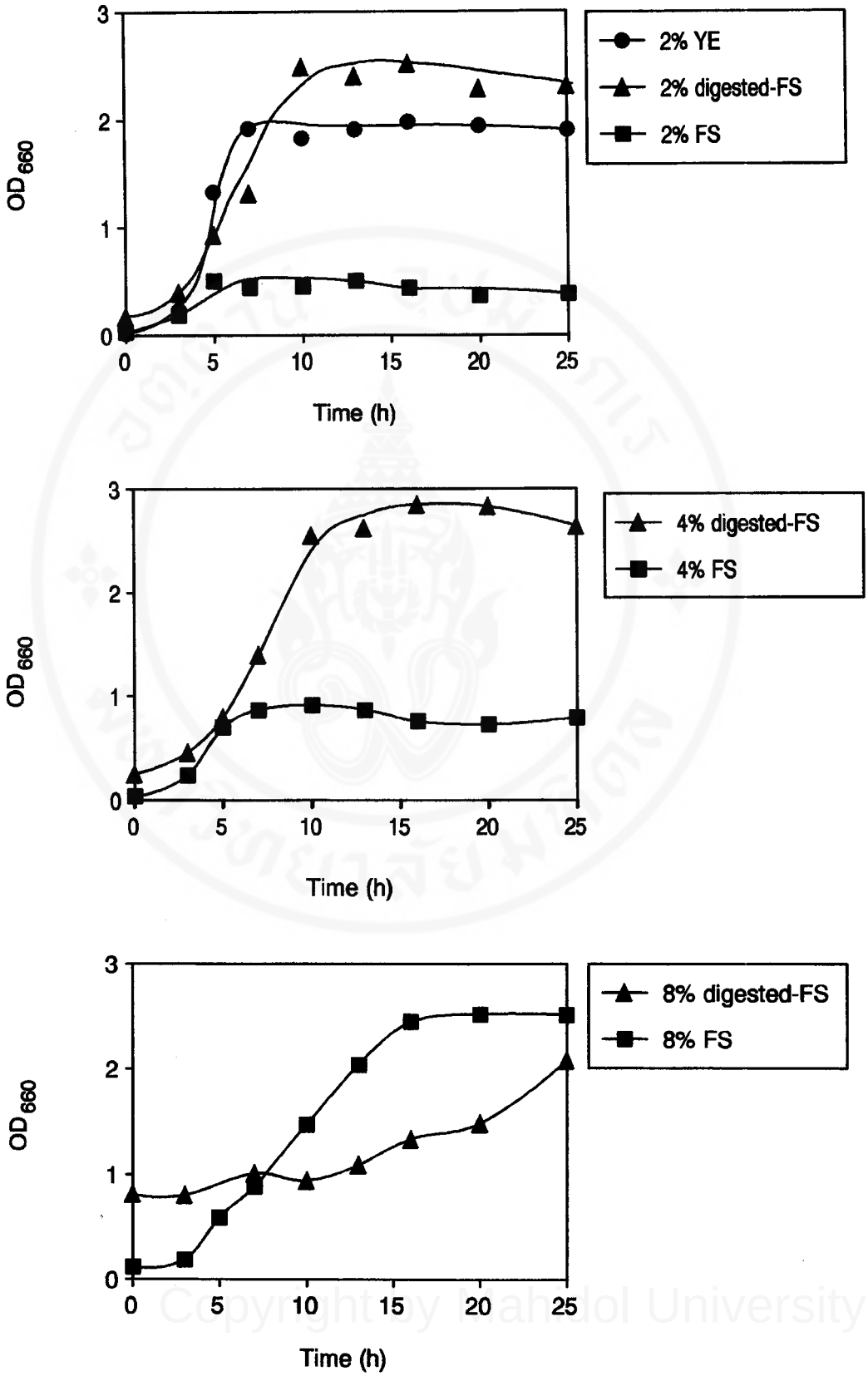

Fig.8 Cultivation of *E. faecium* using fish soluble protein as C-source. The media of 2,4 and 8% were prepared on the basis of equivalent total nitrogen to those of 2, 4 and 8% of yeast extract.

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Table 4. Comparison of the cell yield obtained by using different types of carbon souces[1].

Carbon-source	Cell density, $OD_{660(max)}$	$OD_{660\ max}/TN^2$	AN/TN
Yeast extract	2.03	9.38	0.64
3h SBMH[3]	1.52	6.90	0.34
9h SBMH	1.74	7.90	0.36
NDFS[4]	0.50	2.33	0.26
DFS[5]	2.52	11.72	0.70

[1] The medium used was modified-MRS medium where 2% w/v glucose was omitted and 0.4% w/v yeast extract was substituted by various carbon source in equivalent to 2% w/v yeast extract.

[2] TN was calculated based on total nitrogen of 2% yeast extract.

[3] SBMH = Soy bean meal hydrolysate with stated digested period.

[4] NDFS = Non digested fish soluble protein.

[5] DFS = Digested fish soluble.

1.3.5 Effect of degree of protein digestion on cell growth

Previous experiment suggested that fish soluble with shorter peptide chains may preferable for cell growth than that with longer peptide chains. Hence, further experiment was investigated by varying degree of digestion of fish soluble (by digestion time) to study whether it can provide more short peptides and thus cell growth. Fish soluble was digested with 5M HCl and the digestion time was varied from 0 to 5 h. It was then used to cultivate the cells base on equal total nitrogen. As can be seen from Fig. 9a. AN/TN-ratio was increased with the digestion time. However, after 1h of digestion the AN/TN-ratio was only slightly increased. Fig. 9b.

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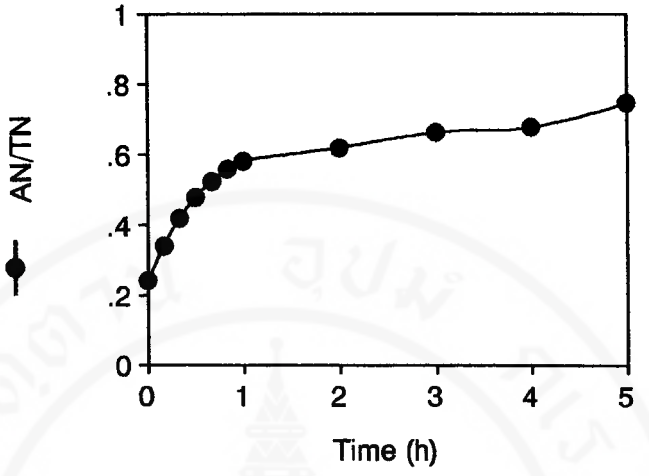

Fig.9a. Time course of AN/TN ratio in sample of fish soluble digested with 5 M HCl. The sample were neutralized to pH 7 by NaOH before analysis of AN and TN.

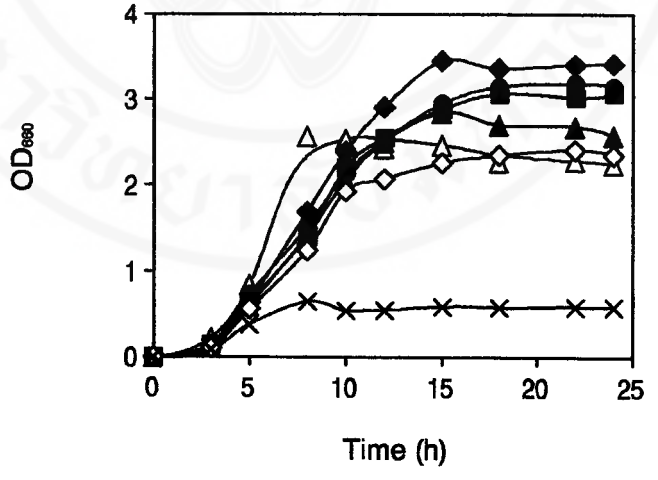

Fig. 9b. Growth of *E. faecium* in fish soluble digested at 5 M HCl for various times. The medium was adjusted to have equal total nitrogen of 2% yeast extract. Symbols: —△— Yeast extract; —×— Non-digested fish soluble; —●— ; —◆— ; —■— ; —▲— ; —◇— ; Fish soluble digested at 10 min, 30 min, 1h, 3 h, and 5 h, respectively.

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shows the growth of *E. faecium* when cultivated using various digested fish soluble. With a digestion time of 30 min, which provided an AN/TN-ratio of 0.5 (considered to contain mainly short peptides), the highest cell density was reached. However, fish soluble digested for 5 h (AN/TN-ratio of 0.7 implying a large pool of free amino acids) poorly supported the growth of *E. faecium.*

1.3.6 Digested fish soluble as sole protein source for cultivation of *E. faecium*

Fish soluble was tested for its capability to be used as sole protein source. The experiment was performed by using digested fish soluble as sole protein source and digested fish soluble supplemented with other protein sources such as meat extract and tryptone for cultivation of *E. faecium*. The protein content of medium in all experiments was adjusted to be equivalent to the total nitrogen of 0.46 % w/v. As shown in Fig. 10 when using digested fish soluble as a sole protein source, cell density was lowest compared with those of digested fish soluble together with other protein sources. This indicated that digested fish soluble might lack some growth-promoting factors necessary for *E. faecium* that are found in meat extract and tryptone. Previously, it was reported that some of essential amino acids, vitamins and trace elements are necessary for growth of lactic acid bacteria (81-82). Hence, mixed amino acids (casamino acids), tryptophan and 5 vitamins which were reported to support cell growth were added into digested fish soluble to investigate their capability to promote cell growth in this study. These were compared with digested fish soluble supplemented with meat extract and tryptone. The result (Fig.10) showed that

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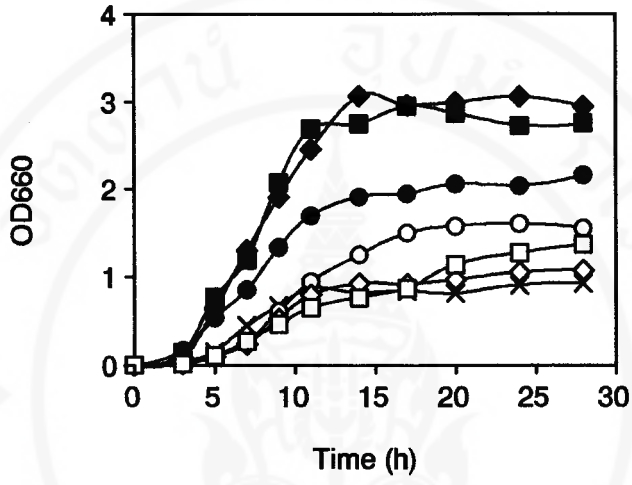

Fig.10 Comparison of growth of *E. faecium* using fish soluble supplemented with various sources of protein source. The medium in all experiment was prepared at equal total nitrogen of 0.46% w/v . Symbols: DFS; DFS+TR; DFS +ME; DFS+ME+TR; DFS+CA+Trp; DFS+CA; DFS+Trp. Abbreviations, DFS= Digested fish soluble, TR=Tryptone, ME=Meat extract, CA= Casamino acids, and Trp=Tryptone.

Note: In the flask experiment with the mixture of DFS and other protein sources, DFS was prepared base on 2% w/v of yeast extract (TN=0.22) and the other protein sources were prepared to total nitrogen of 0.24 % w/v.

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supplement of casamino acids (which contain most free amino acids except amino acid which has aromatic ring residue *e.g.* tryptophan) could not significantly improve cell growth. However, cell growth was improved by supplying with tryptophan. This could be suggested that tryptophan might be essential amino acid which necessary for supporting cell growth. In case of vitamins supplements (Fig. 11), cell density was much lower than that of using meat extract as growth supplement. From the experiment, it can be concluded that the most suitable growth promoter for *E. faecium* when concomitant with digested fish soluble were much contained in meat extract and tryptone, respectively.

1.3.7 Utilization of other alternative protein sources as growth promoting factors for cultivation of *E. faecium*

Even though meat extract and tryptone were shown to be most suitable for supporting cell growth, for industrial scale cultivation these protein sources might not be suitable because of their high cost. It would be beneficial to find other protein sources that showed these characteristics of growth promoting factors. As we know that lactic acid bacteria are generally used in the production of diary products like yogurt and cheese. The main by-product from cheese industry is whey which is considered to be nutrients-rich substrate and might be suitable for cultivation of the cells. Thus, fresh whey from cheese process was investigated for possibility to replace meat extract and tryptone in cultivation of *E. faecium*. Whey and skim milk was used as carbon source together with digested fish soluble for cultivation of the cells and compared with digested fish soluble supplemented with meat extract and tryptone.

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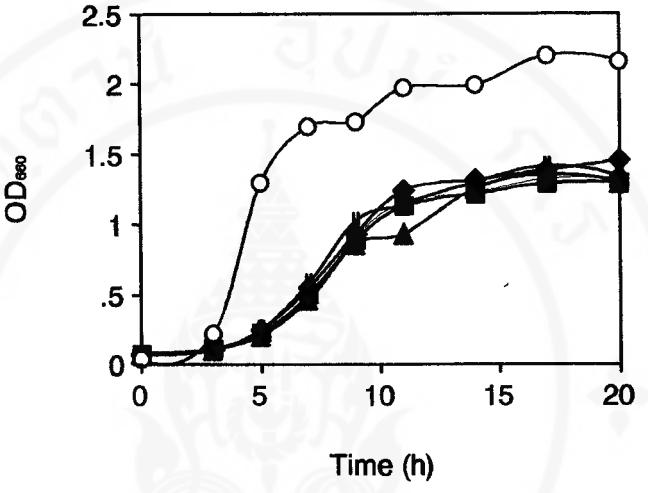

Fig. 11 Effect of vitamins addition on growth of *E. faecium* . The medium in all experiment was prepared at equal total nitrogen of 2% yeast extract. Symbols; —▲— DFS+Biotin; —●— DFS+Nicotinate; —◆— DFS+Pantothenare; —■— DFS+Pyridoxine; —‖— DFS+Riboflavin; —○— DFS+Meat extract. The amount of vitamins in 100 ml broth use was as following: 0.1 g biotin, 0.01 g nicotinate, 0.01 g pantothenate, 0.05 g pyridoxine, and 0.01 g riboflavin.

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As shown in Fig. 12a, viable cell counts when using fresh whey as a sole C-source was about 5 times higher than that of using skim milk, $2x10^9$ and $4x10^8$ cfu/ml, respectively. However, when using fresh whey together with digested fish soluble and skim milk together with digested fish soluble, viable cell was much improved as compared with just merely fresh whey and skim milk. Among these, whey supplemented with digested fish soluble gave the highest viable cell of all, $7.85x10^9$ cfu/ml. However, lactic acid production was high as well, 18 g/L (Fig. 12b). This resulted from high content of lactose in whey that can promote lactic acid production.

Because of the low total nitrogen in fresh whey which directly obtain from the cheese process, 0.13 %w/v, thus, whey in powder form, total nitrogen of 1.14%w/w, was used to increase total nitrogen content. The experiment was performed by comparing fresh whey obtained from the cheese process with whey in powder form, based on equal total nitrogen. It was found that fresh whey in solution form gave higher viable cell than that of whey in powder form (Fig. 13). It is possible that fresh whey in solution form might contain more nutritional factors than in powder form, which have already passed drying process. During drying process some necessary growth-promoting factors or vitamins containing in whey might be destroyed. However, because of the total nitrogen of whey in solution form was low it might be useful to concentrate whey by evaporation at low temperature.

2. Cell cultivation in fermentor

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In the flask experiment, using protein as carbon source in cultivation *E. faecium* under aerating conditions, has merit in improving cell growth and the lower

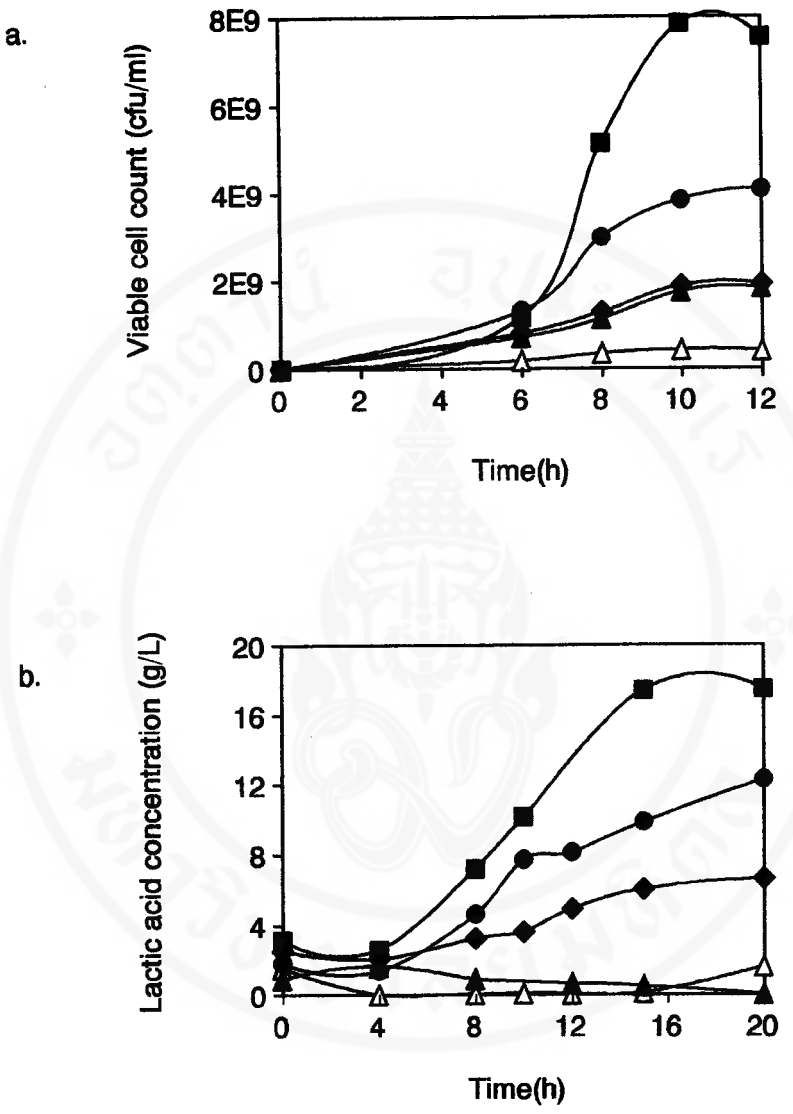

Fig.12 a) Growth of *E. faecium* on various sources of milk based protein.[1] The protein content of medium in all experiment was adjusted to be equivalent in total nitrogen at 0.46 % w/v.[2] b) Lactic acid formation .Symbols: —△— Skim milk; —●— Skim milk+DFS; —◆— Whey; —■— Whey+DFS, —▲— DFS+ME+TR.

Note: 1) Calcium carbonate was added to medium in order to maintain neutral pH during cultivation.

2) In the flask experiment with the mixture of DFS and other protein sources, DFS was prepared base on 2% w/v of yeast extract (TN=0.22) and the other protien sources were prepared to total nitrogen of 0.24% w/v.

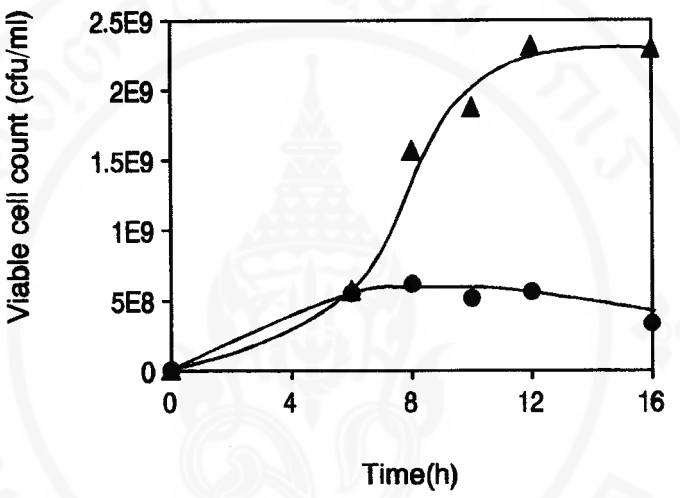

Fig. 13 Growth of *E. faecium* in modified MRS medium using whey in fresh solution and powder form as C-source. The media was prepared base on total nitrogen to that in 2% w/v yeast extract. Calcium carbonate was added to medium in order to maintain neutral pH during cultivation. Symbols: —▲— whey in solution form; —●— whey in powder form.

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the lactic acid production by the cells. The highest viable cell count, $1.58x10^{10}$ cfu/ml, was obtained with 12% w/v yeast extract. However, according to total nitrogen analysis in culture broth at the beginning and the end of cultivation, the total nitrogen utilized was not significantly decreased (Fig. 14). Thus, in order to achieve high cell density of *E. faecium*, a large amount of protein source would have to be applied. Furthermore, to promote protein utilization of the cells more efficiently, detailed study of the peptide size which is suitable for cell uptake was necessary.

In contrast, glucose, a common carbon source, can be easily and completely utilized by the cells. Nevertheless, a large amount of lactic acid production would be observed. To cope with this problem, it is well-known that fed-batch cultivation technique can be used to control nutrient feeding to provide enough nutrients required for cell growth but less for acid production. Thus, it is possible to control supplementation of glucose by using fed-batch technique. According to this, lactic acid production by the cells would be suppressed. So, fed-batch technique was investigated for its applicability in high cell density cultivation of *E. faecium* in the fermentor together with controlling of pH, aeration rate and agitation speed.

It could also be noted that in the previous experiment whey can effectively replace meat extract and tryptone in the cultivation of *E. faecium*. Furthermore it was not only cheap and available in country, its usage also considered as a mean to reduces the biological oxygen demand (BOD) of the waste. However, one major problem that could be observed was the precipitation of protein in whey after heat sterilization. This would interfere with measurement of cell density by spectophotometric method. Since the monitoring of cell growth by spectrophotometric

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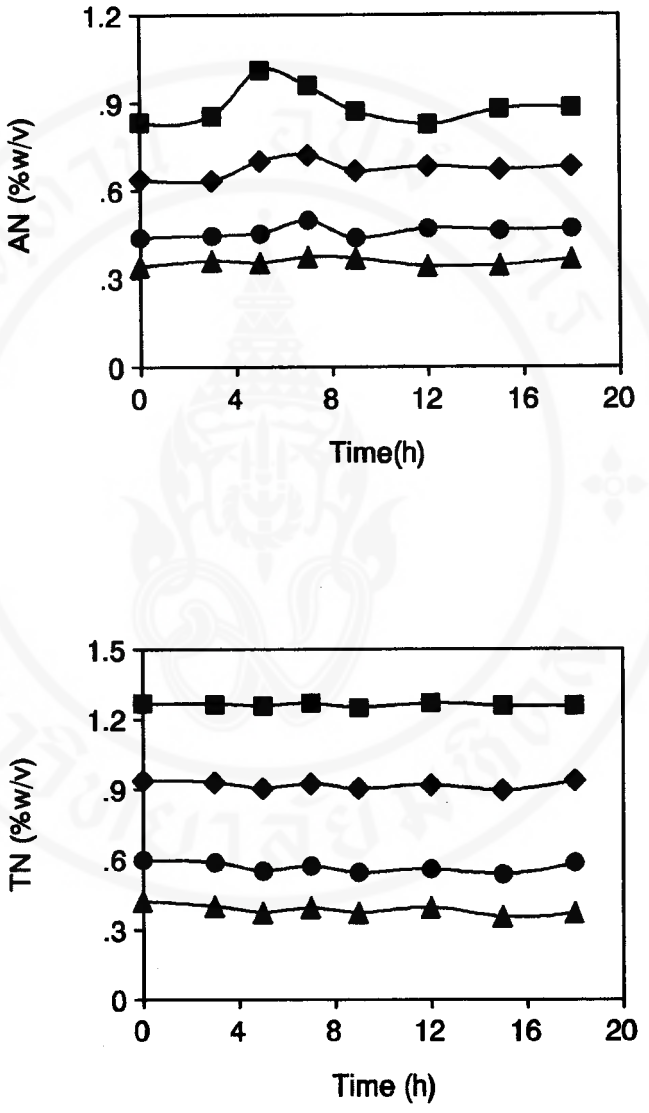

Fig. 14 Time course of amino nitrogen and total nitrogen in supernatant of culture broth using yeast extract at different concentration as C-source instead of glucose in MRS based medium. Symbols: —▲— ; —●— ; —◆— ; —■— ; 2%, 4%, 8%, and 12% yeast extract, respectively.

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method was one important parameter during cultivation of the cells. Likewise, whey was also contain high sugar content, total sugar 40-50 g/L, which could promote lactic acid production by the cells. According to these facts, it would be too complicate to use whey as protein source for the model study in the fermentor. Hence, meat extract and tryptone were used in stead of whey together with digested fish soluble for cultivation of cells in the fermentor. This would facilitate cell measurement and control of lactic acid production.

2.1 Batch cultivation of *E. faecium*

At first, cultivation of *E. faecium* was carried out batchwise in the pH-controlled fermentor. Cells were cultivated in standard MRS medium and were compared with that of the flask experiment. In this case, cell growth as well as product formation were monitored throughout the experiment. It was found that cell growth (OD_{660}) was higher than that obtained from flask cultivation, 7.0 and 1.9, respectively (Fig. 15). In addition, when cultivate the cells in batch cultivation using digested fish soluble in modified MRS medium (medium composition is shown in appendix B, Table 1), higher cell growth could be observed (OD_{660}=13.4) and lactic acid concentration was reduced after glucose depletion (Fig.16). This result was in full agreement with the previous flask experiment which implied that lactic acid was re-utilized after a certain time. This would then result in lowering lactic acid concentration at the end of cultivation. However, no cell growth could be observed at this stage.

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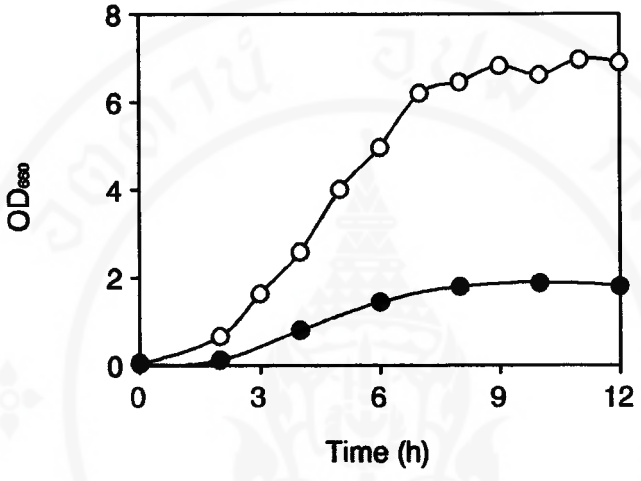

Fig. 15 Growth comparison of *E. faecium* cultivated in shaken flask and pH controlled fermentor. Symbol: —●— shaken flask, —○— ; pH controlled fermentor.

Note: Standard MRS medium were used as cultured medium in both experiment.

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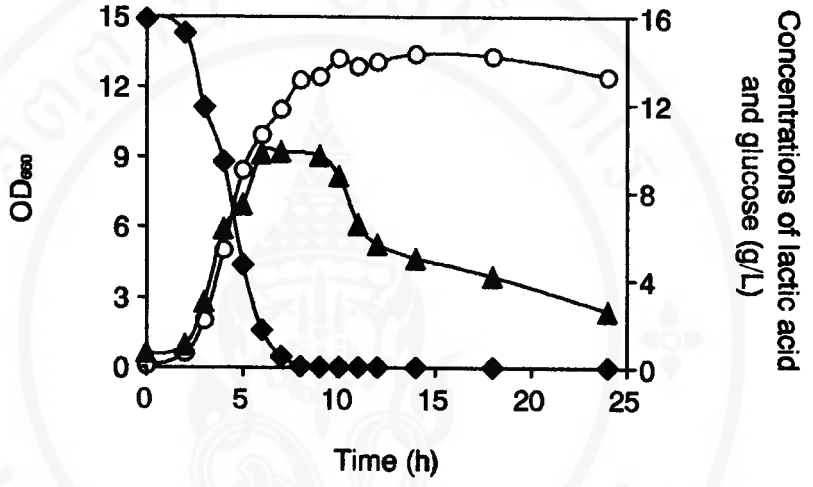

Fig. 16 Time course of growth, lactic acid production and glucose consumption of *E. faecium* in batch cultivation using digested fish soluble protein in modified MRS medium. Cultivation was performed in fermentor with controlling of pH, temperature and dissolved oxygen. Medium composition is shown in Table 1 in appendix B. Symbols: —O— OD_{660}, —▲— lactic acid concentration (g/L), —◆— glucose consumption (g/L).

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2.2 Fed-batch cultivation of *E. faecium*

2.2.1 Fed-batch cultivation with constant feeding strategy

Fed-batch operation was carried out using modified MRS medium as starting medium and glucose solution (245 g/L) as feeding medium. Starting medium composition is shown in Table 1 (appendix B). In this case glucose was limited by feeding at constant feed rate to avoid over production of lactic acid. The feed was not added until the glucose in the medium was depleted (when the pH value as well as dissolved oxygen started to increase). It was found that cell growth increased along with the glucose feeding (from 4-12 h), (Fig. 17). According to glucose analysis, glucose concentration in the medium remained nearly zero throughout the feeding period. Meanwhile, lactic acid was slightly decreased after 7 h of cultivation even glucose was continually fed. Furthermore, after 10 h of cultivation the cell density tend to level off even though there was continued supply of glucose. This implied that using merely glucose as feeding medium was not enough to support growth of *E. faecium*. Hence, it was considered that the combination between protein source and glucose might prolong cell growth than with glucose only. The mixture of glucose and protein source was fed continually at constant feed rate (medium composition is shown in Table 2, appendix B), from 4-18 h, the growth of cells can prolong during feeding period and was terminated at 18h when both nutrient sources were exhausted. Likewise, cell density was nearly two times higher than that of experiment with glucose feeding only, OD_{660} of 23.4 and 12.6, respectively (Fig. 18a). This indicated that protein source was necessary for cell growth, especially in the later stage of cultivation. Lactic acid production (Fig. 18b.) was decreased after 9h of cultivation

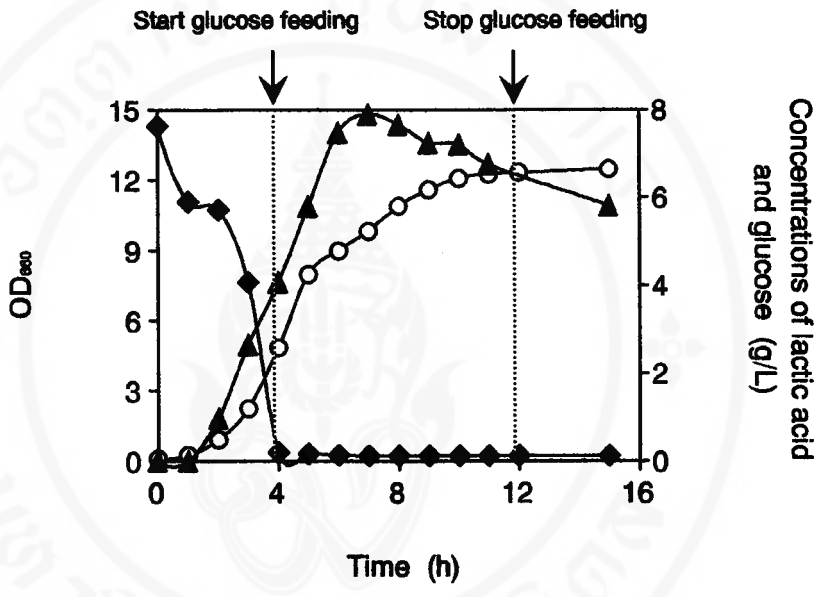

Fig. 17 Time course of growth, lactic acid production and glucose consumption of *E. faecium* in fed-batch cultivation with constant feeding of glucose. Medium composition is shown in Table 1 in appendix B. Symbols: —○— ;OD_{650}, —▲— ; lactic acid concentration (g/L), —◆— ;glucose concentration (g/L).

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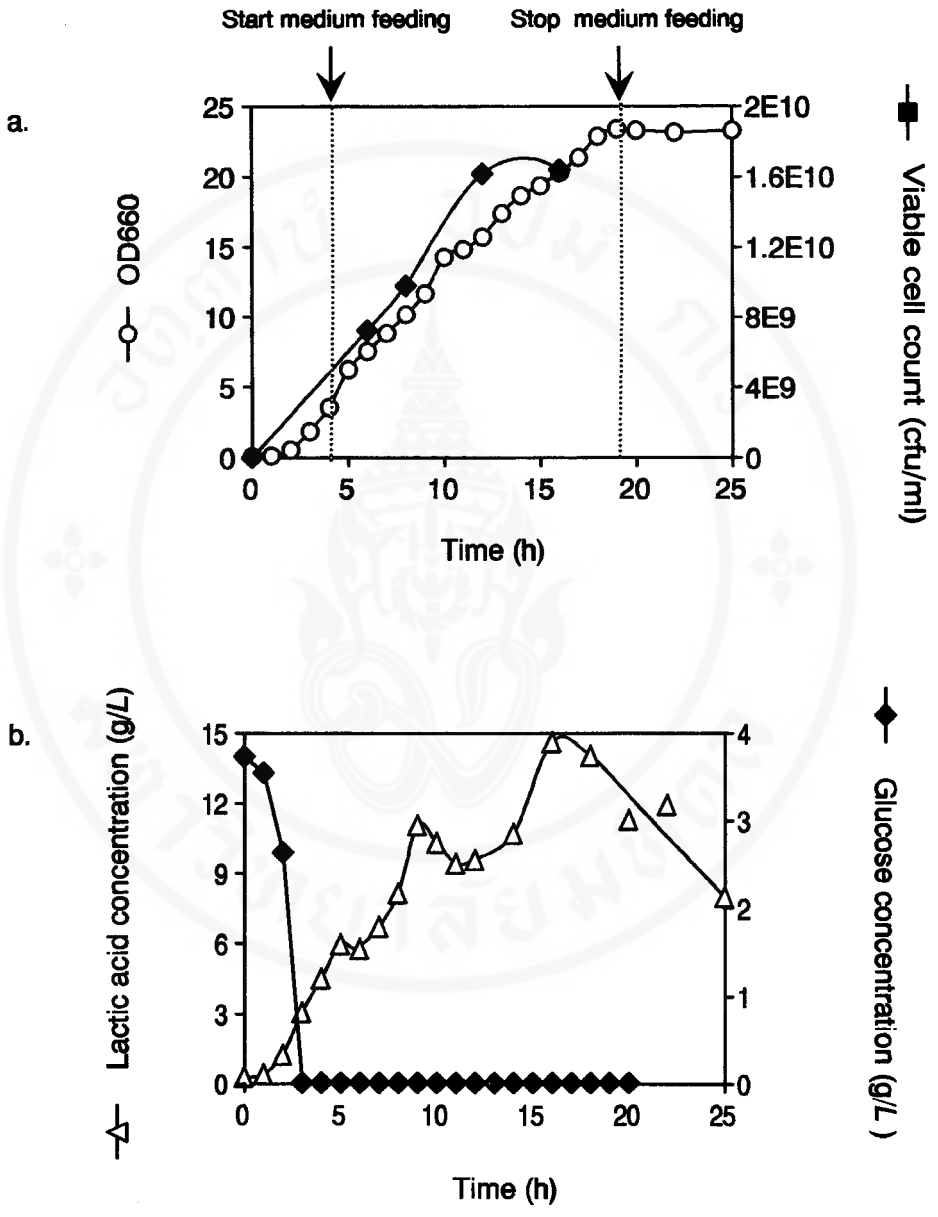

Fig. 18 Fed-batch cultivation with constant feeding of glucose and proteins. a) cell density and viable cell count. b) lactic acid and glucose concentration. For medium composition see Table 2 in appendix B.

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and tended to increase when nutrient feed rate was increased at 13h. After nutrient feeding was exhausted, lactic acid concentration was decreased. According to this observation, it is possible to control lactic acid production by controlling of nutrient feeding. It was also found that growth determined by viable cell count was terminated at 10h of cultivation even though optical density was continuously increased till 18h. This might result from the lack of some nutrients which are necessary for promoting living cell or some cells might be dead.

In order to sustain viable cells, more protein source from digested fish soluble, meat extract and tryptone were dosed. Medium composition is shown in Table 3 (appendix B). Feeding medium 1, containing glucose and protein source, was supplied in the first feeding period after 4 h of cultivation while feeding medium 2, containing protein source as majority was start to supply when the cultivation time was 7h, at the exponential phase of the cells which was hypothesized to be a critical point to sustain viable cells. It was found that cell growth increased continually up to 18 h when all feeding were stopped (Fig. 19). Viable cells can be sustained up to 12 h of cultivation, which was the same time interval of medium 2 supplement. Both cell density and viability were much improved, OD_{660} of 33.8 and 3.11×10^{10} cfu/ml, respectively. Thus it could be stated that growth of lactic acid bacteria, especially viable cell count, can be further improved by fed-batch cultivation with glucose and protein as feeding medium. It was considered that protein might play a major role as growth promoting factor in maintaining cell viability.

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It was found that lactic acid was produced at the beginning of cultivation and then drastically decreased after 7 h of cultivation, when feeding rate was low but was

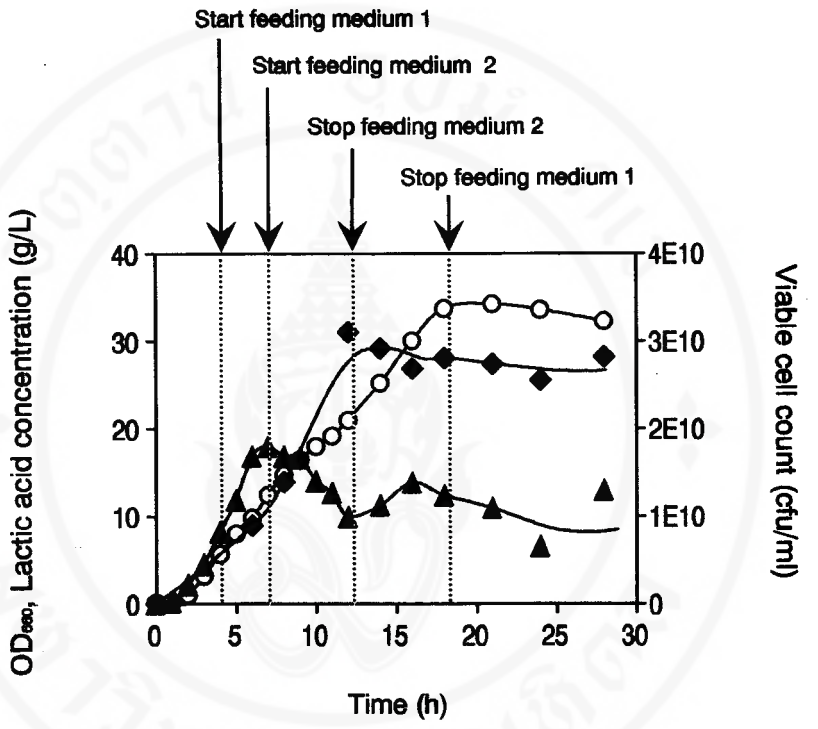

Fig. 19 Fed-batch cultivation with increment feeding of glucose and proteins. The medium was fed at low feed rate at the beginning of cultivation (4-7 h) and then feeding rate was increased along with the cell growth (7-18 h). Detailed in feeding rate see Table 3 in appendix B. Symbols: —○— OD_{600} ; —▲— lactic acid concentration (g/L); —◆— viable cell count (cfu/ml).

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started to increase again after 12 h when feeding rate was elevated. It was also noticed that after feeding medium 2 was depleted, lactic acid was started to produced again. The similar experiment as shown in Fig. 19 was carried out with the 8 times increased of digested fish soluble in feeding medium 2 to study the effect of additional protein source on the viable cell as well as the reduction of lactic acid. Medium composition is shown in Table 4, appendix B). In contrast to our expectation, cell density was not improved and viable cell could not be sustained (Fig. 20). This indicates that apart from digested fish soluble supplementation, meat extract and tryptone which contain growth-promoting factors should be taken into account, as mentioned previously for the flask experiment. It was also found that lactic acid production was significantly decreased, in comparison with the previous experiment, which resulted from the increased concentration of digested fish soluble.

2.2.2 Fed-batch cultivation with exponential feeding strategy

In the previous experiment it has been found that glucose and protein source are necessary for promoting cell growth. However, increase concentration of digested fish soluble was not result in sustainment of viable cell. This might result from the lack of some growth-promoting factors in digested fish soluble. Hence, the next experiments were carried out by increasing amounts of meat extract and tryptone together with using exponential feeding strategies to check whether they can improve cell viability as well as cell growth. Exponential feeding strategies could be done by preset specific growth rate at constant value in feeding equation (see Materials and Methods). In this case, meat extract, tryptone, and digested fish soluble were 2.4, 2.4

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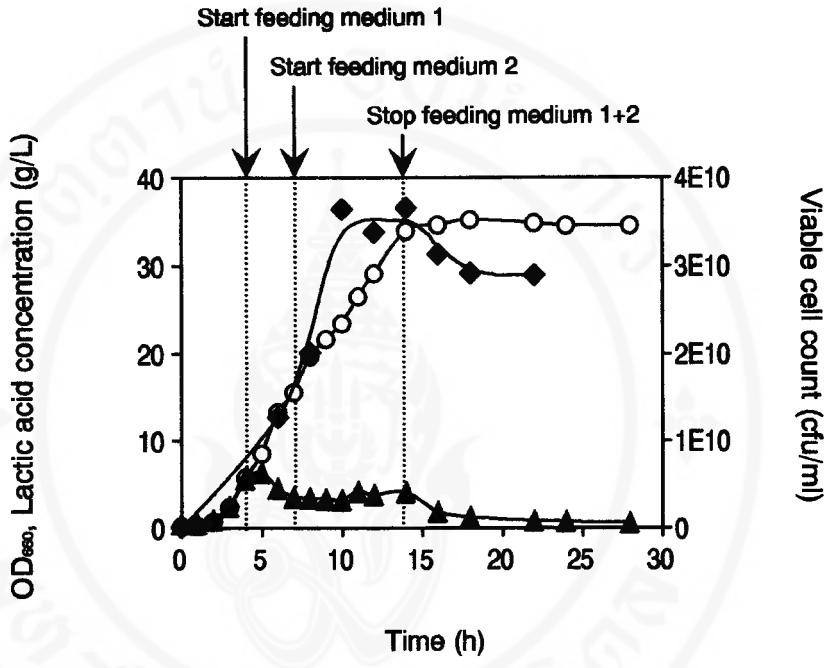

Fig. 20 Fed-batch cultivation with increment feeding of glucose and proteins, and high DFS supplement (8 times in amount of that used in cultivation in Fig. 19). The medium was fed at low feed rate at the beginning of cultivation (4-7 h) and then feeding rate was increased along with the cell growth (7-14 h). Detailed in feeding rate see Table.4 in appendix B. Symbols: —○— OD_{600} ; —▲— lactic acid concentration (g/L); —◆— viable cell count (cfu/ml).

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and 1.5 times, respectively, higher than that used previously Glucose and protein source were fed separately. Medium composition is shown in Table 5 (appendix B). In the previous experiments, 5M HCl was used to digest fish soluble, and as a result high salt concentration (20.4% w/v) was found. In order to reduce salt content in fish soluble, 2M HCl was introduced in the digestion process. The obtained digested fish soluble contain about 50% less salt which was suitable for cultivation of the cell when using large amount of digested fish soluble, even though the AN/TN ratio and cell yield were 0.67 times and 85% of those of DFS with 5M HCl digestion, respectively.

A value of specific growth rates at 0.2, 0.3, and 0.4 h^{-1}, respectively, were investigated to find out the most suitable exponential feeding strategies which appropriate for promoting cell growth but lowering lactic acid production.

Fig. 21a shows fed-batch cultivation by exponential feeding strategies at high feed rate of glucose and protein. The specific growth rate value in feeding equation was preset at 0.4 h^{-1} . It was found that maximum cell density was reached 31.6 within 11 h of cultivation. Furthermore, maximum viable cell count, 3.0×10^{10} cfu/ml, was achieved within 12 h. Nevertheless, cells stopped growing as 11h, even though glucose and protein were fed continually up to 15h. It was also found that glucose in the culture medium accumulated after 9h of cultivation. This indicated that there might be some inhibitors in the culture medium which influence cell growth. According to acid analysis, lactic acid concentration was rapidly increased since the feeding mediums were added and the maximum lactic acid concentration was about 32.7 g/l, at 8h after cultivation. As is demonstrated in Fig. 21b, even though with high medium feed rate,

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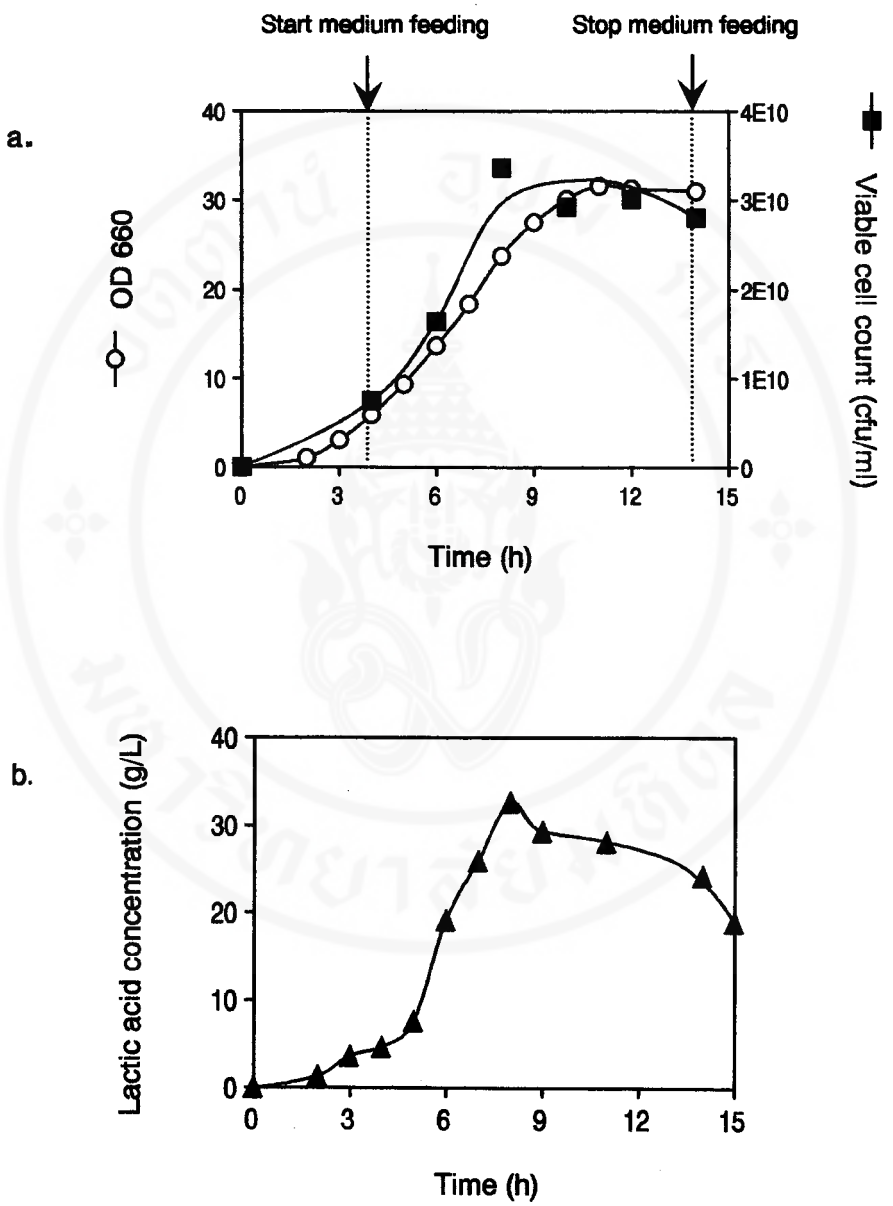

Fig. 21 Fed-batch cultivation with exponential feeding of glucose and proteins at high feed rate. Specific growth rate was preset at 0.4 h-1. a) cell density and viable cell count. b) lactic acid concentration (g/L).

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high cell density was achieved, high lactic acid production was observed as well which was known to inhibit cell growth.

In the fermentor in which the pH was controlled at the value of 6.5, most of the lactic acid (pKa = 3.86) was in the form of lactate anion. Although it was mentioned elsewhere (83-84) that lactate ion, a dissociated form of lactic acid, plays a minor role in inhibition of cell growth. But some authors (85) reported that in case of *Streptococcus lactis*, dissociated lactic acid anions (lactate) also inhibits fermentation and growth ceases beyond a lactate concentration of 80-100 g/L. Hence, in our case, flask experiment was performed to study the effect of lactate ion on the growth of *E. faecium*. It was found that lactate ion at more than 25 g/L can play a role in inhibition of the cell growth. This value is lower than in case of *Streptococcus lactis* as mentioned previously. Therefore it could be ascribed by the fact that at lactic acid concentration of 32.7 g/L, which most of lactic acid was in the form of lactate ion, would play a role in inhibition of the cell growth.

Further experiment was carried out by reducing medium feed rate to prolong cell growth as well as reduce lactic acid production. The specific growth rate value was preset at 0.3 h^{-1}. As is demonstrated in Fig. 22, lactic acid production could be slowed down, however, lactic acid concentration was still high, 33 g/L at 11 h. Cell growth leveled off after 12 h of cultivation, with the highest cell density of 35.7. The other experiment was further investigated to reduce lactic acid production by using the preset value of specific growth rate at 0.2 h^{-1}. As shown in Fig. 23, low medium feed rate resulted in the decreased of lactic acid production by the cells and prolonged cell growth up to 17 h. However, maximum cell density was low, 26.3 at 17 h, in

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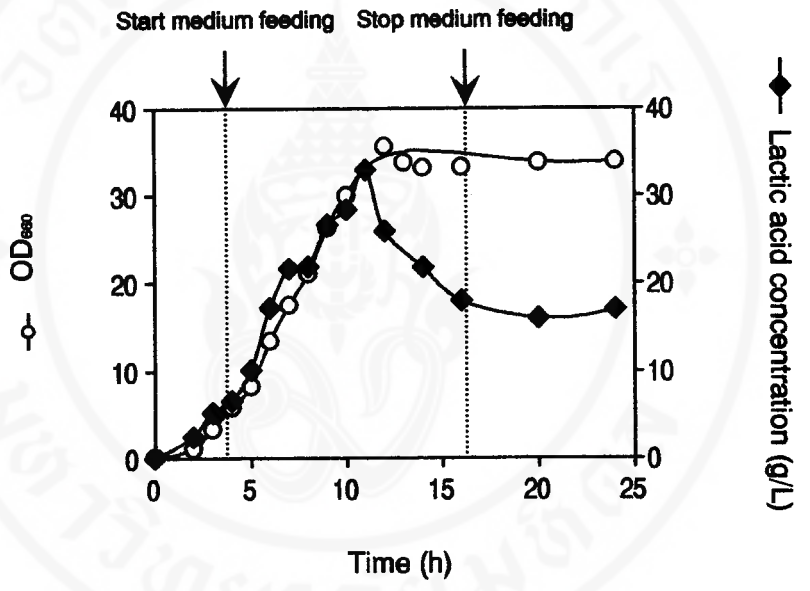

Fig. 22 Fed-batch cultivation with exponential feeding of glucose and protiens at medium feed rate. Specific growth rate was preset at 0.3 h-1.

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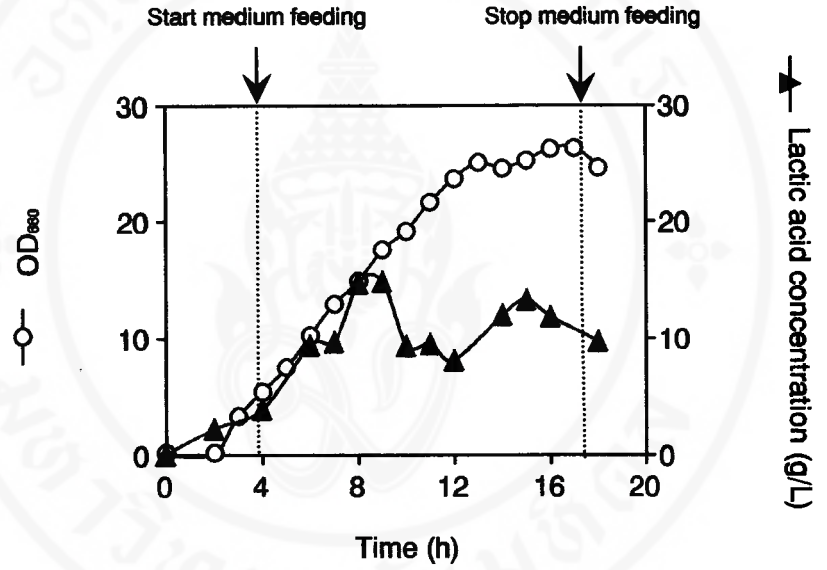

Fig. 23 Fed-batch cultivation with exponential feeding of glucose and proteins at low feed rate. Specific growth rate was preset at 0.2 h-1.

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comparison with high feed rate. This might result from the lower amount of protein supply at the later stage of cultivation which is necessary for cell growth.

Nonetheless, according to the data analysis of previous experiment performed by batch and fed-batch cultivation (Fig. 24), it was found that yield of lactic acid formation per cell density ($Y_{la/x}$) tend to decrease along with the stage of cell growth ($Y_{la/x}$ was high at the first stage of cultivation and then decreased with time). This indicated that lactic acid production tend to be decreased as the cultivation time went on.

The above analysis result coincided with experimental observations reported in literature (86-88). Experiments were carried out with lactic acid bacteria which were cultivated by continuous cultivation. The experiments clearly demonstrated that when the specific growth rate was controlled at a low value, as a result, lactic acid production was low. Hence, from this physiological fact, it could be concluded that the most suitable feeding strategies is to lower feed rate at the beginning of cultivation, during 4 to 10 h, to reduce lactic acid productivity which was high at this stage of cultivation, and then increase feed rate exponentially to provide sufficient nutrients to the cells when cells have lower lactic acid producing capability at the later stage of cultivation.

By this optimal feeding strategy, high cell counts, but low lactic acid production should be obtained. Hence, fed-batch experiment with optimal feeding strategy was performed by presetting a specific growth rate at 0.15 h^{-1} at the beginning and thereafter gradually increased to 0.3 h^{-1} after 10 h of cultivation. According to this feeding strategy, lactic acid production was effectively controlled, as shown in Fig. 25,

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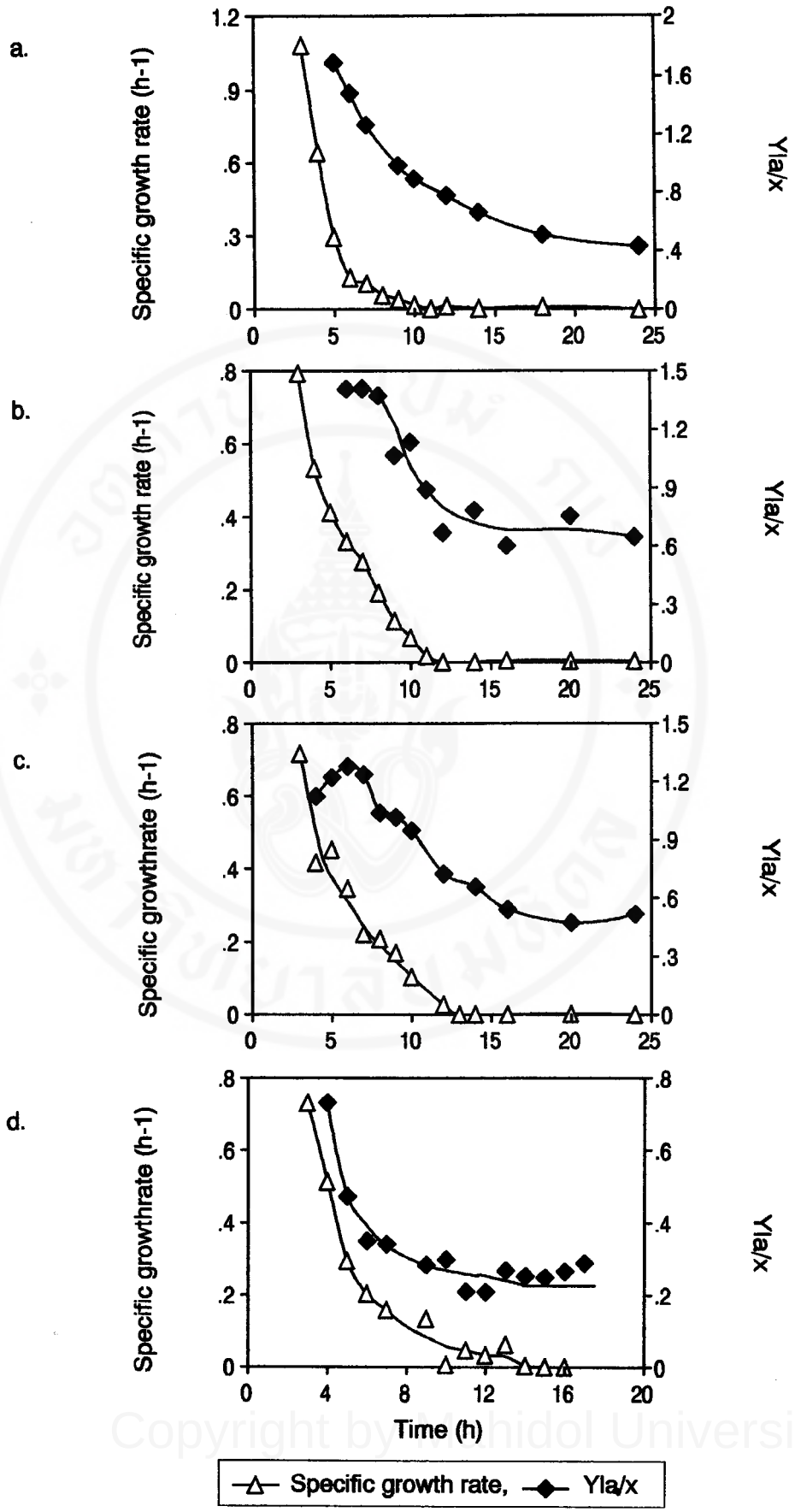

Fig. 24 Time course of specific growth rate and yield of lactic acid formation per cell (Yla/x). Data were obtained from various batch and fed-batch experiments. a) batch experiment b), c), and d) are fed-batch experiment with high, medium and low feed rate, respectively, as metioned previouly.

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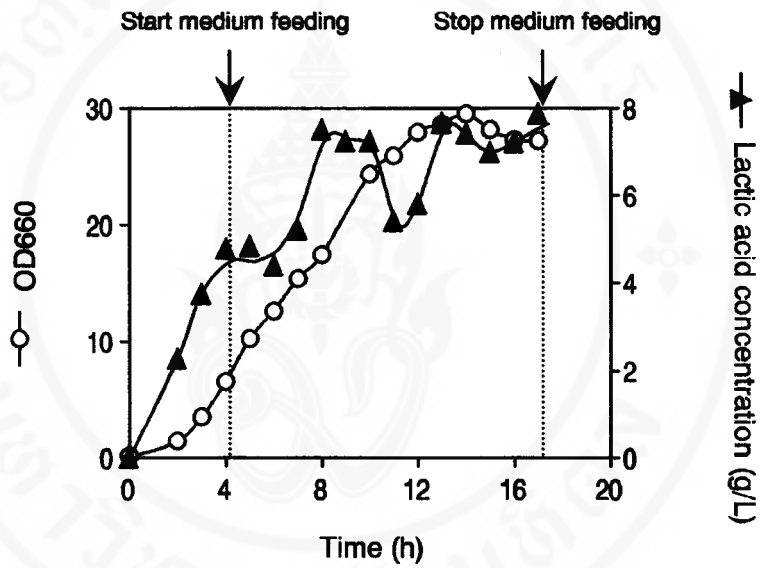

Fig. 25 Fed-batch cultivation with optimal exponential feeding strategy of glucose and protein. Specific growth rate was controlled at 0.15 h-1 from 4-9 h and then increased to 0.3 h-1 after 10 h of cultivation.

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and the highest lactic acid concentration was only 8 g/L. Nevertheless, it could not sustain cell growth even though high a medium feed rate was continually applied at the later stage of cultivation.

Fish soluble digested with 2 M HCl showed a preferable trend to use as protein source because of its lower salt content. However, as mentioned previously from the flask experiment, fish soluble digested with 2 M HCl provided AN/TN ratio and cell yield 0.67 times and 85%, respectively, of those of DFS digested with 5 M HCl. Hence, further fed-batch fermentation experiment was carried out by using fish soluble digested at 5 M HCl as feeding medium instead and it was fed according to optimal feeding strategies as mentioned previously to increase cell density. Contrary to the expectation (Fig. 26a.), cell density was low with a maximum cell density of 25.6 and was terminated after 13 h of cultivation. According to experimental result, both fish soluble digested with 2 M and 5 M HCl showed the same results; a low cell density was achieved while lactic acid concentration was not so high. Furthermore, even though a high medium feed rate was continually applied at the later stage of cultivation, it could not sustain cell growth. It could be mentioned also that calculated salt concentration in broth at final stage was about 1.9 % w/v for experiment with 2 M DFS in which the salt concentration was less than that in experiment (Fig. 25) with 5 M DFS that cell growth could be observed. This suggested that the termination of cell growth before stopping of the feeding may not be due to the nutrient limitation or lactic acid inhibition or salt concentration but might be from other inhibitors in the culture broth. Among the possible inhibitors, Browning reaction products was considered since relatively high amounts of protein was utilized for medium feeding.

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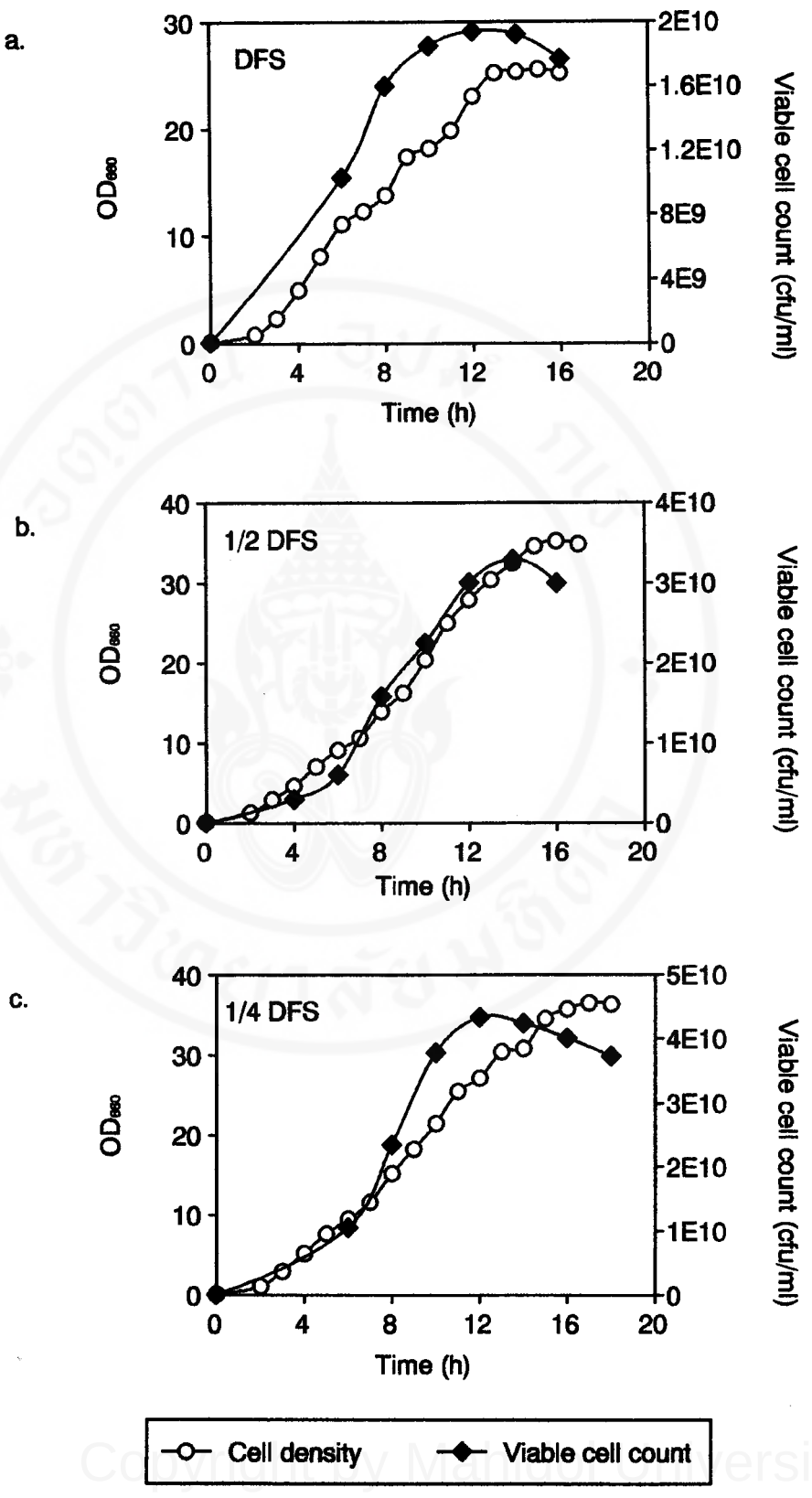

Fig.26 Effect of DFS content on cell growth and viability in fed-batch cultivation using optimal feeding (see details in Table 5). a) DFS at 13.0 % (w/v) total nitrogen, b) 1/2 DFS, and c) 1/4 DFS.

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To prove this hypothesis, further experiments were performed by reduction of the amount of digested fish soluble (DFS) in the medium into half and a quarter to reduce Browning reaction between free amino acids and reducing sugar. As shown in Fig. 25a, b and c there are much improved in cell density and viability compared with using the full amount of DFS.

It is possible that high levels of Browning reaction products could be produced from the large amounts of amino acids and reducing sugar in meat extract and tryptone at high concentrations. Hence, in order to reduce Browning reaction products as well as cost of production, an optimum ratio between DFS to meat extract and tryptone should be investigated for effective utilization of protein sources. It was found that (Fig. 27a.) at the digested fish soluble equivalent to total nitrogen of 2 g of yeast extract, the cell density was maximum. However, according to data analysis, $Y_{x/s}$ and $Y_{x/tn}$ (Fig. 27b), it was found that digested fish soluble equivalent to total nitrogen of 0.8g of yeast extract showed the highest yields. Hence, the most suitable optimal ratio of meat extract to DFS was 1.4 and tryptone to DFS was 1.5, based on total nitrogen.

Regarding to Browning reactions, many factors which are involved in promoting the reaction like temperature, time, and phosphate and citrate concentration have been reported (89). Then a flask experiment was performed to study the effect of these factors on the Browning reaction product formation and thus for cell growth. As demonstrated in Fig. 28, at higher sterilization temperature, an increase in cell growth inhibition could be observed. Moreover, the effect of phosphate and citrate concentration on the formation of Browning reaction products and cell growth was studied and the result is shown in Fig. 29a and b, respectively. At high amounts of

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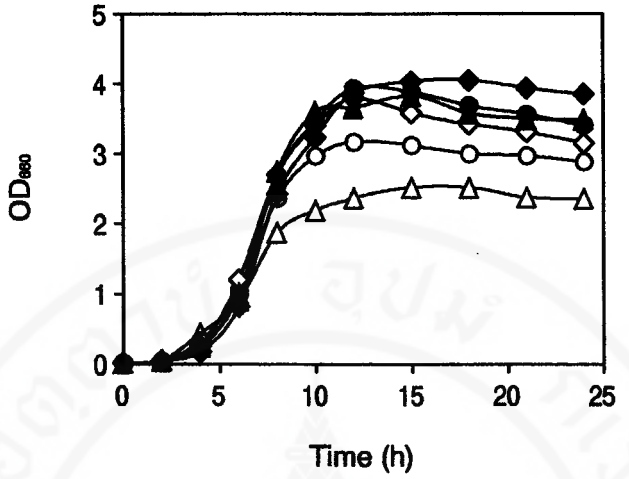

Fig. 27a. Optimal ratio of DFS to ME and to TR for optimal growth of *E. faecium*. 2% w/v of glucose was supplemented for C-source. Concentrations of meat extract and tryptone were fixed (according to ratio in MRS medium content) while varying DFS. DFS was prepared in equivalent to total nitrogen of 0.4, 0.8, 1.2, 1.6, and 2.0 g/100 ml of yeast extract (YE),respectively. Symbols: —△— DFS=0.0 g YE; —○— DFS=0.4 g YE; —◇—DFS= 0.8 g YE; —▲— DFS=1.2 g YE; —●— DFS=1.6 g YE; —◆— ,DFS= 2.0 g YE. The experiments were conducted by shaken flask experiment (see Materials and Methods for medium preparation).

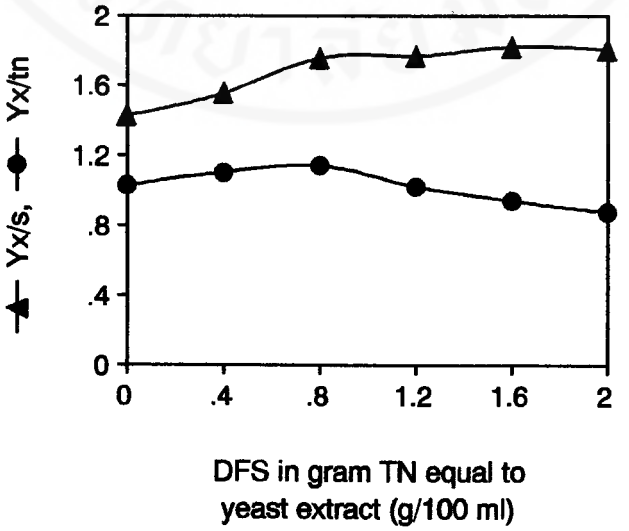

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Fig. 27b The amount of DFS (in gram TN equal to yeast extract) supplemented to fixed ratio of meat extract and tryptone which can provide the optimal ratio of Yx/s and Yx/tn.

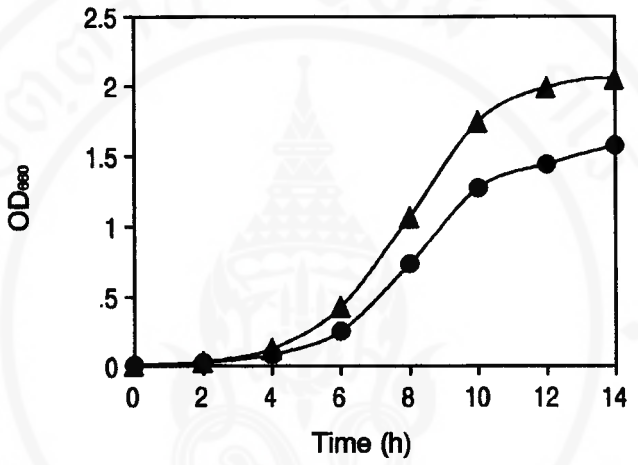

Fig. 28 Growth inhibition of *E. faecium* by Browning reaction products. Experiment was conducted by flask cultivation. Medium was sterilized at 110 °C 10min (—▲—) and 121° C, 15 min (—●—). Medium preparation is shown in Materials and Methods.

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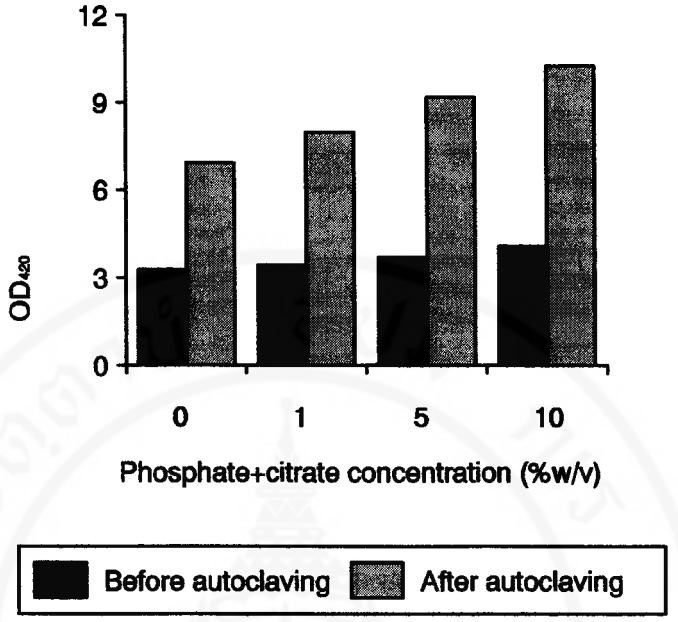

Fig.29a. Effect of phosphate and citrate concentration on formation of Browning reaction products. The medium contained phosphate and citrate and was autoclaved at 121°C for 15 min. Browning reaction product was determined spectrophotometrically at OD_{420} (See medium preparation in Materials and Methods).

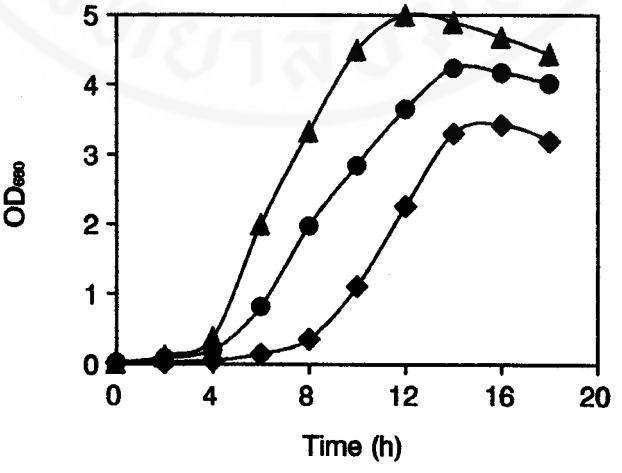

Fig.29b. Growth inhibition effect by Browining reaction products formed in media containing different citrated and phosphate concentrations. Symbols: ▲ ; ● ; ◆ ,1%, 5% and 10% w/v of citrate and phosphate (see medium preparation in Materials and Methods).

phosphate and citrate together with meat extract and tryptone, the formation of Browning reaction products and its inhibition of cell growth was increased.

According to the obtained data, feeding medium was prepared by using optimal ratio's between meat extract, tryptone and DFS. By this optimal ratio between DFS to meat extract and tryptone, the total amount of utilized meat extract and tryptone was reduced by half compared with those in the previous experiment, and phosphate and citrate were autoclaved separately. Medium composition is shown in Table 6 (appendix B). It was found that there were much improved in both cell density and viability (Fig. 30). Moreover, cell growth could be prolonged up to 18 h with the maximum cell density of 36.1 and viable cell count of 4.35×10^{10} cfu/ml. This suggested that cell growth could be further improved by lowering the Browning reaction products.

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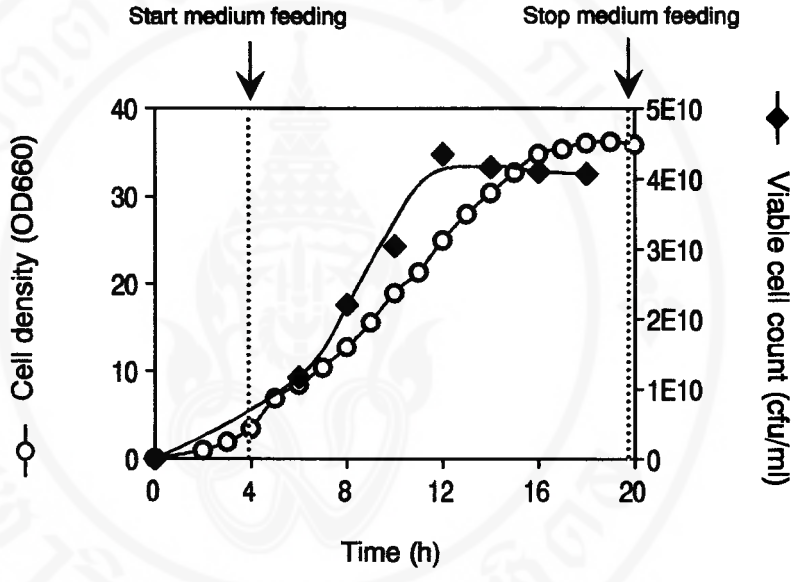

Fig. 30 Fed-batch cultivation with optimal exponential feeding strategy. The medium was prepared base on optimal ratio of DFS to meat extract and tryptone obtained from flask experiment and were autoclaved separately from citrate and phosphate.

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CHAPTER V

DISCUSSION

1. The effect of physical condition and culture media on cell physiology of *E. faecium*

1.1 Effect of aeration and protein source on growth and lactic acid production

From the experimental result of culturing cell in different aeration conditions, it was found that *E. faecium* can grow well under aerobic conditions. Furthermore, when using yeast extract as sole C-source lactic acid production was reduced after a certain period of cultivation. According to previous reports (57-58, 60-61), this might be due to the activation of NADH oxidase during aerobic conditions. NADH is responsible for NAD^+ recovery, which is necessary for energy production in glycolysis pathway. The produced NAD^+ can enter another round of glycolysis. As a consequence, glycolysis can continue and ATP is continually generated while lactic acid formation pathway plays less role for NAD^+/NADH recovery thus resulting in less formation of lactic acid. At the late cultivation stage when the glucose was depleted and flux of glucose through glycolysis was reduced, the increase in the NAD^+-level due to NADH oxidase would result in the reverse reaction of lactic acid to pyruvate by coupling with NAD^+. Hence, lactic acid could be assimilated and converted into cell mass, as a result, lactic acid

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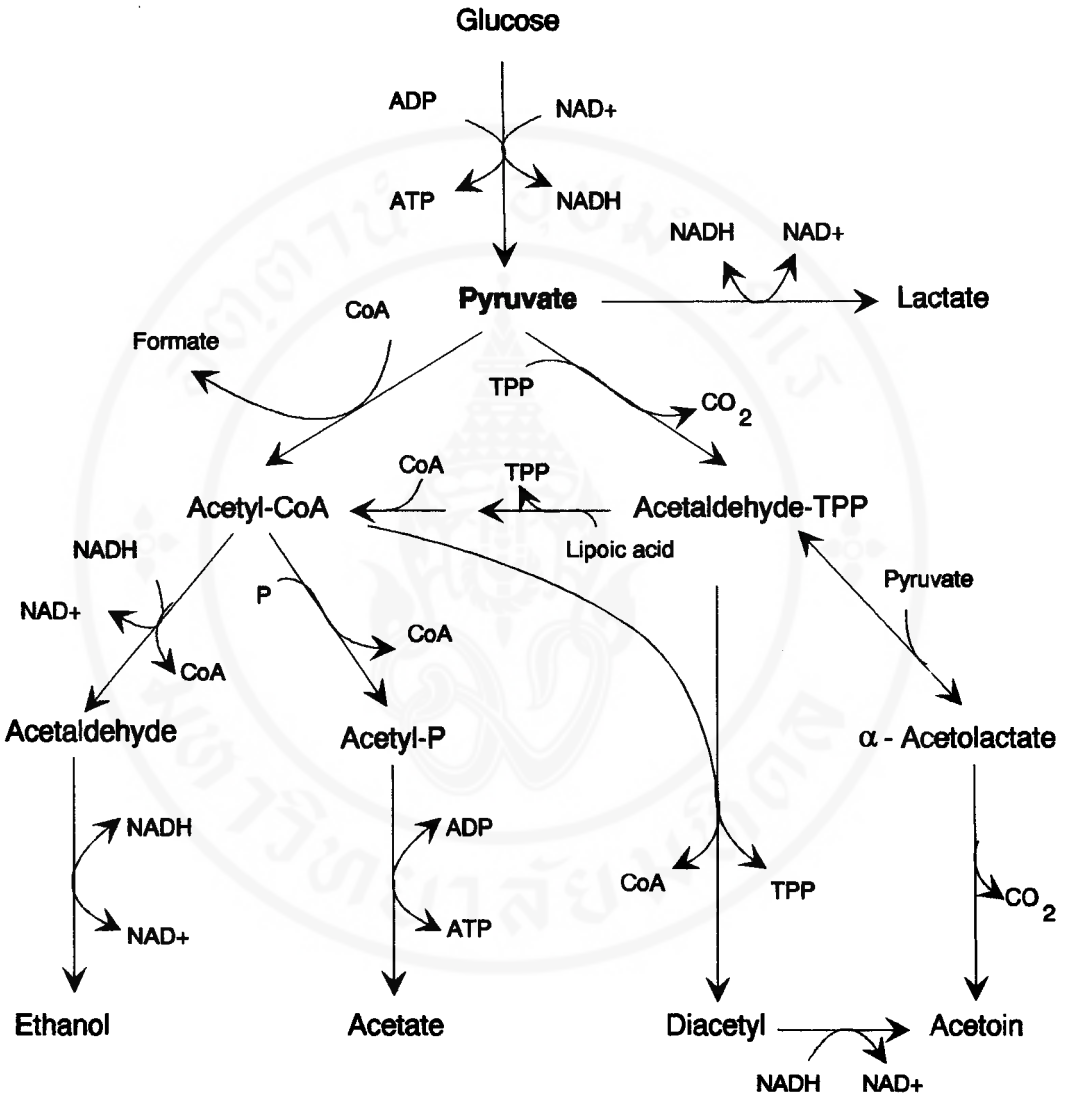

Fig. 31 Alternative pathways of pyruvate metabolism in homofermentative lactic acid bacteria (adapted from Thomas 1979) (90)

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concentration was low. In addition, some amino acids from yeast extract can by-pass the glycolysis pathway and converted directly into intermediates of the glycolysis pathway and then produce energy. These mechanism would result in supporting faster cell growth and greater biomass production of the cells (60). Moreover, as was reported in case of *Leuconostoc mesenteroides* (60), in the presence of oxygen the specific activity of acetate kinase, which responsible for conversion of acetyl phosphate to acetate and yield ATP (Fig. 31), is high (acetic acid could be observed in our case). Hence, an increased in biomass level was obtained. In addition, in case of yeast extract, free amino acids present might be applied directly to preserve energy from amino acids biosynthesis by the cells. Nevertheless, the utilization of yeast extract as sole C-source still could not completely prevent the production of lactic acid due to two possibilities, one is from the total carbohydrate in yeast extract and another from some amino acids which can be converted to pyruvate and then on lactic acid formation.

Regarding to results mentioned above, the proper condition for growing *E. faecium* with less lactic acid accumulation is to use protein source as C-source concomitant with cultivation of the cells under aerobic conditions to reduce lactic acid production by the cells.

1.2 Alternative protein sources and their modification for optimal growth of *E. faecium*

Among C-sources (glucose, molasses, glycerol and yeast extract) that were used to cultivate the cells, yeast extract was the most suitable C-source for promoting cell growth and effectively lower lactic acid production, about 10 times lower than that of

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using glucose and molasses as C-source. Furthermore, with increasing concentrations of yeast extract, higher cell growth could be observed. Likewise, at 12% w/v of yeast extract, the viable cell was about ten times higher than that with 2% w/v glucose, $1.58x10^{10}$ and $1.24x10^{9}$ cfu/ml, respectively. Even though yeast extract can support better cell growth, however, because of the high cost of yeast extract, other alternative cheap proteineous sources which are available in the country have been investigated for applicability.

Among alternative C-sources, soy bean meal and fish soluble which are cheap were used as C-source for cultivation of *E.faecium*. Fish soluble has advantage over soy bean meal in terms of lower sugar content which would decrease lactic acid production by the cells. It was found that digested fish soluble gave higher cell density than that of non-digested fish soluble. Moreover, it can provide higher cell growth than that of soy bean meal hydrolysate. This indicated that protein in short peptide form would be more preferential for being uptake than the long one. The similar result was also confirmed by using other enzyme digested protein source such as meat extract. It was observed that enzyme digested meat extract could provide higher cell growth than that of non-enzyme digested meat extract. However, acid digestion process was more preferable for digestion than enzyme digestion due to its efficient in digestion.

The experiment to investigate the effect of degree of protein digestion on cell growth was further conducted. The result showed that fish soluble digested with 5 M HCl with a digestion time of 30 min (AN/TN ratio of 0.5 considered to contain mainly short peptide chains) could support better cell growth than that with a digestion time of 5 h (AN/TN ratio of 0.7 implying a large pool of free amino acids). This suggested

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that there might be some suitable size of peptides that preferable for supporting cell growth. There was reported that growth of some lactic acid bacteria largely depends more on oligopeptides than free amino acids as nitrogen source (91). It has been found that more than 100 different oligopeptides could be obtained according to the cleavage of peptide bonds by cell enveloped proteinase, PrtP in *Lactococcus lactis* (92). Further study revealed that only certain types of peptides could be taken up and hydrolyzed internally by peptidase to amino acids (91). They also found that growth of *L. lactis* in milk depends on oligopeptides, especially those contain 4 to 8 amino acids residues, as nitrogen source for 98% of growth and the oligopeptides transport system play a crucial role in the utilization of these peptides. Berg *et al.*(93) reported the stimulation of growth of *Lactobacillus sanfrancisco* by a small peptide with a molecular weight of approximately 1,065, containing aspatic acid, cysteine, glutamic acid, glycine and lysine. There was reported elsewhere that peptides serve only to supply a growth-limiting amino acid that is unable to enter the bacterial cells in sufficient quantity because of competition from other amino acids for specific transport sites (94).

Another work on ATP utilization during peptide uptake was also reported by Konings *et al.* (95). They found that the uptake of peptides would require the same amount of ATP as those of free amino acids, thus the uptake of peptides would be preferable in terms of bioenergetics for less energy consumption per amino acid uptake. According to this, compare with that of free amino acid, a higher growth yield could be achieved by peptide utilization.

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Hence, the digestion of fish soluble at certain period of time would provide appropriate oligopeptides which could be taken up by the cells and can provide higher

cell growth and yield. However, the detailed work to identify the peptides as well as peptide size those are specific for growth promoting factor of the cells is complex.

Although digested fish soluble can provide higher cell growth than that of yeast extract based on equal total nitrogen, NaCl content in digested fish soluble should be concerned. It was found that salt concentration of equal to or more than 2% w/v has an inhibitory effect on the growth of *E. faecium*.

Even though digested fish soluble was shown to be an effective protein source for cultivation of *E. faecium*, it could not be used as a sole proteineous source for cultivation of the cells. Other additional protein sources such as meat extract and tryptone should be supplemented. These protein sources might contain specific compounds and specific peptides which play a crucial role in supporting cell growth as was reported previously (91, 96-98).

Among the alternative protein sources which were investigated to replace meat extract and tryptone, whey together with digested fish soluble could promote more cell density and viability than those of using merely whey and even better than those of fish soluble supplemented with meat extract and tryptone. This might indicated that whey would contain some growth promoting factors necessary for promoting of the cell growth as well as cell viability. However, high sugar content in whey should be taken into consideration in order to avoid lactic acid formation during the cultivation of the cells

Although many protein sources were shown to be potential C-sources for high cell density cultivation of *E. faecium*, it could be noted from total nitrogen and amino nitrogen analysis at the end of cultivation that, only a small portion of both were

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consumed. This result was in full agreement with a former report (91) which claimed that no decrease in amino acids occurred after cultivation of *L. lactis* in casein. This means that at the end of this growth phase, not all amino acids and peptides were used, indicating that the remaining free amino acids and peptides were unable to sustain growth. Juillard *et. al* (91) reported that, during the first growth phase, cell do not significantly degrade large peptide or protein because of the absence of proteinase synthesis. The cell wall proteinase synthesis and activity are repressed by peptides which are present during the first growth phase (99-100). During the second growth phase, the proteolytic system of the cells is active and the content of free amino acids and peptides was increased. However, the consumption of free amino acids was very low, suggesting that these types of nitrogen sources play only a minor role in growth. They also found that cell growth was not affected by amino acid supplementation. The other possible reasons for low efficiency in peptide utilization would be due to several factors such as the complexity of peptides, size exclusion limits of oligopeptide transporter, competition of the peptides for entry via a single oligopeptide transport system and hydrophobicity of peptides (91).

The experimental results indicate that when using protein as sole C-source for cultivation of *E.faecium*, only small amounts of protein could be utilized. To promote cell growth and increasing the protein utilization of the cells, it could be considered to supplement glucose, for ATP production in the glycolysis pathway, together with protein source. However, the amount of glucose applied to the cells should be controlled to suppress lactic acid formation. Therefore, fed-batch cultivation technique

was investigated for its applicability to control sugar concentration in the culture medium as well as lactic acid formation .

2. Process optimization for high cell density cultivation of *E. faecium*

E. faecium was cultivated batchwise in fermentor under controlled condition *i.e.* temperature, pH, agitation and aeration. pH control at 6.5 in the fermentor was found to result in extending of cell growth. This is because of the dissociation of most lactic acid into lactate ion (at the pH higher than pKa value of 3.86) which plays a minor role in inhibition of the cell growth (83-84). However, at high lactate ion concentration, 80-100 g/L in case of *Streptococcus lactis,* inhibition of the cell growth was reported by Venkatesh (85). They demonstrated that, in case of *S. lactis*, when lactate was not added in the broth, the flux distribution showed the accumulation of fructose diphosphate (FRUDP) and resulted in high ATP formation. When 75 g/L of lactate was added the flux distribution is completely altered by non accumulation of FRUDP, a positive effector of pyruvate kinase (PK), due to the high concentration of lactate ion and ATP formation is also reduced drastically and thus there is accumulation of inorganic phosphate (Pi), a negative effector of PK. It also found that the formation of pyruvate from phosphoenol pyruvate (PEP) by PK is inhibited strongly, as a consequence, there is an accumulation of PEP inside the cell. Thus in the presence of lactate ion, cells were in a starved state in which Pi inhibited the formation of pyruvate and the positive effector, FRUDP was absent (101-102). As a result, inhibition of the cell growth could be observed.

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With the aim to control lactic acid formation, fed-batch cultivation technique was studied for its applicability in high cell density cultivation of *E. faecium*. At first, fed-batch technique with constant glucose feeding was investigated. It was found that cell growth was increased slightly compared with the batch experiment and lactic acid concentration was reduced in the later stage of cultivation. However, after 10 h of cultivation, even though glucose was fed continually, no more cell growth could be observed. This indicated that the cells might need some additional nutrients apart from glucose for promoting cell growth. However, the result was contrast to the previous experiment reported by Desmons *et al.* (103) that fed-batch cultivation of *Lactobacillus brevis* with glucose feeding only provide higher cell growth than that with MRS medium as feeding medium. Hence, protein source was introduced with the hypothesis that protein would be required as growth promoting factors, a mixture of protein and glucose was used as feeding medium and was fed at a constant rate. The cell density obtained was much improved, about 2 times higher than that of batch cultivation. This confirmed that a protein source is necessary for supporting cell growth. A further experiment was performed by increasing the amount of digested fish soluble. The result was contrary to our expectation, the cell density was not improved and viable cell count could not be sustained. This led us to suggest that the source of protein that contain growth-promoting factors *i.e.* meat extract and tryptone should be taken into account. However, it was observed that a supplement with a high content of free amino acids (by DFS) could efficiently suppress the lactic acid formation. The next experiments were carried out by increasing amounts of meat extract and tryptone together with using exponential feeding strategies to improve cell growth as well as

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cell viability. A value for the specific growth rate (μ) in the feeding equation for substrate was preset and applied for controlling the medium feed rate and then growth rate. Protein and glucose were fed separately. The set points of growth yield on glucose ($Y_{x/s}$) and protein ($Y_{x/tn}$) were 0.6 and 1.5, respectively, which were derived from the previous fed-batch experiments. Fish soluble digested with 2 M HCl was used instead of fish soluble digested with 5 M HCl in order to lower the salt content. Fed-batch experiment with exponential feeding of medium at various preset values of μ were performed in order to investigate the proper μ and feeding strategies. It was found that at high medium feed rate (μ=0.4 h^{-1}), lactic acid was rapidly produced and finally growth inhibition was observed. While at low feed rate (μ=0.2 h^{-1}) lactic acid formation could be well controlled, but lower cell growth was obtained. According to the experimental results of batch and fed-batch experiments, lactic acid productivity as well as yield of lactic acid production ($Y_{la/x}$) was high at growth stages when the specific growth rate was high; thereafter, it gradually decreased together with specific growth rate at later stage. By this physiological fact it is possible to establish strategy to control lactic acid production by controlling of feed rate according to cell physiological stage. Then optimal feeding strategy could be considered by lower feed rate of medium at the first stage when lactic acid producing capability was high and then gradually increase medium feed rate to provide sufficient nutrient to the cells at a later stage of cultivation when lactic acid producing capability of cells was reduced. By this feeding strategy, in principle high viable cell counts but low lactic acid production should be obtained. However, at the late stage of cultivation it was observed that cell growth terminated even before medium feeding was stopped, although the lactic acid

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and salt concentration was considered to be not so high 10 g/L and 38 g/L, respectively. This led us to question about other inhibitors apart from nutrient limitation, salt inhibition as well as lactic acid inhibition.

Among the possible inhibitors, Browning reaction products were considered to be potent, since relatively high amounts of protein was used for feeding medium preparation. By reduction of the amount of DFS into half and a quarter to reduce Browning reaction between free amino acids in DFS and reducing sugar in meat extract and tryptone, both cell density and viability were much improved compared with using full amount of digested fish soluble. Furthermore, it is possible that Browning reaction products might be produced from free amino acids and reducing sugar in meat extract and tryptone. Hence, optimum ratio of meat extract, tryptone and digested fish soluble was investigated to reduce amount of meat extract, tryptone and thus Browning reaction products. The optimal ratio of meat extract and tryptone to digested fish soluble were 1.4 and 1.5 (gTN/gTN), respectively.

One of the non-enzymatic Browning reactions, the Maillard reaction, is considered to be important in food industry, especially in the production of flavor compounds in a wide variety of food products. The Maillard reaction is a reaction between amino groups, which are found in free amino acids or proteins, and the reducing sugars. Maillard reaction involves a series of reactions as discussed by Nagodiwathana (89). The first step is the condenstation of amino group with a reducing sugar. Following this initial condensation step, the product undergoes rearrangement, fragmentation, degradation, dehydration and other reactions resulting in a large number of compounds that eventually impart in an intense dark color to the

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food product. Numerous factors determine the outcome of the browning reaction. This include a) the abundance of carbonyl and amino groups in medium, b) high pH, c) high temperature (especially from 100-140 °C), d) water activity, e) reaction time, f) rate of heating, g) dicarbonyl and other browning intermediates formed during prior processing, h) metal catalysis, i) certain acid species for catalysis and j) presence of inhibitors or antioxidants. Some research has investigated the effect of Maillard reaction products on the inhibition of bacterial growth (104-106).

Based on the literature review, several factors were investigated for their influences in promoting formation of Browning reaction products and cell growth inhibition. Both temperature and heating time play an important role in inhibition of the cell growth. The same positive effect was found with increasing concentrations of phosphate and citrate. Thus, the next fed-batch experiment was performed according to optimal ratio of meat extract and tryptone to digested fish soluble while phosphate and citrate were autoclaved separately. In this case, there were improvements in both cell density and viability. The maximum viable cell count was 4.3×10^{10} cfu/ml which is about 1.8 times higher than that of cultivation influenced by Browning reaction products (2.4×10^{9} cfu/ml). This suggested that cell growth of *E. faecium* might be further improved by lowering Browning reaction products. Further study on the process development to reduce Browning reaction products might necessary to achieve the cell cultivation at high cell density and viability.

Table 5 shows the comparison of growth, lactic acid and yields obtained in experiments using different cultivation techniques. Using protein as sole C-source for cultivation of *E. faecium* can result in lower lactic acid production in all cases. In flask

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cultivation $Y_{x/tn}$ ratio is relatively low which indicates low efficiency in protein utilization by cells. Nevertheless, $Y_{x/tn}$ could be improved in Batch 1 experiment when glucose was supplied in the medium. This might be due to glucose may partly replace catabolism of amino acids as energy source and then promote more protein uptake in coupling for cell growth. However, lactic acid production can not be controlled in this case. In fed-batch experiment with glucose feeding only (Fed-batch 1), lactic acid formation was lower than that of batch cultivation. This indicates the effectiveness in controlling of lactic production by controlling of feed rate of fed-batch experiment over batch experiment. In fed-batch cultivation in which glucose and protein were used as feeding medium (Fed-batch 2), both cell density and viability are much improved compared with those with just merely glucose feeding (Fed-batch 1). Moreover, in the fed-batch experiment with optimal ratio of meat extract, tryptone and DFS and controlling of proper feed rate of both glucose and protein, cell density and viability are much improved. Nevertheless, it could be noted that $Y_{x/tn}$ value tend to decrease as the total amount of supplied nitrogen sources increases in cell cultivation at higher cell density. This might be due to the accumulation of inhibitors such as lactic acid, salt, and Browning reaction products. In addition, when the cells was cultivated using batch experiment (Batch 2) with the same amount of protein as those of fed-batch cultivation (Fed-batch 2), lactic acid production was high while $Y_{x/s}$ and $Y_{x/tn}$ was lower than that of all fed-batch cultivation. This showed that fed-batch techniques are preferential for cultivation of the cells at high concentration since it could provide better cell yield in comparison with batch experiment.

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Table 5 Comparison of the growth, lactic acid and yields of experiments using different cultivation techniques.

Cultivation	OD_{660}	Viable cells ($x10^9$ cfu/ml)	Max. lactic acid concentration (g/L)	Total nitrogen (TN) (g/L)	$Y_{x/s}$ at max. OD (OD/ g/L glucose)	$Y_{x/TN}$ at max. OD (OD/ g/L TN)
Flask [a]	3.5	-	0.5	4.62	-	0.75
Batch 1[b]	13.4	7.0	9.8	4.15	0.37	3.22
Fed-batch 1[c]	12.6	10.8	7.9	4.16	0.44	3.03
Fed-batch 2[d]	23.4	18.2	14.6	9.70	0.55	2.42
Fed-batch 3[e]	36.2	43.5	16.1	18.83	0.50	1.92
Batch 2[f]	15.8	18.0	20.4	9.80	0.34	1.61

[a] Flask cultivation using protein as sole C-source.

[b] Batch cultivation with glucose and protein source.

[c] Fed-batch cultivation with glucose feeding only ;and glucose was fed at constant feed rate.

[d] Fed-batch cultivation with glucose and protein feeding and both were fed at constant feed rate.

[e] Fed-batch cultivation with optimal ratio of DFS to ME and TR. Glucose and protein were fed according to optimal feeding strategy.

[f] Batch cultivation with same amount of protein as those of fed-batch 2 experiment.

Note: All experiments in bioreactor were performed under pH and temperature controlle

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CHAPTER VI

CONCLUSIONS

The knowledge that can be derived from the experiments are:

1. Protein source can be used as C-source to support cell growth as well as lower lactic acid production.
2. Suitable peptide sizes might play a significant role in promoting cell growth and uptake by the cells.
3. Whey protein together with digested fish soluble show promise as a potential protein source to cultivate *E. faecium*. Likewise, they can replace meat extract and tryptone in cultivation of the cells.
4. Using protein as sole C-source results in low effective protein utilization for cell growth. Thus, in order to achieve high cell density, a large amount of protein should be introduced. Nonetheless, it could be considered to supply sugar as C-source to improve protein utilization by the cells. In this case, lactic acid formation from utilization of sugar should be controlled.
5. Fed-batch cultivation using glucose and protein source as feeding medium can promote higher cell growth while lactic acid production could be controlled. Moreover, the additional feeding of protein source, especially amino acids, can suppress lactic acid formation significantly.

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6. In fed-batch cultivation, the most suitable feeding strategy is to lower the medium feed rate at the first stage of cultivation and then gradually increase medium feed rate to provide sufficient nutrients to the cell when the cells have lower lactic acid production capability at the later stage.
7. In preparation of feeding medium with high protein concentration, Browning reaction products should be taken into account. The further study to reduce Browning reaction products during medium preparation might be very beneficial to improve the cell yield and overall process productivity.
8. The possible inhibitors that might play a role in inhibition of the cell growth are as follow: lactic acid, salt and Browning reaction products.
9. Technology established in this study was shown for its capability to be adapted for high cell density cultivation of other lactic acid bacteria and would be applicable to the cell production in industrial sectors.

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APPENDIX A

Sample analysis

1. Determination of total nitrogen (TN) by Kjeldahl's method

Principle and Application

The product is digested with concentrated sulfuric acid, using copper sulfate and potassium sulfate as a catalyst, to convert nitrogen to ammonium ions. Alkali is added and the liberated ammonia distilled into an excess of boric acid solution. The distillate is titrated with hydrochloric acid to determine the amount of ammonia absorbed in the boric acid.

Chemical (A.R. grade)

1. Concentrated sulfuric acid (95-97% H_2SO_4)
2. Catalyst (each tablet contains 0.5 g $CuSO_4.5H_2O$ and 3.5 g K_2SO_4)
3. 40% NaOH
4. 0.1 N Standard HCl
5. Methyl red and Bromocresol green indicator
6. 95% Ethanol
7. 4% Boric acid
8. Distilled water

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Procedure in determination of total nitrogen content

1. Weigh out accurately 0.10-0.50 g. of dry sample or 1-5 ml. of liquid sample into digestion tube.
2. Add 2 tablets of catalyst.
3. Pour 15 ml. of conc. H_2SO_4 into the digestion tube in such a way as to wash down any solid adhering to the neck of tube).
4. Feed the water into the scrubber unit and then start up the scrubber unit and Kjeldahltherm. Heat the digestion tube at a temperature of 420°C for 45 min until the clear of green color had occurred.
5. Allow the solution to cool.
6. Pour distilled water into the digestion tube.
7. Fix the distillation tube with steam pipe of the distillation set.
8. Dip the rubber pipe of the condenser of distillation set into Erlenmeyer flask which contains 25 ml of boric acid and a few drop of mixed indicator.
9. Pump 100 ml. of 40% NaOH into the distillation tube. The solution will become black.
10. Boil the solution in the distillation tube for 4 min. The solution in the Erlenmeyer flask will change from violet-blue to green.
11. Titrate the solution in the Erlenmeyer flask with standard 0.1 N HCl until the end point, the solution will change from green to violet-blue.

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Calculation

$$\% \text{ Total Nitrogen} = \frac{14.01 \times V \times N}{g_{sample} \text{ or } v_{sample}}$$

N - Normality of HCl

V - Volume of HCl

g_{sample} - Gram of sample

v_{sample} - Volume of sample

$$\% \text{ Protein} = \% \text{ Total Nitrogen} \times 6.25^{*}$$

* The crude protein value is calculated from total nitrogen using and appropriate factor, generally 6.25, for milk products 6.38 and for cereal products 7.70.

2. Determination of amino nitrogen by indirect formol titration (AN) (Melnick *et al.* 1949) (79)

1. Add 1 ml sample (of 1% solids, adjust volume if necessary) to a small beaker containing a magnetic bar
2. Make up volume to 10 ml with distilled water, stir continuously, insert pH-electrode
3. Adjust the pH of the mixture to approximately 7.0 with a few drops of 0.10 N NaOH (conveniently added from the burette).
4. Add 10 ml of the formaldehyde solution to the beaker and let mix for 1 minute (the pH will decrease, then become constant).

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5. Note the volume in the burette and titrate (slowly) to pH 9.5 by 0.10 N NaOH (The titration volume should be at least 3 ml or so for sufficient accuracy).
6. From the volume of alkali used in the titration calculate the AN content.
7. Wash the beaker extensively before performing the next assay.

Calculation

%w/v AN = 1.0 sample vol. x 14x 0.10 x vol NaOH x dilution rate/100

Note This method can not differentiate ammonium from amino acids.

3. Total carbohydrate assay (phenol-sulfuric acid reaction.) (Dubois *et al.* 1956) (77)

Reagents:

1. Sulfuric acid, reagent grade 98% conforming to ACS specifications, specific gravity 1.84.
2. 5% Phenol solution, (prepare fresh each week, store in dark bottle).

Methods:

1. Diluted sample to the appropriate concentration (approx. concentration, 0.1-0.01 g/L).
2. Add 0.5 ml and 1 ml of dilute sample into 2 tubes and make up to 2 ml with distilled water.
3. Add 1 ml of 5% phenol solution, mixed gently.

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4. 5 ml of concentrated sulfuric acid is added rapidly. Direct the flow on the center of liquid surface of the tube and keep the pipette approx. 1 cm above the surface of the mixture.
5. The tubes are allowed to stand in a relatively dark place for 40 min.
6. Mix the tubes content vigorously, assay absorbance at 490 nm. The blanks are prepared by substituting distilled water for the sugar solution.

Standard curves.

1. Diluted the glucose standard solution 10x (0.1 g/L).
2. Add, 0, 0.25, 0.5, 0.75, 1 and 1.5 ml of the dilute standard solution and make up to 2 ml with distilled water.
3. Follow steps 3-6 as above method.

4. Use of dinitrosalicylic acid (DNS) reagent for determination of reducing sugar (Miller 1959) (78)

DNS preparation:

1. Dissolve 16 grams of NaOH in 200 ml distilled water.
2. Slowly add 10 grams of 3,5-dinitrosalicylic acid .
3. Add 500 ml of distilled water.
4. Slowly add 300 grams of sodium potassium tartrate .
5. Adjust to final volume of 1000 ml.
6. Leave in dark condition for 2-3 nights.

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Methods:

1. Pipette 5 ml of suitably diluted sample and add 1 ml of DNS reagent.
2. Boil at 100 °C for 5 min. Cool, then.
3. Add 10 ml of distilled water, mix vigorously and measure OD at 520 nm.

5. Enzymatic glucose assay with Trinder-method.

The procedure has been modified to 1) reduce the amount of reagent require and 2) to increase the sample volume.

1. Prepare a glucose standard (dry glucose first) of 0.80 g/L
2. Prepare reaction mixture by taking 75 ml of buffer solution, add in 1 bottle, which contains the enzymes and color reagent, and mix thoroughly. Keep this mixture in a dark bottle.
3. Pipette 0, 25 and 50 µl of the glucose standard into test tubes and make up the 50 µl with distilled water.
4. Pipette 50 µl of suitably diluted samples (*i.e.* diluted to ca. 0.2-0.8 g/L glucose) into test tubes.
5. Add 1.5 ml of reagent mixture into test tubes for standard glucose and sample and mix. Incubate at 37°C for 20 min or 20°C for 40 min.
6. Measure OD at 550 nm.
7. Calculate the glucose concentration from the calibration curve and multiply with the dilution.

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Note: after mixing the buffer solution and the enzymes/color reagent (point 2), this reaction mixture can be kept in a refrigerator at ca. 4°C for up to 3 weeks. The calibration curve should be a straight line with a high correlation coefficient (r^2>0.99).

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APPENDIX B

Medium composition used in batch and fed-batch cultivation of *E. faecium* in fermentor

Table i Modified MRS composition for use in batch and fed-batch cultivation[1] of *E. faecium.* (Fig. 16, 17)

Composition	Starting medium (g/L)
Tryptone	15
Extract	12
Glucose	5
Sodium acetate	5
Di-ammonium hydrogen citrate	2
Di-potassium hydrogen phosphate	2
Tween 80	2
$MgSO_4.7H_2O$	0.3
$MnSO_4.H_2O$	0.06
DFS[2]	4

[1] In fed-batch experiment with just glucose feeding, the glucose concentration was 245 g/L and was fed at constant feed rate (approx. 25 ml/h) from 4 to 12 h.

[2] DFS was prepared based on equal total nitrogen to that in 4 g/L of yeast extract.

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Table ii Medium composition in fed-batch cultivation using mixed feeding of glucose and protein. The medium was fed at constant feed rate from 4 to 18 h. (Fig. 18)

Composition	Starting medium (g/L)	Feeding medium (in 600 ml)
Tryptone	15	60
Meat Extract	12	48
Glucose	5	120
Sodium acetate	5	-
Di-potassium hydrogen citrate	2	12
Di-ammonium hydrogen citrate	2	12
Tween 80	2	6
$MgSO_4.7H_2O$	0.3	1.2
$MnSO_4.H_2O$	0.06	0.24
DFS[1]	4	12

[1] DFS was prepared based on total nitrogen equal to that of 4 g/L of yeast extract.

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Table iii Medium composition in fed-batch cultivation with subsequent usage of two feeding media. Feeding medium1 and 2 were fed from 4 to 18 h and from 7-to 12 h, respectively. The feeding medium 1 was fed at low feed rate (40 ml/h) at the beginning of cultivation (4-7 h) and then feeding rate was increased (60 ml/h) along with the cell growth (7-18 h). The feeding medium 2 was fed at 80 ml/h from 7 to 12 h of cultivation. (Fig. 19)

Composition	Starting medium (g/L)	Feeding medium 1 (in 600 ml)	Feeding medium 2 (in 400 ml)
Glucose	5	200	-
Tryptone	15	60	60
Meat Extract	12	48	48
Sodium acetate	5	-	-
Di-potassium hydrogen phosphate	2	12	4
Di-ammonium hydrogen citrate	2	12	4
Tween 80	2	6	2
$MgSO_4.7H_2O$	0.3	1.2	0.4
$MnSO_4.H_2O$	0.06	0.24	0.08
DFS[1]	4	12	12

[1] DFS was prepared base on total nitrogen equal to that of 4 and 12 g/L of yeast extract.

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Table iv Medium composition in fed-batch cultivation with subsequent usage of two feeding media using high dose of DFS[1]. Feeding medium1 and 2 were fed from 4 to 15 h and from 7 to 14 h, respectively. The feeding medium 1 was fed at low feed rate (40 ml/h) at the beginning of cultivation (4-7 h) and then feeding rate was increased (60 ml/h) along with the cell growth (7-14 h). The feeding medium 2 was fed at 80 ml/h from 7 to 14 h of cultivation. (Fig. 20)

Composition	Starting medium (g/L)	Feeding medium 1 (in 600 ml)	Feeding medium 2 (in 400 ml)
Glucose	5	200	-
Tryptone	15	60	60
Meat Extract	12	48	48
Sodium acetate	5	-	-
Di-potassium hydrogen phosphate	2	12	4
Di-ammonium hydrogen citrate	2	12	4
Tween 80	2	6	2
$MgSO_4.7H_2O$	0.3	1.2	0.4
$MnSO_4.H_2O$	0.06	0.24	0.08
DFS[1]	4	12	32

[1] DFS was prepared base on total nitrogen equal to that in 4, 12 and 32 g/L of yeast extract.

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Table v Medium composition fed-batch cultivation by exponential feeding at different specific growth rate settings[1, 2,3]. (Fig.21, 22, 23, 25 and 26a)

Composition	Starting medium (g/L)	Feeding medium 1 (in 1000 ml)	Feeding medium 2 (in 1000 ml)
Tryptone	15		290
Meat Extract	12		232
Glucose	5	333	-
Sodium acetate	5		-
Di-potassium hydrogen phosphate	2		32
Di-ammonium hydrogen citrate	2		32
Tween 80	2		12
$MgSO_4.7H_2O$	0.3		3.2
$MnSO_4.H_2O$	0.06		0.64
DFS[4]	4		122

[1] Exponential feeding strategies was done by preset specific growth rate at constant value in the feeding equation. The specific growth rates used were 0.4, 0.3 and 0.2 h^{-1} for high, medium and low feed rates, respectively.

[2] In the fed-batch experiment with optimal exponential feeding strategy, the specific growth rate was preset at different values in the feeding equation as follows: 0.15 (4-9h) and 0.3 h^{-1} (10-18h), respectively.

[3] In fed-batch experiment with optimal feeding strategy to check the effect of DFS on the cell growth, the amount of DFS in the medium was reduced based on total nitrogen equal to that in 61 and 30.5 g/L yeast extract.

[4] DFS was prepared based on total nitrogen equal to that in 4 and 225 g/L of yeast extract.

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Table vi Medium composition in fed-batch cultivation using feeding medium with optimal ratio of DFS to meat extract and tryptone[1]. Medium was fed according to optimal feeding strategy. (Fig.30)

Composition	Starting medium (g/L)	Feeding medium 1 (in 1000 ml)	Feeding medium 2 (in 1000 ml)
Tryptone	15		155
Meat Extract	12		124
Glucose	5	333	-
Sodium acetate	5		-
Di-potassium hydrogen phosphate	2		21
Di-ammonium hydrogen citrate	2		21
Tween 80	2		8
$MgSO_4.7H_2O$	0.3		2.1
$MnSO_4.H_2O$	0.06		0.43
DFS[2]	4		122

[1] The feeding medium was prepared by using optimal ratio of DFS to meat extract and tryptone achieved from flask experiment and were autoclaved separately from di-potassium hydrogen phosphate and di-ammonium hydrogen citrate.

[2] DFS was prepared based on total nitrogen equal to 4 and 122 g/L of yeast extract.

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