PRODUCTION OF LACTIC ACID FROM CASSAVA SOLID WASTE

Miss Orawan Cheunkum

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตร์มหาบัณฑิต
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PRODUCTION OF LACTIC ACID FROM CASSAVA SOLID WASTE

Suranaree University of Technology Council has approved this thesis submitted in partial fulfillments of the requirements for the Master's Degree

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การศึกษาในครั้งนี้มีวัตถุประสงค์คือการผลิตกรดแลคติกจากกากมันสำปะหลังซึ่งเป็นของเสียที่มีมูลค่าต่ำมาก โดยปกติโรงงานอาจมีการขายกากมันนี้ให้แก่โรงงานผลิตอาหารสัตว์ แต่ถ้ามีมากเกินไปจะกำจัดโดยการเผาทิ้งซึ่งเป็นการกำจัดที่ไม่ถูกต้อง การนำกากมันไปแปรสภาพนอกจากจะช่วยลดปัญหาการกำจัดแล้วยังได้ผลผลิตที่มีมูลค่านั่นคือกรดแลคติกในการทดลองนี้จุลินทรีย์ที่สำคัญที่ใช้ในการผลิตกรดแลคติกคือ แลคโตคอคคัส I-5 ขั้นแรกของการทดลองคือการสกัดแป้งจากกากมันสำปะหลังแล้วนำไปทำแห้งโดยเครื่องรีดแป้ง ขั้นตอนต่อมาคือ หาสภาวะที่เหมาะสมในแง่ของอาหาร (yeast extract และ urea) ค่าความเป็นกรดเป็นด่าง (pH) และ อุณหภูมิ ที่จุลินทรีย์จะผลิตกรดแลคติกออกมามากที่สุด พบว่าสภาวะที่เหมาะสมคือที่ pH เท่ากับ 7.0 อุณหภูมิบ่มที่ 35 องศาเซลเซียส ปริมาณสารอาหารที่เหมาะสมในสูตร RAM โดยใช้ yeast extract 0.5% และ urea 0.2% ต่อมานำไปทำการหมักโดยใช้กากมันที่เป็นของแข็งในถังหมักขนาด 2 ลิตร ภายใต้อุณหภูมิห้องและไม่ควบคุมค่า pH จะพบว่า pH มีการลดลงจาก 7.0 มาที่ 4.5 ส่วนผลผลิตของกรดแลคติกที่เกิดขึ้นนั้นมีค่าน้อยและเกิดขึ้นช้ามากในขณะที่กลูโคสซึ่งมีค่ามากและเกิดขึ้นเร็ว หลังจากทำการหมักไปแล้ว 10 วันมีโปรตีนเพิ่มขึ้นจาก 0.59% เป็น 1.49% ของโปรตีนในกากมันเดิม กรดแลคติกที่เกิดขึ้นคือ 0.6 กรัมต่อลิตร (yield = 4 mg lactic/g solid) และความเข้มข้นของกลูโคสที่เกิดขึ้น คือ 16 กรัมต่อลิตร (yield = 126 mg glucose/g solid) และพบว่าระบบเริ่มมีการปนเปื้อนจากจุลินทรีย์ชนิดอื่น เช่น เชื้อรา เป็นต้น สำหรับการเพิ่มผลผลิตที่ต้องการ ได้มีการปรับปรุงการทดลองโดยนำแป้งที่สกัดมาจากกากมันมาใช้ในอาหารสูตร RAM ซึ่งเป็นการหมักในสภาวะของเหลว โดยมีการแปรเปลี่ยนความเข้มข้นของแป้งจาก 0% - 5% พบว่าที่ความเข้มข้น 1% เกิดผลผลิตจากกรดแลคติกต่อปริมาณแป้งที่ใช้สูงสุด จากการหาค่าสัมประสิทธิ์ทางจลนศาสตร์ของสมการ Monod พบว่าอัตราการเจริญเติบโตจำเพาะสูงสุด (μ_m)ที่ได้คือ 3.94 ต่อชั่วโมง และค่าคงที่อิ่มตัวในการใช้สารอาหาร (K_s) เท่ากับ 78.07 กรัมต่อลิตร

สาขาวิชาวิศวกรรมสิ่งแวดล้อม
ปีการศึกษา 2544

ลายมือชื่อนักศึกษา……..…….……
ลายมือชื่ออาจารย์ที่ปรึกษา……..……
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..

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LACTIC ACID/PRODUCTION/CASSAVA SOLID WASTE/FERMENTATION

This study is aimed at producing the highest amount lactic acid from fermentation of cassava solid waste. Waste disposal practice and simple management of the factories for this waste are usually selling to the animal food industries or open-burning due to the over-exceeding quantity. Recycling of the waste could reduce the disposal problem and produce valuable conversion product of lactic acid. The microorganism used for the fermentation was lactococcus *sp*. I-5. In the first part of the experiment, the optimum conditions for lactic acid bacteria (LAB) were determined to be at pH 7.0, temperature 35°C, 0.5% of yeast extract concentration and 0.2% of urea concentration. Next, the fermentation was conducted in solid state using urea and yeast extract as nitrogen source and other product for trace element. Under room temperature and uncontrolled pH, which decreased freely from 7.0 to 4.5, lactic acid production increased slowly. Glucose increased rapidly and had a higher value than lactic acid. After 10 days, the protein content increased from 0.59% to 1.49%. Lactic acid produced was 0.6 g/l (the yield was 4 mg lactic/ g solid) and glucose concentration was 16 g/l (the yield was 126 mg glucose/g solid). During the production, the fermenter system was contaminated from other microorganism such as mold and yeast from outside system. For increasing the production to the preferred amount, liquid starch fermentation was used by varying starch extract from 0%-5% in RAM medium. It was found that the 1% starch batch gave the highest value of yield. From the Monod's kinetic coefficients obtained, maximum specific growth rate (μ_m) was 3.94 per hour and saturated constant for substrate (K_s) was 78.07 g/l.

สาขาวิชาวิศวกรรมสิ่งแวดล้อม ลายมือชื่อนักศึกษา……..…..……

ปีการศึกษา 2544 ลายมือชื่ออาจารย์ที่ปรึกษา…..……..……

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..…….....

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Orawan Cheunkum

Table of Contents

Page

Table of Contents (Cont.)

Page

Table of Contents (Cont.)

Page

List of Tables

Table **Page**

List of Figures

Figures **Page**

List of Figures (Cont.)

Figures **Page**

List of Abbreviations

cm	=	centimeter
°C	=	degree Celsius
CFU	=	Colony Forming Unit
et al.	=	et alia (and others)
Fig.	=	Figure
g	=	gram
h	=	hour
K_s	=	Monod’ s Constant
l	=	liter
LAB	=	lactic acid bacteria
mg	=	milligram
min	=	minute
ml	=	milliliter
N	=	Normal
OD	=	Optical Density
RAM	=	Rogasa Agar Modified Medium
t_d	=	Doubling time
μ	=	Specific growth rate
μ_m	=	maximum specific growth rate
%	=	Percent

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Thailand has maintained its position as the world's leader in production and export of agricultural products. Tapioca starch industry is one of the major industries of Thailand. In 1998, tapioca starch export was worth 5 billion bahts. About 40% of starch were used domestically for the production of modified starch, sweetener, and monosodium glutamate; while the remaining 60% were mostly exported (Tanticharoen *et al*., 1990). Efficient production process, low production cost, and the development of value-added products are vital to the starch industry and the farming sectors of totally 1.3 million ha.(8,125,000 Rai) plant area. Wastewater discharge varies from 13 to 50 m^3/ton of starch produced with an average of 20 m^3/ton (Wangnai *et al*., 1990). Solid waste from the starch production still contains 50% of starch (dry weight) and it is used as animal feed after starch extraction. However, tapioca starch is not suitable for the production of feeds requiring high protein content.

Cassava waste is a waste matter that usually does not have appropriate treatment or disposal. This creates order problem and unpleasant appearance at and around the area of its disposal. Waste disposal practice and simple management of the factories for this waste are usually selling to the animal food industries or open-burning due to the over-exceeding quantity. However, several carbohydrates such as tapioca starch, potato starch, and molasses could be used for the production of lactic acid. The starch has to be saccharified into sugars before fermentation (Balagopalan et al, 1988). About 50% of lactic acid were produced by fermentation and the remainder was manufactured by chemical synthesis. Therefore, this research examines the recycling potential of cassava solid waste to produce lactic acid. Such recycling not only could reduce the disposal problem but also could yield valuable return.

In this study, the microorganism used was in-group of lactic acid bacteria (LAB), which could ferment cassava solid waste for lactic acid production. The LAB are all Gram-positive anaerobic, micro-aerophyllic or aero-tolerant; catalase negative and non-motile; rods or cocci. Most importantly, they all produce lactic acid as sole, major or an important product from the energy-yielding fermentation of starch.

1.2 Objective of the Study

1.2.1. To investigate the lactic acid production by fermentation of cassava solid waste.

1.2.2. To examine the kinetics of cassava fermentation.

1.3 Scope of the Study

1.3.1 In this investigation, the experiments were set up to produce lactic acid in batch operation. The pH of cassava solid waste was adjusted for optimum culture growth in initial experiment. Typically, fermentation in fermenters ranging from 50 ml to 1800 ml in size were filled with medium and sterilized in an autoclave at 121°C for15 min. After the addition of nutrients, the pH was adjusted to 6.5-7.0 and the culture was inoculated in a controlled environment to prevent contamination.

1.3.2. Evaluation of the effect of optimum N-source was done using 0.1-0.5% of urea concentration for culture growth.

1.3.3. Investigation of cassava fermentation was done by varying yeast extract concentration from 0.1 % to 0.5%.

1.3.4. Parameters studied are pH, temperature, culture density, lactic acid, carbohydrate, protein, solid and ash.

1.3.5. Cultivation kinetics such as specific growth rate (μ_m) and Monod's constant (K_s) are determined.

CHAPTER 2

LITERATURE REVIEW

2.1 Cassava and Their Manufacturing Process

2.1.1 Characteristics of cassava

The cassava plant is a perennial that grows under cultivation to a height of about 2-4 m. The large, palmate leaves ordinarily have five to seven lobes born on a long slender petiole. They grow only toward the end of the branches. The plant usually grows into three branches, which are divided similarly. The roots or tubers radiate from the stem just below the surface of the ground. Feeder roots growing vertically from the stem and from the storage roots penetrate the soil to depth of 50-100 cm. This capacity of the cassava plant to obtain nutrients from soil below the surface may help to explain its growth on inferior soils. Male and female flowers arranged in loose plumes are produced on the same plant. The triangular-shape fruit contains three seeds, which are viable and can be used for the propagation of the plant. The number of tuberous roots and their dimensions vary greatly among the different varieties. The roots may reach a size of 30- 120 cm long and 4-15 cm. in diameter, and a weight of 1-8 kg or more.

Cassava is frequently cultivated as temporary shade plant in young plantations of cocoa, coffee, rubber or oil palm. Cassava is a typical tropical plant and grows best on light sandy loam's or on loamy sands that are moist, fertile, and deep; but it also does well in soils ranging in texture from the sands to the clays and on soils of relatively low fertility. No fertilization is required when the land is freshly cleared or when there is enough land to enable the cultivator to substitute new land for old when yield fall. Table 2.1 is base on an analysis made in Madagascar comparing the cassava root with the potato.

Table 2.1 Average composition of the cassava root and the potato (Grace, 1998).

%	Moisture	Starch	Sugar	Protein	Fats	Fiber	Ash
Cassava	70.25	21.45	5.13	1.12	0.41	1.11	0.54
Potato	75.80	19.90	0.40	1.80	0.20	1.10	0.90

2.1.2 Manufacturing Process for Tapioca Starch

Tapioca starch is produced using two types of processes. The first grade product is produced using centrifugation filtration and spray drying as the basic unit processes. Their processes are low capital and low labor intensive and use more water. The first grade product is directed to oversea markets. In contrast, very little mechanization is utilized by the second grade starch producers, which are often small private enterprise operations. Their processes are low capital intensive and using very simple methods of separation by cloth filtration, gravity settling, decanting, and drying on heated concrete slabs.

A flow diagram of the first grade starch plant is illustrated in Fig. 2.1. Roots are transported by truck to the plant, which normally processes them within 24 hours to avoid degradation of starch. The sand is removed by dry rasping in a revolving drum and mechanical tumbling in a wash basin from which the root wash water is derived does the peeling. The roots are then mechanically crushed to release the starch granules from their surrounding cellulose matrix. Most of the cellulose materials are removed by centrifugal means in the Jet extractor and then by continuous centrifugation. The cellulose material or pulp is sold as duck feed provided that it is fresh or dewatered, dried and sold as filter feed if not. After primary centrifugation, the starch milk is sieved through a series of three sieve decreasing in pore size to assist in separating the starch from the small amount of pulp remaining. The pulp thereby recovered is recycled to the Jet extractor and the processes starch milk fed to a second continuous centrifuge from which wastewater. After dewatering to a paste-like substance in a basket centrifuge, the product is spray dried and package.

In second grade tapioca refineries (Fig 2.2), the initial stages of processing also include dry removal of sand root washing and rasping. Starch separation from the cellulosic pulp is made in only one step by screening it through fine nylon mesh supported by a large cylindrical drum. The starch is sprayed through and the pulp slowly drawn off and collected for dewatering the starch milk is then sieved again and released into large 1.2 m. deep settling basins. After 24 hours settling, the supernatant is removed by decantation and discharged to a river. The surface of the starch caked on the bottom is washed, the wash water from which is discharge to an outdoor lagoon. The starch is then resuspended and pumped to the second sedimentation basin again 1.2 m. deep and 24 hours, detention time. The supernatant is decanted and discharged to the same lagoon as the first surface wash water. The starch is removed in large cakey chunks to the subsurface hot air concrete pad for spreading and drying. After drying, it is packed and markets.

2.1.3 Solid Waste from Tapioca Process

During the processing of cassava flour, the residual pulp is occurred, that called cassava solid waste. The waste can be defined as those materials that have not been shown to have an economical value through utilization and conversion by animals into valuable products for human benefit, which is separated from the starch in the screening process is used as an animal feed. It is usually utilized wet (75-80 percent moisture content) in the neighborhood of the processing factory but is sometimes sun dried before it is sold. This product is considered a by-product of the cassava starch industry and represents about 10 percents by weight of the cassava roots. An important property of starch granules is that they swell when heated in the presence of water in the process of gelatinization. Gelatinization involves the breaking of hydrogen bonds between the starch, chains, especially in the crystalline regions of the granule (Blanshard, 1987). The range of temperatures over which gelatinization occurs varies with the source of the starch. Cassava starch gelatinizes over the range 58.5-70.0° C, (Balagopalan *et al.*, 1988). Table 2.2 showed the approximate analysis of dry solid waste from tapioca process.

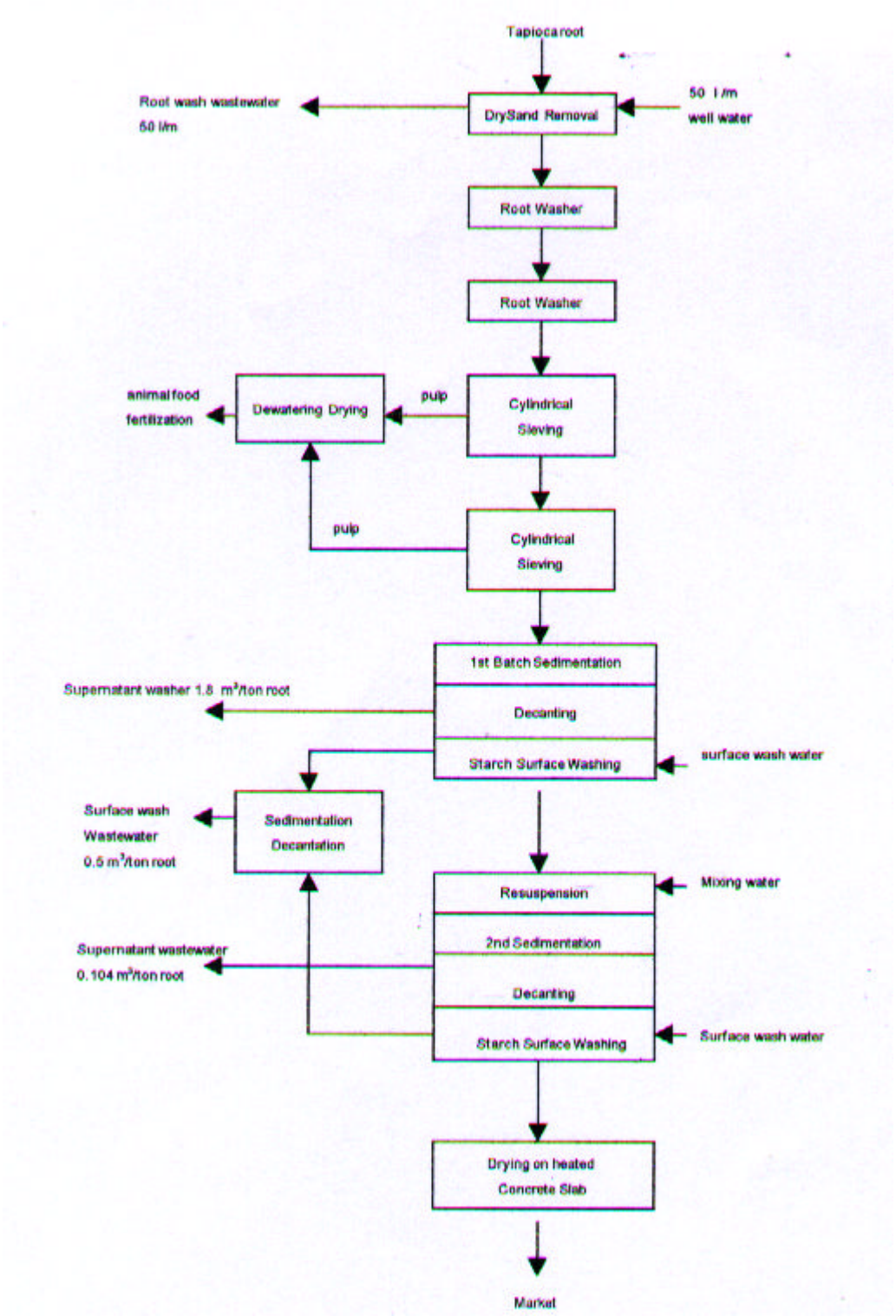


Figure 2.1 First grade tapioca starch process flow diagram (Tanticharoen, 1999).

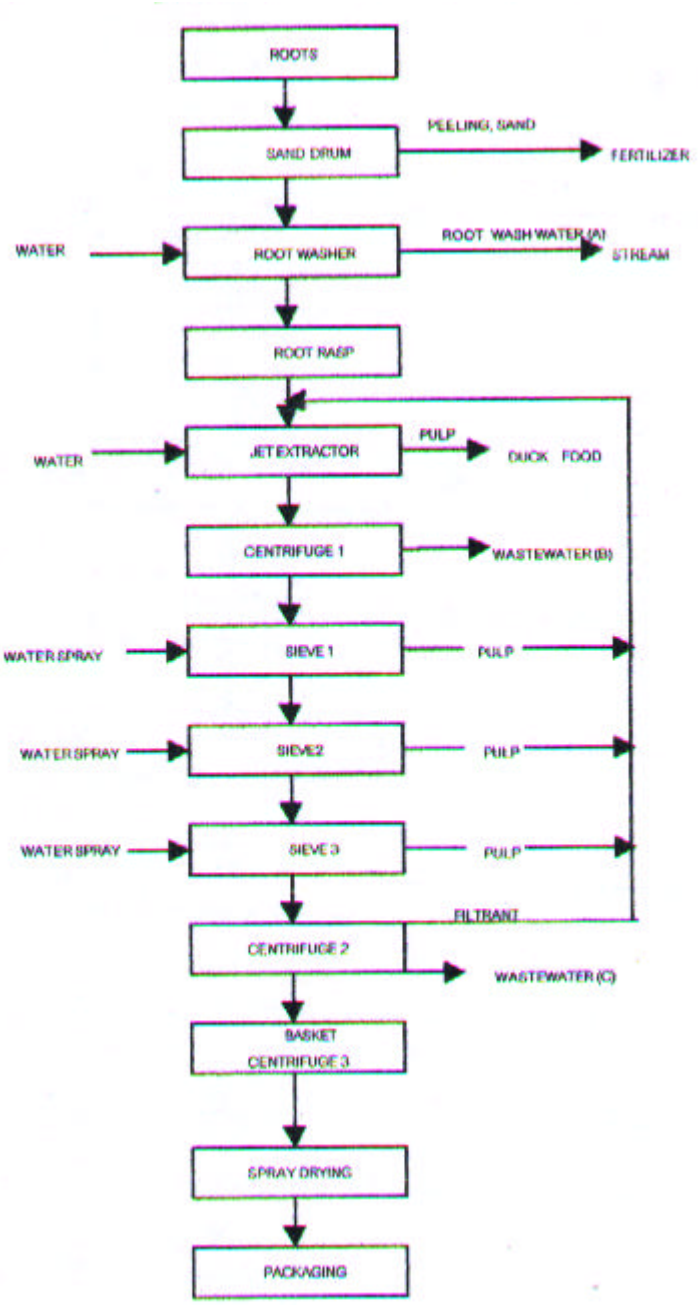


Figure 2.2 Second grade tapioca starch production (Tanticharoen, 1999).

2.1.4 Solid Substrate Fermentation

Solid-substrate fermentations were generally characterized by the growth of microorganisms on water insoluble substrates in presence of varying amounts of free water. In order to avoid the confusion existing in the literature in regard to free water, Moo-Young and co-worker (1983) proposed the term solid-state fermentation for all those processes, which utilize water insoluble materials for microbial growth in the absence of free water. With increasing amounts of free water, solid substrate fermentations progress from solid-state fermentations through slurry fermentations to fermentation of suspensions of solid particle.

Starchy substrates are harvested as agricultural products in the form of the roots, tubers or grains. These may be used directly as substrates for solid substrate cultivation with a minimum of pretreatment, such as grinding or cracking, followed by gelatinization. In all cases, gelatinization represents an essential pretreatment step. In practical situations, gelatinization is achieved by cooking in water or by steaming. (Doelle, Mitchell and Rolz, 1992).

Table 2.2 The approximate analysis of solid waste from cassava (Grace, 1998).

	Protein	Starch	Fat	Ash	Fiber	Total
%	5.3	56.0	0.1	2.7	35.9	100

Cassava products have long been used for animal feeding. Large quantities of cassava roots and cassava waste are utilized in the cassava-producing countries for this purpose. Imports of dried cassava roots and meal into European markets for the supply of the compound feed industry are also increasing. Tapioca, however, is not suitable for the production of feeds requiring high protein content. Attempts have been made for protein enrichment using various microorganisms such as *Aspergillus* and *Rhizopus.* Nevertheless, the economic feasibility is still in doubt and further technological development is needed.

2.2 Bacteria for Cassava Solid Waste Fermentation

2.2.1 Lactic Acid Bacteria

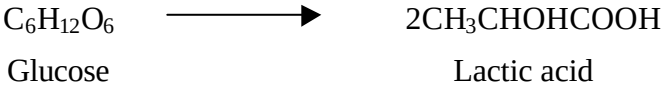
Lactic acid bacteria (LAB) are bacteria that give rise to lactic acid; however, this simple definition would be to include nearly all bacteria, because in general are able to produce lactic acid to a greater or lesser extent. Narrowing the definition to the production of large quantities of lactic acid, in addition to bacteria, *Rhizopus* fungi have the ability to produce lactic acid on an industrial scale. In this group of beneficial bacteria which ferment sugars as an energy source to produce large quantities of lactic acid, and although they may decompose protein they do not give putrefaction (proteolytic) products. Lactic acid bacteria are a group of Gram-positive bacteria united by a constellation of morphological, metabolic and physiological characteristics. The general description of the bacteria included in the group is Gram-positive, nonsporing, nonrespiring cocci or rod, which produce lactic acid as the major end product during the fermentative of carbohydrates. The boundaries of the group have been subject, to some controversy, but these have been general agreement that the genera *Lactobacillus, Leuconostoc* and *Streptococcus* form the core of the group. Recent taxonomic revisions of these genera suggest that the lactic acid bacteria comprise the following; *Aerococcus, Carnobacterium, Enterococcus, lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus,* and *Vagacoccus*.

Lactic acid bacteria are found in foods (dairy products, fermented meat, sour dough, fermented vegetables, silage, beverages), on plants, in sewage, but also in the genial, intestinal and respiratory tracts of man and animals (Hammes *et al.*, 1991). As mentioned, the basis for the classification of the LAB in different genera has essentially remained unchanged. Although morphology is regarded as questionable as a key character in bacterial taxonomy (Woese, 1987), it is still very important in the current descriptions of the LAB genera. Furthermore, cell division in two planes, leading to tetrad formation, is used as a key characteristic in the differentiation of the cocci. An important characteristic used in the differentiation of the LAB genera is the mode of glucose fermentation under standard condition, i.e., nonlimiting concentrations of glucose and growth factors (amino acid, Vitamins and nucleic acid).

LAB is involved in the production of many traditional fermented foods and is therefore so considered as generally recognized as safe organisms. Several lactic bacterial strains with amylolytic activity have been described, (Pintado *et al.*, 1999). Almost LAB is those, which may be poisoned by oxygen, cannot grow in an air atmosphere, and do not use oxygen for energy yielding chemical reactions. Some anaerobes can tolerate low concentrations of oxygen. The toxicity of oxygen for strict anaerobes is due to certain molecules produced during reactions involving oxygen. Some of these reactions result in the addition of a single electron to an oxygen molecule, forming a superoxide radical ($O_2^{\bullet}$). Superoxide radicals may cause damage to cells, but they also give rise to hydrogen peroxide, H_2O_2, and hydroxyl radicals, $OH^{\bullet}$, both of which can destroy vital cell components.

2.2.2 Type of Lactic Acid Fermentation

The type of fermentation carried out by lactic acid bacteria. They occur as cocci or rods and generally lack catalase, although pseudo-catalase can be found in rare cases. Fermentable carbohydrates are used as energy source, "Lactic acid fermentation", can be broadly divided into homo-lactic-acid fermentation, and heterolactic-acid fermentation. In homo-lactic-acid fermentation, glucose is fermented to lactic acid according to the following equation, with virtually 100% conversion and the Embden-Meyerhof pathway is used (Fig.2.3). Lactic acid bacteria that carry out such as fermentation are called homofermentative lactic acid bacteria.



In hetero-lactic-acid fermentation on the other hand, fermentation of glucose produces lactic acid and products other than lactic acid: normally glucose is fermented to lactic acid, carbon dioxide, acetic acid and alcohol. Lactic acid bacteria that carry out such fermentation are called hetero-fermentative lactic acid bacteria.

$$C_6H_{12}O_6 \longrightarrow 2CH_3CHOHCOOH + C_2H_5OH + CO_2$$

Glucose	Lactic acid	alcohol (ethanol)	carbon dioxide

Currently lactic acid bacteria are classified into 5 genera, *Streptococcus* (including *Lacctococcus*), *Pedisococcus*, *Lactobacillus* and *Bifidobacterium*, on the basis of type of fermentation, morphology, aerobic growth habit, the optical rotation of the lactic acid, etc. The effectiveness of a process can be measured as the concentration of lactic acid (LA) produced, as the yield of lactic acid based on substrate and as the productivity (LA product rate). The yields were calculated as gram LA per gram substrate provided ($Y_{LA/tot}$), and the productivity are given as the maximum volumetric productivity (Qv) in g LA per liter per hour.

2.2.3 Characteristics of *Lactococcus* Bacteria

Members of the *Lactococcus* genus are Gram-positive cocci that can, depending on growth conditions, appear ovoid and are typically 0.5-1.5 μm in size. They do not form spores in pairs or in short chains and unlike many members of the *Streptococcus* genus, these organisms do not growth in long chain. They have a fermentative metabolism and expected for lactic acid bacteria, they produce amounts for lactic acid. They have complicated nutritional requirements and are auxotrophic for a number of amino acids and vitamins. Their optimum growth temperature is 30°C and they can grow at temperatures as low as 10°C but not at 45°C. They also cannot grow in 0.5% NaCl. Both their maximum growth temperature and their failure to tolerate salt are somewhat diagnostic of this genus as compared to closely related members of the *Streptococcus* genus, most notably *S. thermophilus.* Lactic acid bacteria have as a common feature: the ability to product lactic acid as a major end product of their fermentation of hexoses or glucose. Initial classification schemes as proposes by Orla-Jensen have proven relatively accurate even in the face of challengers raised by the advent of molecular classification methods (Carl, 1999).

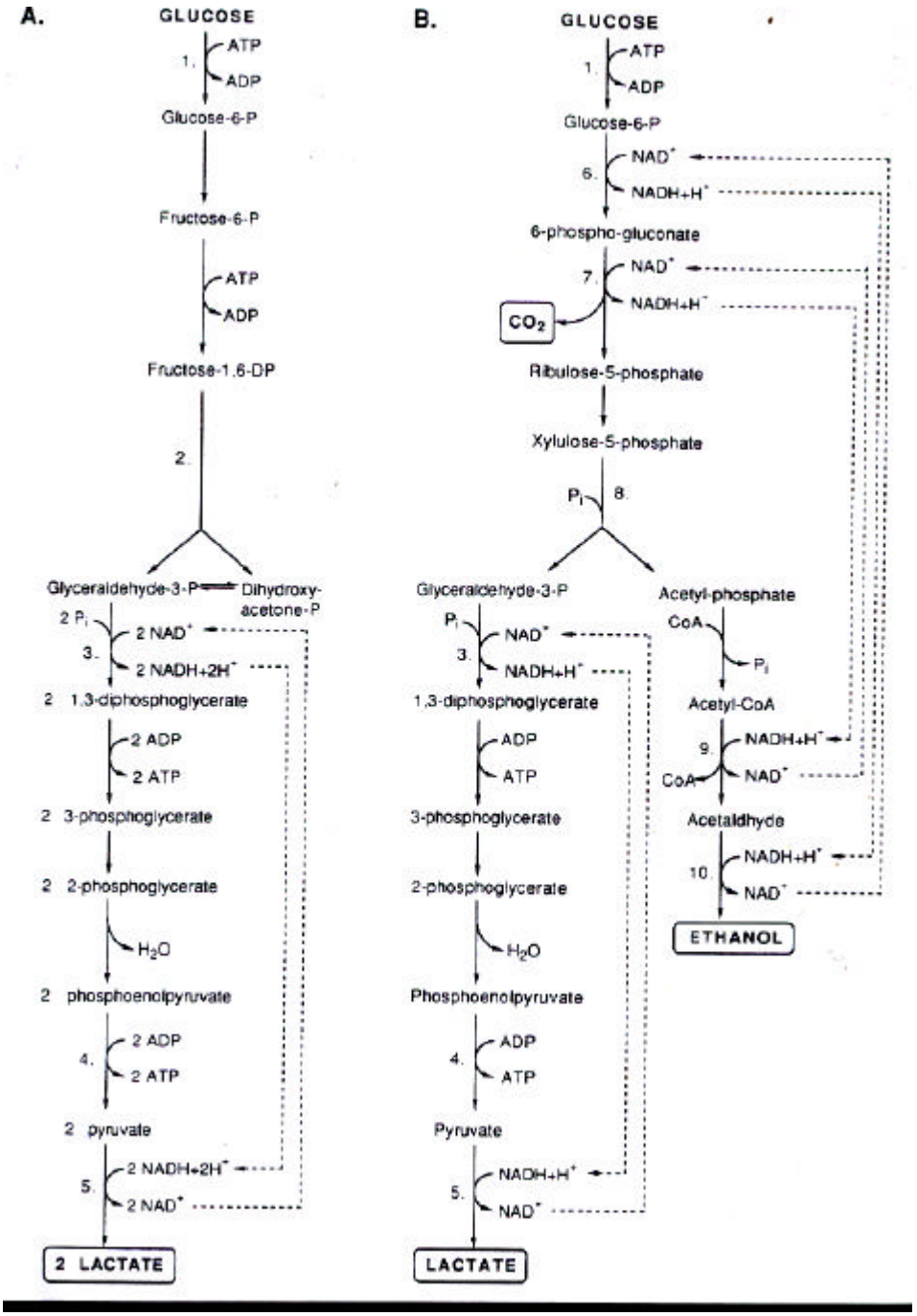


Figure 2.3 Major fermentation pathways of glucose:

(A) homolactic fermentation (glycolysis, Embden-Meyerhof Pathways) ;(B) heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway). Selected enzymes are numbered: 1. Glucokinase; 2. Fructose-1,6-diphosphate aldose; 3. Glyceraldehyde-3-phosphate dehydrogenase; 4. Pyruvate kinase; 5. Lactate dehydrogenase; 6. Glucose-6-phosphate dehydrogenase; 7. 6-phosphoglyconate dehydrogenase; 8. Phosphoketolase; 9. Acetaldehyde dehydrogenase; 10. Alcohol dehydrogenase.

The genus *Lactococcus* is relatively new and most members of this genus previously belonged to the genera *Streptococcus* and *Lactobacillus.* In 1985, Schleifer and colleagues proposed the genus *Lactococcus* and included the species formerly known as *Streptococcus lactis, Lactobacillus hordniae* and *Lactobacillus xylosus.* The fact that the latter two species were formerly classified within the genus *Lactobacillus* is curious and suggests that the pleomorphic nature of these organisms can confound classification based on cell shape. Whereas some lactic acid bacteria produce D-lactic acid, L-lactic acid and/or a combination of D and L, the lactococci produce only L-lactic acid.

2.3 Metabolism of Lactic Acid Bacteria

Microbial growth depends on the cell's ability to use the nutrients in its surroundings to synthesize the macromolecular components of cellular structures and also, the many low molecular weight compounds required for cellular activity. Intermediary metabolism is concerned with the reactions, which transform carbon and nitrogen compounds entering the cell either into new cell material, or into products which are excreted. The synthesis of these compounds requires energy and most of the cells involved to industrial fermentations are heterotrophs, which obtain this energy from breakdown of organic compounds. In aerobic or respiratory processes, organisms are able to completely oxidize some of the substrates to CO_2 and H_2O, resulting in the provision of maximum energy for conversion of the remaining substrates into new cell mass. In anaerobic or fermentative metabolism, cells are less efficient in converting organic substrates into cellular material and usually excrete partially degraded intermediates. Energy producing or catabolic pathways generate ATP and reduced coenzymes needed for various biosynthetic react ions and chemical intermediates used as starting points for biosynthesis. The essential feature of LAB metabolism is efficient carbohydrate fermentation coupled to substrate-level phosphorylation. The generated ATP is subsequently used for biosynthetic purpose. LAB as a group exhibits an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is, of course, lactic acid (> 50% of sugar carbon). It is clear, however, that LAB adapts to various conditions

and change their metabolism accordingly. This may lead to significantly different end-product patterns. Rapid metabolism of lactose to lactic acid is a desirable trait in dairy starter cultures. Under conditions of carbon excess, *Lactococcus* strains fermented sugars by homolactic fermentation. Thus, mainly L (+)-lactic acid is produced from the lactose in milk. Lactic acid production is the major contributor to the preservation of fermented dairy products. The resulting low pH inhibits the growth of many spoilage and pathogenic bacteria. Under carbon-limiting conditions, homolactic fermentation may shift to a mixed acid fermentation yielding acetate, ethanol and CO_2, in addition to lactate. Some *Lactococcus* strains can assimilate simple sugary into complex polysaccharides that are exported and remain associated with the cell surface (exopolysaccharides). These components provide a thick texture to some products and can act as stabilizers. Exopolysaccharides may also increase moisture retention and yield in some cheeses.

2.4 Lactic Acid and Applications

2.4.1 Lactic Acid

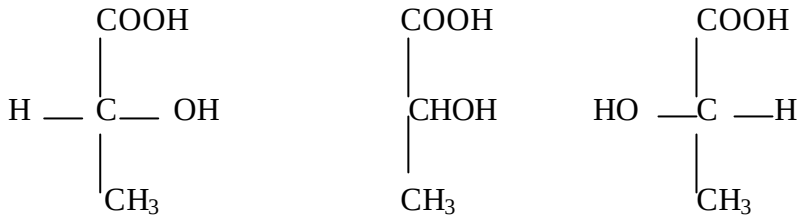
Lactic acid, 2-hydroxypropionic acid ($CH_3CHOHCOOH$) (Fig. 2.4), was isolated and identified by Scheele in 1780. In 1881, Charles E. Avery set up the first commercial lactic acid fermentation plant in Littleton. Currently, lactic acid is produced synthetically as well as via fermentation, with a total worldwide market of about 100 million lb/year, of which the United States consumes roughly 40%. Lactic acid is the simplest, 2-hydroxyacisd that has chiral center, and exists as two anantiomers, L(+)-lactic acid and D(-)-lactic acid. L-lactic acid is enantiomer involved in normal human metabolism, and D-lactic acid is metabolized differently from L-lactic acid by humans. Differentiation scheme for other bacteria can show at Table 2.3. The presence of D-lactic acid to acidified milk formulas was found to cause infant acidosis, an abnormally high level of acidity of the blood and body tissues. Health concern regarding D-lactic acid prompted manufactures of fermentation lactic acid to switch to production of the L-isomer. Currently, all commercial fermentation lactic acid is L-lactic acid. However, synthetic DL-lactic

acid is still used in the baking industry and generally as a food additive. The synthetic process involves reacted hydrogen cyanide with acetaldehyde to form lactonitrile, which is subsequently hydrolyzed to produce racemic DL-lactic acid.

Table 2.3 Differentiation scheme for *lactococci, pediococci* and *leuconostoc* (Teuber *et al.*, 1991).

Fermentation Products of Glucose	Genus
L(+)-Lactic acid	*Lactococcus*
D(-)-Lactic acid, CO_2, acetic, ethanol	*Leuconostoc*
DL-Lactic acid	*Pediococcus*

The conventional fermentation process typically involves fermenting suitable carbohydrates to lactic acid, which is neutralized to form calcium lactate, followed by optional crystallization of calcium lactate, and addition of sulfuric acid to form free lactic acid and calcium sulfate. In comparison, the synthetic process has a higher raw material cost, whereas purification of fermentation lactic acid to make a heat-stable product is difficult. Recently, lactic acid has received strong interest as the feedstock for making degradable polylactic acid plastic and coating. Several companies are starting commercial lactic acid production via fermentation using newly developed proprietary processes. It is expected that lactic acid will soon be available at a much lower cost. This may stimulate wider applications of lactic acid in food and nonfood areas. Lactic acid is also possible to use renewable resources as substrates, such as starch and cellulose in fermentative production. Renewable resources do not give any net contribution of carbon dioxide to the atmosphere, as do the limited oil and fossil-fuel-based sources. Cellulose, hemicellulose and starch are the most abundant compounds in the world, and when hydrolyzed to mainly glucose they are fermentable by a number of microorganisms, (Karin and Barbel, 2000).



D-Lactic Acid DL-Lactic Acid L-Lactic Acid

Figure 2.4 The chemical structure of lactic acid (Budavari, 1996).

2.4.2 Applications of Lactic Acid

Batch fermentations have been the method used industrially up to now. Lactic acid is used in many food and nonfood applications. Of the 33 million lb. of the lactic acid consumed in the U.S. market in 1989, 16.8 million lb. were used for the manufacture of emulsifiers, 9.8 million lb. in food additives, 3.7 million lb. for pharmaceutical and cosmetics applications, and the balance for industrial and miscellaneous uses. Lactic acid is used as an acidulant and a flavor enhanced in many kind use of synthetic and heat-stable lactic acid is in the manufacture of sodium and calcium stearoyl lactylate and other lactylated emulsifiers. Stearoyl lactylate is used in the baking industry as a dough conditioner.

Fermentation conditions were different for each industrial producer, depending on the raw materials and strains in use. In every case, pH and temperatures were kept in the range previously described, while the final lactic acid concentration and fermentation time on the carbohydrate source. When glucose or hydrolyzed starch was the sugar source, *L. delbrueckii* was the organism in use, with initial sugar concentrations between 12 and 15% and fermentation times from 3 to 6 days. When milk whey was the raw material, with initial sugar concentrations of around 5%, fermentation were carried out with *L. bulgaricus* in 1 to 2 days (Mobley, 1994). A maximum productivity of 1-2 g/L/h was achieved. In these commercial processes, little attention was paid to sterility, as lactic acid is inhibitory to most other microorganisms. In recent years, important improvements in fermentation rates have been achieved through better equipment design and process control. Especially for

temperature and pH, and also through strain development. There has also been change in focus on the process, both from the raw materials side and the final product side.

2.5 The Process of Fermentation

2.5.1 Fermenter and Bioreactor configurations

The cylindrical tank, either stirred or unstirred, is the most common reactor in bioprocessing. Yet, a vase array of fermenter configurations is in use in different bioprocess industries. Novel bioreactors are constantly being developed for special applications and new forms of biocatalyst such as plant and animal tissue and immobilized cells and enzymes. Must of the challenge in reactor design lies in the provision of adequate mixing and aeration for the large proportion of fermentation requiring oxygen; reactors for anaerobic culture are usually very simple in construction without sparging or agitation. In the following discussion of bioreactor configuration, aerobic operation will be assumed.

2.5.1.1 Shake flasks and bottles

Shake flasks find a variety of uses in the fermentation laboratory, including initial strain screening, multivariable testing, and inoculum growth. These pieces of glassware can vary in size and form, and in some instances have been designed and developed for specialist applications. Shake flasks have a number of significant disadvantages relative to cultivation in a fermenter, including lower oxygen transfer rates, less closely controlled environmental conditions, and the difficulty of withdrawing samples aseptically. The lower volume of medium in the shake flasks, the better will be the oxygen transfer rate. The volume will be dependent on the medium and type of culture. The standard 250-ml Erlenmeyer flask is cheap and simple; most of the shaker tables are designed to use these flasks, although there are tables, which can be adapted to allow other shapes or bigger flasks. Different plugs can be made of cotton wool, glass wool, polyurethane foam, and gauze or synthetic fibrous material (an aluminium foil cap can sometimes be used in conjunction with these plugs). The plug has to prevent airborne microorganism from

getting into the medium while at the same allowing free flow of air into the flask and for this reason it must not be allowed to become wet (Fig. 2.5).

Figure 2.5 Shake flask fermenter (Iirvine, 1990).

2.5.1.2 Stirred tank

Basically, the stirred tank fermenter consists of a cylindrical tube with a top-driven or bottom-driven agitator. The stirred tank with a top-drive assembly is the most commonly used fermenter because of its ease of operation, neat design, reliability, and robustness. For smaller mini- fermenter (bench-top), borosilicate glass is used as the cylindrical tank and a top plate of stainless steel clamped on. A motor is fixed above the top plate and is attached in an autoclave. The vessel, medium and probes are usually sterilized together, minimizing the number of aseptic operations required. These glass vessels can vary in size from one litre to 30 litre capacity. The vessel itself will have a specific impeller design, baffles, an air sparger, and sample port(s). Figure 2.6 shows the basic configuration.

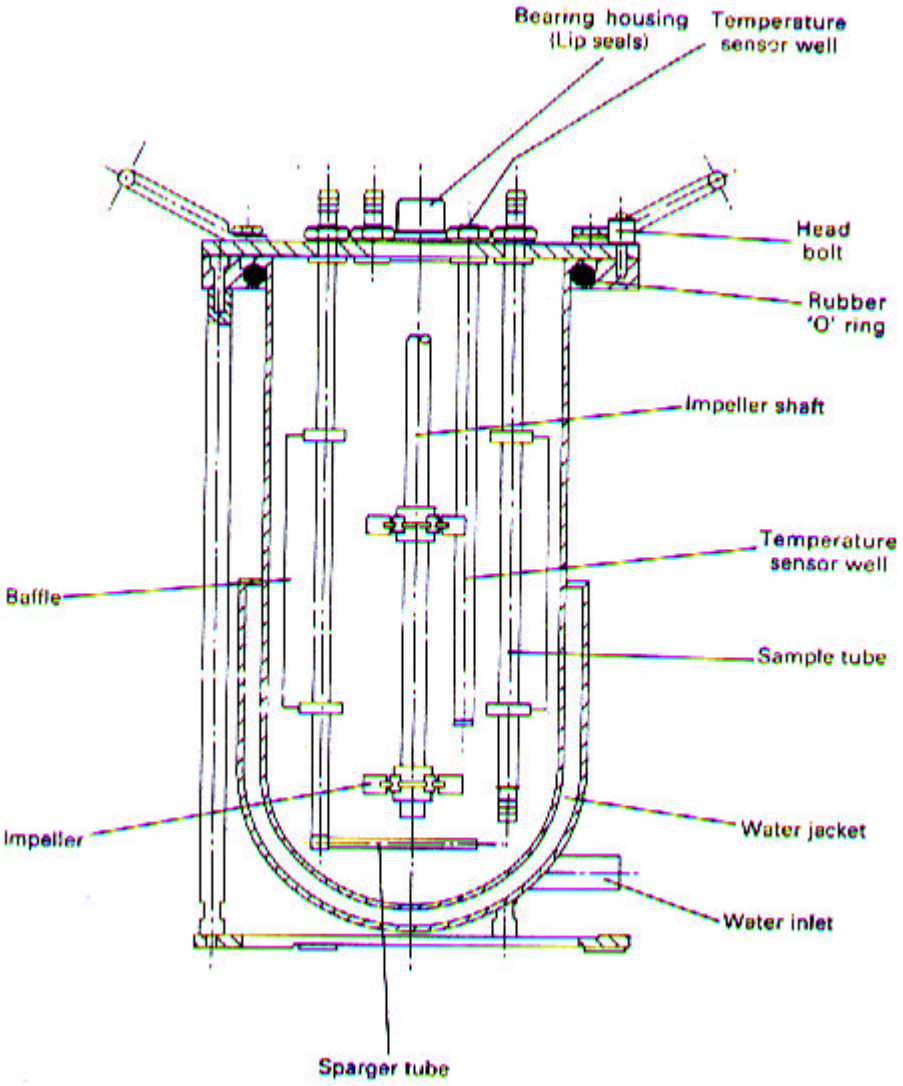


Figure 2.6 Typical stirred-tank fermenter (Glass fermentation vessel) (Iirvine, 1990).

2.5.1.3 Bubble column

Alternatives to the stirred reactor include vessels with no mechanical agitation. In bubble-column reactors, aeration and mixing are achieved by gas sparging (Fig2.7); this requires less energy than mechanical stirring. Bubble columns are applied industrially for production of bakers' yeast, beer and vinegar, and for treatment of wastewater.

Bubble columns are structurally very simple. They are generally cylindrical vessels with height greater than twice the diameter. Other than a sparger for entry of compressed air, bubble columns typically have no internal structures. A height-to diameter ratio of about 3:1 is common in bakers' yeast production; for other applications, towers with height-to-diameter ratios of 6:1 have been used. Perforated horizontal plates are sometimes installed in tall bubble columns to bread up and redistribute coalesced bubbles. Advantages of bubble column include low capital cost, lack of moving parts, and satisfactory heat-and mass transfer performance. As in stirred vessels, foaming can be a problem requiring mechanical dispersal or addition of antifoam to the medium.

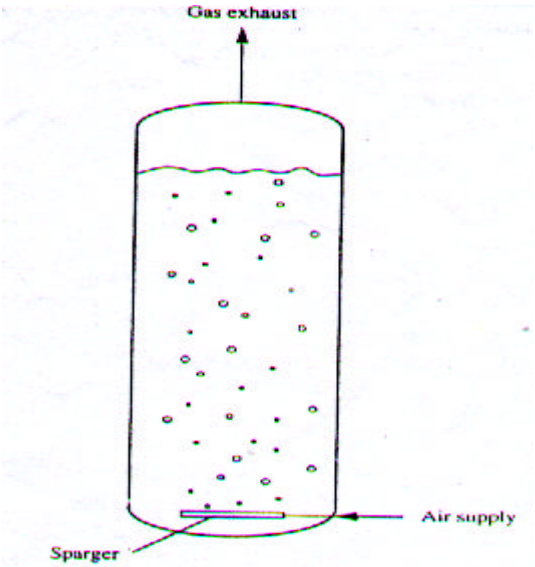


Figure 2.7 Bubble-column bioreactor (Pauline, 1995).

2.5.1.4 Airlift reactor

In all aerobic fermentations, air is an essential requirement. Air-lift fermenters have no mechanical agitation system but utilize the air circulating within the fermenter to bring about the mixing of the medium. This rather gentler system of mixing is ideal for plant and animal cell cultures. The air-lift fermenter is designed in such a way that aeration provides both the agitation of the broth and dispersal of the oxygen into the broth. Power draw is very much reduced and the main power source is the air compressor.

Several types of air-lift reactor in use, their distinguishing feature compared with the bubble column is that patterns of liquid flow are more defined owing to the physical separation of up-flowing and down-flowing streams. Gas is sparged into only part of the vessel cross-section called the riser. Gas hold-up and decreased fluid density cause liquid in the riser to move upwards. Gas disengages at the top of the vessel leaving heavier bubble-free liquid to recirculate through the downcomer. Liquid circulates in air-lift reactors as a result of the density difference between riser and downcomer. A variation of the air-lift fermenter principle is the tubular loop fermenter. This type of fermenter can be used to increase the volume of the fermentation while maintaining the residence time (Fig 2.8).

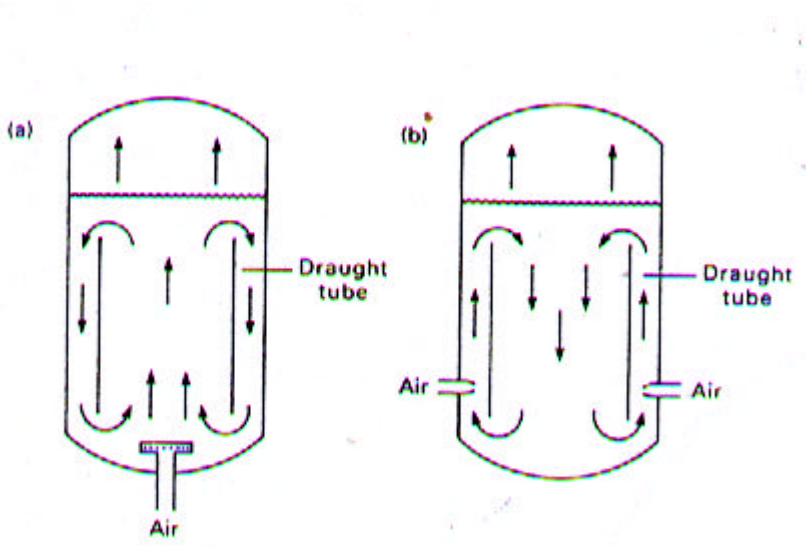


Figure 2.8 Airlift reactor configurations (Iirvine, 1990).

2.5.1.5 Fluidized beds

The use of inert dense particles has been further developed in fluidized beds. These reactors are hollow chambers in which dens particles containing a microbial film or microbial mass are mixed or recycled. The immobilized biomass consists of microorganisms, which adhere to large particles such as rocks, glass beads, or plastic beads. The quantity of biomass is dependent upon the inert particle surface area, sloughing effect, aeration and recycle efficiency. A typical example is show in Figure 2.9. One of the oldest fermentations known to man, vinegar fermentation, utilizes this principle.

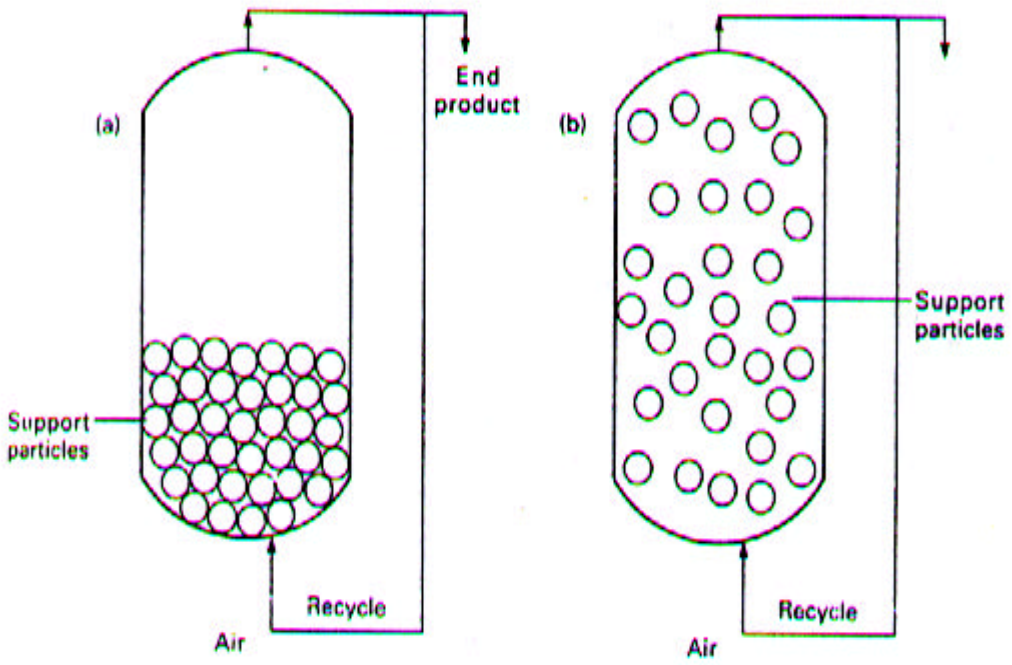


Figure 2.9 Fluidized beds reactor (Iirvine, 1990).

2.5.2 Reactions of Fermentation

2.5.2.1 Homogeneous reactions

When the temperature and all concentration in system are uniform. Most fermentation and enzyme reaction carried out in mixed vessels fall into category. The extent to which reactants are converted to products is expressed as the reaction yield. Yield is the amount of the product formed or accumulated per amount of reactant provided or consumed.

2.5.2.2 Heterogeneous reactions

Reactions occurring in the presence of significant concentration or temperature gradients is call heterogeneous reactions. Because biological reaction are not generally associated with large temperature gradients. When heterogeneous reactions occur in solid catalysts, not all-reactive molecules are available for immediate conversion. Reaction lakes place only after reactants are transported to the site of reaction. Thus, mass transfer process can have a considerable influence on the over all conversion rate. Because rate of reaction is generally dependent on substrate concentration, when concentrations in the system vary, kinetic analysis becomes more complex.

2.6 Kinetics

2.6.1 Kinetics in Batch Growth

The kinetics of cell growth is expressed using equations similar to those for enzymes. From a mathematical point of view, there is little difference between the kinetic equations for enzymes and cells, after all, cell metabolism depends on the integrated action of a multitude of enzymes. Several phase of cell growth are observed in batch culture; a typical growth curve is show in Figure 2.10. The different phases of growth are more readily distinguished when the natural logarithm of viable cell concentration is plotted against time; alternatively, a semi-log plot can be used. Rate of growth varies depending on the growth phase, during the lag phase immediately after inoculation, rate of growth is essentially zero, cells use the lag phase to adapt to their new environment; new enzymes or structural components may be synthesized. Following the lag period, growth starts in the acceleration phase and

continues through the growth and decline phases. If growth is exponential, the growth phase appears as a straight line on a semi-log plot. As nutrients in culture medium become depleted or inhibitory products accumulate, growth slows down and the cells enter the decline phase. After this transition period, the stationary phase is reached during which no further growth occurs. Some cultures exhibit a death phase as the cells lose viability or are destroyed by lysis. Table 2.4 provides a summary of growth and metabolic activity during the phases of batch culture.

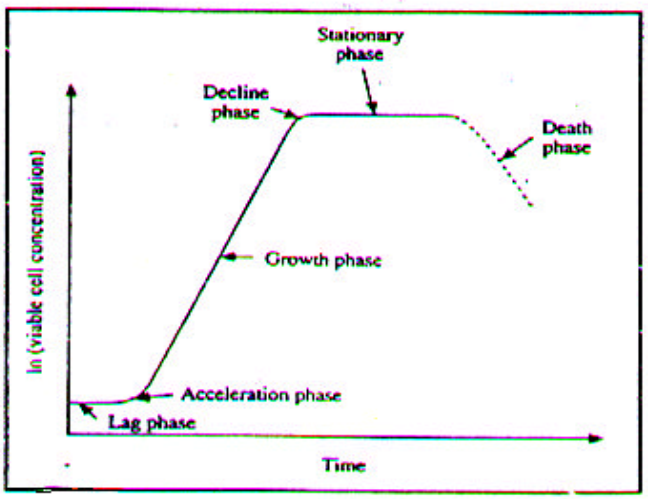


Figure 2.10 Typical batch growth curve (Pauline, 1995).

Table 2.4 Summary of batch cell growth

Phase	Description	Specific growth rate
Lag	Cell adapt to the new Environment; no or very little growth	$\mu = 0$
Acceleration	Growth starts	$\mu < \mu_{max}$
Growth	Growth achieves its maximum rate	$\mu \approx \mu_{max}$
Decline	Growth slows due to nutrient exhaustion and build-up of inhibitory products	$\mu < \mu_{max}$
Stationary	Growth ceases	$\mu = 0$
Death	Cells lose viability and lyse	$\mu < 0$

During the growth and decline phases, rate of cell growth is described by the equation,

$$r_x = \mu x \tag{2.1}$$

Where r_x is the volumetric rate of biomass production with units of $kg/m^3/s$, for example; x is viable cell concentration with units of, for example, kg/m^3, and μ is the specific growth rate, with the unit s^{-1}, for example. Cell growth is therefore considered a first-order autocatalytic reaction. In a closed system where growth is the only process affecting cell concentration, $r_x = dx/dt$ and integration of Equation (2.1) gives an expression for x as function of time. If μ is constant, the integrate directly with initial conditions were used; $x = x_o$ at $t = 0$ to give:

$$x = x_0 e^{\mu t} \tag{2.2}$$

Where x_0 is the viable cell concentration at time zero. Equation 2.2 represents exponential growth. Taking natural logarithms:

$$\ln x = \ln x_0 + \mu t \tag{2.3}$$

According to Eq.(2.3), a plot of ln x versus time gives a straight line with slope μ.. Because the relationship of Eq.(2.3) is strictly valid only if μ is unchanging, a plot of ln x versus t is often used to assess whether the specific growth rate is constant. As shown in Figure 2.10, μ is usually constant during the growth phase. It is always advisable to prepare a semi-log plot of cell concentration before identifying phase of growth. Growth always appears much slower at the beginning of culture because the number of cells is present is small.

Cell growth rates are often expressed in terms of the doubling time (t_d) an expression for doubling time can be derived from Equation.(2.2), starting with a cell concentration of x_o, the concentration at $t = t_d$ is $2x_0$. Substituting these values into Equation 2.2.

$$2x_0 = x_0 e^{\mu t d} \tag{2.4}$$

And canceling x_0 gives;

$$2 = e^{\mu td} \tag{2.5}$$

Taking the natural logarithm of bothsides;

$$\ln 2 = \mu t_d \tag{2.6}$$

or $$t_d = \ln 2/\mu \tag{2.7}$$

2.6.2 Effect of Substrate Concentration

During the growth and decline phases of batch culture, the specific growth rate of cells is dependent on the concentration of nutrients in the medium. Often, a single substrate exerts a dominant influence on rate of growth; this component is knows as the growth-rate-limiting substrate or, more simply, the growth-limiting substrate. The growth-limiting substrate is often the carbon or nitrogen source, although in some cases it is oxygen or another oxidant such as nitrate. During balanced growth, the specific growth rate is related to the concentration of growth-limiting substrate by the Monod equation a homologue of Michaelis-Menten expression (McNeil and Harvey, 1990):

In Eq. (2.8), S is the concentration of growth-limiting substrate, μ_m is the maximum specific growth rate, and K_s is the substrate constant. μ_m has dimension T^{-1}, K_s has the same dimensions as substrate concentration.

Estimation of μ_m and k_s

$$r_x = dx/dt = \mu_m \,.\, s \,.\, x /(K_s + s) \tag{2.8}$$

A common expression is the Monod equation, which relates the rate r_x, to two variables that can measure, carbon substrate concentration, s, and cell concentration, x. The expression also contains two parameter, μ_m and K_s, which are assumed to be constants for a particular microorganism at constant environmental condition,

temperature, pH, etc. Since that have the products of variables, e.g. s, x and reciprocals, e.g. x /(K_s + s), it cannot be handled straight forwardly. The trick is to rearrange the variables in groups or change them in some way so that the equation looks like something we are used to handling. The simplest equation to work with is the one which when plotted is a straight line (y = mx + c, where y and x are the variables, m is the slope, and c is intercept). The first obvious grouping is to devide both sides of eq. 2.8 by x to give

$$r_x / x = \mu_m . s /(K_s + s) \quad (2.9)$$

From $r_x / x = \mu$ and $dx/dt = \mu_m . s . x/(K_s + s)$, we can reducing the number of variables from three untransformed to two transformed so that,

$$\mu = r_x / x = (dx/dt)/x \quad (2.10)$$

(For a closed batch process only)

Remember that μ is variable even though it is called the specific growth rate that it depends on the value of s. We could now plot μ against s and then find values of the parameters μ_m and k_s which give the theoretical curve which best fits the data. If we take the reciprocal of both sides of equation (2.9)

$$1/\mu = (K_s+s)/(\mu_m. s) \quad (2.11)$$

$$1/\mu = (K_s/(\mu_m.s)) + 1/\mu_m \quad (2.12)$$

From straight line y = mX + C, so against 1/S on arithemetic graph paper then we should get a straight line with slope k_s/μ_m and intercept on the 1/μ axis of $1/\mu_m$. Hense we can easily find k_s and μ_m. This plot is called a Lineweaver-Bürke plot. In addition, because of the geometry of the plot, a value for $1/k_s$ can be read directly off the abscissa (horizontal axis). Figure 2.11 shows an example of the plot.

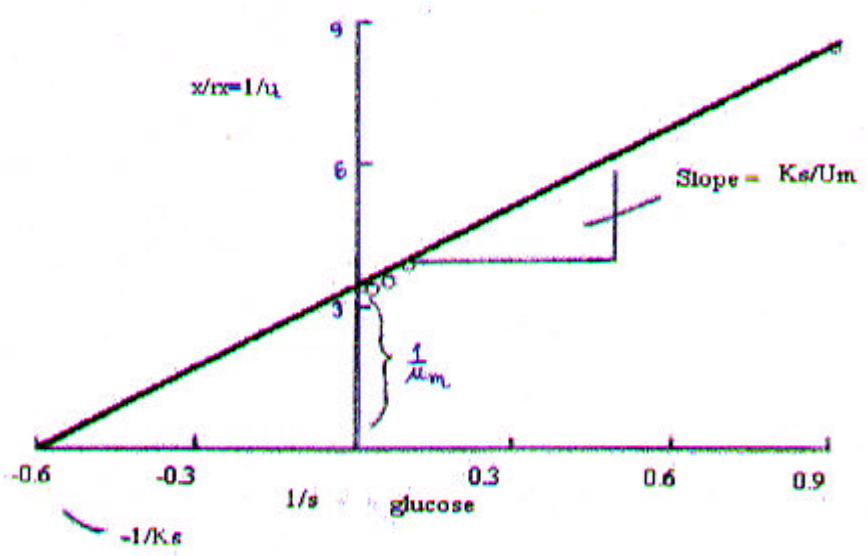


Figure 2.11. Determination of μ_m and K_s by Lineweaver-Burk plot.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and reagents

3.1.1 Iodine solution

- Dissolve 30 g KI and 3 g I_2 in 160 ml distilled water.

3.1.2 Reagents for Kjeldahl method

- Concentrated H_2S0_4
- Catalyst ($CuS0_4$:K_2S0_4) 1:10 (w/w)
    - Mixed indicator; dissolved 0.066 g of methyl red and 0.099 g of bromocresol green 0.099 g in 95% ethanol 80 ml, adjust color to green with 0.1 M NaOH (pH 4.2) then add 95%ethanol to 100ml.
    - Boric indicator; add H_3BO_3 60 g into 1800 ml distilled water mix until dissolved and add mixed indicator 2.5 ml. The color solution will be change from violet to red-violet.
    - 40% NaOH, dissolved 400 g NaOH in 900 ml distilled water and adjust to 1000 ml with distilled water.
- 0.025 M H_2S0_4.

3.1.3 Solution for standard HPLC

- 8 5% Lactic acid
- 98.0% Propionic acid
- 99% Butyric acid
- 99.8% Acetic acid
- Solvent mobile phase; 0.005 M H_2S0_4

3.2 Culture Medium

Medium for stock culture, Rogasa Agar Modified (RAM), was modified by Asst. Prof. Dr. Sureelak Rodtong, Institute of Science, SUT. The composition Table

3.1, were suspended in 1 litter distilled or deionized water and heated to boil until completely dissolved. The medium was dispensed into tubes or flasks as desired and manually sterilized by autoclaving for 15 minutes at the pressure of 15 lbs (121° C).

Table 3.1 Rogasa Agar Modified (RAM) medium

RAM medium[1]	%	g/l
Sodium acetate anhydrous (CH_3COONa)	0.1	1.00
Pancreatic digest of casein	0.5	5.00
Potassium hydrogen phosphate (K_2HPO_4)	0.6	6.00
Yeast extract	0.3	3.00
Tri-ammonium citrate $(NH_4)_3$-$C_6H_{17}N_30_7$	0.1	1.00
Magnesium sulfate ($MgS0_4.7H_20$)	0.007	0.07
Manganese sulfate ($MnS0_4.H_20$)	0.0076	0.076
Ferrous sulfate ($FeS0_4.7H_20$)	0.005	0.05
Cassava starch	2.0	20.00

[1]Final pH 7.00±0.1 at room temperature for RAM medium

3.3 Cultivation of microorganisms

Lactic Acid Bacteria (LAB) *Lactococcus sp.* I-5 was isolated from cassava solid waste. These microorganism were obtained from local factories, Korat Flour Industries, by Dr. Sureelak Rodtong. The starch-utilizing microorganism was cultured on 2% cassava in RAM medium and carried out at 37° C in an incubator at a duration time of 1 day, using starch as a carbon source for inoculation. The stock culture is grown in the medium and stocked by freezing. The method for storing the culture was dissolving 10 g of skimmed milk by 100 ml distilled water and sterilizing by autoclaving for 10 minutes at 115° C. The mixture, which contains stock culture and skimmed milk at 1:1 ratio in the microtubes of volume 1.5 ml, are stored at -4° C before being used as the inoculum for different trials.

Using the plate count method and the spectrophotometric method did the growth measurement. In the first method, the culture was diluted to 10^{-4}, 10^{-5}, 10^{-6},

and 10^{-7} CFU/ml then each dilution was pipetted into individual plates with RAM agar and incubated in the incubator at 37° C. The plates were checked for progress every hour that for 16 hours, then the numbers of colonies were counted. In the spectrophotometric method, the undiluted culture was put into tubes with RAM medium (liquid) and examined every hour for 16 hours in the incubator. After incubation, it was measured with the spectrophotometer at a wavelength of 650 nm. The standard curve for culture growth between colonies of culture and optical density (OD) from spectrophotometer is then plotted to show results. Between two methods, it is more covinient to count the culture by using spectrophotometer.

3.4 Starch Extraction from Cassava Solid Waste

The characteristics of cassava solid waste can be described as small pieces of solid, with whitish-gray color and sour smelling odor (Fig.3.1). The solid waste from cassava was extracted using hot water with ratio 1:1 (w/v) at 70° C for 10 minutes. It is at the gelatinizes temperature when the amylose leaches out of the granule. Upon cooling this result in swollen granules of amylopectin suspended in an amylose matrix (Morris, 1990). When mixed completely, gelatinization may be done with just sufficient water to achieve the desired water content. Alternatively, excess water may be removed by drum drying after the gelatinization process at 130° C for temperature. Drum drier has two rollers and use condensed water to raise the temperature. Flow diagram for solid waste extraction and steps of experiment were Figure 3.2 and Figure 3.3, respectively.

Figure 3.1 Characteristic of cassava solid waste from tapioca flour process

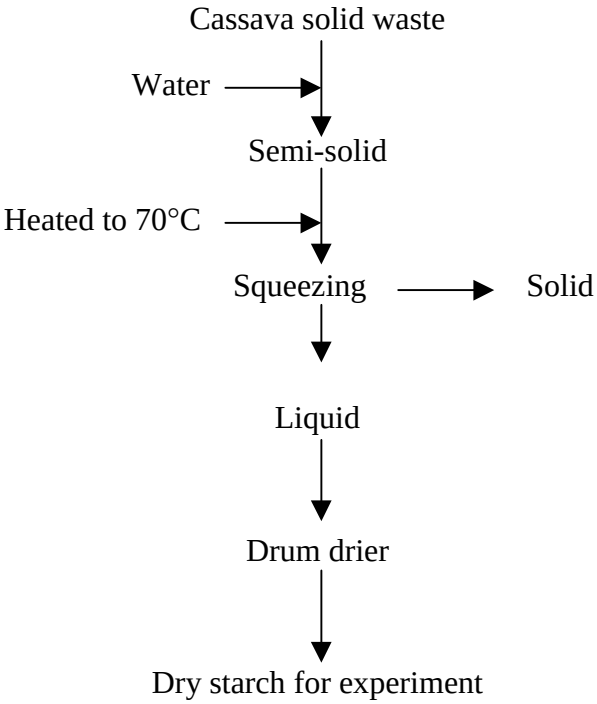


Figure 3.2 Starch extraction process for experimental from cassava solid waste

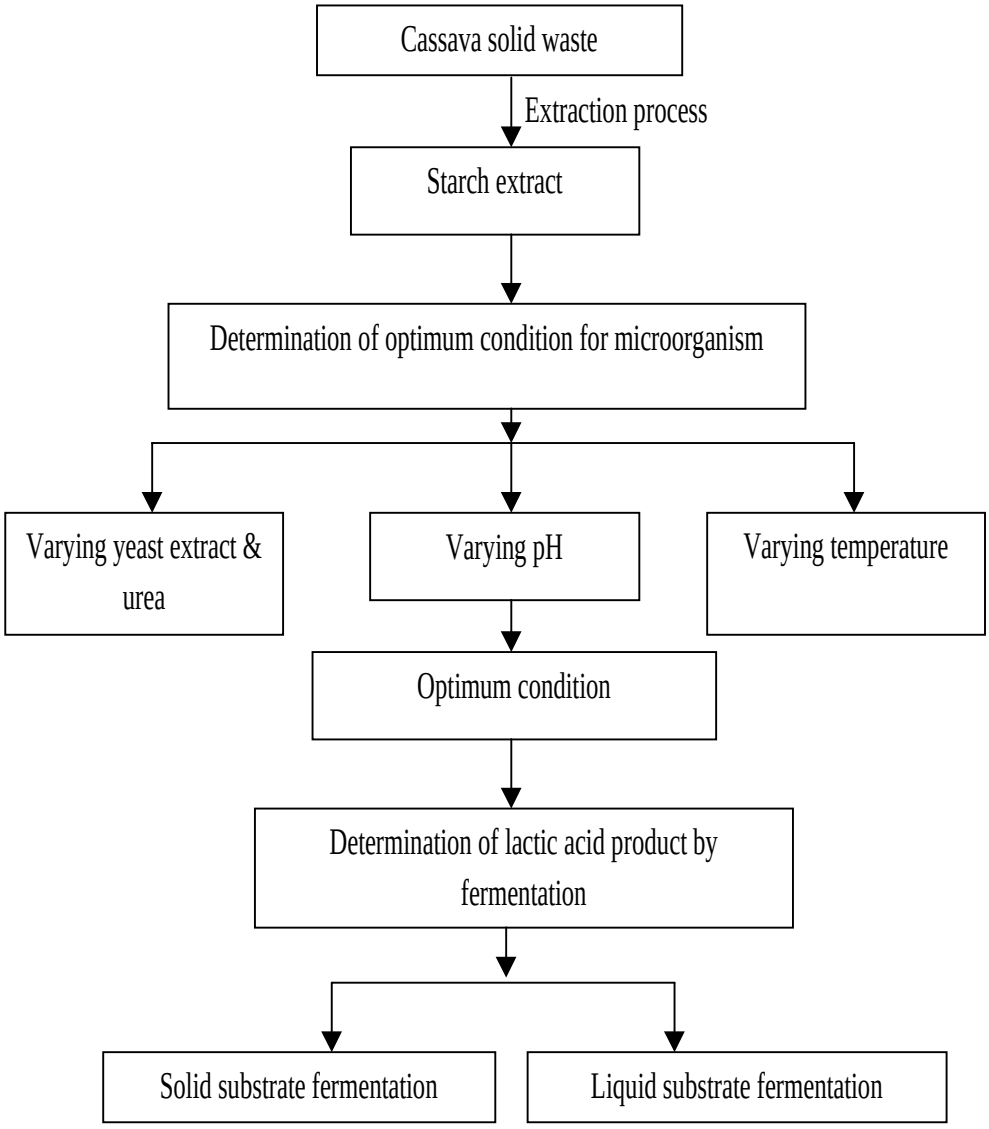


Figure 3.3 Flow diagram for experiment of cassava solid waste fermentation

3.5 Determination of Optimum Condition for Microorganism

Three procedures were developed in this study. The first trial methodology examined the nutrient variation to optimum concentration for the best condition of culture growth. RAM medium was added with 0.1%-0.5% of yeast extract and urea concentration. The medium was then sterilized by autoclaving at 15 min, 121° C.

Stock culture was added into the flask at 10% (v/v) and incubated at 37° C for 2 days under anaerobic condition. After the incubation was completed, the remaining starch and the culture concentration was measured using the spectrophotometric method with the wavelength of 620 and 650 nm. The product after fermentation was lactic acid, which was detectable by Biosensor the YS1 2300 SELECT Analyzer. The optimum concentration of yeast extract and urea was chosen for the next experiment. From the variety of nutrients, 36 samples examined are shown in Table 3.2.

Table 3.2 Number of samples for various yeast extract and urea concentration

%Yeast Extract / %Urea	**0**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
0	1[1]	7	13	19	25	31
0.1	2	8	14	20	26	32
0.2	3	9	15	21	27	33
0.3	4	10	16	22	28	34
0.4	5	11	17	23	29	35
0.5	6	12	18	24	30	36

[1]Remark: 1-36 are the sample numbers

The second protocol was developed from the first protocol. This method was to determine several pH values ranging from 4.0-8.0 with 2% starch in RAM medium. It was done by adding the stock culture into the flask and incubating it at 37° C for 2 days with optimum yeast extract and urea concentration in the RAM medium. The method for checking final products was the same as the first protocol. To obtain the efficient experimental results, duplicates of experiment were used.

The third method was selection of an optimum temperature between 30°- 40° C. The other conditions were used by optimum of yeast extract and urea concentration in RAM medium and the optimum pH as in the 1st and 2nd protocols.

3.6 Determination of Lactic Acid Product by Fermentation

3.6.1 Solid-Substrate Fermentation

a) The solid substrate fermentation was performed in a 2 liter beaker. In the preparation of a solid substrate, nutrients were added to the cassava solid waste before culturing of the microorganisms. Pieces of cassava were pretreated to decrease the particle size and increase the availability of fraction between 0.5-1.0 mm. In some cases, additional nutrients were added. The pH was adjusted at this stage to be 7.0 with 0.5% and 0.2% yeast extract and urea concentration, respectively.

b) The second step was to sterilize or at least pasteurize the substrate, which would cause the absorption of water into the substrate particles. In this step, the solid waste was autoclaved for sterilizing at 121°C for 15 minutes.

c) Pure culture technique was used for inoculum in oder to raise the concentration to a suitable ratio of 10% (v/v).

d) The cassava solid wastes were inoculated and incubated at room temperature over period of time.

e) During the process, the substrate changes into lactic acid and glucose products. Samples were collected everyday during the fermentation.

f) The product was dried and tested for moisture content.

3.6.2 Liquid-Substrate Fermentation

A batch fermentation unit used six concentrations of starch extract from waste by varying it at 0-5% in RAM medium. It is the optimum condition among yeast extract concentration, urea concentration, pH and temperature. The dry starch was dissolved in RAM medium and pH was adjusted by HCl and KOH for optimum value, then sterilized by autoclaving for 15 minutes at 121° C. The medium was pored into the 2-L fermenter (Figure 3.4) and the temperature was adjusted by using fermenter controller. When the system was ready, 10% culture (LAB) was transferred into the fermenter using aseptic technique. The liquid was collected for to check

parameters by sampling every hour for 10 hours or until result became constant. Then the collection was done every 2-4 hours until fermentation period was completed. The important parameters were culture, starch, pH, lactic acid and glucose.

Figure 3.4 Two litter of reactor for liquid fermentation

3.7 Kinetic Determinations

The extent to which reactions were converted to products is expressed as the reaction yield. Yield is the amount of product formed or accumulated per amount of reactant provided. Several different yield parameters are applied to different situations. This experiment had two types of yield, which are production yield ($Y_{p/s}$) and biomass yield ($Y_{x/s}$).

$$Y_{p/s} = \text{gram production/ gram substrate} \quad (3.1)$$

$$Y_{x/s} = \text{gram Biomass/ gram substrate} \quad (3.2)$$

It is usual to start off by making a several simple assumptions. After an initial look at the results of the first experimental exercise, some of these assumptions are removed and a more complex experimental set-up evolves. The assumptions made for the experiment were:

(i) the laboratory fermenter is well mixed, that is, conditions are uniform throughout the whole of its interior;

(ii) the microorganism (LAB) cells do not die or become non-viable;

(iii) the only two substrates of importance are the carbon source (sugar or starch) and nitrogen.

Initial rates of soluble starch hydrolysis were determined at various starch substrate concentrations (0-5% starch). The kinetic constant K_s and μ_{max} were estimated by the method of Lineweaver-Burk Plotted. Graphs were plotted between 1/S and 1/μ. The Y-intercept was $1/\mu_{max}$ and the slope was K_s/μ_m. The specific growth (μ) and the Monod constant were calculated as a function of time that was

$$\ln (X/X_o) = \mu t \quad (3.3)$$

$$\text{so} \qquad (\ln X - \ln X_o)/t = \mu \quad (3.4)$$

3.8 Analytical Methods

3.8.1 Moisture content

The dry matter and the moisture content were determined according to the method of Tinsley and Nowakowski (AOAC, 1990). A 50 g mass of the sample was heated on evaporation dishes in an electric oven at 105° C for 24 hours. It is weighed until a constant weight is obtained. The difference in the weight of material after drying is the moisture content of the material.

3.8.2 Ash

Three duplicate 5 g of dried samples were prepared, weighted on alundum crucibles, and heated in an electric furnace at 550° C for 30 minutes. The organic

solids burnt off on ignition are the volatile solids (or organic matter), while the residue contributes to the ash content.

3.8.3 pH

The pH was measured by pH meter in a mixture homogenization of 1 g sample diluted in 5 ml of distilled water in solid substrate fermentation. In liquid substrate fermentation pH was measured by putting the probe into the solution.

3.8.4 Starch

Calorimetric method was used for starch analysis. Drop 0.5 ml iodine solution into 1 ml sample then add 2 ml of distilled water. After it is well mix, the absorbency was determined using spectrophotometer at the wavelength of 620 nm. Starch content was obtained using standard curve tapioca starch.

3.8.5 Nitrogen and crude protein

Total nitrogen was determined by Kjeldahl method. The proteins were expressed in term of total real nitrogenous matter (TRNM) and obtained by multiplying total nitrogen by 6.25 following AOAC guidebook, 1990.

3.8.6 Culture

The microorganism was determined using spectrophotometric method by setting the wavelength at 650 nm. This wavelength was used for yellow to brown solutions (Wistreich, 1997). The value of culture was determined using the standard curve that compares optical density (OD) by spectrophotometric method and plate count method.

3.8.7 Lactic acid and glucose products of fermentation

Lactic acid and glucose were final products from fermentation that was detected by Biosensor YS1 2300 SELECT Analyzer in form calcium lactate and glucose, respectively. In solid substrate fermentation was measured considering product by homogenization of 1 g of sample diluted in 5 ml distilled water or same ratio. The results of lactic acid could show in form of yield (g lactic/ g solid).

3.8.8 Other organic acids

The sample was filtered through a 0.45 μm. membrane before analyzed through HPLC (high performance liquid chromatography) using UV 6000 LP A 210 nm. as the detector and Polyspher® OA HY for column. The injection volume used was 10 μ L and 0.005 M H_2SO_4 for the mobile phase at a flow rate of 0.6 ml/min. The temperature was set at 50° C in the system. Propionic, Butyric and acetic acid were identified and quantified using external standard solutions.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characteristics of Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are a group of Gram-positive bacteria united by a constellation of morphological, metabolic and physiological characteristics. The general description of the bacteria included in the group can be described as Gram-positive, nonsporing, nonrespiring cocci or rods, of nonaerobic habit but aerotolerant, acid tolerant, which produce lactic acid as the major end product during the fermentation of carbohydrates (Lars, 1993). The lactic acid bacteria used in the experiment was in genus *Lactococcus sp.* that could grow very fast in RAM medium with 2% starch for carbon source at 37°C for 24 hours. At first, the color of RAM medium was clear yellow, after complete incubation (Figure 4.1) the color changed from clear yellow to cloudy yellow and culture was suspended at the bottom of medium. The smell was sour like the smell of acid.

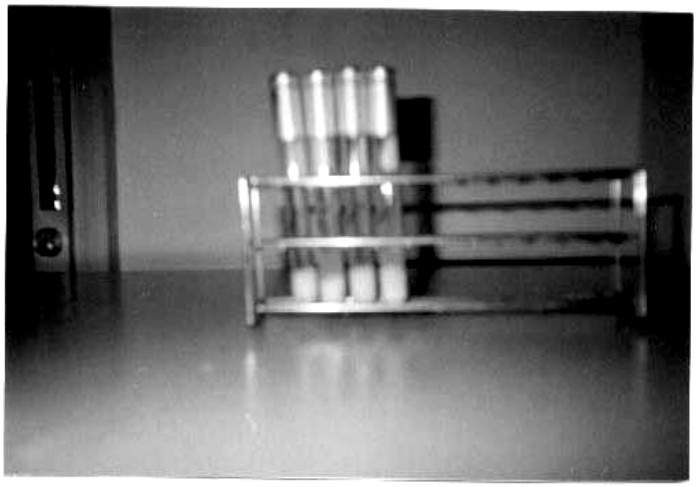


Figure 4.1 Characteristic of Lactic Acid Bacteria (LAB)

4.2 Characteristics and Extraction of Cassava Solid Waste

The cassava solid waste was white-gray in color. It was in small pieces and it had the pH value of 4.9, as shown in Table 4.1. The low values of nitrogen and crude protein in this waste make it not appropriate for use as animal feed. Cassava solid waste after extraction still contains 50% of starch by dry weight (Tanticharoen, 1999). The solid waste is used as a feed component to the fermenter and it consists of glucose molecule or starch that bacteria use as carbon source, extraction for starch will be necessary. The cassava solid waste was extracted for starch by boiling at 70°C (Table 4.2). Extraction of starch from cassava involves direct cell rupture to give a suspension of starch granules and cell wall materials. The total mass of waste used was 96 kg and the starch obtained from extraction was 4.3 kg, so the percent of extractive was 4.48%. Cassava and its waste have low nutrients but high carbohydrate content. The main amino acids present in cassava protein are arginine, histidine, isoleucien, leucine and lysine, (Chumkhunthod, 2000).

Table 4.1 The Characteristic of cassava solid waste

Characteristics	Average Value
pH	4.9
Moisture content	60.9%
Nitrogen	0.034%
Protein	0.213%
Ash	1.160%

Table 4.2 Component for process of extraction starch from cassava solid waste

Characteristics	Value
Waste from cassava	96 kg
Water added	134 L
Temperature for extraction	70°C
Temperature (dry starch)	130°C
Starch from extract	4.3 kg
Extraction yield	4.48%

4.3 Effects of Nutrients, Temperature and pH for Optimum Condition

4.3.1 Effects of Nutrient for Maximum Product

For maximum products of culture growth and lactic acid yield, various yeast extract concentrations and urea concentrations of 0%, 1 %, 2%, 3%, 4% and 5% as substrates were used. Starch was often supplemented with nutrients, mostly in the form of yeast extract or peptone. Two percents starch extract and 10% stock culture were used in the experiment. The incubation period was prolonged for 2 days and the temperature was set at 37° C. The final product containing lactic acid and glucose were analyzed to determine the suitable concentration for culture growth (see data at Appendix A). The results show trends of culture growth that decreased when the urea concentration increased. On the other hand, the growth increased when the yeast extract concentration increased, as indicated in Figure 4.2 (see number of sample in Table 3.2). In Figure 4.3, results show lactic acid product variation, which follow the similar pattern as the culture growth, but not as clear. The results in figure 4.4 shown urea and yeast extract variation indicated by glucose that found the urea concentration had effects to first 18 samples and few effects when higher value of yeast extract concentration. But the glucose concentration not exactly results because glucose was the intermediate substrate from starch to lactic acid in anaerobic fermentation. The microorganism could change starch to glucose and used it to produced lactic acid production. In the glucose cycle, the glucose occurred was not the total glucose in the system. The results of starch utilized (Figure 4.5) were the same as culture growth such as if the culture growth is well, the starch utilization was high. However, when comparing between culture growth, final products (lactic acid, glucose) and starch remained it was found that supplements with 0.5% yeast extract and 0.2% urea is the optimum combination to exploit the contribution of cell growth to lactic acid production. Kulozik and Wild (1999) reported, from the effects of supplementation with yeast extract on cell growth were investigated. Addition to 10 g/l (1%) yeast extract was required to optimized the productivity. Higher yeast extract levels further improved cell growth, but lactic acid production remained unchanged.

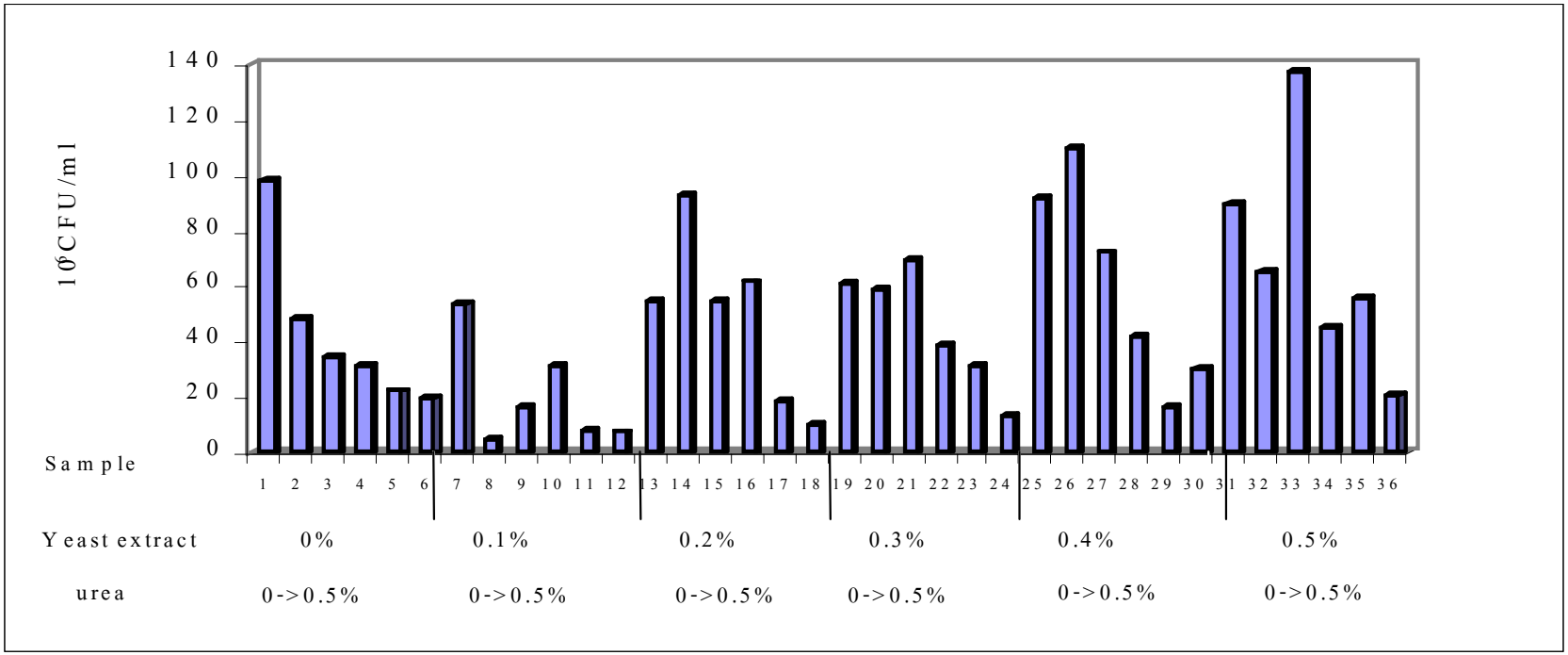


Figure 4.2 Comparison of various urea and yeast extract with indicated by culture growth

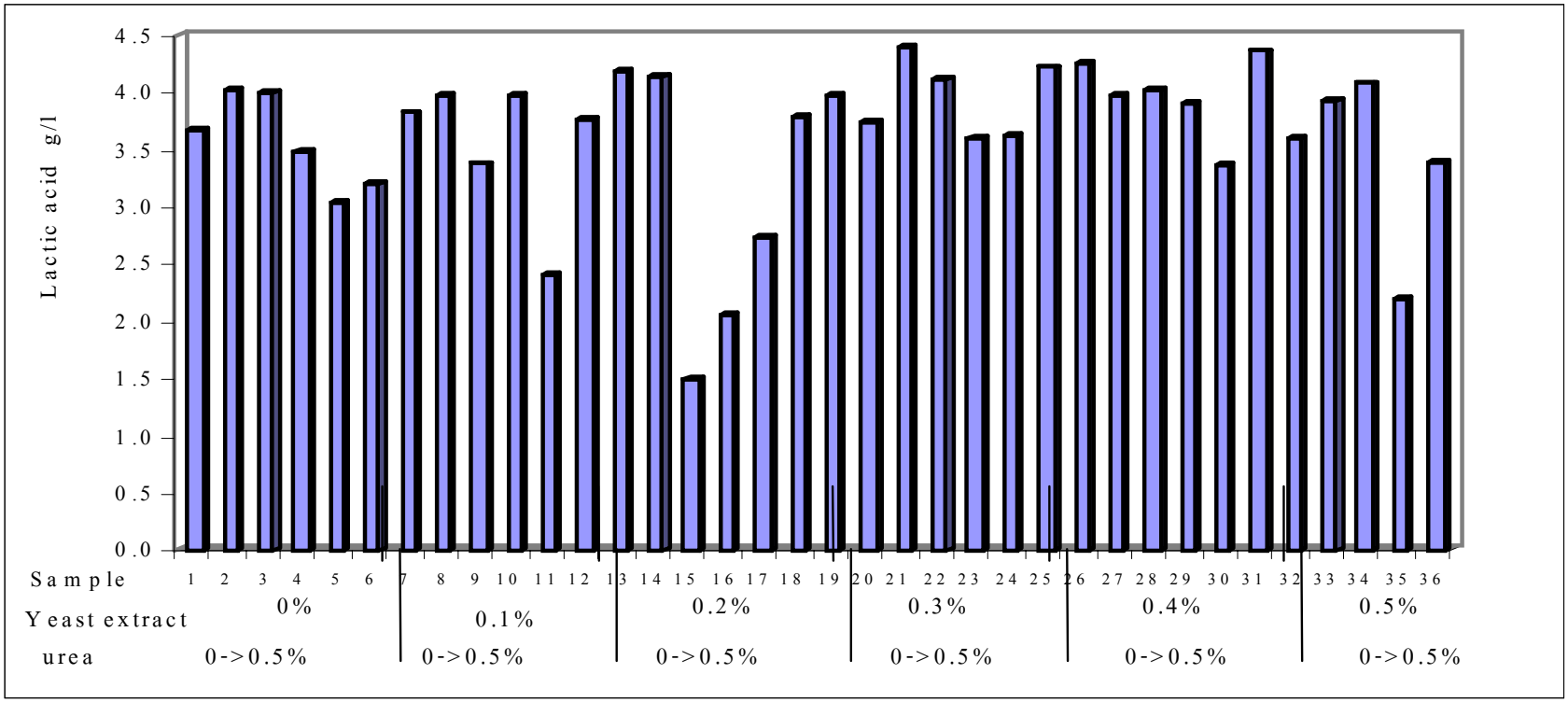


Figure 4.3 Comparison of various urea and yeast extract with indicated by lactic acid product.

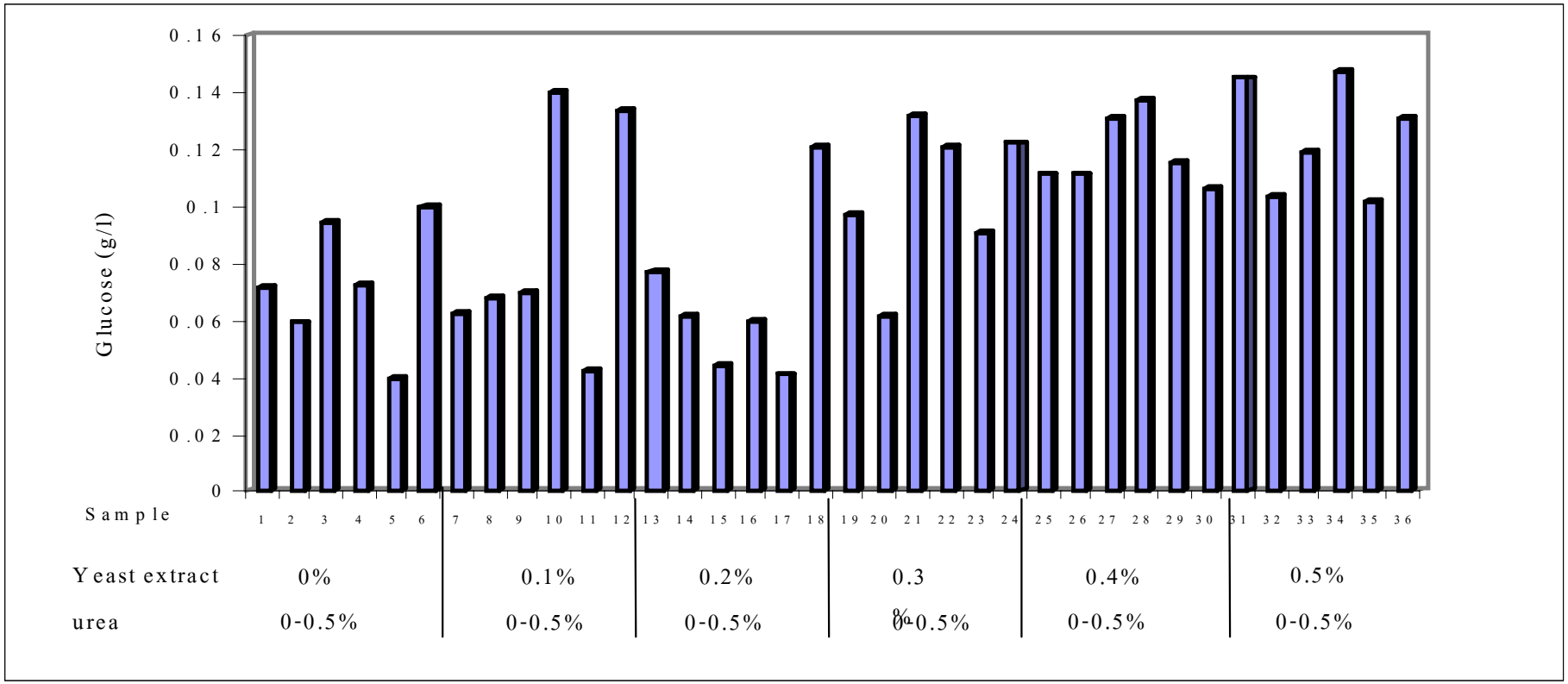


Figure 4.4 Comparison of various urea and yeast extract with indicated by glucose

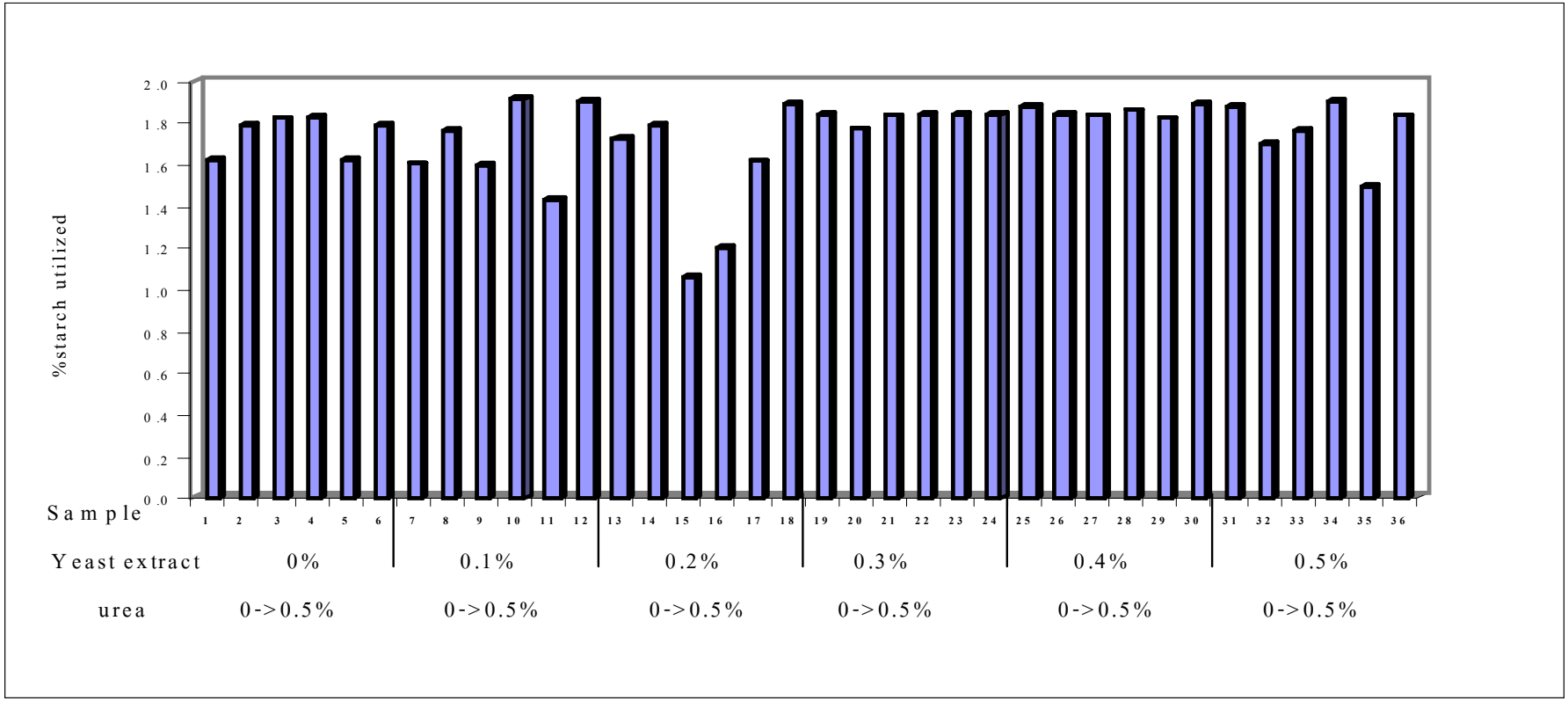


Figure 4.5 Comparison of various urea and yeast extract with indicated by starch utilized

4.3.2 Effects of Temperature

This experiment used optimum conditions of nutrients, which was 10% stock culture (v/v) for 2 days and initial pH of 7.0 under anaerobic condition. RAM medium was modified using urea and yeast extract concentration of 0.2% and 0.5%, respectively. The temperatures were varied at 30° C, 33° C, 35° C, 37° C and 39° C. The results are shown in Figure 4.6. The culture growth peaked at 35° C. and the concentration of lactic acid product was increased when the temperature increased and decreased after reaching the limiting point temperature of the product. The best temperature from experiment was 35°C. base on lactic acid concentration.

4.3.3 Effects of pH

The pH of culture may change in response to microbial metabolic activities due the several reasons. The most obvious reason is the secretion of organic acid such as acetic or lactic acid, which causes the decrease in pH value. In the initial stage of fermentation the pH value was set at 7.00. When fermentation was completed, the pH value decreased due to acid production. The effect of pH was studied by fermenting at various pH values. Various pH values of 4, 5, 6, 7 and 8 were used as initial pH with 0.2% of urea concentration and 0.5% of yeast extract concentration. Temperature selection was 35° C for 2 days under anaerobic condition. Trend of culture growth (Figure 4.7) was linear until pH value reaches 6.0 but pH 7.0 and 8.0 the culture growth rate began to constant in form linear line. The results show that culture growth increases with increasing pH. The trend of lactic acid concentration was a nonlinear line. The peak of lactic acid concentration was pH 7 as same as glucose occurred that shows this condition was optimum for the microorganism. The value of 7.0 was optimum pH for initial pH. Annica and Marc had reported that most of the works have been done with lactococci, and for them the optimum pH is in the range of 6.0 to 6.5. However, bacteria in general have an optimum pH between 6.5 to 7.5 with lower and upper limits for growth usually between pH 4.0 and pH 9.0 (van Demark and Batzing, 1987).

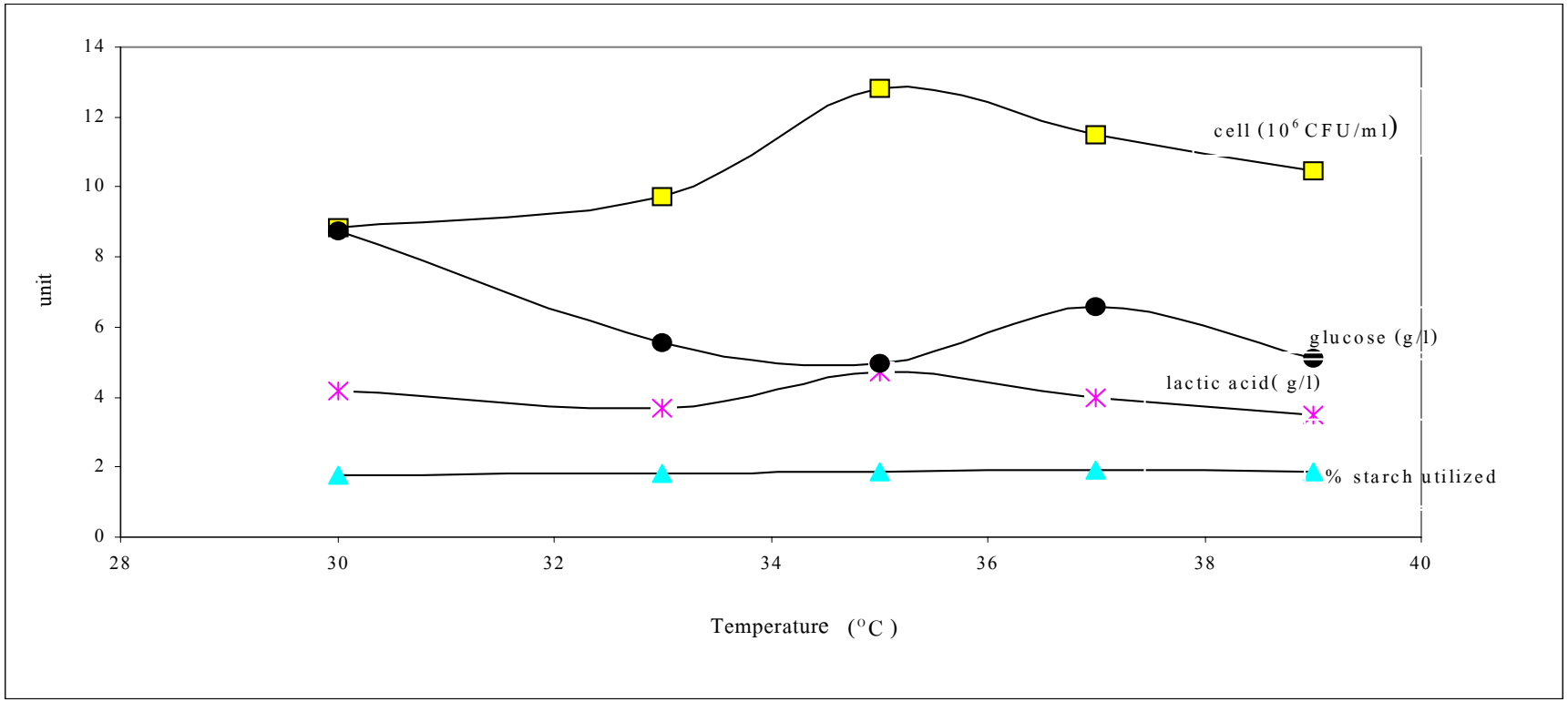


Figure 4.6 Effects of temperature on RAM medium after incubation

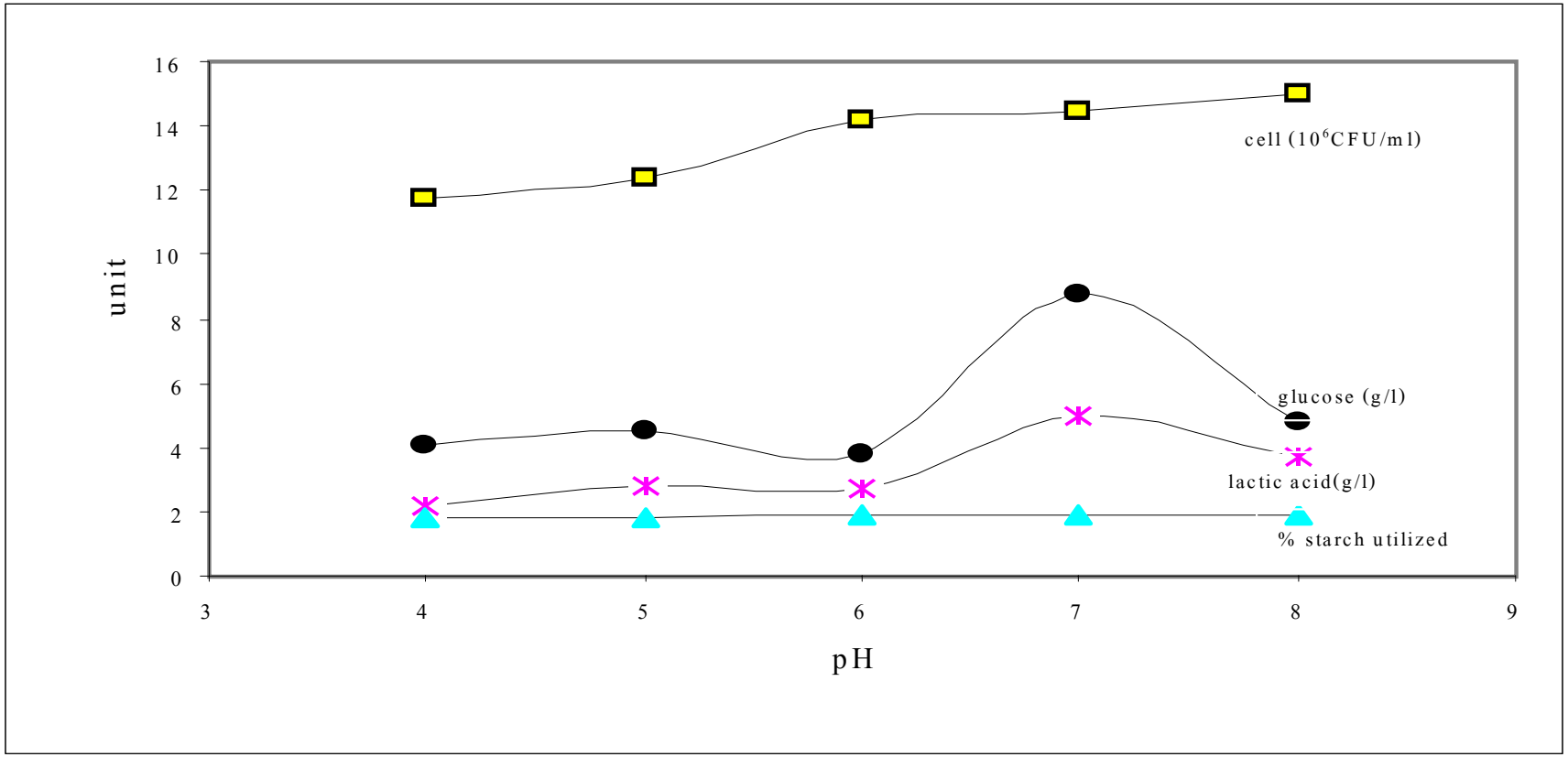


Figure 4.7 Effects of pH on RAM medium after incubation

4.4 Solid Substrate Fermentation

Results obtained through optimization condition could be concluded as autoclaving cassava solid waste being the most suitable carbon source. Urea powder was considered to be nitrogen source at 0.2% of urea concentration and 0.5% yeast extract. The waste from cassava were sterilized by autoclaving for 15 min at 121° C. This could prevent the initial contamination from other microorganisms. The fermentation of cassava solid waste was done using simple process. Ten percents stock culture was added into 2-liter beaker containing cassava solid waste. The initial pH was controlled at 7.0 and the fermentation process was kept under anaerobic condition (cover by plastic film) at room temperature. The initial moisture content of the cassava solid waste with added stock culture before incubation was 80.5%. The protein content of cassava, which is generally about to 0.59%, was increased to 1.49% on dry mass basis. Nitrogen was determined by Kjeldahl method and biomass protein was calculated as 6.25 times (Doelle and co-worker, 1992). This process was not controlled the temperature and pH. Without pH control, pH levels (Figure 4.8) decreased from the initial pH at 7.00 and became constant at 4.5-4.6. After the fermentation, the moisture increased to 83.2%. From Figure 4.8 the trend of lactic acid concentration shows slow increase while the glucose concentration increased very fast. Lactic acid product was less due to tapioca flour production processes, which has chemical addition. H_2SO_4 or HCL was added during the process to extract starch from the tapioca. Chemical addition may cause other impurities, which may be the prohibitory lactic acid product. The starch must be changed into glucose by LAB before it could produce lactic acid, since the microorganism needs to prepare sufficient substrate before producing lactic acid products. Thus, the lactic acid product occurred slowly in the initial stages of the experiment. Forced aeration was often easier to pass into solid-state because the inter-particle spaces allow transfer of fresh air to thin films of water at the substrate surface. Agitation from outside of the system made toxicity for LAB by O_2. For this reason LAB and lactic production were decreased. In addition, it was very difficult to ensure even distribution of any substances added during the process. This experiment could not be used over long period because while sampling the system will be contaminated by other micro

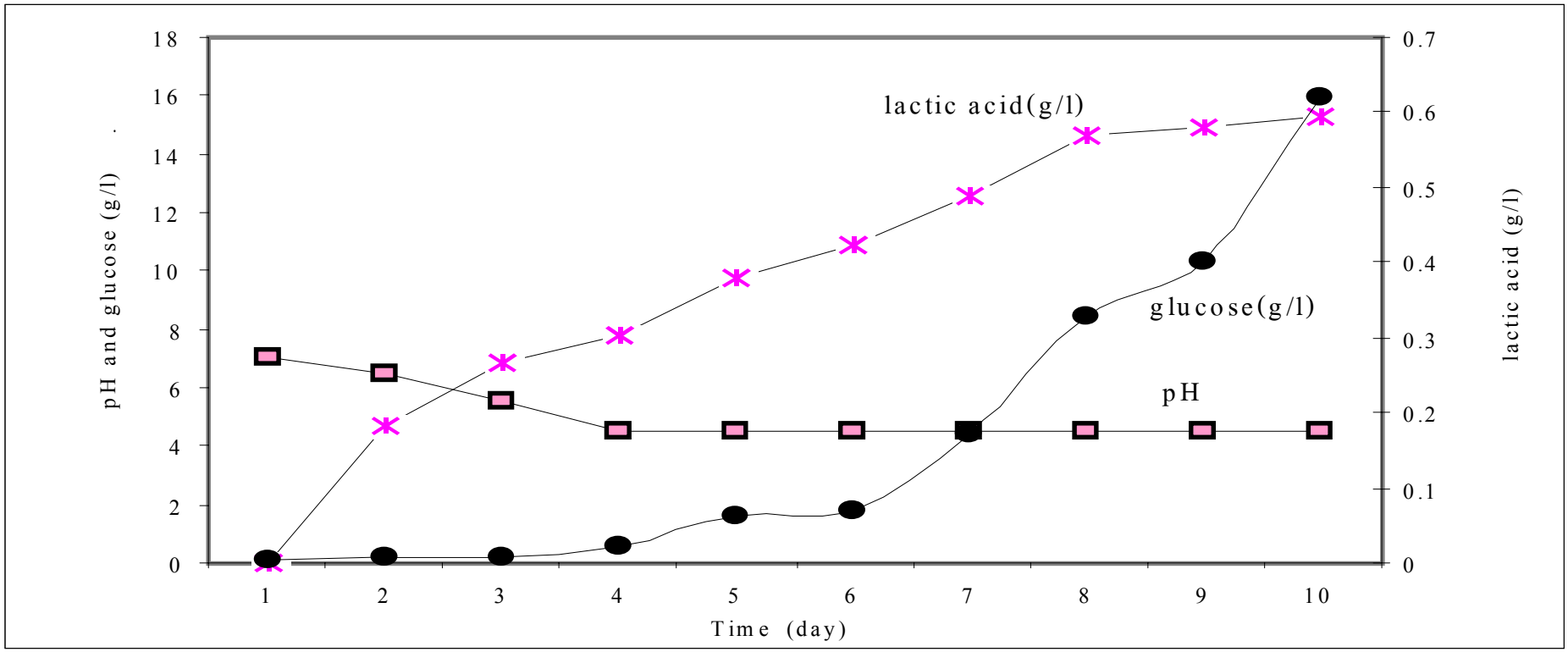


Figure 4.8 Efficiency of cassava solid waste fermentation (10 days)

organism such as mold or fungi. The restricted availability of water may help protect against undesirable contaminants, especially bacteria and yeast. Although contamination by other fungi may also become problem. Fungi grow well in solid-state culture because the conditions were similar to the natural habitats in which fungi were found.

4.5 Comparison of Lactic Acid Product among Starch Substrate

The different concentration of substrate in RAM medium were 0%, 1%, 2%, 3%, 4% and 5% starch. The incubation period was prolonged for 3 days and the temperature was controlled at 35° C. In the pre-experiment fermentation (see data in Appendix A), the substrate used was in the range of 0%-5%. The flask containing 100 ml medium was added with 10% stock culture and a catalyst (anaerocult A) and used for anaerobic condition. The initial pH was adjusted to 7.0 and the temperature was controlled at 35° C for the prolonged 3 days. The trend of pH shows a decrease from 7.0 to 4.5 in most batches except the batch with 0% starch that the pH dropped to 6.0. In Figure 4.9 shows lactic acid productions in batches with 2% and 4% starch substrate were of higher value than other batches. Considering starch utilization, it was found that starch utilization increased with the percent of starch substrate.

After the pre-experiment fermentation, the percents of starch were selected to be 0%-5%. The experimental results of each batch are shown and discussed below.

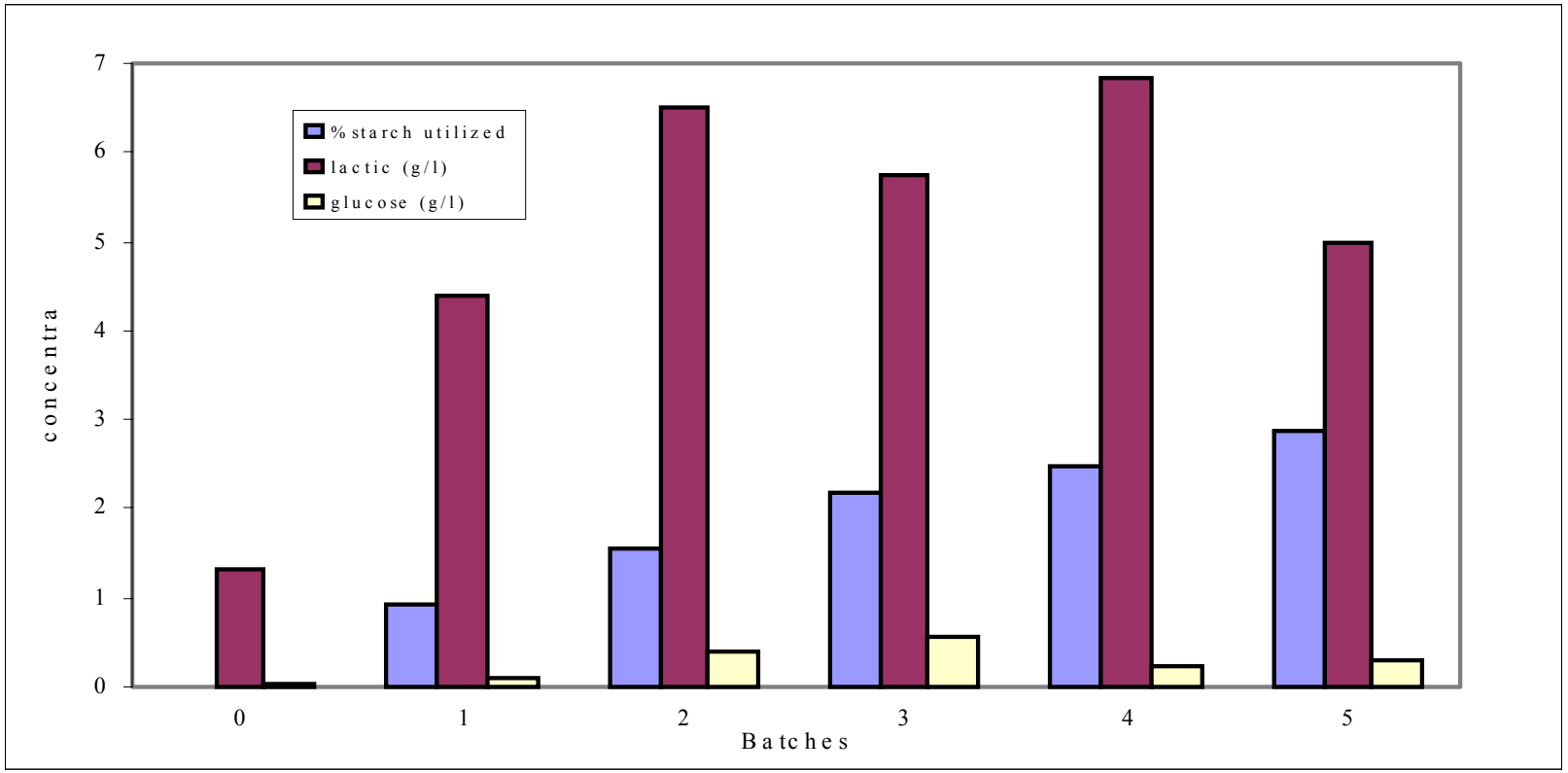


Figure 4.9 Pre-experiment for fermentation by various starch substrate for 0%-5%

4.5.1 Batch 0 % Starch (Figure 4.10)

Culture growth and lactic acid productions were low because the medium did not have starch to generate a carbon-source. Yet, the microorganism could obtain carbon from yeast extract to produced lactic acid product. Yeast extract was consistently found to give the best results by far (Ulrich and Jürgen, 1999). When depletion of carbon source occurs because of microorganism consumption the production of lactic acid becomes the source of carbon for generation of lactic acid bacteria. From this reason, after 10 hours from the beginning the lactic acid production will decrease giving low quantities of product. The amount of bacteria was rapidly increase during the first period but eventually it will decrease according to time, as well as lactic acid. During the first period, bacteria can survive by utilizing other nutrients in RAM medium but at one point, it will start using carbon source within lactic acid molecule. Under lactic acid decreasing condition, the dropping pH will increase. However, the bacteria can not survive with will decrease the member of cell.

4.5.2 Batch 1 % Starch (Figure 4.11)

The pH value decreased very fast, from 6.7 to 4.7, within 3 hours from initial and the final pH after 3 day was 4.26. Through observation, the starch consumption by culture was extremely high in the initial phase of cell growth. After that, in the stationary phase, the concentration of lactic acid production increased slowly while glucose concentration showed high increase. The final lactic acid product after the fermentation was completed was 4.26 g/1 while culture growth was 13.938×10^6 CFU/ml and starch utilizing was 0.886% from 1% starch substrate in RAM medium. Lactic acid bacteria (LAB) utilized starch substrate to produced lactic acid but it could be detected only when fermentation finished.

4.5.3 Batch 2% Starch (Figure 4.12)

The pH values and starch utilized decreased very fast with in the first 3 hours just as 1% starch batch. Glucose concentration after finish was increased to 0.166 g/1 and the microorganism used it to produced lactic acid. Therefore, the glucose concentration was decreased and finally become constant. The culture growth of this

batch was higher than culture growth in batch with 1 % starch. The final product yield of lactic acid was 4.375 g/1 and 17.34x10^6 CFU/ml for culture growth.

4.5.4 Batch 3% Starch (Figure 4.13)

Trends of parameter in this batch were like the parameter in the batch with 2% starch, with the pH decreasing from 7.0 to 4.2. The maximum starch utilization was 2.541%, which occurred in 3 hours. Lactic acid was produced up to 4.385 g/1 as final products. Culture growth was increased to maximum value at 15.93 x10^6 CFU/ml after 5 hours and become constant until finished.

4.5.5 Batch 4% Starch (Figure 4.14)

The pH value decreased from 7.0 to 4.2 as batch 3% starch. The culture growth and lactic acid products were increased and did not become constant when fermentation complete. At the end of the 3 days period, the culture growth was 19.673x10^6 CFU/ml and lactic acid product was 8.095 g/l. Both the culture growth and lactic acid product in the batch with 4% starch were higher in value from other batch. However, starch utilization was 3.457% from 4% starch in RAM medium.

4.5.6 Batch 5% Starch (Figure 4.15)

The pH value in this batch was decreased from 6.9 to 4.2. After three days, the culture growth was 15.624x10^6 CFU/ml and lactic acid product was 4.645 g/1. The starch utilized was 3.16% from 5% starch. This is due to the exceed amount of starch. The microorganism continuously utilizes substrate. Once whatever substrate remain was over needed, the microorganisms start dying due to lack of other required substrates. This condition is called limiting factor, which prohibit microorganism growth.

When considering the parameters in initial phase or log phase (0-5 hours) of bacterial growth, the numbers of cell growth were likely the reason for lactic acid production, starch utilization, and pH drop. It was found that the uptake of starch by the culture was rapid. It directly transformed the starch into lactic acid (Pintado *et al*, 1999), reaching higher acid value that bring lower pH. The lactic acid was the main

production of the exponential phase of growth in batches 1%-5% starch and reached its maximum at the stationary phase. Because of LAB utilizes it for producing lactic acid. In the batch 0% starch, there is no starch for bacteria utilization for growth or production of lactic acid. Therefore, production was low. For comparison purpose, an experiment was done using 1% tapioca starch in RAM medium and the same condition as the cassava solid waste. The maximum yield (product**:**substrate), batch 1% starch from cassava solid waste to compared with 1 % tapioca starch. The fermentation results of 1 % tapioca flour (Figure 4.16) were the pH value dropping from 6.9 to 4.6. Lactic acid produced was 4.36 g/l. The culture growth was $17.11x10^6$ CFU/ml and starch remained was 0.049% from 1% starch (or 0.951% starch utilizes). However, the cassava solid waste was replaced by tapioca starch.

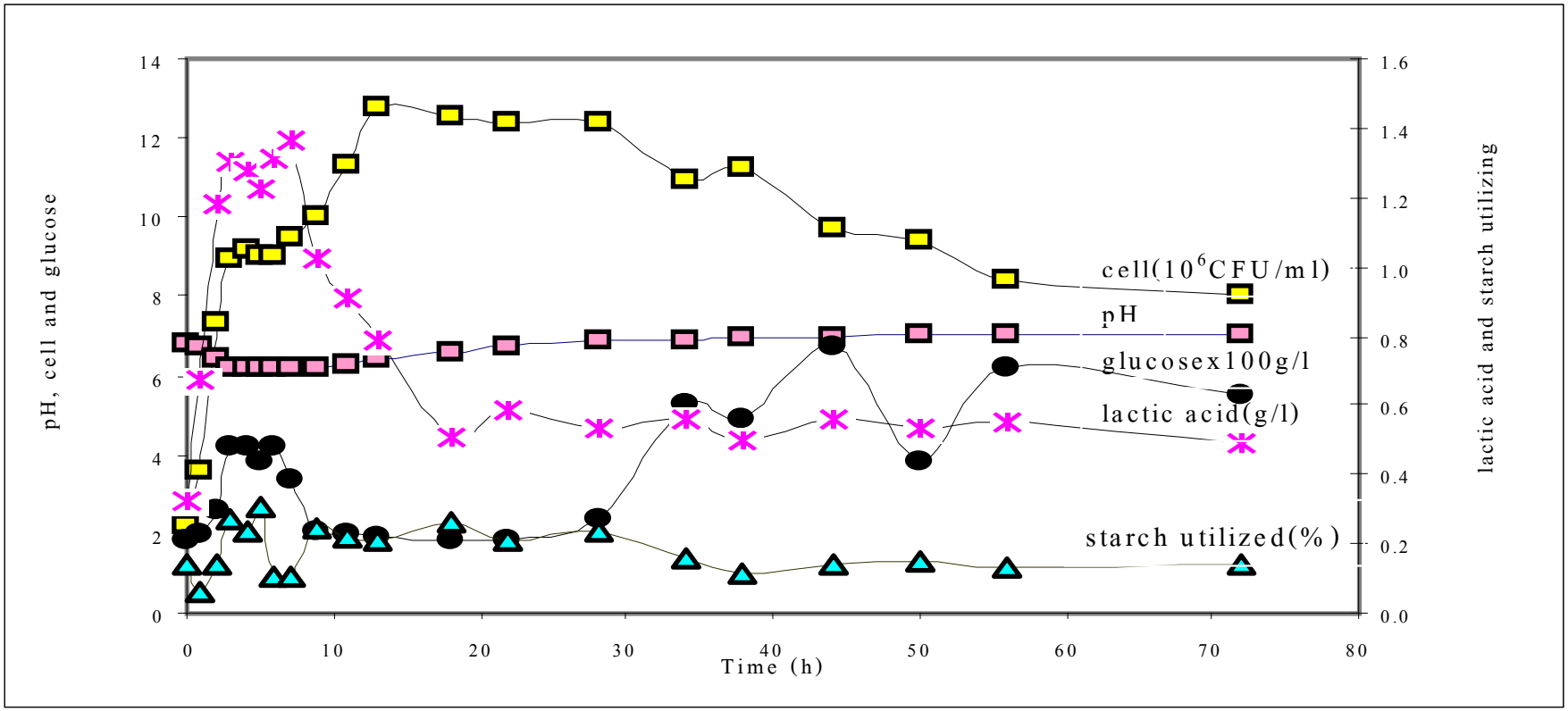


Figure 4.10 Efficiency of 0% starch in RAM medium

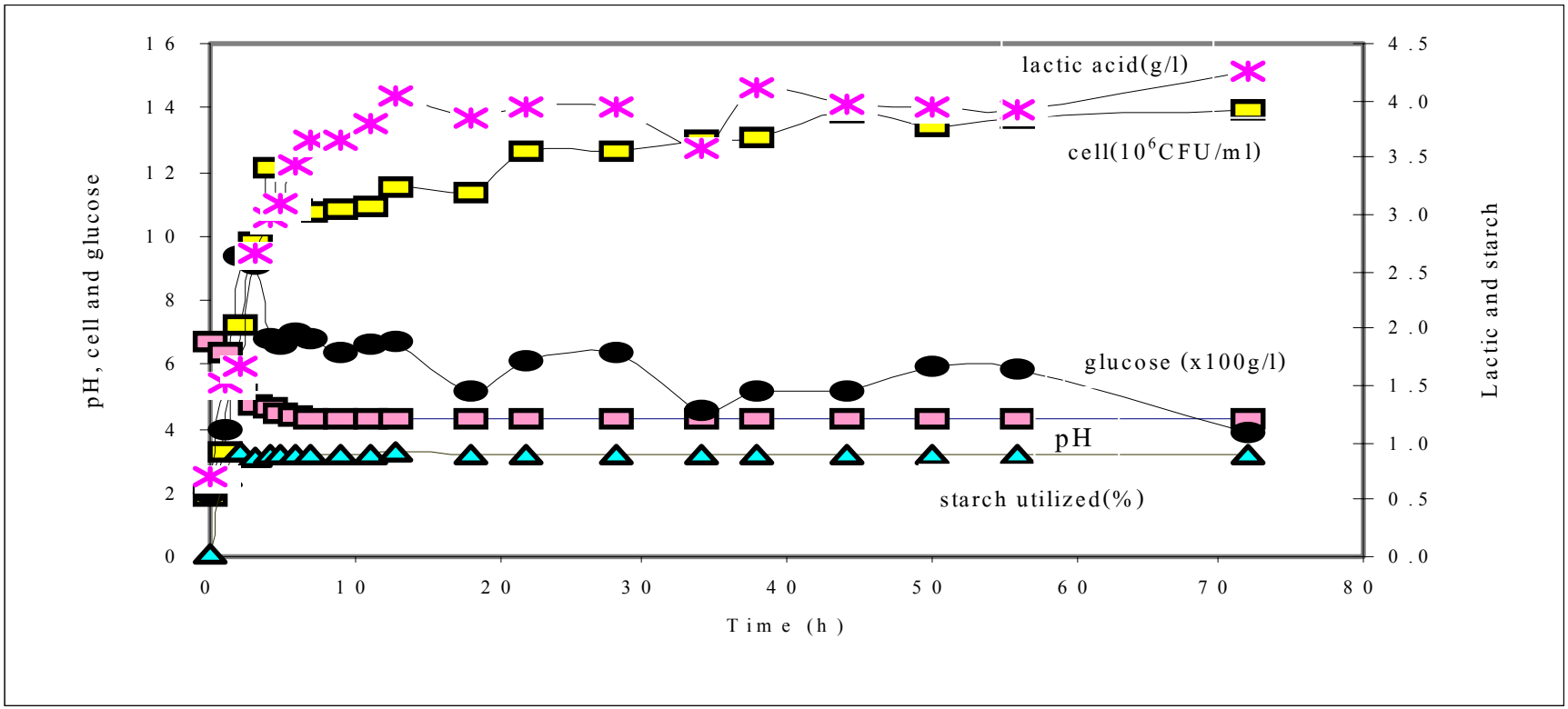


Figure 4.11 Efficiency of 1% starch in RAM medium

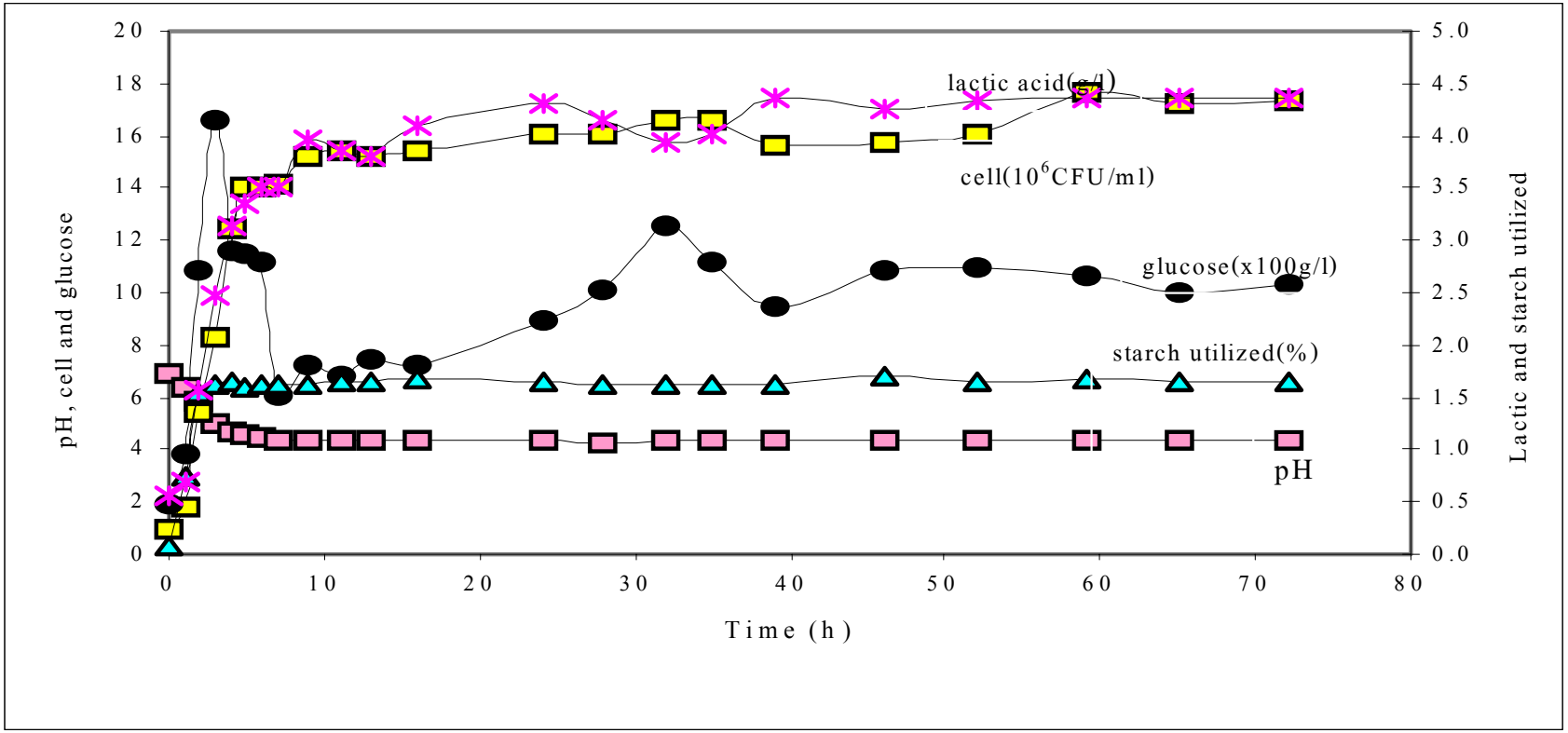


Figure 4.12 Efficiency of 2% starch in RAM medium

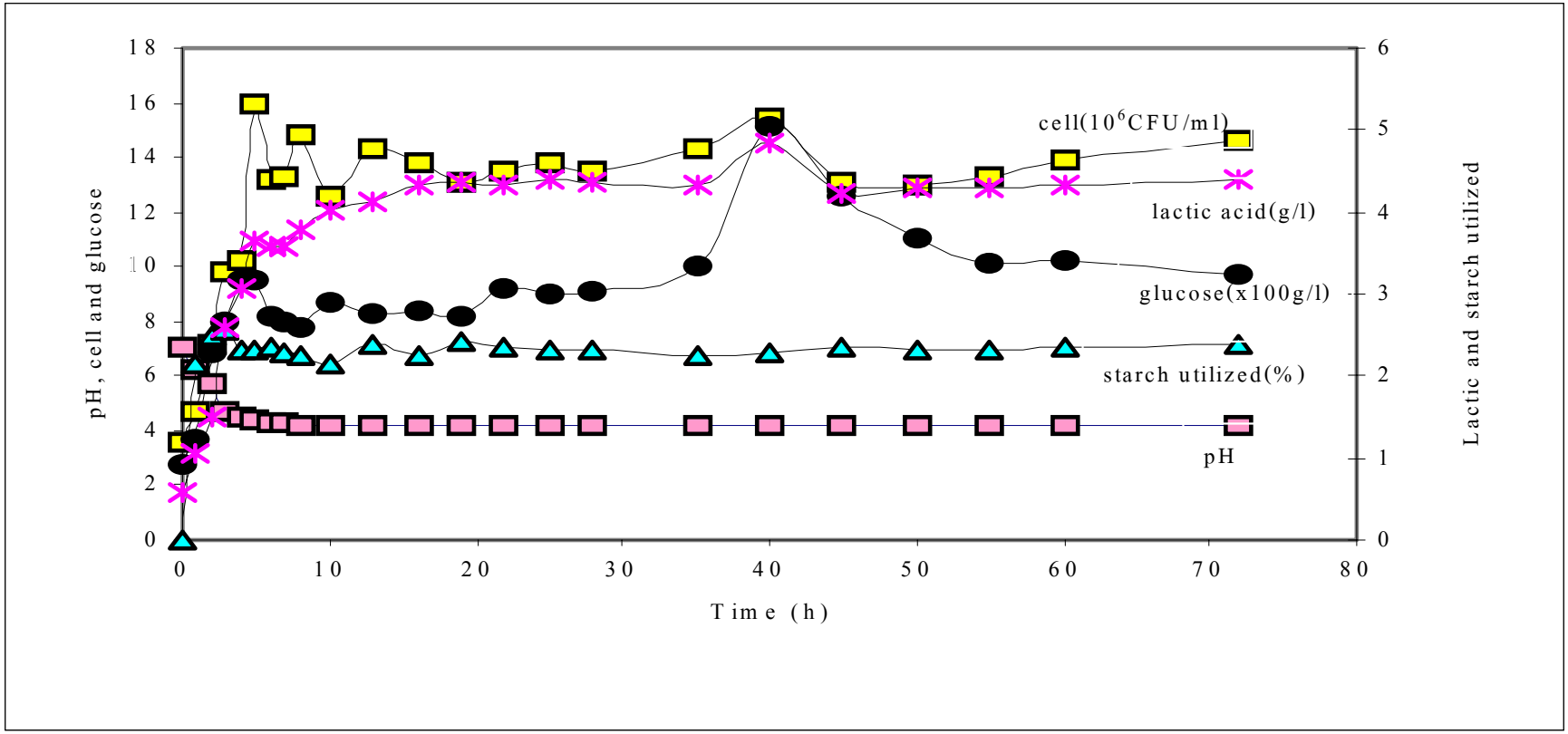


Figure 4.13 Efficiency of 3% starch in RAM medium

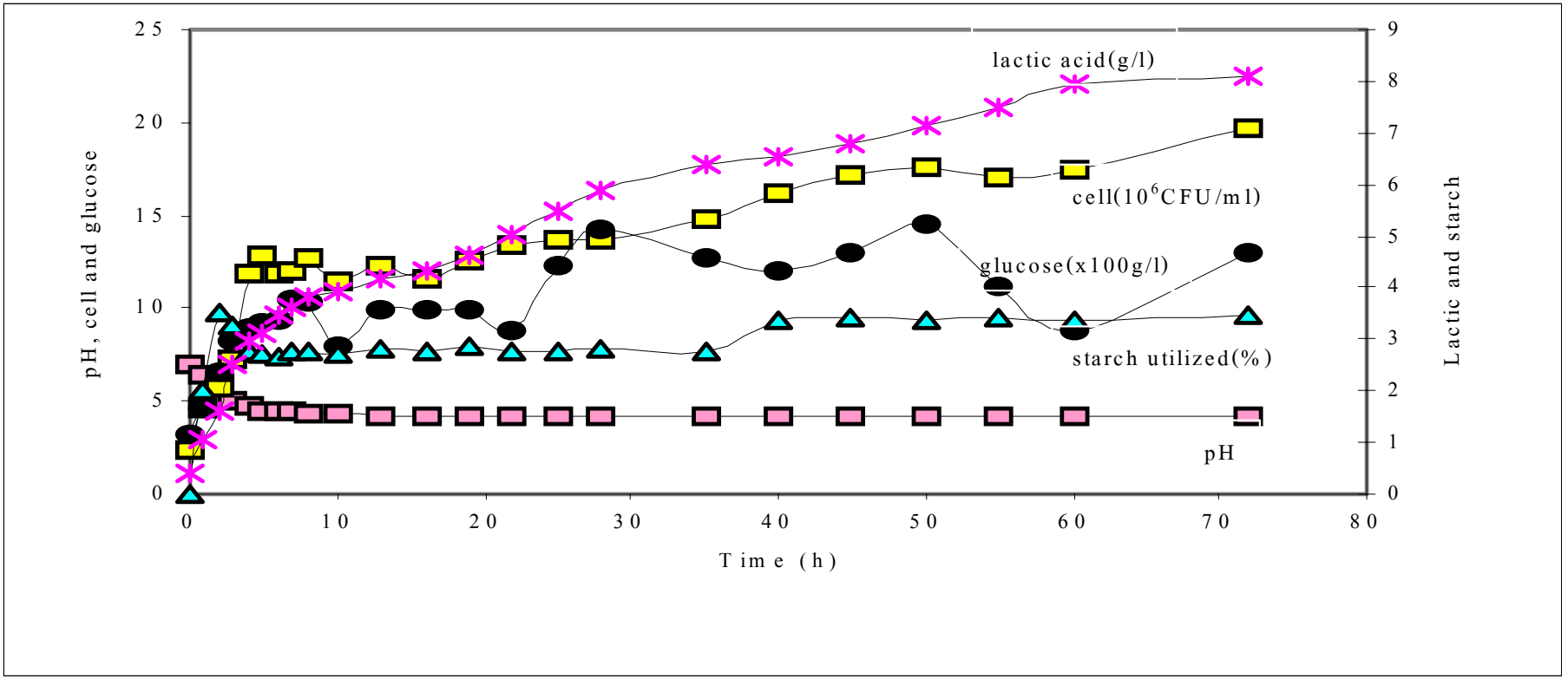


Figure 4.14 Efficiency of 4% starch in RAM medium

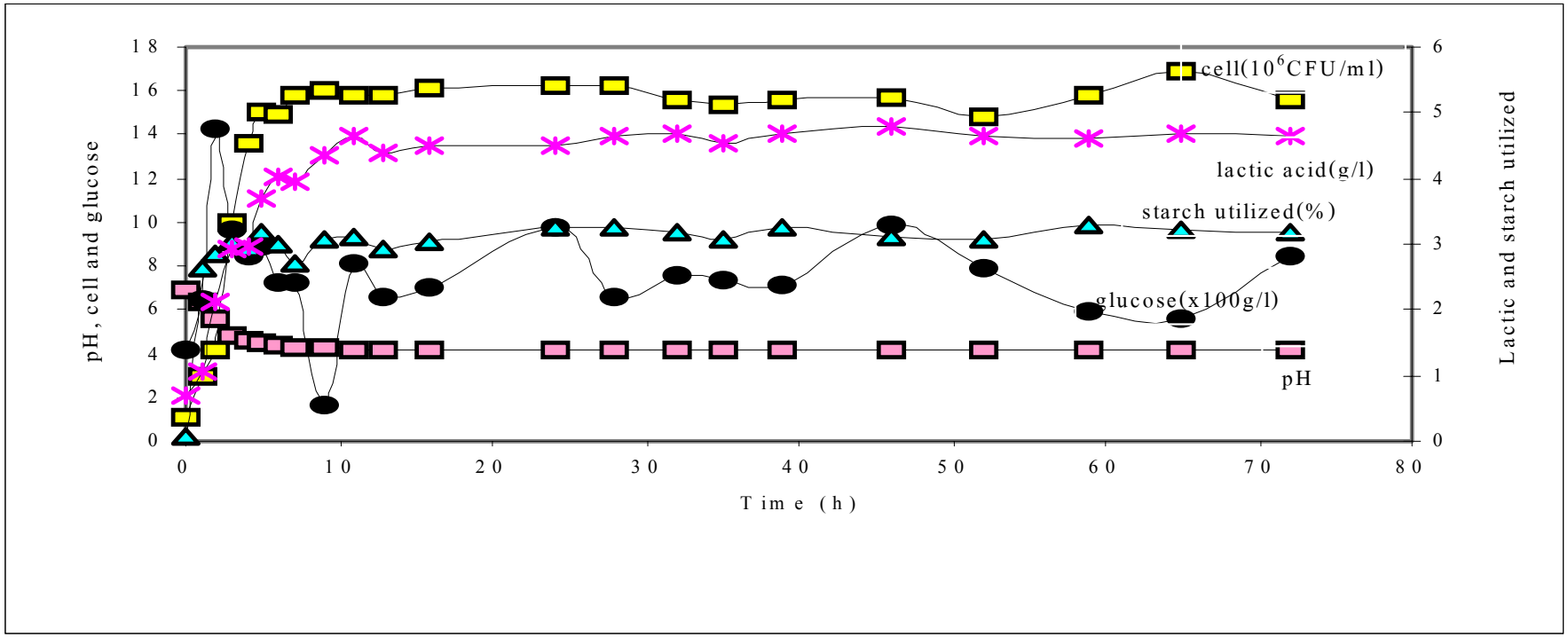


Figure 4.15 Efficiency of 5% starch in RAM medium

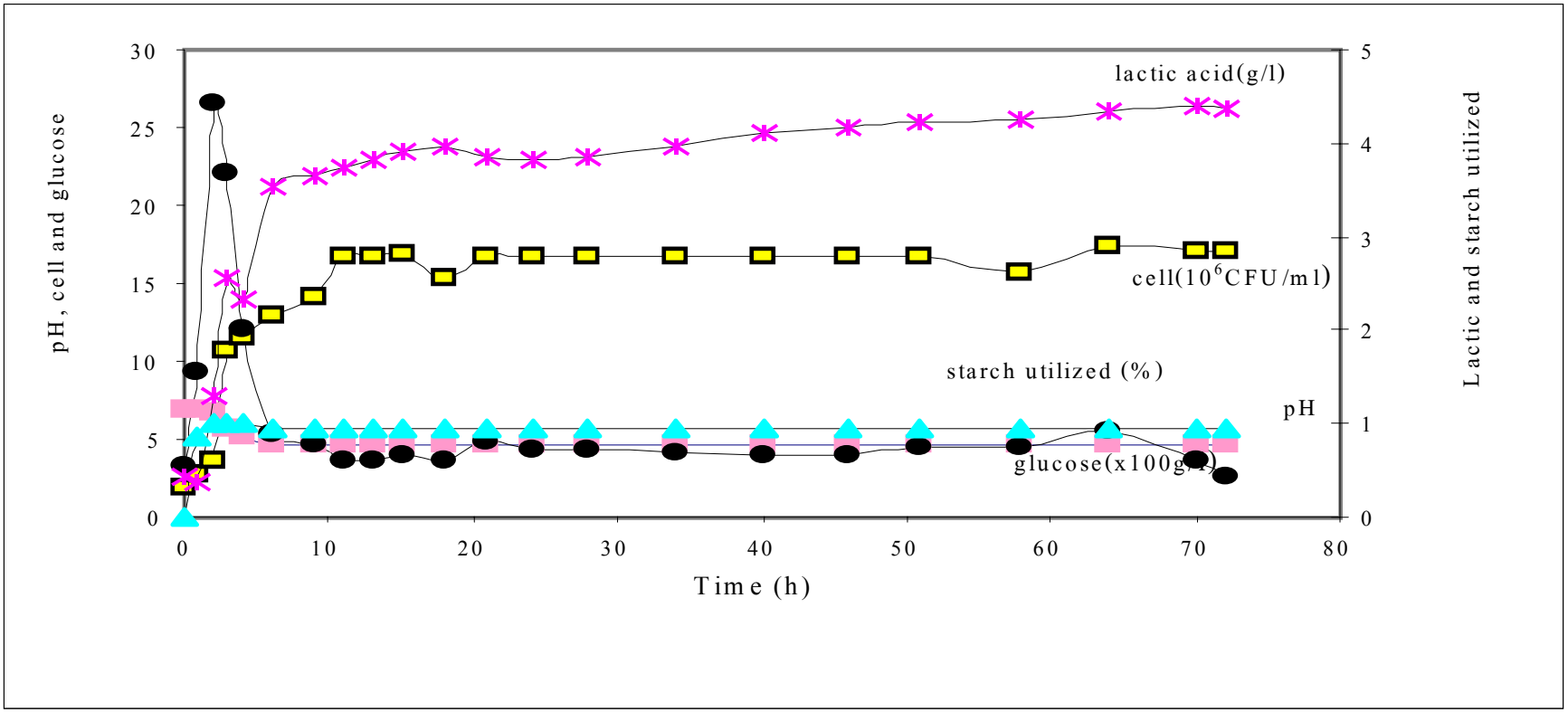


Figure 4.16 Efficiency of 1% tapioca starch in RAM medium

4.6 Other Products from Extracted Starch Fermentation

Apart form lactic acid and glucose production, the process produces acetic acid, propionic acid, and butyric acid during anaerobic fermentation, which were analyzed by HPLC analysis (High Performance Liquid Chromatograph) in Table 4.3. Zero percent of starch could produce butyric acid ($C_4H_8O_2$) more than other product and propionic acid ($C_3H_6O_2$) occurred at 5% starch batch. Batch 4% starch could produces more acetic acid ($C_2H_4O_2$) than other batches. Mobley, 1994 found that under glucose limitation, homolactic fermentation can become heterolactic with the formation of acetate, ethanol, butyric. For example, at low glucose concentration *L. plantarum* metabolizes most pyruvate to acetate.

When compared lactic acid concentration between Biosensor (Lactate and Glucose analyze) and HPLC analysis, the data from HPLC analysis was higher. The objective was for testing the accuracy of the data by preparing standard lactic acid concentration and taking it to Biosensor and HPLC measurement. From this experiment found the value from HPLC analyze had higher value from Biosensor for 4 times but can not indicated for true data. Because of the results were occurred by Biosensor that were the linear range of lactate concentration was 2.67 g/l. If the concentration of soluble more over this limit that was diluted, which this reason it could be error. Another reason from Biosensor that had many reducing agents would give rise to a false signal current (and falsely elevated reading). Most of these are excluded from the probe by the cellulose acetate layers of the membrane. While mistake from HPLC analyze does not occurred from HPLC measurement but occurred from time of samples conservation. The samples were kept in refrigerator that could assume the microorganism utilized starch remain for viability and the product will occurred in this time.

Table 4.3 Other products from starch fermentation

Batch	Lactic acid (g/l)	Acetic acid (g/l)	Propionic acid (g/l)	Butyric acid (g/l)
0%starch	3.918	4.459	4.730	7.544
1%starch	10.045	3.362	6.712	1.069
2%starch	10.469	3.531	5.768	1.119
3%starch	9.801	3.049	4.525	1.269
4%starch	23.029	8.520	6.391	1.040
5%starch	19.805	4.449	7.057	1.748
1% tapioca	12.545	7.875	6.172	0.112
Cassava waste	15.490	1.605	2.792	2.245

4.7 Kinetics for Fermentation of Cassava Solid Waste

The purpose of fermentation modeling is to use the obtained data, which results from the practical fermentation experiment, to analyze for the kinetic constants. One of the principle uses of a fermentation model is to design large-scale fermentation processes using data obtained from small-scale fermentation. The kinetic parameters of starch fermentation were studied using 16 hours-old culture in RAM medium and a reactor with controlled temperature 35° C and an initial pH 7.0. Six concentrations of starch were studied. The kinetics is show at Table 4.4. These experiments had two types of yield, which were production yield ($Y_{p/s}$) and biomass yield ($Y_{x/s}$). For production yield, the yield of batch with 1% starch from waste was higher than other batches (0.426 g lactic/ g substrate) and the value was close to that of batch 1% starch from tapioca (0.46 g lactic/g substrate). The lowest value of yield was 0.093 g lactic/ g substrate in batch 5% starch from waste. The other type of yield, biomass yield, was calculated between amount of culture and substrate. It was found that batch 1%

starch from tapioca gave the highest value of biomass yield at $1.711x10^6$ CFU / g substrate.

Table 4.4 Product Yield ($Y_{p/s}$) and Biomass Yield ($Y_{x/s}$)

Batch (waste)	Cell conc. x (10^6CFU/ml)	Substrate s (g/l)	Products P (g/l)	Product yield $Y_{p/s}$ (g/g)	Biomass yield$Y_{x/s}$ (10^6 CFU/g)
1% starch	13.938	10	4.26	0.426	1.394
2% starch	17.34	20	4.38	0.219	0.867
3% starch	14.675	30	4.38	0.146	0.489
4% starch	19.673	40	8.08	0.202	0.492
5% starch	15.624	50	4.65	0.093	0.312
1% tapioca	17.11	10	4.60	0.460	1.711

The rate of lactic acid formation was both a function of cell growth and cell concentration. The specific growth rate (μ) was calculated as a function of time using the following equation

$$\ln (x/x_o) = \mu t$$

Maximum specific growth rate (μ_m) and Monod constant (K_s) were calculated from a graph with 1/s on the X-axis and 1/μ on the Y-axis (Appendix B). The Y intercept was $1/\mu_m$ and slope was K_s/μ_m. The values of μ_m and Ks that obtained from the plot were 3.94 hr^{-1} and 78.06 g/l, respectively (Table 4.5).

Cell growth rates were often expressed in term of the doubling time (t_d) that derived from

$$t_d = \ln 2/ \mu$$

From Table 4.5, the highest doubling time (t_d) was found in batch 3% starch from waste. In batch 1% starch from waste and 1% starch from tapioca flour, the

values for t_d were similar (1.04 h. and 1.21 h., respectively). Considering batch 0% starch, it was found that the t_d value was 1.2 h for this batch, which was close to the values from other batches except batch 3%. From these results, it can be concluded that the carbon source for the substrate was poor. The microorganism will use the other substrate for carbon source such as yeast extract.

Table 4.5 Summary of kinetic parameters of cassava solid waste fermentation

Batch (waste)	Starch(g/l) (waste)	$Y_{p/s}$ (g./g.)	$Y_{x/s}$ 10^6CFU/g	t_d (hour)	μ ($hour^{-1}$)
0% starch	0	-	-	1.2	-
1% starch	10	0.426	1.394	1.04	0.47
2% starch	20	0.219	0.867	0.82	0.61
3% starch	30	0.146	0.489	2.01	1.31
4% starch	40	0.202	0.492	1.67	1.953
5% starch	50	0.093	0.312	1.06	1.31
1% tapioca	10	0.460	1.711	1.21	0.47

CHAPTER 5

CONCLUSION

The lactic acid bacteria used in the experiment are *Lactococcus sp.* code I-5. The cassava solid waste was brought from Korat Flour Industries. Cassava solid waste has 60.9% moisture content, 0.034% nitrogen (0.2125% protein), and pH value range of 4.9-5.2. The *Lactococcus sp.* I-5 is an anaerobic bacterium. It utilizes starch for production of lactic acid. Optimum conditions for culture growth were determined for nutrients, temperature, and pH. Variation of 0.1-0.5% yeast extract and urea concentration was considered for optimum nutrients. The combination of 0.5% yeast extract and 0.2% urea proved to be the best for lactic acid producing and maximum culture growth in 2 days of inoculum. Optimum temperature and pH was found at 35° C and pH 7.0. These are the best conditions to produce maximum lactic acid product and culture growth.

In solid fermentation, wastes from cassava were used with 0.5% yeast extract and 0.2% urea powder. It did not use RAM medium formula. The pH was not controlled in the system and was decreased from 7.0 to 4.5. During 10 days of fermentation, glucose concentration was rapidly increased and reached a high value of 16 g/1 or yield was 0.126 mg glucose/g solid, while lactic acid production value was 0.6 g/1 or yield was 4 mg lactic / g solid. Bacteria used enzyme Amylase to digest starch to be glucose and use it as lactic acid. It was found that the potential of lactic acid production would increase with the increasing of glucose. However, longer period of experimental time, lactic acid increasing rate will decrease due to the contamination of other microorganisms, i.e., fungi, with use glucose in its metabolism process and produce acetic acid. The system can produce less lactic acid. Protein content was increased from 0.59% to 1.49% (dry mass).

For comparison between different concentration of substrate, 0-5% starch was selected for use in RAM medium for fermentation. The initial pH was at 6.9-7.15, where after the fermentation was completed the pH value dropped to 4.5-4.6. When

considering the yield from product and substrate, batch 1% starch gave the best yield of all batches. Doubling time of microorganism closed to 0.82-2.01 hours. From yield and substrate, Monod's constants (μ_m and Ks) were obtained by Lineweaver-Burk Plots. The average values of μ_m and K_s of starch extracted from cassava solid waste were 3.94 hr^{-1} and 78.06 g/1, respectively. Consideration of phase of substrate for lactic acid product reveals that the best yield of starch extracted was 0.426 g lactic/ g substrate in batch 1% starch, which was similar to the value from 1% tapioca starch.

For future research, it is very interesting to look into and develop a fermenter and substrate. In developing the fermenter, turbine should be used inside the fermenter for solid substrate to prevent oxygen leakage and contaminate from other microorganism. In the part of substrate development, we should use tapioca starch in RAM medium rather than starch extracted because it is more convenience for lactic acid production. From the study of lactic acid production, it was found that starch cassava solid waste would produce less acid when comparing with direct fermentation. The consideration between starch extracted from the cassava solid waste and instant tapioca starch indicated that the amount of lactic acid produced were the same approximately. If consider from starch extraction, utilization of instant tapioca starch will be more convenience. Therefore, direct lactic acid producing from cassava solid waste was not appropriate due to process and production. However, alternatives in disposal of cassava waste should be considered, i.e., the natural digestion container for small tree.

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APPENDIX A
DATA FROM EXPERIMENT

1. Experiment for optimum condition by use RAM medium formula

Varying yeast extract and urea concentration in range 0.1-0.5%

Sample	Yeast (%)	Urea (%)	Cell 10^6 CFU/ml	%starch utilized	Lactic (g/l)	Glucose (g/l)
1	0	0	98.422	1.616	3.66	0.071
2	0	0.1	48.026	1.783	4.015	0.059
3	0	0.2	34.412	1.816	3.985	0.095
4	0	0.3	31.616	1.823	3.48	0.073
5	0	0.4	22.273	1.619	3.03	0.040
6	0	0.5	19.559	1.787	3.205	0.1
7	0.1	0	53.469	1.599	3.815	0.062
8	0.1	0.1	4.327	1.762	3.97	0.068
9	0.1	0.2	16.417	1.591	3.37	0.070
10	0.1	0.3	31.085	1.907	3.96	0.140
11	0.1	0.4	7.571	1.423	2.405	0.042
12	0.1	0.5	7.402	1.898	3.755	0.134
13	0.2	0	55.001	1.721	4.17	0.077
14	0.2	0.1	93.016	1.789	4.12	0.061
15	0.2	0.2	54.383	1.053	1.49	0.044
16	0.2	0.3	61.580	1.201	2.05	0.060
17	0.2	0.4	18.381	1.611	2.735	0.041
18	0.2	0.5	10.388	1.884	3.78	0.121
19	0.3	0	60.545	1.835	3.97	0.097
20	0.3	0.1	58.859	1.765	3.73	0.062
21	0.3	0.2	69.730	1.829	4.39	0.132

Sample	Yeast (%)	Urea (%)	Cell 10^6 CFU/ml	%starch utilized	Lactic (g/l)	Glucose (g/l)
22	0.3	0.3	38.967	1.836	4.105	0.121
23	0.3	0.4	31.438	1.837	3.585	0.091
24	0.3	0.5	12.949	1.839	3.625	0.122
25	0.4	0	91.452	1.872	4.21	0.111
26	0.4	0.1	109.575	1.835	4.255	0.111
27	0.4	0.2	72.134	1.829	3.965	0.131
28	0.4	0.3	41.937	1.855	4.015	0.138
29	0.4	0.4	16.792	1.816	3.905	0.116
30	0.4	0.5	30.049	1.882	3.35	0.106
31	0.5	0	89.915	1.872	4.35	0.145
32	0.5	0.1	64.792	1.692	3.6	0.104
33	0.5	0.2	137.359	1.761	3.925	0.119
34	0.5	0.3	45.133	1.898	4.07	0.147
35	0.5	0.4	55.626	1.490	2.185	0.1015
36	0.5	0.5	20.464	1.829	3.39	0.131

Varying Temperature optimum condition form 30°C-39° C

Temp. (°C)	Growth (10^6 CFU/ml)	%starch utilized	Lactic (g/l)	Glucose (g/l)
30	8.819	1.791	4.175	0.087
33	9.707	1.812	3.685	0.0555
35	12.824	1.876	4.7	0.0495
37	11.499	1.895	3.975	0.066
39	10.46	1.879	3.465	0.051

Varying pH from pH 4 to pH 8

pH	Growth (10^6 CFU/ml)	%starch utilized	Lactic (g/l)	Glucose (g/l)
4	11.77	1.819	2.195	0.041
5	12.387	1.831	2.775	0.045
6	14.1878	1.873	2.745	0.0376
7	14.464	1.871	4.965	0.0875
8	14.961	1.888	3.69	0.0475

Solid substrate fermentation of cassava solid waste

Time (day)	pH	Lactic (g/l)	Glucose (g/l)
0	7	0	0.1215
2	6.5	0.1825	0.2215
3	5.5	0.267	0.209
4	4.5	0.3015	0.5905
5	4.5	0.3805	1.555
6	4.5	0.4245	1.78
7	4.5	0.49	4.415
8	4.5	0.5695	8.42
9	4.5	0.58	10.335
10	4.5	0.595	15.955

3 Fermentation

Fermentation of starch extract from cassava solid waste by using flask pre-experiment

% initial starch	%starch utilized	Growth (10^6 CFU/ml)	Lactic (g/l)	Glucose (g/l)	Final pH
0	0	9.19	1.335	0.035	6
1	0.914	77.8	4.39	0.102	4.5
2	1.56	82.79	6.51	0.382	4.5
3	2.18	9.51	5.735	0.555	4.5
4	2.48	37.97	6.825	0.215	4.5
5	2.86	7.29	4.99	0.281	4.5

Fermentation of RAM medium by using 0% starch extract

Time (h)	pH	#cell 10^6 CFU/ml	%starch utilized	Lactic (g/l)	Glucose (g/l)	Temp. (°C)
0	6.78	2.241	0.138	0.325	0.018	35.0
1	6.73	3.611	0.060	0.670	0.020	35.1
2	6.43	7.374	0.136	1.180	0.026	35.1
3	6.18	8.925	0.269	1.300	0.042	35.0
4	6.18	9.180	0.239	1.280	0.042	35.0
5	6.18	9.030	0.305	1.220	0.038	35.1
6	6.18	9.030	0.105	1.305	0.042	34.9
7	6.18	9.467	0.101	1.360	0.034	34.9
9	6.19	9.993	0.247	1.015	0.021	35.1
11	6.25	11.318	0.218	0.905	0.020	35.2
13	6.4	12.809	0.213	0.785	0.019	35.0
18	6.6	12.583	0.265	0.505	0.018	35.0
22	6.72	12.357	0.212	0.585	0.018	35.1
28	6.85	12.357	0.238	0.525	0.024	35.0
34	6.89	10.957	0.160	0.560	0.053	34.9
38	6.93	11.258	0.113	0.500	0.049	35.0
44	6.99	9.707	0.140	0.555	0.067	35.1
50	7.03	9.391	0.148	0.525	0.038	35.1
56	7.06	8.383	0.128	0.550	0.062	35.1
72	7.05	8.067	0.143	0.493	0.055	35.1

Fermentation of RAM medium by using 1% starch extract

Time (h)	pH	#cell *10^6 CFU/ml	%starch utilized	Lactic (g/l)	Glucose (g/l)	Temp. (ºC)
0	6.71	1.909	0.027	0.71	0.019	34.9
1	6.34	3.279	0.700	1.52	0.039	34.9
2	5.49	7.254	0.914	1.68	0.094	34.9
3	4.71	9.828	0.883	2.66	0.092	34.9
4	4.63	12.146	0.891	2.98	0.068	35.0
5	4.51	10.882	0.904	3.11	0.067	35.0
6	4.41	10.836	0.907	3.44	0.070	35.0
7	4.30	10.791	0.903	3.66	0.068	35.0
9	4.30	10.806	0.900	3.67	0.064	35.1
11	4.30	10.897	0.907	3.81	0.067	35.1
13	4.29	11.499	0.910	4.05	0.067	35.1
18	4.29	11.333	0.903	3.85	0.052	35.1
22	4.29	12.628	0.901	3.95	0.062	35.0
28	4.29	12.643	0.897	3.95	0.064	35.0
34	4.29	13.019	0.902	3.59	0.046	35.1
38	4.29	13.079	0.893	4.11	0.052	35.1
44	4.26	13.892	0.898	3.98	0.052	35.1
50	4.26	13.441	0.896	3.94	0.060	35.1
56	4.26	13.682	0.893	3.93	0.059	35.1
72	4.26	13.938	0.886	4.26	0.038	35.1

Fermentation of RAM medium by using 2% starch extract

Time (h)	pH	#cell 10^6 CFU/ml	%starch utilized	Lactic (g/l)	Glucose (g/l)	Temp. (° C)
0	6.91	1.006	0.072	0.57	0.019	35.0
1	6.41	1.789	0.748	0.69	0.039	35.0
2	5.85	5.432	1.552	1.57	0.108	34.4
3	5.05	8.337	1.619	2.49	0.166	34.9
4	4.70	12.402	1.650	3.14	0.116	35.0
5	4.59	13.998	1.604	3.36	0.115	35.0
6	4.47	14.043	1.628	3.51	0.112	34.9
7	4.39	14.118	1.621	3.50	0.061	35.0
9	4.35	15.247	1.635	3.97	0.073	35.0
11	4.34	15.398	1.637	3.85	0.068	35.0
13	4.31	15.262	1.645	3.79	0.075	35.0
16	4.31	15.428	1.665	4.10	0.072	34.9
24	4.31	16.105	1.643	4.31	0.089	35.0
28	4.30	16.090	1.625	4.16	0.101	35.1
32	4.31	16.617	1.617	3.95	0.126	35.0
35	4.31	16.632	1.622	4.03	0.112	35.0
39	4.31	15.654	1.622	4.37	0.095	35.0
46	4.32	15.714	1.696	4.26	0.109	35.0
52	4.31	16.105	1.646	4.33	0.110	35.0
59	4.31	17.626	1.663	4.38	0.106	35.0
65	4.31	17.280	1.653	4.37	0.100	35.0
72	4.31	17.340	1.652	4.38	0.103	34.9

Fermentation of RAM medium by using 3% starch extract

Time (h)	pH	#cell 10^6 CFU/ml	% starch utilized	Lactic (g/l)	Glucose (g/l)	Temp. (°C)
0	7.01	3.595	0.000	0.58	0.028	35.0
1	6.26	4.679	2.163	1.07	0.037	35.0
2	5.76	7.148	2.473	1.49	0.069	35.1
3	4.69	9.783	2.541	2.61	0.080	35.1
4	4.48	10.234	2.307	3.09	0.095	35.1
5	4.37	15.925	2.313	3.64	0.095	35.0
6	4.32	13.215	2.363	3.57	0.082	35.1
7	4.27	13.245	2.301	3.57	0.080	35.0
8	4.24	14.811	2.262	3.79	0.078	34.9
10	4.21	12.628	2.157	4.03	0.087	35.0
13	4.20	14.359	2.379	4.14	0.083	35.0
16	4.20	13.817	2.250	4.33	0.084	35.0
19	4.20	13.110	2.423	4.36	0.082	35.1
22	4.20	13.486	2.340	4.32	0.092	35.0
25	4.20	13.832	2.320	4.41	0.090	35.0
28	4.20	13.456	2.317	4.36	0.091	35.0
35	4.20	14.284	2.255	4.34	0.100	35.1
40	4.21	15.428	2.278	4.85	0.151	35.0
45	4.21	13.079	2.345	4.23	0.126	35.0
50	4.21	13.019	2.318	4.29	0.110	35.0
55	4.22	13.305	2.321	4.31	0.101	35.1
60	4.22	13.923	2.343	4.35	0.102	35.1
72	4.22	14.675	2.382	4.39	0.097	35.1

Fermentation of RAM medium by using 4% starch extract

Time (h)	pH	#cell 10^6 CFU/ml	% starch utilized	Lactic (g/l)	Glucose (g/l)	Temp. (°C)
0	6.99	3.370	0.000	0.42	0.032	35.0
1	6.37	4.589	2.016	1.07	0.046	35.0
2	6.02	7.733	3.516	1.60	0.066	35.0
3	4.99	9.208	3.256	2.50	0.082	35.0
4	4.70	11.875	2.772	2.96	0.090	35.0
5	4.51	12.854	2.697	3.12	0.092	35.0
6	4.45	11.890	2.651	3.49	0.094	35.0
7	4.40	12.056	2.777	3.61	0.105	35.0
8	4.32	12.733	2.751	3.80	0.104	35.0
10	4.26	11.484	2.740	3.91	0.080	35.0
13	4.25	12.282	2.821	4.16	0.100	35.1
16	4.25	11.559	2.770	4.32	0.100	35.0
19	4.25	12.507	2.874	4.62	0.098	35.1
22	4.24	13.471	2.764	5.01	0.088	35.0
25	4.24	13.667	2.766	5.48	0.124	35.0
28	4.24	13.697	2.822	5.90	0.143	35.0
35	4.25	14.750	2.788	6.39	0.127	35.0
40	4.24	16.166	3.361	6.53	0.120	35.0
45	4.24	17.114	3.429	6.80	0.131	35.0
50	4.23	17.578	3.386	7.12	0.146	35.0
55	4.24	17.054	3.430	7.51	0.112	35.0
60	4.24	17.415	3.376	7.95	0.088	35.0
72	4.24	19.673	3.457	8.10	0.130	35.0

Fermentation of RAM medium by using 5% starch extract

Time (h)	pH	#cell 10^6 CFU/ml	%starch utilized	Lactic (g/l)	Glucose (g/l)	Temp. (°C)
0	6.92	1.127	0.0707	0.71	0.0414	35.0
1	6.40	1.978	2.469	1.06	0.065	35.0
2	5.60	4.122	2.881	2.12	0.143	35.0
3	4.85	9.933	3.0283	2.95	0.097	35.0
4	4.56	13.576	2.9167	2.95	0.085	35.0
5	4.46	15.037	3.1771	3.70	0.089	35.0
6	4.38	14.931	3.0159	4.02	0.072	35.0
7	4.32	15.819	2.6935	3.95	0.072	35.0
9	4.25	15.985	3.0903	4.37	0.016	35.0
11	4.22	15.819	3.1089	4.64	0.081	35.0
13	4.21	15.849	2.9353	4.41	0.066	35.0
16	4.21	16.151	3.0283	4.51	0.0705	35.0
24	4.21	16.211	3.2577	4.50	0.0975	35.0
28	4.21	16.271	3.2639	4.66	0.066	35.0
32	4.20	15.533	3.1647	4.69	0.0755	34.9
35	4.20	15.353	3.0779	4.54	0.074	35.0
39	4.20	15.578	3.2701	4.67	0.071	35.0
46	4.20	15.729	3.1151	4.78	0.099	35.1
52	4.20	14.781	3.0593	4.65	0.079	35.1
59	4.20	15.789	3.3073	4.62	0.059	35.1
65	4.20	16.903	3.2143	4.69	0.0555	35.0
72	4.20	15.624	3.1647	4.65	0.085	35.0

Fermentation of RAM medium by using 1% tapioca starch

Time (h)	pH	#cell 10^6 CFU/ml	%starch utilized	Lactic (g/l)	Glucose (g/l)	Temp. (°C)
0	6.93	1.91	0	0.417	0.0329	34.9
1	6.90	2.74	0.849	0.372	0.093	34.9
2	6.76	3.64	0.995	1.295	0.2655	35.0
3	5.73	10.64	0.995	2.545	0.221	35.0
4	5.09	11.59	0.989	2.34	0.12	35.0
6	4.72	13.00	0.958	3.535	0.054	35.0
9	4.65	14.12	0.958	3.65	0.046	35.0
11	4.62	16.80	0.959	3.725	0.0363	35.0
13	4.60	16.75	0.960	3.81	0.0365	35.0
15	4.60	16.84	0.960	3.895	0.039	35.0
18	4.60	15.26	0.959	3.955	0.03665	35.0
21	4.59	16.80	0.957	3.845	0.04835	35.0
24	4.58	16.80	0.959	3.825	0.04335	35.0
28	4.59	16.80	0.958	3.85	0.0425	35.0
34	4.59	16.80	0.958	3.975	0.0409	35.0
40	4.59	16.80	0.957	4.11	0.0401	35.0
46	4.59	16.80	0.955	4.165	0.03945	35.0
51	4.59	16.80	0.955	4.215	0.0443	35.0
58	4.59	15.68	0.953	4.258	0.04565	35.0
64	4.59	17.42	0.952	4.35	0.0545	35.0
70	4.59	17.05	0.951	4.398	0.0368	35.0
72	4.59	17.11	0.951	4.36	0.0267	35.0

4. HPLC Analysis

Standard solutions for HPLC analysis were 85% lactic acid, 99.8% acetic acid, 98% propionic acid and 99% butyric acid.

Batch (waste)	% Lactic acid	%Acetic acid	%Propionic acid	%Butyric acid
0%starch	0.32653	0.42474	0.47725	0.78752
1%starch	0.83707	0.32026	0.67597	0.11153
2%starch	0.87242	0.33636	0.58087	0.11687
3%starch	0.81675	0.29039	0.45571	0.13250
4%starch	1.91910	0.81146	0.64355	0.10859
5%starch	1.65040	0.42377	0.71088	0.18250
1% tapioca flour	1.04540	0.75011	0.62154	0.01169
Cassava waste	1.29080	0.15234	0.28117	0.23439

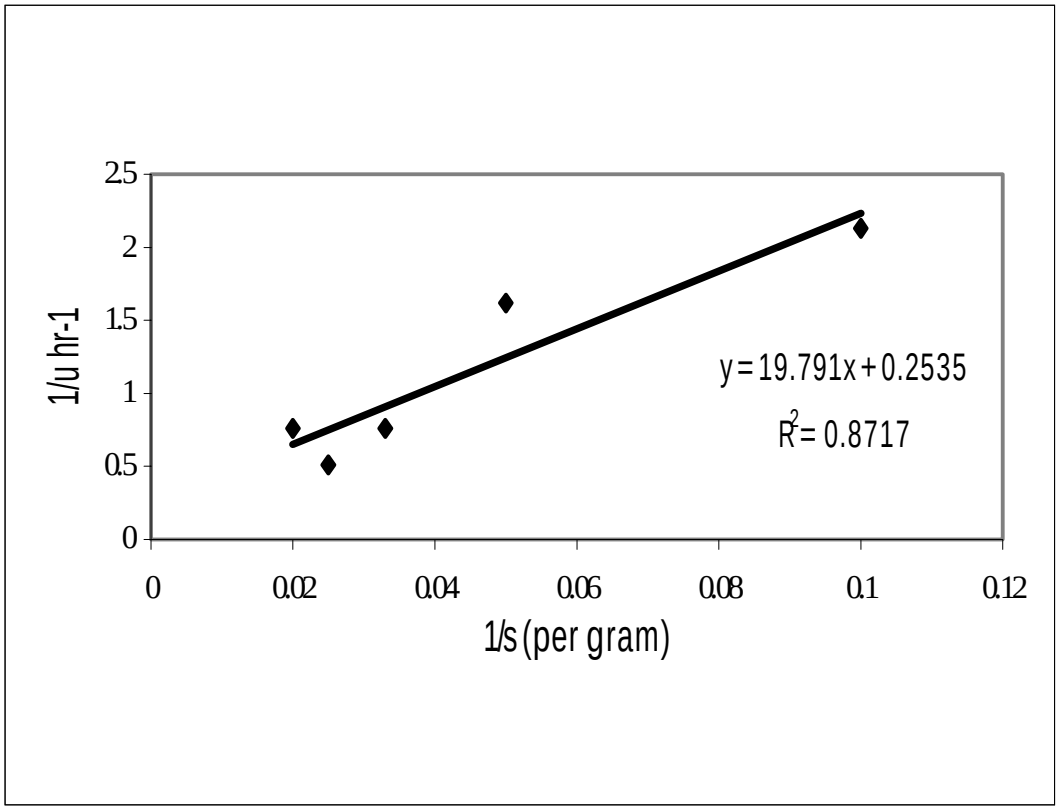
Lactic acid concentration compares between Biosensor and HPLC analysis

Batch	Biosensor anaysis g/l	HPLC analysis g/l	different value (times)
0.1%	0.31	1.37	4.42
0.5%	1.51	5.99	3.96
1.0%	3.1	12.052	3.89
3.0%	9.09	36.16	3.98
5.0%	14.98	60.6	4.05

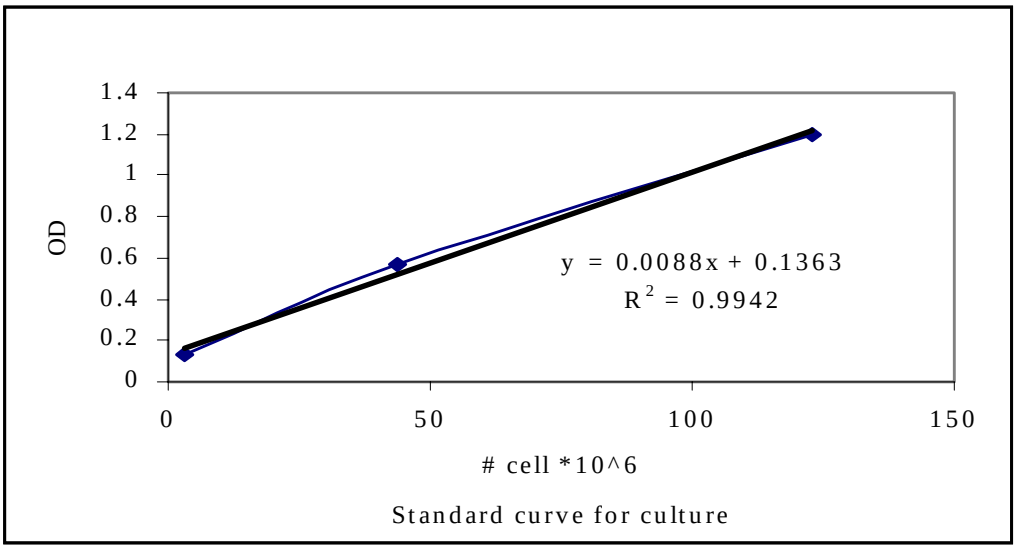
APPENDIX B
KINETICS FOR EXPERIMENT

Kinetic Parameters in Batch 1-5 % Starch from Waste

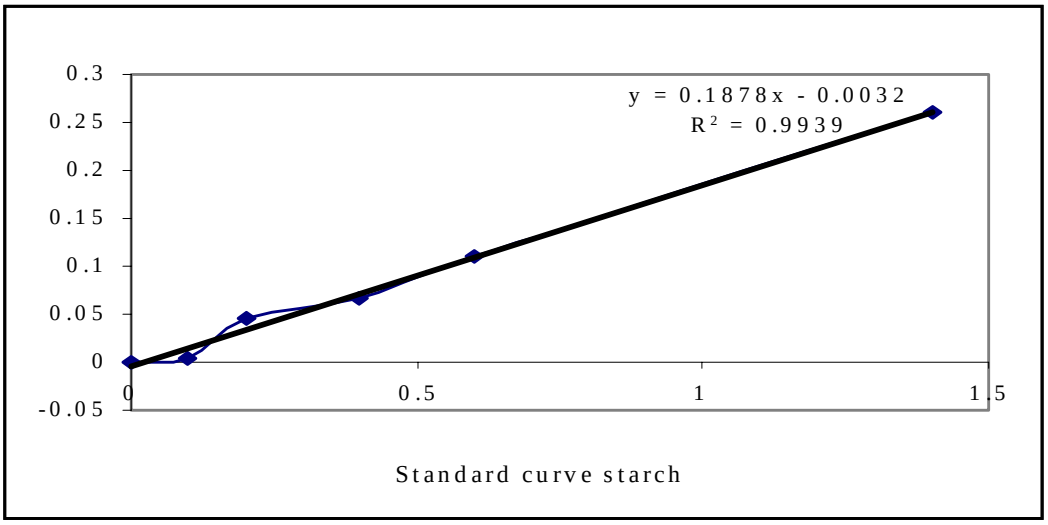
Batch	s g/l	μ h^{-1}	1/s	1/μ
1%starch	10	0.47	0.1	2.13
2% starch	20	0.61	0.05	1.62
3% starch	30	1.31	0.033	0.76
4% starch	40	1.95	0.025	0.51
5% starch	50	1.31	0.02	0.76



Lineweaver-Burk plots for μ_m and K_s analysis

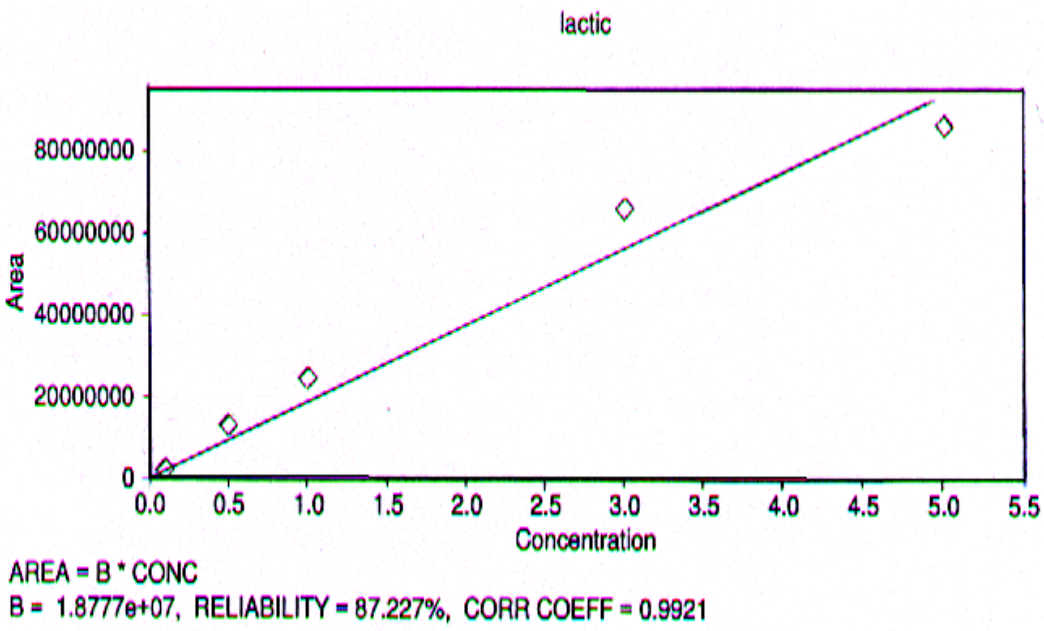


Standard Curve for culture analysis

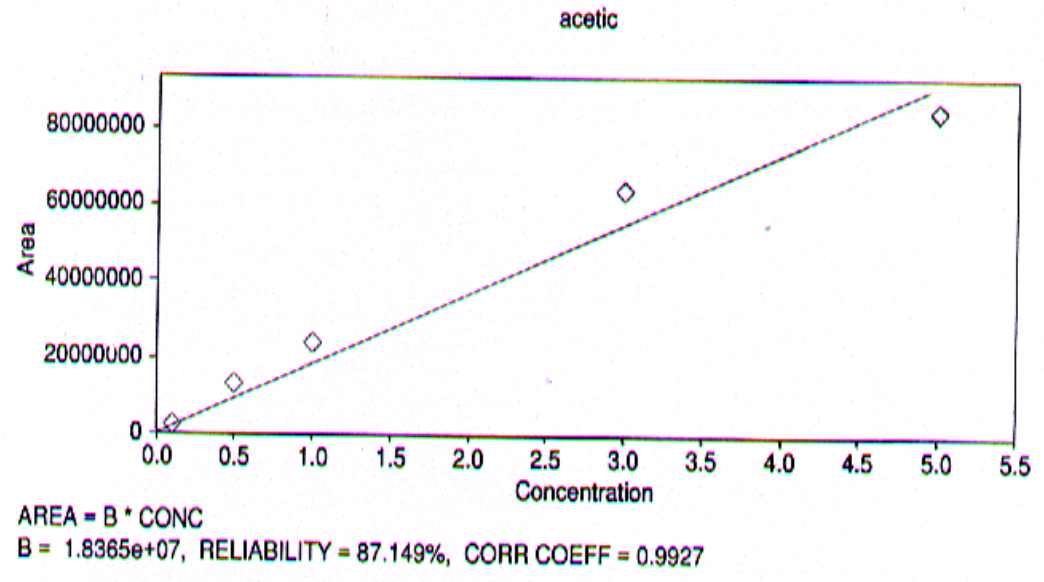


Standard Curve for starch analysis

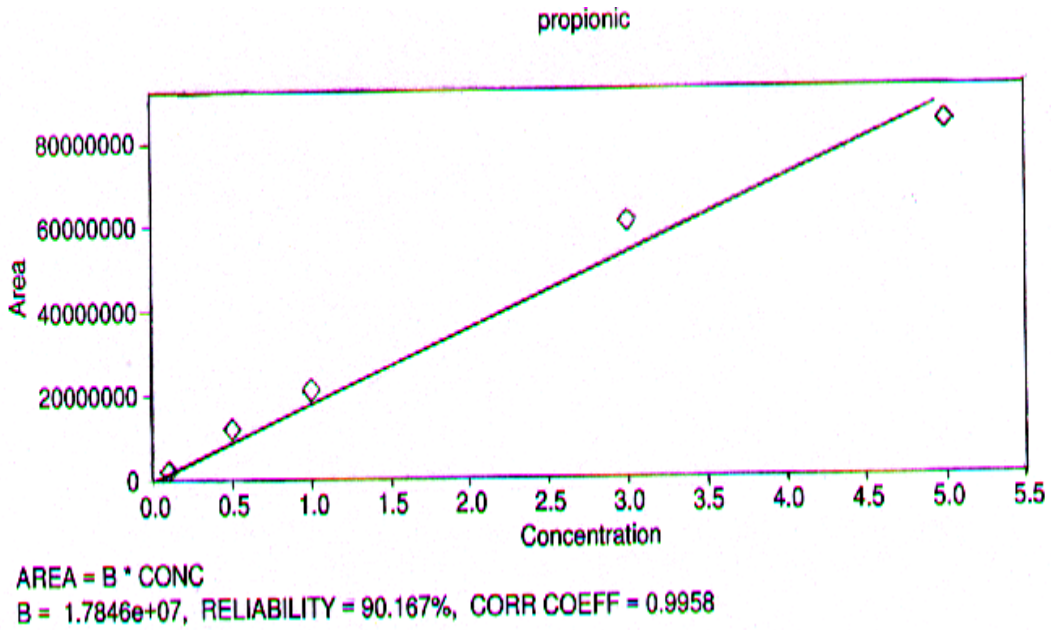
APPENDIX C
DATA FROM HPLC ANALYSIS



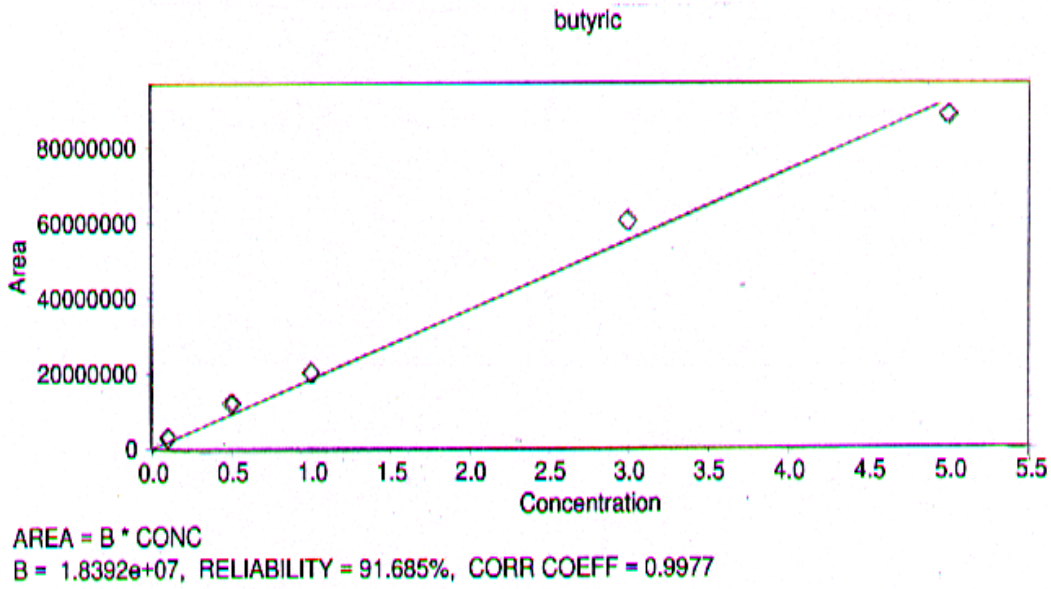
Standard curve for lactic acid by HPLC analysis



Standard curve for acetic acid by HPLC analysis



Standard curve for propionic acid by HPLC analysis



Standard curve for butyric acid by HPLC analysis

Analysis Report

Name: Sample 01
Type: Sample
Injection Volume: 10.0 µL

Vial: A06

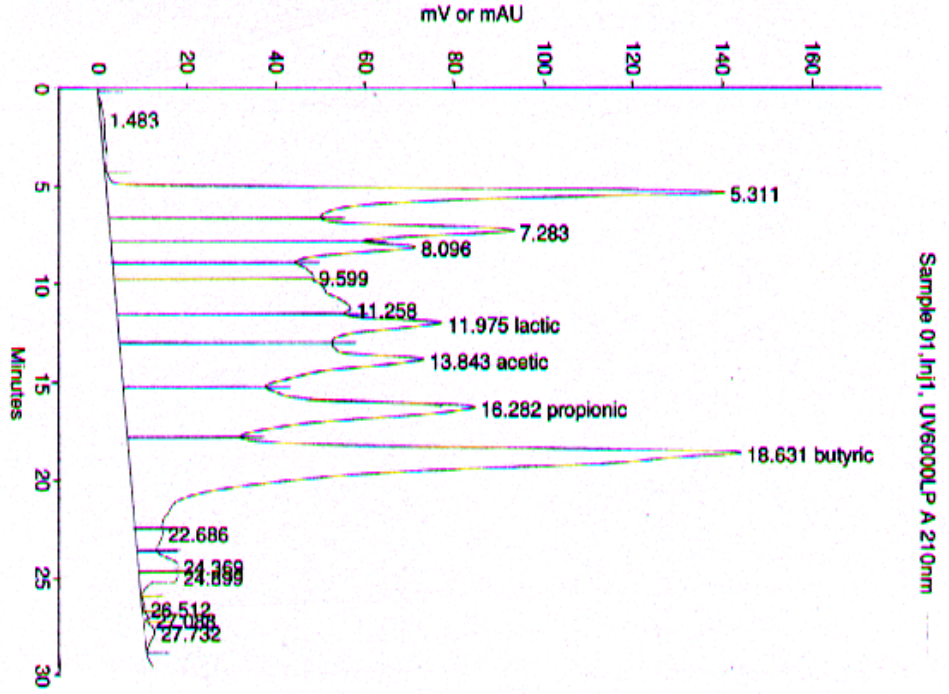
Injection: 1 of 1
Injected On: 07-12-01 10:24:42

Acquisition Log
Column Pressure: N/A
Noise (microAU): 1e+02
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): 9e+02

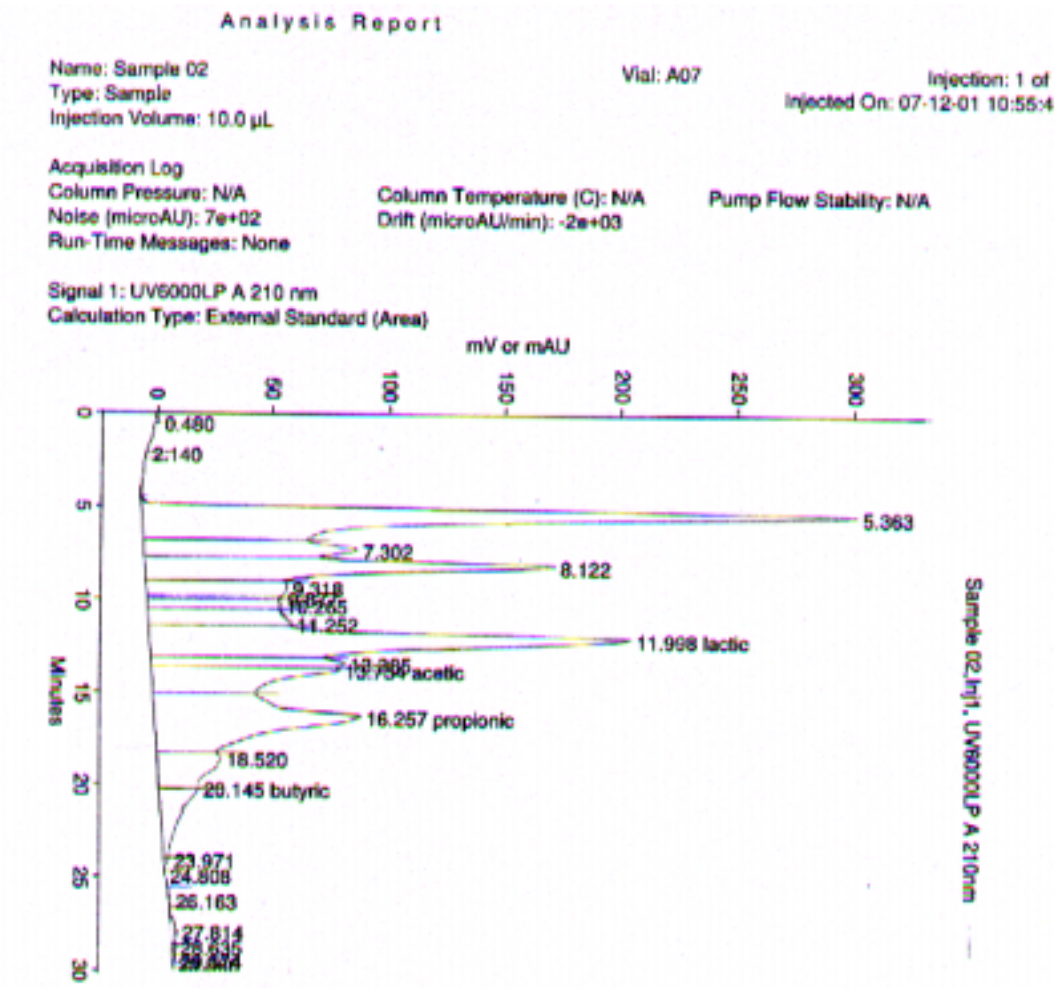
Pump Flow Stability: N/A

Signal 1: UV6000LP A 210 nm
Calculation Type: External Standard (Area)



Component	RT(min)	Area	Height	%	Peak Type
Unident0001	1.483	150801	866	0.0	Fused
Unident0002	5.311	7485768	138781	0.0	Fused
Unident0003	7.283	4894796	90748	0.0	Fused
Unident0004	8.096	3579691	68422	0.0	Fused
Unident0005	9.599	2162299	44838	0.0	Fused
Unident0006	11.258	5258655	52669	0.0	Fused
lactic	11.975	5070683	72874	0.32653	Fused
acetic	13.843	6601673	68128	0.42474	Fused
propionic	16.282	7276915	78543	0.47725	Fused
butyric	18.631	12431427	137469	0.78752	Fused
Unident0011	22.686	372390	6114	0.0	Fused
Unident0012	24.369	477503	8886	0.0	Fused
Unident0013	24.899	312443	8606	0.0	Fused
Unident0014	26.512	16328	567	0.0	Fused
Unident0015	27.088	27764	1526	0.0	Fused
Unident0016	27.732	123909	2377	0.0	Fused
Totals		56243045	781414	2.016	

Data analysis for batch 0% starch by HPLC analyzer



Component	RT(min)	Area	Height	%	Peak Type
Unident0001	0.480	32869	1861	0.0	Resolved
Unident0002	2.140	6983	603	0.0	Resolved
Unident0003	5.363	16309936	308239	0.0	Fused
Unident0004	7.302	4540219	92157	0.0	Fused
Unident0005	8.122	8451414	176784	0.0	Fused
Unident0006	9.318	2941814	61084	0.0	Fused
Unident0007	9.877	599898	58902	0.0	Fused
Unident0008	10.265	1739850	57632	0.0	Fused
Unident0009	11.252	3173363	61755	0.0	Fused
lactic	11.998	12998744	207249	0.83707	Fused
Unident0011	13.385	2501665	83325	0.0	Fused
acetic	13.734	4977745	90834	0.32026	Fused
propionic	16.257	10306849	88596	0.67597	Fused
Unident0014	18.520	2582198	27588	0.0	Fused
butyric	20.145	1760572	16859	0.11153	Fused
Unident0016	23.971	40653	2068	0.0	Fused
Unident0017	24.808	2523	212	0.0	Fused
Unident0018	26.163	21766	740	0.0	Fused
Unident0019	27.814	164565	2658	0.0	Fused
Unident0020	28.635	44118	1788	0.0	Fused

Data analysis for batch 1% starch by HPLC analyzer

Analysis Report

Name: Sample 03
Type: Sample
Injection Volume: 10.0 µL

Vial: A08

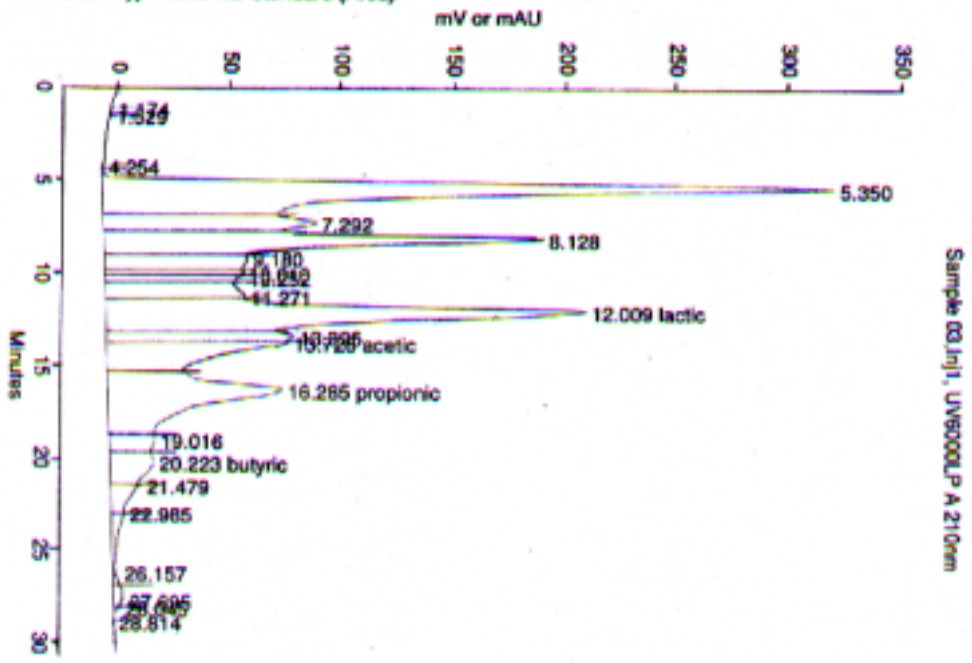
Injection: 1 of
Injected On: 07-12-01 11:26:4

Acquisition Log
Column Pressure: N/A
Noise (microAU): 4e+02
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): -4e+03

Pump Flow Stability: N/A

Signal 1: UV6000LP A 210 nm
Calculation Type: External Standard (Area)



Component	RT(min)	Area	Height	%	Peak Type
Unident0001	1.174	7633	949	0.0	Resolved
Unident0002	1.529	885	250	0.0	Resolved
Unident0003	4.254	1785	315	0.0	Resolved
Unident000-	5.350	17921345	328098	0.0	Fused
Unident0005	7.292	4556435	96056	0.0	Fused
Unident0006	8.128	9463982	198917	0.0	Fused
Unident0007	9.180	3099034	64462	0.0	Fused
Unident0008	10.010	1094203	62392	0.0	Fused
Unident0009	10.252	1485332	62554	0.0	Fused
Unident0010	11.271	3001039	62827	0.0	Fused
lactic	12.009	13547685	215393	0.87242	Fused
Unident0012	13.395	2555423	84885	0.0	Fused
acetic	13.728	5228040	82275	0.33636	Fused
propionic	16.285	8856875	79012	0.56987	Fused
Unident0015	19.016	1173558	21182	0.0	Fused
butyric	20.223	1844880	20366	0.11687	Fused
Unident0017	21.479	880082	14655	0.0	Fused
Unident0018	22.985	502188	6602	0.0	Fused
Unident0019	26.157	399341	3573	0.0	Fused
Unident0020	27.695	287673	5331	0.0	Fused

Data analysis for batch 2% starch by HPLC analyzer

Analysis Report

Name: Sample 04
Type: Sample
Injection Volume: 10.0 µL

Vial: A09

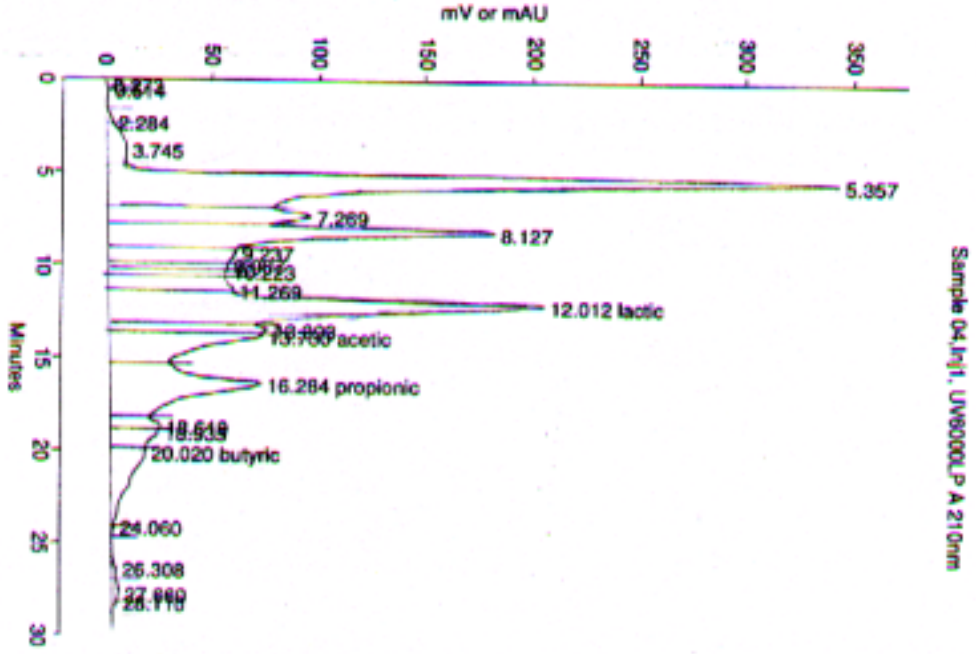
Injection: 1 of 1
Injected On: 07-12-01 11:57:41

Acquisition Log
Column Pressure: N/A
Noise (microAU): 2e+02
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): 2e+03

Pump Flow Stability: N/A

Signal 1: UV6000LP A 210 nm
Calculation Type: External Standard (Area)



Component	RT(min)	Area	Height	%	Peak Type
Unident0001	0.273	2897	300	0.0	Fused
Unident0002	0.514	2997	433	0.0	Fused
Unident0003	2.284	40172	2079	0.0	Fused
Unident0004	3.745	918469	8662	0.0	Fused
Unident0005	5.357	18254823	342001	0.0	Fused
Unident0006	7.269	4580960	94837	0.0	Fused
Unident0007	8.127	8661937	180068	0.0	Fused
Unident0008	9.237	2989947	59844	0.0	Fused
Unident0009	9.907	1023334	55893	0.0	Fused
Unident0010	10.223	1095329	55362	0.0	Fused
Unident0011	11.269	2875213	58211	0.0	Fused
lactic	12.012	12683269	202763	0.81675	Fused
Unident0013	13.393	2126109	74191	0.0	Fused
acetic	13.730	4513543	71807	0.29039	Fused
propionic	16.284	6948462	71062	0.45571	Fused
Unident0016	18.619	877305	23539	0.0	Fused
Unident0017	18.935	1212162	22919	0.0	Fused
butyric	20.020	2091665	16593	0.1325	Fused
Unident0019	24.060	30770	1556	0.0	Fused
Unident0020	26.308	163436	2700	0.0	Fused

Data analysis for batch 3% starch by HPLC analyzer

Analysis Report

Name: Sample 05
Type: Sample
Injection Volume: 10.0 µL

Vial: A10

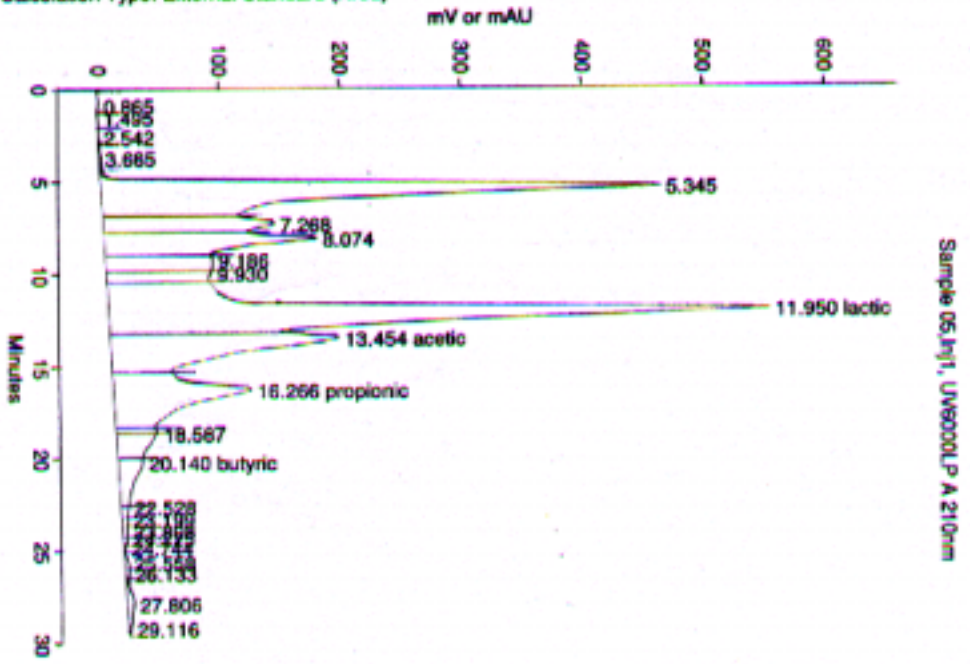
Injection: 1 of 1
Injected On: 07-13-01 00:34:57

Acquisition Log
Column Pressure: N/A
Noise (microAU): 2e+02
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): 9e+02

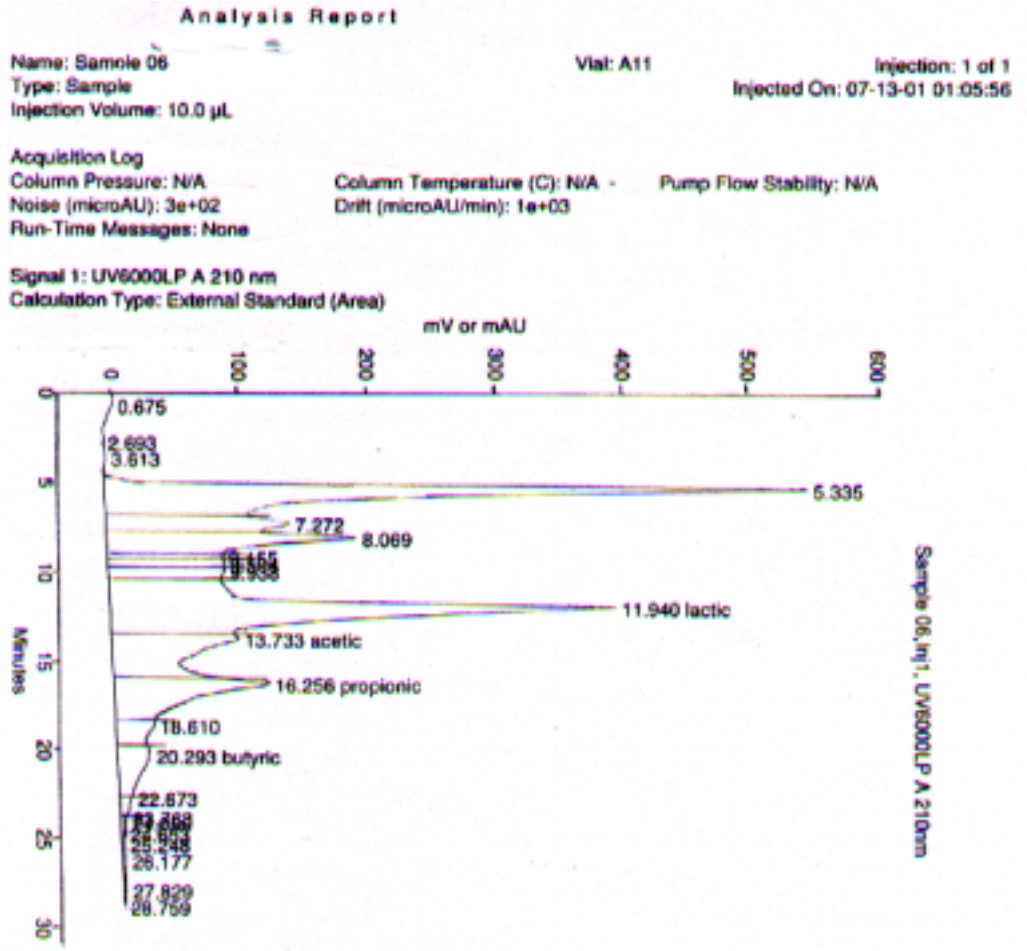
Pump Flow Stability: N/A

Signal 1: UV6000LP A 210 nm
Calculation Type: External Standard (Area)



Component	RT(min)	Area	Height	%	Peak Type
Unident0001	0.865	22883	873	0.0	Fused
Unident0002	1.495	7924	284	0.0	Fused
Unident0003	2.542	13408	433	0.0	Fused
Unident0004	3.665	29382	718	0.0	Fused
Unident0005	5.345	26149657	463160	0.0	Fused
Unident0006	7.268	6979102	142469	0.0	Fused
Unident0007	8.074	9325109	176821	0.0	Fused
Unident0008	9.186	4528197	89494	0.0	Fused
Unident0009	9.930	2903689	87849	0.0	Fused
lactic	11.950	36034615	546906	1.9191	Fused
acetic	13.454	14902682	189129	0.81146	Fused
propionic	16.266	11484881	114350	0.64355	Fused
Unident0013	18.567	529863	34383	0.0	Fused
Unident0014	18.587	2197072	34496	0.0	Fused
butyric	20.140	1997192	20662	0.10859	Fused
Unident0016	22.528	189128	5830	0.0	Fused
Unident0017	23.199	104858	4328	0.0	Fused
Unident0018	23.808	132706	3811	0.0	Fused
Unident0019	24.225	57595	2949	0.0	Fused
Unident0020	24.744	16738	1335	0.0	Fused

Data analysis for batch 4% starch by HPLC analyzer



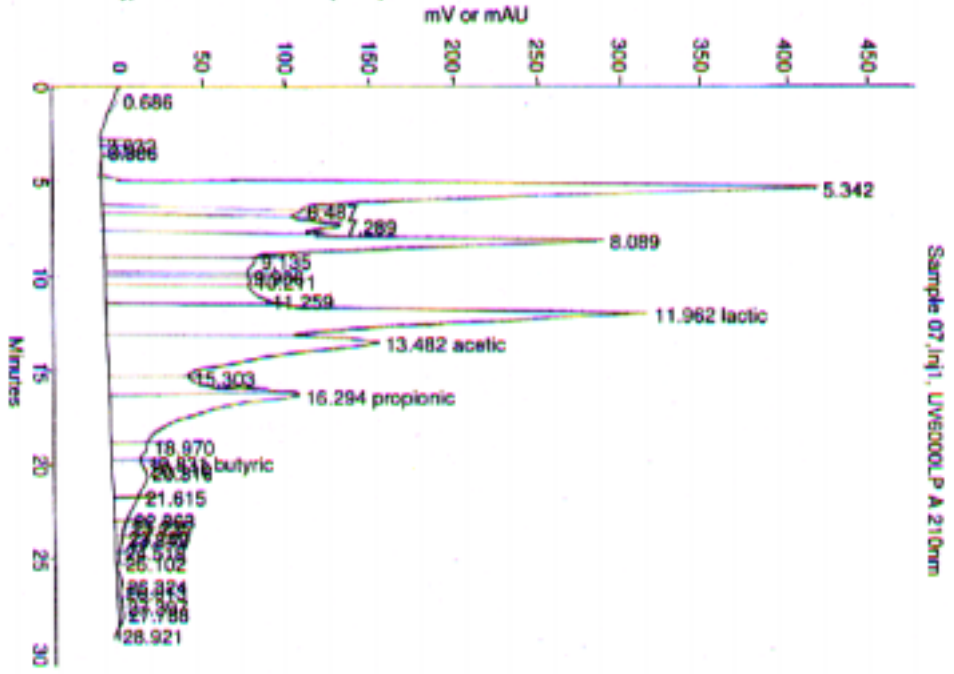
Component	RT(min)	Area	Height	%	Peak Type
Unident0001	0.675	173389	3071	0.0	Resolved
Unident0002	2.693	503	220	0.0	Fused
Unident0003	3.613	141719	2692	0.0	Fused
Unident0004	5.335	28373068	552958	0.0	Fused
Unident0005	7.272	7069909	144836	0.0	Fused
Unident0006	8.069	10570978	197012	0.0	Fused
Unident0007	9.155	1537475	91708	0.0	Fused
Unident0008	9.504	2491634	92021	0.0	Fused
Unident0009	9.938	3612440	92083	0.0	Fused
lactic	11.940	30989662	396985	1.6504	Fused
acetic	13.733	7782631	100521	0.42377	Fused
propionic	16.256	12682958	122422	0.71068	Fused
Unident0013	18.610	2328898	30638	0.0	Fused
butyric	20.293	3356567	26681	0.1825	Fused
Unident0015	22.673	471195	10047	0.0	Fused
Unident0016	23.763	69338	5514	0.0	Fused
Unident0017	24.066	138685	4562	0.0	Fused
Unident0018	24.653	34440	2596	0.0	Fused
Unident0019	25.248	93570	2491	0.0	Fused
Unident0020	26.177	183657	3183	0.0	Fused

Data analysis for batch 5% starch by HPLC analyzer

Name: Sample 07 Vial: A12 Injection: 1 of 1
Type: Sample Injected On: 07-13-01 01:36:55
Injection Volume: 10.0 µL

Acquisition Log
Column Pressure: N/A Column Temperature (C): N/A Pump Flow Stability: N/A
Noise (microAU): 3e+02 Drift (microAU/min): -2e+03
Run-Time Messages: None

Signal 1: UV6000LP A 210 nm
Calculation Type: External Standard (Area)



Component	RT(min)	Area	Height	%	Peak Type
Unident0001	0.686	162694	2874	0.0	Resolved
Unident0002	3.032	2395	244	0.0	Fused
Unident0003	3.386	15075	717	0.0	Fused
Unident0004	5.342	22259880	432932	0.0	Fused
Unident0005	6.487	2595753	122190	0.0	Fused
Unident0006	7.289	6827726	145199	0.0	Fused
Unident0007	8.089	14084690	302768	0.0	Fused
Unident0008	9.135	4587087	93225	0.0	Fused
Unident0009	9.966	809130	88302	0.0	Fused
Unident0010	10.211	2405379	89065	0.0	Fused
Unident0011	11.259	5295356	98635	0.0	Fused
lactic	11.962	19630147	327942	1.0454	Fused
acetic	13.482	13775812	165044	0.75011	Fused
Unident0014	15.303	276413	49534	0.0	Fused
propionic	16.294	11984319	115715	0.67154	Fused
Unident0016	18.970	1172378	22950	0.0	Fused
butyric	19.831	214969	18831	0.011688	Fused
Unident0018	20.110	301322	19962	0.0	Fused
Unident0019	20.316	1713327	21277	0.0	Fused
Unident0020	21.615	890428	16358	0.0	Fused

Data analysis for batch 1% starch (tapioca) by HPLC analyzer

Bibliography

Miss Orawan Cheunkum was borned on October 26 1974 in Bangkok. She graduated high school from Horwang School. She received her Bachelor's Degree in Environmental Engineering from Suranaree University of Technology (SUT), Nakhorn Ratchasima in 1996 and started Master's Degree Program in the same field and Campus since 1998.