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PRODUCTION OF ORGANIC ACID FROM LOCAL RAW MATERIALS

(PENGHASILAN ASID ORGANIK DARI BAHAN MENTAH TEMPATAN)

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ABSTRACT

PRODUCTION OF ORGANIC ACID FROM LOCAL RAW MATERIALS

(Keywords: Organic Acid; lactic acid; Fermentation; Pineapple Wastes)

The liquid pineapple wastes from pineapple cannery industries contain mainly sucrose, glucose, fructose and other nutrients. Lactic acid, a normal organic acid, has long been of use in the pharmaceutical, chemical, cosmetic and food industry. Recently, lactic acid has been considered as an important raw material for production of biodegradable lactide polymer. The objective of this project is to study the feasibility of lactic acid production from liquid pineapple waste as a substrate using *Lactobacillus delbrueckii*. Batch fermentation of pineapple waste was studied in a 3-litre stirred fermenter (Biostat B Model). In order to know the physical and chemical properties of liquid pineapple waste, characterisation study was carried out. During the fermentation process, 0.5 ml sample was withdrawn from the fermentation broth at regular time intervals in order to measure the variation of sugar concentration, lactic acid concentration, pH and biomass with fermentation time. The microbial cells were separated by centrifugation for dry biomass determination. The supernatant was immediately frozen for further determination of the concentrations of lactic acid, glucose, fructose and sucrose. Effects of some parameters such as temperature, pH, inoculum size, substrate concentrations and nitrogen source were studied. Mathematical modelling and kinetic parameters estimation as a function of pH and inoculum size were also studied. By using the concept of material balance, an unstructured model based on a Monod type kinetic equation for cell growth, substrate utilisation and product formation were developed. During fermentation, the concentrations of the substrate, product and cell were measured. The data were used for the estimation of kinetic parameters in the differential equation for the balance of the substrate, cell and product by computer program. To obtain the best fitting, a nonlinear regression analysis combined with a Runge-Kutta method was used. The standard deviation and standard error between the measured and calculated concentrations of lactic acid are used as statistical criterion for testing the adequacy of the model. Optimisation studies were also carried out for selected parameters in the Erlenmeyer flask containing 100 ml of production medium. $CaCO_3$ (3% w/v) was added to control the pH in the shake flask fermentation. A fractional factorial central composite design (FFCCD) was used to determine the optimum values of the process variables such as temperature, speed, concentration of yeast extract, concentration of substrate and the time by the response surface methodology (RSM) for obtaining the maximum yield of lactic acid. The result of the second order response surface model fitting was tested for adequacy by the analysis of variance. The optimal values of tested variables for maximal lactic acid production were found to be: temperature 40°C, speed 50 rpm, yeast extract 10 g/l, sugar concentration 52.5 g/l and time 7

days. A techno-economic evaluation of the process understudy showed that the utilisation of pineapple waste as substrate for lactic acid production using lactobacillus delbruekii is feasible process even though its profitability is lower than molasses and wheat flour. This is due to the fact that the yield obtained from this study was only 79 % compared to industrial yield is about 97 %. Therefore, increasing the yield through continuous or fed-batch fermentation couple with immobilised cells could enhance the profitability of the process.

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ABSTRAK

PENGHASILAN ASID ORGANIK DARI BAHAN MENTAH TEMPATAN

(Kata Kunci: Asid Organik; Asid Laktik; Fermentasi; Sisa Nanas)

Bahan buangan cecair daripada industri pengetinan nenas mengandungi bahan berguna seperti sukrosa, glukosa, fruktosa dan nutrien lain. Asid laktik telah lama digunakan dalam industri farmasi, kimia, kosmetik, plastik dan makanan. Baru-baru ini, asid laktik telah diambilkira sebagai suatu bahan mentah yang penting untuk penghasilan polimer laktida yang terbiodegradasi. Oleh itu objektif projek ini adalah untuk mengkaji kemungkinan pengeluaran asid laktik daripada buangan cecair nenas sebagai suatu substrak penapaian dengan menggunakan *Lactobacillus delbrueckii*. Penapain kelompok dikaji menggunakan penapai berpengaduk (Biostat B Model) 3-liter. Demi mengetahui sifat-sifat fizikal dan kimia bagi buangan cecair nenas, kajian pencirian telah dilakukan. Semasa proses penapaian, 0.5 ml sampel dikeluarkan dalam selang masa tertentu untuk mengukur perbezaan kepekatan gula, kepekatan asid laktik, pH dan biojisim dengan masa penapaian. Sel-sel mikrob dipisahkan dengan daya emparan untuk menentukan biojisim yang kering. Supernatant itu dibekukan dengan segera untuk penentuan kepekatan asid laktik, glukosa, fruktosa dan sukrosa selanjutnya. Kesan-kesan sesetengah parameter seperti suhu, pH, saiz inokulum, kepekatn substrak dan sumber nitrogen telah dikaji. Pengmodelan matematik dan penganggaran parameter kinetik sebagai fungsi terhadap pH dan saiz inokulum juga dikaji. Dengan menggunakan konsep imbangan bahan, suatu model tak berstruktur berdasarkan persamaan kinetik jenis Monod bagi pertumbuhan sel, penggunaan substrak dan pembentukan produk telah dibangunkan. Semasa penapaian, kepekatan substrak, produk dan sel diukur. Data-data ini digunakan untuk penganggaran parameter kinetik dalam persamaan pembezaan demi keseimbangan substrak, sel dan produk dengan menggunakan program komputer. Untuk mendapatkan keputusan yang paling sepadan, gabungan analisis regresi tidak linear denga kaedah Runge-Kutta telah digunakan. *Standard deviation* dan *standard error* antara kepekatan asid laktik yang diukur dan yang dikira telah digunakan sebagai kriteria statistik untuk menguji ketepatan model ini. Kajian pengoptimuman juga dilaksanakan bagi parameter-parameter terpilih dalam kelalang *Erlenmeyer* yang mengandungi 100 ml media pengeluaran. $CaCO_3$ (3% w/v) telah ditambah untuk mengawal pH dalam penapaian kelalang bergoncang tadi. Suatu rekabentuk komposit pusat faktor pecahan (FFCCD) telah digunakan untuk menentukan nilai optimum bagi pembolehubah proses seperti suhu, kelajuan, kepekatan ekstrak yis, kepekatan substrak dan masa dengan menggunakan metodologi permukaan balasan (RSM) untuk mendapatkan penghasilan asid laktik maksimum. Keputusan model permukaan balasan tertib kedua diuji untuk kesepadanannya dengan analysis ketaksamaan. Nilai-nilai optimum untuk pembolehubah yang diuji bagi penghasilan

asid laktik maksimum ialah: suhu 40°C, kelajuan 50 rpm, ekstrak yis 10g/l, kepekatan gula 52.5 g/l dan masa 7 hari. Sau penilaian tekno-ekonomik terhadap proses yang dikaji menunjukkkan bahawa penggunaan sisa nanas sebagai substrak bagi proses penghasilan asid laktik menggunakan *Lactobacillus delbrueckii* adalah merupakan proses yang berjaya walaupun keberuntunganya adalah lebih rendah berbanding dengan molases dan tepung gandung. Ini disebabkan oleh kerana *yield* yang diperolehi hanya 79 % berbanding dengan *industrial yield* adalah 97%. Oleh itu dengan meningkatkan *yield* melalui proses fementasi *continuous* atau *fed-batch* dan dengan menggunakan sel tersekatgerak boleh mempertingkatkan keberuntungan proses.

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LIST OF ABBREVIATIONS

ADP	Adenosine-5-diphosphate
ATCC	American type culture collection, Rockville, Maryland, USA
ATP	Adenosine-5-triphosphate
DNS	Dinitrosalicilic acid
DSMZ	Deutcdche samlung von organismen und zelkulturen
FELCRA	Federal land consolidation and rehabilitation authority
FFCCD	Fractional factorial central composite design
Fru	Fructose
Gal	Gallactose
Glu	Glucose
HPLC	High performance liquid chromatography
IFO	Institute for fermentation, Osaka, Japan
KPUM	Kementerian perusahaan utama Malaysia
LAB	Lactic acid bacteria
Lac	Lactose
LSF	Liquid substrate fermentation
Mal	Maltose
Man	Mannose
MPIM	Ministry of primary industries Malaysia
MRS	De Man, Rogosa and Sharpe
NAD^+	Nicotinamide adenine dinucleotide (oxidised state)
NADH	Nicotinamide adenine dinucleotide (reduced state)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised state)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced state)
NRIC	Nodai research institute culture, University of agriculture, Tokyo, Japan
NRLL	Northern regional research laboratory, US department agriculture, Peoria, Illinois, USA
RI	Refractive index

RNA	Ribonucleic acid
RSM	Response surface methodology
SSF	Solid state fermentation
Suc	Sucrose
USP	United State pharmaceutical
UV	Ultra violet

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CHAPTER I

INTRODUCTION

1.1   Background

The canned pineapple industry in Malaysia is the oldest agro based export oriented industry dating back to 1888. Although pineapple can be grown all over the country, the planting of pineapple for canning purpose is presently confined to the peat soil area located only in Johor which is the major producer of canned pineapple in Malaysia. In 1997, the production of canned pineapple was estimated at about 48 million standard cases as against 41 million standard case tonnes in 1996, an increase of almost 16%. Major world producers of canned pineapple are Thailand (39%), Philippines (23%), Indonesia (13%) and Kenya (8%) which altogether contribute to more than 80% of total world canned pineapple production in 1997. Malaysia's production amounting to 1,563,291 standard cases would be equivalent to 3.3% of total world production (MPIM, 1998).

The growing, harvesting and canning of pineapple are a marvel of a modern science and technology. Specialised plant breeding, planting, fertilising and cultivating techniques are employed in raising the crop to the uniform quality and maturity. The fruit is harvested fully ripe and is canned within a few hours. Upon receipt at the cannery the fruit is graded according to size and fed into the inventor machine in which the cell and ends are removed. The fruit is cut into cylinder to fit the selected can size and the appropriate diameter core is removed. The cylinders are then cut into slices of uniform thickness. Broken slices may be cut into titbits or

other small pieces. Trimmings from the various operations are used to make crushed pineapple or juice. Refined juice may be used as the liquid packing medium for slices or other pieces. In general, the stages of pineapple canning include grading and sorting, peeling and coring, slicing and canning (Jackson and Shinnery, 1979). These processes especially peeling and coring of the fruits will produce unwanted materials which are discarded while primary fruits are used for further processing.

Food processing operation consumes enormous quantities of water which are consequently discharged as polluted effluent. These wastes contain high concentration of biodegradable organic materials and suspended solids. As a result it has a high BOD and extremes of pH conditions (Buckle, 1989). The solid waste from pineapple canning process was estimated about 40 - 50 % from fresh fruit as pineapple peals and cores (Bardiya et al., 1996; Moon and Woodroof, 1986; Viswanath, 1992). Bisaria (1991), Kosaric and Velayudhan (1991) have reported that the wastes from agricultural industries contain cellulose and sugar which can be potentially processed to food, fuel, feed and variety of chemicals.

The wastes generated by pineapple canning industries that are located in tropical region such as Malaysia, Thailand and Indonesia produce large quantity of solid and liquid waste. In the world, it was estimated that more than 1,651,672 tonnes of pineapple waste are generated by canning industries, which are respectively 50% liquid waste and 50% solid waste each year (Sasaki et al., 1991). If these wastes discharge to the environment untreated, they could cause serious environmental problems. Beside their pollution and hazard aspects in many cases, food processing waste such as pineapple waste might have a potential for recycling to get valuable raw material or for conversion into useful and of higher value added products, or even as raw material for other industries, or for use as food or feed after biological treatment (Kroyer, 1991).

This waste contains valuable components which are mainly sucrose, glucose, fructose and other nutrients (Sasaki et al., 1991). An attempt has been made by many researchers to utilise the waste for producing high value added chemicals such as single cell protein (SCP), ethanol, acetic acid, oxalic acid and methane (Sasaki et al.,

1991; Vimal and Adsole, 1976; Bardiya et al., 1996). So far, there are no studies reported on the application of pineapple waste for lactic acid production.

Lactic acid is the one of the most widely used organic acids in the food industry and is a very common substrate for chemical synthesis. Recently, there has been increased interest in lactate because it can be used as raw material for production of biodegradable polymers with application in food industries for packaging as well as in medical and pharmaceutical fields (Wang et al., 1995; Payot et al., 1999).

The world production of lactic acid is approximately 50,000 tonnes per year and the commercial price of lactic acid (1999) ranges from US$1.40/kg for 50% to US$1.90/kg for 88% food grade lactic acid. The fermentative production of lactic acid is interesting due to the prospect of using cheap polysaccharide raw materials such as starch or cellulose. However, the production of lactic acid, using this process, suffers from a number of drawbacks. The raw material must be exposed to some form of pre-treatment before fermentation to produce a suitable fermentation medium (Akerberg and Zacchi, 2000).

Lactic acid is generally produced from glucose, maltose, sucrose or lactose. Starches especially those from corn and potatoes are hydrolysed by enzymes or acid to maltose and glucose before they are used for lactic acid fermentation (Atkinson and Mavituna, 1991; Blanch and Clark, 1997). Lactic acid can be produced by microbial fermentation or by chemical synthesis but in recent years fermentation process has become more industrially successful because of the increasing demand for naturally produced lactic acid. So far, the lactic acid is produced by fermentation process using whey permeate by *Lactobacillus helviticus* (Kulozik and Wilde, 1999; Fu and Mathews, 1999) and molasses by *Lactobacillus delbrueckii* (Payot et al., 1999; Monteagudo et al., 1994) as a carbon source.

1.2   Objectives And Scopes

The physical and chemical characteristics of pineapple waste produced from canning process will vary according to the process as well as the areas and seasons for pineapple plantation. Therefore, the characterisation of the waste is important to be carried out in order to determine the physical and chemical composition such as sugar content which influences the fermentation process. Hence, the first objective of this study is the determination of sugar content such as glucose, fructose and sucrose along with soluble protein and total nitrogen. Acidity of the organic acids such as citric acid and malic acid, macro elements such as phosphorus, sulphates, magnesium and potassium, and micro elements such as sodium, calcium, chlorides, ferro, mangan and zinc as well as pH will also be investigated in this study.

The successful implementation of fermentation processes depends on the effect of environmental parameters on cell growth and product formation. Since the lactic acid fermentation depends on various parameters such as temperature, pH, concentration of nutrient, nitrogen source and inoculum size, the second objective is to study the effect of controlled and uncontrolled pH, temperature, initial sugar concentration, inoculum size and types of nitrogen source on microbial growth, sugar utilisation and lactic acid production.

The third objective of this study is the mathematical modelling and the estimation of kinetic parameters for microbial growth, sugar utilisation and lactic acid production. This is important since knowledge of the kinetics of fermentation process is necessary in order to size the fermentor and its associated equipment, and this information is normally obtained from laboratory experimentation with one- to three- litres fermentors. In batch fermentation, the kinetic model provides information to predict the rate of cell mass or product formation. A process might be developed on a trial and error basis but that is an extremely costly approach both in terms of time and equipment. A more profitable approach is to use mathematical modelling for the process. Then one can examine the consequences of changing parameters without the expense of running costly experiment.

The fermentation process normally takes several weeks and therefore it is required to solve some related problems such as time-consuming process and high

cost of production. Many variables may potentially affect the productivity of lactic acid, so the sample size has to be minimised in order to save time and money. This problem can be solved by reducing variables through the identification of several important factors, the optimisation process and the confirmation of the result. There are no theoretical models which can be used to explain process performance. Consequently, successful research is characterised by effective empirical problem solving. Statistical problem solving provides a set of powerful tools which can be used to maximise the efficiency and productivity. Development of mathematical models can be used to predict the lactic acid yields as a function of factors. This mathematical model provides a prediction of the best setting of factors which should produce the optimum value of lactic acid. Therefore the fourth objective of this study is the optimisation of some parameters by using fractional factorial central composite design with response surface methodology.

The lactic acid fermentation was carried out at shake flask and small fermentor with working volume of 100 ml and 1000 ml respectively. The process is to be conducted on large scale to produce commercial quantities of lactic acid. Therefore the fifth objective of this study is fermentation scale-up from 100 ml to 5 litres at optimised fermentation conditions such as initial sugar concentration, concentration of yeast extract, time of fermentation, temperature and stirring speed. Last but not least, the sixth objective of this study is a techno-economic evaluation of lactic acid production from liquid pineapple waste.

1.3   Report Outline

Respective chapters of this report can be broadly identified with one of the objectives outline in Section 1.2. The report contains eight chapters in which each chapter is an entity containing its own introduction and descriptions to achieve the respective objective of each chapter and the report as a whole.

Chapter I describes the research background, objectives and scopes of the work, and the report outline. This will be followed by Chapter II which describes the historical aspects, world pineapple production, nutritive aspect of pineapple, characteristics and utilisation of solid and liquid pineapple waste, lactic acid industry, physical and chemical properties, lactic acid application, biochemistry of sugar metabolism, selection of micro-organism, conditions and modes of lactic acid fermentation. This chapter also describes a theoretical background of modelling and optimisation of lactic acid fermentation.

In order to achieve the objectives discussed in Section 1.2, the materials and the experimental as well as analytical methods are presented in Chapter III. This chapter includes some materials that are used for lactic acid production, experimental methods such as pineapple waste treatment, extraction of sugar from solid waste, bath and fed-batch fermentation. The analytical methods are also presented in this chapter which contains the determination of physical and chemical properties of pineapple waste such as the measurement of pH, sugar, protein, organic acid, cations, anions and product analysis such as biomass, sugar, lactic acid and other organic acids.

The findings of the parameteric study are presented in Chapter IV. This chapter comprises several characteristics of pineapple waste, and also effects of parameters understudied such as inoculum size, temperature, controlled and uncontrolled pH, concentration of initial sugar and type of nitrogen source in fermentation system followed by fermentation optimisation using response surface methodology. This chapter presents also results of comparative study between the pineapple waste as substate with pure sugar and mixed sugar, as well as batch fermentation with fed-batch fermentation.

In order to simulate the results presented in Chapter IV, the mathematical model, parameters kinetic estimation and sensitivity analysis are described in Chapter V followed by fermentation optimisation using response surface methodology and fermentation scale-up which will be presented in Chapter VI. A detailed techno-economic evaluation of the pineapple waste utilization for lactic acid

production will be presented in Chapter VII. Last but not least, Chapter VIII gives a summary of this study and recommendations for future research.

CHAPTER II

LITERATURE REVIEW

2.1   Pineapple Industry

2.1.1   Historical Aspects

The pineapple (***Ananas comusus***) is a native of South America from which is now also cultivated in other areas and becomes an important fruit of the tropic regions. It is a multiple fruit collection of small flowers and fused ovaries with clusters of flowers on a single stalk coming out from the base of the flower stalk. It is available fresh, canned and dried, and is also used to produce juice. It may also be an ingredient of fruit salads in cans (Arthey, 1995).

Wild species of pineapple still grow in the dry uplands of Brazil and Paraguay and it is there that the cultivated pineapple probably arose, although it cannot be found in its present form wild today. Following the discovery of America, the Spanish and Portuguese very quickly took the pineapples they found growing there and introduced them to other parts of the tropics such as Southeast Asia and Africa (Shewfelt, 1986).

Towards the ends of the eighteenth and early nineteenth, pineapples in great variety were grown in English greenhouse. During the latter century large-scale field production started and rapidly increased in Hawaii, Queensland, South Africa, The Azores, Malaysia and Florida. The development of canning process from the middle of the century onwards greatly stimulated production of pineapple and its related product. Pineapples are now also grown on a large scale in Ceylon, Madagascar,

Philippines, tropical Central America and both the East and West Indies (Swabey, 1975).

2.1.2   World Pineapple Production

World canned pineapple and pineapple juice production in 1998 was estimated at 62.5 million standard cases equivalent to 1.25 million tonnes, a decrease of 5.3% compared to 1997 (66 million standard cases). Major world producers of canned pineapple in 1998 are Thailand (39%), Philippines (23%), Indonesia (14%) and Kenya (8%) which all together contribute to more than 80% of total world canned pineapple production. Malaysia's production was estimated in 1998 about 1,563,291 standard cases which would be equivalent to 2.2% of total world production (MPIM, 1999).

In 1998, world export of canned pineapple stood at 49.7 million standard cases as shown in Figure 2.1. Thailand, the world's exporter, exported about 19 million standard cases or 38.2% of the total world export, followed by the Philippines (11.3 million standard cases), Indonesia (7 million standard cases), Kenya (4.1million standard cases) and South Africa (1.7million standard cases). While these countries registered an increase in their exports over the previous year, Malaysia which exported 1.2 million standard cases in 1998, recorded a decrease of 26.6% compared to 1997. The world's major canned pineapple exporter in 1998 is given in Figure 2.2.
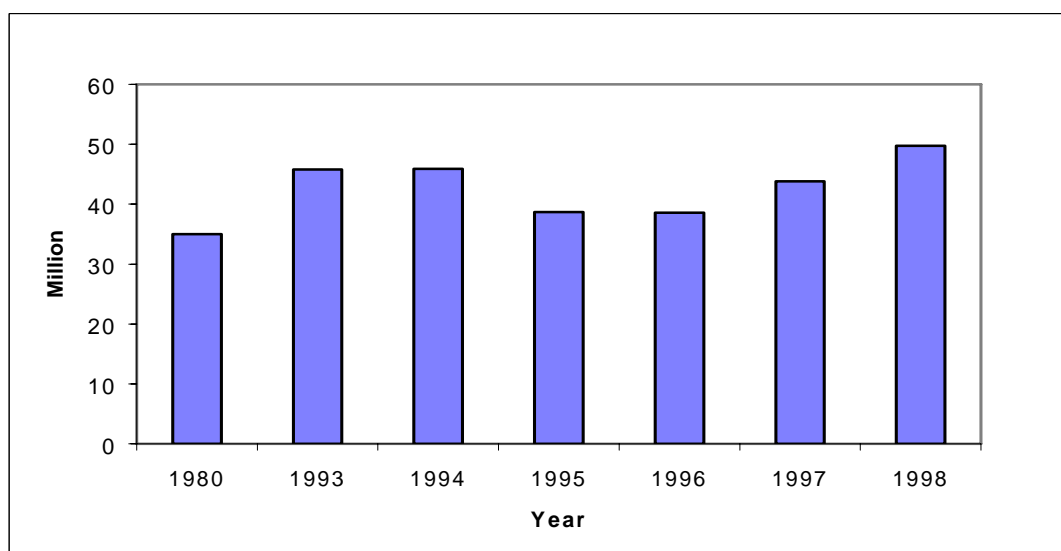
*One standard case contains 24 cans (weighing 20 ounces each).

Figure 2.1: World canned pineapple and juice export by major countries (MPIM, 1999).
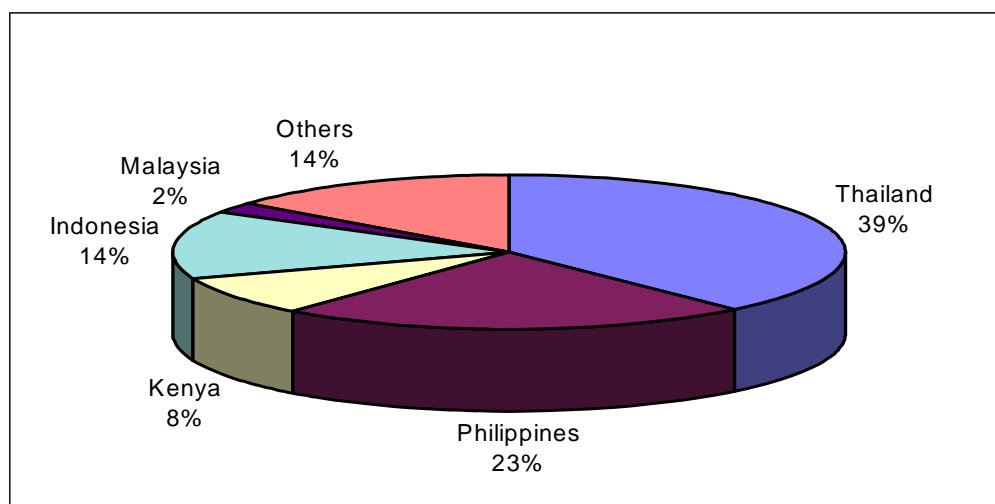
Figure 2.2: Major world producers of canned pineapple in 1998 (MPIM, 1999).

2.1.3   Pineapple Industries In Malaysia

In view of the good market opportunities for canned pineapple in the world, there is prospect for Malaysia to step up its pineapple production. The abolition of the Japanese global quota with effect from April, 1990 will give added impetus. Likewise, the industry will have to take the necessary steps to increase production and export of canned pineapple to compete in the growing world market. The structure of the pineapple planting will be further improved whereby estate planting will be extended and encouraged to achieve higher production yield as well as greater competitiveness. In the small grower sector, FELCRA will organise farmers to get smallholder production on a more organised basis. With the production of better quality fruits, recovery in processing will improve which will contribute towards improving Malaysia's competitiveness in the world market (KPUM, 1990).

In 1989, Malaysia's production of fresh fruit for canning amounted to 179,600 tonnes, a decrease of 6% from 168,300 tonnes produced in 1990. The production had been on the declining trend since 1992. The total production declined by 23.2% from 119,825 tonnes in 1997 to 92,035 tonnes in 1998. The production for the period 1989-1998 is shown in Figure 2.3. A drastic reduction of 18.7% was recorded in smallholder's production from 9,216 tonnes in 1997 to 7,495 tonnes in 1998. This was due to the shortage of planting materials, higher labour cost, poor farm practices and conversion to other crops (MPIM, 1999). The production trend of the canned pineapple and juice production for 1989-1998 is also given in Figure 2.3. The canning sector is very much dependent upon the availability of fresh fruits. Hence, in line with the decline in fruit production, the production of canned and pineapple juice had dropped by 9.7% from 42,871 tonnes in 1995 to 38.702 tonnes in 1996. It further declines by 10.4% to 34,660 tonnes in 1997 compared with the previous year. The production of canned pineapple decreased by 29.1% from 34,660 tonnes in 1997 to 27,629 tonnes in 1998. On the other hand, the production of pineapple juice increased by 62.1% from 3351 tonnes in 1997 to 5431 tonnes in 1998 (MPIM, 1999).
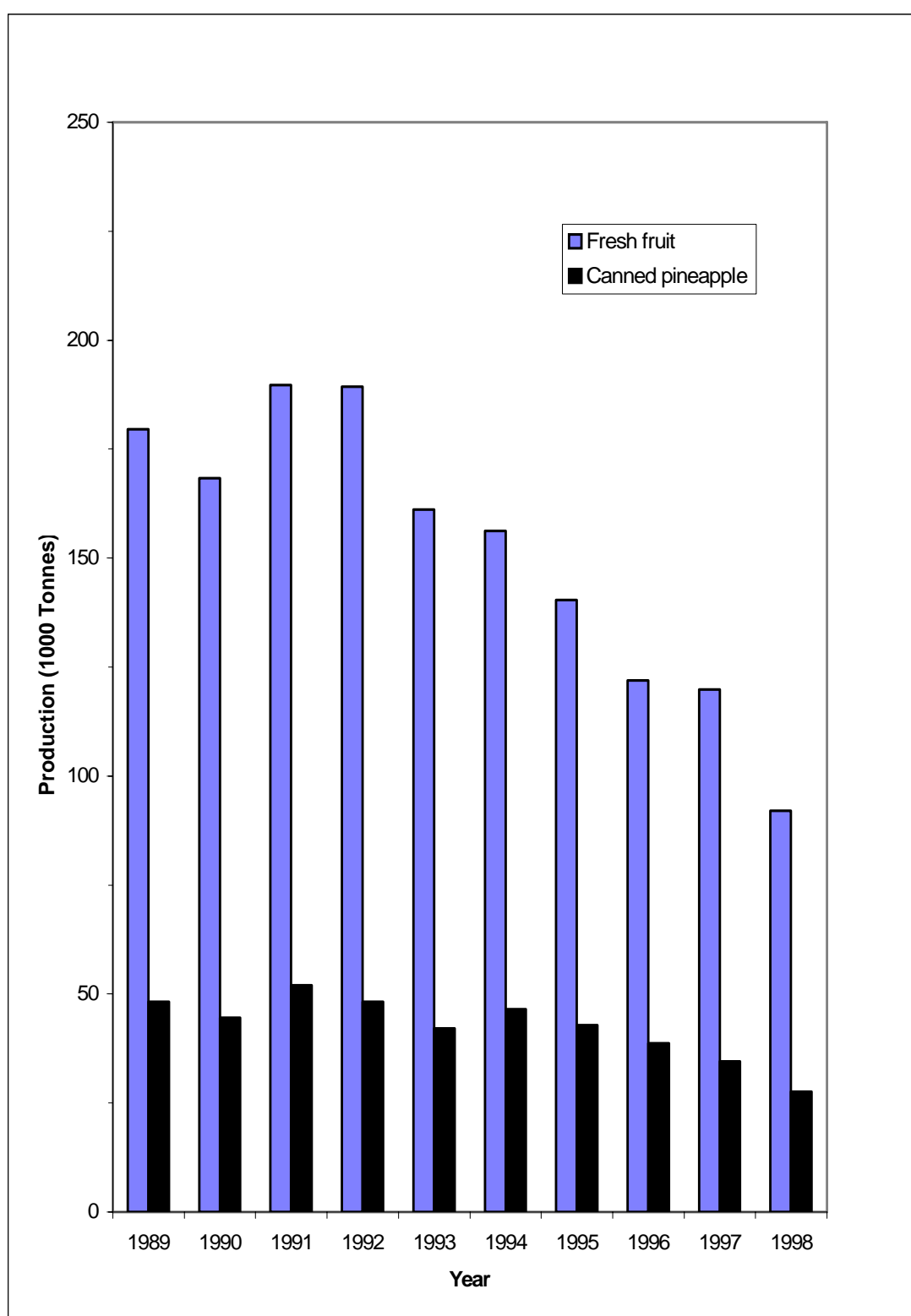
Figure 2.3: Malaysian production of fresh fruit and export canned pineapple and juice from 1989-1998 (MPIM, 1999).

2.1.4   Nutritive Aspects Of Pineapple

The edible portion of most type of fruit contains 75-95% water. Fruits are low in protein but in general, contain substantial carbohydrates. The latter may include various proportions of glucose, fructose, sucrose and starch according to the type of fruit and its maturity. The main acids, in fruits are citric, tartaric and malic acids. The total acidity often decreases during repining and storage. The pH of fruits is usually from to 2.5-to 4.5. Other constituents of fruits include cellulose and woody fibres, mineral salts, pectin, gums, tannins, pigments and volatile constituents. From the nutritional point of view, fruits also have the valuable sources of vitamins A and C (Young and How, 1986). Moon and Woodroof (1986) reported that the pH of food products categorised as, high acid foods (< 3.7), acid foods (3.7-4.6), medium acid foods (4.6-5.3) and low or non acid foods (> 5.3). The pH of food products can change during ripening, processing and storage.

As in other fruits of this group, sucrose is the major sugar present in pineapples. Citric acid is the predominant acid with malic and oxalic acids are also present. A volatile flavour component unique to pineapple is 2,5 dimethyl-4-hydroxy-2,3-dihydro-3-furanon, ethyl-3-methylthiopropionate, ethyl butirate and ethyl hexanoate. Acetic acid, furfural, formaldehyde, acetal dehyde, acetone and the ethyl, isobutyl, methyl, and propyl esters of acetic acid and formic acid are the major volatile constituents containing in canned pineapple juice (Shewfelt, 1986).

Krueger et al. (1992) reported that major constituents of fresh pineapple juice are glucose, fructose, sucrose, citric acid, malic acid and mineral potassium. The dominant sugar is sucrose, the glucose and fructose levels are similar to each other with fructose is slightly higher than glucose. The composition of sugar depends of geographical origins and varying degrees of ripeness. The composition of fresh pineapple and juice are given in Tables 2.1 and 2.2.

Table 2.1: The composition and nutritive value of pineapple (Young and How, 1986).

Composition	Pineapple (100 g)	
	Raw	Juice (canned)
Water (g)	86.5	85.5
Food energy (kcal)	49.0	56.0
Protein (g)	0.39	0.32
Total lipid (g)	0.43	0.08
Total carbohydrate (g)	12.4	13.8
Fibre (g)	0.54	0.10
Ash (g)	0.29	0.30
Calcium (mg	7.00	17.00
Iron (mg)	0.37	0.26
Magnesium (mg)	14.0	13.0
Phosphorus (mg)	7.00	8.00
Potassium (mg)	113	134
Sodium (mg)	1.00	1.00
Zinc (mg)	0.08	0.11
Copper (mg)	0.11	0.09
Manganese (mg)	1.65	0.99
Ascorbic acid (mg)	15.4	10.7
Thiamine (mg)	0.09	0.06
Riboflavin (mg)	0.04	0.02
Niacin (mg)	0.42	0.26
Panthothenic acid (mg)	0.16	0.10
Vitamin B_6 (mg)	0.09	0.10
Folacin (µg)	10.6	23.1
Vitamin A (IU)	23.0	5.00

Table 2.2: The composition of pineapple juice (Krueger et al., 1992).

Composition	Quantity (g/l)
Soluble solid	112-162
Acidity (as citric acid)	4.60-12.10
Fructose	17.20-47.50
Glucose	12.10-45.20
Sucrose	24.70-97.30
Citric acid	4.39-11.51
Malic acid	0.73-3.91
Isocitric acid	0.80-2.65
Potassium	8.30-14.10

2.2   Pineapple Waste

2.2.1   Pineapple Canning Industry

When the fresh pineapple fruits arrive in the canning factory, the fruits will be graded into several sizes according to the fruit diameter. Then they will be peeled, core removed, sliced, sorted and canned. All the peeled skin, unwanted fruits and the core will be sent to the crush machine for crushing. After crushing, the solid waste will be sent to cattle feeding while the liquid waste is send to the storage for fermentation process (Koshy, 1990). Figure 2.4 shows the pineapple canning process.

```
          ┌──────────┐
          │  FRESH   │
          │  FRUIT   │
          └────┬─────┘
               │
               ▼
          ┌──────────┐
          │ GRADING  │
          │    &     │
          │ SORTING  │
          └────┬─────┘
               │
               ▼
     ┌──────────┐         ┌──────────┐        ┌──────────┐
     │ PEELING  │────────▶│ SLICING  │───────▶│ CANNING  │
     │    &     │         └────┬─────┘        └──────────┘
     │ CORING   │              │
     └────┬─────┘              ▼
          │             ┌────────────┐
          │             │ UNDERGRADED│
          │             │   SLICE    │
          │             └─────┬──────┘
          │                   │
          │                   ▼
          ▼             ┌────────────┐
     ┌──────────┐◀──────│   JUICE    │
     │  WASTE   │       │ PRODUCTION │
     └────┬─────┘       └────────────┘
          │
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     │  CRUSH   │
     └────┬─────┘
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     ┌──────────┐
     │  PRESS   │
     └──┬────┬──┘
        │    │
        ▼    ▼
  ┌────────┐ ┌────────┐
  │ LIQUID │ │ SOLID  │
  │ WASTE  │ │ WASTE  │
  └────────┘ └────────┘
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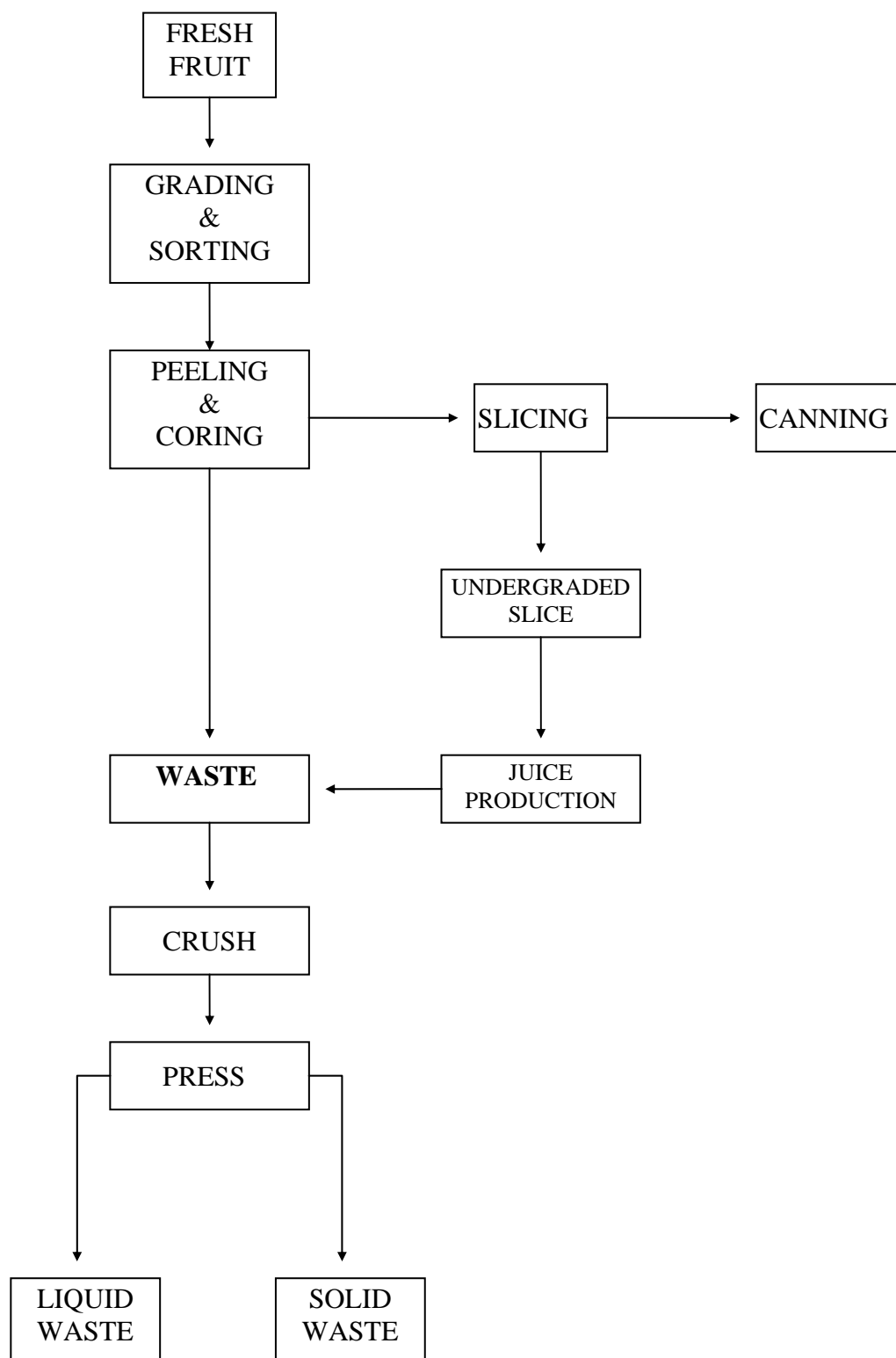
Figure 2.4: The pineapple canning process (Jackson and Shinnery, 1979).

2.2.2   Pineapple Waste Characteristics

        The wastes generated by fruits processing are primarily solid in the forms of peels, stems, pits, culls and organic matter in suspension. The first stage in the optimisation of waste reduction is to identify and characterise the wastes (solid and liquid) produced. Each particular food industry generates specific type and amount of wastes. For example, the fruit and vegetables industry generates much more solid waste than the dairy industry. The characteristics of the waste load of various fruit processing industries indicate the problem of suspended organic matter in the waste water (Moon and Woodroof, 1986). The comparison between pineapple waste to other fruit processing wastes is given in Table 2.3. The solid waste from pineapple processing was about 45% from fresh fruit, followed by citrus, apple, pear, peach and cherry were 43, 32, 30, 24, 17, and 14 % respectively. For pineapple processing, the suspended and organic matter in the waste water is higher than other fruits processing. It can be indicated by the BOD and suspended solid contained in the rinse water which are 4.8 kg/m^3 and 2.4 kg/m^3 respectively.

Table 2.3: The comparison between pineapple waste to other fruit processing wastes (Moon and Woodroof, 1986).

Fruit	Raw (tonnes)	Waste water (m^3)	BOD (kg/m^3)	Suspended solid (kg/m^3)	Solid residual (tonnes)
Apple	1,000,000	18,920,000	0.95	0.11	320,000
Apricot	120, 000	2,270,000	1.39	0.20	21,000
Cherry	190,000	1,130,000	1.60	0.40	27,000
Citrus	7,800,000	87,050,000	0.16	0.28	3,390,000
Peach	1,100,000	16,650,000	1.79	0.29	270,000
Pear	400,000	6,050,000	2.09	0.74	120,000
Pineapple	1,000,000	1,890,000	4.80	2.40	450,000

The characteristics of solid waste from pineapple processing are shown in Table 2.4 reported by different authors. The moisture content of solid waste was found to be at the range of 87.50 - 92.80 %. The difference of moisture content might be due to the sample obtained from various geographical origins and of varying degree of ripeness. The total nitrogen and ash content in the wastes were between 0.90-0.95 % and 3.9-10.6 %, respectively.

Table 2.4: The characteristics of solid pineapple waste reported by different authors.

Composition	Bardiya et al. (1996)	Viswanath (1992)	Chandapillai and Selvarajah (1978)
Moisture	92.80	87.69	89.70
Total solid	7.80	12.31	10.30
Ash	10.60	6.20	3.90
Organic carbon	51.85	38.9	-
Nitrogen free extract	-	-	75.10
Total carbohydrates	35.00	-	-
Ether extract	-	-	0.20
Cellulose	19.80	-	-
Crude fibre	-	-	14.70
Hemicellulose	11.70	-	-
Phosphorus		0.08	0.10
Total soluble	30.00	-	-
Total nitrogen	0.95	0.90	-
Crude protein	-	-	6.10

The characteristics of liquid waste from pineapple processing are given in Table 2.5 (Sasaki et al., 1991). The compositions vary considerably with the season, area and canning process. The waste contains mainly sucrose, glucose and fructose while dextrin, raffinose and galactose exist as minor components.

Table 2.5: The characteristics of liquid pineapple waste (Sasaki et al., 1991).

Composition	Liquid Waste	
	Before sterilisation	After sterilisation
COD (g/l)	100.8	103.7
Reducing sugar (g/l)	39.20	41.20
Total sugar (g/l)	100.0	100.9
Dextran (g/l)	1.50	1.50
Raffinose (g/l)	2.60	1.50
Sucrose (g/l)	40.1	40.1
Glucose (g/l)	23.6	23.6
Galactose (g/l)	1.70	2.10
Fructose (g/l)	14.0	15.6
Soluble protein (g/l)	0.90	-
Kjeldahl nitrogen (g/l)	0.20	-
Fe (mg/l)	5.43	-
Ca (mg/l)	3.31	-
Mn (mg/l)	13.97	-
Mg (mg/l)	62.50	-
Co (mg/l)	0.07	-
Cu (mg/l)	2.02	-
Cd (mg/l)	0.03	-
Na (mg/l)	8.61	-
SO_4^{2-} (mg/l)	169.7	-
PO_4^{3-} (mg/l)	223.8	-
pH	4.00	4.00

2.2.3   Pineapple Waste Utilisation

All of the raw materials that contain sugar can be processed to high value added products such as biomass, methane, alcohols, enzymes, antibiotics and organic acids (Kosaric and Velayudhan, 1991). The potential product from sugar waste is given in Figure 2.5.
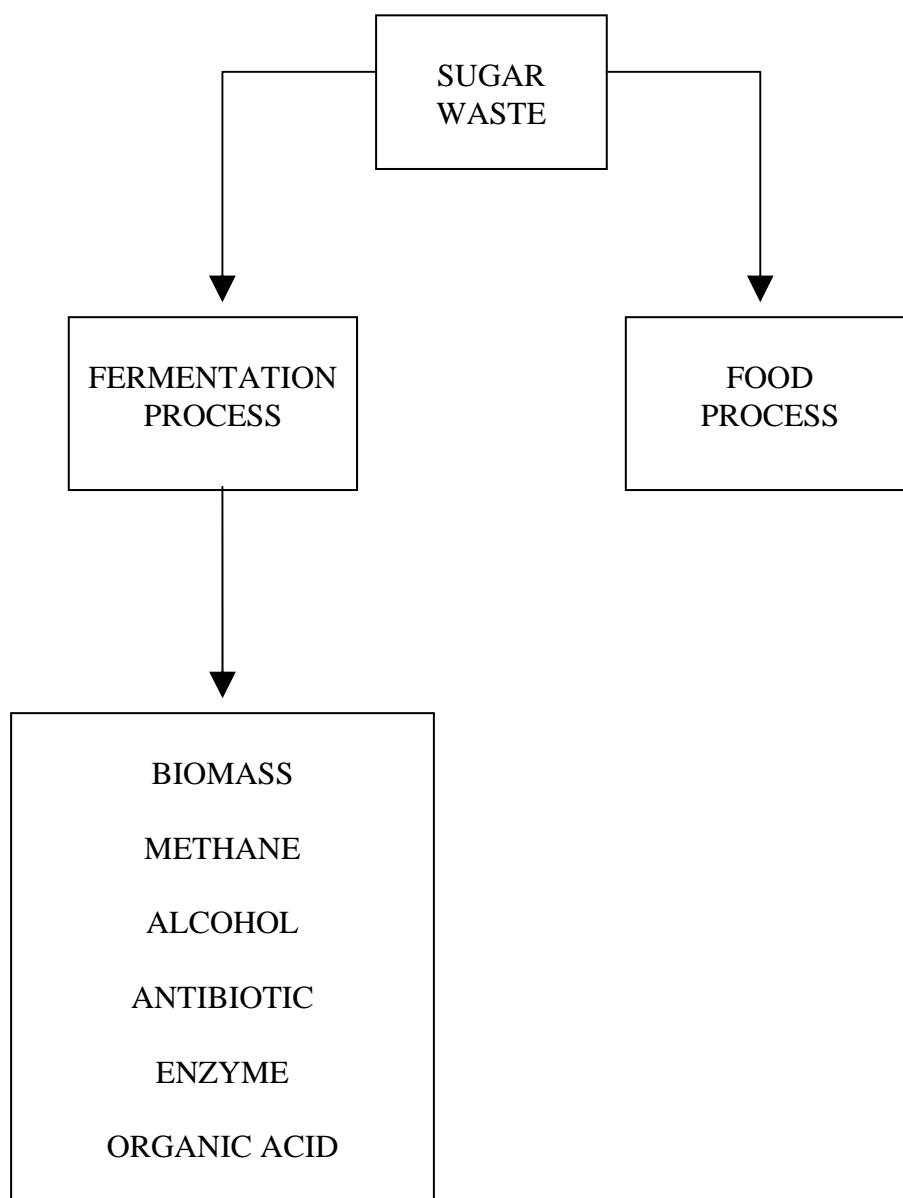
Figure 2.5: A schematic diagram of the potential products from sugar waste (Kosaric and Velayudhan, 1991).

Vimal and Adsole (1976) reported that the juice extracted from comminuted skin and cores of pineapple when mixed with an equal quantity of fresh juice, can be used for the production of acetic acid by fermentation. The residual left after juice extraction can be utilised a cattle feed after drying. Bardiya et al. (1996) studied about the biomethanation of pineapple waste in aerobic digestion. The digester volume is 1.8 litres and it could be operated at 10 days and 58 % substrate. The rate of gas production maximum is 0.93 vol/vol/day. The liquid waste from pineapple peel can also be used for the production of single cell protein by strain of *Rhodobacter Sphaeroides*. Pineapple peel juice was obtained by a press dehydration of the peel and cores followed by filtration before used. The cultivation was composed of peel filtrate supplemented with diammonium sulphate, diammonium hydrogen phosphate and some vitamins. The culture yielded 26.5 g dry cell / litre after 60 hours of cultivation with the maximum specific growth rate of 0.31 / hour and growth yield at 0.45 g cell / g sugar; COD removal 85.3 % (Sasaki et al., 1991). Pineapple waste was also employed for the production of oxalic acid by oxidation with nitric acid in the presence of vanadium pentoxide as a catalyst. Oxalic acid produced about 75-80 % yield on dry weight basis (Vimal and Adsole, 1976). Pineapple waste juice can also be used to produce citric acid using *Candida lypolytica ATCC 8661*. The maximum citric acid production obtained was 3.2 g/l under submerged fermentation after four days (Koshy, 1990). Lategan and Botes (1984) studied the production of ethanol by semi solid state fermentation of pineapple waste. Pineapple peel was macerated to 3 mm size and 100 ml water added to 800 g peel plus 100 ml yeast Inocolum. Using optimal parameters such as pH, temperature and combination of a high concentration of yeast in the inoculum and recirculating of the yeast, the yield of ethanol is approximately 4 ml ethanol per 100 g of pineapple peel.

2.3   Lactic Acid Industry

2.3.1   Historical Perspective

Lactic acid is the simplest hydroxyl acid having an asymmetric carbon atom. It occurs naturally either in the form of the dextrorotatory, L (+), levorotatory, D (-) or as a racemic of both. Lactic acid occurs widely in nature, being found in man, animals, plants and microorganisms. It was discovered in 1780 by The Swedish chemist Scheele in sour milk (Holten, 1971). Blondeau in 1847 recognised lactic acid as the final product of fermentation process (Paturau, 1982).

D-lactic acid sometimes known as sarcolactic acid, is present in muscle and can be obtained from meat extract by extraction with ether. L-lactic acid does not occur naturally, and must be obtained by the separation of optically active components of D,L-lactic acid. D,L-lactic acid is present in sour milk and can be obtained by fermentation of glucose, lactose or sucrose. The two optically active lactic acids are given in Figure 2.6 (Brown, 1957).

$$\underset{\text{D - lactic acid}}{\overset{\displaystyle COOH}{\underset{\displaystyle CH_3}{H-C-OH}}} \qquad\qquad \underset{\text{L -lactic acid}}{\overset{\displaystyle COOH}{\underset{\displaystyle CH_3}{HO-C-H}}}$$
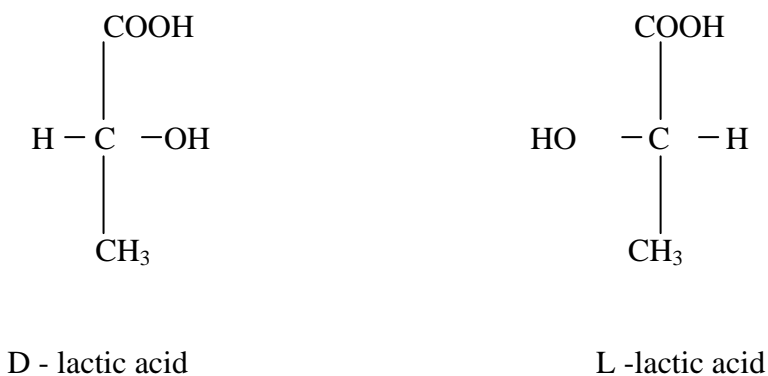
Figure 2.6: Lactic acid structure and isomers (Brown, 1957).

In animal and human cells, L-lactic acid is present only in the form of L-lactate dehydrogenate and therefore configuration of lactic acid is very important from the nutritional point of view. The intake of larger amounts of D-lactic or D, L-lactic acid can result in an enrichment of D-lactic acid in the blood and hyperacidity

of the urine may occur. These findings caused the WHO to limit human consumption of D-lactic acid to 100 mg/kg/d (Buchta, 1983). Lactic acid is possible to make lactic acid copolymer that emulates many of the thermoplastics now being used in packaging and consumer goods. The rate of biodegradation can be controlled. Thus, lactic acid could become the source of a family of environmentally friendly thermoplastics benign polymer for use in packaging and other applications that affect the quality of our life. The potential industrial products from lactic acid are shown in Figure 2.7 (Lipinsky and Sinclair, 1986).

Lactic acid was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881 (Van Ness, 1984). The first successful use in the leather and textile industries began in 1894 and the production levels were about 5.0 tonnes per year. In 1942, about half of the 2,700 tonnes per year produced in the US was used by leather industry, and an emerging use in food product. During the World War II, US production peaked at 4,100 tonnes per year (Vickroy, 1983). In 1982 worldwide production of lactic acid is 26 tonnes per year. More than 50 % of lactic acid produced is used in food as an acidulant and a preservative. Fermentation is presently used to make about half of the world's total production of lactic acid (Vickroy, 1983; Blanch and Clark, 1997).

The present annual world production has a total volume of nearly 30,000 ton, about half of this production is made using chemical synthetic, and it is rapidly becoming regarded as a commodity chemical. Compared with the increasing price of petroleum, lactic acid produced by fermentation is cheaper. This has created an increased demand for knowledge to improve the fermentation and recovery process (Van Ness, 1984; Blanch and Clark, 1997). In economic terms, the foremost parameters to be considered are price and the volume of production. The world production of lactic acid is relatively small compared to other biotechnological products and in term of price per tonne, it is relatively expensive (Hacking, 1989).
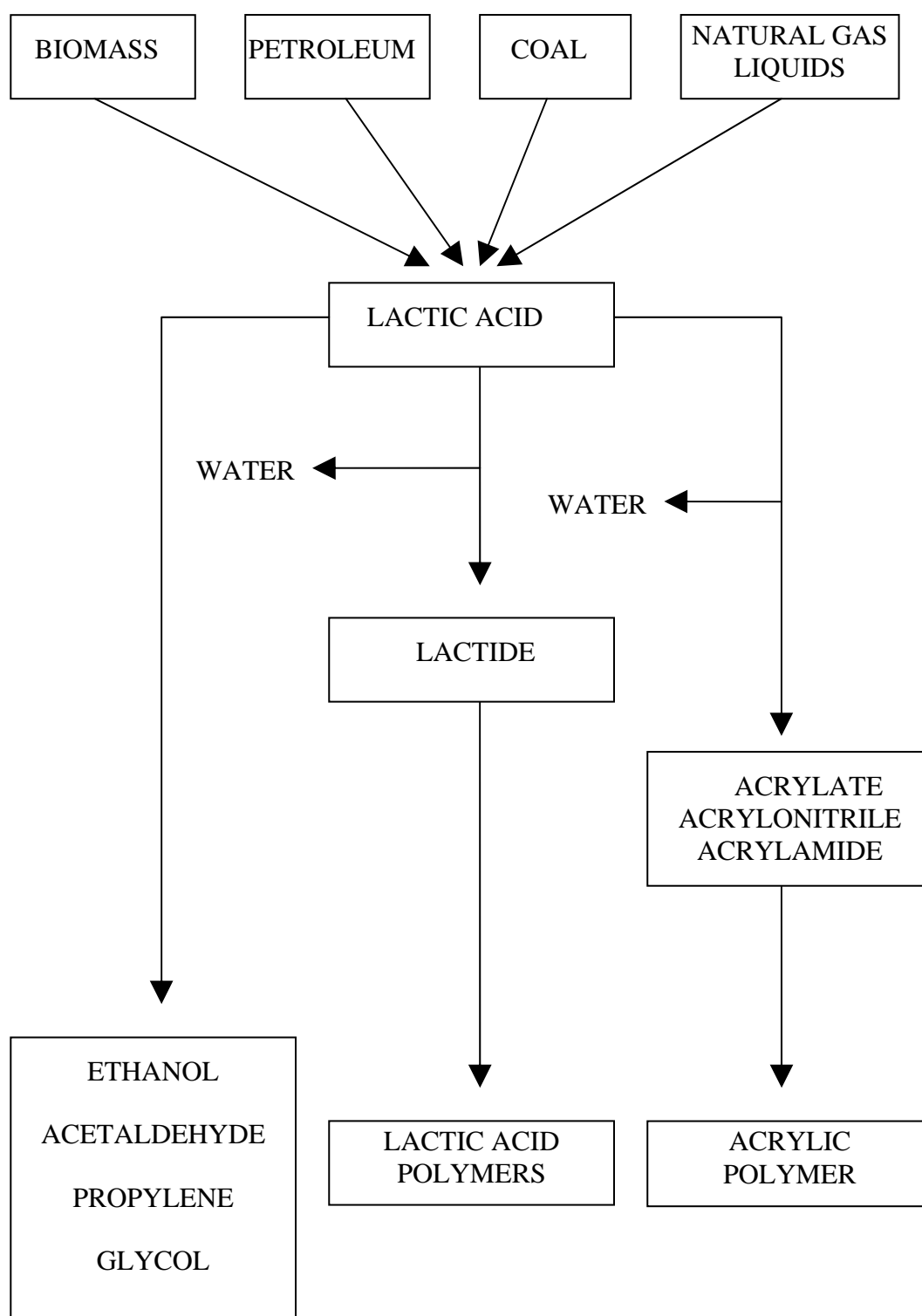
Figure 2.7: The potential industrial products from lactic acid (Lipinsky and Sinclair, 1986).

2.3.2   Physical And Chemical Properties

Lactic acid is a colourless, sour taste odourless, syrup liquid miscible in all proportions in water, alcohol and ether but insoluble in chloroform. It does not crystallise from solution as do other acids. It is a weak acid with good solvent properties and low melting point (Casida, 1964).

Three forms of lactic acid are known; D-lactic acid, L- lactic acid and DL-lactic acid. D-lactic acid and L-lactic acid are both optically active, and DL-lactic acid is optically inactive. The optically inactive form of lactic acid is simply an equi-molecular mixture of the both forms and denoted as racemic mixture (Brown, 1957).

The commercial product of lactic acid is usually optically inactive. Lactic acid is very corrosive, therefore corrosion resistant material must be used for its production. The material can be used are high-molybdate stainless steel, ceramic, porcelain or glass lined vessel (Paturau, 1982). The physical properties of different lactic acid forms are shown in Table 2.6.

Table 2.6: The physical properties of three forms of lactic acid (Weast and Astle, 1985; Holten, 1971).

Physical properties	Formula		
	D-lactic acid	L-lactic acid	DL-lactic acid
Melting point	53	26	18
Boiling point	103	-	122
Density	-	-	1.206
Refractive index (88.6%)	-	-	1.432
Viscosity (88.6%)	-	-	36.9
Colour	White	White	Yellow

Table 2.6: The physical properties of three forms of lactic acid (Continued).

Physical properties	Formula		
	D-lactic acid	L-lactic acid	DL-lactic acid
Specific rotation	-2.3	+3.8	-
Solubility	Water, Ethanol	Water, Ethanol	Water, Ethanol, Ether
Dissociation constant	-	-	0.000138

Lactic acid has both a hydroxyl group and a carboxylic acid, it is optically active and these attributes should provide lactic acid with a high ranking in the top organic chemicals. The existence of a hydroxyl group and a carboxylic acid in lactic acid enable it to be converted into polyesters directly. Dehydration of the hydroxyl group generates unsaturation that is conjugated with the carboxyl group. Therefore lactic acid has not only multiple functional groups but they are capable of co-operating with each other effectively (Holten, 1971).

Lactic acid is one of the smallest molecules that exhibits optical activity. It is not only feasible to manufacture D, L, DL lactic acid but also there are distinct differences in the physical and chemical properties of polymers and other derivatives, depending on the choice of optical polymer (Vickroy, 1985).

Lactic acid can be converted to acrylic material (acrylates, acrylonitrile and acrylamide), small molecule such as propylene glycol and ethanol, and lactic acid polymers (polylactic acid) that emulate many of the thermoplastics now being used in packaging and consumer goods with controllable rate of biodegradation (Lipinsky and Sinclair, 1986).

The reaction of lactic acid can be classified into the following main types: oxidation, disintegration, reduction, condensation and substitution of the alcohol

group and esterification. Oxidation of lactic acid with potassium permanganate in sulphuric acid results in the formation of acetic acid, water and carbon dioxide, but in alkaline condition with addition sodium hydroxide, it produces oxalate, potassium carbonate and water. Lactic acid is decomposed by heat in the presence of sulphuric acid to acetal dehyde and formic acid. It can be reduced by hydrogen iodide forming propionic acid, liberated iodine and water. Lactic acid can also be substituted by hydrogen bromide to 2-bromo propionic acid and water (Holten, 1971).

Polylactic acid can be prepared by either condensation polymerisation of the free acid or by catalic, ring opening polymerisation of lactide, which is the dilactone of lactic acid and its resulting polyesters are usually named polylactic acid (Lipinsky and Sinclair, 1986). Esterification of lactic acid to isopropyl lactate can be performed by addition of isopropyl alcohol with catalyse zeolite in benzene and the operating temperature at 71 $^{\circ}$C. The ester is a colourless liquid and decomposition can occur if it is boiled at 157 $^{\circ}$C (Vogel, 1973).

2.3.3   Lactic Acid Uses And Applications

Lactic acid, a normal organic acid, has long been of use in the pharmaceutical, chemical, cosmetic and food industry. Recently, lactic acid has been considered to be an important material for production of biodegradable lactide polymer (Wang et al., 1995). Lactic acid, a highly hygroscopes, syrupy liquid is commercially available at different grades (qualities). Technical grade lactic acid is used in deliming hides, in the textiles industry, and in the manufacturing of esters that are used as solvents and plasticiser. Food grade lactic acid is used as an additive in the manufacturing of beverages, essences, fruit juices and syrups; and as an acidulant in jams, jellies and confectionery, in the canning industry and in bakeries to produce sour flours and dough's, respectively (Vickroy, 1985).

Lactic acid of pharmacopoeia grade is used for treatments of the intestine, in hygienic preparations as well as for the manufacturing of pure pharmaceutical and

other derivatives of lactic acid such as calcium lactate. Recently the use of lactic acid in hand scrubbing before surgery has been recommended. Plastic grade lactic acid is used to manufacture various lacquers, varnishes, and impregnating agents as well as polymers (Kascak et al., 1996). Fermentation lactic acid generally has a yellow colour that is darkest for the concentrated technical grade, pale yellow for the food grade and colourless for pharmaceutical grade (Vickroy, 1983). There are altogether four grades of lactic acid and their characteristics are given in Table 2.7 (Paturau, 1982).

Table 2.7: The characteristics of the four grades of lactic acid (Paturau, 1982).

Item	Technical	Edible	Plastics	USP*
Total acidity	44	50	50	85
Free acidity	40-42	46-48	47-49	76-78
Volatile acids	1-2	1-2	1-2	2-3
Ash	0.6-0.7	0.4-0.5	0.005-0.01	0.05-0.1
Carbonisable organic matter	Present	Present	none	none
Sulphates	Present	Trace	none	none
Chlorides	Present	Present	none	none
Colour	Yellow to brown	Faint straw yellow	Colourless	Colourless
Iron	Present	< 3 ppm	Trace	Trace
Copper	Present	Trace	Trace	Trace

*USP: United state pharmaceutical

2.3.4   Lactic Acid Production

The methods for the lactic acid production can be categorised into two groups, biochemical and chemical processes. The commercial production has until recently only been performed by fermentation, but some chemical processes have also been used to manufacture lactic acid (Holten, 1971). Lactic acid is generally produced from glucose, maltose, sucrose or lactose. Starches, especially those from corn and potatoes, are hydrolysed by enzymes or by acid to maltose and glucose before used in the lactic acid fermentation (Atkinson and Mavituna, 1991; Blanch and Clark, 1997). A large number carbohydrate material have been used, tested or proposed for the lactic acid production by fermentation. It is useful to compare feed stock on the basis of the following desirable qualities, namely: low cost, low level contaminant, fast fermentation rate, high lactic yield, little or no by product formation, ability to ferment with little or no pre-treatment, and year round ability. Crude feed stocks have historically been avoided because high level of extraneous materials can cause separation problems in the recovery stage of lactic acid (Vickroy, 1985).

Starch or sugar containing substances can be used as raw material. Starches as raw materials have to be degraded first enzymatically or by means of acid, because the lactobacilli does not have amylolytic enzymes. This again means a higher cost for addition step which also brings in impurities (Buchta, 1983). Sucrose from cane and beet sugar, whey containing lactose and maltose, and dextrose from hydrolysed starch are presently used commercially (Vickroy, 1983). The carbohydrates available for lactic acid fermentation of most fruit and vegetables consist almost exclusively of glucose, fructose and sucrose (Fleming et al., 1985). An anaerobic batch fermentation using *Lactobacillus delbrueckii* or *Lactobacillus bulgaricus* is generally employed. The batch process is not subjected to significant contamination, apart from butiric acid bacteria, and often the fermenters are simply steamed or washed with boiling water (Blanch and Clark, 1997).

2.4   Lactic Acid Fermentation

2.4.1   General

Fermentation processes are characterised by biological degradation of substrate (glucose) by a population of micro-organism (biomass) into metabolites such as ethanol, citric acid and lactic acid (Maher et al., 1995). Lactic acid is produced from mono or disaccharida via the Embden Mayerhof glycolysis. Under anaerobic condition, the pyruvic acid produced is reduced to lactic acid by the enzyme lactic dehydrogenase (Milson, 1987).

Metabolism is referred to the chemical reactions which occur in living cells. Most chemical reactions in the cell do not occur spontaneously because of high kinetic barriers. The cell uses enzymes as catalysts to overcome these barriers, thus making possible energetically favourable reaction to take place at sufficiently high rates (Singleton, 1995). The metabolism of the cell aims at maintaining and multiplying the cell substance, which include the synthesis of the cell component. Cell synthesis requires not only structural components but also energy (Prave and Fauset, 1987). There are numerous species of bacteria and fungi that are capable to produce relatively large amount of lactic acid from carbohydrates (Atkinson and Mavituna, 1991). However in industrial fermentation, the use of various species of lactobacillus is preferred because of their higher conversion, yield and rate of metabolism (Mercier et al., 1992).

Two different types of lactic acid fermentation from carbohydrates are known, namely homolactic fermentation and heterolactic fermentation. Pure lactic or homolactic fermentation is characteristics of microorganism which form lactic acid exclusively or predominantly.

In mixed lactic acid or heterolactic fermentation, considerable amount of other fermentation products are formed as well as lactic acid, for instances, acetic acid, ethanol, formic acid and carbon dioxide (Holten, 1971).

2.4.2   Biochemistry Of Lactic Acid Fermentation

2.4.2.1 Biochemistry Of Glucose Metabolism

Two different types of lactic acid fermentation from carbohydrates are known namely homolactic fermentation and heterolactic fermentation. Pure lactic or homolactic fermentation is essentially performed by the homolactic *lactobacteriaceae*. The *lactobacilli* have the enzyme aldolase and lack the enzyme phosphoketolase, by glycolytic pathway (Embden-Meyerhof pathway) more than 85% glucose is converted to lactic acid. The equation (2.1) represents the overall equation for the metabolism of glucose to lactate under anaerobic condition (Zubay, 1984). The heterolactic fermentation lacks the enzyme aldolase and by phosketholase pathway, it produces equimolar amount of carbon dioxide, lactic acid, and acetic acid and or ethanol (Hammes and Whiley, 1993). The equation (2.2) represents the overall equation for the hetero lactic fermentation of glucose producing acetic acid and ethanol (Zubay, 1984). The type of fermentation occurring depends on the presence of certain bacteria. However homolactic fermentation in some cases can be converted into heterolactic type by changing the fermentation condition (Buchta, 1983; Hammes and Whiley, 1993).

$$\text{Glucose} + 2\,\text{ADP} + 2\text{P}_i \quad \rightarrow \quad 2\,\text{Lactic Acid} + 2\,\text{ATP} \qquad \text{……. (2.1)}$$
$$\text{(homo)}$$

$$\text{Glucose} + 2\,\text{ADP} + 2\text{P}_i \quad \rightarrow \quad \text{Acetic Acid} + \text{Ethanol} + CO_2 + 2\,\text{ATP}$$
$$\text{(hetero)}$$

$$\text{…… (2.2)}$$
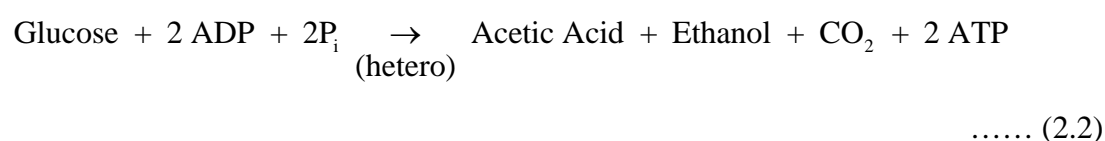
where ADP, ATP and P_i are Adenosine-5-diphosphate, Adenosibne-5-triphosphate and the inorganic phosphorus, respectively.

The first reaction (glycolysis) is phophorylation of glucose on carbon atom 6 by enzyme *hexokinase* to glucose 6-phosphate and the second reaction is the isomerisation by *phosphoglucoisomerase* to fructose-6-phosphate. This is followed by the third reaction which is phosporilation by *phospho-fructokinase* on carbon

atom 1 yielding the doubly phophorylated sugar fructose-1,6-diphosphate. The forth reaction occurs where fructose-1,6-diphosphate is split by *aldolase* to yield two three carbon sugar that turn out to be isomers of other namely dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The fifth is oxidation of glyceraldehyde-3-phosphate by *glyceraldehyde-3-phosphate dehydrogenase* to 1,3-diphosphoglyserate. The sixth is oxidation by *phosphoglycerokinase* yield 3- phosphoglycerate and subsequently the seventh is movement of the phosphate group from its original position on carbon 3 to a new location on carbon 2 by *phosphoglyceromutase* yield 2-phosphoglycerate. The eighth reaction is dehydration by *enolase* to phosphoenolpyruvate followed by the ninth reaction which is oxidation of phosphoenolpyruvate by *pyruvate kinase* to pyruvate. The tenth is reduction of pyruvate by *lactate dehydrogenase* to lactate. The metabolic pathways of glucose by lactic acid bacteria are shown in Figure 2.8 (Buchta, 1983; Zubay, 1984).

Glucose

ATP → 1 → ADP   NADP+ H$^+$

2 ← Glu-6-P   11 → 6-P-gluconic acid

Fru-6-P   NADP$^+$ NADP$^+$

3 ATP   12

ADP CO_2

Fru - 1.6 - P   NADPH+H$^+$

4 → Dihydroxy Acetone-P   Ribulose-5-P

Glyceral dehyde-3-P   13

Pi NAD$^+$ Xyulose-5-P

5   14   Pi

NADH+H$^+$ ATP ADP

1-3-di-P-glyserate   15   Acetyl-P

Acetic acid ←   NADH+H$^+$ 16

6 ATP   Pi

ADP   NAD$^+$

3-P-glyserate   Acetaldehyde

7 → H$_2$0   NADH+H$^+$ 17

2-P-Glyserate   NAD$^+$

8 → H$_2$0   Ethanol

Phosphoenol pyruvate

9 ATP   NADH+H$^+$ NAD

ADP

Pyruvate ————————————

10 Lactic acid

(homolactic)   (heterolactic)

Figure 2.8: The metabolic pathway of glucose (Buchta, 1983; Zubay, 1984).
The enzymes catalysing numbered reactions are: 1. Hexokinase, 2.
Phosphoglucoisomerase, 3. Phosphofructokinase, 4. Aldolase, 5. Triose
phosphate isomemerase, 6. Phosphoglycerokinase,
7. Phosphoglyseromutase, 8. Enolase, 9. Pyruvate kinase, 10. Lactic
dehydrogenase, 11. Glucose-6- phosphate dehydrogenase, 12. 6-phospho
gluconate dehydrogenase, 13.Ribolosephosphate-3-epimerase,
14. Phosphoketolase, 15. Acetokinase, 16.Aldehyde dehydrogenase and
17. Alcohol dehydrogenase.

2.4.2.2   Biochemistry Of Fructose Metabolism

Fructose is most commonly obtained by ingestion hydrolysis of sucrose. There are two pathways for metabolism of fructose. First, catalysed by the enzyme fructokinase, the phophorylation of fructose by ATP at position of carbon 1 (C1) to form fructose 1-phosphate (2.3). Second, the fructose is phosphorylated at position of carbon 6 by the enzyme hexokinase to form fructose-6-phosphate (2.4) and followed by the phophorylation using phosphofructokinase to form fructose-1,6-diphosphate (2.5).

1. Fructose + ATP $\longrightarrow$ Fructose-1-phosphate + ADP

       enzyme fructokinase

                                                          ………. (2.3)

2. Fructose + ATP $\longrightarrow$ Fructose-6-phosphate + ADP

       enzyme hexokinase

                                                          ………   (2.4)

3. Fructose-6-phosphate + ATP $\longrightarrow$ Fructose-1,6-biphosphate + ADP

                                                          ..……   (2.5)
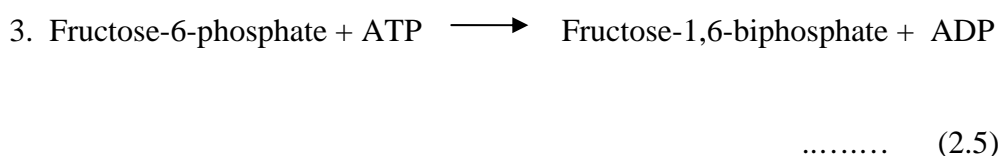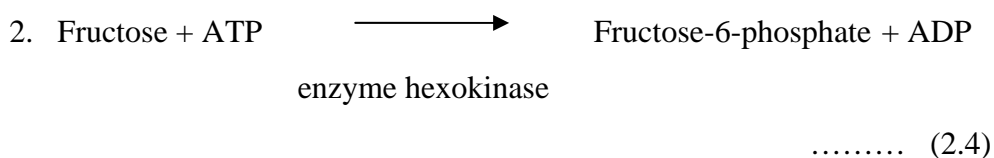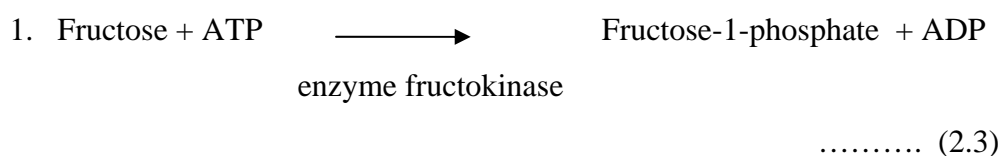
The fructose 1-phosphate is split by fructose 1-phosphate aldolase to dihydroxyacetone phosphate and glyceraldehyde. The glyceraldehyde formed is converted to glyceraldehyde-3-phosphate by phophorylation of ATP through the action of glyseraldehyde kinase. The fructose-1,6-diphosphate is split by aldolase to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The glyceraldehyde-3-phosphate is then undergone catabolism by means of the glycolytic pathway to produce lactic acid. The metabolic pathways of fructose by lactic acid bacteria are shown in Figure 2.9 (Freeman, 1985; Zubay, 1984; Moat, 1985).

ATP   ATP

Fru-6-P   1   Fructose   10   Fruc-1-P

ATP   11

2   ADP   ADP

Fru - 1.6 - P   Glyseral dehyde

3   Dihydroxy-Acetone-P   ATP   12

Glyceral dehyde-3-P

ADP

Pi   4 NAD^+

$NADH + H^+$

1-3-di-P-glyserate

ATP

5   ADP

3-P-glyserate

6   H_2O

2-P-glyserate

7   H_2O

Phosphoenol pyruvate

8   ATP

ADP $NADH+H^+$ NAD

Pyruvate   9   Lactic acid
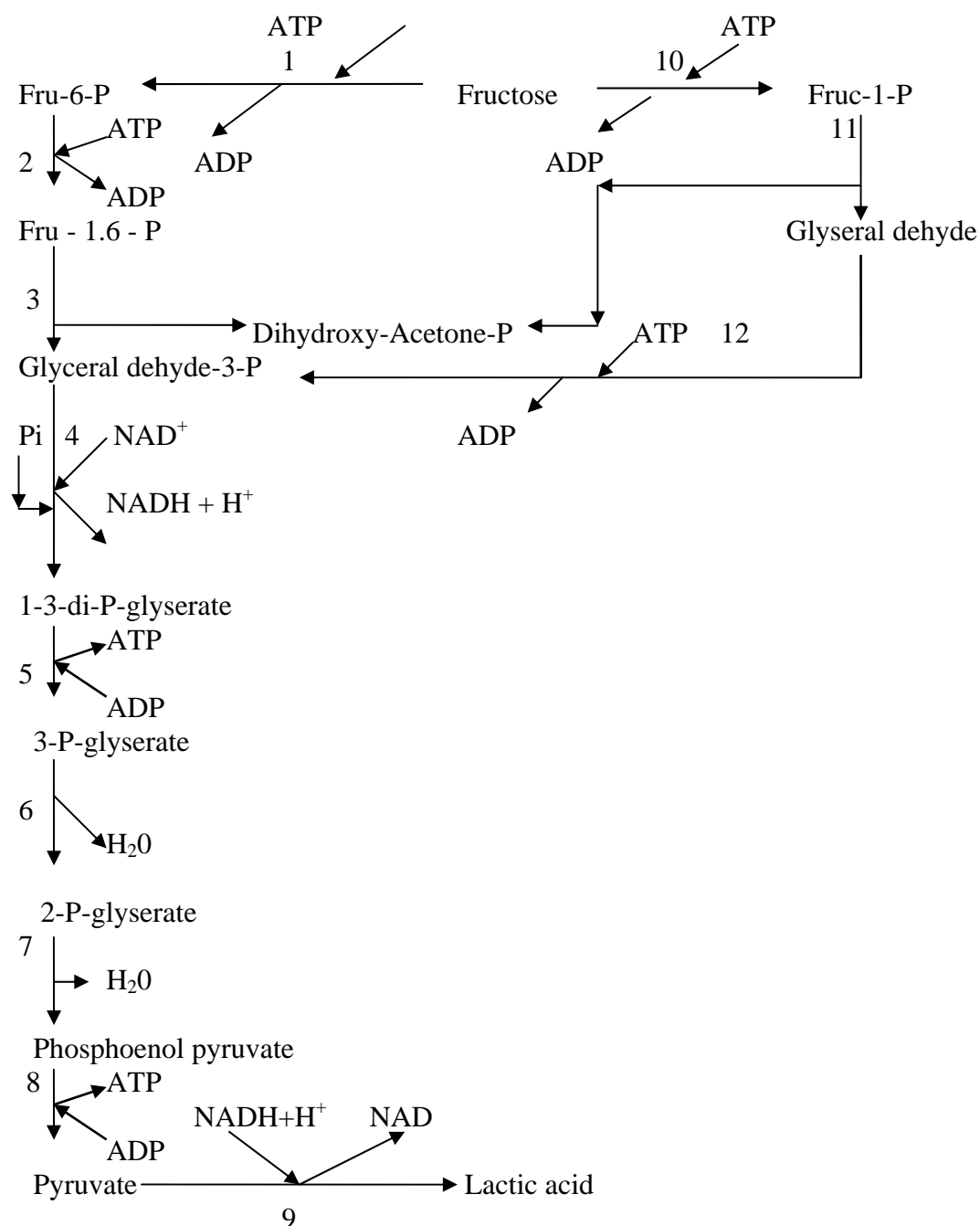
Figure 2.9: The metabolic pathway of fructose (Freeman, 1985; Zubay, 1984; Moat, 1985). The enzymes catalysing the numbered reactions are: 1. Hexokinase; 2. Phosphofructokinase; 3. Aldolase; 4.Triose phosphate isomemerase; 5. Phosphoglycerokinase; 6. Phosphoglyseromutase; 7. Enolase; 8. Pyruvate kinase; 9. Lactic dehydrogenase; 10. Fructokinase; 11. Fructose 1-phosphatealdolase; and 12. Glyseraldehyde kinase.

2.4.2.3 Biochemistry Of Sucrose Metabolism

Many *lactobaciili* species produce levans sucrase in response to growth on sucrose. Depending on the enzymes present in a given strain, the disaccharide sucrose (α-glucose-1,2-β-fructose) can be cleaved by two alternatives namely hydrolysis by enzymes invertase or levansucrase (2.6) yield glucose and fructose (Zubay, 1984; Moat, 1985).

$$\text{Sucrose (glucose-fructose)} \xrightarrow{\text{levan sucrase}} \text{Glucose} + \text{Fructose} \quad \dots\dots \quad (2.6)$$
$$\text{invertase}$$
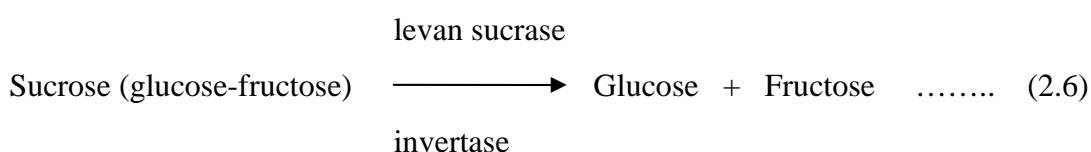
A few organisms also have phosphorolytic enzymes that phosphorylyze the sucrose to glucose-1-phosphate and fructose (2.7). The tranferase reaction of glucose-1-phosphate by the enzyme phospho glucomutase yields glucose-6-phosphate (Freeman, 1985; Zubay, 1984).

$$\text{Sucrose (glucose-fructose)} \longrightarrow \text{Glucose-1-phosphate} + \text{Fructose} \dots\dots (2.7)$$
$$\text{sucrosephosphorylase}$$

$$\text{Glucose-1-phosphate} \longrightarrow \text{Glucose-6-phosphate} \quad \dots\dots (2.8)$$
$$\text{phosphoglucomutase}$$
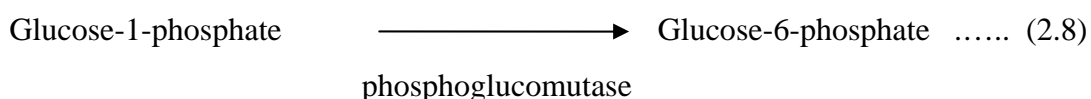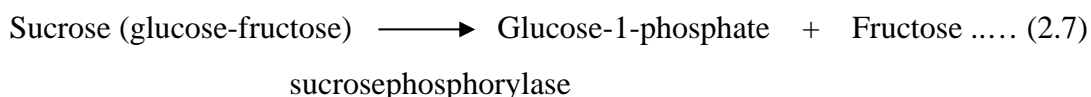
The glucose-6-phosphate entry is gained into the glycolytic pathway to produce lactic acid. The fructose is phosphorylated at position of carbon 6 by hexokinase and then phoporilation by phosphofructokinase. The fructose-1,6-diphosphate is split by aldolase to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The further catabolism by means of the glycolytic pathway produces lactic acid. The metabolic pathways of sucrose by lactic acid bacteria are shown in Figure 2.10 (Freeman, 1985; Zubay, 1984; Moat, 1985).

Fructose ◄──── Sucrose ────► Glucose

1(2) 2 1

3 ATP

ADP

Glucose-1-P

ATP | 4

5

Fru-6-P ◄──── Glucose-6-P

ATP 3 ATP

4 ADP

ADP ADP

Fru - 1.6 - P

5

──────► Dihidroxy-Acetone-P

Glyceral dehyde-3-P

6 NAD^+

Pi

$NADH + H^+$

1-3-di-P-glyserate

ATP

7

ADP

3-P-glyserate

8

H_2O

2-P-glyserate

9

H_2O

Phosphoenol pyruvate

10 ATP

$NADH+H^+$ NAD

ADP

Pyruvate ──────────► Lactic acid

11
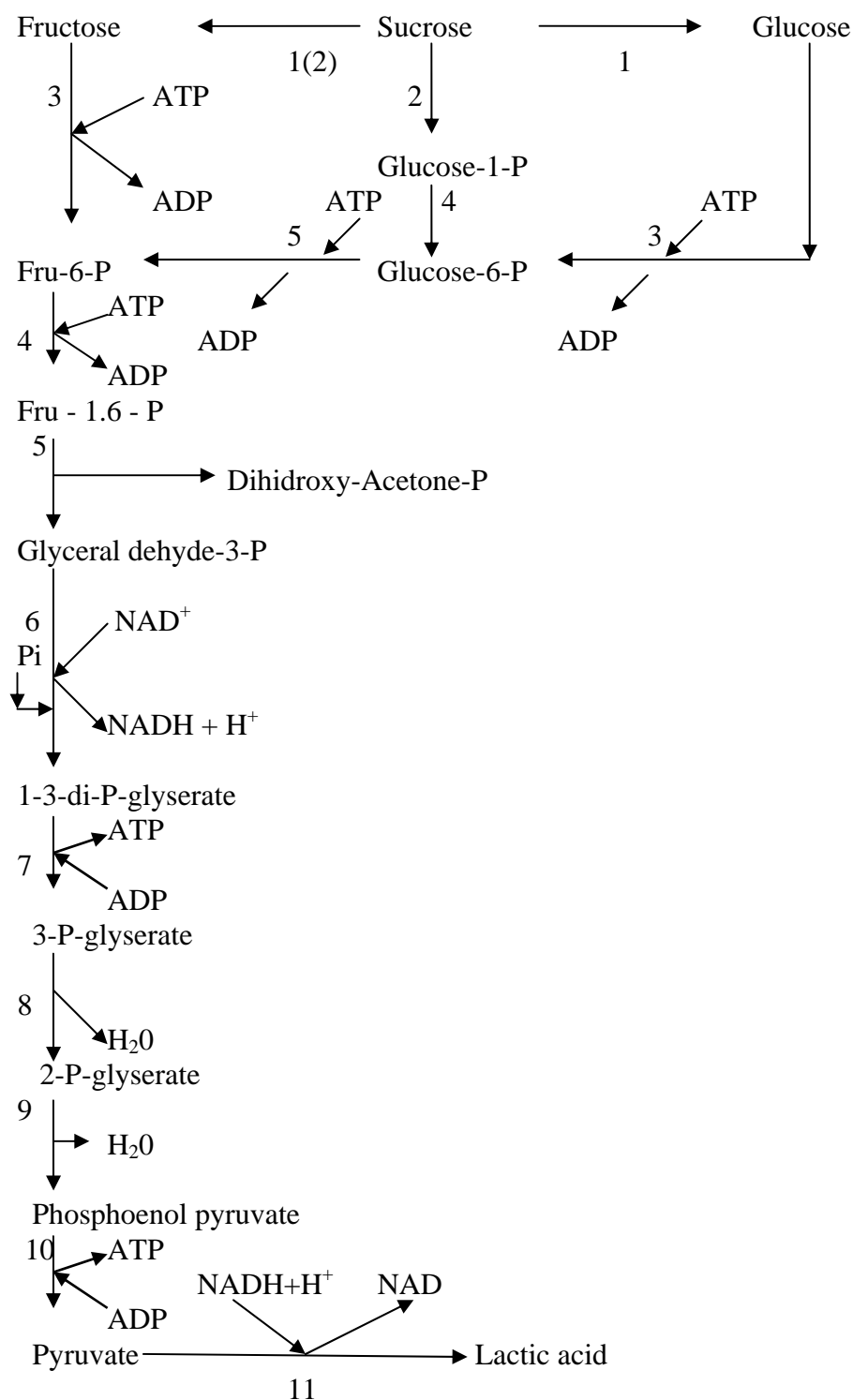
Figure 2.10: The Metabolic pathway of sucrose (Freeman, 1985; Zubay, 1984 ;
Moat, 1985). The enzymes catalysing the numbered reactions are:
1. invertase (levansucrase); 2. Sucrose phosphorylase; 3.Hexokinase;
4. Phosphoglucomutase; 5.Phosphoglucoisomerase;
6. Phosphofructokinase; 7. Aldolase; 8. Triose phosphate isomemerase;
9. Phosphoglycerokinase; 10. Phosphoglyseromutase; 11. Enolase;
12. Pyruvate kinase; and 13. Lactic dehydrogenase.

2.4.3   Fermentation Operation Conditions

Lactic acid fermentation has been studied since 1935 using different types of microorganism and fermentation operation conditions such as carbon source, pH, temperature, inoculum size, initial substrate conditions and nitrogen source (Atkinson and Mavituna, 1991; Hofvendahl and Hagerdal, 1997). In this section, the types of microorganism and the range of operation conditions used will be described briefly in order to provide the background for the present study which will be helpful in selecting the appropriate microorganism and operational conditions for lactic acid fermentation of pineapple waste.

2.4.3.1 Types Of Microorganism

There are numerous species of bacteria and fungi that are capable to produce large amounts of lactic acid from carbohydrate, and the selection of an organism to be applied and selected for higher productivity largely depends primarily on the carbohydrate to be fermented (Atkinson and Mavituna, 1991). The most important producers of lactic acid belong to the family of *Lactobacillaae* and they are classified into four genera: *Pediococcus*, *Lactobacillus*, *Streptococcus Leoconostoc* and *Bifido bacterium* (Buchta, 1983).

Lactic Acid Bacteria (LAB) are group of gram positive, non spora forming, anaerobic bacteria which excrete lactic acid as the main fermentation product into the medium if supplied with suitable carbohydrates. Therefore, in practice, low oxygen tensions should be maintained but exclusion of oxygen (air) is not an absolute requirement (Teuber, 1993). The different types of microorganism have been used for lactic acid fermentation such as *L. delbrueckii* and *L. plantarum* (Samuel and Lee, 1980), *L. bulgaricus* (Gadgil and Venkatesh, 1997) and *L. amylophylus* (Merciers et al., 1992).

Basically, the criteria for selection of bacteria depend on carbohydrate to be fermented and higher productivity. In this work, the type of microorganism used was *L. delbrueckii subspecies delbrueckii,* because of their higher productivity and only lactic acid is produced. Furthermore, it uses not only glucose as raw material, but sucrose and fructose are utilised as well. Therefore, it is suitable for the fermentation of sucrose and fructose such as pineapple waste (Teuber, 1993; Hammes and Whiley, 1993). As reported in literature, the *L. delbrueckii* is the best strain utilising glucose, fructose and sucrose with sugar conversion of 90% (Kascak et al., 1996; Carr et al., 1975).

2.4.3.2 Carbon Sources

According to Luedeking and Piret (1959), the rate of lactic acid formation is a function of cell growth and cell concentration, which is characterised by increasing, cell mass and/or number. It occurs only when certain chemical and physical conditions are satisfied, such as acceptable temperature and pH as well as the availability of required nutrients (Wang et al., 1979).

The carbon source is important for microbial growth. In lactic acid fermentation, glucose, fructose and sucrose have been used as carbon source in the pure substrate or in the form of complex system such as whey, molasses and starch. The type of carbon source chosen is significant since it will affect the lactic acid production as well as metabolic pathway.

Inhibitory levels of microbial growth vary depending on the types of substrate, such as glucose which may be inhibitory at concentrations above 200 g/l. probably due to a reduction in water activity (Shuler and Kargi, 1992). In industrial fermentation, the sugar concentration in the medium is initially adjusted to 5 - 20 % but usually not exceed 12 % (Atkinson and Mavituna, 1991). The concentration of carbon source such as glucose has an effect on the lactic acid production where the maximum productivity obtained was 0.34 g/l.h at sugar concentration of 60 g/l

(Buyukgungor et al., 1984). The effect of sucrose concentration on lactic acid production from beet molasses by *L. delbrueckii* shows that the highest yield achieved was at 78.2 g/l sugar concentration (Goksungur and Guvenc, 1997).

2.4.3.3 Nitrogen Sources

Nitrogen is important for microbial growth, and is second to carbon in terms of quantity and economic importance. It serves as the building block for the synthesis of proteins and other cellular macro-molecules. Sources of nitrogen in fermentation media include corn steep liquor, fish meal, yeast extract, and protein hydrolysate (Posten and Cooney, 1993). The types and concentration of nitrogen sources affect the lactic acid fermentation. Yeast extract is the most commonly used nitrogen source in lactic acid fermentation. Lactic acid production increases with increasing concentration of the supplement especially yeast extract. The highest production rate was found with addition of 5-15 g/l yeast extract (Goksungur and Guvenc, 1997; Lund et al., 1992). Yeast extract yielded the highest final level of lactic acid when compared with the other nitrogen sources such as malt sprout, corn steep liquor, soy flour, urea, cotton oil cake, tryptone, grass extract, NZ case plus, NZ amine YT, a mixture of diammonium hydrogen phosphates and ammonium dihydrogen phosphates (1:2), casein hydrolisate, and distillers waste (Hujanen and Linko, 1996; Lund et al., 1992). However, addition of yeast extract during large scale fermentation is unrealistic due to the extra cost introduced for the fermentation process, in combination with the low value of lactic acid. It was reported that the ratio of $(NH_4)_2 SO_4$ to yeast extract was 3:1, the lactic acid yield was as in whey supplemented with 20 g/l yeast extract (Arasaratnam et al., 1996).

2.4.3.4 Macro Elements

Macro elements such as phosphorus, potassium, magnesium and sulphur are important for microbial growth. Phosphorus is present in the microbial cell as phosphate sugar, nucleic acids and nucleotides. Potassium is required for maintenance of ionic balance across the cell membranes, and as stabilising component RNA. Magnesium is an essential enzyme activator and component of the cell membrane meanwhile sulphur is an important component of amino acids and coenzymes (Posten and Cooney, 1993).

Optimisation studies were carried out by Monteagudo et al. (1993) to determine the most suitable medium for obtaining a maximum cell concentration in the lactic acid fermentation of beet molasses by *L. delbrueckii*. The selected macro elements were dipotassium dihydrogen phosphates, potassium dihydrogen phosphates as well as magnesium sulphate; yeast extract and peptone were added as nitrogen source. They found that the effect of macro elements addition (K, P and S) under experimental condition is insignificant, except yeast extract and peptone. Therefore, it could be concluded that the addition of potassium dihydrogen phosphates (KH_2PO_4), dipotassium hydrogen phosphates (K_2HPO_4) and magnesium sulphate ($MgSO_4$) does not give significant effect in lactic acid production. These results seem to be opposite to what of one expected.

2.4.3.5 Micro Elements (Trace)

Microbial growth needs, beside the carbon source, macro elements, nitrogen and minerals, micro elements such as folic acid, vitamin B and serine. Micro elements are required in extremely small amount, for example, in the scale of mM or μM. They are important as effectors of enzymes and coenzymes which are normally present in complex media and also in tap water in varying amounts (Posten and Cooney, 1993).

Lactic acid bacteria have complex nutritional requirements especially vitamins B, which are usually met by enrichment of the medium with crude

vegetables sources, such as malt sprouts (Atkinson and Mavituna, 1991). The types of micro elements required depend on fermentation media used. For example, many lactic acid bacteria need the addition of aspartic acid for growth in a biotin-deficient medium. However in biotin rich medium no aspartic acid is required. A similar relationship exists between folic acid and serine. Vitamin B_6 is very important in the biosynthesis of amino acids for lactic acid bacteria (Buchta, 1983). Even though the micro elements required for microbial growth as reported in literature indicated that there was no significant effect of vitamin B complex (Arasaratnam et al., 1996), mangan and ferrum (Monteagudo et al., 1993) on lactic acid production.

One of the most important factors in optimisation of a fermentation process is the design of the growth medium. The medium must meet the needs for synthesis of cell materials and for biosynthetic process as well as for environmental requirements of the microorganism. Several mediums for growth of lactic acid bacteria have proposed, but many researchers using MRS medium for cultivation of lactobacilli. The design of 1 litre medium for growth of lactobacilli is given by different authors in Table 2.8

Table 2.8: The design media for growth of lactic acid bacteria published by different authors

Composition	Medium RMS (De Man et al., 1960)	Medium LC (Teuber, 1993)	Medium SY (Cejka, 1985)
Peptone (g)	10.00	-	-
Trypticase (g)		10.00	
Meat extract (g)	10.00	-	-
Yeast extract (g)	5.00	10.00	20.00
K_2HPO_4 (g)	2.00	-	-
KH_2PO_4 (g)		6.00	2.50
Diammonium citrate (g)	2.00	2.00	-
Glucose (g)	20.00	20.00	-

Sucrose (g)	-	-	100.00
Tween 80 (mL)	1	1	-
Sodium acetate (g)	5.00	25.00	-
$MgSO_4.7H_2O$ (g)	0.58	0.58	-
$MnSO_4.4H_2O$ (g)	0.25	0.15	-
$FeSO_4\ 7H_2O$ (g)	-	0.03	-
Agar (g)	15	15	15

2.4.3.6 Agitation

As a basic operation of fermentation, stirring can be carried out mechanically, pneumatically or hydrodynamically. The functions of stirring and mixing processes are to disperse the entering and rising bubbles of air, to homogenise the nutrient medium, to produce sufficiently high turbulence for the heat transfer as well as to maintain high relative velocities between air bubbles, substrate, and microorganism for the optimum exchange of matter (Prave and Fauset, 1987).

The power requirement of agitation systems represents a significant cost in running large scale fermentations. The changes of impeller type or relative size need to be justified by either a reduction in power cost for the same productivity or an increase in productivity at the same cost (Amannullah et al., 1998).

Konda et al. (1997) reported that the productivity of bacterial cellulose is affected by agitator configuration and agitator speed because by using certain types of agitator, they mix culture broth well and have large mass transfer coefficient. The lactic acid fermentation of cheese whey permeate by *lactobacillus helviticus* was studied by Fairbrother (1991). By factorial design, they found that the optimum condition for lactic acid formation and cell growth was at the speed of 200 rpm.

Effect of agitation speed in Xanthan fermentations has been studied by Amannullah et al. (1998) and the result shows that Xanthan production is not influenced by changes of agitation speed. They also studied the effect of the ratio of impeller diameter and fermentor diameter (D/T) on productivity of Xanthan fermentation. The result of this study shows that the productivity increases with increasing of D/T from 0.33 to 0.42. If the value of D/T is further increased to 0.54, the productivity will increase from 0.56 to 0.68 g/l.h.

L. delbrueckii is facultative anaerobic bacteria, therefore in practice low oxygen tensions should be maintained. Based on literature study, the fermentor was run with the chosen agitation speed at 50 rpm. The low agitator speed was operated to prevent cell damage and to prevent aeration so that an anaerobic process can be maintained.

2.4.3.7 Temperature

Microbial growth rate, as all chemical reaction, is a function of temperature. In general, microorganism will grow over a temperature range of 25-30 $^{\circ}$C. However, it is important to note that there are, in nature, microorganisms which can grow at temperatures below 0 $^{\circ}$C and above 90 $^{\circ}$C, with the primary requirement of liquid water (Singleton, 1995). Most of bacteria and fungi are killed at temperature around 60 $^{\circ}$C within 15-20 minutes, yeast and fungal spore are killed only above 80 $^{\circ}$C, while bacterial spores need about 15-20 minutes to be destroyed at 121 $^{\circ}$C (Teuber, 1993).

Temperature also impinges on the efficiency of the carbon energy substrate conversion to cell mass. The maximum conversion yield occurs at temperature that is less than the temperature for maximum growth rate. This temperature is particularly important in process optimisation when it is desired to maximise yield but not growth rate. Temperature also affects product formation. However the temperature for growth and product formation may be different (Shuler and Kargi, 1992).

Lactic acid bacteria are classified as thermophilic or mesophilic. The temperature range for optimal growth of mesophilic is 25-45 ^{o}C and the thermophilic bacrteria 45-62 ^{o}C. *L. delbrueckii* is a kind of mesophilic bacteria which grows in the mid range of ambient temperature and has optimal temperature between 37 and 45 ^{o}C (Taylor, 1992).

The effect of temperature on lactic acid production using *L. delbrueckii* has been studied by Goksungur and Guvenc (1997). The optimum temperature achieved was at 45 ^{o}C and lactic acid concentration produced was 4.9%. If the temperature was decreased to 40 ^{o}C, the production obtained was 4.2%. While at temperature 50 ^{o}C, the lactic acid concentration was only 2.1%. Optimisation of the conditions in the fermentation of beet molasses to lactic acid by *L. delbrueckii* was investigated by Monteagudo et al. (1994). A central composite design was used to determine the optimal values of the process variable. The temperature was studied at a range of 45-50 ^{o}C. The optimal value obtained was at 50 ^{o}C and at maximum yield of 87.8 %.

2.4.3.8 pH

Most bacteria grow best at or near pH 7 (neutral), and the majority cannot grow under strongly acidic or strongly alkaline condition. In lactic acid production by fermentation, the pH is kept neutralised by either sodium hydroxide, calcium hydroxide or calcium carbonate. For rapid and complete fermentation the optimal pH ranges between 5.5 - 6.0 (Buchta, 1983). Hydrogen ion concentration (pH) affects the activity of enzymes and therefore also affects the microbial growth rate. The optimal pH for growth may be different from that for product formation. Different organisms have different pH optima, but the optimum pH for many bacteria ranges from pH 3 to 8 (Shuler and Kargi, 1992).

The effect of pH on the production of lactic acid from beet molasses by *L. delbrueckii* was also studied by Goksungur and Guvenc (1997). The optimal pH for

growth and lactic acid production was found to be 6.0 with 94.7 % yield. If the pH were increased to 6.5, the yield would decrease to 86.3 %. While at pH 5.5, the yield was only 67.1 %. Effect of pH on the fermentation of beet molasses to lactic acid by *L. delbrueckii* has also been investigated by Monteagudo et al. (1994). The pH studied was between 5.5-6.5. The results showed that the effect of pH, with experimental condition under study was found to be significant with an optimal pH obtained at 5.5 and a maximum yield at 87.8 %.

2.4.3.9 Dissolved Oxygen

Some bacteria need oxygen for growth. Bacteria that require oxygen for growth are called " strict " or " obligate " aerobes in order to emphases their absolute need for oxygen. Strict or obligated anaerobes grow only when oxygen is present. These organisms exist in river mud and in the rumen. Bacteria which normally grow in the presence of oxygen but which can still grow under anaerobic condition (absence of oxygen) are called facultative anaerobes. Similarly, those which normally grow anaerobically but which can grow in the present of oxygen are called facultative aerobes (Singleton, 1995). For all organisms, including obligate aerobes, oxygen may be toxic at any concentration. The mechanism of oxygen toxicity undergoes the formation of single oxygen, superoxida radicals O_2^-, peroxida O_2^{2-} or hydroxy free radical OH^- which are destructive to many cell component (Teuber, 1993).

Lactic acid bacteria are facultative anaerobic or microaerophilic that grow poorly in the presence of oxygen. Sakamoto et al. (1998) reported that the growth of 22 strains of lactic acid bacteria was observed under aerobic conditions and the result showed that most strains grow well under aerobic condition except for *L. fermentum* and *L. delbrueckii*. They grew well under anaerobic condition but in contrast aerobically. The reason could be that these two strains accumulated at high concentration of hydrogen peroxide in the culture medium under aerobic condition, which caused the growth to cease due to its toxicity.

The end product of glucose metabolism using *L. delbrueckii* has also been studied by Sakamoto and Komagata (1996). The result showed that in aerobic condition, it produced lactic acid and acetic acid, but in anaerobic condition only lactic acid was produced.

2.4.4   Modes Of Lactic Acid Fermentation

Fermentation process can be grouped based on native of substrate used which is either solid substrate fermentation or liquid state fermentation. Solid substrate fermentation (SSF) process has been used by man for many centuries. The term SSF describes the biological transformation within particles of solid substrate where the liquid contents bond with them. If the substrate is solublised or suspended as fine particles in a large volume of water, it is called liquid state fermentation (LSF) or submerged fermentation. There are many advantages of LSF such as all types of organism (fungi, some yeast, some bacteria, and streptomycetes) can grow at reduced water activity, no technical problem in controlling the heat generated during fermentation, high product yield, fast fermentation, and ease of using reliable devices to measure or control some of the fermentation parameters. Therefore, in general the submerged fermentation is better than solid state fermentation (Lopez and Solio, 1991).

In term of native of bacteria used in fermentation process, it can be in immobilised and free cell form. Application of immobilised cell as biocatalyst is a new and rapidly growing area in biotechnology. Immobilised cells exhibit many advantages over free cells, such as stable activity, high productivity, reusability and the possibility of continuous operation. However in immobilised cell systems, mass transfer limitations can reduce productivity due to nutrient starvation or inhibitory product build-up. Particularly, in the case of immobilised growing cells, their productivity will be influenced greatly by the mass transfer limitation due to the increase of diffusional resistance with cell growth inside the immobilising support (Wang et al., 1996).

The use of cell in free solution is wasteful although not necessarily uneconomic. To prevent loss, cell may be immobilised by association with insoluble materials. The balance of economic factors that have to be taken into account to establish the feasibility of immobilisation are cost of cells, extent of cell purification required, cost of immobilisation process, cell stability, and inhibition and poisoning effect (Atkinson and Mavituna, 1991).

The productivity of lactic acid production by *L. delbrueckii NRLL B445* was studied by Buyukgungor et al. (1984) in free and immobilised cell. The productivity of immobilised cell was higher than free cell due to the cell can be maintained at stable and viable state. The production of lactic acid from beet molasses by calcium alginate immobilised *L. delbrueckii IFO3202* was also studied by Goksungur and Guvenc (1999) in batch fermentation. The highest yield obtained was 82%, while only 81% if using free cell at similar fermentation conditions. Cells immobilised in Ca-alginate gels offer rapid, mild, simple, cheap and versatile technique which may be applied to a wide range of cell (Goksungur and Guvenc, 1997).

Based on the fermentation operation, the fermentation can be operated either batch, fed batch or continuous operation. In batch fermentation, conditions within the fermentor change during the fermentation cycle, with the increase of product and cell concentration and the depletion of the substrate (Russel, 1987).

Batch fermentation is less suitable, if substrate inhibition or growth dependent inhibitory by product formation occurs (Kascak et al., 1996). Some of the problems of batch fermentation can be overcome with fed batch operation where the substrates are permanently fed to the reactor without removal of fermentation broth and thus the volume of the broth increases (Roukas and Kotzekidou, 1998).

In a continuous fermentation, the condition in the fermentor remains constant during operation so that fermentation is controlled with same cell, substrate and product concentration. Continuous operation is normally chosen for production at low value and high volume production (Russel, 1987). In continuous fermentation

processes, considerably higher productivity is achieved, and thus they have been performed in various forms. Based on laboratory results, two or three stage continuous fermentation could reduce the fermentation time up to 40% compared to that required for a batch process (Vickroy, 1985).

2.4.5   Lactic Acid Fermentation Models

Knowledge of the kinetics of fermentation is necessary in order to size the fermentor and its associated equipment, and this information is normally obtained from laboratory experimentation with one to three litres fermentor. In batch fermentation, the kinetic model provides information to predict the rate of cell mass or product generation while in continuous fermentation it will predict the rate of product formation under given conditions (Russel, 1987).

The kinetic models play an important role in monitoring and predicting fermentation process. The models contain kinetic of growth, substrate utilisation and product formation. According to this view of the cell, growth models can be divided into unstructured and structured types. Unstructured models are the simplest type. They take the cell mass as a uniform quantity without internal dynamics where the reaction rate depends only upon the conditions in the liquid phase of the reactor (Nielsen et al., 1991).

If the internal state of the cell is considered, it is then called structured models. Hence changes of structured models in the microorganism composition are considered and sometimes needed. One of the problems associated with the construction of structured model is the complexity of the model. In principle an extensive number of compositional variables can be attributed to biomass. If this is pursued to the extreme, very complex models were developed (Bailey and Ollis, 1977).

Most of the available mathematical models for lactic acid fermentation process are unstructured. The biomass is considered as one entity described only by

its concentration and the linear relations link the product formation to the biomass formation. This model contains a small number of parameters which can be easily estimated on the basis of steady state experiments and open ended, and can also be extended to describe more complex systems (Roels, 1983).

2.4.5.1 Kinetics Of Microbial Growth

Many unstructured models of bacterial growth on lactic acid fermentation have been presented in the literature where changes in the composition of the biomass are totally ignored or the rate of increase is only a function of cell number or cell concentration (Nielsen et al., 1991). The growth rate can be described as:

$$\frac{dX}{dt} = \mu X \qquad\qquad ......(2.9)$$

where X is cell mass concentration (gram / litre), t is time (hour) and μ is specific growth rate (hour^{-1}).

The kinetic of microbial growth in lactic acid fermentation has been studied by Mercier et al. (1992) and Norton et al. (1994). They used the logistic models that express the relationship of the rate of growth and two kinetic parameters such as the maximum specific growth rate (μ_{max}) and the maximum biomass concentration (X_{max}). The two parameters were estimated by non-linear regression using the least square methods. The model is described in the following form:

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}} \right) \qquad\qquad .....(2.10)$$

Integration of equation (2.10), gives

$$X_t = \frac{X_o X_m \exp(\mu_{max} t)}{X_m - X_o + X_o \exp(\mu_{max} t)} \qquad \text{....(2.11)}$$

Parajo et al. (1996) also proposed a similar expression for modelling of lactic acid from wood. By statistic method, the logistic models for lactic acid fermentation were found to be in good agreement with the experimental data. They obtained the kinetic parameters of X_m and μ_{max} which were 2.95 g/l and 0.737 h^{-1} respectively but Mercier et al. (1992) only obtained 2.06 g/l and 0.29 h^{-1}, respectively. This difference is due to the substrate and strain used in lactic acid fermentation.

An unstructured model which is frequently used in the kinetic description of microbial growth is the Monod equation. This model expresses that the specific growth rate of micro organism increases if the substrate concentration in the medium is increased. However the increase in specific growth rate becomes progressively less if the substrate concentration level is higher. The equation has the following form:

$$\mu = \mu_m \left[\frac{S}{K_s + S} \right] \qquad \text{.....(2.12)}$$

Hanson and Tsao (1972) also proposed the kinetics of microbial growth by combining equation (2.9) with (2.12) as shown in the following model:

$$\frac{dX}{dt} = \mu_{max} \frac{S}{K_s + S} X \qquad \text{.....(2.13)}$$

Similar model has also been proposed by Suscovic et al. (1992), but they assumed that the specific death rate cannot be neglected and therefore they simulate the equation as follows:

$$\frac{dX}{dt} = \mu_{max} \frac{S}{K_s + S} X - k_d X \qquad \text{.....(2.14)}$$

The kinetic models for microbial growth and estimated parameter values have been reported in literature and are summarised in Table 2.9. The different parameter values due to the different substrates and operation conditions were used in lactic acid fermentation.

Table 2.9: The proposed kinetic models of microbial growth and estimated parameter values.

Strains	Model	X_m (g/l)	μ_{max} (hr^{-1})	k_d (hr^{-1})	K_s (g/l)	Authors
L. amylophilus	$\frac{dX}{dt} = \mu_{max}X\left(1 - \frac{X}{X_{max}}\right)$	2.06	0.290	-	-	Mercier et al. (1992)
L. delbrueckii	$\frac{dX}{dt} = \mu_{max}X\left(1 - \frac{X}{X_{max}}\right)$	2.95	0.732	-	-	Parajo et al. (1996)
L. delbrueckii	$\frac{dX}{dt} = \mu_{max}\frac{S}{K_s + S}X$	-	0.150	-	10.5	Buyukgungor et al. (1984)
L. xylosus	$\frac{dX}{dt} = \mu_{max}\frac{S}{K_s + S}X$	-	0.722	-	-	Tyree et al. (1990)
L. delbrueckii	$\frac{dX}{dt} = \mu_{max}\frac{S}{K_s + S}X$	-	0.831	-	-	Monteagudo et al. (1997)
L. bulgaricus	$\frac{dX}{dt} = \mu_{max}\frac{S}{K_s + S}X$	-	0.700	-	1.8	Gadgil and Venkatesh (1997)
L. delbrueckii	$\frac{dX}{dt} = \mu_{max}\frac{S}{K_s + S}X - k_dX$	-	0.827	0.17	36.3	Suscovic et al. (1992)

2.4.5.2 Kinetics Model Of Substrate Utilisation

The substrate utilisation kinetics for lactic acid fermentation using *L. delbrueckii* may be expressed by an equation proposed by Monteagudo et al. (1997) which considers both substrate consumption for maintenance and substrate conversion to biomass and product. The rate of substrate utilisation is related stochiometrically to the rate of biomass and lactic acid formation. The substrate requirement to provide energy for maintenance is usually assumed to be first order with respect to biomass concentration, mX. This gives:

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\frac{dX}{dt} + \frac{1}{Y_{p/s}}\frac{dP}{dt} + mX \qquad .....(2.15)$$

The parameters of the biomass yield on the utilised substrate ($Y_{x/s}$), the product yield on the utilised substrate ($Y_{p/s}$) and maintenance coefficient (m) were estimated by non linear regression analysis. A similar model was used for the kinetics of substrate utilisation in lactic acid fermentation using *L. amylophilus* by Mercier et al. (1992) and *Streptococcus cremoris* by Aborhey and Williamson (1977).

Simpler model has also been proposed by Parajo et al. (1996) and Yeh et al. (1991). They assumed that the maintenance coefficient is very much smaller than the specific growth rate and therefore the substrate utilisation is only for the conversion of biomass and product. The equation is then given as:

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\frac{dX}{dt} + \frac{1}{Y_{p/s}}\frac{dP}{dt} \qquad .....(2.16)$$

The simplest model has been proposed by Suscovic et al. (1992). They assumed that the substrate utilisation is only for the conversion of biomass. By combining Monod equation to this model, the following equation can be obtained:

$$\frac{dS}{dt} = -\frac{1}{Y_{x/s}} \mu_{max} \left(\frac{S}{K_s + S} \right) X \qquad .....(2.17)$$

The parameters of biomass yield on the utilised substrate ($Y_{x/s}$,) and saturation constant (K_s) can be estimated using linear regression analysis.

Samuel and Lee (1980) have proposed the multi-substrate utilisation on lactic acid fermentation by *L. bulgaricus and L. plantarum* using sorghum extract. Despite the complexity of substrate utilisation, the relationship of total carbohydrate concentration and fermentation time has repeatedly shown an exponential (first order) decay of substrate. This gives:

$$-\frac{dS}{dt} = k_3 S \qquad .....(2.18)$$

The parameter of first-order rate constant (k_3) was estimated using linear regression analysis.

The glucose utilisation on lactic acid fermentation by *L. delbrueckii* has also been proposed by Hanson and Tsao (1972). The fermentation was analysed generally with irreversible biological reaction as follows:

$$X + S \rightarrow 2X + P \qquad ....(2.19)$$

The kinetic model for substrate utilisation can be expressed according to the following equation:

$$-\frac{dS}{dt} = k_s SX \qquad .....(2.20)$$

where, k_s is a substrate rate constant.

The kinetic models for substrate utilisation and kinetic parameter values have been reported in literature and are summarised in Table 2.10.

Table 2.10: The proposed kinetic models of substrate utilisation and estimated parameter values.

Strains	Model	$Y_{x/s}$	$Y_{p/s}$	m (hr^{-1})	Authors
L. amylophilus	$-\dfrac{dS}{dt} = \dfrac{1}{Y_x}\dfrac{dX}{dt} + \dfrac{1}{Y_p}\dfrac{dP}{dt} + mX$	0.250	0.620	0.001	Mercier et al. (1992)
L. delbreuckii	$-\dfrac{dS}{dt} = \dfrac{1}{Y_x}\dfrac{dX}{dt} + \dfrac{1}{Y_p}\dfrac{dP}{dt} + mX$	0.270	0.910	0.090	Monteagudo et al. (1997)
L. xylosus	$-\dfrac{dS}{dt} = \dfrac{1}{Y_x}\dfrac{dX}{dt} + \dfrac{1}{Y_p}\dfrac{dP}{dt} + mX$	0.209	0.880	0.740	Tyree et al. (1990)
L. delbreuckii	$-\dfrac{dS}{dt} = \dfrac{1}{Y_x}\dfrac{dX}{dt} + \dfrac{1}{Y_p}\dfrac{dP}{dt}$	0.246	0.910	-	Parajo et al. (1996)

2.4.5.3 Kinetics Of Lactic Acid Production

In some fermentation processes, the growth and the formation of product are only partly linked (mixed growth associated), especially in lactic acid fermentation that was described by Luedeking and Piret (1959). Norton et al. (1994) also reported that lactic acid production was strongly linked to biomass production. The enormous variety of fermentation processes known can be reduced in complexity using formal kinetic concept. With this approach, basically three types of fermentation can be distinguished such as growth associated product formation, mixed growth associated product formation and non growth associated product formation (Moser, 1983).

Many researchers have used the mixed growth associated product formation for the study of the kinetics of lactic acid production. They used the model described by Luedeking and Piret (1959). This model has a simple relationship between the rate of lactic acid production to both the growth rate and the biomass concentration. The equation is given as follows:

$$\frac{dp}{dt} = k_1 \frac{dX}{dt} + k_2 X \qquad\qquad .....(2.21)$$

where $\frac{dp}{dt}$ is volumetric product formation rate (gram /litre hour), k_1 is growth associated product formation (g product /g cell), and k_2 is non growth associated product formation (g product /h. g cell).

A great number of mathematical models for lactic acid production have been reported in literature. They proposed similar models to that developed by Luedeking and Piret (1959). These models represent lactic acid fermentation process using different strains and substrates.

Mathematical modelling and estimation of kinetic parameters for lactic acid production using high-glucose, high fructose and high-sucrose syrup by *L.delbrueckii* have been studied by Suscovic et al. (1992). The growth associated lactic acid production constant (k_1) and non growth associated product formation constant (k_2) were estimated by linear regression and the values of k_1 obtained were always higher than k_2. Similar result was also reported by Norton et al. (1994) but they used *L. helviticus* and whey permeate as a substrate.

Samuel and Lee (1980) also studied the production of lactic acid by *L.bulgaricus* and *L. plantarum* on sorghum extract. They used the same model and the kinetic parameters were also estimated by linear regression on integral balance equation.

Monteagudo et al. (1997) too proposed the kinetic model for lactic acid production on beet molasses using *L. delbrueckii.* Using Luedeking and Piret's model, improvement was made by the addition of a term indicating the dependence of the rate of acid production on inhibitor concentration. This model is developed as follows:

$$\frac{dp}{dt} = (k_1 \frac{dX}{dt} + k_2 X)(1 - \frac{P}{P_{max}})$$ …..(2.22)

The parameters were estimated by non-linear regression analysis, and similar results were also obtained as reported by previous researchers.

Mercier et al. (1992) proposed the kinetic model for lactic acid production by analogy with the microbial growth model and it is given by:

$$\frac{dp}{dt} = P_o' P (1 - \frac{P}{P_{max}})$$ …..(2.23)

where P_{max} is the maximum concentration and P'_o is defined as the ratio between the initial volumetric rate of product formation (r_p) and the initial product concentration P_o . The equation (2.23) can be directly solved to give the following expression:

$$P = \frac{P_o P_m \exp(P'_o t)}{P_m - P_o + P_o \exp(P'_o t)}$$ ….(2.24)

Similar expression is also proposed by Parajo et al. (1996) and this logistic model adequately describes the kinetics of lactic acid production in the glucose fermentation.

Hanson and Tsao (1972) proposed a kinetic model for lactic acid production based on the general bioreactios as given in equation (2.19). Similar with the substrate utilisation, the lactic acid production rate may be stated as:

$$\frac{dP}{dt} = k_p SX \qquad\qquad\qquad\qquad\qquad …..(2.25)$$

where k_p is product rate constant.

The kinetic models for lactic acid production and estimated kinetic parameter values as reported in literature are summarised in Table 2.11.

Table 2.11: The proposed kinetic models of lactic acid production and estimated parameter values.

Strains	Model	P_o' (g/l)	P_m (hr^{-1})	k_1 (g/g)	k_2 (g/h.g)	Authors
L. amylophilus	$P = \dfrac{P_o P_m \exp(P'_o t)}{P_m - P_o + P_o \exp(P'_o t)}$	0.29	21.4		-	Mercier et al. (1992)
L. delbreuckii	$P = \dfrac{P_o P_m \exp(P'_o t)}{P_m - P_o + P_o \exp(P'_o t)}$	0.17	15.5		-	Parajo et al. (1996)
L. bulgaricus	$\dfrac{dp}{dt} = k_1 \dfrac{dX}{dt} + k_2 X$	-	-	18.0	0.19	Samuel and Lee (1980)
L. xylosus	$\dfrac{dp}{dt} = k_1 \dfrac{dX}{dt} + k_2 X$	-	-	3.17	0.91	Tyree et al. (1990)
L. delbrueckii	$\dfrac{dp}{dt} = k_1 \dfrac{dX}{dt} + k_2 X$	-	-	0.23	0.09	Monteagudo et al. (1997)
L. plantarum	$\dfrac{dp}{dt} = k_1 \dfrac{dX}{dt} + k_2 X$	-	-	15.6	0.44	Samuel and Lee (1997)

2.4.6   Optimisation In Lactic Acid Fermentation

2.4.6.1 Introduction

The experimentation is required to solve problems but it is always cost and time consuming. When an experiment is planned, it has different objectives depending on what is known so far about the solution to the problems. There are three stages of experimentations such as screening, optimisation and verification. Screening experiments are small which include many variables and play an important role in the early stages of an investigation. Their objectives are to reduce problems, to focus on the important variables and to find out more about their best setting. The purpose of optimisation experiments is to build a mathematical model which can be used to predict the behaviour of the process being investigated and it aims at producing specific optimal values for the experimental factors. The simplest type of verification experiment shows that the predicted optimal process performance can be reproduced in a second experiment (Haaland, 1989).

2.4.6.2 Optimisation Through Experiment

Experimental optimisation of chemical reactions has always been of strong economic concern to chemical engineers and industrial mathematicians (Deming and Morgan, 1973). The optimisation through experimentation cannot be isolated so far with the experimental design, model building and optimisation. Even then it has been apparent that there are essential interrelationship among the topics of statistics, models and optimisation (Biles and Swain, 1980). In order to determine the optimum values of the process variables, the statistic of experimental design and the optimisation techniques are used and these will be presented and discussed in following sections.

a)   Experimental Design

The statistical design of the experiments as a proven technique continues to show increasing use in chemical and biochemical process industries. Experimental designs are frequently performed in the study of empirical relationship, in terms of mathematical model, between one or more measured responses and a number of variables factors. They have been successfully used a long time ago and these allow us to express the response as a polynomial model (Murphy, 1977; Monteagudo et al., 1994).

A factorial experimental design approach was used to optimise the composition of medium in fermentation process by many researchers. They used two-level full factorial design consisted of 2^n experiments (n is the number of factor). Machucha and Duran (1996) used 2^4 factorial designs to optimise some parameters affecting the growth rate of *Thermoacus aurantiacus*. It is a very powerful tool because it provides information about all main effect and two factors interaction.

Study about optimisation of the fermentation media for maximisation of surfactine production was carried out by Sen (1997). A 2^4 full factorial composite design was used. This procedure required 30 experiments consisted of 16 factorial designs with 8 stars points and six replicates at the centre points. The design was employed to fit the second order polynomial model. The result indicated that the central composite design is a useful design to acquire data to fit this polynomial.

Many researchers also employed full factorial design for 2, 3, and 4 variables to optimise the nutrient medium in liquid fermentation (Vazquez and Martin, 1998; Oijkaas et al. 1999). However, designs that are used most frequently for screening experiments are two level fractional factorial designs especially for 5 factors to reduce the sample size. Fractional designs are very efficient because of their smaller sample sizes (Haaaland, 1989; Box et al., 1978; Karthikeyan et al., 1996).

The number of experiments can be reduced by using only part of the factorial design (fractional factorial design). Many researchers applied two-level fractional

factorial designs to reduce the sample size, which become very efficient (Haaaland, 1989; Box et al., 1987; Karthikeyan et al., 1996). To estimate the error and check on the linearity, all of the designs were always further expanded to central composite design (CCD) with added star and centre points (Strange, 1990; Murphy, 1977; Hakkarainen et al., 1984).

A 2^{5-1} fractional factorial central composite experimental design was used by Karthikeyan et al. (1996) to optimise the nutrient for dextran production. In these designs there are 32 experiments comprised 16 factorial designs (-1/+1) with ten star points (-2/+2) and six replicates at centre point (0). Similar design was also utilised by Sinha (1998) to optimise the medium constituents for endogluconase production by *Trichoderma reesei*. Application of 2^{5-1} central composite experimental design was also reported by Carvalho et al. (1997) to optimise the conditions for transestrification reaction using cutinase in AOT-reversed micelles. They reported that application of fractional factorial central composite design allowed the attainment of information about each factor and the interaction among different factors. The identification of optimum value was easier with this methodology and further works were not required provided that the range of experimental conditions was well defined. Also, it was always possible to determine the more favourable conditions within the range studied when there was an optimum.

Many researchers utilised a central composite design to examine the influence of variables. The central composite design allowed us: 1) to show which of the variables significantly affect each response and 2) to optimise the values of variables that were found significantly in stage 1 (Monteagudo et al., 1992; Sung and Huang, 2000). The central composite design consists of 32 experiments for five experimental factors with ten stars points and six replicates at the centre points to allow estimation of the error and to provide a check on linearity (Strange, 1990; Murphy, 1977; Hakkarainen et al., 1984).

b)   Optimisation Techniques

Various procedures have been applied in combining optimisation and experimentation to seek the optimum solution of single response as a function of multiple independence variables, which are direct search and response surface methods (Biles and Swain, 1980).

Myers (1971) reported a response surface method to optimise the yield as a function of three independent variables in fermentation process for producing an antibiotic. The similar optimisation was also done by Biles and Swain (1980) but they used direct search methods to obtain the optimal yield. This solution was almost similar to the one found by Myers (1971) but interactions between factors could not be clearly examined.

Several optimisation works have also been reported by Sung and Huang (2000) and Liu and Tzeng (1998). The conventional methods of optimisation involve the changing of one independent factor while fixing the others at a certain level. These methods for multiple factor experimental design are time consuming and are incapable of detecting the true optimum, due to the interaction among factors especially. It was recognised that employing response surface method (RSM) can significantly minimise the number of experiments, evaluate mutual interactions between multiple variables, and optimise objective functions to predict the conditions for the best result. RSM had been successfully employed for optimising the medium ingredients and operating conditions in many bioprocesses (Lee and Chen, 1997; Sen, 1997; Liu and Tzeng, 1997; Karthikeyan et al., 1996).

c)   Response Surface Methods (RSM)

Response surface method (RSM) is an experimental strategy that was initially developed and described by Box and Wilson in 1951. It has been widely employed in the development of physical and chemical processes because it provides data to (1)

estimate linear, curvature and interaction effects of the variables studied, (2) optimise or evaluate multiple responses, and (3) generate statistically valid mathematical models which can be utilised for graphic interpretation of the process under study (Burtis et al., 1981).

Response surface methods (RSM) consist of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and responses are measured according to one or more selected criteria. The maximum values were taken as the response of the design experiments. The optimal conditions of the factors were obtained by solving the regression equation and also by analysing the response surface contour plots (Sen, 1997).

The optimisation of batch fermentation for lactic acid production by *L. delbrueckii* using response surface methods has been studied by Monteagudo et al. (1994) and Hakkarainen et al. (1984). The results of this study illustrated the ability of the method to satisfactorily predict and optimise biotechnological processes. Monteagudo et al. (1993) also studied the determination of the best nutrient medium for the production of lactic acid. The results also indicated that response surface methodology is well suited for process optimisation of nutrient medium and lactic acid fermentation.

CHAPTER III

MATERIALS AND METHODS

3.1   Introduction

In order to achieve the outlines of the objectives and the scopes of the research, several materials along with experimental and analytical procedures used in this study are presented and discussed with more details in the following sections.

3.2   Materials

3.2.1   Strain

The microorganism used in this study was *Lactobacillus delbrueckii subsp. delbrueckii ATCC 9649,* a mesophilic homo fermentative lactic acid bacterium. It was obtained from DSMZ Germany.

3.2.2   Stock Culture

In stock culture, the cell was maintained at - 80 ^{o}C in MRS broth containing 15 % (v/v) glycerol solution until it was ready for used. *Lactobacillus delbrueckii*

was maintained on MRS agar plate at 37 ^{o}C for 24 hours and transferred to fresh medium every month (Goksungur and Guvenc, 1997). The MRS medium was suggested by DSMZ catalogue (1993). The composition of 1 litre MRS medium are as follows: yeast extract, 5g; meat extract, 5g; peptone, 10g; K_2HPO_4, 2g; diammonium citrate, 5g; glucose, 20g; sodium acetate, 2g; $MgSO_4.7H_2O$, 0.58g; $MnSO_4.4H_2O$, 0.25g and 1 ml of Tween-80. All chemicals used were of analytical grade and used as received without pre-treatment or stated otherwise.

3.2.3   Substrates

The fermentation media used to carry out the fermentation process were solid and liquid pineapple waste juices obtained from Malaysian Cannery of Malaysia Sdn. Bhd. In order to study the effect of different types of sugar, glucose, fructose and sucrose were purchased from several suppliers (Sigma, Fluka and Merck). All chemicals used were of analytical grade and used as received.

3.3   Experimental Methods

3.3.1   Pineapple Waste Treatment

The liquid pineapple waste contains undissolved and suspended particulate matters that need to be separated before used to avoid any interference in biomass determination. The solution was boiled for 5 minutes resulting particulate flocs which settled rapidly upon cooling at room temperature. The particulate was then separated by centrifugation at 4000 rpm for 15 minutes. The clear supernatant was stored at -18^{o}C (Lazaro et al., 1989; Samuel and Lee, 1980). Before use, the samples were carefully defrosted using a microwave oven and filtered through Whatman No. 54 filter paper under vacuum (Blake and Clarke, 1987). The solid pineapple waste sample was dried in the oven at 55^{o}C for a week, reduced the size by blender and

then screened with screener (Lazaro et al., 1989; Bryan, 1990). To analyse the sugar content in solid waste, 10g of sample was transferred to glass beaker where 100 ml of solvent mixture of alcohol:water (1:1) was added and then weighed for example, x gram. The mixture was placed in water bath at 80-85^{o}C for 25 minutes, stirred occasionally, then cooled at room temperature and added with the solvent until the original weigh (x gram). The mixture was then centrifuged for 10 minutes at 2000 rpm. If the mixture is not clear, it will be recentrifuged for 5 minutes at 3500 rpm and filtered through 0.45-0.7 μm filter paper. The filtrate was used to determine the sugar content in the solid waste (Zygmunt, 1982).

3.3.2 Extraction Of Sugar From Solid Waste

The solid pineapple wastes were extracted by mixing 10g solid in 90g distilled water and then being shaken in the incubator shaker at 200 rpm for one hour which was adequate to achieve the equilibrium. The mixture was then filtered to remove the solid particles followed by centrifugation. The supernatant was used to determine the physical and chemical properties which represent the characteristics of the solid waste.

3.3.3 Inoculum Media Preparation

Each fermentation process was initiated by transferring a small amount of biomass to a 250 ml Erlenmeyer flask containing 50ml of liquid MRS medium. Anaerobic condition was created by flushing with nitrogen and sealing them with tight-fitting rubber stopper. The flask was then incubated in incubator shaker at 37^{o}C, 150 rpm for 24 hours (Sakamoto and Komagata, 1996; Mercier, et al., 1992; Chatterjee and Chakrabarty, 1997).

3.3.4 Batch Fermentation

Parameter studies such as pH, temperature, initial sugar concentration, inoculum size and nitrogen source were carried out in 3-litre fermentor (Biostat B Model, Braun, Germany). The fermentor was equipped with pH, temperature and dissolved oxygen controllers. The fermentor containing 950 ml of substrate was first sterilised at $121^{o}C$ for 15 minutes. 50 ml of inoculum was sterilised separately and added aseptically to the fermentor. Anaerobic system was produced by sparged the fermentor by nitrogen at 6.5 ml/minute and stirring speed at 50 rpm (Lund et al., 1992). Samples of 10-20ml were withdrawn from the fermentor at regular time intervals. The microbial cells were separated by centrifugation for dry biomass determination. The supernatant was immediately frozen for further determination of the lactic acid, glucose, fructose and sucrose concentrations (Mercier et al., 1992). The flow chart of liquid pineapple waste fermentation to lactic acid by *L. delbrueckii* is shown in Figure 3.1

The optimisation study was carried out in incubator shaker (New Brunswick Scientific, Innova 4080 Model). The shake flask fermentation was performed by transferring 5 ml of inoculum to a 250 ml Erlenmeyer flask containing 95 ml of fermentation medium and, by adding 3g of $CaCO_3$ (3% w/v) to control the pH (Goksungur and Guvenc, 1997; Vahvaselha and Linko, 1987). The flask was then incubated in incubator shaker at certain temperature and speed for 10 days. The fermentation broth was then separated by centrifugation and the supernatant was used for the determination of lactic acid and residual sugar.

In studying the effect of size and type of fermentor, the culture vessels of B2 and B5 of the BIOSTAT B were used with total volume of 3 and 6.6 litres, respectively. The geometric of two fermentors is shown in Figure 3.2.

Liquid Waste

| Boiled

Glass Beaker (2 litres)

t = 5 minutes

| Separated

Centrifuge

N = 4000 rpm

t = 15 minutes

| Stored

Freezer

T = - 18^{o}C

| Defrosted

Microwave Oven

| Separated

Vacuum Filter

Inoculum media (5% v/v) →

Yeast extract (0.5 % g/v) →
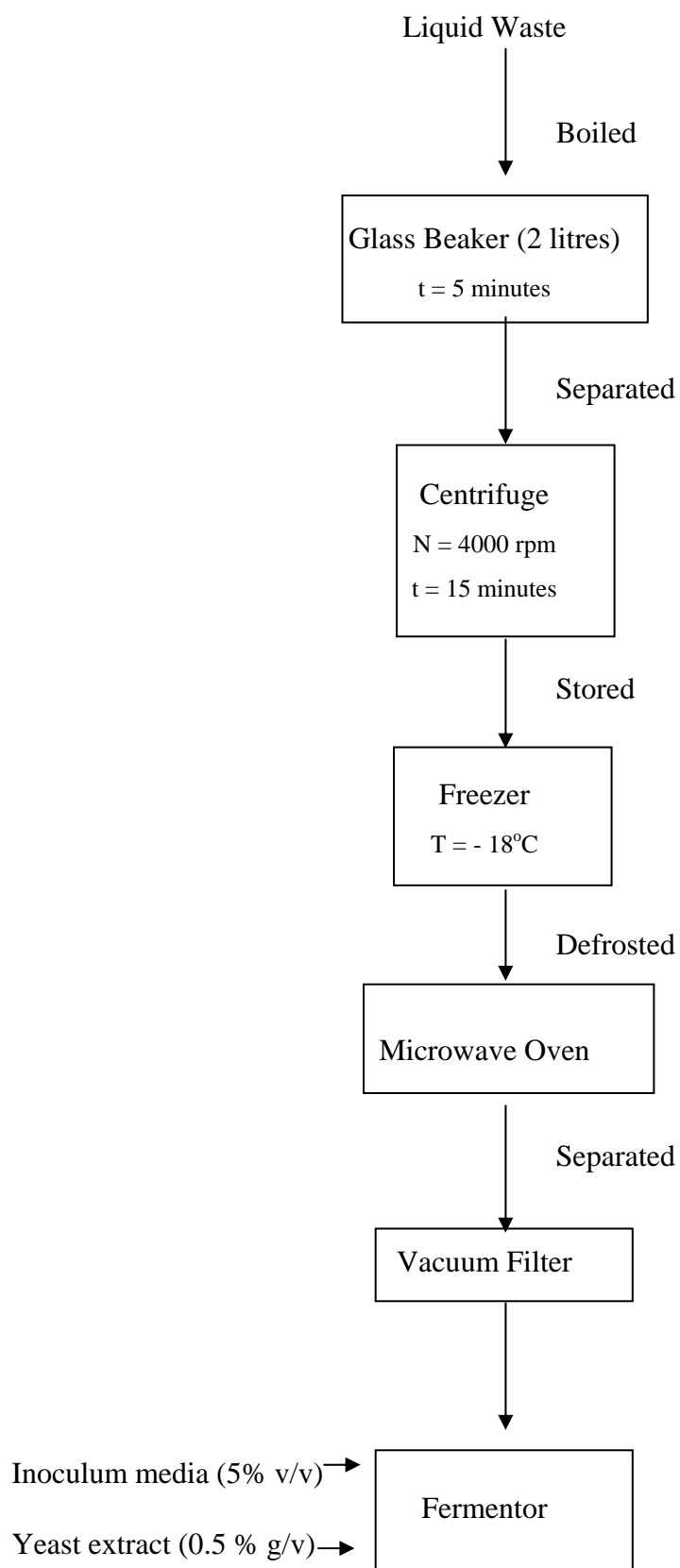
Fermentor

Figure 3.1: Flow chart for lactic acid production from liquid pineapple waste.
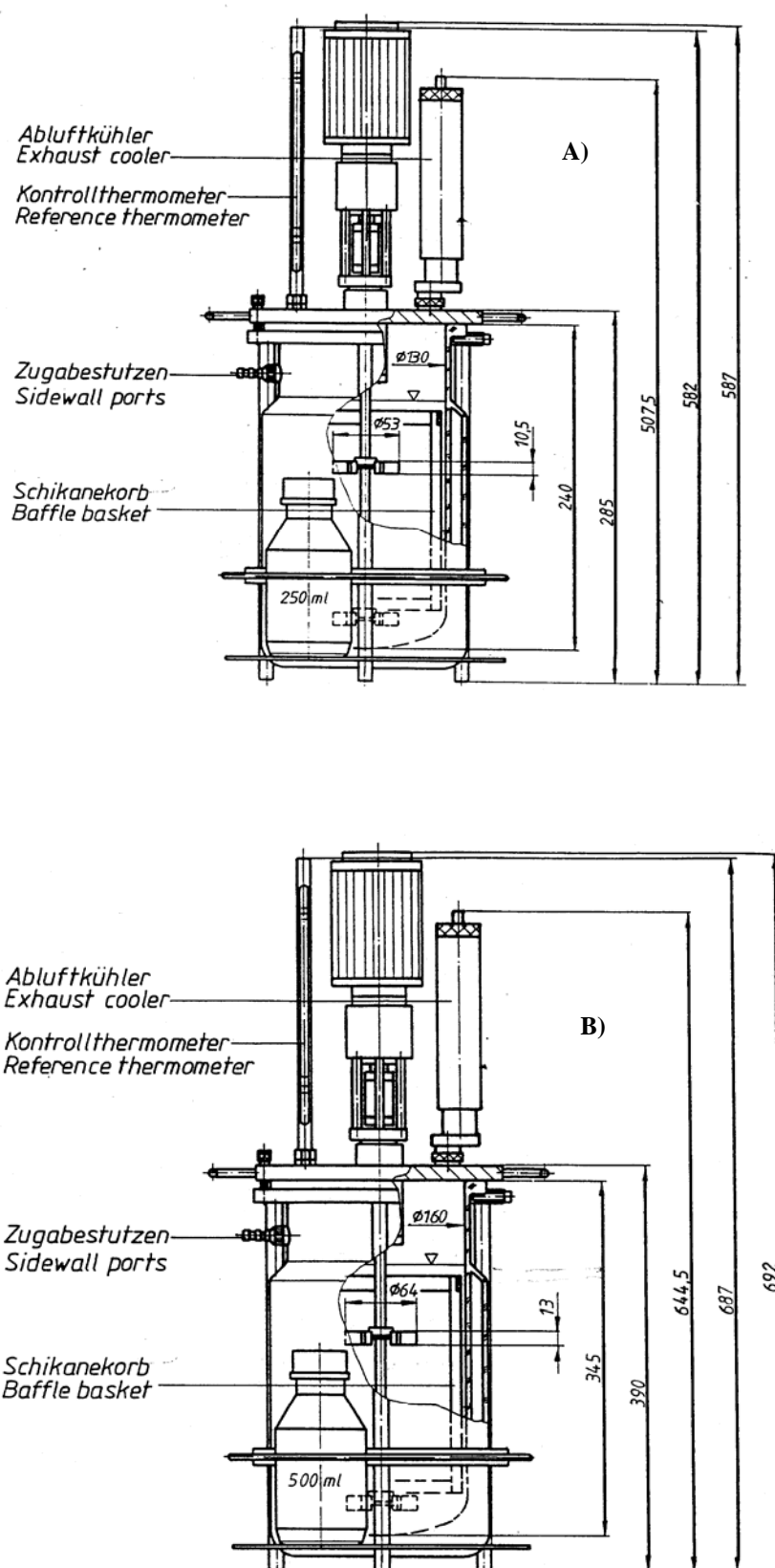
Figure 3.2: The culture vessel with total volume A) 3 litres and B) 5 litres.

3.3.5   Fed Batch Fermentation

The fermentation was carried out in 3-litre fermentor (Biostat B Model, Braun, Germany) with initial broth of 0.70 litre. The fermentation was performed in two phases: in the first phase, the lactic acid bacteria were grown in batch culture until the late of the exponential growth phase (20 hours), and in the second phase, the medium was continuously added into the fermentor using peristaltic pump at a constant feeding rate of 14 ml/h until the total volume of liquid reaches to 2.5 litres. The fermentation conditions used were similar to that described in batch fermentation (Roukas and Kotzekidou, 1998; Chen and Zhang, 1997).

3.4   Analytical Methods

3.4.1   Pineapple Waste Characterisation

3.4.1.1 Metal Content

The metal concentration was determined according to the method described by Clesceri et al. (1989). The method of Atomic Absorption Spectrophotometer (model: Philips PU 9200) and Direct Air-Acetylene Flame was used. In this measurement, the hallow cathode lamp was used where each specific lamp was selected for each element being measured. The measurement was carried out at least in triplicates and the standard deviation for the measurement was 0.002.

3.4.1.2 Anion Content

Sulphate, nitrate, phosphate and chlorine ions were measured by using the Ion Chromatography (model LC20) equipped with Dionex DX 500 Column and the electric chemical detector ED40. Fig. 3.3 shows the chromatogram for liquid

pineapple waste. The measurement was carried out at least in triplicates and the standard deviation for the measurement was 1.17.
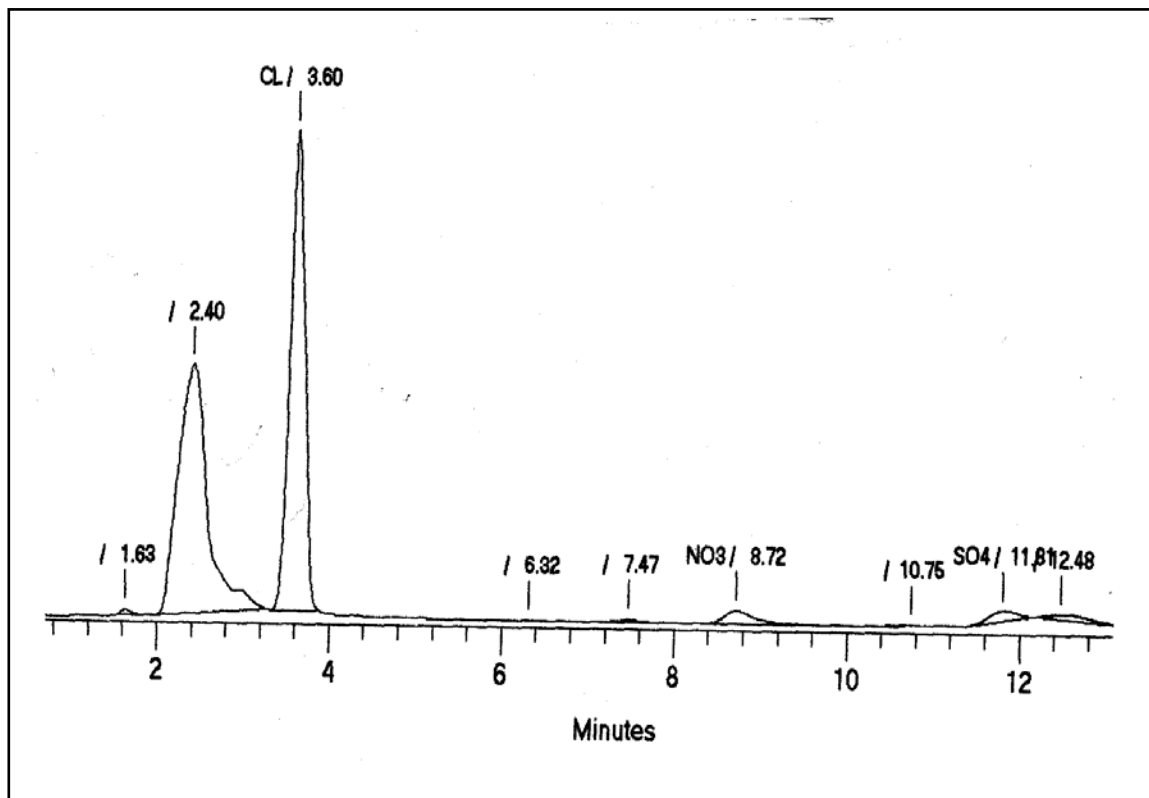
Figure 3.3: A typical chromatogram for liquid pineapple waste sample.

3.4.1.3 pH

The pH was measured by the Cyberscan 1000 pH meter supplied by Chemopharm Sdn. Bhd. The electrode assembly should be checked at least daily with buffer solution of pH = 7, and samples for ordinary control work should be cooled to room temperature. The electrode assembly was rinsed thoroughly with the test solution, and immersed in the solution to sufficient depth to cover the bulbs of the electrode. The reading of pH should be taken after a minimum period of five minutes. The measurement was carried out at least in triplicates and the standard deviation for the measurement was 0.01.

3.4.1.4 Moisture Content

Moisture content measurement was carried out according to Malaysian Standard (1973). 5g of sample were accurately weighed into a dish and dried in an air oven at $105 \pm 2\ ^{o}C$. The sample was then cooled in a desiccator and weighed. Drying, cooling and weighing were repeated until two consecutive weighing should not deviate by more than 1 milligram. The moisture content was calculated according to equation (3.1).

$$\text{Moisture content (\%)} = \left(\frac{W_1 - W_2}{W_1 - W}\right) \times 100 \qquad \qquad \ldots\ldots(3.1)$$

where W_1 is weight in g of dish and sample before drying, W_2 is weight in g of dish and sample after drying and W is weight in g of empty dish. The measurement was carried out at least in triplicates and the standard deviation for the measurement was 0.21.

3.4.1.5 Ash Content

Ash content was determined according to Malaysian Standard (1973). 5g of sample were accurately weighed into a dish and the material was ignited with the flame of a suitable burner for about 1 hour. The sample was then transferred into a muffle furnace until grey ash was obtained. The sample was then cooled in a desiccator and weighed. Igniting, cooling and weighing were repeated at half-hour intervals until two consecutive weighing should not deviate by more than 1 milligram. The ash content was calculated according to equation (3.2).

$$\text{Ash content (\%)} = \left(\frac{W_2 - W}{W_1 - W}\right) \times 100 \qquad \qquad \ldots\ldots(3.2)$$

where W_1 is weight of sample and dish in g before ashing, W_2 is weight of dish and ash in g, and W is weight of empty dish in g. The measurement was repeated at least three times and the standard deviation for the measurement was 0.24.

3.4.1.6 Crude Fibre Content

Crude fibre content of the sample was determined according to Malaysian Standard (1973). 2.5 g of sample were accurately weighed and placed in a Soxchlet apparatus. The sample was then extracted for about 1 hour with petroleum ether. The sample which was free of the fat material was then transferred to the flask and 200 ml of hot dilute sulphuric acid were added to the flask. The flask was then connected to a water-cooled reflux condenser and boiled for 30 minutes. After that, the content of the flask was filtered through a fine linen hold in funnel. The residue was washed on the linen with boiling water until the washings were no longer acidic to litmus. The residue was transferred into the flask with 200 ml of boiling sodium hydroxide solution. The condenser was fitted to the flask and reflux for 30 minutes. The condenser was then removed and the contents of the flask were filtered through the filtering cloth. The residue was thoroughly washed with boiling water and then with 15 ml of ethanol 95 % and finally transferred to a Gooch crucible. The crucible was dried in an air oven at $105 \pm 2\ ^oC$, then cooled in a desiccator and weighed (W_1). The contents of the crucible were ignited in the muffle furnace at $600 \pm 20\ ^oC$ until all carbonaceous matters were burnt. The crucible containing the ash was cooled in a desiccator and weighed (W_2). The crude fibre content was calculated according to equation (3.3).

$$\text{Crude fibre content} (\%) = \left(\frac{W_1 - W_2}{W} \right) \times 100 \qquad \text{……(3.3)}$$

where W_1 is weight in g of crucible and contents before ashing, W_2 is weight in g of crucible containing ash, and W is weight in g of sample. The measurement was carried out at least in triplicates and the standard deviation for the measurement was 0.51.

3.4.1.7 Crude Protein Content

Crude protein content was carried out according to Malaysian Standard (1973) using the Kjeldahl method. 1g of sample was accurately weighed and transferred to the Kjeldahl flask containing 25 ml of concentrated sulphuric acid, 10g of potassium sulphate, 0.3g of copper sulphate, and 0.1g of zinc metal. The mixture was than digested until the solution turned green or light green in colour (30-60 minutes). The flask and the content were cooled slightly before being transferred to the distillation unit. 250ml of water and 50ml of 50 % sodium hydroxide were added into the flask. The distillate was collected in 50ml of sulphuric acid (0.1N) containing the methyl red indicator. The distillate was then titrated with 0.1 N of the standard sodium hydroxide until an end point was observed. The blank sample containing 1g of pure sucrose was determined and the titrate deducted from the sample titrate. The crude protein or total nitrogen content was calculated according to equation (3.4).

$$\text{Nitrogen content} (\%) = 1.4 \left(\frac{V_2 - V_1}{W} \right) N \qquad\qquad ……(3.4)$$

$$\text{Crude protein} = \% \text{ Nitrogen} \times 6.25$$

where V_2 is volume in ml of sodium hydroxide required for the blank determination, V_1 is volume in ml of sodium hydroxide required for the sample determination, N is the exact normality of sodium hydroxide standard volumetric solution and W is 1.0 g. The measurement was carried out at least in triplicates and the standard deviation for the measurement was 0.053.

3.4.1.8 Reducing Sugar

Reduction sugar was measured by using an alkaline 3,5-dinitrosalicilioc acid (DNS) method. 1.0 ml of distilled water was transferred into a test tube (blank) and 1.0 ml of standard glucose solution (0.25-1.5 g/l) was also transferred into 5 other

labelled test tubes. 1.0 ml of DNS reagent and 2 ml of distilled water were added to each tube using pipettes. All tubes were heated in boiling water bath for 5 minutes to allow the reaction between glucose and DNS. The mixture was then cooled and made up to 10 ml accurately with distilled water using pipette or burette. The mixture was then mixed well and the absorbance of the solution was measured at 540 nm (Ceirwyn, 1995). The concentration of reduction sugar was determined by standard curve of glucose concentration (Figure 3.4). The reduction sugar measurement was carried out at least in triplicates with the calculated standard deviation was 0.28.
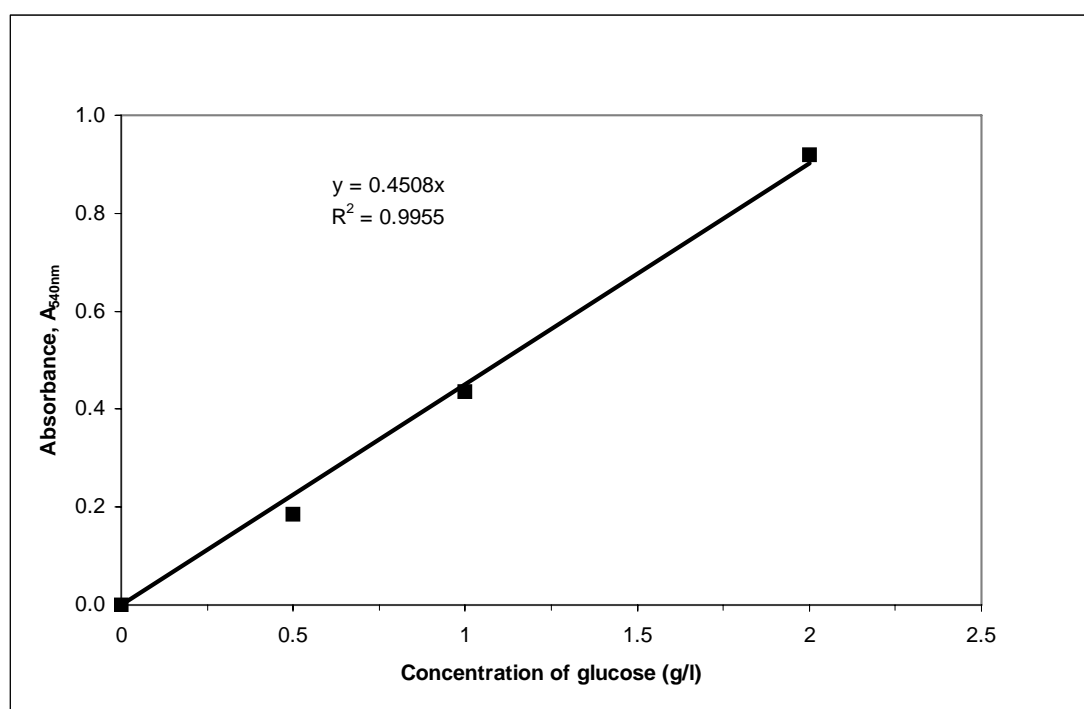
Figure 3.4: A calibration curve for glucose determination.

3.4.1.9 Total Sugar

The total sugar determination was carried out at first, by hydrolysing the non reducing sugar to reducing sugar. This was carried out by transferring 25.0 ml of sample into volumetric flask (50ml). The sample was then added with 2.5 ml of HCl 2 M and boiled for 5 minutes. The mixture was cooled and then neutralised with 10%

NaOH and made up to 50 ml using distilled water. This hydrolysate was then determined for the reduction sugar by DNS method (Ceirwyn, 1995). The absorbance of the solution was measured by using UV/VIS spectrophotometer at 540 nm. The total sugar measurement was carried out at least in triplicates with the calculated standard deviation of 0.42.

3.4.1.10 Phosphorus

Phosphorus was measured by the molybdenum blue calorimetric method. 0.00, 0.25, 0.5, 1.0 and 2.0 ml of the standard phosphate solution containing 0.1mg P per ml were prepared and 1 ml of each of the sample were transferred to a 100 ml of volumetric flask. To each flask 1 drop of phenolphthalein was added, neutralised with ammonia (1:4) and made up to 85 ml with distilled water. The mixture was added with 4 ml of ammonium molybdate reagent (4% ammonium molybdate in sulphuric acid) and 0.7 ml of 2% stanno chloride solution. The solution was then shaken, made up to 100 ml and kept in a cupboard for about 20 minutes to allow the blue colour to develope which will give the maximum absorption intensity. The absorbance of each solution was measured at 710 nm using UV/VIS spectrophotometer (Ceirwyn, 1995). The concentration of phosphorus was determined by standard curve of phosphorus concentration (Figure 3.5). The measurement was repeated at least three times and the standard deviation for the measurement was 0.050.
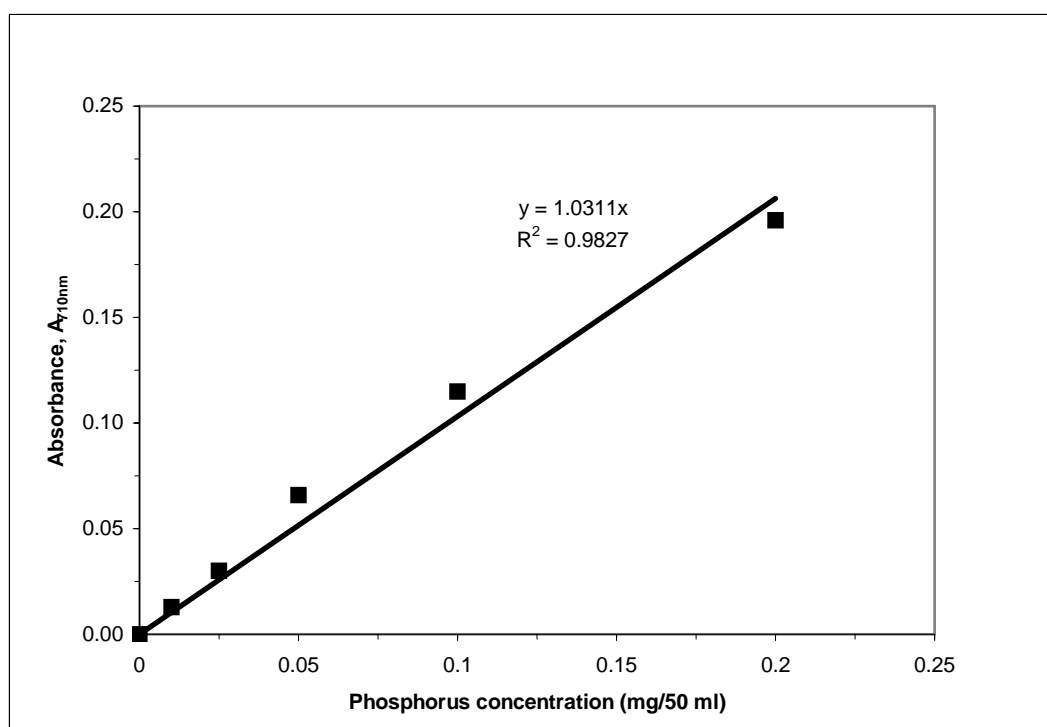
Figure 3.5: A calibration curve for phosphorus determination.

3.4.1.11 Soluble Protein

Soluble protein was measured according to Lowry method (Regensteine, 1984). 2 ml of Biuret reagent were added to 0.4 ml of the sample. This reagent consisted of 1ml of 1% cupric sulphates, 1 ml of 2% sodium potassium tartrate and 100 ml of 2% sodium carbonate. The solution was then mixed and cooled for 10 minutes at room temperature. After that, the solution was mixed rapidly within 1 second using 0.2 ml of Folin-Ciocalteu reagent. The absorbance of each solution was measured at 750 nm after 30 minutes. The soluble protein was estimated from the standard curve prepared with bovine serum albumin (Figure 3.6). The measurement was repeated at least three times and the standard deviation for the measurement was 0.036.
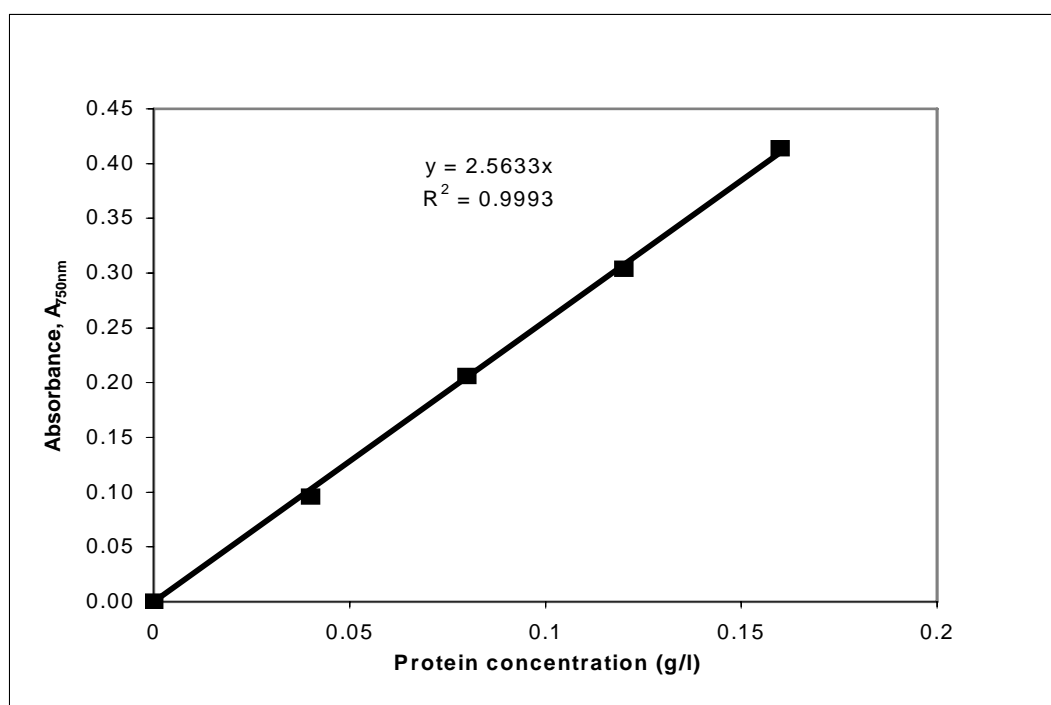
Figure 3.6: A calibration curve for protein determination.

3.4.1.12 Acidity

Total acidity was measured by Official Method of Analysis (AOAC, 1984). The pineapple waste contains a number of fairly simple organic acids such as malic acid and citric acid which are readily neutralised by strong bases and can be titrated against standard bases such as sodium hydroxide. The total acidity is then expressed as concentration of citric acid (g/l). 10 ml of sample were transferred into a 100 ml volumetric flask and made up to 100 ml with distilled water.10 ml of diluted sample were then transferred into 100 ml Erlenmeyer flask and 0.3 ml of phenolpthaline indicator was added to the Erlenmeyer. The solution was titrated to a faint pink end point with 0.1N sodium hydroxide.1 ml 0.1N NaOH is equivalent to 0.018 g citric acid. The total acidity was calculated according to equation (3.5).

$$\text{Total acidity (g/l)} = \text{ml 0.1N NaOH} \times 100 \qquad \qquad \ldots\ldots(3.5)$$

The measurement was repeated at least three times and the standard deviation for the measurement was 0.015.

3.4.1.13 Sugar

The individual sugar concentration was determined according to the method described by Official Method of Analysis (AOAC, 1984). Glucose, fructose and sucrose content were measured by HPLC (Waters TM 600), using a 300mm×4 mm ID μ Bondapak/Carbohydrate column (Waters) with RI detector. The eluent used was a mixture of acetonitrile:water (80:20) at a flow rate of 2 ml per minute and at ambient temperature. The samples were diluted with water before analysed. If a clear solution was not obtained, the solution was centrifuged at 500 rpm for 10 minutes. The samples was then mixed with acetonitrile:water (50:50) and filtered through 0.45 μm paper filter. 10 μl of the clear solution were injected into HPLC to obtain peak height or peak areas. Similar method was also performed on standard sugar solution. The sample sugar concentration was determined by comparing peak areas with those of the standard curve at various sugar concentrations as given in Figure 3.7. Hence the amount of each sugar in the sample taking was determined into the account of the dilution made.
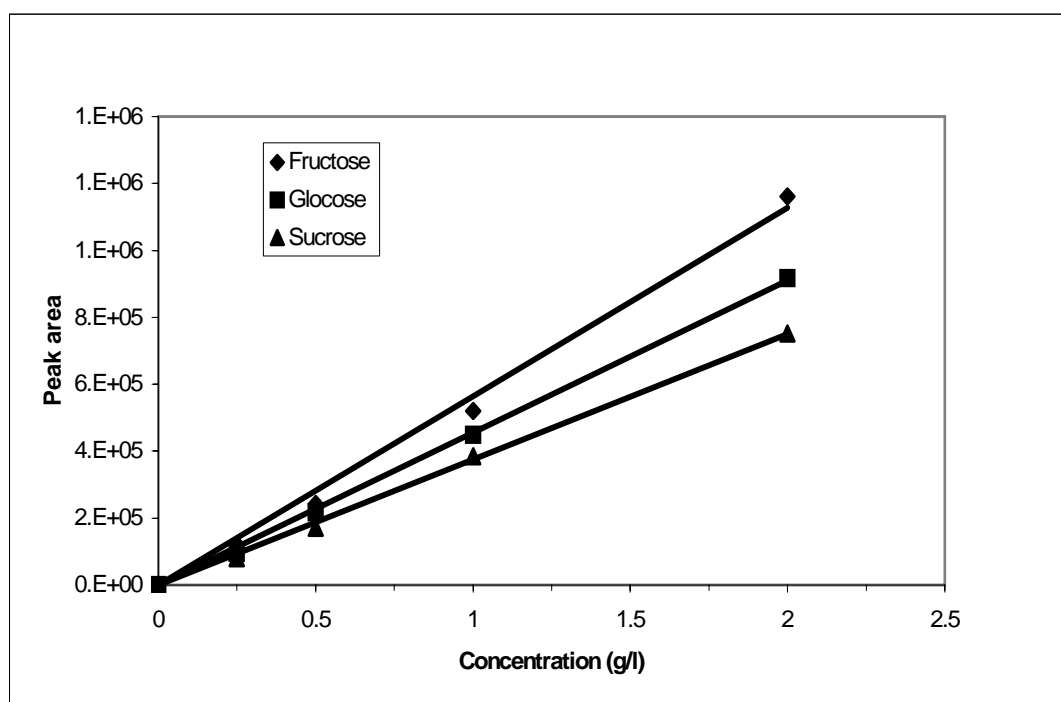
Figure 3.7: The calibration curves for fructose, glucose and sucrose determination.

3.4.1.14 Organic Acids

The organic acid concentration was determined using the method described by Brogley et al.(1993). The organic acid concentration was measured using HPLC (Waters TM 600). A 250mm×4.6 mm ID Spherisob Octyl column (Waters) with UV detector at maximum wavelength of 210 nm was used. The eluent used was 0.2 M phosphoric acid at flow rate of 0.8 ml per minute at ambient temperature. The samples were diluted with water before analysed. If a clear solution was not obtained, the solution was centrifuged at 500 rpm for 10 minutes. The samples were then mixed with 0.2 M phosphoric acid and filtered through 0.45 μm paper filter. 10 μl of the clear solution were injected into HPLC to obtain peak highs or peak areas. Similar method was also performed on standard organic acid solution. The concentration of the organic acid was determined by comparing peak areas with

those of the standard curves at various organic acid concentrations as given in Figure 3.8.
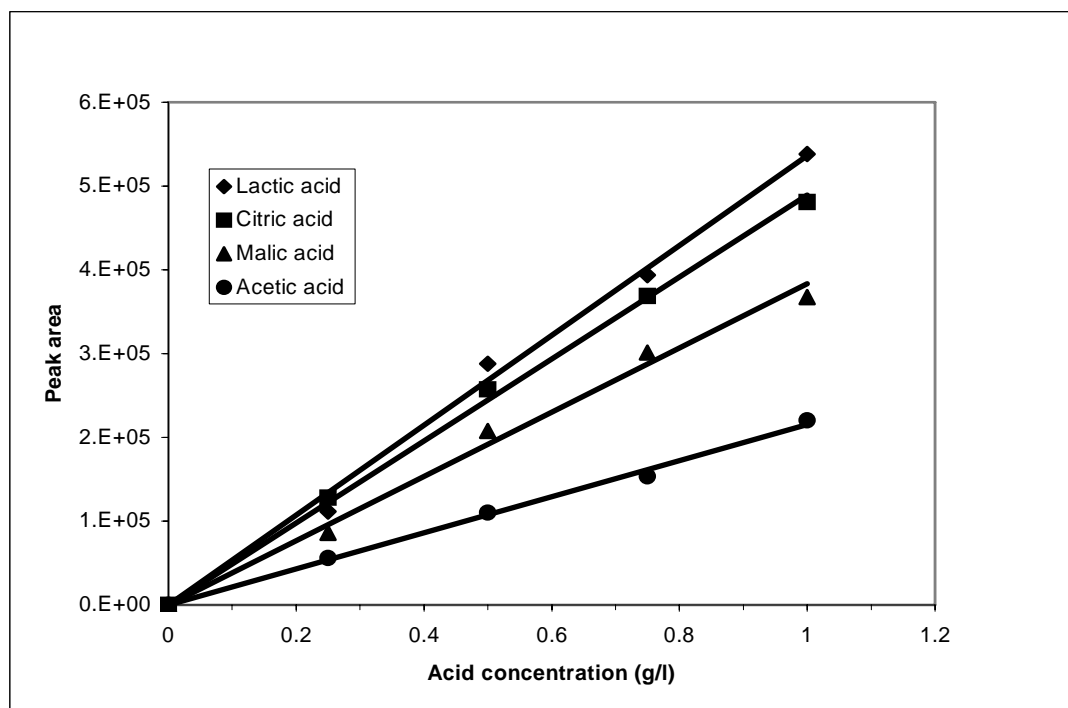
Figure 3.8: The calibration curves for organic acid determination.

3.4.2   Fermentation Product Analysis

3.4.2.1 Reducing Sugar

The reducing sugar in the culture broth was determined by DNS method described in Section 3.4.1.8. Each experiment was repeated at least three times and the calculation of standard deviation for the measurement was 0.24

3.4.2.2 Total Sugar

The total sugar in culture broth was determined at first by hydrolysing the non reducing sugar to reducing sugar as described in Section 3.4.1.9. This hydrolysate was then determined by DNS method described in Section 3.4.1.8. Each experiment was repeated at least three times and the calculation of standard deviation for the measurement was 0.41

3.4.2.3 Sugar

Fructose, glucose and sucrose in the culture broth were determined using the method described in Section 3.4.1.13. The chromatogram of sugar standard solution, pineapple waste, and fermentation sample is given in Figure 3.9. The measurement was repeated at three times and the calculation of standard deviation for the measurement was 0.2

3.4.2.4 Organic Acids

The lactic, acetic, citric and malic acid in the culture broth were determined by the method described in Section 3.4.1.14. The chromatograms of standard solution and fermentation sample are given in Figure 3.10. Determination of the organic acids was carried out in triplicates with the calculated standard deviation of 0.15.
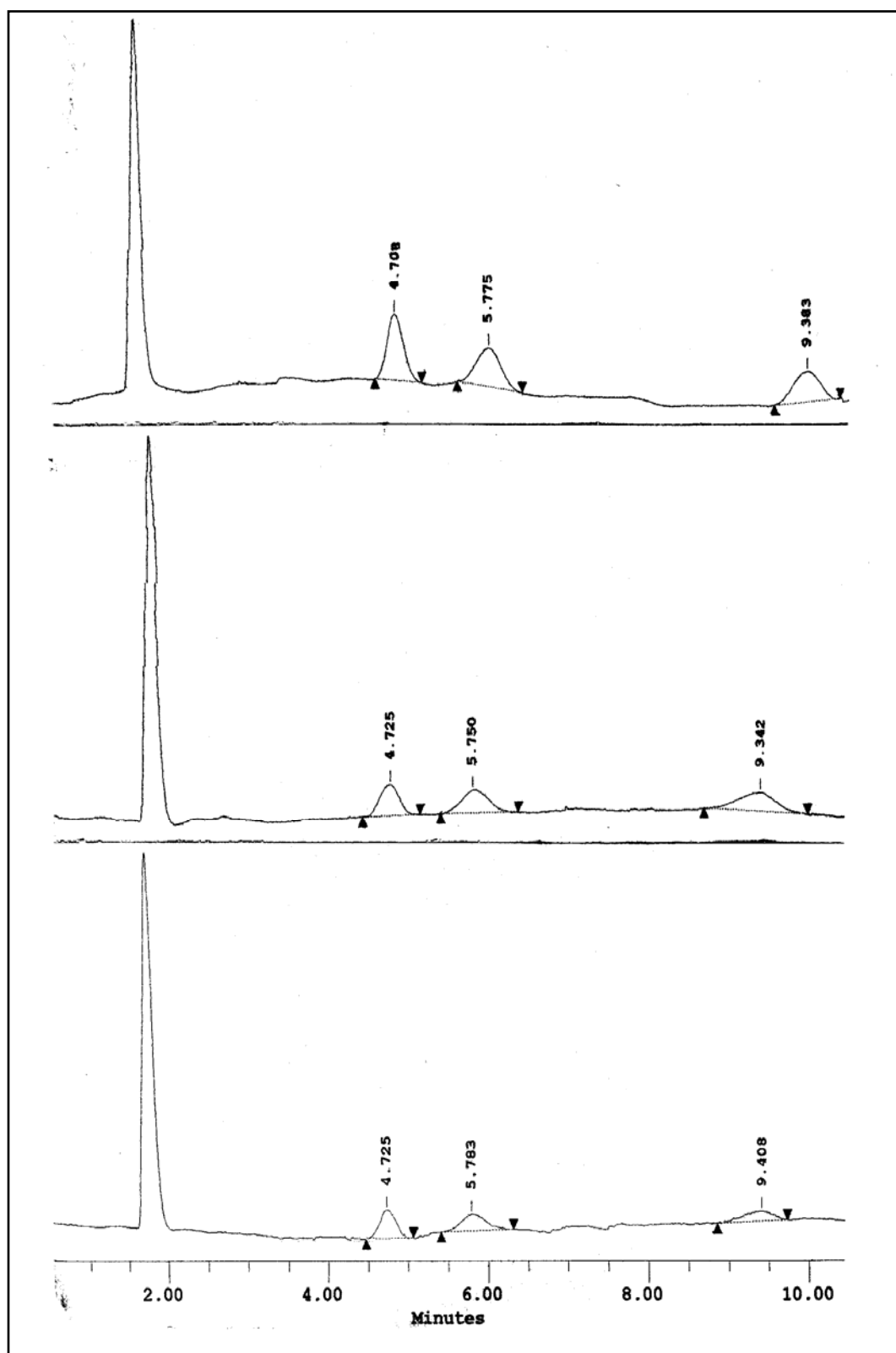
Figure 3.9: The chromatograms for standard sugar solution, pineapple waste, and fermentation samples. Retention time for acetonitrile (solvent), fructose, glucose and sucrose are 1.7, 4.71, 5.80 and 9.40 minutes, respectively.
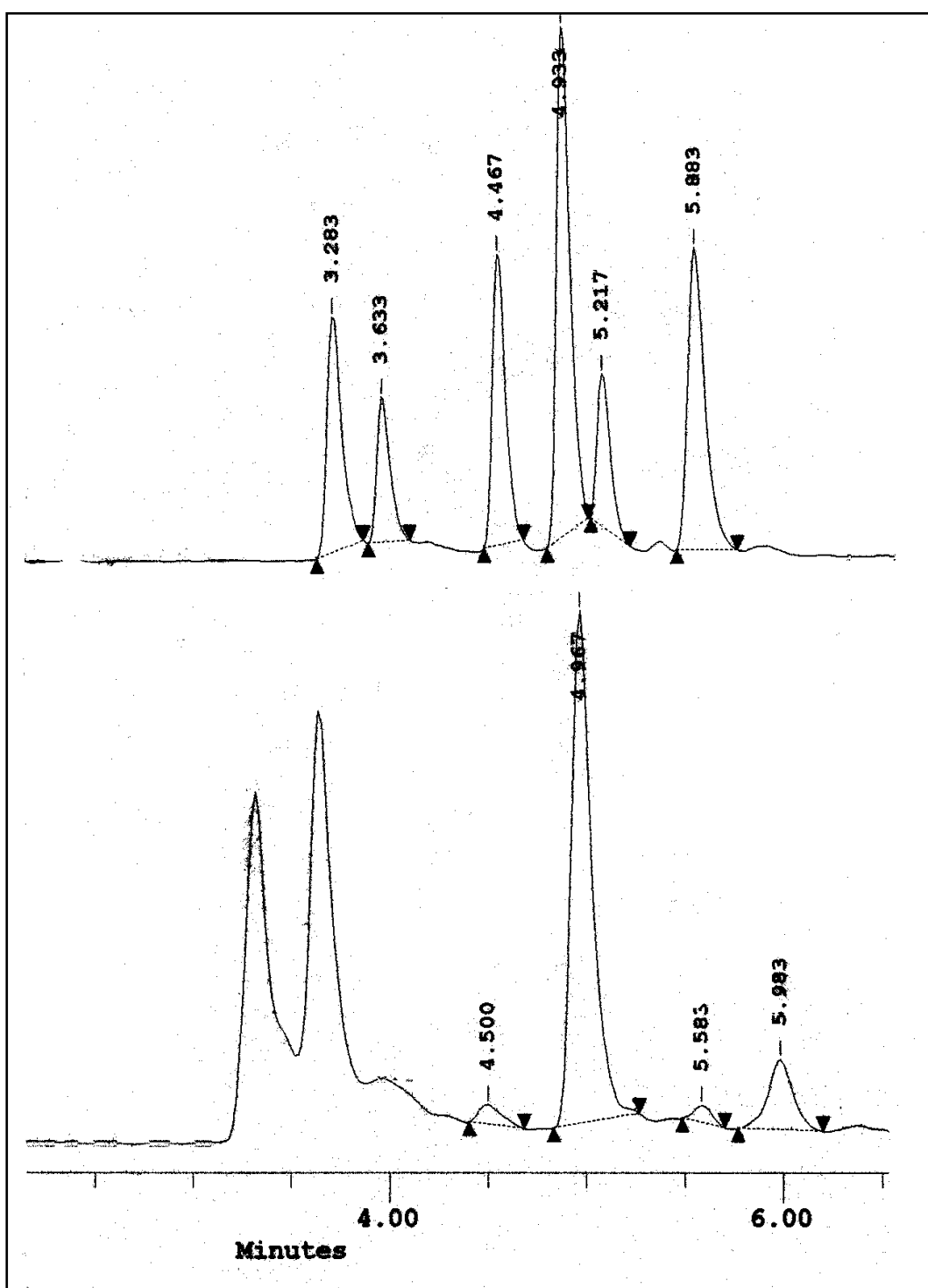
Figure 3.10: The chromatograms for the standard organic acids solution and fermentation samples. Retention time for phosphoric acid, water (solvent), malic acid, lactic acid, acetic acid and citric acid are 3.28, 3.63, 4.46, 4.93, 5.21 and 5.88 minutes, respectively.

3.4.2.5 Biomass

Cell concentration was measured by constructing a calibration curve of optical density as a function of dry cell weight. Dry cell weight was determined by centrifugation of the sample at 4000 rpm for 15 minutes. The supernatant was carefully removed by pipette and the pellet was washed twice with distilled water and recentrifuged. The washed cell was dried to a constant weight at 103 ^{o}C in the centrifuge tube. The optical density was measured using UV/VIS spectrophotometer (UV-1601 Model) at 620 nm (Aeschlimann and Stockar, 1987; Monteagudo et al., 1997). The dry cell weight was estimated from the standard curve of dry cell weight concentration versus optical density as given in Figure 3.11. The measurement was repeated at least three times with the standard deviation of measurement obtained at 0.31.
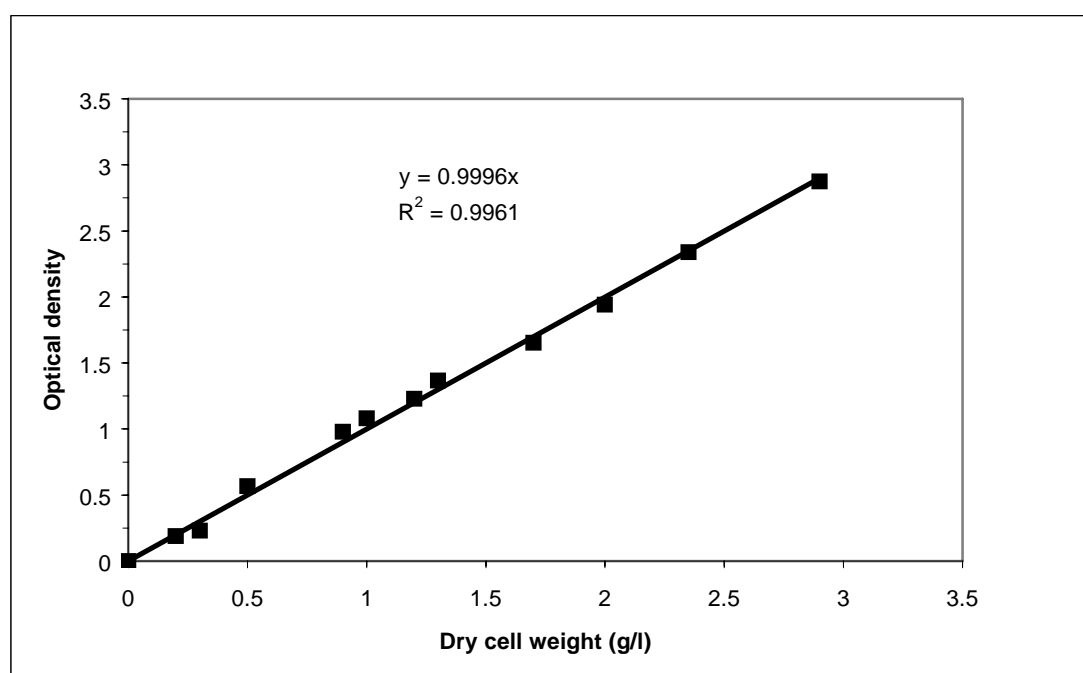
Figure 3.11: A calibration curve for dry cell weight (biomass) determination.

CHAPTER IV

PARAMETRIC STUDY OF LACTIC ACID FERMENTATION

4.1   Introduction

Several parameters such as temperature, pH, inoculum size, initial substrate concentration and types of nitrogen sources that affect the lactic acid fermentation as reported in the literature were reviewed and discussed in Chapter II. In this chapter, the effect of those parameters on lactic acid fermentation of pineapple waste as well as on pure and mixed sugar is presented and discussed. Since the pineapple waste contains not just carbon source such as glucose, fructose and sucrose as reported in the literature, the characterisation of this waste is important in order to know the actual composition of the waste which will definitely affect the results of the fermentation process. Therefore the characterisation results are presented and discussed in this chapter.

4.2   The Characteristics Of Pineapple Wastes

In this study, three types of pineapple waste were characterised which were the liquid, solid and liquid extract pineapple wastes. This characterisation study is important since the compositions of the wastes especially from food processing factories are varied and are dependent on both the nature of the product and the

production technique employed. Hence, the characteristics of the waste will affect the lactic acid fermentation process.

4.2.1   The Characteristics Of Liquid Pineapple Waste

The variation of physical and chemical composition of liquid pineapple waste for different sampling times from pineapple cannery is given in Table 4.1. Table 4.2 shows the comparison of physical and chemical compositions of liquid pineapple waste between the present work and the report of Sasaki et al. (1991). Analysis of sugar indicates that the liquid pineapple waste contains sugar mainly sucrose, glucose and fructose. The fructose concentration was slightly higher than glucose concentration. These results are similar to those for pineapple juice as reported by Krueger et al. (1992) as given in Table 2.2, but it is different with those reported by Sasaki et al. (1991) for liquid pineapple waste stating that the sucrose content is higher than glucose's and the fructose content is smaller than glucose's. The difference in sugar composition is probably due to wastes generated by different processes, season and area. Analytical results of soluble protein and total nitrogen obtained were at the range of 1.13-2.38 g/l and 0.64-1.4 g/l, respectively.

The pH of the liquid pineapple waste is between 4.0 to 4.5, which is quite similar to the pH of pineapple juice as reported by Moon and Woodroof (1986) and Sasaki et al. (1991) (Table 2.5). Organic acid analysis by liquid chromatography indicated that the acidity is mainly due to citric acid and malic acid. The value of acidity in pineapple waste is ranging from 2.6-4.8 g/l with the ratio of citric acid and malic acid between 4.2-8.7. These results are similar to those reported by Krueger et al. (1992) where the acidity in pineapple juice is between 4.6-12 g/l and the ratio of citric acid and malic acid is between 1.8 and 8.2. The pH decreases with increasing total acidity indicated by increasing organic acid content in the liquid pineapple waste.

The waste contains very little nitrogen and soluble protein. The elements such as Fe, Ca, Mn, Zn, Cu, Cd, Na and K are also present in the waste. Potassium content is the highest (425-612 mg/l), but it is lower than the potassium content in pineapple juice as reported by Krueger et al (1992). The chlorine ion concentration is higher than sulphate and nitrate, but the phosphate ion concentration is nil for all samples analysed.

Table 4.1: The characteristics of the liquid pineapple waste at different sampling times.

Composition	Parameters	Nov. (1997)	June (1998)	June (1999)	March (2000)
Sugars	Reducing sugar (g/l)	17.65	39.62	39.64	42.38
	Sucrose (g/l)	6.54	15.51	13.89	14.37
	Glucose (g/l)	9.00	20.00	19.54	20.29
	Fructose (g/l)	9.88	20.76	20.18	21.47
	Total sugar (g/l)	30.86	70.20	66.18	65.04
Proteins	Soluble protein (g/l)	1.36	1.10	2.38	0.82
	Total nitrogen (g/l)	0.86	0.53	1.097	0.46
Organic Acids	Acidity, as citric acid (g/l)	4.80	2.93	2.55	3.30
	Citric acid (g/l)	3.10	2.38	2.12	2.76
	Malic acid (g/l)	0.96	0.36	0.29	0.39
Cations	Fe (mg/l)	18.90	3.70	6.60	7.20
	Ca (mg/l)	89.00	145.0	44.0	82.00
	Mn (mg/l)	1.70	3.10	1.30	2.48
	Mg (mg/l)	53.0	55.60	69.0	55.40
	Zn (mg/l)	5.80	8.00	3.90	4.60
	Cu (mg/l)	1.00	1.80	0.80	1.30

Table 4.1: The characteristics of the liquid pineapple waste at different sampling times (Continued).

Compositions	Parameters	Nov. (1997)	June (1998)	June (1999)	March (2000)
Cations	Cd (mg/l)	0.00	0.00	0.00	0.00
	Na (mg/l)	382.0	346.0	310.4	307.0
	K (mg/l)	425.0	561.0	571.0	612.0
Anions	SO_4^{2-} (mg/l)	5.64	25.60	19.18	28.40
	PO_4^{3-} (mg/l)	0.00	0.00	0.00	0.00
	NO_3^{1-} (mg/l)	12.40	6.50	17.80	5.35
	Cl^{1-} (mg/l)	105.0	43.20	96.0	87.5
	Phosphorus (mg/l)	12.00	24.41	8..67	18.14
pH		4.00	4.30	4.50	4.10

Table 4.2: The comparison of the liquid pineapple waste composition between this work and as reported by Sasaki et al. (1991).

Composition	Parameters	This Work	Sasaki et al., 1991
Sugars	Reducing sugar (g/l)	40.62	39.20
	Sucrose (g/l)	15.51	40.10
	Glucose (g/l)	20.00	23.60
	Fructose (g/l)	20.76	14.0
	Total sugar (g/l)	70.20	100.00

Table 4.2: The comparison of the liquid pineapple waste composition between this work and as reported by Sasaki et al. (1991) (Continued).

Composition	Parameters	This work	Sasaki et al., 1991
Proteins	Soluble protein (g/l)	1.10	0.90
	Kjeldahl nitrogen (g/l)	0.53	0.20
Organic Acids	Acidity, as citric acid (g/l)	2.93	-
	Citric acid (g/l)	2.38	-
	Malic acid (g/l)	0.36	-
Cations	Fe (mg/l)	3.70	5.43
	Ca (mg/l)	145.0	3.31
	Mn (mg/l)	3.10	13.97
	Mg (mg/l)	55.60	62.50
	Zn (mg/l)	8.00	-
	Cu (mg/l)	1.80	2.02
	Cd (mg/l)	0.00	0.00
	Na (mg/l)	346.0	8.61
	K (mg/l)	561.0	-
Anions	SO_4^{2-} (mg/l)	25.60	169.7
	PO_4^{3-} (mg/l)	0.00	223.8
	NO_3^{1-} (mg/l)	6.50	-
	Cl^{1-} (mg/l)	43.20	-
	Phosphorus (mg/l)	24.41	-
pH		4.30	4.00

4.2.2   The Characteristics Of Solid Pineapple Waste

The compositions of solid pineapple waste obtained from this work and published by different authors are given in Table 4.3. The moisture content of the solid waste is ranging from 87.50-92.20 %. The difference of moisture content obtained by other researchers as reported in literature might result from the different samples obtained from various geographical origins and of varying degree of pineapple ripeness. Ash and phosphorus contents are 4 and 1%, respectively. This is quite similar to those reported by Chandapillai and Selvarajah (1978) because the wastes are obtained from the same area (Johor). The total nitrogen content in waste is 0.9 %, which is similar to those previously reported by other researchers.

The determination of sugar content in solid pineapple waste was carried out by extracting 10 g of solid waste using 100 ml of ethanol-air mixture (1:1). The solution was heated at 80 $^{\circ}$C for 15 minutes (Zygmunt, 1982). The glucose and fructose content in solid waste was found to be 8.24 and 12.17 %, respectively. Sucrose was not detected and this might be due to the sucrose conversion to glucose and fructose, and degradation and polymerisation of sucrose forming brown colour of glucosan polymer (Chen, 1993). The highest mineral constituent in the waste was potassium which was 4%, and it is similar to those previously reported by other researchers.

Table 4.3: The characteristics of solid pineapple waste.

Composition (% w/w)	This Work (1998)	Bardiya et al. (1996)	Viswanath (1992)	Chandapillai and Selvarajah (1978)
Moisture	87.50	92.20	87.69	89.70
Total solid	12.50	7.80	12.31	10.30
Ash	4.05	10.60	6.20	3.90
Organic carbon	-	51.85	38.90	-

Table 4.3: The characteristics of solid pineapple waste (Continued).

Composition (% w/w)	This work (1998)	Bardiya et al. (1996)	Viswanath (1992)	Chandapillai and Selvarajah (1978)
Total carbohydrates	-	35.00	-	-
Reducing sugar	20.93	-	-	-
Glucose	8.24	-	-	-
Fructose	12.17	-	-	-
Sucrose	0.00	-	-	-
Cellulose	-	19.80	-	-
Crude fibre	10.57	-	-	14.70
Hemicellulose	-	11.70	-	-
Total soluble	-	30.00	-	-
Total nitrogen	0.83	0.95	0.90	0.97
Crude protein	5.18	-	-	6.10
Ether extract	0.15	-	-	0.20
Phosphorus	0.14	-	0.08	0.10
Fe	0.20	-	-	-
Ca	0.26	-	-	-
Mn	0.01	-	-	-
Mg	0.40	-	-	-
Zn	0.02	-	-	-
Cu	0.03	-	-	-
Cd	0.00	-	-	-
Na	0.30	-	-	-
K	4.00	-	-	-
SO_4^{2-}	0.23	-	-	-
PO_4^{3-}	0.00	-	-	-
NO_3^{1-}	0.06	-	-	-
Cl^{1-}	0.38	-	-	-

4.2.3   The Characteristics Of Liquid Pineapple Waste Extract

The liquid pineapple waste extract was obtained by extracting the solid waste using water at a ratio of solid to water of 5, 10 and 15% (w/w). The extraction was carried out by shaking the mixture at 150 rpm and at room temperature for 100 minutes. This time is enough to extract the solid waste constituents. Table 4.4 shows the composition of liquid pineapple waste extract for different percentage of solid present. The concentration of sugar increases proportionally with increasing percentage of solid waste used. The result also indicates that the sucrose content is nil. Sucrose inversion during drying could be the reason to this. The inversion is a decomposition of sucrose to glucose and fructose under acidic condition, and the rate of reaction depends on temperature, pH, time and sugar concentration (Chen, 1993).

The mineral and soluble protein contents increase proportionally with increasing percentage of solid except for calcium. This might due to the limited solubility of calcium salt in water. The potassium content in the liquid pineapple waste extract was the highest which was similar to the liquid pineapple waste's. There was no change of pH if the percentage of solid was increased. The extraction process could be carried out up to 15% of solid waste only and thus the mixture was very viscous and it was very difficult to perform extraction.

Table 4.4: The characteristics of liquid pineapple waste extract.

Parameters	Solid (% w/w)		
	5	10	15
Fructose (g/l)	6.80	13.01	19.12
Glucose (g/l)	5.01	10.33	15.44
Sucrose (g/l)	0.00	0.00	0.00
Reducing sugar (g/l)	11.43	24.20	36.88
Soluble protein (g/l)	2.38	4.93	7.54
Fe (mg/l)	6.20	14.5	17.2
Ca (mg/l)	170.0	146.0	123.0
Mn (mg/l)	2.20	3.40	4.50
Mg (mg/l)	105.0	141.0	197.0
Zn (mg/l)	9.70	9.80	11.40
Cu (mg/l)	3.20	3.70	6.90
Cd (mg/l)	0.00	0.00	0.00
Na (mg/l)	276.0	411.0	500.0
K (mg/l)	758.0	1058	1205
SO_4^{2-} (mg/l)	125.0	191.0	227.0
PO_4^{3-} (mg/l)	0.00	0.00	0.00
NO_3^{1-} (mg/l)	32.00	115.0	197.0
Cl^{1-} (mg/l)	20.50	39.00	76.00
Phosphorus	6.00	10.3 0	22.4 0
pH	4.0 0	4.00	4.00

4.2.4 Sterilisation

All fermentation media such as substrate were sterilised to prevent contamination during fermentation process. Steam was used almost universally for the sterilisation process which was carried out in an autoclave at 120 ^{o}C and a pressure of 15 lb/in^2 for 15 minutes. Effect of sterilisation on pineapple waste composition is shown in Table 4.5.

The concentrations of glucose and fructose after sterilisation were constant but the concentration of sucrose decreased probably due to the caramelisation process. The caramelisation is the degradation of sucrose at high temperature (above 80 ^{o}C) and followed by polymerisation resulting in the formation of glucosan polymer with brown colour. Sasaki et al. (1991) results show that the contents of glucose, fructose, sucrose and total sugar after sterilisation are relatively constant, but in this work, only the contents of glucose and fructose are constant whereas total sugar and sucrose decreased. This result is different due to difference in the waste generated, season and area.

The protein concentration after sterilisation decreased because the protein is expected to denature at 55 ^{o}C. The acidity or organic acid content was not affected by heating and this is indicated by the constant pH value. The cation concentrations after sterilisation increased but anion concentrations decreased. This phenomenon cannot be explained at present therefore further investigation is required.

Table 4.5: Effect of sterilisation on pineapple waste compositions.

Composition	Parameters	This Work	
		A	B
Sugars	Reducing sugar (g/l)	40.40	40.62
	Sucrose (g/l)	16.75	15.51
	Glucose (g/l)	19.72	20.00
	Fructose (g/l)	20.62	20.76
	Total sugar (g/l)	73.76	70.20
Proteins	Soluble protein (g/l)	1.13	1.10
	Kjeldahl nitrogen (g/l)	0.64	0.53
Organic Acids	Acidity, as citric acid (g/l)	2.95	2.83
	Citric acid (g/l)	2.82	2.78
	Malic acid (g/l)	0.19	0.16
Cations	Fe (mg/l)	3.30	3.70
	Ca (mg/l)	194.0	245.0
	Mn (mg/l)	3.60	3.90
	Mg (mg/l)	47.70	55.60
	Zn (mg/l)	5.80	8.00
	Cu (mg/l)	1.40	1.80
	Cd (mg/l)	0.00	0.00
	Na (mg/l)	294.0	346.0
	K (mg/l)	526.0	561.0
Anions	SO_4^{2-} (mg/l)	25.60	19.50
	PO_4^{3-} (mg/l)	0.00	0.00
	NO_3^{1-} (mg/l)	8.20	6.50
	Cl^{1-} (mg/l)	256.0	243.20
	Phosphorus (mg/l)	27.40	24.41
pH		4.30	4.30

A: Before sterilisation   B: After sterilisation

4.3   Lactic Acid Fermentation Of Pineapple Wastes

Several parameters that affect the lactic acid fermentation process as reported in literature such as temperature, pH, inoculum size, initial substrate concentration and types of nitrogen sources were studied. In this section, the pineapple waste was used as an alternative to the conventional carbon sources such as glucose, fructose and sucrose. The results of this study are presented and discussed in the following sub-sections.

4.3.1   Liquid Pineapple Waste

The liquid pineapple waste after sterilisation contains 15 g/l, sucrose; 20 g/l, fructose; 20 g/l, glucose and 70 g/l, total sugar. In industrial fermentation of lactic acid, the carbohydrate containing medium has been enriched with nutrient such as yeast extract to achieve the optimal growth conditions for the demand of nutrient on lactic acid bacteria (Lund et al., 1992). Based on the characteristics of pineapple waste, it is known that the liquid pineapple waste has little nitrogen and soluble protein therefore the substrate must be supplemented with yeast extract as a nitrogen source. In this initial study, 0.5 % of yeast extract was supplemented to the liquid pineapple waste. The fermentations were carried out in the fermentor at $40^{o}C$; pH, 6.0; stirring speed, 50 rpm; inoculum, 5% and concentration of yeast extract, 0.5%. The results of the microbial growth, sugar utilisation and lactic acid production are given in Figure 4.1.

A profile of dry cell weight concentration shows that the lag phase ended approximately after 8 hours, followed by the logarithmic growth phase until 48 hours with the maximum concentration of biomass of 1.6 g/l. The stationary growth phase was obtained after 4 hours, followed by gradual decline of cell concentration (death phase). The decreasing of the cell concentration might be due to inhibition of lactic acid production and lack of the nutrient to maintain the cell growth. Although the growth ceased, the bacteria still produce lactic acid. The similar results were also

reported by Monteagudo et al. (1997) on the subject of lactic acid fermentation using beet molasses by *L. delbrueckii*. The maximum lactic acid concentration obtained was 54.97 g/l or 79 % yield at 168 hours. It was found that sucrose was diminished completely after 62 hours. During the fermentation process the microorganisms which have phosphorolytic enzymes will phosphorylyze the sucrose to glucose-1-phosphate and fructose (Freeman, 1985) and in acidic condition, the sucrose will be hydrolysed to glucose and fructose (Chen, 1993). As a result, the concentrations of glucose and fructose increase. This also indicates that the rate of sucrose hydrolysis is faster than fermentation process. The glucose consumption is better than fructose's, but both sugars were not completely utilised even after 240 hours. The sucrose was completely utilised after 48 hours.
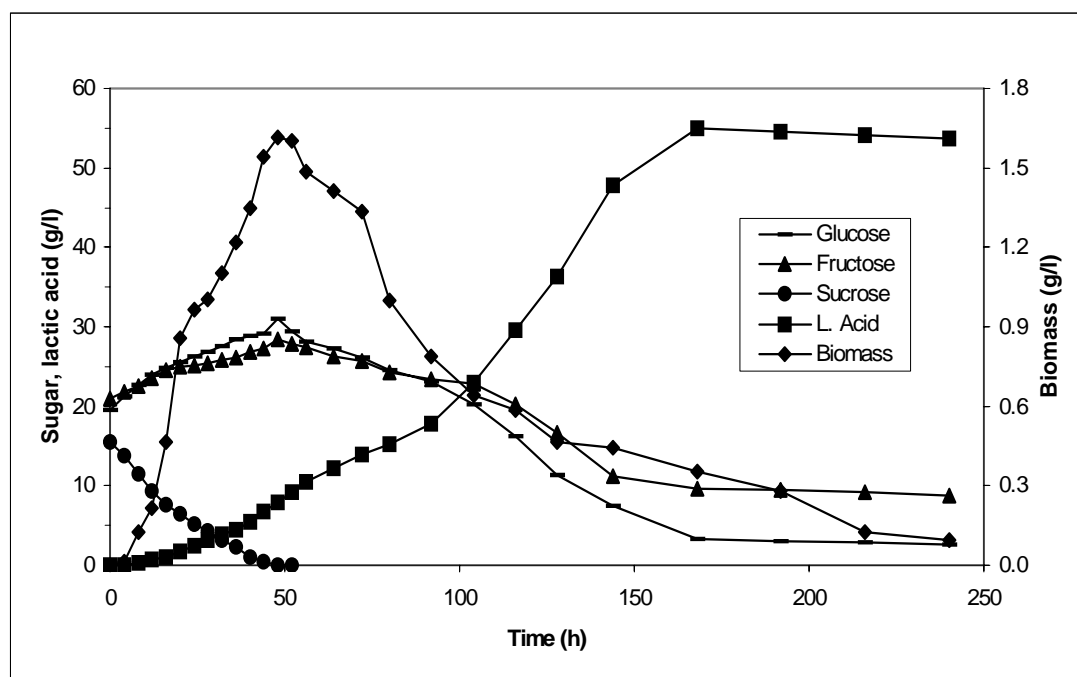
Figure 4.1: The time course of biomass, sugar (glucose, fructose and sucrose) and lactic acid concentrations during fermentation of liquid pineapple waste. Experimental conditions: T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

4.3.2. Liquid Pineapple Waste Extract

The liquid pineapple waste extract was obtained by extracting the solid waste (10 % w/w). The substrate was then supplemented with 5 g of yeast extract and the fermentations were carried out at similar conditions for liquid pineapple waste fermentation. The bacterial growth, sugar utilisation and lactic acid production are given in Figure 4.2.

During the lag phase (8 hours), the concentration of glucose and fructose decreased as a result of sugar utilisation for growth and maintenance of the cell by *L. delbrueckii*. This is followed by the exponential growth phase until 32 hours with the maximum concentration of biomass of 1.32 g/l. The stationary growth phase was obtained after 12 hours, followed by gradual decline of cell concentration occurred in the death phase.

The initial sugar concentration of liquid pineapple waste extract was 23.33 g/l, containing only glucose and fructose. After 6 days of fermentation, the concentration of glucose and fructose in the fermentation medium decreased from 10.26 to 1.24 and 12.84 to 4.45 g/l, respectively. This shows that glucose utilisation was better than fructose's but both sugars were not completely utilised. The utilisations of glucose and fructose were only 87.9 % and 65.34%, correspondingly. The lactic acid production was only 13.1 g/l or 56% yield based on initial sugar concentration.
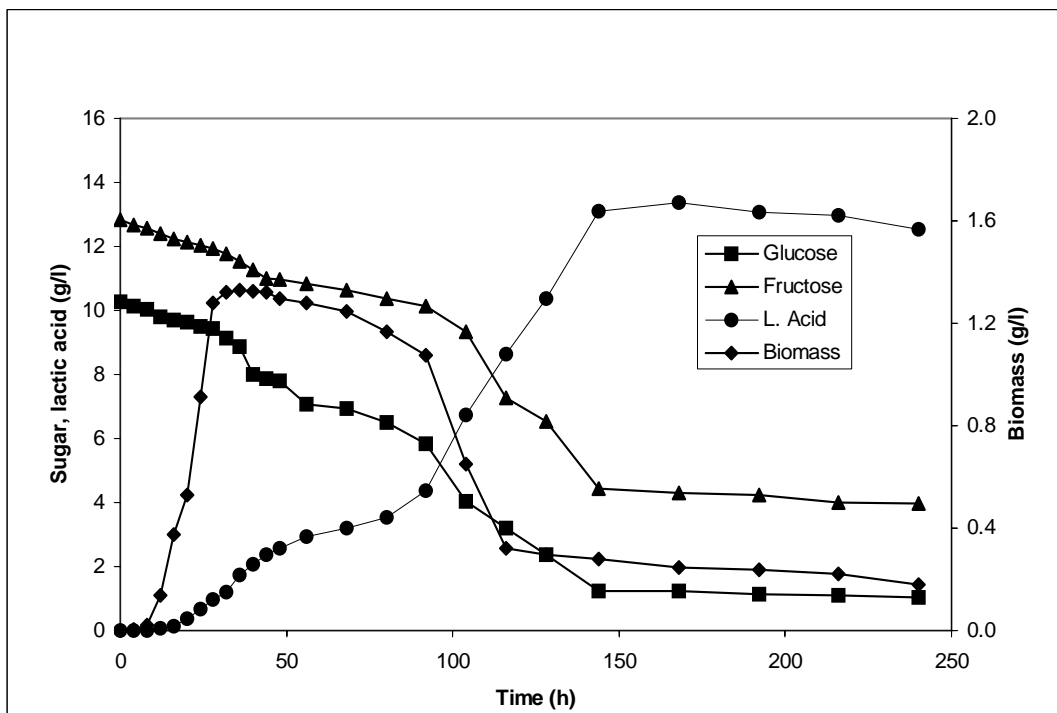
Figure 4.2: The time course of biomass, sugar (glucose and fructose) and lactic acid
concentrations during fermentation of liquid pineapple waste extract.
Experimental conditions: T, 40°C; pH, 6.0; inoculum, 5%; and stirring
speed, 50 rpm.

4.3.3 Solid Waste

The substrate was obtained by mixing 100 g of solid waste with 900 g of
water. The substrate contains mainly the reducing sugar and crude fibre such as
cellulose, hemicellulose, legnocellulose and lignin (Table 4.2). Initial sugar
concentration was about 15.72 g/l containing glucose and fructose only. The time
course of sugar utilisation and lactic acid production on solid waste fermentation is
shown in Figure 4.3. After 24 hours of fermentation, the concentration of sugar
increased to 18.24 g/l. This indicates that the hydrolyses of cellulose to glucose by
enzymes were still carried on. The glucose utilisation is better than fructose's, but
both sugars were completely utilised after 144 and 216 hours, respectively.

In this experiment, the biomass concentration was not measured because the solid pineapple waste used as substrate will interfere on the biomass measurement. The lactic acid production obtained was 22.51 g/l or 93% yield, based on initial sugar concentration. The yield was higher than liquid pineapple waste extract (56%), which indicates that the solid pineapple waste medium is better than liquid pineapple waste extract because the extraction process is longer and the complete solubility of nutrient from the solid waste into the liquid fermentation medium is taken place.
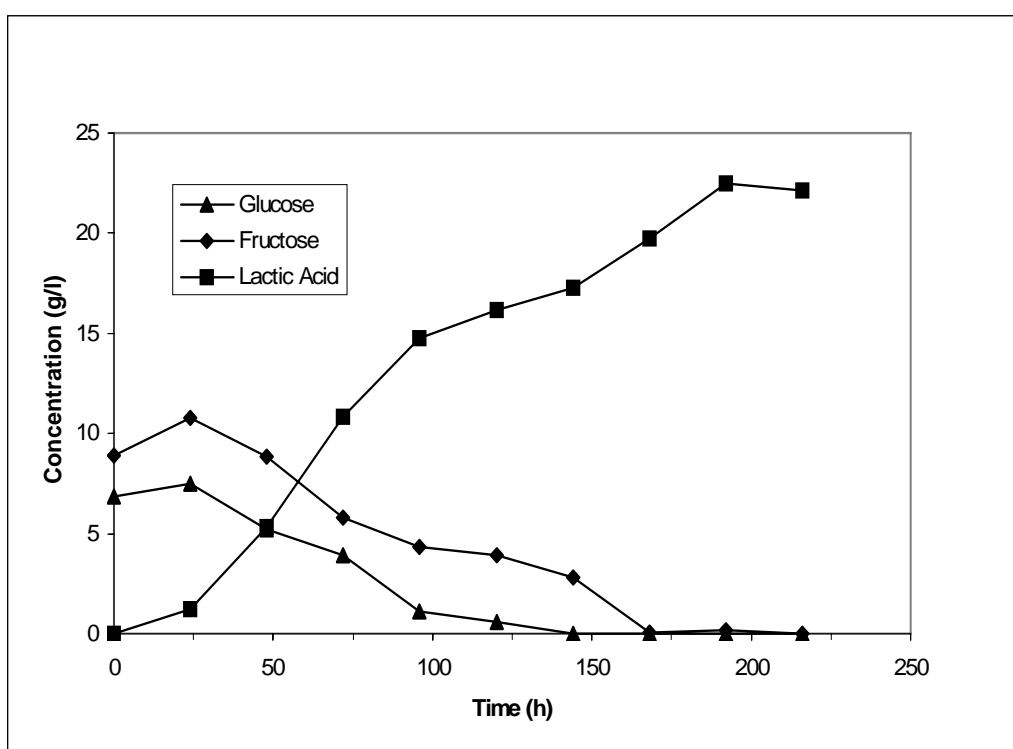
Figure 4.3: Profile of sugar (glucose and fructose) and lactic acid concentrations during fermentation of solid waste. Experimental conditions: T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

4.4   Parameter Study On Lactic Acid Fermentation Of Liquid Pineapple Waste

4.4.1   Effect Of pH

The optimal pH for the growth of *lactobacillus* has been reported to be at pH 5.5-6.0. Fermentation is strongly inhibited at pH below 5.0 and ceases at pH 4.5 (Buchta, 1983). In lactic acid fermentation, pH can cause reduction of lactic acid productivity due to the formation of lactic acid inhibitor. This lactic acid inhibitor is higher than ammonium lactate, sodium lactate or calcium lactate. The effect of pH inhibitor on cell growth of *L. delbrueckii* is very significant. Hence the production of lactic acid must be neutralised with alkaline such as ammonium hydroxide, sodium hydroxide and calcium carbonate (Tyagi et al., 1991). Calcium carbonate cannot be used because it is insoluble in the substrate and thus the suspended particulate matter will form where it will interfere with bacterial density measurement. Sodium hydroxide is the most suitable alkaline for pH control agent in fermentation process.

4.4.1.1 Controlled pH vs. Uncontrolled pH

Effect of controlled and uncontrolled pH on *L. delbrueckii* growth can be seen in Figure 4.4. During the early stages of the fermentation (lag growth phase), lactic acid was not produced (pH=6.0), but the concentration of sucrose decreases while the concentrations of glucose and fructose are increasing. It is perhaps due to hydrolysis of sucrose to glucose and fructose. After 8 hours, the exponential growth was attained followed by lactic acid production, decrease of pH (5.95) and increase of the concentrations of glucose and fructose because of the enzymatic hydrolysis of sucrose to glucose and fructose.

After 24 hours, the biomass concentration for controlled pH was 0.96 g/l, while for uncontrolled pH the biomass concentration was only 0.33 g/l and the pH dropped from 6.0 to 5.8. The concentrations of glucose and fructose still increased

most likely for the reason that the hydrolysis of sucrose was faster than the conversion of glucose and fructose to lactic acid. The maximum growth for controlled pH was achieved after 48 hours with biomass concentration of 1.6 g/l, but for uncontrolled pH only 0.8 g/l was obtained at 56 hours and pH 5.5. The lactic acid production was found to be 10.41 and 5.85 g/l, for controlled and uncontrolled pH respectively. This implies that the growth for uncontrolled pH was inhibited by lactic acid production. Therefore, the addition of alkaline to the medium was needed to obtain higher biomass concentration.

After 56 hours of fermentation, for controlled pH, the death phase was occurred but the lactic acid production still increased with decline of concentrations for glucose and fructose. Similar result was also obtained for uncontrolled pH where it diminished gradually from pH 5.5 to 5.1 with the maximum lactic acid production obtained at 13.5 g/l for 192 hours.
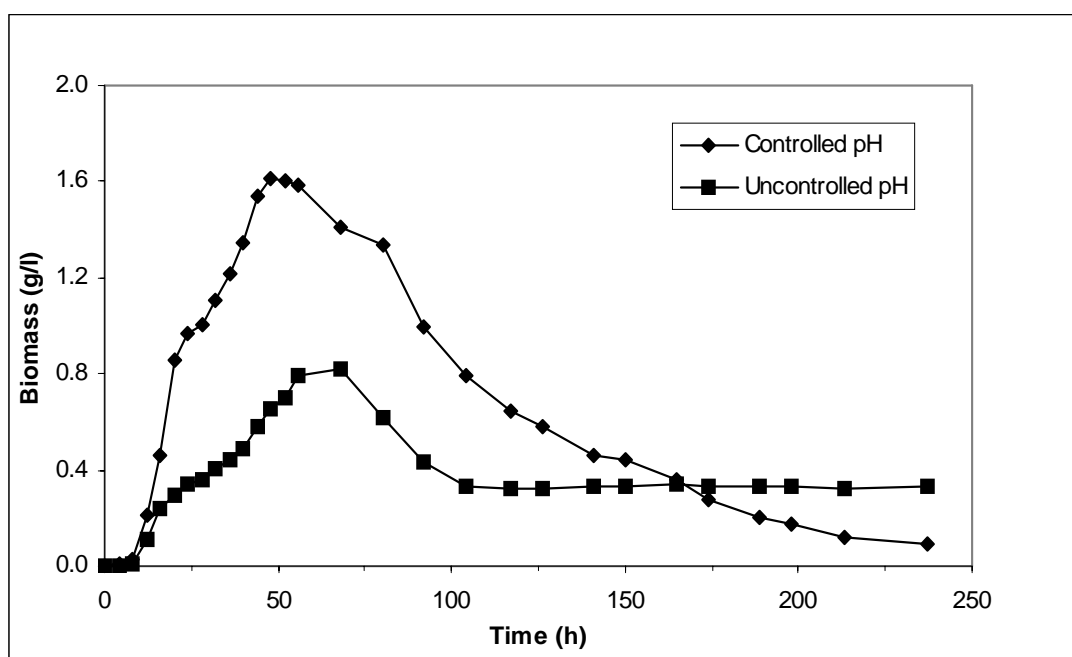
Figure 4.4: Effect of controlled and uncontrolled pHs on *L. delbrueckii* growth of lactic acid fermentation. Experimental conditions: T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

The profile of sugar consumption during lactic acid fermentation using liquid pineapple waste by *L. delbrueckii* is shown in Figure 4.5. During the first 48 hours, glucose and fructose accumulated in the medium due to higher rate of sucrose hydrolysis to glucose and fructose than the conversion of these substrates to lactic acid. In controlled and uncontrolled pH, the glucose utilisation was better than fructose's. The sucrose consumption during lactic acid fermentation was almost analogous for controlled and uncontrolled pH and was completely utilised after 40 and 56 hours, respectively.

The concentration of lactic acid produced depends on whether the pH of the fermentation was controlled or not. Figure 4.6 illustrates that if the pH was controlled, lactic acid yield after 168 hours was found to be 54.97 g/l or 78.52 %, while for uncontrolled pH the lactic acid yield was only about 13.52 g/l or 19.31 %. If the pH were not controlled, the pH itself would drop from 6.0 to 5.1 within 168 hours. After that no more lactic acid was produced. This result shows that continuous control of pH is advantageous since the yield and the rate of sugar utilisation can be increased thereby.
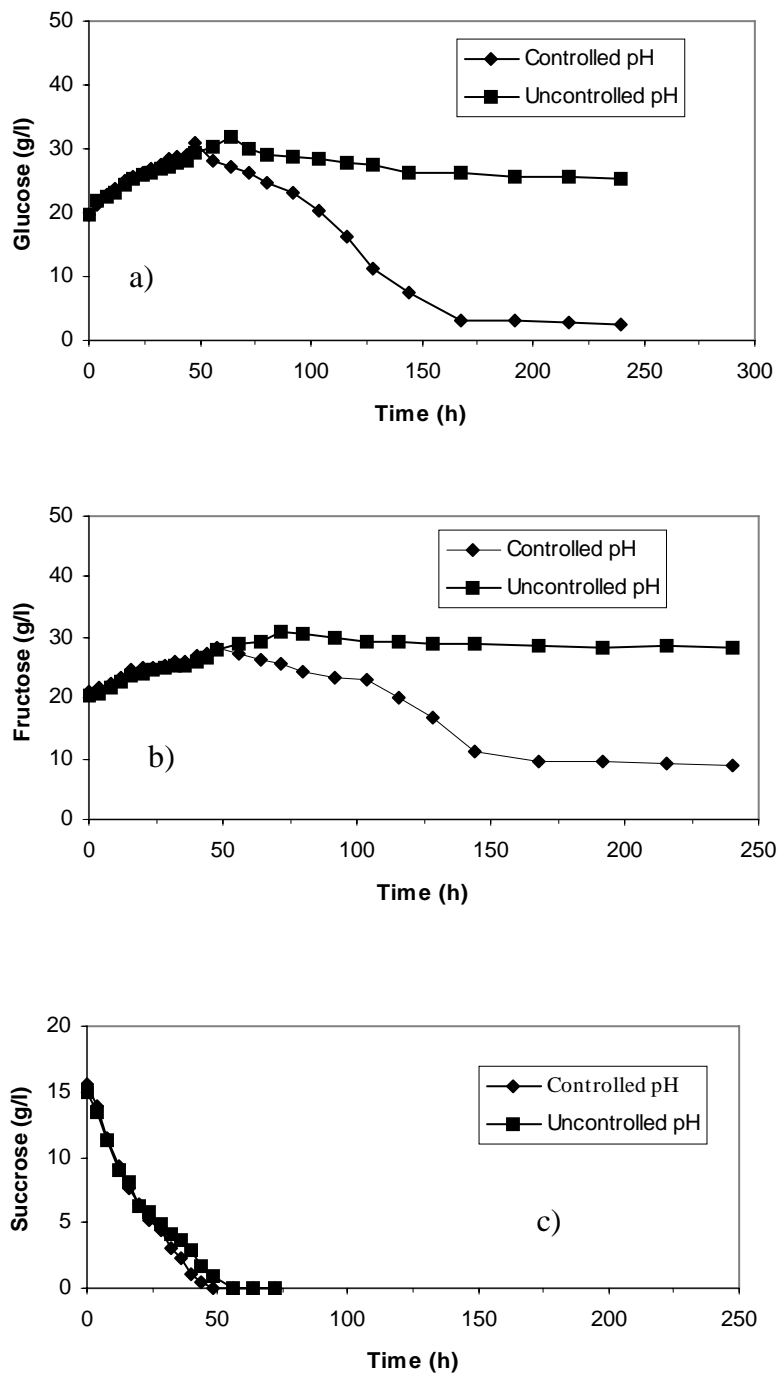
Figure 4.5: Effect of controlled and uncontrolled pHs on sugar consumption during
lactic acid fermentation: a) glucose, b) fructose and c) sucrose.
Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring
speed: 50 rpm.
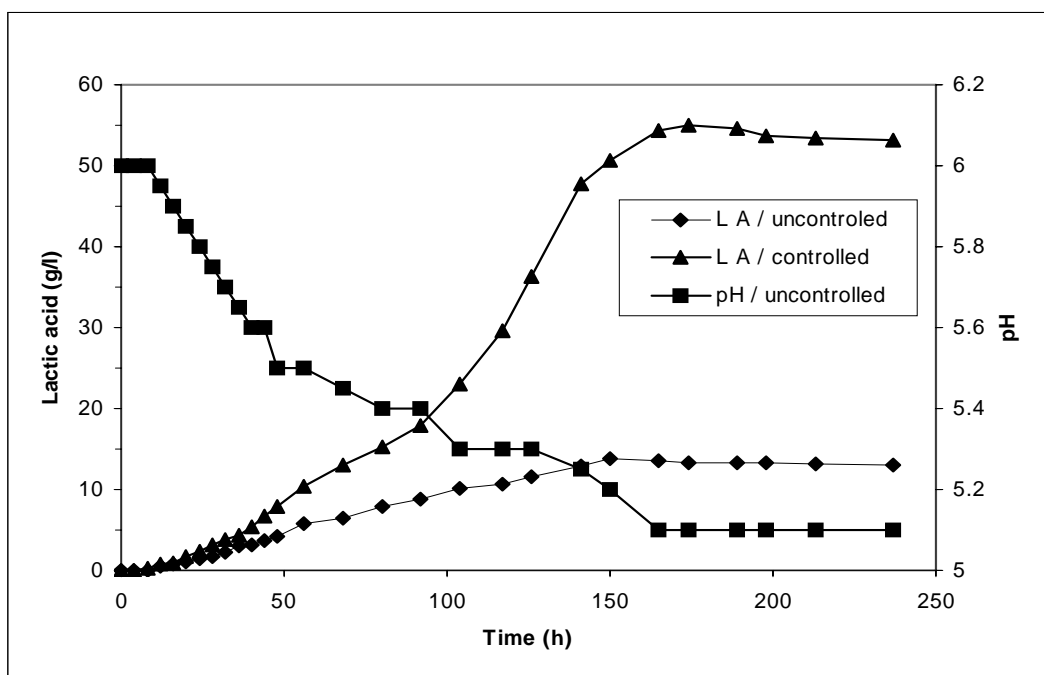
Figure 4.6: Effect of controlled and uncontrolled pHs on lactic acid production
during lactic acid fermentation. Experimental conditions: T, 40^{o}C; pH,
6.0, inoculum, 5% and stirring speed, 50 rpm.

4.4.1.2 Controlled pH

Effects of controlled pH were conducted in 3-litre fermentor with working
volume of 1 litre at 40^{o}C using liquid pineapple waste containing 70 g/l sugar. The
pH of the fermentation medium was controlled by automatic pH controller using 2 M
NaOH. The pHs of 5.5, 6.0 and 6.5 were studied and the result of bacterial growth,
sugar utilisation and lactic acid production are shown in Figures 4.7, 4.8 and 4.9.

The effect of pH on bacterial growth is given in Figure 4.7. The lag phase of
bacterial growth at pH 6.5 and 5.5 are longer than at pH 6.0, followed by exponential
phase after 4, 24, 24 hours at pH 6.0, 6.5 and 5.5, and the maximum concentrations
of dry cell weight were 1.6, 0.81 and 0.52 g/l, respectively. Following the stationary
phase, a gradual decline of cell concentration occurred in the death phase. The death
phases for pH 6.0, 6.5 and 5.5 were found to last for approximately 52-216, 72-192

and 144-192 hours, respectively. Therefore the optimal growth of the liquid pineapple waste fermentation using *L. delbrueckii* was pH 6.0, which is similar to those reported by Goksungur and Guvenc (1997) using beet molasses as a substrate.
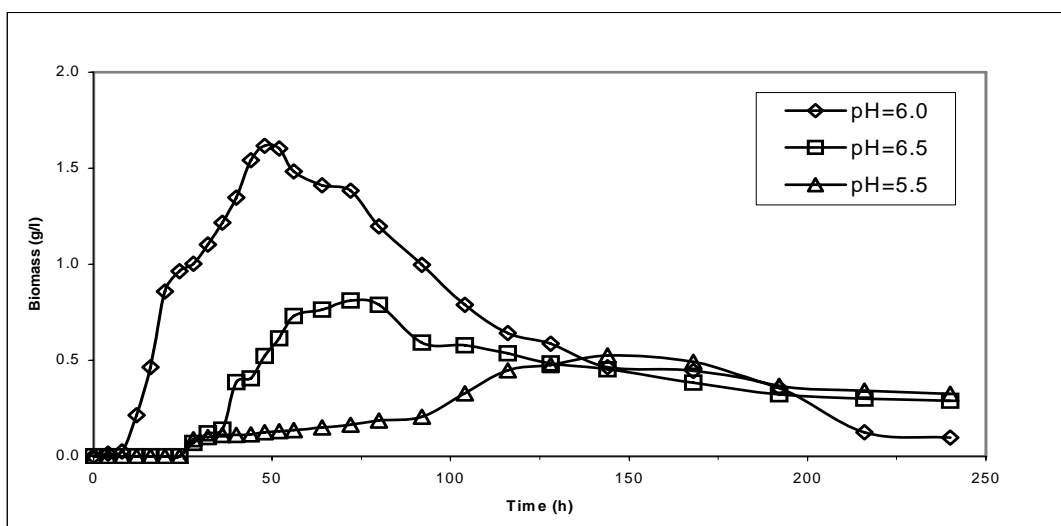
Figure 4.7: Effect of controlled pH on *L. delbrueckii* growth during lactic acid fermentation. Experimental conditions: T, 40^{o}C; inoculum, 5%; and stirring speed, 50 rpm.

Figure 4.8 shows the consumption pattern of the sugar during fermentation of the liquid pineapple waste. During the first 48 hours of fermentation, at pH 6.0 and pH 6.5 the glucose and fructose accumulated in the medium due to the assumption that hydrolysis of sucrose to glucose and fructose was faster than the conversion of these substrates to lactic acid. For pH 5.0, the accumulation of glucose and fructose in the medium was obtained before 104 hours. This was due to the slower rate of sugar conversion to lactic acid compared to pH 6.0 and 6.5.

The maximum concentrations of glucose and fructose accumulated in the medium for pH 6.0, 6.5 and 5.5 were 30.9, 31.9, 32.6 g/l and 28.3, 30.8, 32.8 g/l, respectively. The glucose utilisation was found to be higher than fructose's. The glucose and fructose were not completely utilised but sucrose was completely utilised for pH 6.0, 6.5 and.5.0 after 48, 56 and 144 hours, respectively.

Effect of controlled pH on lactic acid production is given in Figure 4.9. The optimal pH for production of lactic acid was found to be at 6.0 with lactic acid production and yield at 54.97 g/l and 78.52 %, respectively. If the pH were increased to 6.5, lactic acid production and the yield obtained would decrease to 21.88 g/l and 31.25 %, respectively. For pH 5.5 however, lactic acid production and the yield were 11.59 g/l and 16.55 %, respectively which were the lowest among the three pHs under study. These results are similar to those reported by Goksungur and Guvenc (1997) using beet molasses as a substrate where the optimal pH obtained was pH 6.0.

Figure 4.8: Effect of controlled pH on sugar consumption during lactic acid fermentation: a) glucose; b) fructose and c) sucrose. Experimental conditions, T: 40°C, inoculum: 5% and stirring speed: 50 rpm.
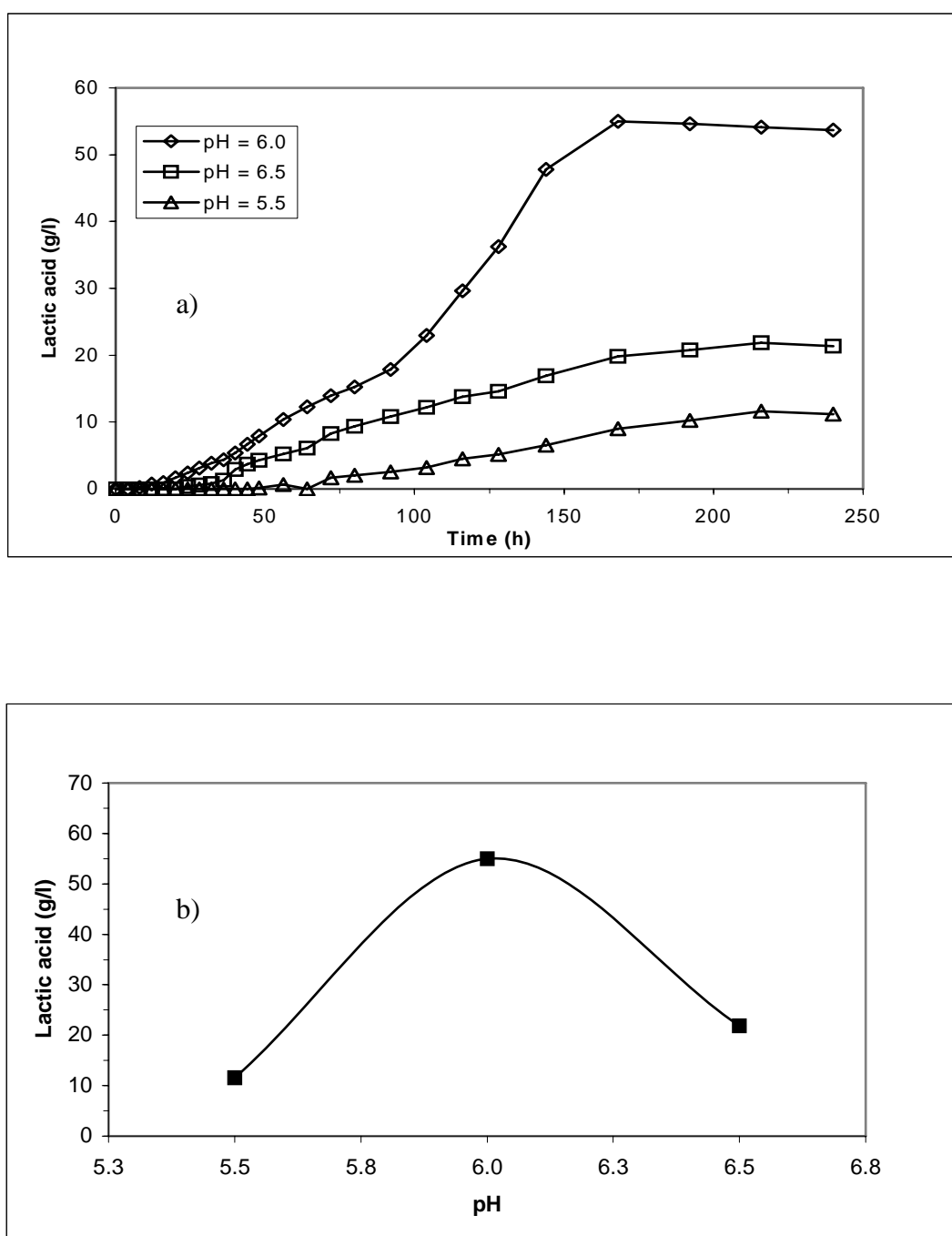
Figure 4.9: Effect of controlled pH on lactic acid production: a) time dependence of lactic acid concentration and b) pH versus maximum lactic acid production. Experimental conditions: T, 40^{o}C; inoculum, 5% and stirring speed, 50 rpm.

4.4.1.3 Initial pH

The effect of initial pH was studied at four different pH values of 6.0, 6.5, 7.0 and 7.5. The results of this study were also used to express the effect of $CaCO_3$ (3% w/v) addition and mechanism for pH control in the shake flask fermentation. The initial studied cannot be carried out on pH below 6.0 due to the fact that addition of 3% calcium carbonate into pineapple waste medium resulted in the pH of the medium to be of measurement at 6.0.

The effect of initial pH to the end results of fermentation process is given in Table 4.6. The last pH values of initial pH 6.0 and 6.5 were similar but the yields were different. This might be due to the initial pH of 6.0 was higher than 6.5 or the lactic acid production for initial pH of 6.0 was higher than pH 6.5 (55.36 and 44.97 g/l). With increasing of initial pH from 7.0 to 7.5, the final pH increases from 6.15 to 6.4. These results indicated that that the calcium carbonate was effective in controlling pH value at about 6.0. The process of neutralisation involves the reaction of alkaline in the fermentation medium with lactic acid produced. As a result, the pH of the fermentation medium was constant. The similar results were also reported by Goksungur and Guvenc (1997) and Vahvaselha and Linko (1987).

The effect of pH on the performance of lactic acid fermentation by *L. delbrueckii* is summarised in Table 4.6

Table 4.6: Summary of fermentation results for effect of pH

pH	End pH	X_m (g/l)	P_m (g/l)	Yield (%)	Productivity (g/l.h)
Uncontrolled pH	5.1	0.80	13.52	19.37	0.082
Controlled pH, 6.0	6.0	1.60	54.97	78.52	0.327
Controlled pH, 5.5	5.5	0.52	11.59	16.55	0.053
Controlled pH, 6.5	6.5	0.81	21.88	31.25	0.101
Initial pH, 6.0	6.02	-	55.36	79.80	0.329
Initial pH, 6.5	6.05	-	46.23	66.04	0.275
Initial pH, 7.0	6.15	-	44.20	63.10	0.263
Initial pH, 7.5	6.40	-	36.25	51.78	0.215

X_m and P_m are maximum concentration of biomass and lactic acid production.

4.4.2   Effect Of Temperature

Temperature is one of the most important factors that impinges on the growth of microorganism. Most species have a characteristic range of temperature in which they can grow, but they do not grow at the same rate over the whole of temperature range. Microbial growth is governed by the rate of chemical reaction catalysed by enzymes within the cell. Lactic acid bacteria are classified as thermophilic or meshopilic bacteria. The *L. delbrueckii* is meshopilic bacteria which grow at 17-50 ^{o}C and have optima growth between 20 to 40 ^{o}C (Taylor, 1992).

The effect of temperature on lactic acid fermentation was studied at 40, 45, and 50 ^{o}C using 70 g/l of sugar concentration, 0.5 % of yeast extract, 5% of inoculum and at pH 6.0. Effect of temperature on bacterial growth is given in Figure 4.10. The lag phase of bacterial growth for 50 ^{o}C was longer than for 40 and 45 ^{o}C, which are only 4 hours, respectively. This longer lag phase was due to the bacteria needed to adapt with their environment. The maximum concentration of dry cell weight

decreases with increment of temperature. This is probably because above 45 ^{o}C the protein enzymes start losing their activity (Taylor, 1992). The maximum concentrations of dry cell weight for 40, 45 and 50 ^{0}C obtained were 1.6, 1.4 and 1.3 g/l, respectively.

The stationary phase of the bacterial growth for 40 ^{o}C was started at 48 to 52 hours, followed by the death phase. For 45 and 50 ^{0}C, the stationary phase started at 92 and 104 hours and ended at 104 and 128 hours, respectively. The stationary phase of bacterial growth for 50 ^{o}C was longer than for 40 and 45 ^{o}C, which were only 4 and 12 hours, respectively. Inhibition by lactic acid production and depletion of nutrient concentration could be the reasons for it.
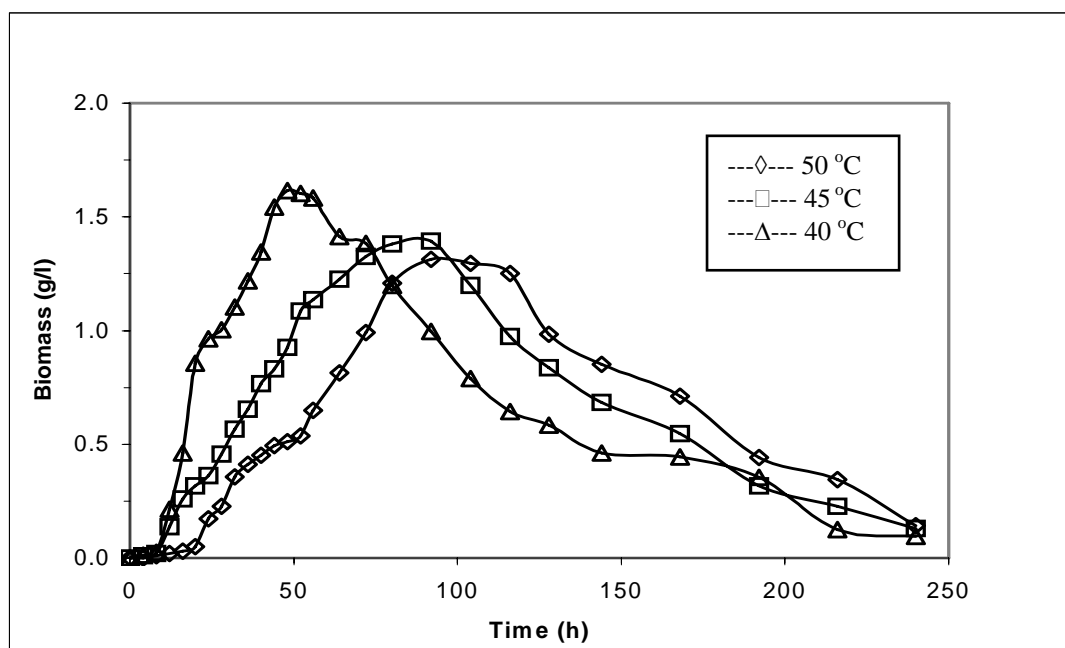
Figure 4.10: Effect of temperature on *L. delbrueckii* growth during lactic acid fermentation. Experimental conditions: T, 40^{o}C; inoculum, 5% and stirring speed, 50 rpm.

At temperature 40, 45 and 50 ^{o}C, the sugar consumption during fermentation were shown in Figure 4.11. During the lag phase, the glucose and fructose concentration increased with decreasing of sucrose concentration. This might be due

to hydrolysis of sucrose to glucose and fructose. At temperature 40, 45 and 50 °C, the sucrose was utilised completely after 40, 44 and 52 hours, respectively.

The lactic acid formation was occurred at exponential growth phase, but the concentration of the sugars still increased. Increase in glucose and fructose concentrations designates that the hydrolysis of sucrose was faster than the conversion of glucose and fructose to lactic acid. The maximum concentration for glucose obtained for temperatures of 40, 45 and 50 °C were 30.6, 31.4 and 33.51 meanwhile for fructose, 30.1, 30.2 and 32.8 g/l, respectively. The glucose utilisation was higher than fructose's and the consumption of sucrose was faster than both sugars. However glucose and fructose were not completely utilised even after 240 hours.

The effect of temperature on the lactic acid production is given in Figure 4.12. The highest lactic acid production and yield obtained were 54.97 g/l with the yield of 78.52 % at 40 °C. Similar observation was obtained if the temperature was increased to 45 °C (53.61 g/l lactic acid or 76.59 % yield). Nevertheless if the temperature were increased to 50 °C, the lactic acid production or yield would decrease rapidly to 25.14 g/l or 35.30 %.

This result indicates that the lactic acid production depends on microbial growth or cell concentration. With increasing cell concentration, the lactic acid production increased as well. However for temperature 50 °C, the growth of bacteria was not optima and therefore the lactic acid production decreased. These results differ from those reported by Goksungur and Guvenc (1997). They obtained the highest yield was at 45 °C. Difference in the substrate and strain used in lactic acid fermentation process could be the explanation.

The effect of temperature on maximum biomass concentration, maximum lactic acid production, yield and productivity on lactic acid fermentation of liquid pineapple waste by *L. delbrueckii* is summarised in Table 4.7.

Figure 4.11: Effect of temperature on sugar consumption during lactic acid fermentation: a) glucose; b) fructose and c) sucrose. Experimental conditions, T: 40^{o}C; inoculum: 5%; and stirring speed: 50 rpm.
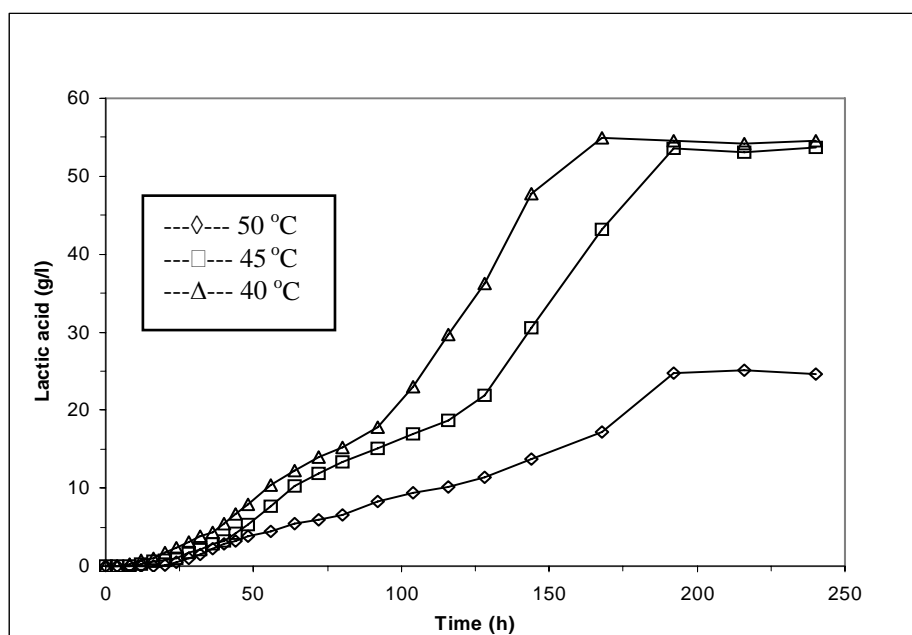
Figure 4.12: Effect of temperature on lactic acid production during lactic acid
fermentation. Experimental conditions: T, $40^{\circ}C$; inoculum, 5% and
stirring speed, 50 rpm.

Table 4.7: Summary of fermentation results for effect of temperature.

Temperature	X_m (g/l)	P_m (g/l)	Yield (%)	Productivity (g/l.h)
$40\ ^{\circ}C$	1.60	54.97	78.52	0.327
$45\ ^{\circ}C$	1.40	53.61	76.59	0.279
$50\ ^{\circ}C$	1.30	25.14	35.30	0.116

4.4.3   Effect Of Nitrogen Source

The lactic acid bacteria require substrates with high nitrogen content and have a particular demand for vitamins B. The nitrogen sources required for the fermentation medium are supplied in the form of malt sprout, corn steep liquor, yeast extract, cotton oil cake, soy flour, tryptone and pepton (Hujanen and Linko, 1996).

Yeast extract is most commonly used as nitrogen source in lactic acid fermentation as it provides a convenient source of growth factors for lactic acid bacteria. Lactic acid production increases with the increasing concentration of supplement especially yeast extract. The highest production rate was found with addition of 5-15g/l of yeast extract. However the addition of yeast extract during large scale fermentation is unrealistic due to the extra cost introduced for the fermentation process in combination with the low value of lactic acid (Lund et al., 1992; Goksungur and Guvenc, 1997).

5 nitrogen sources such as yeast extract, corn steep liquor, malt sprout, ammonium sulphate and urea were chosen for this study on the basis of their nitrogen content and cost. The quantity of nitrogen sources added to the fermentation medium depends on nitrogen sources used by the total nitrogen that were kept constant with equivalent to 5 g/l of yeast extract (0.5 % w/v). Table 4.8 shows the amount of nitrogen sources required to give the nitrogen content equivalent to 0.5 % (w/v) of yeast extract.

Table 4.8: The amount of nitrogen source used for lactic acid fermentation.

Nitrogen source	Nitrogen content (% w/w)	Total required (g)	Total nitrogen in medium (g/l)
Yeast extract	11.0	5.0	0.55
Corn steep liquor	3.5	15.71	0.55
Malt sprout	4.0	13.75	0.55
Ammonium sulphates	21.0	2.61	0.55
Urea	46.7	1.17	0.55

The effect of different nitrogen sources on lactic acid fermentation was studied using 70 g/l of sugar concentration at pH, 6.0; temperature, 40 $^{\circ}$C; inoculum, 5 % and stirring speed, 50 rpm. The effect of nitrogen sources on bacterial growth is given in Figure 4.13.

The fermentation using yeast extract as nitrogen source gave the shortest lag phase (4 hours). The lag phases for other nitrogen sources such as corn steep liquor, malt sprout, ammonium sulphate and urea were up to 12 hours. During the lag phase, the concentration of glucose and fructose increased but the concentration of sucrose decreased. This points out that during this period the sucrose was hydrolysed to glucose and fructose.

After the lag phase, exponential growth phase and simultaneous biosynthetic of lactic acid with growth were observed. During the exponential growth, the lactic acid production increased while the concentrations of glucose and fructose also increased. This might be due to the sucrose hydrolysis was faster than conversion of glucose and fructose to lactic acid. The maximum concentration of dry cell weight obtained was 1.6 g/l followed by urea, corn steep liquor, malt sprout and ammonium sulphates with each maximum concentration at 0.90, 0.82, 0.60 and 0.46 g/l, respectively. The death phase occurred after 52 hours. Although the growth ceased, the bacteria still produced lactic acid.
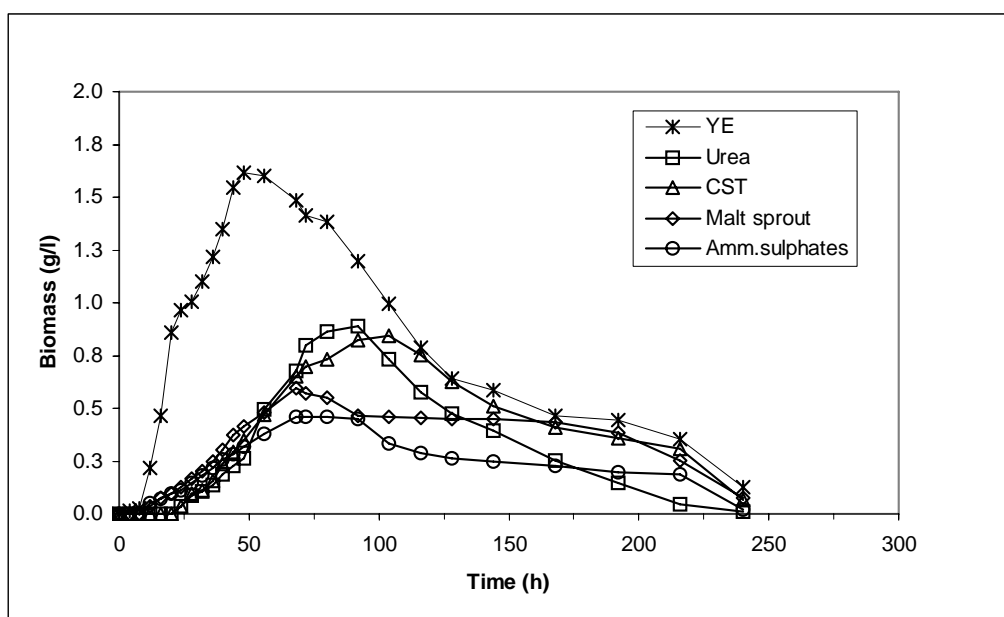
Figure 4.13: Effect of nitrogen source on *L. delbrueckii* growth during lactic acid fermentation. Experimental conditions: T, 40^{o}C; inoculum, 5% and stirring speed, 50 rpm.

The effect of nitrogen source on sugar utilisation is given in Figure 4.14. The trends of sugar utilisation were similar with other effects such as temperature and pH. Glucose utilisation was higher than fructose's whereas the sucrose consumption was faster than both sugars'. The glucose and fructose were not completely utilised but sucrose was completely utilised for yeast extract, urea, corn steep liquor, malt sprout and ammonium sulphates after 48, 64, 72, 80 and 92 hours, respectively.

The residual sugar concentrations at the end of fermentation time for yeast extract, urea, corn steep liquor, malt sprout and ammonium sulphates were 2.49, 21.38, 24.66, 26.85 and 28.32 g/l for glucose, and 9.11, 26.64, 27.79, 30.34 and 30.57 g/l for fructose, respectively, along with sugar conversion to lactic acid at 93, 83, 70, 64 and 32 % respectively.
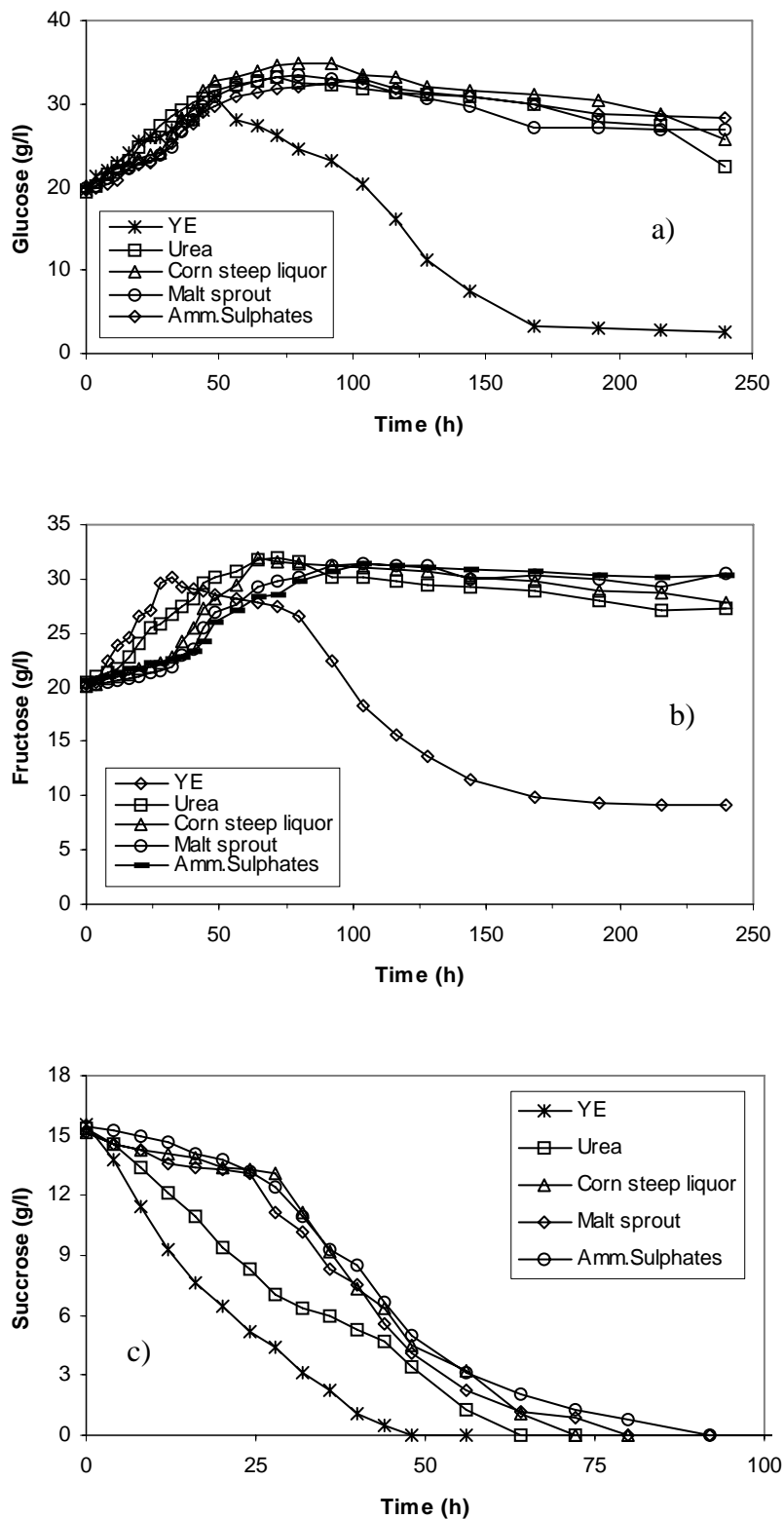
Figure 4.14: Effect of nitrogen source on sugar consumption during lactic acid
fermentation: a) glucose; b) fructose and c) sucrose. Experimental
conditions, T: 40^{o}C, inoculum: 5% and stirring speed: 50 rpm.

The effect of nitrogen source on lactic acid production is given in Figure 4.15. The yeast extract gave the highest lactic acid production followed by urea, corn steep liquor, malt sprout and ammonium sulphates with maximum lactic acid concentration each at 54.97, 18.34, 13.25, 9.30, and 3.65 g/l. Table 4.9 also indicates the effect of nitrogen source on the performance of lactic acid fermentation by *L. delbrueckii.* The yeast extract exhibited the highest volumetric productivity, followed by urea, corn steep liquor, malt sprout and ammonium sulphates. Therefore the yeast extract is the best nitrogen source for lactic acid production using *L. delbrueckii.* Lund et al. (1992), Arasaratnam et al. (1996) as well as Hujanen and Linko (1996) reported similar findings as well.
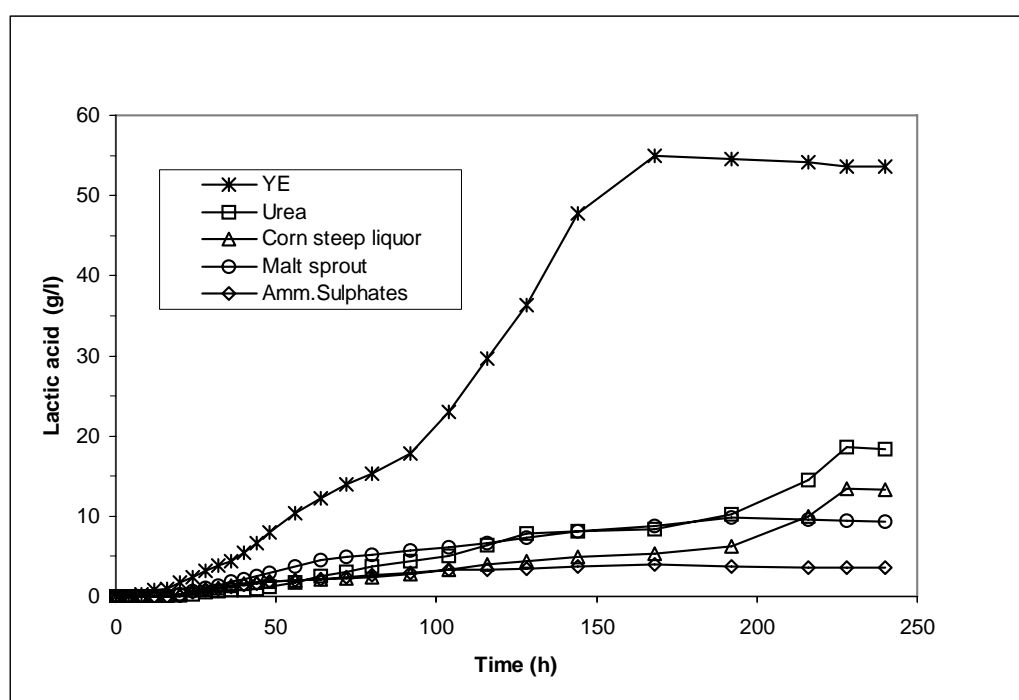
Figure 4.15: Effect of nitrogen sources on lactic acid production during lactic acid fermentation. Experimental conditions: T, 40^{o}C; inoculum, 5% and stirring speed, 50 rpm.

The use of yeast extract as nitrogen source not only increases the bacterial yield but also reduces the time required for the completion of fermentation. This could be due to the yeast extract contains substances such as amino acid, peptides, vitamins, and several organic acids including pyruvic and glyseric acid which are needed for the *L. delbrueckii* growth. According to Lund et al. (1992) and Arasaratnam et al. (1996), yeast extract is the best nitrogen source for *L. delbrueckii* growth. However it is expensive compared to other nitrogen sources.

The fermentation results of nitrogen source effect on lactic acid fermentation using liquid pineapple waste by *L. delbrueckii* is summarised in Table 4.9.

Table 4.9: Summary of fermentation results for effect of nitrogen source.

Nitrogen source	X_m (g/l)	P_m (g/l)	Yield (%)	Productivity (g/l.h)
Yeast extract	1.60	54.97	78.52	0.327
Urea	0.90	18.34	26.68	0.08
Corn steep liquor	0.82	13.52	19.14	0.06
Malt sprout	0.60	9.30	14.10	0.05
Ammonium sulphates	0.46	3.65	5.60	0.02

4.4.4 Effect Of Inoculum Size

The main objective of inoculum development for bacterial fermentation is to produce an active culture which will give the shortest lag phase in the subsequent culture. A long lag phase is disadvantageous because it is time consuming and the medium is used in maintaining a viable culture prior to growth. The length of the lag phase is affected by the size of the inoculum and its physiological condition. The inoculum size normally ranges between 3 to 10% (v/v) of the culture volume

(Stanbury, 1984). In industrial lactic acid fermentation process, the inoculum volume is usually about 5% (v/v) of the fermentation broth volume (Atkinson and Mavituna, 1991; Blanch and Clark, 1997 and Casida, 1964).

The maximum concentrations of lactic acid and biomass can be achieved depending on quantity or size of inoculum added in the substrate. In this study, the inoculum sizes of 5%, 10% and 15 % (v/v) were used. Effect of bacterial growth on inoculum size is given in Figure 4. 16. The growth started at 4 hours for 5% of inoculum followed by 10 % and 15 % of inoculum at 12 and 20 hours, respectively. Therefore the shortest lag phase for bacterial growth was obtained at 5% of inoculum size.

During the lag phase the concentration of glucose and fructose increased while the concentration of sucrose decreased. The concentration of both sugars at 5%, 10% and 15 % of inoculum size were 21.83, 22.4 and 23.35 g/l for glucose, and 21.30, 22.15 and 22.95 g/l for fructose, respectively. Increase of both sugars was probably owing to sucrose hydrolysis to glucose and fructose. The lag phase was followed by exponential phase with the maximum concentrations of dry cell weight obtained for 5 and 10 % of inoculum were similar (1.6 g/l), but only 1 g/l was attained if 15 % of inoculum was used.

However 5% of inoculum performed better than 10 % because the length of lag phase for 10 % inoculum caused higher consumption of sugar to maintain the growth. As a result, the production of lactic acid decreased. During this period, the growth associated with lactic acid production occurred but the concentrations of both sugars still increased. This, again, might be due to the hydrolysis of sucrose to glucose and fructose was faster than the conversion of both sugars to lactic acid. The short stationary phases were observed, followed by the death phase respectively after 52, 72 and 64 hours for 5%, 10% and 15 % inoculum sizes. During death phase, the lactic acid production still continued and this indicates that the bacteria were still able to produce lactic acid even after cease of growth. Similar results were also reported by Monteagudo et al. (1997) on lactic acid fermentation using beet molasses as a carbon source.
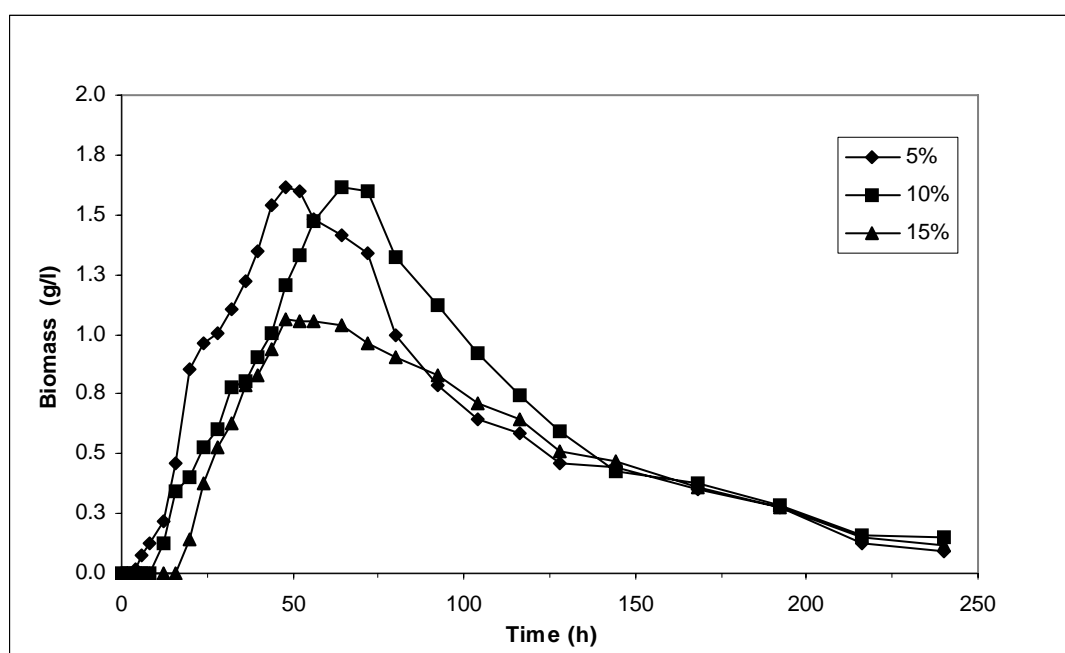
Figure 4.16: Effect of inoculum size on *L. delbrueckii* growth during lactic acid

fermentation. Experimental conditions: T, 40°C; pH, 6.0 and

stirring speed, 50 rpm.

Figure 4.17 shows the consumption pattern of the sugar during fermentation of pineapple waste at different inoculum sizes. The trends of glucose and fructose utilisation were similar to the effect of temperature and pH on sugar utilisation. The results showed that the accumulation of glucose in the medium until 48, 72 and 80 hours acquired maximum concentrations of 30.96. 31.37 and 31.58 g/l for 5, 10 and 15% inoculum sizes, respectively while the maximum concentrations of fructose were 28.38, 28.42 and 28.64 g/l, respectively. These might be due to the rate of hydrolysis of sucrose to glucose and fructose was faster than the conversion of these substrates to lactic acid. This denotes that hydrolysis of sucrose caused higher glucose concentration than fructose's. The glucose utilisation was again faster than fructose's whereas the sucrose consumption was faster than both sugars'. Glucose and fructose were not completely utilised but sucrose was completely utilised at 48, 56 and 64 hours for 5%, 10% and 15% of inoculum sizes, respectively.

Figure 4.18 gives the time course of the lactic acid production for three different inoculum sizes. The highest lactic acid concentration was 54.97 g/l, produced after 168 hours. When the inoculum sizes were increased, the lactic acid production decreased to 51.8 and 44.84 g/l for 10% and 15 % inoculum sizes, respectively.

The effect of inoculum size on the performance of lactic acid fermentation by *L. delbrueckii* is given in Table 4.10. As illustrated, the best inoculum size for lactic acid production was 5% with the highest maximum volumetric productivity of lactic acid obtained at 0.327 g/l.

Optimisation studies were carried out by Monteagudo et al. (1994) for the lactic acid production from the beet molasses using *L.delbrueckii*. They found that the maximum yield of lactic acid production obtained was 87.8% at 5.14 % of inoculum concentration. In comparison of the result obtained in this study to those reported in literature, the optimal inoculum size for lactic acid production by *L. delbrueckii* is quite similar for both.
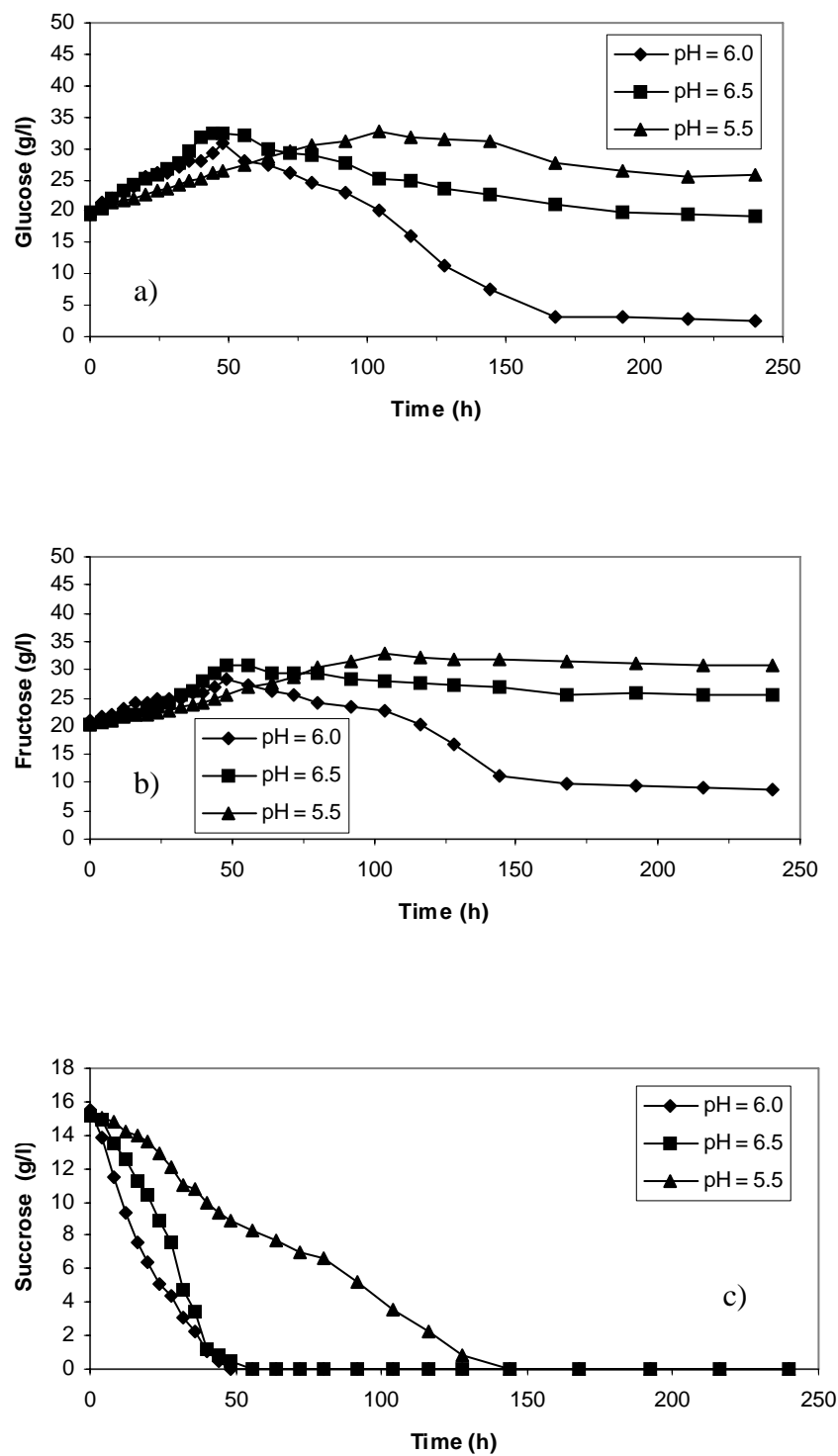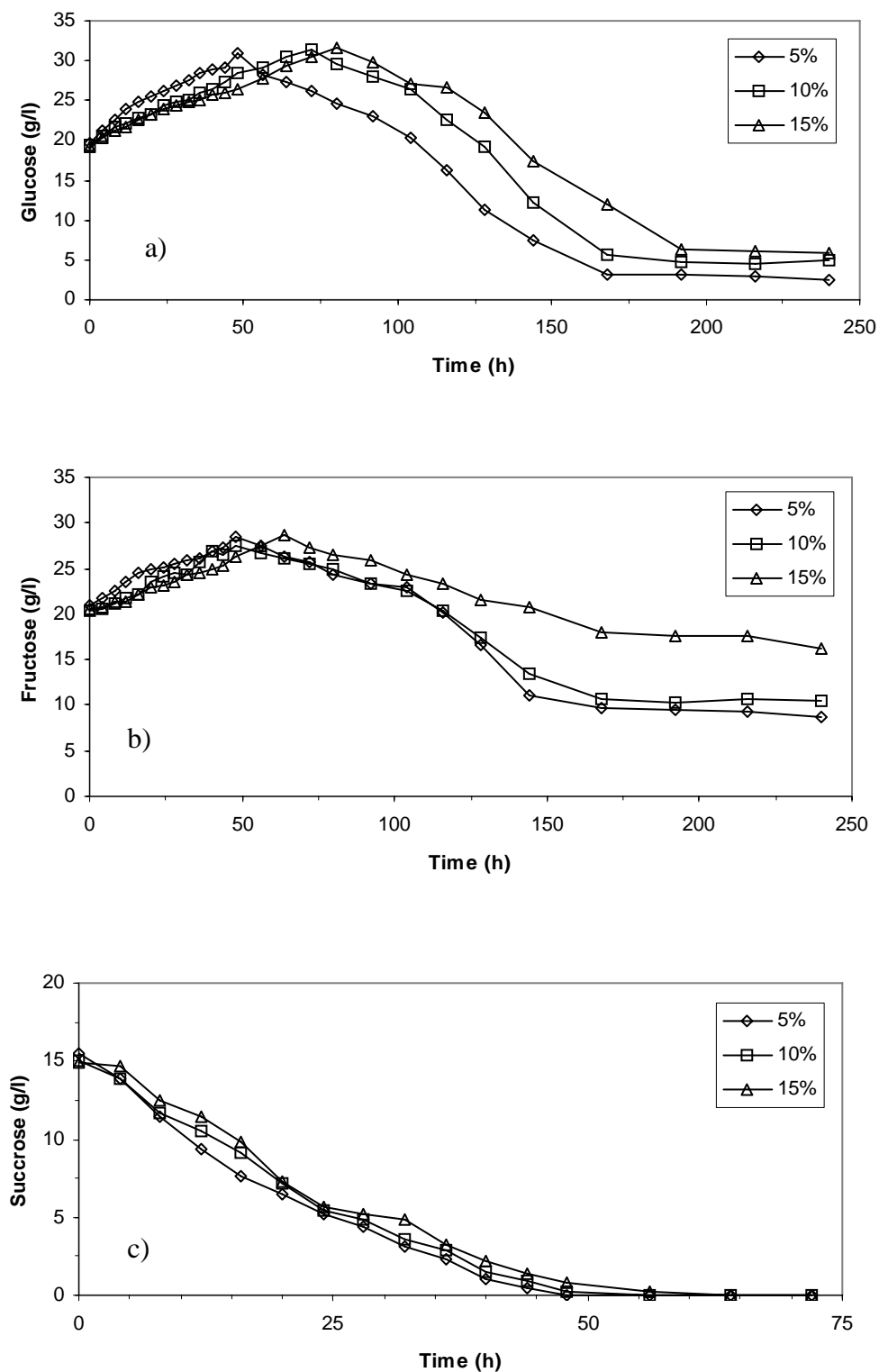
Figure 4.17: Effect of inoculum size on sugar consumption during lactic acid fermentation: a) glucose; b) fructose and c) sucrose. Experimental conditions, T: 40°C, pH: 6.0 and stirring speed: 50 rpm.
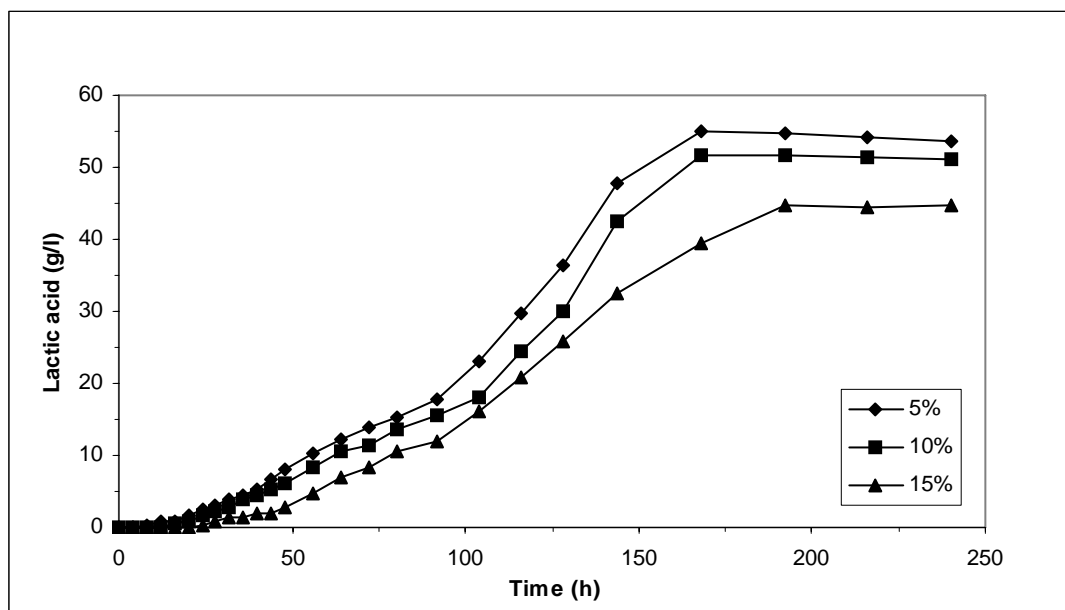
Figure 4.18: Effect of inoculum size on lactic acid production during lactic acid fermentation. Experimental conditions: T, 40°C; pH, 6.0 and stirring speed, 50 rpm.

Table 4.10: Summary of fermentation results for effect of inoculum size.

Inoculum size	X_m (g/l)	P_m (g/l)	Yield (%)	Productivity (g/l.h)
5 %	1.60	54.97	78.52	0.327
10 %	1.60	51.80	74.00	0.308
15 %	1.00	44.84	64.05	0.233

4.4.5   Effect Of Initial Sugar Concentration

The initial sugar concentrations used in this study varied between 7.0 and 13.5% by addition of pure sugar to liquid pineapple waste with proportional quantity according to composition of sugar in original pineapple waste. The composition of three initial sugar concentrations is given in Table 4.11.

Table 4.11: The composition of three initial sugar concentrations

Types of sugar	Initial sugar concentration (g/l)		
	70.0	95.0	135.0
Glucose	20.0	29.0	43.5
Fructose	20.0	29.0	43.5
Sucrose	15.0	22.0	33.0
Others	15.0	15.0	15.0
Total sugar	70.0	95.0	135.0

Effects of initial sugar concentrations on *L. delbrueckii* growth are illustrated in Figure 4.19. The lag phase of bacterial growth was achieved for 4 hours for initial sugar concentration of 70 g/l. When the concentration was increased to 95 and 135 g/l, the lag phase increased to 12 and 16 hours, respectively. It is obvious that higher initial sugar concentration increased the lag phase due to the bacteria needed longer time to adapt to their environment. This result is similar to those reported by Goncalves et al. (1991). During this period, the concentrations of glucose and fructose increased contrary to sucrose. The increasing of both sugars was probably because fo hydrolysis of sucrose to glucose and fructose.

The lag phase was followed by the exponential phase where the growth for the initial sugar concentration of 70 g/l was faster than the initial sugar concentration of 95 and 135 g/l. This indicates that the bacteria growth was inhibited by higher sugar concentration which was more than 70 g/l. The maximum concentrations of biomass achieved were 1.6, 2.5 and 2.3 g/l at 48, 116 and 168 hours, respectively.

Although the growth occurred simultaneously with biosynthetics of lactic acid production, the concentration of glucose and fructose still increased. Higher rate of hydrolysis of sucrose to glucose and fructose than conversion of both sugars to lactic acid was again the possible explanation to the above occurrence. The short stationary phase for the concentrations of 70, 95 and 135 g/l were occurred until 52, 124 and 190 hours, respectively. Following the stationary phase was the death phase where the growth rate began to decrease due to depletion of nutrient or inhibitory effect due to product accumulation, but the bacteria were still able to produce lactic acid.
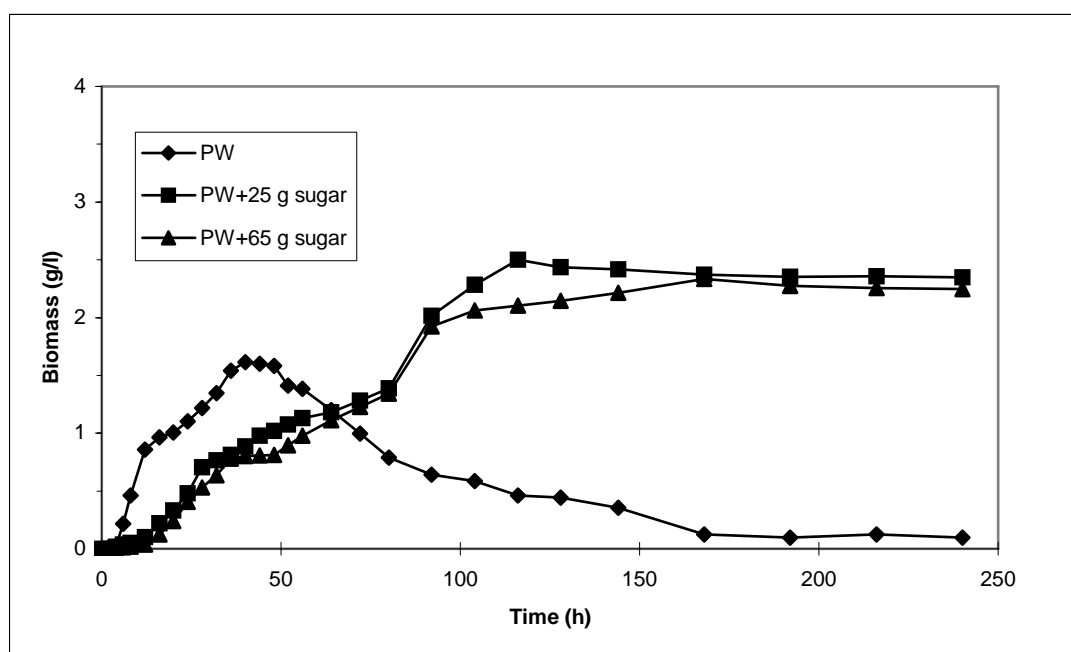
Figure 4.19: Effect of initial sugar concentrations on *L. delbrueckii* growth during lactic acid fermentation. Experimental conditions: T, 40^{o}C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

Figure 4.20 shows the consumption pattern of the sugar during the fermentation of pineapple waste at different initial sugar concentrations. The trends of glucose and fructose consumption were similar to the effect of temperatures, inoculum sizes and pHs on sugar consumption. During the first hours of fermentation, the glucose concentration accumulated in the medium until 48, 32, and

28 hours, and fructose until 48, 24 and 20 hours for initial sugar concentration of 70, 95 and 135 g/l, respectively. The maximum concentration of sugars in the medium is given in Table 4.12.

Table 4.12: The maximum sugar concentration accumulated in the medium for different initial sugar concentration.

Initial sugar concentration (g/l)	Maximum sugar concentration accumulated			
	Glucose		Fructose	
	Concentration (g/l)	Time (hr)	Concentration (g/l)	Time (hr)
70.0	30.96	48	28.38	48
95.0	44.42	32	38.42	24
135.0	67.94	28	53.05	20

The accumulation of both sugars was due to the rate of hydrolysis of sucrose to glucose and fructose was faster than the conversion of these substrates to lactic acid. The concentration of glucose in the medium was higher than fructose, which indicates that hydrolysis of sucrose resulted in higher concentration of glucose than fructose. The glucose utilisation was higher than fructose's, and the sucrose consumption was faster than both sugars'. Both glucose and fructose were not completely consumed but sucrose was fully utilised after 48 and 64 hours for initial sugar concentrations of 70 and 95 g/l, but for the initial sugar concentration of 135 g/l, the sucrose was not totally utilised. This might be due to the enzyme activity to hydrolyse the sucrose was slower at higher sugar concentration.
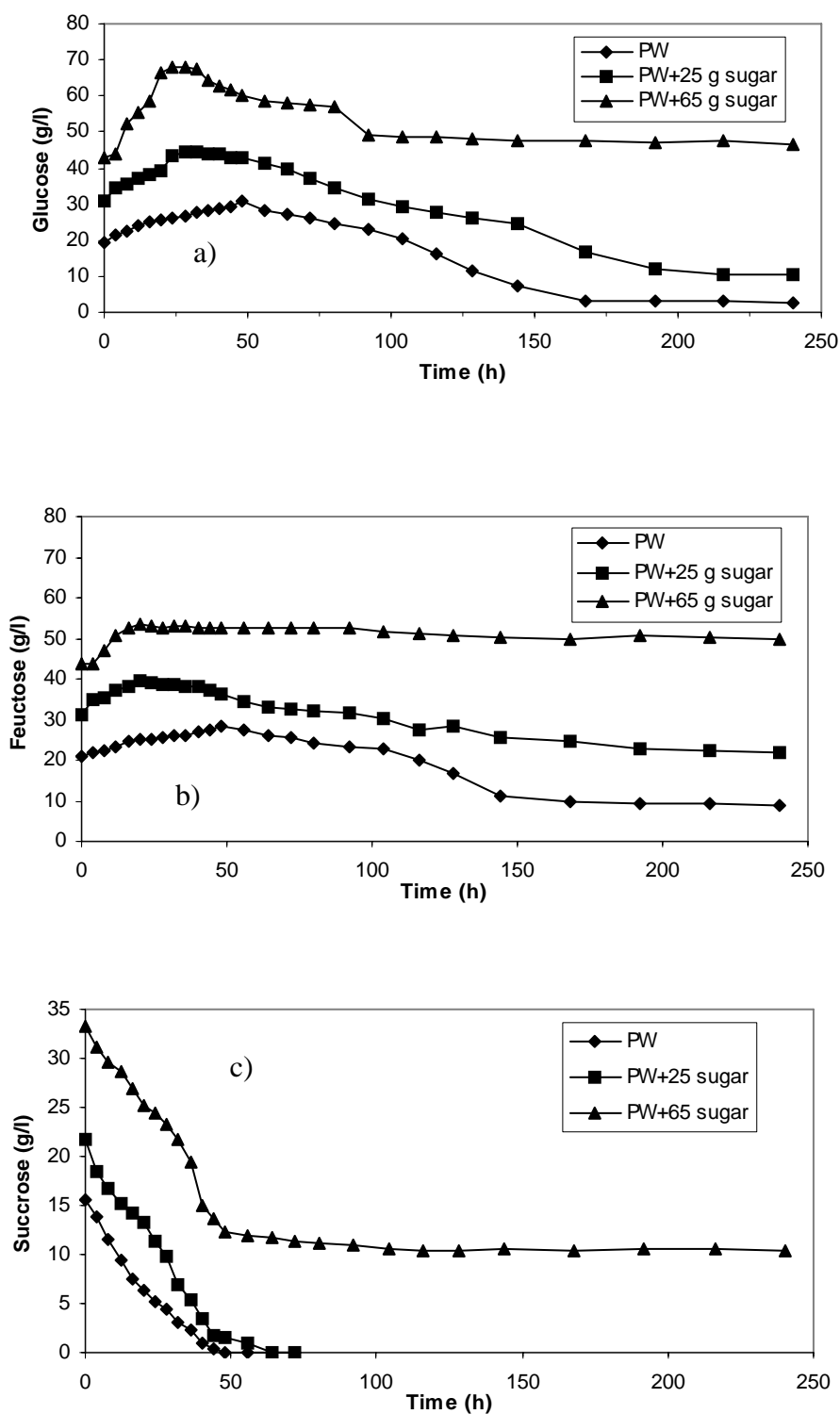
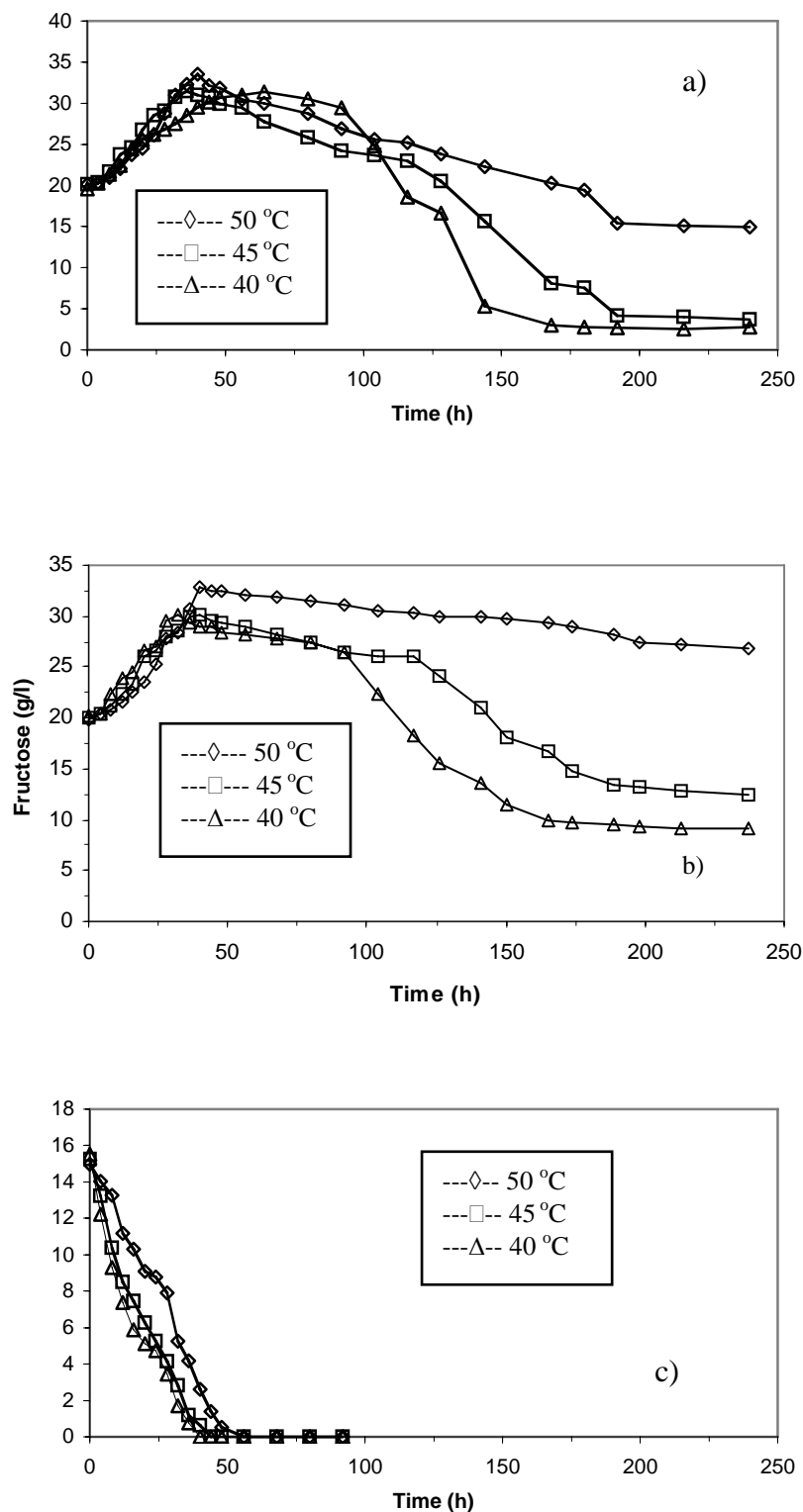Figure 4.20: Effect of initial sugar concentration on sugar consumption during lactic acid fermentation: a) glucose; b) fructose and c) sucrose. Experimental conditions: T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

Effect of initial sugar concentration on lactic acid production is given in Figure 4.21. The highest lactic acid production obtained was 54.97 g/l with the yield of 78.52 % for 70 g/l sugar concentration. When the concentration of sugar was increased to 95 g/l, the lactic acid production or yield decreased to 51.53 g/l or 54.24 %. And when the initial sugar concentration was increased to 13.5 %, the lactic acid production or yield decreased rapidly to 19.92 g/l or 14.75 %. Although the cell concentration increased with increasing of the initial sugar concentration, but the lactic acid yield decreased. The decreasing of yield might be due to inhibition by higher sugar concentration.
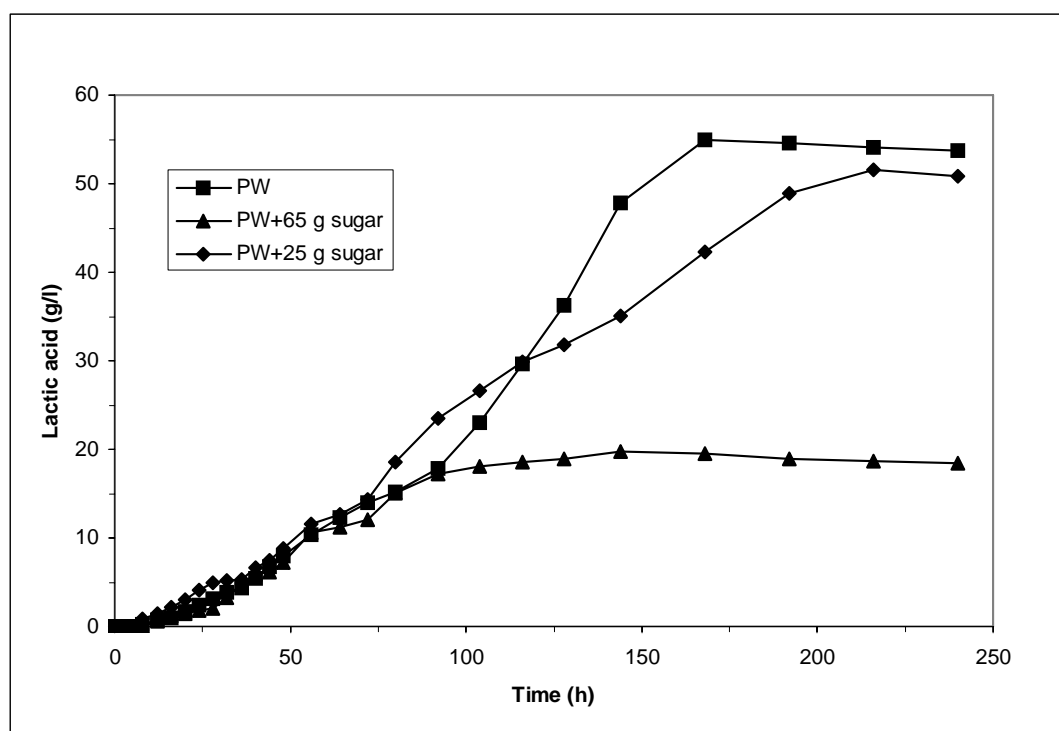
Figure 4.21: Effect of initial sugar concentration on lactic acid production during lactic acid fermentation. Experimental conditions: T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

Effect of initial sugar concentration was also studied in shake flask fermentation by diluting the pineapple waste giving the final sugar concentrations of 23.3, 35.0 and 52.5 g/l. The results can be seen in Table 4.13 and Figure 4.22. The yield increased with increasing of the initial sugar concentration, and the yields obtained were 82.25, 82.6, 85.65 and 79.8 % each. When the initial sugar concentration exceeded 52.5 g/l, the yield decreased due to inhibition caused by high sugar concentration. This is a characteristic of batch culture fermentation.

In this experiment, the highest yield obtained was 85.65 % for the initial sugar concentration of 52.50 g/l. This result was almost similar to those reported by Monteagudo et al. (1994). The maximum yield obtained was 87.8 % for 58.8 g/l of initial sugar concentration. Goksungur and Guvenc (1997) also reported that the highest yield obtained was 81 % for the initial sugar concentration of 78.2 g/l. This difference might be due to different substrate and operation condition used in the study.

The effect of initial sugar concentration on the performance of lactic acid fermentation using liquid pineapple waste by *L.delbrueckii* is summarised in Table 4.13.

Table 4.13: Summary of fermentation results for effect of initial sugar concentration.

Initial sugar concentration (g/l)	X_m (g/l)	P_m (g/l)	Yield (%)	Productivity (g/l.h)
23.33	-	19.19	82.25	0.114
35.00	-	28.94	82.60	0.172
52.50	-	44.97	85.65	0.267
70.00	1.6	54.79	78.52	0.327
95.00	2.5	51.53	54.24	0.238
135.00	2.3	19.92	14.75	0.115
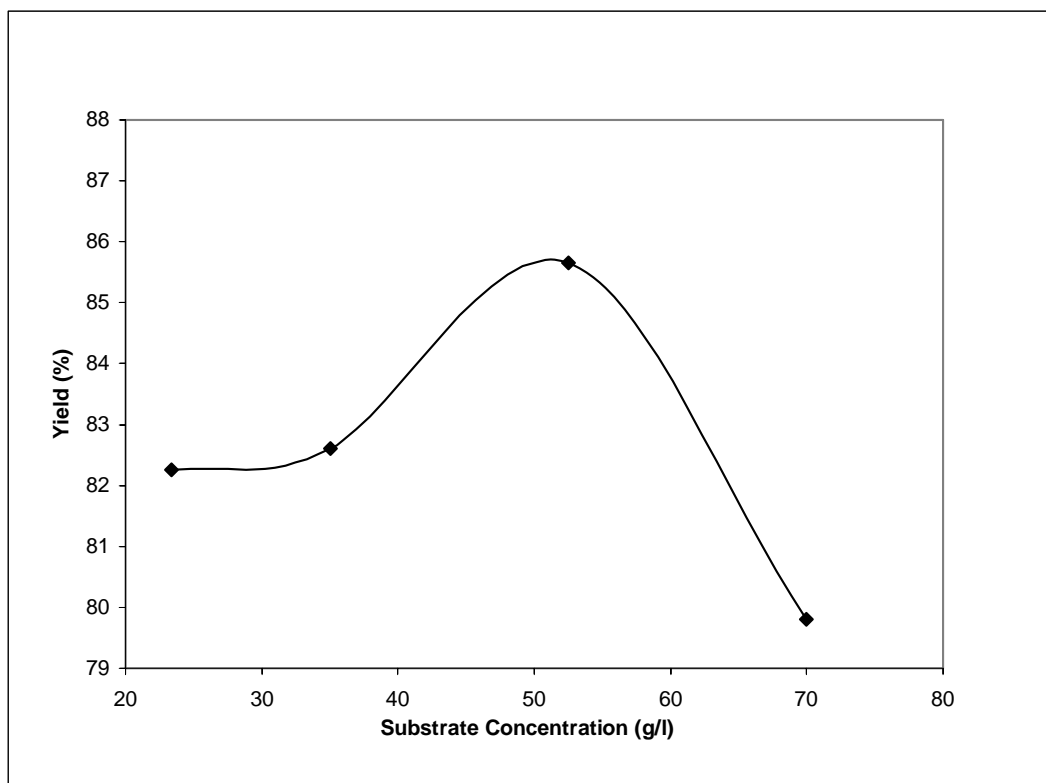
Figure 4.22: Effect of initial sugar concentration on the yield of lactic acid fermentation. Experimental conditions: T, 40^{o}C; pH, 6.0; inoculum, 5% and stirring speed, 50 rpm.

4.5 Fermentation Characteristics Of Different Carbon Sources

As presented and discussed in Section 4.4, the main objective of this study was to use pineapple waste as a carbon source. Since the pineapple waste contains more than single sugar component, hence it is important to understand the fermentation characteristics of these components as a carbon source. As discussed in Section 4.2, the liquid pineapple contains mainly glucose, fructose and sucrose. Therefore fermentation study on pure and mixed sugar will help to understand the effect of pineapple waste sugar constituents on lactic acid fermentation.

4.5.1   Single Sugar vs. Mixed Sugar

In order to understand the fermentation characteristics of different carbon sources, three types of sugars: glucose, fructose and sucrose were chosen due to their existent in the pineapple waste. The study on mixed sugar system is important to understand the effect of sugar types and their concentrations on lactic acid fermentation of pineapple waste. This can be done by comparing mixed sugar system to pure sugar system. The concentration of sugar used was the same as the composition of inoculum broth which was 20 g/l of glucose because of the low resistance of the microorganism to the acidity of medium and the efficiency of the microorganism diminished in highly acidic medium in which the sugar level is higher than 25-30 g/l (Buyukgungor et al., 1984). The results of the microbial growth, sugar utilisation and lactic acid production are given in Figures 4.23, 4.24 and 4.25.

4.5.1.1 Bacterial Growth

The profiles of dry cell weight concentration with fermentation time on several of sugar types are given in Figure 4.23. The profiles of biomass concentration with time of fermentation show that the lag phases, which are the adaptation period for bacteria to fermentation environment, were up to 4 hours for all types of sugars. The exponential growth phase of glucose medium ended at 68 hours followed by mixed sugar, sucrose and fructose at 80, 116 and 128 hours, respectively. The exponential phase is followed by stationary phase with the maximum biomass concentration achieved at 2.28, 2.16, 1.44 and 1.36 g dry cell weight/l, respectively. This denotes that the optimal growth of *L. delbrueckii* was obtained when glucose was used as a carbon source, followed by mixed sugar, sucrose and fructose.
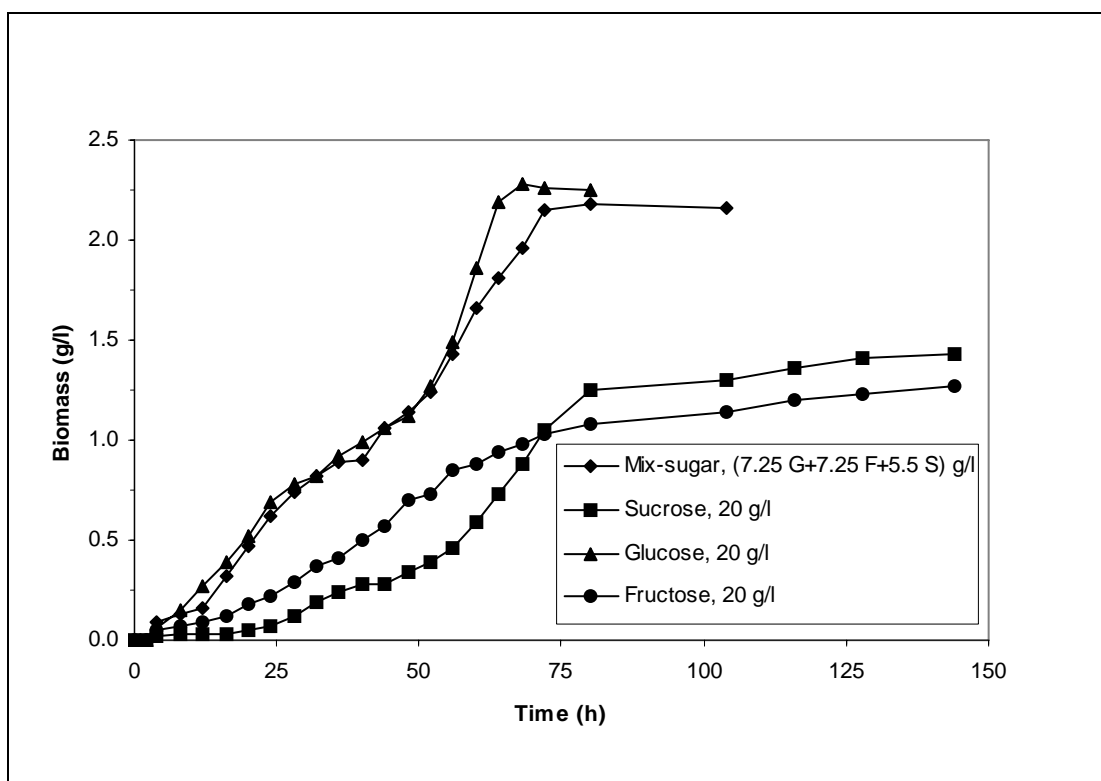
Figure 4.23: Time course of biomass concentration during lactic acid

fermentation of single and mixed sugar. Experimental conditions:

T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

4.5.1.2 Sugar Utilisation

The glucose, fructose, sucrose and mixed sugar (7.25g fructose, 7.25g glucose and 5.5g sucrose) concentrations used were 20 g/l. Figure 4.24 shows that the sucrose was completely utilised at 56 hours, followed by glucose and fructose at 68 and 104 hours, respectively. The consumption pattern of the mixed sugar during the first 8 hours of fermentation indicates that the glucose and fructose concentrations increased in the medium due to the rate of hydrolysis of sucrose to glucose and fructose was higher than the conversion of these substrates. The maximum concentrations of glucose and fructose were 8.48 and 7.84 g/l, respectively. The sucrose, glucose and fructose consumption were entirely utilised at 24, 56 and 72 hours. Detailed discussion on sugar utilisation selectivity will be presented in Section 4.5.
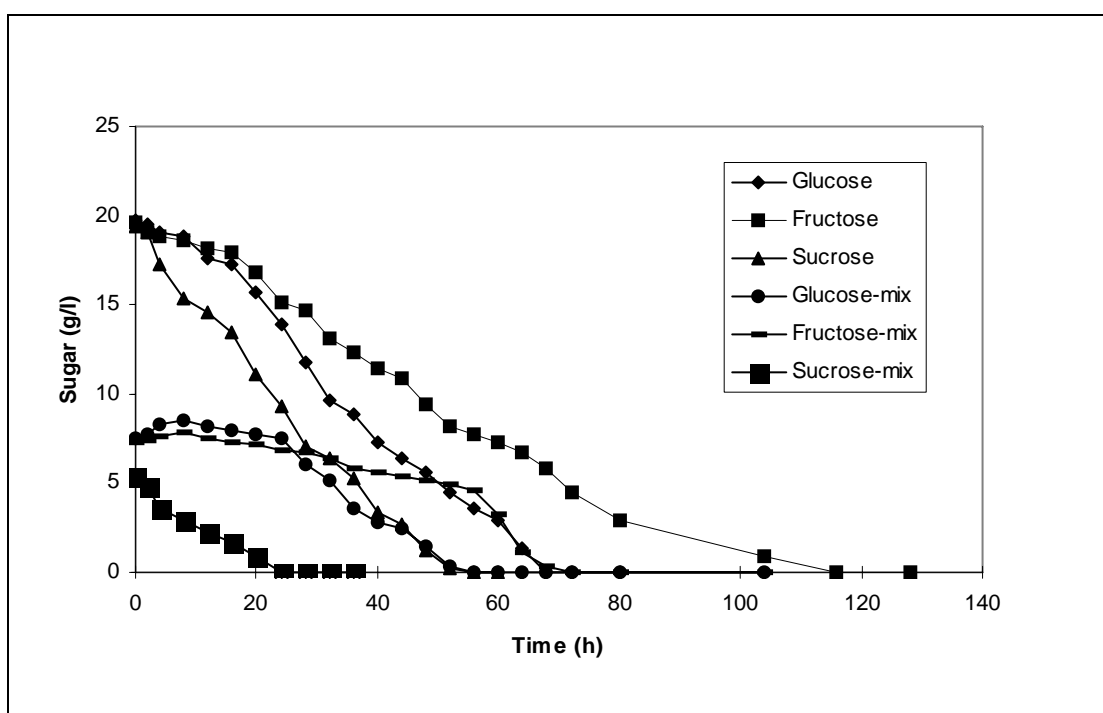
Figure 4.24: Time course of sugar concentration during lactic acid
fermentation of single and mixed sugar. Experimental conditions:
T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

4.5.1.3 Lactic Acid Production

Effect of the sugar types used on the lactic acid production is given in Figure 4.25. The maximum concentration of lactic acid obtained for glucose medium was 18.25 g/l or 92 % of yield at 68 hours. The maximum concentration of lactic acid (yield) for mixed sugar, fructose and sucrose obtained were 18.41 g/l (93 %), 18.3 g/l (93 %) and 18.22 g/l (92%) at 72, 104 and 116 hours, respectively. Although maximum lactic acid concentrations obtained were almost similar for each, the productivities were different. Maximum lactic acid productivities for glucose, mixed sugar, fructose and sucrose were 0.27, 0.26, 0.19 and 0.17 g /l.h, respectively.

Buyukgungor et al. (1984) has reported that the production of lactic acid using *L. delbrueckii* on glucose was 85%. The results differ because of the difference in fermentation process conditions used.
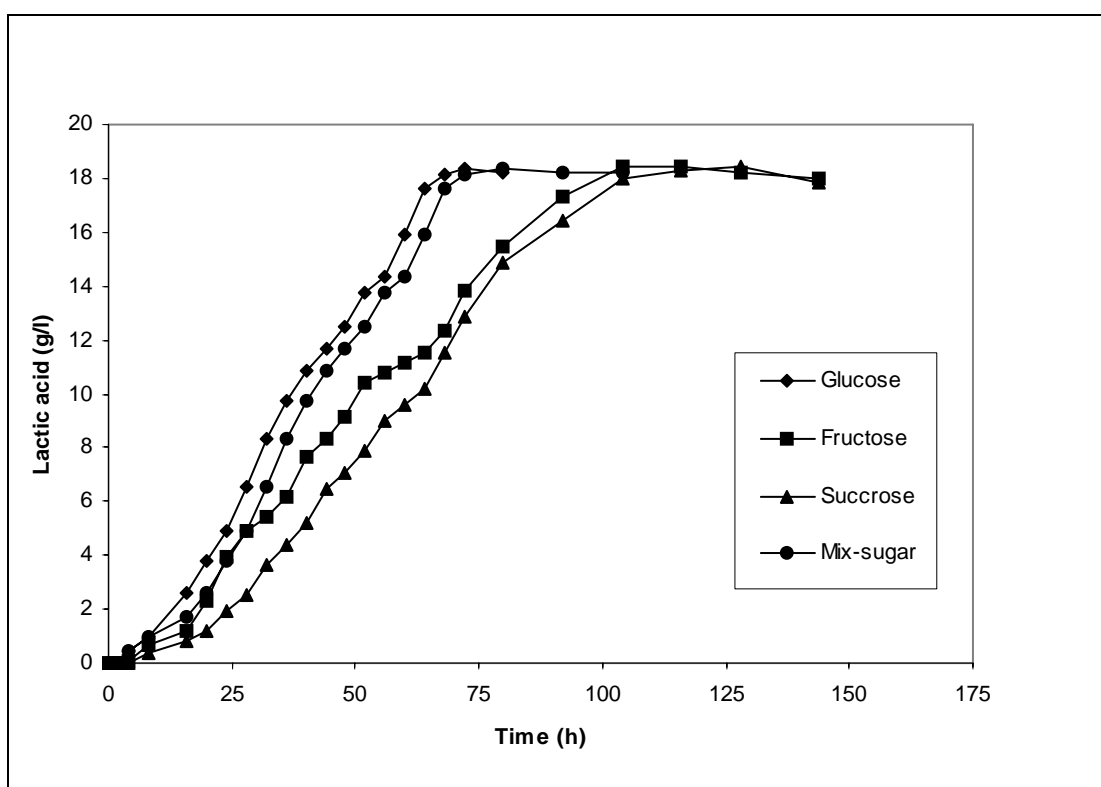
Figure 4.25: Time course of lactic acid concentration during lactic acid fermentation of single and mixed sugar. Experimental conditions: T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

The effect of different carbon sources on the performance of lactic acid fermentation by *L. delbrueckii* is summarised in Table 4.14.

Table 4.14: Summary of fermentation results for fermentation characteristics of different carbon sources.

Fermentation	X_m (g/l)	P_m (g/l)	Fermentation time (hr)	Yield (%)	Productivity (g/l.h)
Glucose (20 g/l)	2.28	18.25	68	92	0.270
Fructose (20 g/l)	1.44	18.41	104	93	0.190
Sucrose (20 g/l)	1.32	18.30	116	93	0.170
Mixture of glu, fru and suc (20 g/l)	2.16	18.22	72	92	0.260

4.5.2 Mixed Sugar vs. Pineapple Waste

The substrate used was pineapple waste, which contains a complex of saccharides such as glucose, fructose and sucrose. This study was to compare the bacterial growth, sugar utilisation and product formation of pineapple waste fermentation with the mixed sugar prepared according to pineapple waste composition.

4.5.2.1 Bacterial Growth

The composition of mixed sugar concentration chosen was proportional to the composition of pineapple waste. The first mixed sugar contains 7.25 g fructose, 7.25 g glucose and 5.5 g sucrose, and the second contains 20 g fructose, 20 g glucose and 15 g sucrose or total sugar of 20 and 55 g/l, respectively. The bacterial growth on

pineapple waste was compared for both 20 and 55 g/l mixed sugar fermentation. The profile of dry cell weight concentration with time of fermentation can be seen in Figure 4.26. This figure shows that the lag phase was achieved until 8 hours for all fermentation media used. The exponential growth phase seemed to last at 48 hours on pineapple waste, similar with 55 g of mixed sugar, and 80 hours for 20 g of mixed sugar.

The maximum biomass concentration obtained for 20 g of mixed sugar was 2.18 g/l. When the concentration of mixed sugar was increased to 55 g/l, the maximum biomass concentration decreased to 1.8 g/l. This was likely due to inhibition by higher mixed sugar concentration. Maximum biomass concentration obtained was only about 1.6 g/l for pineapple waste. This may reflect the complex nature of pineapple waste. This result shows that the most favourable growth of *L. delbrueckii* was at mixed sugar concentration of 20 g/l. Following by the stationary phase, a gradual decline of cell concentration (death phase) was observed approximately after 104, 64 and 55 hours for 20 g of mixed sugar, pineapple waste and 55 of mixed sugar, respectively.
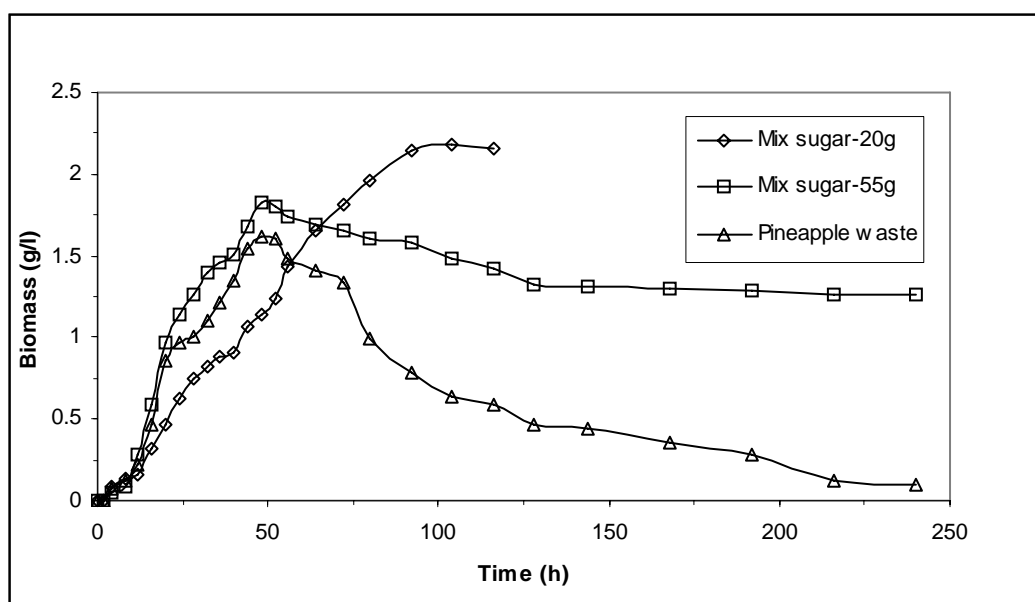
Figure 4.26: Time course of biomass concentration during lactic acid fermentation of mixed sugar and pineapple waste. Experimental conditions: T, 40°C; pH, 6.0; inoculum, 5% and stirring speed, 50 rpm.

4.5.2.2 Sugar Utilisation

The trends of glucose and fructose consumption are given in Figure 4.27. During the first hours of fermentation, the glucose and fructose accumulated in the medium until 8, 16, and 48 hours for 20 g of mixed sugar, 55 g of mixed sugar and pineapple waste, respectively. The maximum sugar concentration accumulated in the medium is given in Table 4.15.

Table 4.15: The maximum sugar concentration accumulated in the mixed sugar and pineapple waste medium.

| Fermentation | Maximum sugar concentration accumulated | | | |
| | Glucose | | Fructose | |
	Concentration (g/l)	Time (hr)	Concentration (g/l)	Time (hr)
Mixed sugar (20 g/l)	8.40	8	7.82	8
Mixed sugar (55 g/l)	24.36	16	23.75	16
Pineapple waste (70 g/l)	30.59	48	28.32	48

The accumulation of these sugars was due to the higher rate of sucrose hydrolysis to glucose and fructose than the conversion of these substrates to lactic acid. The concentration of glucose in the medium was higher than fructose's, which designates that hydrolysis of sucrose resulted in higher concentration of glucose than fructose. The glucose utilisation is so far higher than fructose whereas the sucrose consumption was faster than both sugars'. The glucose and fructose in 20 g/l of mixed sugar medium were completely utilised but only glucose in 55 g/l of mixed sugar was wholly utilised. On the other hand both sugars were not completely utilised in the case of pineapple waste. Sucrose was fully consumed for all mediums used.
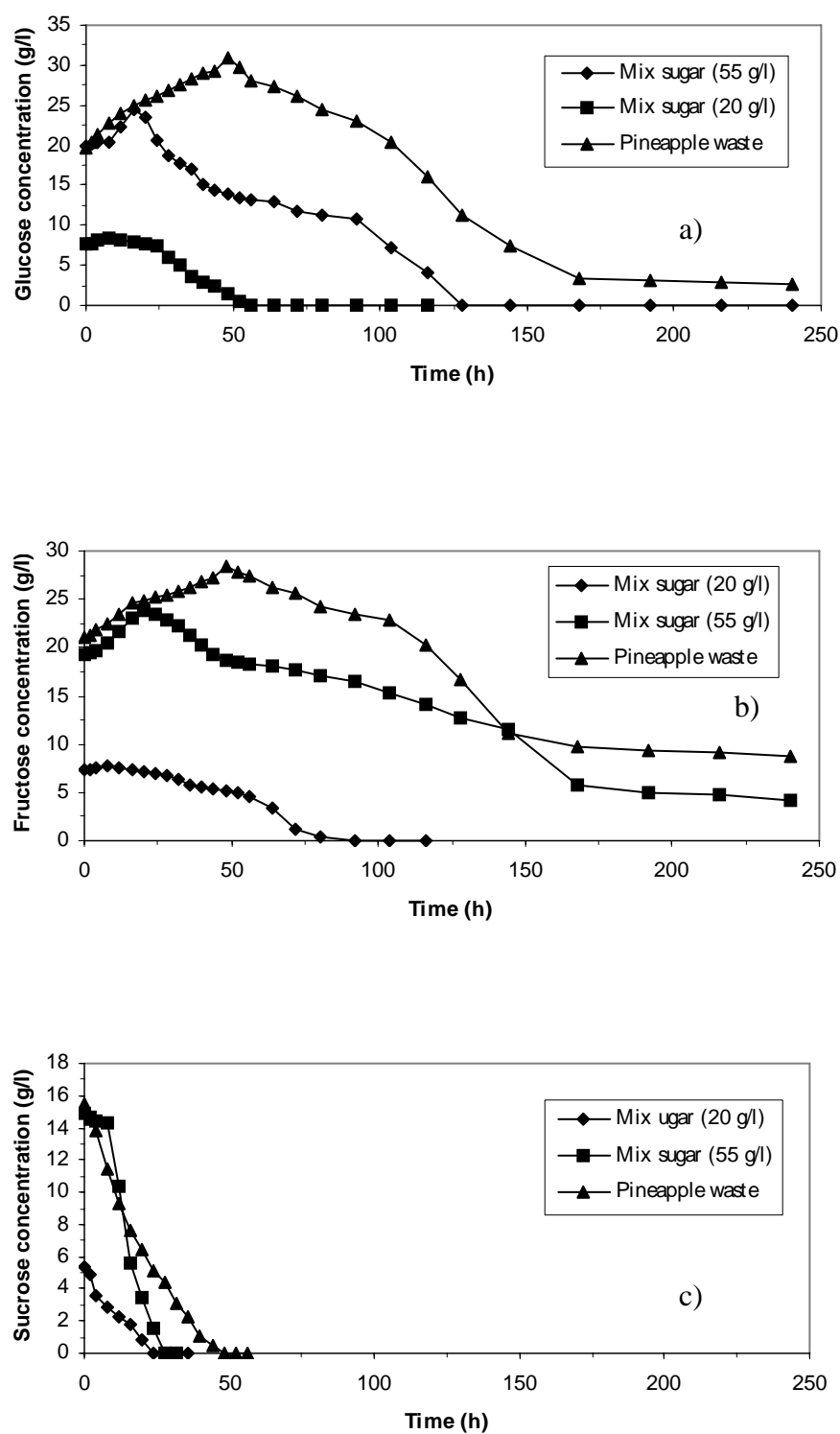
Figure 4.27: Time course of sugar concentration during lactic acid fermentation of mixed sugar and pineapple waste. a) glucose; b) fructose and c) sucrose. Experimental conditions: T, 40^{o}C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

4.5.2.3 Lactic Acid Production

The time dependence of lactic acid production during lactic acid fermentation of mixed sugar medium is given in Figure 4.28. Comparing the yield of pineapple waste fermentation to 20 and 50 g/l of mixed sugar fermentation, increasing mixed sugar concentration from 20 to 55 g/l made the lactic acid production increased from 18.22 to 47.65 g/l and the yield decreased from 92 to 87 %. The lactic acid yield decreased due to inhibition as a result of higher sugar concentration. The lactic acid yield was smaller using pineapple waste as a substrate, which contains 70 g/l total sugar, compared to pure mixed sugar which was only 54.97 g/l or 78.54 % of yield. This may reflect the complex nature of pineapple waste that inhibited the lactic acid production.
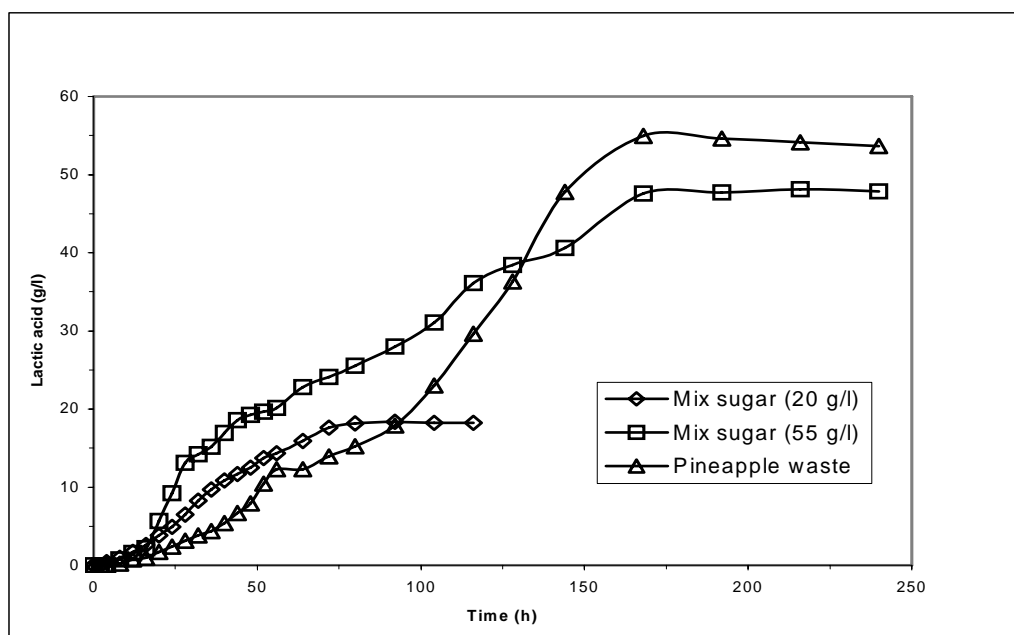
Figure 4.28: Time course of lactic acid concentration during lactic acid
fermentation of mixed sugar and pineapple waste. Experimental
conditions: T, 40°C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

The effect of different sugar concentrations of lactic acid fermentation by *L. delbrueckii* is summarised in Table 4.16.

Table 4.16: Summary of fermentation results for fermentation characteristics of different concentrations of carbon source.

Fermentation	X_m (g/l)	P_m (g/l)	Fermentation time (hr)	Yield (%)	Productivity (g/l.h)
Mixed sugar (20 g/l)	2.18	18.25	72	92.00	0.260
Mixed sugar (55 g/l)	1.80	47.65	168	86.63	0.280
Pineapple waste (70 g/l)	1.60	54.97	168	78.54	0.327

4.5.3   Substrate Utilisation Selectivity

In general, lactic acid bacteria use carbon sources such as glucose, fructose, lactose, maltose and sucrose for growth and lactic acid production. Starch cannot be utilised but there are several reports stating that certain members of lactic acid bacteria can be used to liquefy the starch. *Lactobacillus delbrueckii ATCC 9649* is the preferred organism for lactic acid production by using glucose, fructose, and sucrose as carbon source. Lactose cannot be utilised by *Lactobacillus delbrueckii* (Atkinson and Mavituna, 1991).

There are three carbon sources which exist in the pineapple waste namely glucose, fructose and sucrose that are potential to be fermented to produce lactic acid. In this section, the selectivity of the sugar utilisation for lactic acid production by *Lactobacillus delbrueckii ATCC 9649* using pure single sugar, mixed sugar and

pineapple waste, as a carbon source will be discussed. The fermentations were performed anaerobically at pH, temperature inoculum and stirring speed of 6.0, 40^{o}C, 5 % and 50 rpm, respectively.

4.5.3.1 Mixed Sugar

Three different substrates were used in this study, which were glucose, fructose and sucrose with initial concentrations of 20 g/l each. The sugar concentration (20g/l) was chosen according to the sugar concentration existed in the pineapple waste. Effect of different substrates and concentrations on bacterial growth, sugar utilisation and lactic acid production are given in Figures 4.29 and 4.30, and Table 4.17

During the first 54 hours of fermentation, the growth rate of *Lactobacillus delbrueckii* was similar for both glucose and mixed sugar medium. After that, the growth for glucose was faster than mixed sugar, and maximum concentrations of biomass were achieved at 2.28 and 2.16 g/l respectively (Figure 4.29a). The growth of *Lactobacillus delbrueckii* for fructose and sucrose medium was slower than mixed sugar medium (Figures 4.29b and 4.29c). A conclusion is drawn that glucose is the best medium for growth of *Lactobacillus delbrueckii,* followed by mixed sugar, fructose and sucrose.

The rate of sugar utilisation was almost similar for glucose and mixed sugar medium, except for the end of fermentation process where the glucose medium performed higher sugar utilisation than mixed sugar medium. Similar trend was also observed in lactic acid production. Although the maximum lactic acid concentration obtained was similar, glucose medium showed faster response than mixed sugar medium at the end of fermentation process (Figures 4.30a and 4.30d).

After 32 hours of fermentation, all lactic acid production was associated with microbial growth. Sucrose utilisation was faster than that of glucose and fructose but

the lactic acid production was slower in general. This indicates that the rate of hydrolysis was higher than the conversion of glucose and fructose to lactic acid.

After 64 hours of fermentation, sucrose was completely utilised (Figure 4.30c) but glucose and fructose were fully utilised only after 68 and 104 hours, respectively (Figures 4.30a and 4.30b). The fructose utilisation was slower than mixed sugar utilisation (Figure 4.30b) but the sucrose utilisation was faster than mixed sugar or glucose utilisation. The sucrose consumption depends on the rate of sucrose hydrolysis. The lactic acid yield obtained for glucose, fructose and sucrose fermentation were 92, 93 and 93 %, respectively with the residual sugar of 7-8% that was utilised for bacterial growth and maintenance requirement of the cell. This result is similar to those reported by Buyukgungor et al. (1984) and Goncalves et al. (1997). They stated that glucose consumption in lactic acid fermentation using *Lactobacillus delbrueckii* was complete for initial glucose concentration from 2 to 10% with the yield of 90 %.
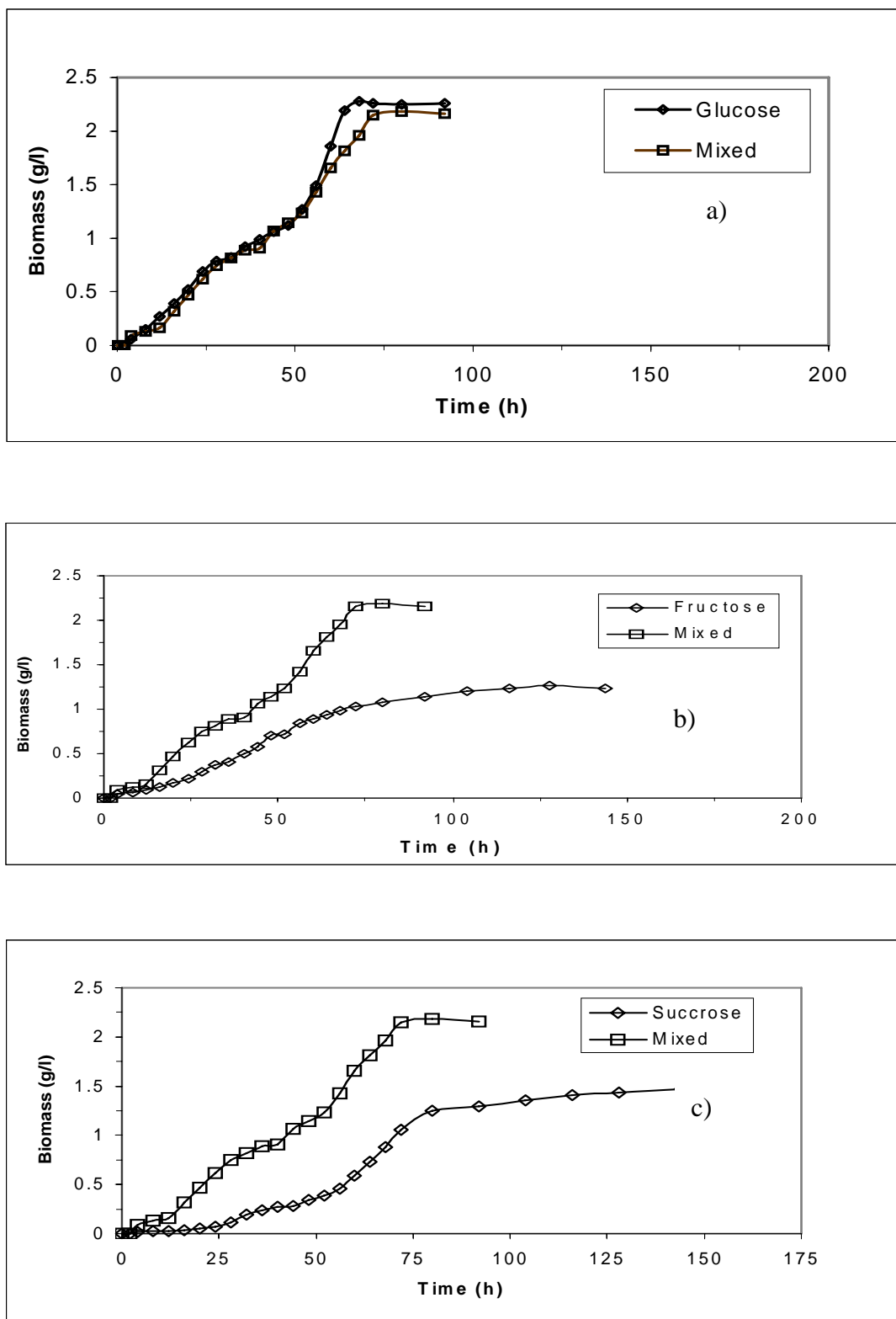
Figure 4.29: Effect of different substrate on *L. delbrueckii* growth of lactic acid
fermentation. Experimental conditions: T, 40^{o}C; pH, 6.0; inoculum,
5%; and stirring speed, 50 rpm.
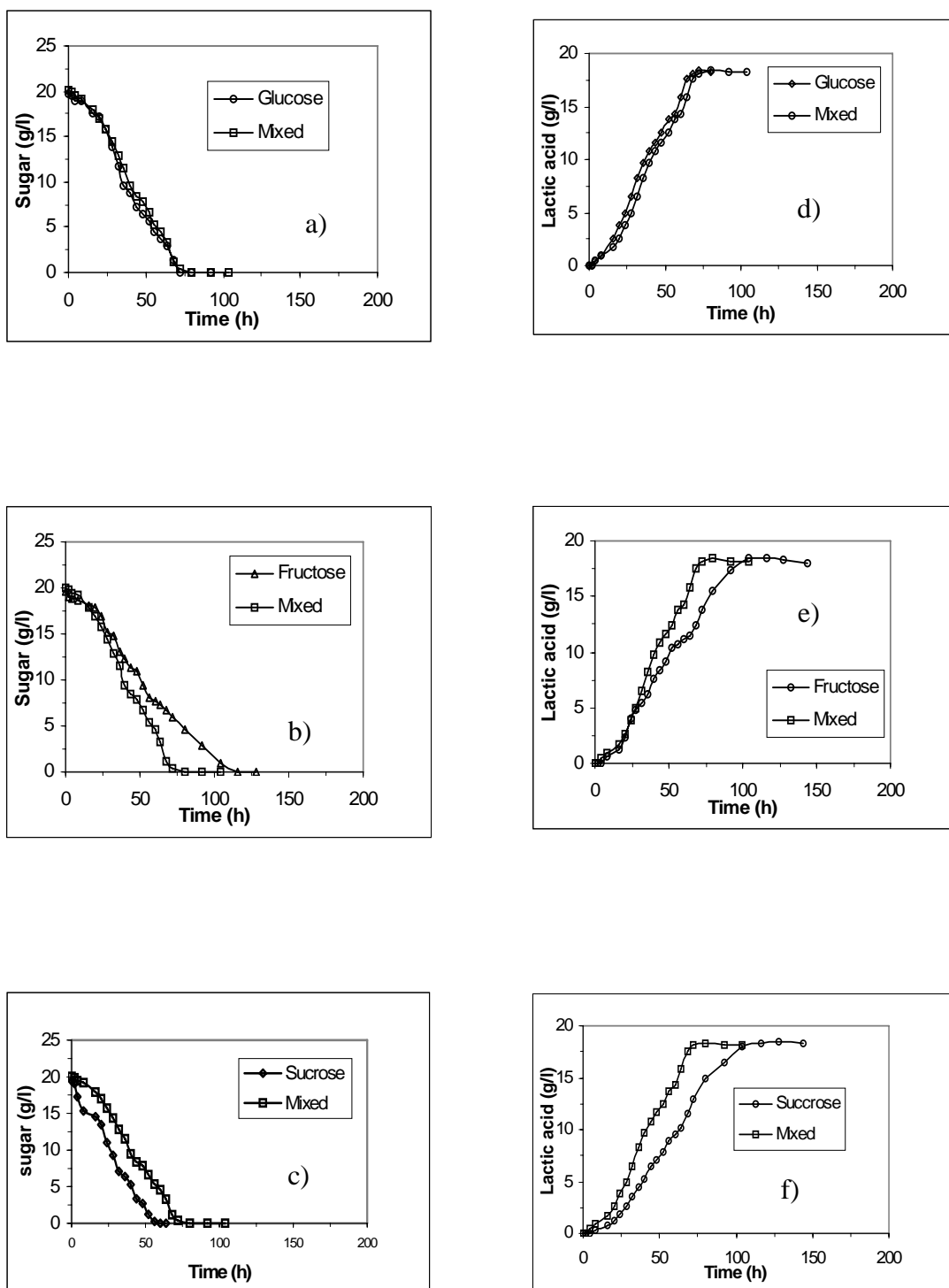
Figure 4.30: Mixed sugar utilisation and lactic acid production compared with pure sugar. Experimental conditions: T, 40^{o}C; pH, 6.0; inoculum, 5%; and stirring speed, 50 rpm.

4.5.3.2 Pineapple Waste

The sugar utilisation, *L. delbrueckii* growth and lactic acid production is given in Table 4.17. During the first 4 hours of fermentation (lag phase), the concentration of the glucose and fructose for sucrose, mixed sugar and pineapple waste increased while the sucrose concentration decreased. It is obvious that sucrose is converted to glucose and fructose. The reason could be that before fermentation process the sucrose was hydrolysed by levanse sucrose enzyme or inverted to glucose and fructose. The exponential phase was achieved after 4 hours of fermentation except for sucrose. During this period, lactic acid was produced but the concentration of glucose and fructose still continued to increase. The increase of these sugars concentration shows that the rate of hydrolysis was higher than the rate of conversion of these sugars to lactic acid. The growth rate of *L. delbrueckii* on mixed sugar (55 g/l) was faster than pineapple waste, glucose, mixed sugar (20 g/l), fructose and sucrose.

A short stationary phase (48-52 hours) was observed for mixed sugar (55 g/l) and pineapple waste, and it was followed by death phase. A long stationary phase was observed for glucose (64-80 hours), mixed sugar (20 g/l) (72-104 hours), fructose (104-144 hours) and sucrose (116-144 hours). The maximum biomass concentrations for glucose, mixed sugar (20 g/l), fructose, sucrose, mixed sugar (55 g/l) and pineapple waste were 2.43, 2.31, 1.41, 1.58, 1.97 and 1.76 g/l, respectively. Although the death phase for mixed sugar (55 g/l) and pineapple waste occurred at a shorter period, the lactic acid production still continued. This denotes that the bacteria were able to produce lactic acid even after growth had ceased. The glucose consumption was faster than fructose's meanwhile the sucrose utilisation is faster than both sugars. All pure sugar and mixed sugar (20 g/l) were completely utilised. When the concentration of mixed sugar was increased to 55 g/l, fructose was not completely converted to lactic acid. However when the concentration was increased further to 70 g/l (pineapple waste), both glucose and fructose were not fully utilised due to inhibition effect as a result of higher sugar concentration. This may reflect the complex nature of pineapple waste, which could inhibit the fermentation process.

Table 4.17: Lactic acid fermentation of multisubstrates with different concentrations.

| Types of sugar | T = 0 hours | | | | | T = 4 hours | | | | | T = 8 hours | | | | |
| | X (g/l) | S (g/l) | | | P (g/l) | X (g/l) | S (g/l) | | | P (g/l) | X (g/l) | S (g/l) | | | P (g/l) |
		Glu	Fru	Suc			Glu	Fru	Suc			Glu	Fru	Suc	
Glucose	0.150	20.00	-	-	0.00	0.156	19.75	-	-	0.23	0.300	18.86	-	-	0.95
Fructose	0.150	-	20.00	-	0.00	0.155	-	19.66	-	0.05	0.219	-	18.56	-	0.67
Sucrose	0.150	-	-	20.00	0.00	0.151	1.12	0.95	17.24	0.00	0.158	2.18	1.64	15.35	0.26
Mixed (20 g/l)	0.150	7.25	7.25	5.50	0.00	0.156	8.25	7.62	3.56	0.18	0.282	8.45	7.83	2.96	0.91
Mixed (55 g/l)	0.150	20.00	20.00	15.00	0.00	0.155	20.59	20.28	14.37	0.06	0.276	21.41	21.37	14.26	0.76
Pine-apple	0.150	20.00	20.76	15.51	0.00	0.152	21.28	21.83	13.48	0.00	0.238	22.68	22.51	11.45	0.22

X, S and P are biomass, sugar and lactic acid concentration (g/l), respectively

Table 4.17: Lactic acid fermentation of multisubstrate with different concentrations (Continued).

Types of sugar	T = 12 hours					T = 16 hours					T = 32 hours				
	X (g/l)	S (g/l)			P (g/l)	X (g/l)	S (g/l)			P (g/l)	X (g/l)	S (g/l)			P (g/l)
		Glu	Fru	Suc			Glu	Fru	Suc			Glu	Fru	Suc	
Glucose	0.370	17.64	-	-	1.74	0.440	17.18	-	-	2.59	0.971	9.66	-	-	8.28
Fructose	0.245	-	18.12	-	0.95	0.271	-	17.97	-	1.18	0.523	-	13.26	-	5.45
Sucrose	0.180	2.72	1.87	14.56	0.54	0.197	3.21	2.28	13.49	0.79	0.344	4.85	4.33	6.38	3.61
Mixed (20 g/l)	0.312	8.18	7.52	3.59	1.58	0.365	7.92	7.33	1.63	2.57	0.890	6.09	6.25	0.00	7.19
Mixed (55 g/l)	0.430	22.37	21.64	8.41	1.56	0.742	23.36	22.16	5.56	2.21	1.54	16.64	21.21	0.00	14.23
Pine-apple	0.365	23.90	23.48	9.35	0.74	0.610	24.88	24.56	7.59	3.82	1.250	27.54	25.89	3.12	3.82

X, S and P are biomass, sugar and lactic acid concentration (g/l), respectively.

Table 4.17: Lactic acid fermentation of multisubstrate with different concentrations (Continued).

Types of sugar	T = 48 hours					T = 64 hours					T = 80 hours				
	X (g/l)	S (g/l)			P (g/l)	X (g/l)	S (g/l)			P (g/l)	X (g/l)	S (g/l)			P (g/l)
		Glu	Fru	Suc			Glu	Fru	Suc			Glu	Fru	Suc	
Glucose	1.270	5.62	-	-	12.48	2.430	1.13	-	-	17.55	2.430	0.00	-	-	18.32
Fructose	0.748	-	9.39	-	9.15	1.087	-	6.72	-	11.54	1.233	-	2.86	0.00	15.47
Sucrose	0.489	5.38	5.07	1.25	7.06	0.880	4.55	4.58	0.00	9.67	1.203	0.58	2.97	0.00	14.89
Mixed (20 g/l)	1.140	1.49	5.16	0.00	12.23	1.960	0.00	1.144	0.00	17.42	2.310	0.00	0.00	0.00	18.20
Mixed (55 g/l)	1.976	13.65	18.77	0.00	19.22	1.843	12.90	18.06	0.00	22.74	1.761	11.24	17.13	0.00	25.51
Pine-apple	1.766	30.96	28.37	0.00	7.95	1.652	27.28	26.24	0.00	13.06	1.147	24.53	24.54	0.00	15.24

X, S and P are biomass, sugar and lactic acid concentration (g/l), respectively.

4.6   Fed Batch Fermentation

4.6.1   Introduction

Batch culture is still the most important process operation used in fermentation industry. There are technical and biological reasons for the choice of this strategy, which include simple operation and less effort in chemical engineering, measurement and control. Batch culture is less suitable if substrate inhibition or growth dependent inhibitory by product formation occurs (Hammes and Whiley, 1993).

Some pitfalls of batch culture can be overcome with fed batch operation. Fed batch culture is a batch culture where the nutrients are fed continuously or sequentially with substrate without the removal of fermentation broth. It is widely used for the production of microbial biomass, ethanol, organic acids, antibiotics, vitamins, enzymes, and other compounds (Roukas and Kotzekidou., 1997).

The rationale for fed batch operations are: the excess of carbon caused inhibition of cell growth, the excess of carbon or nitrogen caused the catabolite repression or relative decrease in the rate of synthesis of a specific enzyme resulting from exposure to rapidly assimilated carbon source, the formation of undesirable product, and unable to initially batch all nutrient (Wang et al., 1979).

The nutrient limiting for the cell growth in a fed-batch bioreactor provides an excellent means of controlling the growth rate and the metabolism of the cell. Thus, fed-batch bioreactor may be operated in a variety of ways by regulating the feed rate in a predetermined manner (feed forward control) or using feed back control. The most commonly used are constantly, exponentially fed, extended and repeated fed-batch cultures. In extended fed-batch culture, the feed rate is regulated to maintain the substrate concentration constant until the bioreactor is full. These modes of operations are limiting cases of the complex optimal feed rate profiles. In a repeated fed-batch culture a part of the broth remaining after a partial removal at the end of a cycle is used as an inoculum for the next cycle (Lim and Lee, 1993)

Effects of constant feeding rate on lactic acid production from deproteinised whey by mixed cultures of *Lactobacillus casei* and *Lactococcus lactis* using fed batch culture have been reported by Roukas and Kotzekidou. (1998). The lactic acid production was performed by feeding substrate containing 75, 100 and 150 g/l of sugar. The result shows that the lactic acid concentration increased slightly with the increase in sugar concentration up to100 g/l, but remained constant beyond this value. Effect of feeding rate was also studied and the result indicated that the lactic concentration decreased if the feeding rate was increased from 250 to 1000 ml/h. The lactic acid productivity and yield remained practically constant with an increase in feeding rate from 250 to 334 ml/h, but decreased at higher feeding rate from 500 to 1000 ml/h.

For the results of batch fermentation, as discussed in Section 4.4.5, the increasing of initial sugar concentration over 70 g/l in lactic acid fermentation of pineapple waste by *L. delbrueckii* caused the decreasing of maximum lactic acid production, yield and productivity. In order to obtain higher lactic acid productivity and lactic acid concentration, fed-batch fermentation study is significant. According to literature, the constantly feed used with slower feed rate to maintain the concentration of substrate does not increase as a result of the lengthy time of fermentation (240 hours). The effect of feeding concentration *on L. delbrueckii* growth, sugar utilisation and lactic acid production are presented and discussed in the following section.

4.6.2   Fed-Batch Culture

The fermentation was performed in a 3-litre stirred fermentor with working volume of 2.5 litres. The fermentation was performed in two phases. In the first phase, 700 ml of pineapple waste containing 65 g/l of sugar was inoculated with 5% (v/v) of inoculum. *L. delbrueckii* were grown in batch culture for 24 hours. In the second phase, the production mediums containing 65, 90, and 115 g/l sugar were continuously added into the fermentor at constant rate of 8.5 ml/h.

Effect of the sugar feeding concentration on *L. delbrueckii* growth is shown in Figure 4.31a. The feeding was begun at exponential growth rate (24 hours) and the *L. delbrueckii* growth after 24 hour shows that the specific growth rates [defined as $(1/X)(dX/dt) = \mu$] determined by the slopes in the logarithmic phase increased from feeding concentration of 65 to 90g/l. If the sugar feeding concentration were further increased from 90 to 115 g/l, the specific growth rates would decrease. The possible reason could be the substrate inhibition as result of higher sugar concentration in the medium. The residual sugar concentration after feeding decreased for feeding concentration of 65 and 90 g/l but for 115 g/l, the residual sugar concentration in the medium was almost constant. The concentrations of sugar in the medium after 56 hours for initial sugar concentration of 65, 90 and 115 g/l were 45.24, 47.75 and 58.34, respectively.

The maximum concentration of biomass obtained for sugar feeding concentration of 90 g/l was similar to 115 g/l as 2.55 g/l. At feeding rate of 65 g/l, the maximum concentration was achieved at 2.0 g cell dry weight /l meanwhile the *L. delbrueckii* growth was faster than that of 115 g/l. However after 80 hours, the growth declined gradually which indicates that at the feeding concentration of 65 g/l, the concentration of nutrient in the medium was not enough to maintain the cell growth. The short stationary phases for three sugar feeding concentration occurred at 56-72, 56-72 and 104-116 hours, respectively. Followed by the death phase, the growth rate began to decrease gradually and the concentrations of biomass reached at the end of fermentation were 0.6, 2.3 and 2.2 g/l, respectively. The decreasing of biomass concentration was due to the increasing of the volume in medium (diluted) or dying. Although the *L. delbrueckii* growth decreased, it continued to produce lactic acid.

Effect of feeding concentration on sugar consumption is given in Figure 4.31b. During the first 24 hours of fermentation (before feeding), the concentration of sugar decreased from 65.0 to 59.4 g/l as a result of sugar utilisation for *L. delbrueckii* growth and lactic acid production. During this period, the microorganism was building the necessary enzymes for the sugar conversion to lactic acid with lactic acid concentration obtained at only 6.8 g/l. After 24 hours, the consumption

pattern of the sugar shows that for sugar feeding concentration of 65 g/l, the sugar concentration continuously decreased while for 115 g/l it was constant although the lactic acid production increased. It might be due to the lactic acid production was faster than sugar addition to the system. A similar pattern was also obtained for sugar feeding concentration of 90 g/l. The sugar concentration for 65 and 90 g/l at 192 hours decreased to 11.9 and 23.2 g/l, respectively. For the sugar feeding concentration of 115 g/l, after 24 hours the concentration of residual sugar was relatively constant until 80 hours. This might be due to the rate of feeding was equal to the conversion rate of sugar to lactic acid. After that, the concentration of sugar decreased which indicates that the conversion of sugar to lactic acid was faster than the increasing of sugar concentration in the medium. After 216 hours, the concentration of the sugar increased while the lactic acid production was constant. This indicates that the addition of sugar to the fermentor was faster than the conversion of sugar to lactic acid.

Figure 4.31c illustrates the effects of sugar feeding concentrations on lactic acid production. The lactic acid production before feeding (24 hours) was 4.8 g. After that lactic acid production for 90 g/l was faster than those of 115 and 65 g/l. The maximum lactic acid production shows that the highest lactic acid concentration observed (122.91 g) was for 90 g/l of sugar, followed by 115 g/l (113.88 g) and 65 g/l (109.63 g). After 216 hours, the lactic acid production was relatively constant but the sugar concentration increased because after this time, the sugar addition was faster than the conversion of sugar to lactic acid, and also due to the increase in substrate volume in the fermentor with the time.
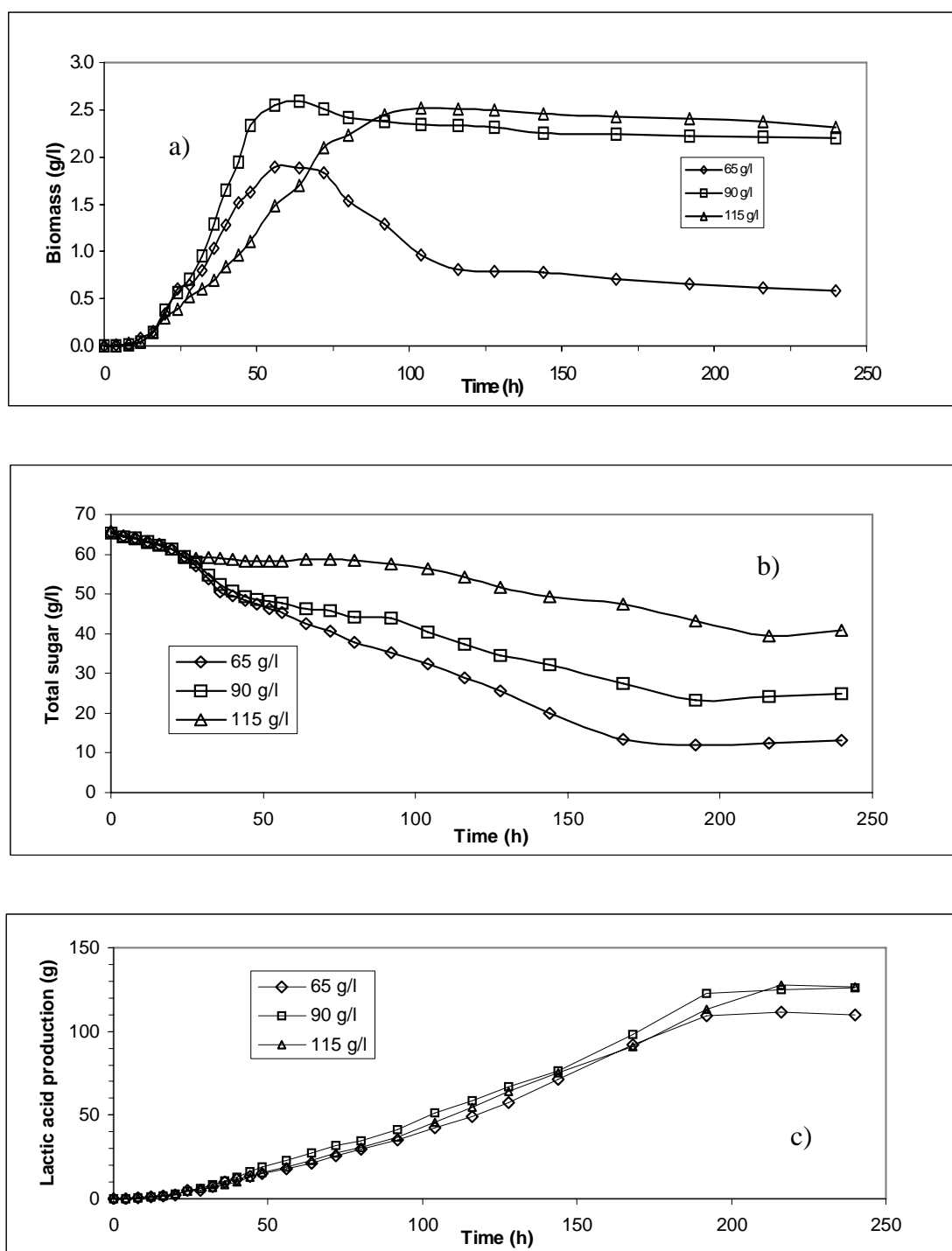
Figure 4.31: Effect of sugar feeding concentration on pineapple waste fermentation

using fed batch culture. a) *L. delbrueckii* growth, b) sugar consumption,

c) lactic acid production. Experimental conditions, T: 40°C, pH: 6.0,

inoculum: 5% and stirring speed: 50 rpm.

Effect of sugar feeding concentration on lactic acid productivity is given in Figure 4.32. By increasing the sugar feeding concentration from 65 to 90 g/l, the productivity increased but if it was further increased to115 g/l, the productivity decreased due to the inhibition by high sugar concentration in the medium. The maximum productivity obtained was 0.44 g/l.h with sugar feeding concentration of 90 g/l at 48 hours of fermentation. This followed by sugar feeding concentration of 115 g/l and 65 g/l. Batch fermentation with the maximum productivity obtained was 0.36, 0.35 and 0.16 g/l h for the three sugar feeding concentrations respectively. These results were different from those reported by Suscovic et al. (1992). They obtained 4 g/l.h at feeding rate of 97.2 g/l after19 hours. This difference might be due to substrate used in lactic acid fermentation. If the results were compared with batch culture, the lactic acid productivity was twice and half fold higher than batch productivity at 48 hours. The fed batch culture also gave higher lactic acid concentration, lactic acid yield, specific lactic acid productivity and biomass concentration compared to batch fermentation.
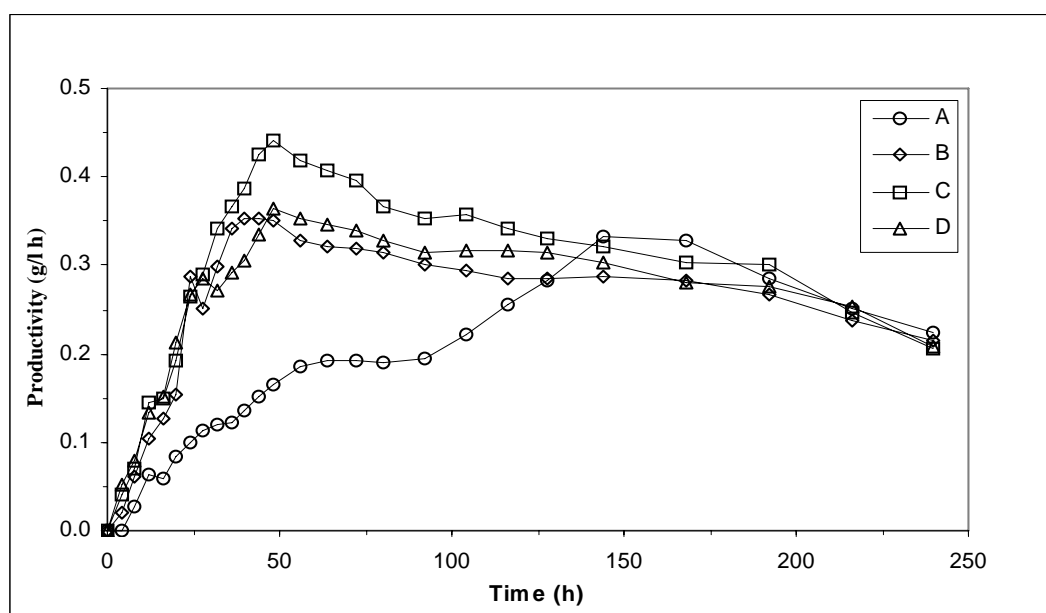
Figure 4.32: Effect of sugar feeding concentration on volumetric productivity of pineapple waste fermentation using batch and fed batch cultures. Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm. A) Batch, B) fed batch (65 g/l), C) fed batch (90 g/l), and D) fed batch (115 g/l).

CHAPTER V

MODELLING Of LACTIC ACID FERMENTATION

5.1   Introduction

Knowledge of the kinetics of fermentation is necessary in order to size the fermentor and its associated equipment, and this information is normally obtained from laboratory experiment using one to three litres of fermentor. In batch fermentation, the kinetic model provides information to predict the rate of cell mass or product generation; while in continuous fermentation it will predict the rate of product formation under given conditions (Russel, 1987). The mathematical model can be constructed by setting up a verbal model, translation into a mathematical model, solving the equations, determination of parameter sensitivity and testing the model. The verbal model or verbal statement is the relationship between variables which are not stated by symbols (Roels, 1983).

In this section, the development of process models on batch fermentation was performed at first by translating a verbal model of the process into a mathematical expression. The kinetic parameters such as specific growth rate and specific product formation rate were determined by application of conservation laws (material balance) for bacterial growth, substrate utilisation and lactic acid production to obtain the models in the form of mathematical equations. The differential equations were solved by integration to estimate the parameters. The validity of the models was tasted using standard deviation between the measured and calculated concentrations of biomass, substrate and lactic acid.

5.2 Model Development

The model can be structured on the basis of biomass concentration, biomass component such as concentrations of metabolite (M), enzymes (E) and RNA (R), by population related variables, describing different morphological types of cells or cell aging (Nielsen et al., 1991). The structured model can be described by

$$\frac{dX}{dt} = f(X, M, E, R, ...) \qquad\qquad \ldots (5.1)$$

where X is cell concentration (g/l) and t is time (hour).

In the simplest approach to model batch culture, the unstructured batch growth models were used. Based on this model, the rate of increase in biomass is a function of the single parameters such as cell number or biomass concentration only (Bailey and Ollis, 1977). Thus,

$$\frac{dX}{dt} = f(X) \qquad\qquad \ldots (5.2)$$

One of the simplest models belonging to the general form given by equation (5.2) is Malthus' law, which is in the form of

$$\frac{dX}{dt} = \mu.X \qquad\qquad \ldots (5.3)$$

where μ is specific growth rate (hour^{-1}).

Unstructured models are the simplest; they take the cell mass as a uniform quantity without internal dynamics where reaction rate depends only on the conditions in the liquid phase of the reactor. Therefore, the models only contain bacterial growth, substrate utilisation and product formation.

5.2.1 Bacterial Growth

Microbial growth is usually characterised by an increase in cell mass and cell number with the time. Mass doubling time may differ from cell doubling time because the cell mass can increase without an increase in cell number. However the interval between cell mass or number doubling is constant with time, the microorganism is growing at an exponential rate. The material balance of cell can be described by,

$$\frac{dX}{dt} = \mu.X - k_{d.}X \qquad \qquad ......(5.4)$$

where $\frac{dX}{dt}$ is microbial growth rate (g l^{-1} h^{-1}) and k_d is specific death rate (hour^{-1}).

In batch fermentation, the specific growth rate is constant and independent of the changing of the nutrient concentration. It is expected that growth rate, as any chemical reaction rate, will depend on the concentration of chemical nutrients. Monod-Type relationship on the specific growth rate μ, is usually expressed as a function of the limiting substrate concentration (S),

$$\mu = \mu_m \left[\frac{S}{K_s + S} \right] \qquad \qquad ......(5.5)$$

where μ_{max} is maximum specific growth rate (h^{-1}), S is substrate concentration (g/l) and K_s is saturation constant (g/l).

Substituting the value of μ from equation (5.5) to (5.4), gives

$$\frac{dX}{dt} = \mu_{max} \left(\frac{S}{K_s + S} \right) X - k_d X \qquad \qquad ......(5.6)$$

or,

$$\frac{dX}{dt} = \left\{ \mu_{\max}\left(\frac{S}{K_s + S}\right) - k_d \right\} X \qquad \ldots\ldots(5.7)$$

5.2.2 Substrate Utilisation

The growth was described in term of simple first order rate equation and then related to substrate utilisation via a stoichiometric relationship. Metabolic product formation can be similarly related to nutrient consumption. Furthermore, product formation cannot occur without the presence of cells. Thus, it is possible to relate substrate consumption and product formation with growth by making material balance as follows:

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\frac{dX}{dt} + \frac{1}{Y_{p/s}}\frac{dP}{dt} + mX , \qquad \ldots\ldots(5.8)$$

where $(\frac{dS}{dt})$ is the specific rate of substrate utilisation, $(\frac{dP}{dt})$ is the specific rate of product formation, $Y_{p/s}$ is the product yield on the utilised substrate (g product / g substrate), $Y_{x/s}$ is biomass yield on the utilised substrate (g cell / g substrate) and m is coefficient of maintenance (g substrate / h g cell).

When the product is associated with energy metabolism as a catabolic of the carbon source (Wang et al., 1979), the equation (5.8) can be written as

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\frac{dX}{dt} + mX \qquad \ldots\ldots(5.9)$$

Frequently the maintenance requirement is low relative to growth $(mX <<< \frac{1}{Y_{x/s}}\frac{dX}{dt})$ (Wang et al., 1979), therefore equation (5.9) becomes,

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\frac{dX}{dt} \qquad \text{.....(5.10)}$$

Substitution equation (5.3) and (5.5) into (5.10), gives

$$\frac{dS}{dt} = -\frac{1}{Y_{x/s}}\mu_{\max}\left(\frac{S}{K_s + S}\right)X \qquad \text{.....(5.11)}$$

5.2.3 Product Formation

The product formation kinetics on lactic acid fermentation by *L.delbrueckii* have been proposed by Luedeking and Piret (1959). This model indicates a simple relationship between the rate of lactic acid production and the growth rate and biomass concentration as given by

$$\frac{dP}{dt} = k_1\frac{dX}{dt} + k_2 X \qquad \text{.....(5.12)}$$

where k_1 is growth associated product formation (g product / g cell) and k_2 is non growth associated product formation (g product / h. g cell).

5.3 Parameter Estimation

Batch fermentation of pineapple waste to lactic acid by *Lactobacillus delbrueckii* was studied in 3-litre stirred fermentor as a function of temperature, pH and size of inoculum. During fermentation process, the product, substrate and cell concentrations were measured. The data were used for the estimation of kinetic parameters such as k_d, $Y_{s/x}$, $\mu_{\max}$, k_1, k_2 and K_s in the differential equations for the cell balance (5.7), substrate balance (5.11) and product balance (5.12) by linear regression analysis.

To calculate the parameters of k_d, $Y_{s/x}$, μ_{max}, and K_s, it can be assumed that the concentration of the substrate (S) at initial stages of fermentation is larger than the saturation constant (K_s). Therefore the value of $(K_s + S \approx S)$ or $[S/(K_s + S) = 1]$, and the equations (5.7) and (5.12) can be expressed as follows:

$$\frac{dS}{dt} = -\frac{1}{Y_{x/s}}\mu_{max}X \qquad \text{.......(5.13)}$$

and

$$\frac{dX}{dt} = (\mu_{max} - k_d)X \qquad \text{........(5.14)}$$

Substitution equation (5.13) into (5.14), gives

$$S_t - S_0 = \gamma_1(X_t - X_0) \qquad \text{....... (5.15)}$$

where S_0 and X_0 are initial sugar and initial cell concentration, S_t and X_t are sugar and cell concentrations after the time interval t.

Integration of the equation (5.14), can be obtained

$$\ln\frac{X_t}{X_0} = \gamma_2.t \qquad \text{........(5.16)}$$

The coefficients in the equation (5.15) and (5.16) have the following groups of parameters,

$$\gamma_2 = (\mu_{max} - k_d) \qquad \text{.........(5.17)}$$

and

$$\gamma_1 = -\frac{\mu_{max}}{\gamma_2 Y_{x/s}} \qquad \text{.........(5.18)}$$

Integration of equation (5.7), gives

$$X_t(\mu_m, k_d) = X_0.\exp.\int h_s.dt \qquad\qquad\qquad ....(5.19)$$

where

$$h_s = \frac{\mu_m.S}{K_s + S} - k_d \qquad\qquad\qquad ....(5.20)$$

Substitution the equation (5.17) with (5.20) becomes

$$h_s = \gamma_2 - \frac{\mu_m.K_s}{K_s + S} \qquad\qquad\qquad ....(5.21)$$

Enters the value of h_s into equation (5.19), becomes

$$X_t(\mu_m, k_d) = X_0.\exp.\int \left(\gamma_2 - \frac{\mu_m.K_s}{K_s + S} \right).dt \qquad\qquad\qquad ....(5.22)$$

If the value of $\gamma_3 = \mu_m K_s$, the equation (5.22) becomes

$$X_t(\mu_m, k_d) = X_0.\exp.\int \left(\gamma_2 - \frac{\gamma_3}{K_s + S} \right).dt \qquad\qquad\qquad ....(5.23)$$

Integration of equation (5.11), gives:

$$S_t = S_o - K_s \ln\frac{S_0}{S_t} + \frac{\mu_m X_o}{\gamma_2.Y_{x/s}} \exp.\gamma_2.t \qquad\qquad\qquad ....(5.24)$$

The equation (5.24) can be written as,

$$S_t = S_o - K_s \ln\frac{S_0}{S_t} + \gamma_4 \exp.\gamma_2.t \qquad\qquad\qquad ....(5.25)$$

The saturation constant (K_s) is estimated by equation (5.25) as follows,

$$K_s = \frac{S_o - S_t + \gamma_4 \exp.\gamma_2.t}{\ln \dfrac{S_o}{S_t}} \qquad\qquad ……..(5.26)$$

where $\gamma_4 = \gamma_1 X_o$ and S_t is concentration of substrate after the time interval t.

The parameters k_1 and k_2 from the Luedeking-Piret model are obtained from measured concentration of lactic acid during complete fermentation. By integration of equation (5.12) gives

$$P = k_1(X_t - X_0) + k_2 \int_0^t X.dt \qquad\qquad ……..(5.27)$$

Substitution value of X_t from equation (5.16) into equation (5.27) will give

$$P(t) = k_1(X_t - X_0) + k_2 \int_0^t X_0 \exp.\gamma_2.t.dt \qquad\qquad …….(5.28)$$

Integration of equation (5.28) gives

$$P(t) = k_1(X_t - X_0) + \frac{k_2 X_o}{\gamma_2} \exp.\gamma_2.t \qquad\qquad …… (5.29)$$

where $P(t)$ is concentration of lactic acid after the time interval t.

5.3.1   Microbial Growth

Growth, which is characterised by increase in cell mass and or number, occurs only where certain chemical and physical conditions are satisfied, such as acceptable temperature and pH as well as the availability of required nutrients. The kinetics of growth and product formation reflects the cell ability to respond to the environment and here in lies the rationale for a study of growth kinetics. In this

section the effect of various parameters such as types of sugar, temperature, pH and inoculum size are presented.

5.3.1.1 Dependence Of The Model Parameters On Types Of Sugar

L. delbreuckii is the best strain to produce lactic acid using glucose, fructose and sucrose as substrates with conversion of 90% (Hammes and Whiley, 1993). Based on the characteristics of pineapple waste, the sugar consists of mainly glucose, fructose and sucrose. The objective of this study is to compare the kinetic parameters for microbial growth on single sugar and mixed sugar with liquid pineapple waste on lactic acid fermentation.

The fermentation data under anaerobic condition at pH, 6.0; temperature, 40 $^{\circ}$C; inoculum size, 5%; and stirring speed, 50 rpm were used to obtain the model parameters on the various of types of sugar. The data obtained from these experiments and predicted model from equation (5.23) are illustrated in Figure 5.1. The parameters for *L. delbrueckii* growth are shown in Table 5.1. The maximum specific growth for glucose was higher than fructose and sucrose. This is also shown in the values of γ_2 (Eqs.5.16), which express that the concentration of biomass at any time t is a function of maximum specific growth rate and specific death rate. If the maximum specific growth rate increases, the rate of biomass production will increase as well. Therefore the glucose medium is the best for the cultivation of *L. delbrueckii* to produce lactic acid.

The maximum specific growth rate for *L. delbrueckii* grown on glucose in this work was 0.103 h^{-1} which is comparative favourably with results obtained by Hakkarainen et al. (1984) who found the maximum specific growth rate of 0.150 h^{-1}. Mercier et al. (1992) also studied about kinetics of lactic acid fermentation on glucose by *L. amylophilus* in which they obtained the maximum specific growth rate of 0.29 h^{-1} at pH, 6; temperature, 30 $^{\circ}$C; and stirring speed, 350 rpm. Meanwhile Tyree et al. (1990) obtained higher maximum specific growth rate than other authors

which was 0.722 h^{-1} using *L. xylosus* with operation conditions of pH: 6.0, temperature: 30 ^{o}C and stirring speed, 150 rpm. The different results might be due to difference of operation conditions and types of strain used.
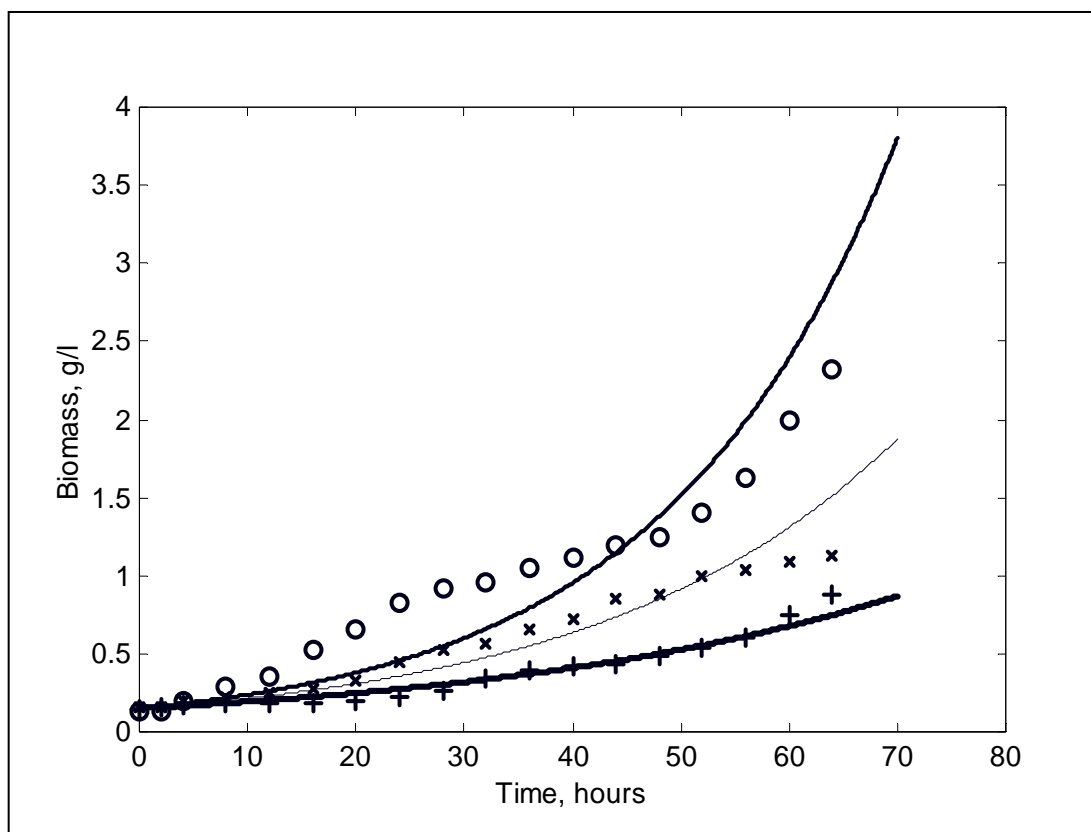
Figure 5.1: Time courses of the *L. delbrueckii* growth in lactic acid fermentation of glucose, fructose and sucrose for experimental and model predicted data. Glucose (— model, o-experiment), fructose (— model, x-experiment) and sucrose (▬ model, +-experiment). Experimental conditions: pH, 6.0; T, 40^{o}C; Stirring speed, 50 rpm and inoculum, 5%.

The sugar contents in liquid pineapple waste are: 20 g/l fructose, 20 g/l glucose and 15 g/l sucrose. To compare with the pure sugar, the fermentation was also carried out for mixed sugar consisting glucose, fructose and sucrose with concentration of 20 and 55 g/l of total sugar at same conditions. The experimental and model predicted data are shown in Figure 5.2.
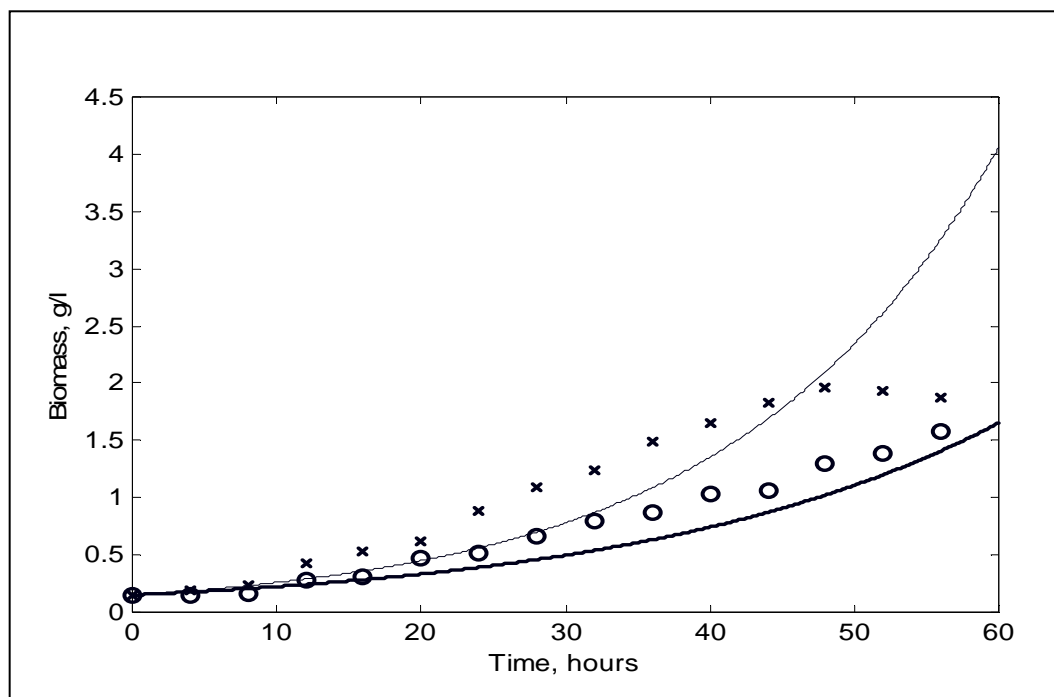
Figure 5.2: Time courses of the *L. delbrueckii* growth in lactic acid fermentation of mixed sugar for experimental and model predicted data. Mixed sugar-20 g/l(—— model, x-experiment) and mixed sugar-55 g/l (—— model, o-experiment). Experimental conditions: pH, 6.0; T, 40°C; stirring speed, 50 rpm; and inoculum, 5%.

The maximum specific growth rate for fermentation using mixed sugar at total sugar concentration of 55 g/l was higher than 20 g/l. This can be seen on the γ_2 value which expresses the function of the maximum specific growth rate and specific death rate (Table 5.1). If the results were compared with single sugar at total sugar concentration of 20 g/l, the value of μ_{max} for mixed sugar was between glucose-fructose and glucose-sucrose. This indicates that the growth rate of *L. delbrueckii* on mixed sugar was slower than glucose, contrary to fructose and sucrose.

When the concentration of mixed sugar was increased to 55 g/l, the maximum specific growth rate (μ_{max}) increased from 0.101 to 0.169 h^{-1}. This is also shown in the values of γ_2 which increased from 0.0380 to 0.0678 h^{-1}. The values of μ_{max} and γ_2 on lactic acid fermentation of pineapple waste were lower than 55 g/l of mixed sugar. This denotes that the growth rate for mixed sugar was faster than liquid pineapple waste.

Table 5.1: Model estimated parameters expressing the kinetics of microbial growth

in lactic acid fermentation for different types of sugar.

Types of sugar	Concentration of sugar (g/l)	$\gamma_2 = \mu_{max}\text{-}k_d$ (h^{-1})	μ_{max} (h^{-1})	k_d (h^{-1})
Single sugar	Glucose (20)	0.0462	0.103	0.0568
	Fructose (20)	0.0316	0.0448	0.0132
	Sucrose (20)	0.0251	0.0398	0.0147
Mixed sugar	Glucose (7.25) + Fructose (7.25) + Sucrose (5.5)	0. 0380	0.101	0.065
	Glucose (20) + Fructose (20) + Sucrose (15)	0.0678	0.169	0.101
Pineapple Waste	Glucose (20) + Fructose (20) + Sucrose (15)	0.0599	0.104	0.0441

5.3.1.2 Dependence Of The Model Parameters On Process Variables

The fermentation of pineapple waste was carried out to obtain the kinetic parameters as a function of process variables. The variables are pH, temperature, and inoculum size ranging from 5.5 to 6.5, 40 to 50 $^{\circ}$C and 5 to 15 %, respectively.

The experimental and model predicted data for kinetics of microbial growth on pineapple waste with different pHs are shown in Figure 5.3. The effect of pH on model estimated parameter is given in Table 5.2.

It was found that the maximum specific growth rate μ_{max} for pH 6.0 was higher than at pHs 6.5 and 5.5. This is illustrated by the slope of curves presented in Figure 5.3 and the value of γ_2. The maximum specific growth rate obtained at pH 6.0 was 0.104 h^{-1}. This figure was smaller than that reported by Monteagudo et al. (1997) which was 0.831 h^{-1}. The reason could be the difference of type of substrate and strain used in fermentation process. They used *L. delbrueckii CECT 286* on lactic acid fermentation of beet molasses.

The specific death rate (k_d) increases with increasing of γ_2 and μ_{max} (Table 5.2). A similar trend was also obtained by Suscovic et al. (1992) in lactic acid fermentation study using sucrose, high-fructose syrup and high-glucose syrup, as a carbon source. They obtained the values of μ_{max} and k_d were 0.671, 0.827, 3.44 and 0.035, 0.177, 2.62 h^{-1}, respectively.

The effect of pH on maximum specific growth rate μ_{max} or γ_2 is illustrated in Figure 5.4, and can be presented by equation:

$$\mu_{max} = -0.3316 \, pH^2 + 4.0028 \, pH - 11.975$$

and

$$\gamma_2 = -0.1722 \, pH^2 + 2.0829 \, pH - 6.2383.$$

Effects of pH on lactic acid fermentation of glucose by *L. amylophilus* have been studied by Mercier et al. (1992) at five different pH values from 5.4 to 7.8. The result was explained by second order polynomial equation of $\mu_{max} = -0.1515 \, pH^2 + 1.415 \, pH - 5.7355$ ($R^2 = 0.985$). Similar trend was also obtained by Yeh et al. (1991) on lactic acid fermentation of glucose by *L. delbrueckii* at five different pH values from 4.5 to 6.0.
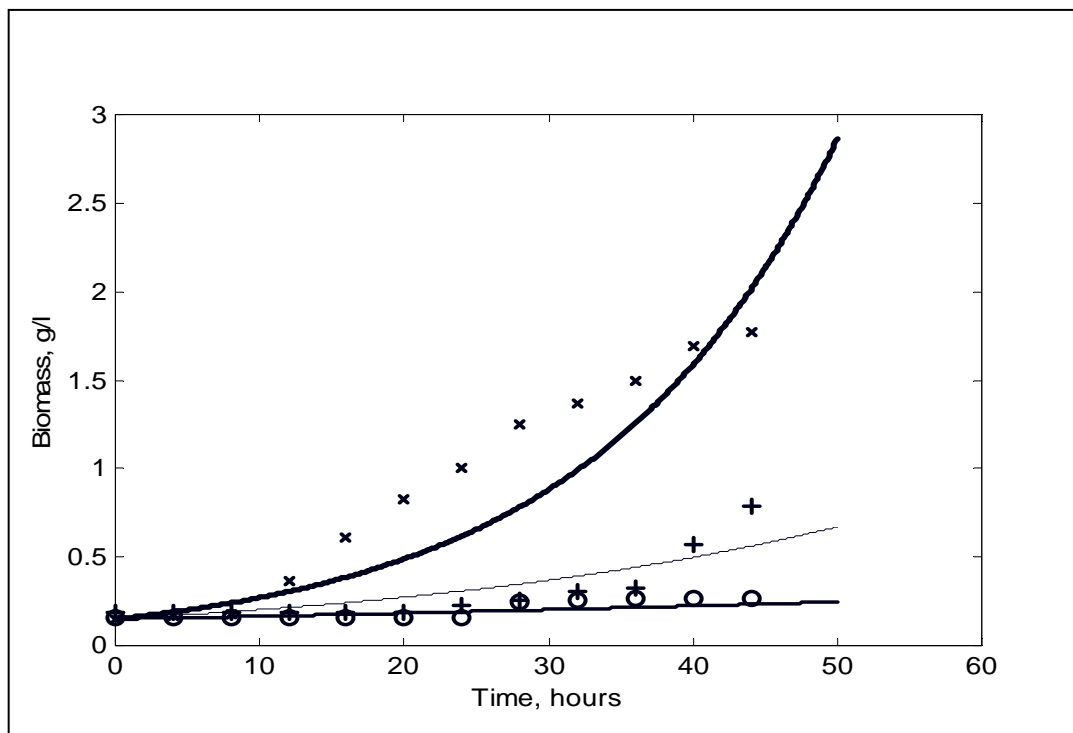
Figure 5.3: Time courses of the *L. delbrueckii* growth in lactic acid fermentation of pineapple waste for experimental and model predicted data at different pHs. pH:5.5 (▬ model, o-experiment), pH:6.0 (▬ model, x-experiment) and pH:6.5 (▬ model, +-experiment). Experimental conditions: Temperature, 40^{o}C; stirring speed, 50 rpm; and inoculum, 5%.

Table 5.2: Model estimated parameters expressing the kinetics of microbial growth in lactic acid fermentation on pineapple waste with different process variables.

Parameters	Values	$\gamma_2 = \mu_{max}-k_d$	μ_{max} (h^{-1})	k_d (h^{-1})
pH	5.5	0.0086	0.0093	0.0007
	6.0	0.0599	0.1040	0.0441
	6.5	0.0251	0.0329	0.0078
Inoculum size	5 %	0.0599	0.1040	0.0441
	10 %	0.0492	0.0899	0.0407
	15 %	0.0462	0.0735	0.0273
Temperature	40 °C	0.0599	0.1040	0.0441
	45 °C	0.0561	0.0870	0.0317
	50 °C	0.0485	0.0760	0.0275
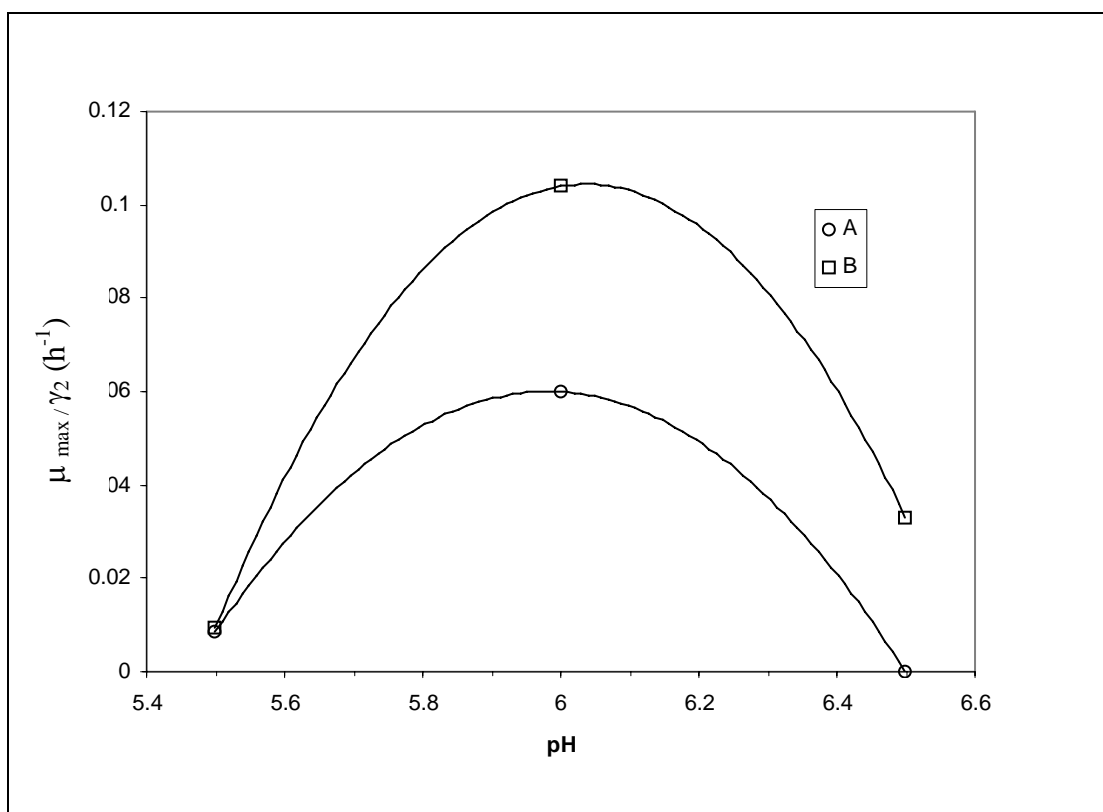
Figure 5.4: pH dependence of maximum specific growth rate (μ_{max}) and γ_2 in lactic acid fermentation of pineapple waste. A) γ_2 and B)μ_{max}
Experimental conditions: Temperature, $40^{o}C$; stirring speed, 50 rpm; and inoculum, 5%.

The experimental and model predicted data for kinetics of microbial growth on pineapple waste for different inoculum sizes and temperatures are illustrated in Figures 5.5 and 5.6. The effect of inoculum size on μ_{max} shows that when the inoculum size was increased from 5 to 10 %, the value of specific growth rate decreased from 0.104 to 0.0899 (Table 5.2). With further increase of inoculum size from 10 % to 15 %, μ_{max} decreased from 0.0899 to 0.0735. Monod equation (5.5) shows that if the concentration of nutrient increased, the value of μ_{max} decreased. It might be due to the concentration of nutrient in inoculum size of 5% was higher than 10 and 15%. This can be indicated with the slope of curves in Figure 5.5. The highest value was found with the inoculum size of 5%.

The effect of temperature on the value of μ_{max} shows that if the temperature was increased, the value of specific growth rate decreased (Table 5.2). Similar effect was obtained for the value of specific death rate. The highest specific growth rate was obtained at 40°C. The process variables such as pHs, temperatures and inoculum sizes influence the μ_{max}, and the highest values were achieved at 40 °C, pH, 6.0; and inoculum, 5%. These results are shown in the Figures 5.3, 5.5 and 5.6.

Figure 5.5: Time courses of *L. delbrueckii* growth in lactic acid fermentation of pineapple waste for experimental and model predicted data for different inoculum sizes. Inoculum size:5 % (▬▬ model, o-experiment), inoculum size:10 % (──── model, x-experiment) and inoculum size:15 % (▬▬ model, +-experiment). Experimental conditions: pH, 6.0; temperature, 40°C; and stirring speed, 50 rpm.
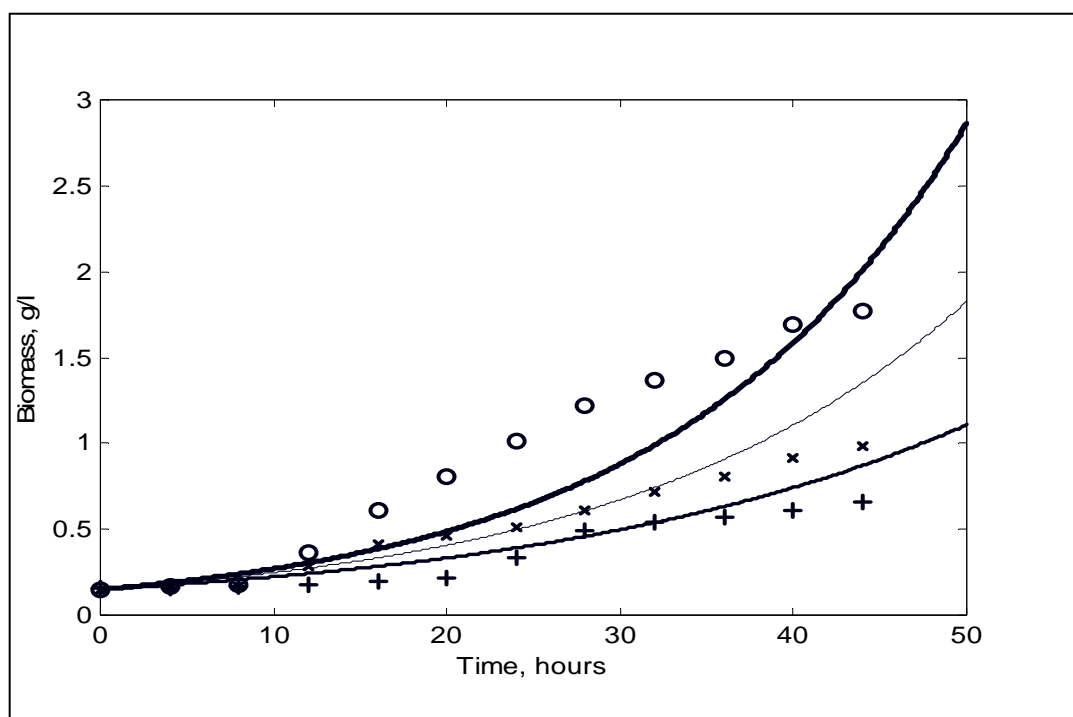
Figure 5.6: Time courses of *L. delbrueckii* growth in lactic acid fermentation of
pineapple waste for experimental and model predicted data for different
temperatures. T: 40 ^{o}C (▬ model, o-experiment), T: 45 ^{o}C (← model,
x-experiment) and T:50 ^{o}C (▬ model, +-experiment).
Experimental conditions: pH, 6.0; stirring speed, 50 rpm; and inoculum
size, 5%.

5.3.2   Sugar Utilisation

The growth was described in terms of a simple first order rate equation and
then related to substrate utilisation via a stoichiometric relationship. Effect of
substrate concentration on growth rate is given by Monod equation which expresses
the hyperbolic relationship between specific growth rate and substrate. The equation
(5.26) was used to obtain the parameters that represent the substrate utilisation in
batch culture for single sugar, mixed sugar and pineapple waste.

5.3.2.1 Dependence Of The Model Parameters On Types Of Sugar

The data obtained from these experiments as well as predicted model (Eqs.5.25) for fructose, glucose and sucrose fermentation are shown in Figure 5.7. The model predicted that all sugars were completely utilised during the fermentation process. The effects of sugar types on saturation constant (K_s) and biomass yield ($Y_{x/s}$) are given in Table 5.3.

The biomass yield ($Y_{x/s}$) had a maximum value of 0.23 g cell / g substrate in glucose fermentation (Table 5.3). This was almost similar to the figure reported by Mercier et al. (1992) in the fermentation of glucose-yeast extract medium using *L. amylophilus*. Table 5.4 also shows that the $Y_{x/s}$ value on sucrose fermentation was smaller than that obtained by Monteagudo et al. (1997). The results were different due to variation in initial substrate concentration used in lactic acid fermentation.

The saturation constants (K_s) of the *L. delbrueckii* for fructose, glucose and sucrose and mixed sugar fermentation at concentration of 20 g/l obtained in this work were almost similar. In glucose fermentation the value of K_s was found to be 2.13 g/l, which was smaller than the figure obtained by Buyukgungor et al. (1984) (Table 5.4). For sucrose fermentation, K_s was higher than the result obtained by Gadgil and Venkatesh (1997), but smaller than that obtained by Suscovic et al. (1992) (Table 5.5). The reason could be difference in types of substrate and strain used in the fermentation process.
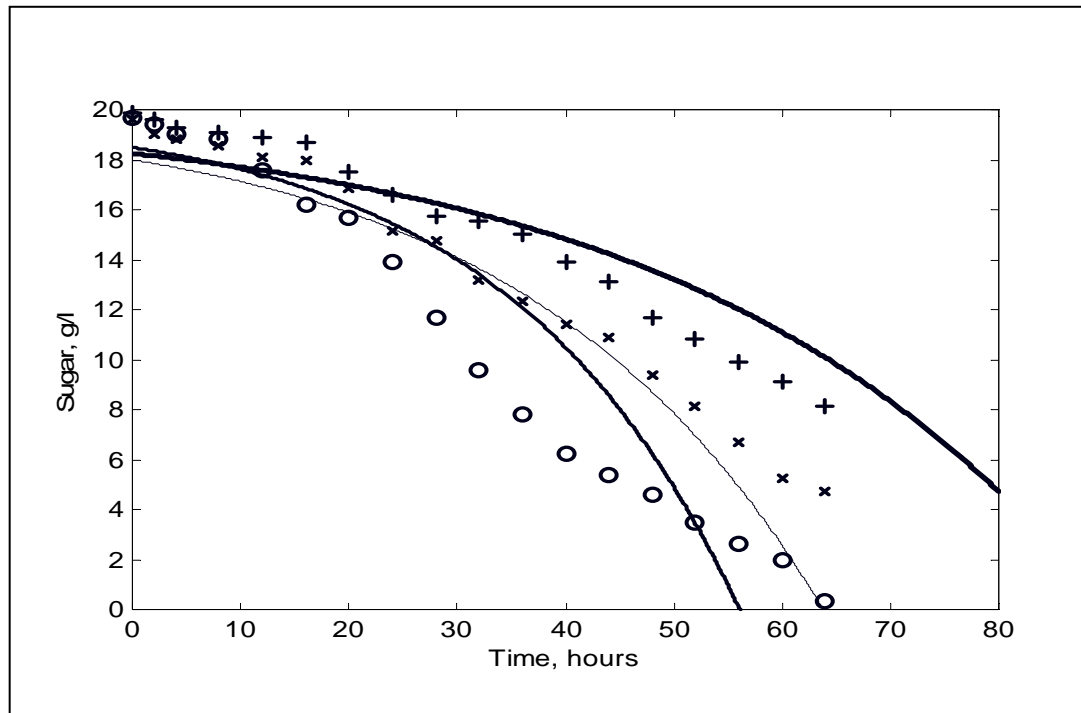
Figure 5.7: Time courses of the sugar utilisation in lactic acid fermentation of pure

sugar (20 g/l) for experimental and model predicted data.

Glucose (—— model, o-experiment), fructose (—— model,

x-experiment) and sucrose (—— model, +-experiment).

Experimental conditions: pH, 6.0; temperature, 40^{o}C; stirring speed, 50

rpm ; and inoculum, 5%.

Table 5.3: Model estimated parameters expressing the kinetics of sugar utilisation in lactic acid fermentation with different types of sugar.

Types of sugar	Sugar concentration (g/l)	$Y_{x/s}$ (Eqs. 5.18) (g biomass / g sugar)	K_s (Eqs.5.26) (g/l)
Single sugar	Glucose (20)	0.230	2.13
	Fructose (20)	0.100	2.67
	Sucrose (20)	0.078	3.32
Mixed sugar	Glucose (7.25) + Fructose (7.25) + Sucrose (5.5)	0.185	2.76
	Glucose (7.25) + Fructose (7.25) + Sucrose (5.5)	0.216	7.57
Pineapple waste	Glucose (20) + Fructose (20) + Sucrose (15)	0.376	10.93

Table 5.4: Comparison of the saturation constant (K_s) and biomass yield ($Y_{x/s}$) on glucose utilisation in lactic acid fermentation.

Strain	pH	T (^{o}C)	Speed (Rpm)	$Y_{x/s}$	K_s	Authors
L. delbrueckii	6.00	40.0	50	0.23	2.31	This work
L. delbrueckii	6.20	44.0	400	-	10.50	Buyukgungor et al. (1984)
L. amylophilus	6.00	30.0	350	0.24	-	Mercier et al. (1991)

Table 5.5: Comparison of the saturation constant (K_s) and biomass yield ($Y_{x/s}$) on sucrose utilisation in lactic acid fermentation.

Strain	pH	T (^{o}C)	Speed (Rpm)	$Y_{x/s}$	K_s	Authors
L. delbrueckii	6.00	40.0	50	0.078	3.32	This work
L. delbrueckii	5.90	49.0	100	0.27	-	Monteagudo et al. (1994)
L. delbrueckii	6.00	49.0	800	-	4.47	Suscovic et al. (1992)
L. bulgaricus	5.60	45.0	400	-	1.80	Gadgil and Venkatesh (1997)

The data obtained from the experiment and predicted model on mixed sugar utilisation is shown in Figure 5.8. The effects of different types of sugar on saturation constant (K_s) and biomass yield ($Y_{x/s}$) are shown in Table 5.3.

Figure 5.8 shows that the model predicted for mixed sugar fermentation at concentration of 20 and 55 g/l was completely utilised. The biomass yield on mixed sugar fermentation at concentration of 20 g/l was found to be 0.185 g cell / g substrate. This value was higher than that of sucrose and fructose fermentation but smaller than the biomass yield for glucose fermentation (Table 5.3). If the concentration of mixed sugar were increased to 55 g/l, the $Y_{x/s}$ would increase as well due to the higher consumption of bacteria on sugar.

The saturation constant increases with increasing sugar concentration in the medium. The values of K_s obtained were 2.76 and 7.57 g/l for 20 and 55 g/l, respectively. When the concentration was further increased to 70 g/l (liquid pineapple waste), the value of K_s increased from 7.57 to 10.93. This can be expressed in equation (5.5) which shows that the saturation constant values depend on sugar concentration in the medium. Suscovic et al. (1992) reported that the value of K_s obtained on lactic acid fermentation of high fructose syrup was 36.3 g/l. This result was higher than for single sugar (20 g/l), mixed sugar (20 g/l) and mixed sugar (55 g/l) because they used 97.2 g/l of sugar concentration.
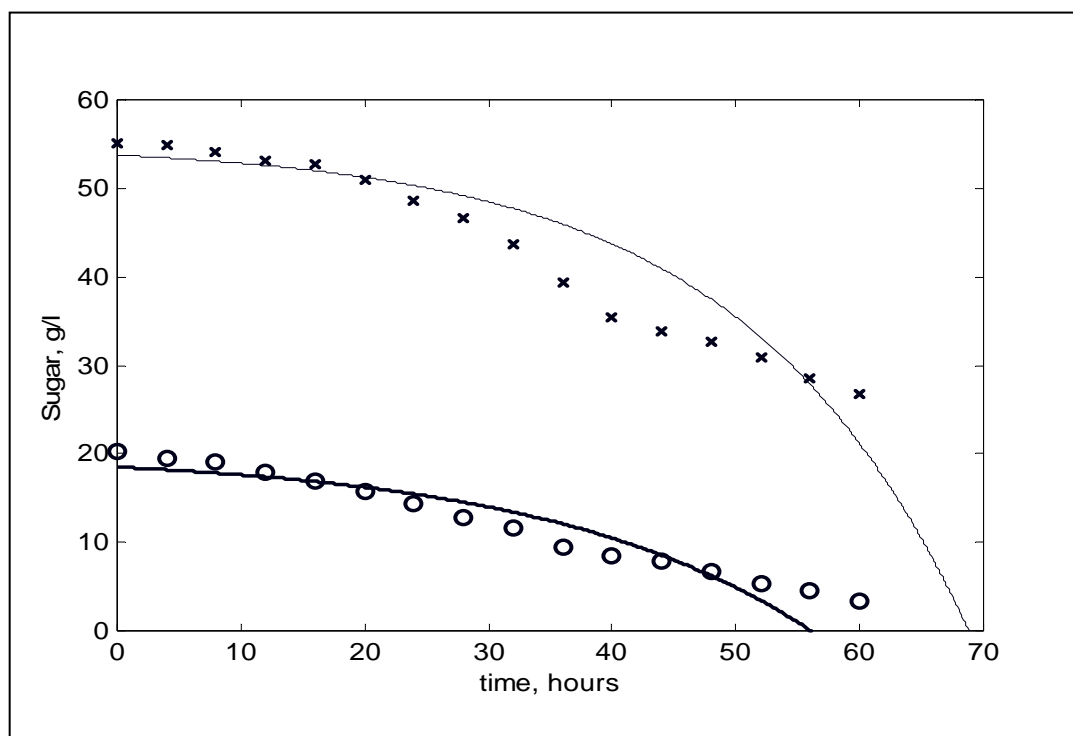
Figure 5.8: Time courses of the sugar utilisation in lactic acid fermentation of mixed sugar for experimental and model predicted data.

Mixed sugar-20 g/l (━━ model, o-experiment) and mixed sugar-55 g/l (── model, x-experiment. Experimental conditions: pH, 6.0; temperature, 40°C; stirring speed, 50 rpm and inoculum, 5%.

5.3.2.2 Dependence Of The Model Parameters On Process Variables

Effects of some parameters such as pH, temperature and inoculum size on substrate utilisation of pineapple waste fermentation were also studied. The experimental and model predicted data were shown in Figures 5.9, 5.10 and 5. 11. The model estimated parameters are given in Table 5.6.

Effects of pHs (5.5, 6.0 and 6.5) on bacterial yield shows that the pH 6.0 gave the highest value of $Y_{x/s}$ which was 0.376 g biomass / g sugar (Table 5.6). When the pH was increased to 6.5, the biomass yield decreased to 0.101 g biomass / g sugar. This can be shown by the maximum specific growth rate or maximum dry cell weight concentration obtained. For instance, for pH 6.0 , the biomass yield was

higher than of pH 6.5. While for pH 5.5, the biomass yield obtained was only 0.059 g biomass / g sugar. Similar result was also reported by Mercier et al. (1992) stating that the effect of pH on bacterial yield was very significant on glucose fermentation by *L. amylophilus.* They obtained the maximum biomass yield of 0.347 g biomass / g sugar at pH 5.4.

Table 5.6 shows that the values of saturation constant (K_s) are not affected by pH from 5.5 to 6.5. The similar result was obtained by Fu and Mathews (1999) which explained that K_s was almost similar when the pH was increased from 5.0 to 7.0. When the pH was decreased from 5.0 to 4.0, K_s increased from 49 to 178 g/l. Gadgil and Venkatesh (1997) also reported that the values of K_s were not affected by pHs. They obtained the value of K_s at 1.8 g/l for pH 4.2-5.6 on lactic acid fermentation of lactose using *L. bulgaricus*. The experimental and model predicted data were shown in Figure 5.9.

The effects of inoculum size on bacterial yield were also similar to the effect of pH. The maximum bacterial yield value was found to be 0.376 g cell / g substrate at inoculum size of 5 %. If the inoculum was increased, the biomass yield decreased and the $Y_{x/s}$ obtained for 10 and 15 % inoculum were 0.306 and 0.285 (g biomass / g sugar), respectively. (K_s) was not affected by inoculum sizes too. For 5 % inoculum size, K_s obtained was 10.93 g/l. If the inoculum size was increased, the values of K_s obtained were almost similar. For 10 and 15 % inoculum sizes, K_s obtained were 12.26 and 12.62 g/l, respectively. The experimental and model predicted data was shown in Figure 5.10.
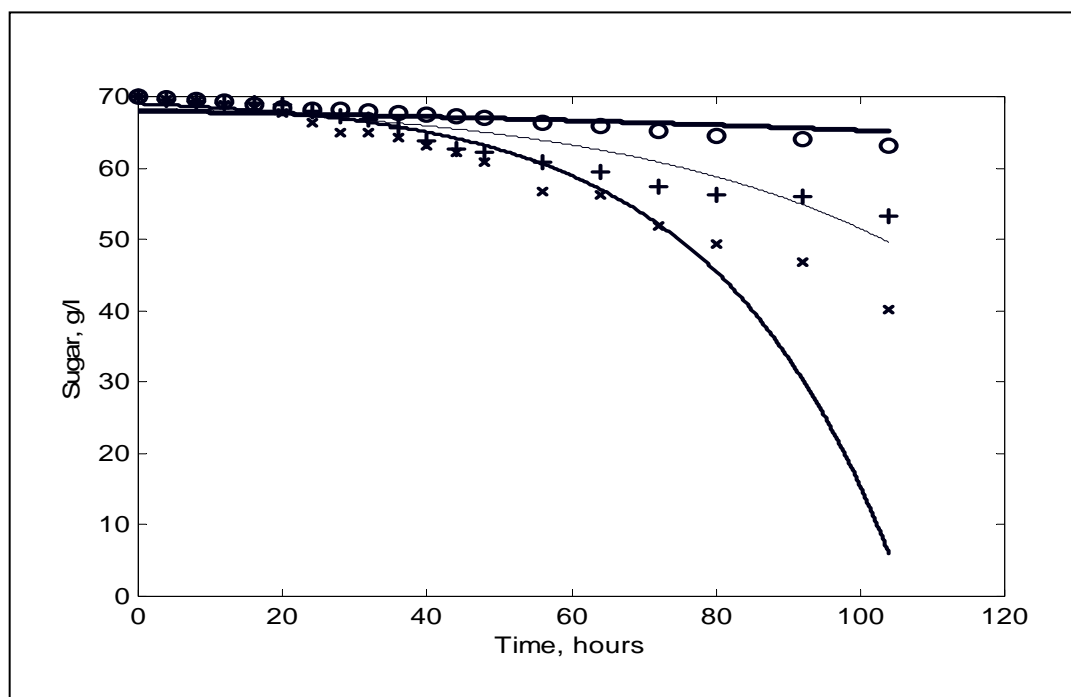
Figure 5.9: Time courses of the sugar utilisation in lactic acid fermentation of
pineapple waste for experimental and model predicted data for different
pHs. pH: 5.5 (▬ model, o-experiment), pH: 6.0 (▬ model,
x-experiment) and pH: 6.5 (▬ model, o-experiment).
Experimental conditions: Temperature, 40°C; stirring speed, 50 rpm and
inoculum, 5%.
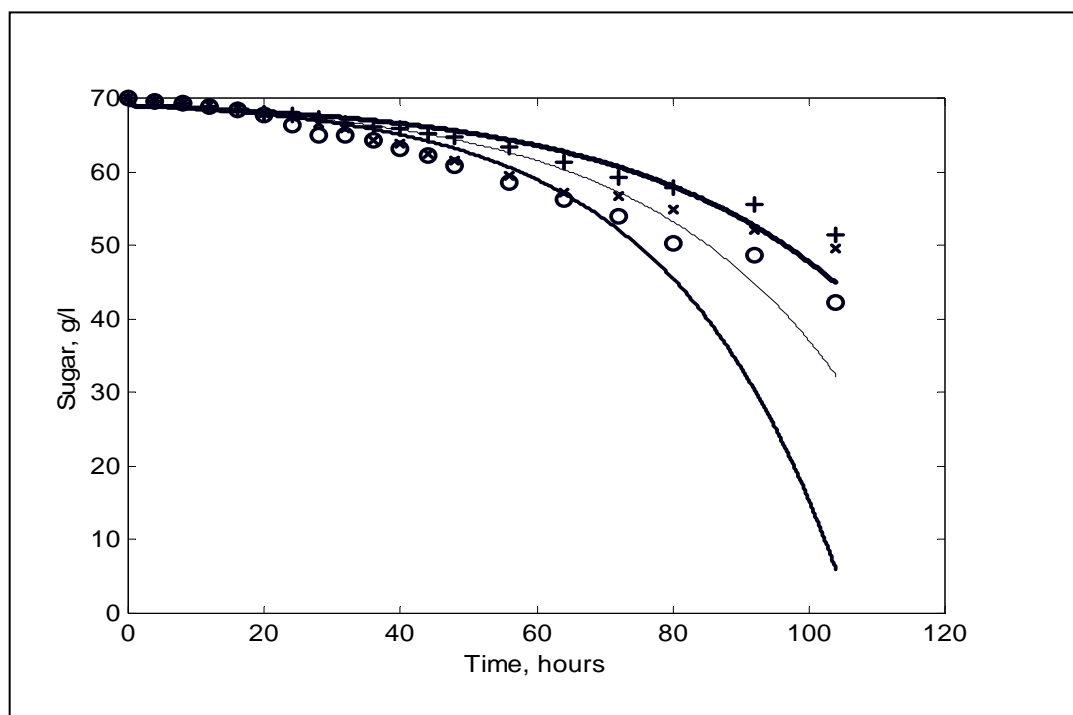
Figure 5.10: Time courses of the sugar utilisation in lactic acid fermentation of pineapple waste for experimental and model predicted data for different inoculum sizes. Inoculum size: 5 % (▬ model, o-experiment), inoculum size: 10 % (model, x-experiment) and inoculum size:15 % (model, +-experiment). Experimental conditions: Temperature, $40^{\circ}C$; stirring speed, 50 rpm; and inoculum 5%.

The effects of temperature on bacterial yield shows that at temperature $40\ ^{\circ}C$, the optimal value of $Y_{x/s}$ was 0.376 g biomass / g sugar (Table 5.6). If temperature was increased, the biomass yield decreased. The $Y_{x/s}$ obtained for 45 and 50 $^{\circ}C$ were 0.277 and 0.157 g biomass / g sugar, respectively. This can be shown by the maximum specific growth rate or maximum dry cell weight concentration. The value of $Y_{x/s}$ obtained for 40 $^{\circ}C$ was higher than for 45 and 50 $^{\circ}C$. The saturation constants (K_s) were affected by temperature from 40 to 50 $^{\circ}C$. The value of K_s obtained for 40 $^{\circ}C$ was 10.93 g/l. When the temperature was increased to 45 and 50 $^{\circ}C$, K_s also increased to 16.62 and 19.81 g/l, respectively. The experimental and model predicted data was shown in Figure 5.11.
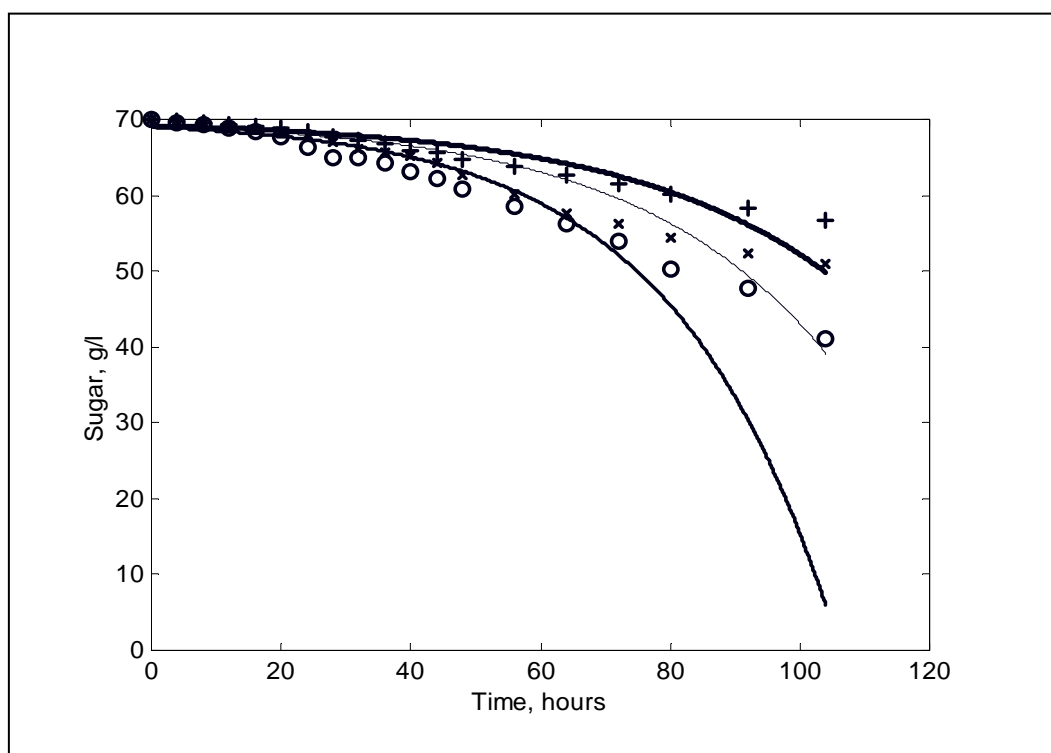
Figure 5.11: Time courses of the sugar utilisation in lactic acid fermentation of
pineapple waste for experimental and model predicted data for
different temperatures. T: 40 ^{o}C (—— model, o-experiment), T:45^{o}C
(—— model, x-experiment) and T: 50 ^{o}C (—— model, +-experiment).
Experimental conditions: pH, 6.0; stirring speed, 50 rpm; and inoculum,
5%.

Table 5.6: Model estimated parameters expressing the kinetics of sugar
utilisation in lactic acid fermentation of pineapple waste for different
process variables.

Parameters	Values	$Y_{x/s}$ (g biomass / g sugar)	K_s (g/l)
pH	5.5	0.059	12.26
	6.0	0.376	10.93
	6.5	0.101	11.41
Inoculum (%)	5	0.376	10.93
	10	0.306	12.26
	15	0.285	12.62
Temperature (^{o}C)	40	0.376	10.93
	45	0.277	16.62
	50	0.157	19.81

5.3.3   Lactic Acid Production

Fermentation kinetics describes growth and product formation by microorganism, not only active cell growth but also the activities of resting and dying cell, since many fermentation products of commercial interest are produced after growth has stopped. Metabolic product formation can be similarly related to nutrient consumption. Furthermore, product formation cannot occur without the presence of cell. Thus it is expected that growth and product formation are closely coupled to nutrient utilisation and product formation will be coupled to growth and/or cell mass concentration. Luedeking and Piret have studied about lactic acid fermentation of glucose by *L. delbrueckii*, which indicated that the product formation kinetics combined growth associated, and non growth-associated. The rate of lactic acid production was shown in equation (5.12), and two parameters can be obtained by integration of this equation.

5.3.3.1 Dependence Of The Model Parameters On Types Of Sugar

The data obtained from the experiments and predicted model of the lactic acid production with glucose, fructose and sucrose as substrate are shown in Figure 5.12. The experiment and predicted model on lactic acid production for mixed sugar are shown in Figure 5.13. The parameters of growth associated product formation constant (k_1) and non growth associated product formation (k_2) are found in Table 5.7.

The k_1 values for mixed sugar and single sugars at concentration of 20 g/l are not constant, but the k_2 values are relatively constant. This indicates that the types of sugar influence the growth associated product formation, but not for portion of non growth associated product formation. The highest k_1 obtained was for glucose fermentation, followed by mixed sugar, fructose and sucrose. This might be due to lactic acid production for glucose was faster than mixed sugar; while for fructose and sucrose, their productions were slower than mixed sugar's as shown in Figure 4.26. The k_1 value for 50 g/l mixed sugar was higher than 20 g/l mixed sugar, and k_1 for liquid pineapple waste was smaller than both mixed sugars. This indicates that the rate of lactic acid production for 50 g/l of mixed sugar was higher than 20 g/l of mixed sugar as shown in Figure 4.29.
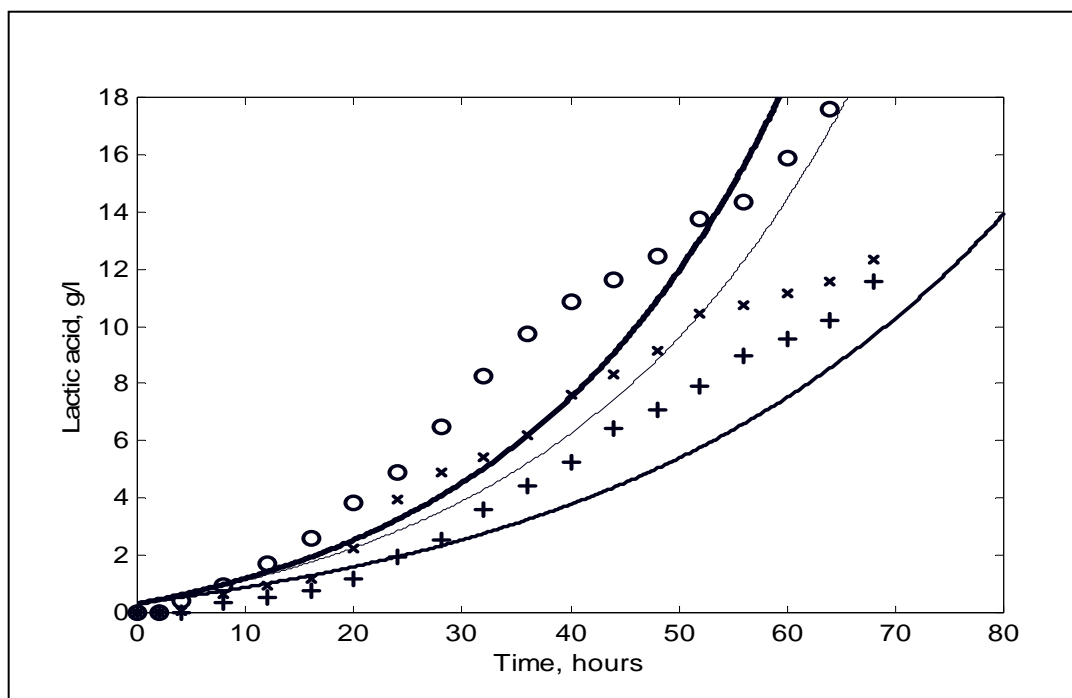
Figure 5.12: Time courses of the lactic acid production in lactic acid fermentation of single sugar (20 g/l) for experimental and model predicted data. Glucose (━ model, o-experiment), fructose (— model, x-experiment) and sucrose (— model, +-experiment).Experimental conditions: pH, 6.0; temperature, $40^{o}C$; stirring speed, 50 rpm; and inoculum, 5%.
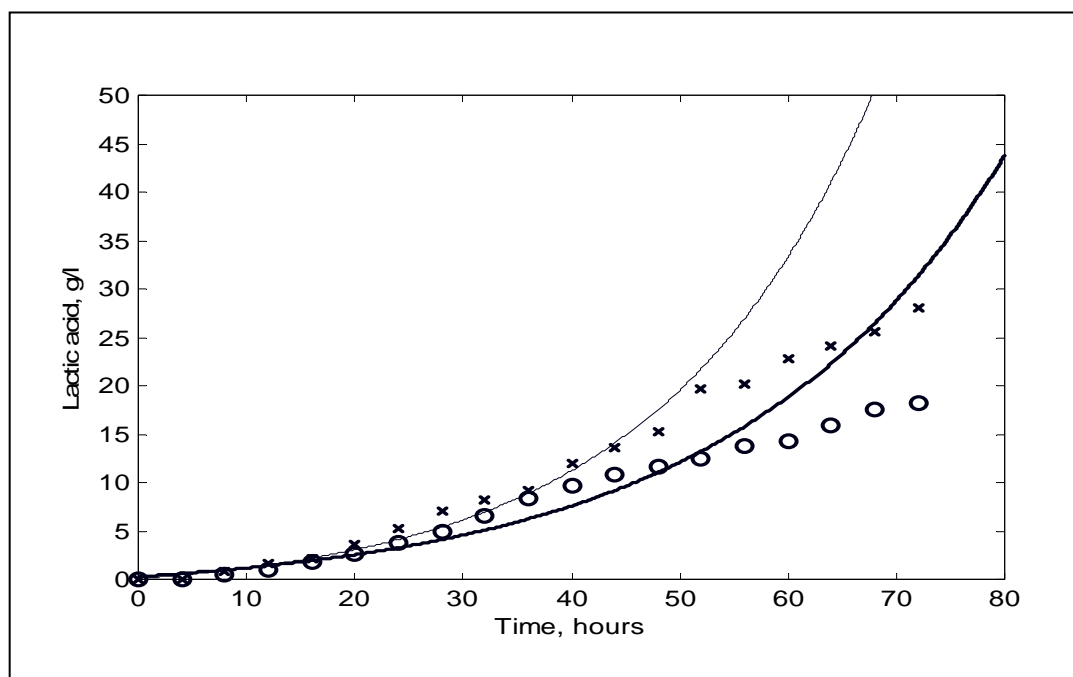
Figure 5.13: Time courses of the lactic acid production in lactic acid fermentation of mixed sugar for experimental and model predicted data. Mixed sugar-20 g/l (— model, o-experiment) and mixed sugar-50 g/l ⟵ model, x-experiment). Experimental conditions: pH, 6.0; temperature, 40°C; stirring speed, 50 rpm; and inoculum, 5%.

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Table 5.7: Model estimated parameters expressing the kinetics of product formation on lactic acid fermentation of different types of sugar.

Types of sugar	Concentration (g/l)	k_1 (g product / g cell)	k_2 (g product / h.g cell)
Single sugar	Glucose (20)	11.162	0.080
	Fructose (20)	10.320	0.079
	Sucrose (20)	9.460	0.061
Mixed sugar	Glucose (7.25) + Fructose (7.25) + Sucrose (5.5)	10.500	0.073
	Glucose (20) + Fructose (20)+ Sucrose (15)	12.250	0.019
Pineapple waste	Glucose (20) + Fructose (20) + Sucrose (15)	8.300	0.0009

5.3.3.2 Dependence Of The Model Parameters On Variables Process

The data obtained from the experiment and predicted model for lactic acid production from pineapple waste with pH, inoculum size and temperature as process variables are illustrated in Figures 5.14, 5.15 and 5.16.

Luedeking and Piret (1959) found that the constant k_1 and k_2 in the model were strongly dependence of pH. The ratio k_1/k_2 obtained was 4.0 in the lactic acid fermentation of glucose by *L. delbrueckii*. In this work at pH 6.00, the k_1 and k_2 values obtained were 8.3 and 0.0009, respectively. This result differs with results obtained by Samuael and Lee (1980) which were 18.05 and 0.19, respectively but k_2 was smaller than k_1 in both cases. This is probably because they used sorghum extract as substrate. Even though sorghum extract consists of glucose, fructose and sucrose, which is similar to liquid pineapple waste, it differs in terms of sugar and other nutrients concentration.

The $k_2 < k_1$ ($k_1/k_2 > 1$) indicates that the growth associated portion was higher than the non growth associated portion of lactic acid formation by *L. delbrueckii*. These bacteria produce lactic acid proportionally to the concentration, without influence of their growth phase. Similar results were also reported by Monteagudo et al. (1997) and Suscovic et al. (1992) where the ratio k_1/k_2 obtained were 2.70 and 96.84, respectively. These results are different since they used beet molasses and high fructose syrup as a substrate in fermentation process.

The k_1 and k_2 values are affected by variable pHs with the highest value at pH 6.0. Table 5.8 shows that the growth associated portion of lactic acid formation by *L. delbrueckii* is favoured by fermentation at controlled pH in the range of 6.0-6.5. Effect of pHs on the values of growth associated product formation constant (k_1) and non growth associated product formation (k_2) obtained in this work as well as that given by Samual and Lee (1980) were also presented in Table 5.9.

The effects of inoculum sizes and temperatures on the parameters estimated in expressing the kinetics of product formation in lactic acid fermentation on pineapple waste were shown in Table 5.8.

Effect of inoculum sizes on the k_1 and k_2 values shows that when the inoculum size was increased from 5 to 15 %, the k_1 and k_2 decreased from 8.30 to 5.70 and from 0.0009 to.0.0006, respectively. The effect of temperatures also gave the same result, where temperature increased from 40 to 50 ^{o}C, followed by decrease

of the k_1 and k_2 values from 8.30 to 5.39 and from 0.0009 to 0.0004, respectively. The values of growth associated product formation constant (k_1) and non growth associated product formation are affected by the process variables understudied. The highest value obtained was at pH, 6.0; inoculum size, 5 % and temperature, 40 ^{o}C.
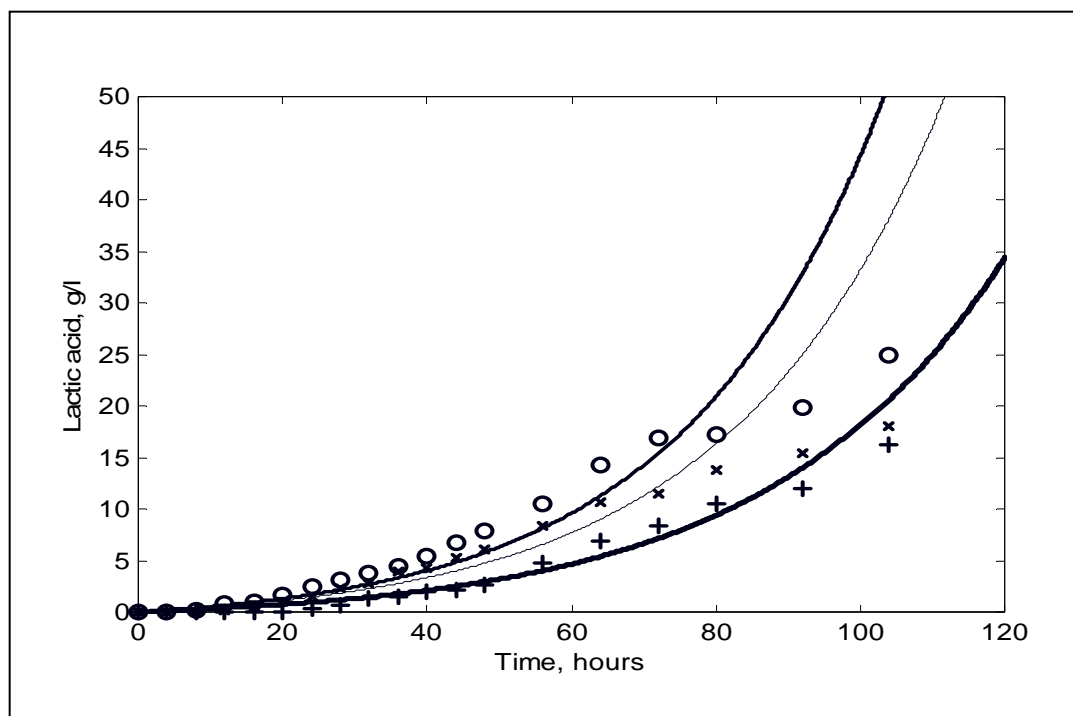
Figure 5.14: Time courses of the product formation in lactic acid fermentation of pineapple waste for experimental and model predicted data for different pHs. pH:5.5 (▬ model, +-experiment), pH:6.0 (▬ model, o-experiment) and pH:6.5 (▬ model, x-experiment).Experimental conditions: Temperature, 40^{o}C; stirring speed, 50 rpm; and inoculum, 5%.
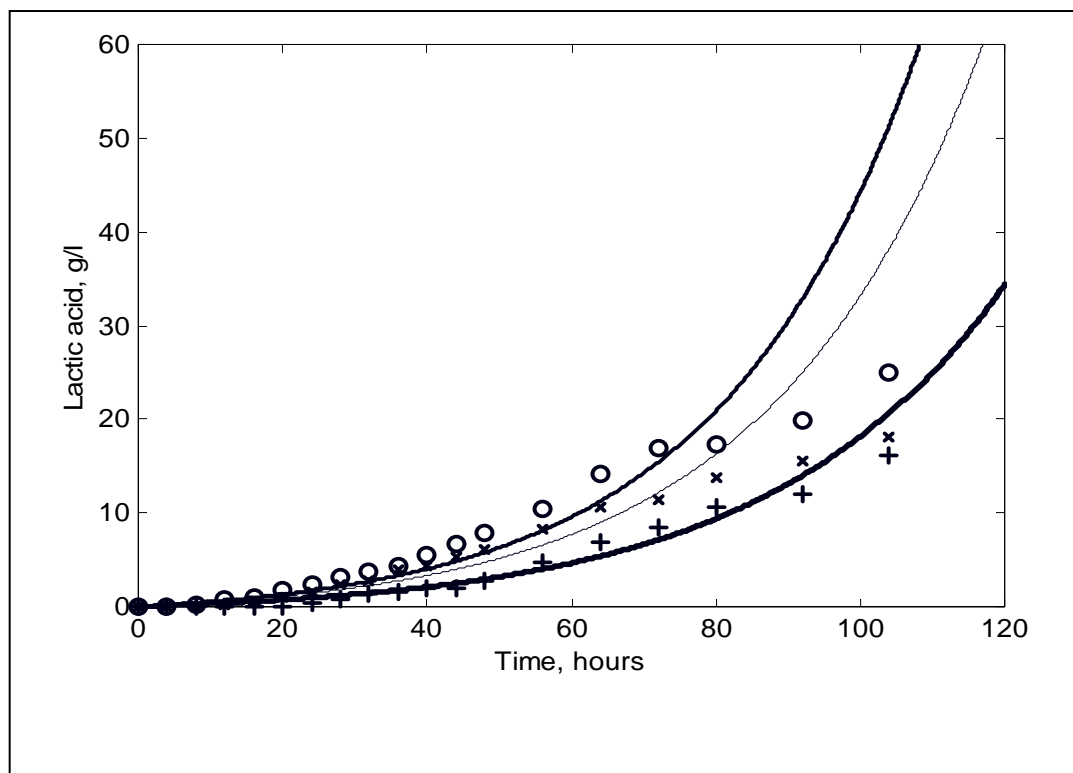
Figure 5.15: Time courses of the lactic acid production in lactic acid fermentation of pineapple waste for experimental and model predicted data for different inoculum sizes. Inoculum size:5 % (━━ model, o-experiment), inoculum size: 10 % (── model, x-experiment) and inoculum size:15 % (━━model, +-experiment). Experimental conditions: pH, 6.0; T, 40°C; and stirring speed, 50 rpm.
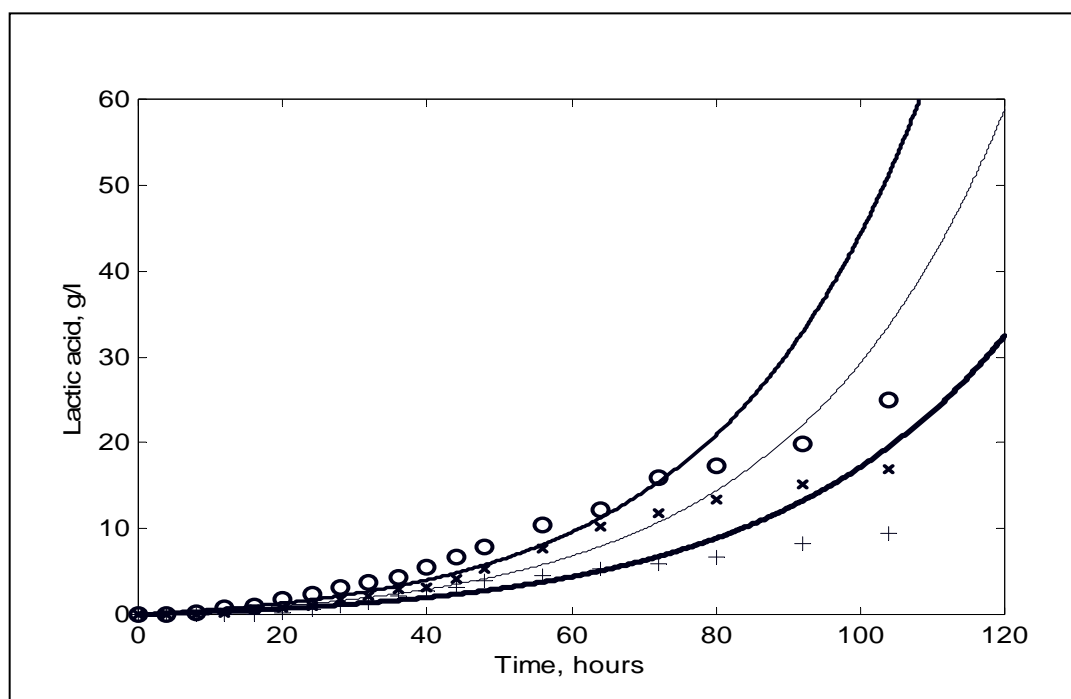
Figure 5.16: Time courses of the lactic acid production in lactic acid fermentation of pineapple waste for experimental and model predicted data for different temperatures. T: 40 °C (▬▬ model, o-experiment), T: 45 °C (▬▬ model, x-experiment) and T:50 °C (▬▬ model, +-experiment). Experimental conditions: pH, 6.0; stirring speed, 50 rpm;and inoculum size, 5%.

Table 5.8: Model estimated parameters expressing the kinetics of product formation
In lactic acid fermentation of pineapple waste for different process
variables.

Parameters	Values	k_1 (g product / g cell)	k_2 (g product /h.g cell)
pH	5.5	6.18	0.0002
	6.0	8.30	0.0009
	6.5	6.79	0.0004
Inoculum (%)	5	8.30	0.0009
	10	7.65	0.0007
	15	5.70	0.0006
Temperature (^{o}C)	40	8.30	0.0009
	45	6.75	0.0008
	50	5.39	0.0004

Table 5.9: The comparison of the values of k_1 and k_2 for different pHs.

pH	*L. delbrueckii* (This work)		*L. bulgaricus* Samuel and Lee. (1980)		*L. plantarum* Samuel and Lee (1980)	
	k_1	k_2	k_1	k_2	k_1	k_2
5.5	6.18	0.0002	16.21	0.35	15.36	0.22
6.0	8.30	0.0009	18.05	0.19	15.46	0.44
6.5	6.79	0.0004	17.67	0.28	17.67	0.31

5.4   Model Evaluation

On the assumption that the models are adequate, an estimation of standard error may be obtained by the root of dividing the residual sum square by its number of data used as given in equation 5.30 (Box et al., 1978). Standard error between the measured and calculated biomass as well as sugar and lactic acid concentration was used as a statistical criterion for testing the adequacy.

$$E = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_i - Y_i^*\right)^2}{n}}$$

....(5.30)

where Y_i and Y_i^* are the original and the predicted value, and n is the number of data used.

The validation of the models and the adequacy of the fitted model to experimental results were also tested by computing the R-square and using statistic F, respectively. The value of R^2 and F can be calculated using equation given by Box et al. (1978) as follow:

$$F = \frac{SSR/(p-1)}{SSE/(N-p)}$$

.....(5.31)

$$R^2 = \frac{SSR}{SST}$$

.....(5.32)

where *SSR* is the sum of squares due to regression, *SSE* is the sum of squares error, *SST* is the sum square total, *N* is the sum of experiment data and *p* is the sum variables and response. The values of *SSR*, *SSE* and *SST* can be calculated using the following equation:

$$SST = \sum_{u=1}^{N} (Y_o - Y_{avg})^2 \qquad\qquad \text{.....(5.33)}$$

$$SSR = \sum_{u=1}^{N} (Y_p - Y_{avg})^2 \qquad\qquad \text{.....(5.34)}$$

$$SSE = \sum_{u=1}^{N} (Y_p - Y_o)^2 \qquad\qquad \text{.....(5.35)}$$

where Y_{avg}, Y_o and Y_p are the average, observed and predicted of biomass, sugar and lactic acid concentrations, respectively.

The values of standard error, F and R^2 for biomass, sugar and lactic acid concentrations are given in Table 5.10. An overall fit of the proposed model can be expressed by the average of standard error obtained for biomass, sugar and lactic acid concentrations which were 0.106, 1.36 and 0.75 g/l respectively. These values indicate the same order of magnitude as the analytical error in measurement of biomass, sugar and lactic acid concentrations. The average values of R-square obtained were 0.87, 0.88 and 0.87, respectively. This designates that only 13% of total variation cannot be explained by the models. The average values of F obtained were 117, 77 and 300, respectively.

The adequacy of the fitted model can be tested using static F. The value of F is compared to the table value $F_{(p-1,N-p,\alpha)}$, which is the upper 100 α percent point of the F distribution with p-1 and N-p degrees of freedom, respectively. From the table $F_{(5,26,.01)}$ obtained was 8.18.

Since the F values exceed from table value of F= 8.18, this indicates that the fitting of the models to the experimental results is good. Thus, the models are able to reproduce the experimental results.

Table 5.10: Standard error, fisher value and r-square for biomass, sugar and lactic acid concentration.

Substrate	Item	Conditions	Biomass			Sugar			Lactic acid		
			E_b	F_b	R_b	E_s	F_s	R_s	E_l	F_l	R_l
Glucose	Concentration (g/l)	20	0.15	20.6	0.85	1.41	23.1	0.79	0.64	119	0.84
Fructose	Concentration (g/l)	20	0.09	47.8	1.26	68.3	0.98	0.91	0.08	683	0.93
Sucrose	Concentration (g/l)	20	0.07	93.5	0.94	0.97	92.5	0.94	0.07	447	0.86
Mixed sugar	Concentration (g/l)	20	0.18	15.7	0.78	1.76	66.0	0.89	0.77	39.5	0.79
		55	0.16	32.5	0.86	1.51	56.7	0.83	1.08	94	0.80
Pineapple waste	Temperature (^{o}C)	40	0.17	48.6	0.82	1.68	64.8	0.82	1.29	86	0.81
		45	0.08	65.9	0.89	0.72	83.9	0.91	1.17	103	0.83
		50	0.01	436	0.92	1.67	36.6	0.96	0.52	940	0.98

Table 5.10: Standard error, fisher value and r-square for biomass, sugar and lactic acid concentration (Continued).

Substrate	Item	Conditions	Biomass			Sugar			Lactic acid		
			E_b	F_b	R_b	E_s	F_s	R_s	E_l	F_l	R_l
Pineapple waste	pH	5.5	0.03	374	0.95	0.55	94.3	0.93	0.65	531	0.96
		6.0	0.17	48.6	0.82	1.68	45.6	0.82	1.29	86	0.81
		6.5	0.08	73.4	0.90	0.98	36.1	0.85	0.46	479	0.94
	Inoculum (%)	5	0.17	48.6	0.82	1.68	35.5	0.82	1.29	86	0.81
		10	0.09	96.5	0.91	1.75	25.9	0.76	0.54	336	0.92
		15	0.04	237	0.94	1.36	22.7	0.88	0.67	182	0.90

Eb, Fb, Rb, Es, Fs, Rs, El, Fl and Rl are standard error, Fisher value and r-square for biomass, sugar and lactic acid concentration, respectively.

5.5 Sensitivity Analysis

5.5.1 Introduction

Before a comparison can be made between the mathematical model and the experimental results, the right values of the different parameters in the model have to be known. Obtaining these parameters can be very time consuming, particularly in biotechnology where the systematic compilation of parameter values is still in its infancy. The influence of the various parameters on model behaviour can be very different. Parameters having a large influence should be known accurately, for others a span of an order of magnitude is often sufficient. Activities to obtain an insight into the influence of parameters are called *parameters sensitivity analysis*. The purpose of these analyses is to avoid unnecessary effort in obtaining accurate values of less relevant parameters. One of the simplest stages for this analysis is obtaining a first estimate of the values of the parameters involved, introduce these parameters values into the model, and vary the values of these parameters one by one then register their influences on the most important output variables of the model (Kossen and Oosterhuis, 1985).

5.5.2 Model Parametric Sensitivity

Lactic acid production and bacterial growth are closely connected. Therefore, the effects of maximum specific growth rate (μ_{max}), specific death rate (k_d) and saturation constant (K_s) on growth kinetics have been first considered instead of the influence of the initial biomass concentrations, since (X_o) is measurable.

In order to quantify these parameters, the sensitivity of P_i to variation on parameter y is defined as follows:

$$P_i = (\frac{Y_i}{X}).(\frac{\Delta X}{\Delta Y})$$

…..(5.36)

where Y_i is the original value of the parameter, ΔY_i is the variation of this parameter, X is the value of the important output variable at the original value of the parameter and ΔX is the variation of this variable due to the variation of the parameter.

The concentration of biomass at resting state (X_m) depends on parameters μ_{max}, k_d and K_s to various extents. The sensitivity of X_m to variations of parameter Y is defined as followed (Amrane and Prigent, 1994).

$$XMPS = (\frac{Y_{obs}}{X_{m,obs}}).(\frac{\Delta X_m}{\Delta Y}) \qquad \text{…..(5.37)}$$

where XMPS is X_m parametric sensitivity (dimensionless), Y_{obs} is the original value of the parameter, ΔY is the variation of this parameter, $X_{m,obs}$ is the observed biomass concentration at resting state and at the original value of the parameter, ΔX_m is the variation of this variable due to the variation of the parameter.

The limit of the lactic acid production P as a function of time tends to proceed towards infinity, for the maximum lactic acid concentration P_m, the parametric sensitivity is defined in the same manner as XMPS in equation (5.37), namely

$$PMPS = (\frac{Y_{obs}}{P_{m,obs}}).(\frac{\Delta P_m}{\Delta Y}) \qquad \text{….(5.38)}$$

where PMPS is P_m parametric sensitivity (dimensionless), Y_{obs} is the original value of the parameter, ΔY is the variation of this parameter, $P_{m,obs}$ is the observed lactic acid concentration at resting state and at the original value of the parameter, ΔP_m is the variation of this variable due to the variation of the parameter.

5.5.2.1 Parametric Sensitivity Analysis For *L. Delbrueckii* Growth

The relationship between biomass concentration and time as shown in equation (5.23) was influenced by the values of γ_2 and γ_3. The model parametric sensitivity for microbial growth in lactic acid fermentation of single sugar can be illustrated in Figures 5.17-5.22.

Figures 5.17 and 5.18 show that the parameters of γ_2 and γ_3 have very little effect on biomass concentration during the fermentation time of 4 and 16 hours, respectively. The parametric sensitivities toward γ_2 and γ_3 for the growth kinetics in lactic acid fermentation of glucose as calculated were 3.37 and 1.24, respectively. Thus, the growth appears to be controlled mainly by the parameter γ_2. In lactic acid fermentation of fructose, the parameters of γ_2 and γ_3 have very little effect on biomass concentration during the fermentation time of 8 and 20 hours, respectively (Figures 5.19 and 5.20), but in sucrose fermentation, the parameters of γ_2 and γ_3 have very little effect during the fermentation time of 4 and 8 hours, respectively (Figures. 5.21 and 5.22). The parametric sensitivities toward γ_2 and γ_3 for the growth kinetics in lactic acid fermentation of fructose and sucrose as calculated were 7.2 and 0.78, 5.86 and 2.16, respectively.

Figures 5.23 and 5.24 show that the γ_2 and γ_3 have very little effect on biomass concentration during 8 and 16 hours of fermentation on the mixed sugar fermentation (20g/l). The parametric sensitivities toward γ_2 and γ_3 for the growth kinetics in lactic acid fermentation of mixed sugar (20g/l) as calculated were 9.91 and 0.09. Figures 5.25 and 5.26 indicate that when mixed sugar concentration was increased to 55 g/l, the parameters of γ_2 and γ_3 also have very little effect on biomass concentration during 4 and 12 hours of fermentation. The parametric sensitivities toward γ_2 and γ_3 as calculated were 14.21 and 1.43, respectively. Thus the growth kinetics for single sugar and mixed sugar appeared to be controlled mainly by maximum specific growth rate during the whole batch.
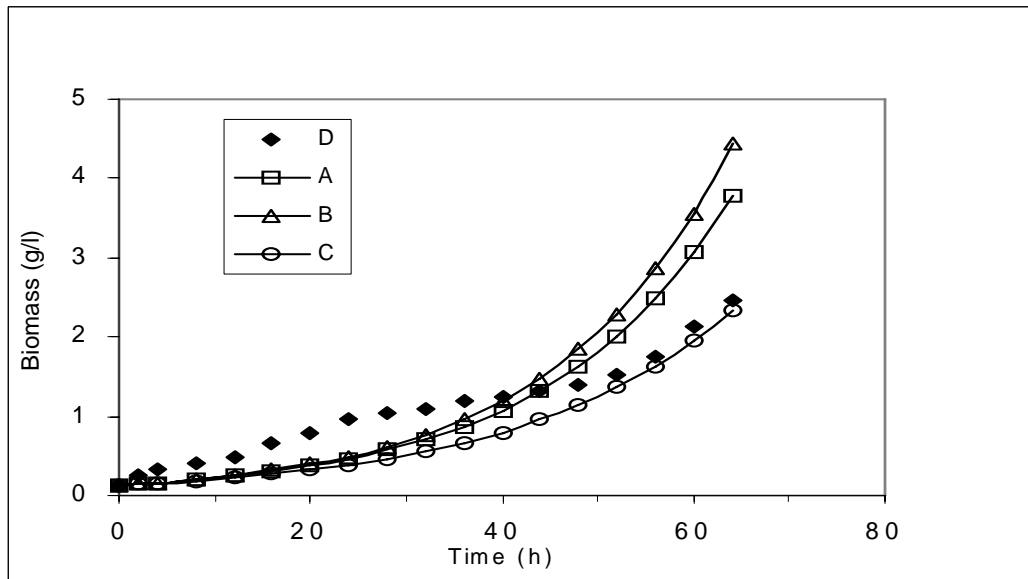
Figure 5.17: Growth model parametric analysis of glucose fermentation with constant level of $\gamma_2 = 0.0599$. A) $\gamma_3 = 0.090$; B) $\gamma_3 = 0.095$; C) $\gamma_3 = 0.1$ and D) observed data. Experimental conditions: pH, 6.0; T, $40^{\circ}C$; stirring speed, 50 rpm; and inoculum, 5%.
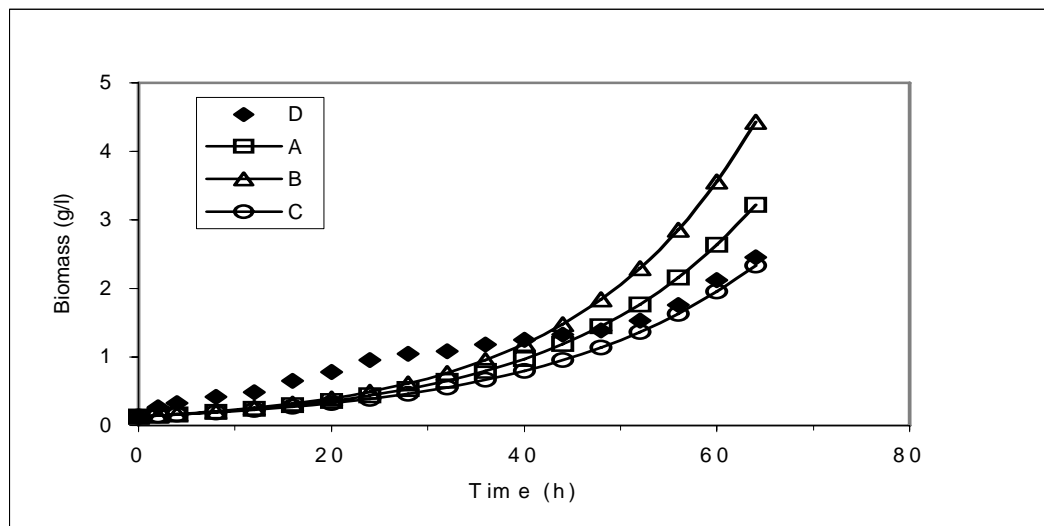
Figure 5.18: Growth model parametric analysis of glucose fermentation with constant level of $\gamma_3 = 0.095$. A) $\gamma_2 = 0.0599$; B) $\gamma_2 = 0.0649$; C) $\gamma_2 = 0.0549$; and D) observed data. Experimental conditions: pH, 6.0; T, $40^{\circ}C$; stirring speed, 50 rpm; and inoculum, 5%.
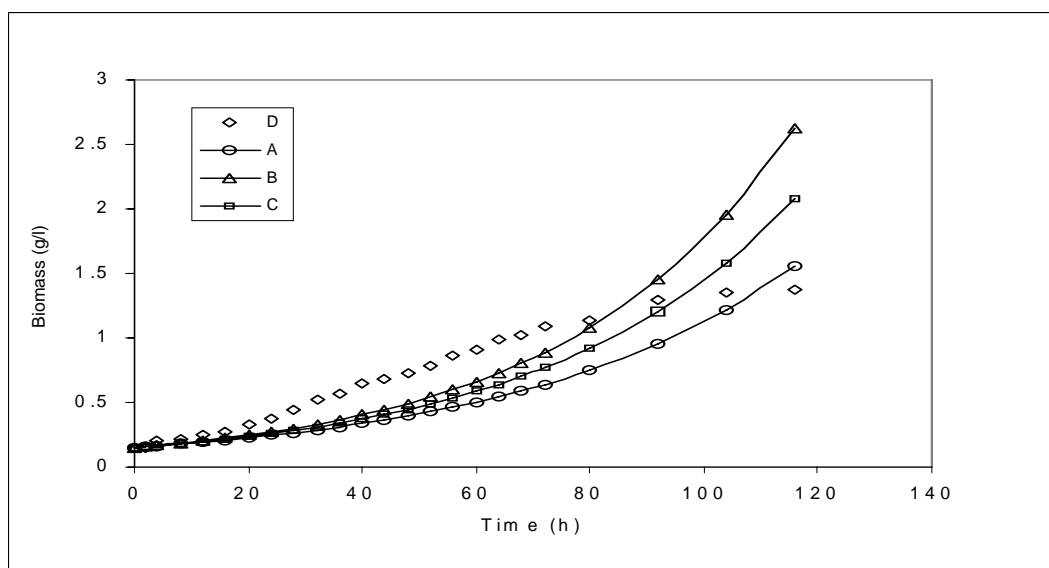
Figure 5.19: Growth model parametric analysis of fructose fermentation with constant level of $\gamma_2 = 0.0251$. A) $\gamma_3 = 0.085$; B) $\gamma_3 = 0.10$; C) $\gamma_3 = 0.0925$ and D) observed data. Experimental conditions: pH, 6.0; T, 40°C;stirring speed, 50 rpm; and inoculum, 5%.
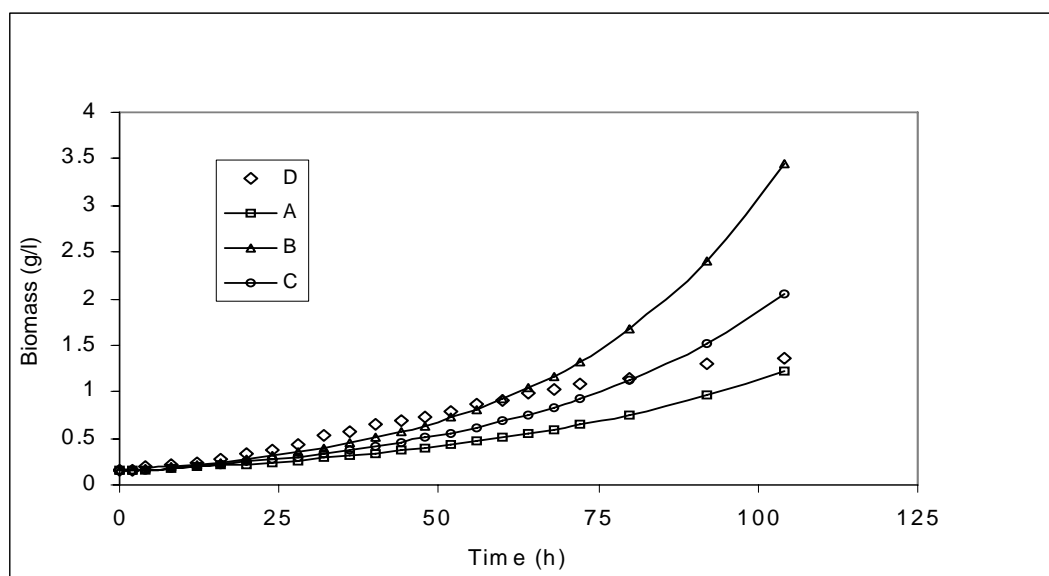
Figure 5.20: Growth model parametric analysis of fructose fermentation with constant level of $\gamma_3 = 0.085$. A) $\gamma_2 = 0.0251$; B) $\gamma_2 = 0.0351$; C) $\gamma_2 = 0.0301$; and D) observed data. Experimental conditions: pH, 6.0; T, 40°C; stirring speed, 50 rpm; and inoculum, 5%.
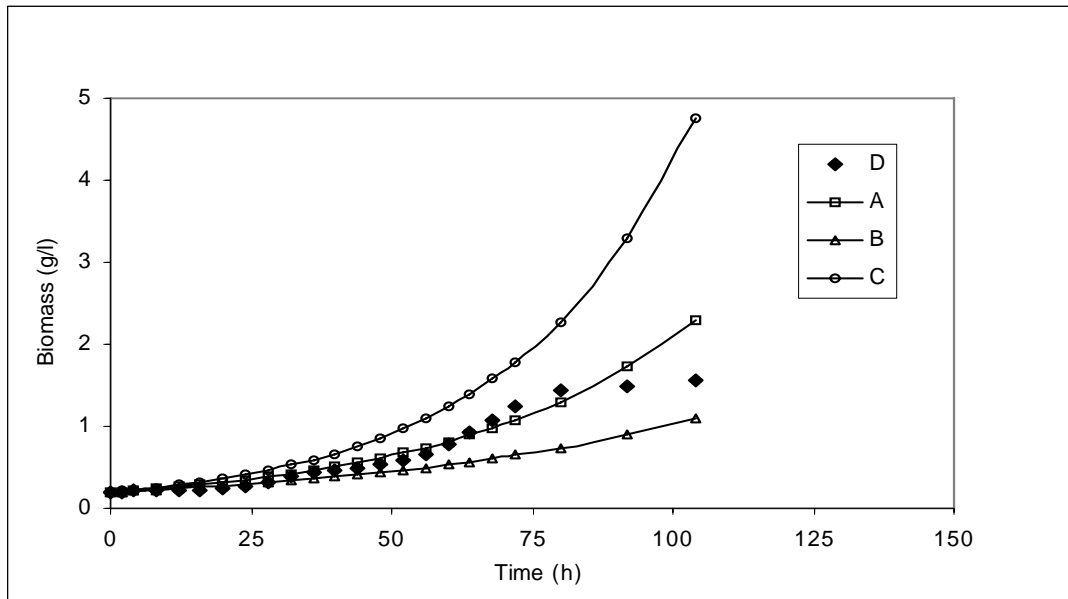
Figure 5.21: Growth model parametric analysis of sucrose fermentation with
constant level of $\gamma_2 = 0.0316$. A) $\gamma_3 = 0.085$; B) $\gamma_3 = 0.10$; C) $\gamma_3 = 0.0925$ and D) observed data. Experimental conditions: pH, 6.0; T, 40^oC; stirring speed, 50 rpm; and inoculum, 5%.
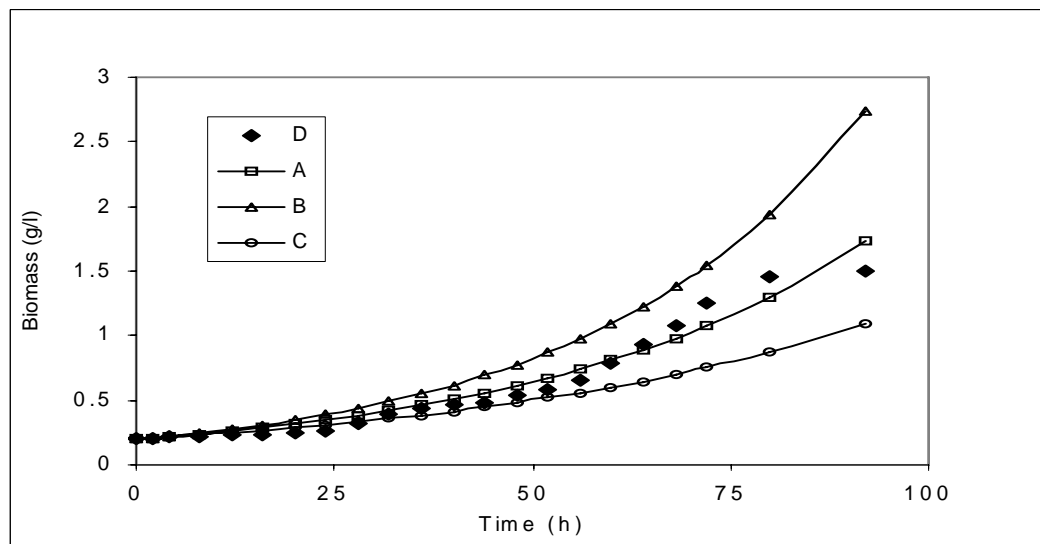
Figure 5.22: Growth model parametric analysis of sucrose fermentation with
constant level of $\gamma_3 = 0.085$. A) $\gamma_2 = 0.0316$; B) $\gamma_2 = 0.0351$; C) $\gamma_2 = 0.0301$; and D) observed data. Experimental conditions: pH, 6.0; T, 40^oC; stirring speed, 50 rpm; and inoculum, 5%.
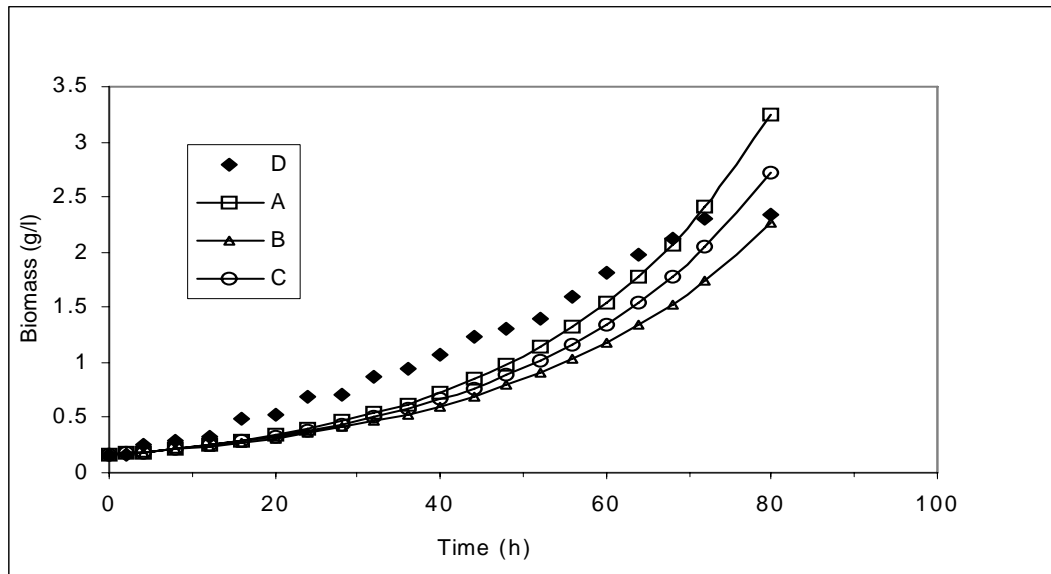
Figure 5.23: Growth model parametric analysis of mixed sugar fermentation (20 g/l) with constant level of $\gamma_2 = 0.0380$. A) $\gamma_3 = 0.10$; B) $\gamma_3 = 0.12$; C) $\gamma_3 = 0.11$ and D) observed data. Experimental conditions: pH, 6.0; T, 40^{o}C; stirring speed, 50 rpm; and inoculum, 5%.
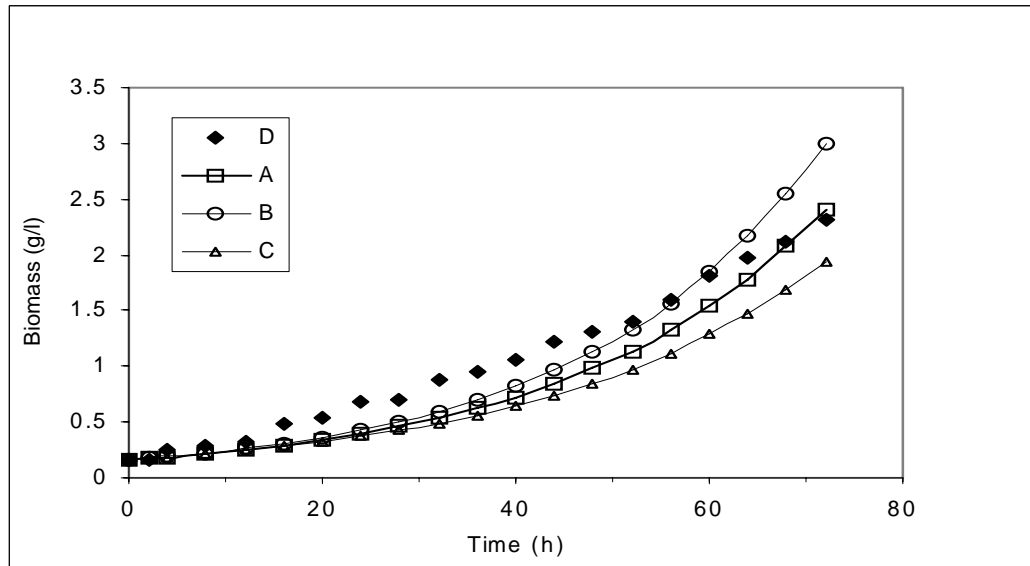
Figure 5.24: Growth model parametric analysis of mixed sugar fermentation (20 g/l) fermentation with constant level of $\gamma_3 = 0.10$. A) $\gamma_2 = 0.038$; B) $\gamma_2 = 0.041$; C) $\gamma_2 = 0.035$; and D) observed data. Experimental conditions: pH, 6.0; T, 40^{o}C; stirring speed, 50 rpm; and inoculum, 5%.
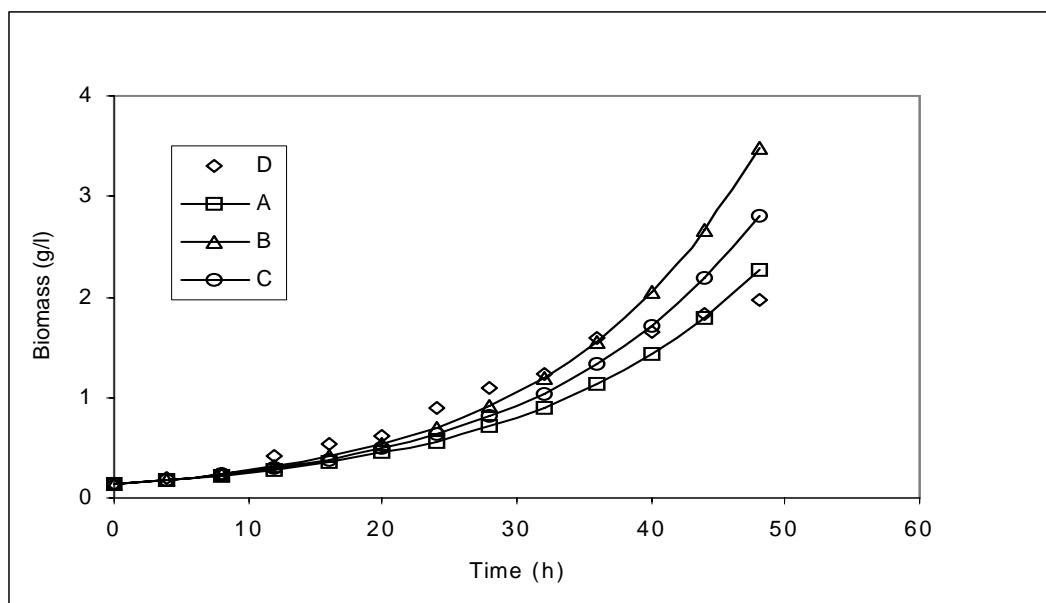
Figure 5.25: Growth model parametric analysis of mixed sugar fermentation (55 g/l) with constant level of $\gamma_2 = 0.0678$. A) $\gamma_3 = 0.51$; B) $\gamma_3 = 0.55$; C) $\gamma_3 = 0.53$ and D) observed data. Experimental conditions: pH,6.0; T, 40°C; stirring speed, 50 rpm; and inoculum, 5%.
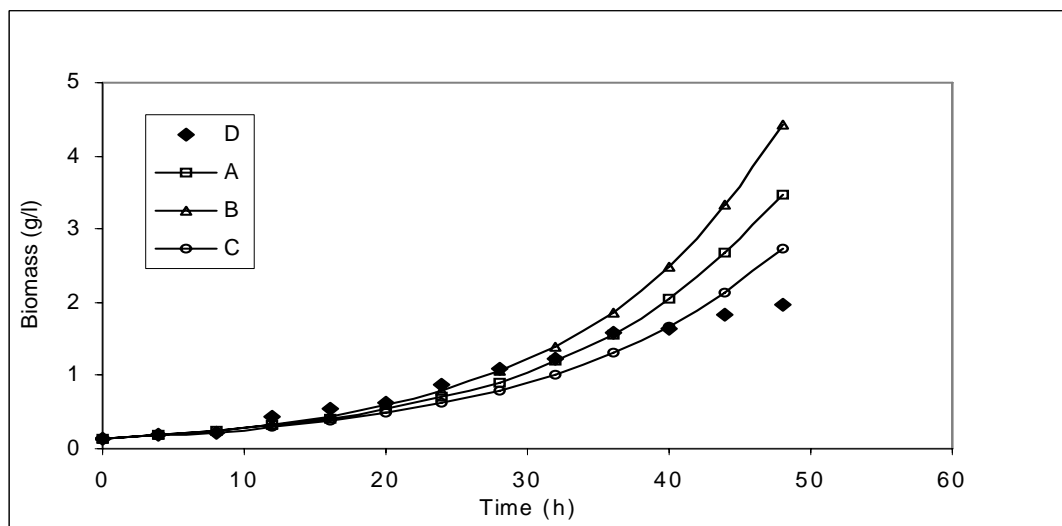
Figure 5.26: Growth model parametric analysis of mixed sugar fermentation (55 g/l) with constant level of $\gamma_3 = 0.51$. A) $\gamma_2 = 0.0678$; B) $\gamma_2 = 0.0728$; C) $\gamma_2 = 0.0628$; and D) observed data. Experimental conditions: pH, 6.0; T, 40°C; stirring speed, 50 rpm; and inoculum, 5%.

5.5.2.2 Parametric Sensitivity Analysis For Lactic Acid Production

The relationship between lactic acid, biomass concentration and time is given by Luedeking and Piret (1959) as follows:

$$P = k_1(X) + k_2 \int_0^t X.dt \qquad \qquad .....(5.36)$$

This equation shows that lactic acid production was affected by k_1 and k_2. As shown in Figures 5.27 and 5.28, the lactic acid production was slightly affected by k_1 and k_2, and the variation of k_1 and k_2 have impact on lactic acid concentration after 20 hours of fermentation ($t \geq 20$ hours). The calculated parametric sensitivities towards k_1 and k_2 for the lactic acid production using glucose as medium were 1.2 and 0.43, respectively. Thus the lactic acid production appeared to be controlled predominantly by k_1.

Figures 5.29 and 5.30 illustrate that the variation of k_1 and k_2 affects lactic acid production for fructose after 20 hours of fermentation ($t \geq 20$ h). When using sucrose as medium, the variation of k_1 and k_2 influences the lactic acid production after 30 hours of fermentation (Figures 5.31 and 5.32). The parametric sensitivities toward k_1 and k_2 for the lactic acid production as calculated were respectively 1.68 and 0.43 for fructose and 0.771 and 0.327 for sucrose. Thus the production of lactic acid from fructose or sucrose appeared to be controlled predominantly by k_1.

The effect of k_1 and k_2 on the lactic acid fermentation using mixed sugar are shown in Figures. 5.33, 5.34, 5.35 and 5.36. These results demonstrate that the variation of k_1 affects the lactic acid production after 10 hours of fermentation, but in Figure 5.34, the variation of k_2 has no effect to lactic acid production. The calculated parametric sensitivities toward k_1 and k_2 for 20 g/l and 55 g/l were 1.77 and 0.33, and 2.55 and 0.498, respectively. Thus, the production of lactic acid from mixed sugar also appeared to be controlled predominantly by k_1.
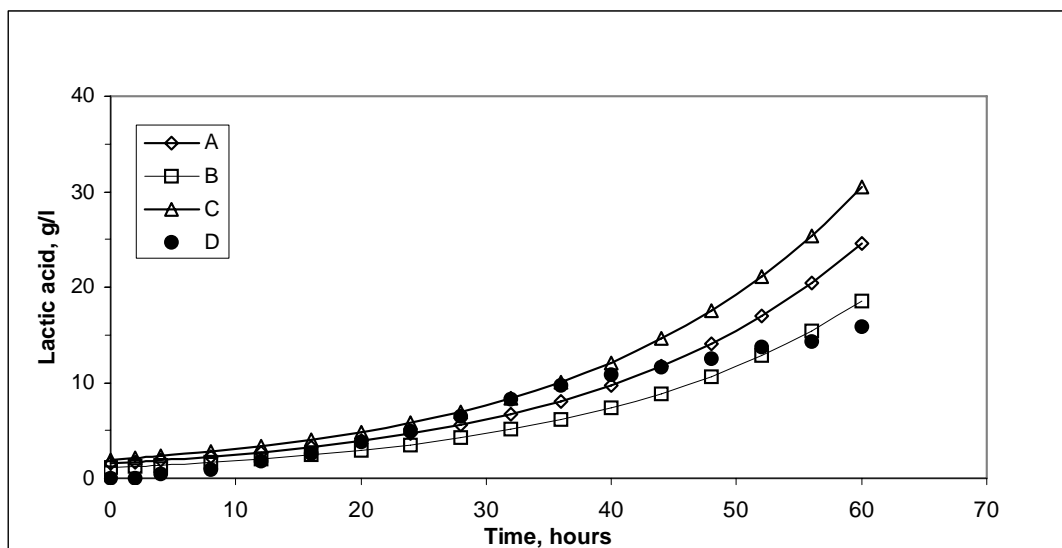
Figure 5.27: Lactic acid production model parametric analysis of glucose fermentation with constant level of $k_2 = 0.08$. A) $k_1 = 8.5$, B) $k_1 = 6.0$, C) $k_1 = 11.0$ and D) observed data. Experimental conditions: pH, 6.0; T, 40^oC; stirring speed, 50 rpm; and inoculum, 5%.
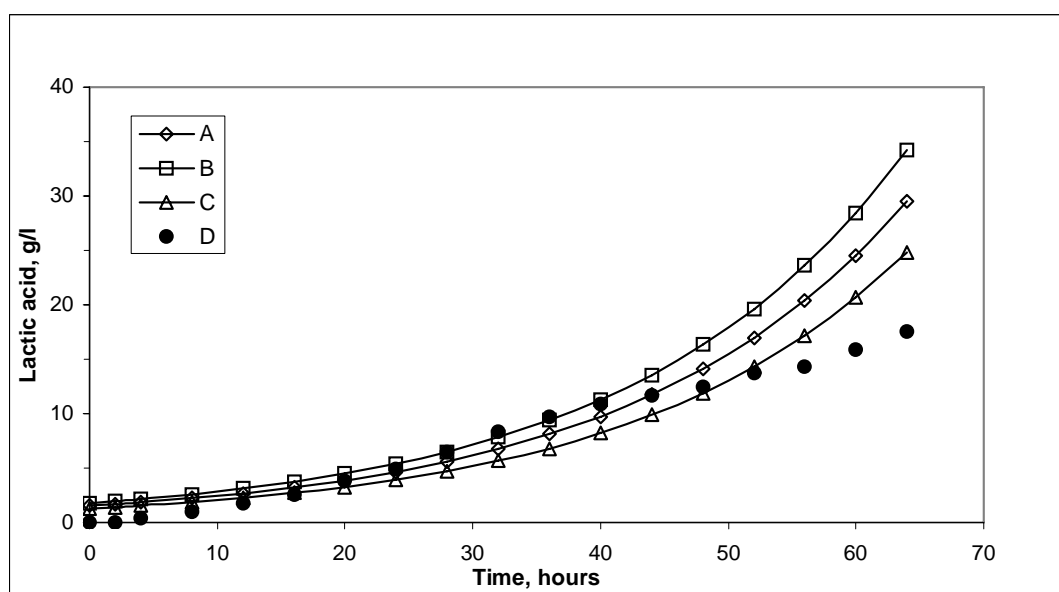
Figure 5.28: Lactic acid production model parametric analysis of glucose fermentation with constant level of $k_1 = 8.5$. A) $k_2 = 0.08$, B) $k_2 = 0.155$, C) $k_2 = 0.005$ and D) observed data. Experimental conditions: pH, 6.0; T, 40^oC; stirring speed, 50 rpm; and inoculum, 5%.
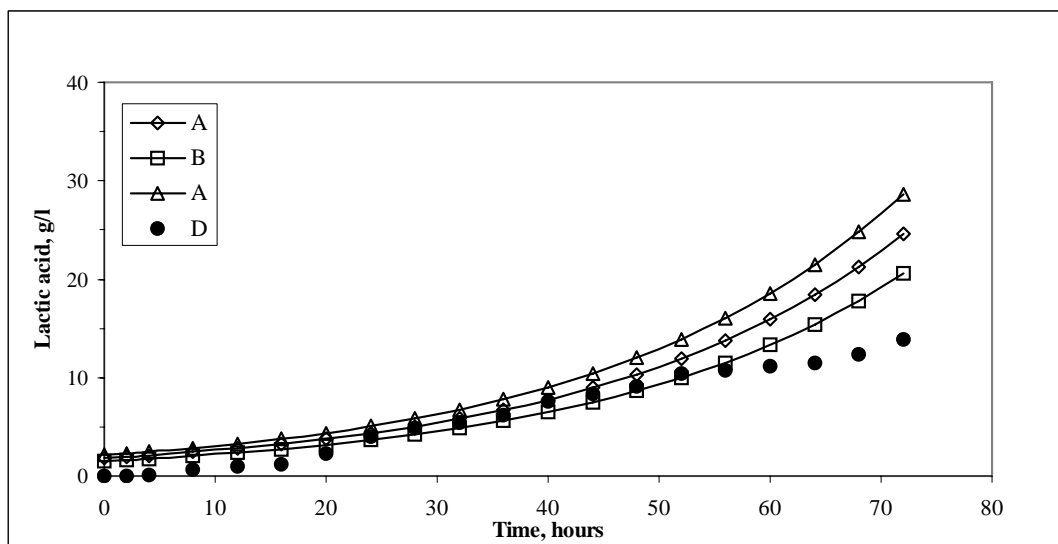
Figure 5.29: Lactic acid production model parametric analysis of fructose
fermentation with constant level of $k_2 = 0.079$. A) $k_1 = 10.0$, B) $k_1 = 8.0$,
C) $k_1 = 12.0$ and D) observed data. Experimental conditions: pH, 6.0; T,
$40^{\circ}C$; stirring speed, 50 rpm, and inoculum, 5%.
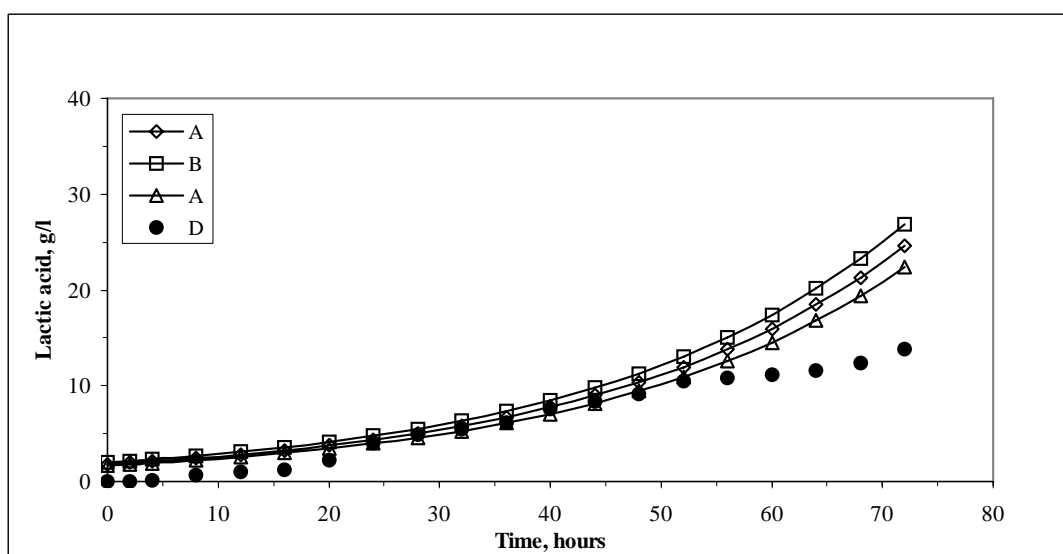
Figure 5.30: Lactic acid production model parametric analysis of fructose
fermentation with constant level of $k_1 = 10.0$. A) $k_2 = 0.08.$, B) $k_2 =$
0.12, C) $k_2 = 0.04$ and D) observed data. Experimental conditions: pH,
6.0; T, $40^{\circ}C$; stirring speed, 50 rpm; and inoculum, 5%.
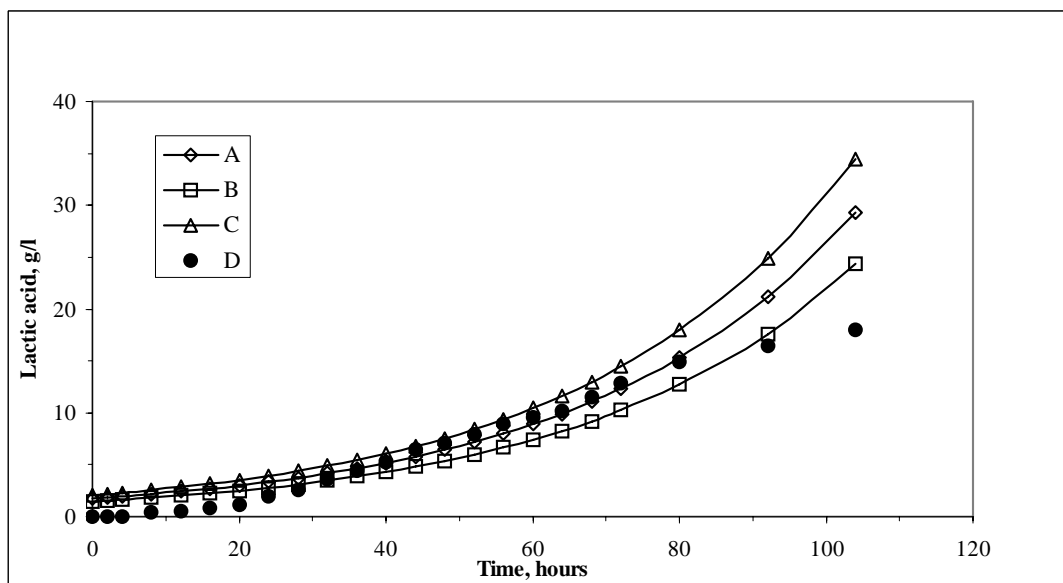
Figure 5.31: Lactic acid production model parametric analysis of sucrose
fermentation with constant level of $k_2 = 0.06$. A) $k_1 = 9.5$, B) $k_1 = 7.5$,
C) $k_1 = 11.5$ and D) observed data. Experimental conditions: pH, 6.0; T,
40^oC; stirring speed, 50 rpm; and inoculum, 5%.
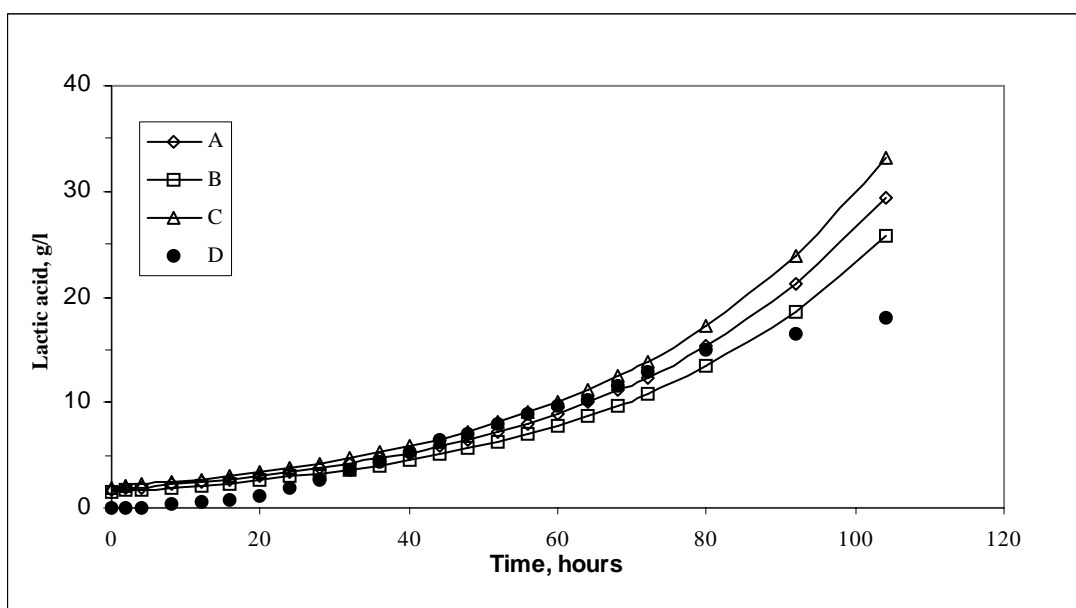
Figure 5.32: Lactic acid production model parametric analysis of sucrose
fermentation with constant level of $k_1 = 9.5$. A) $k_2 = 0.08$., B) $k_2 = 0.04$,
C) $k_2 = 0.06$ and D) observed data. Experimental conditions: pH, 6.0; T,
40^oC; stirring speed, 50 rpm; and inoculum, 5%.
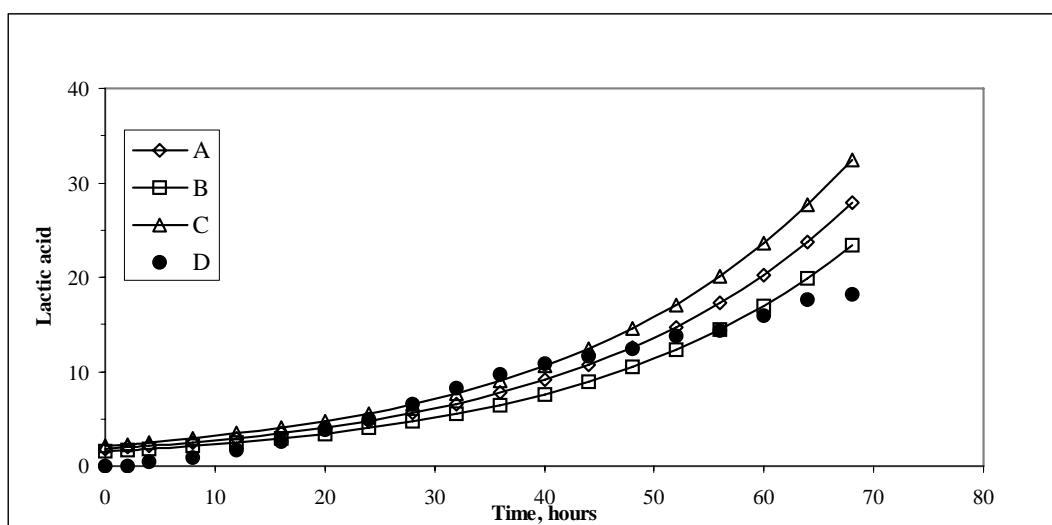
Figure 5.33: Production model parametric analysis on mixed sugar fermentation (20 g) with constant level of $k_2 = 0.07$. A) $k_1 = 10.5$, B) $k_1 = 8.5$, C) $k_1 = 12.5$ and D) observed data. Experimental conditions: pH, 6.0; T: 40^{o}C; stirring speed, 50 rpm and inoculum, 5%.
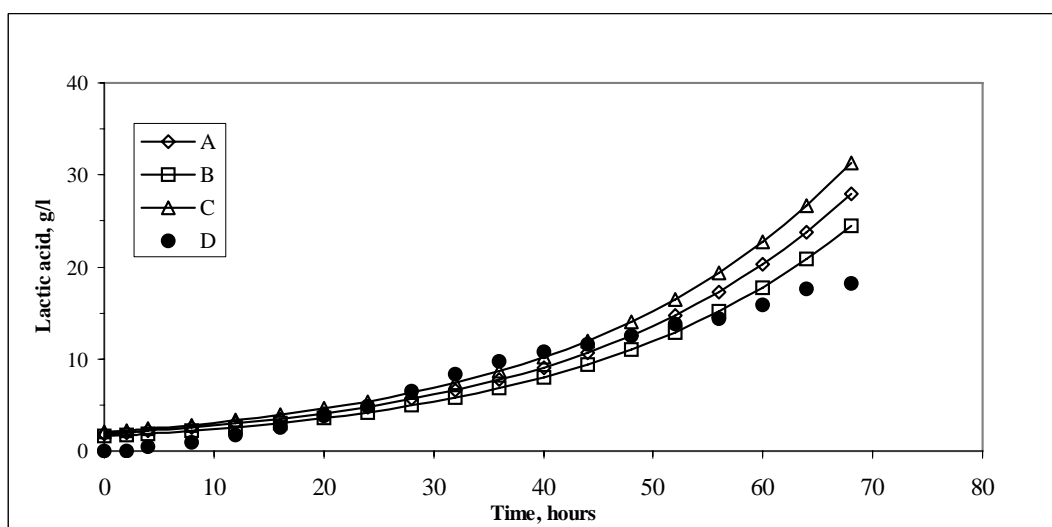
Figure 5.34: Production model parametric analysis on mixed sugar fermentation (20 g) with constant level of $k_1 = 10.5$. A) $k_2 = 0.07$, B) $k_2 = 0.1$, C) $k_2 = 0.04$ and D) observed data. Experimental conditions: pH, 6.0; T, 40^{o}C; stirring speed, 50 rpm and inoculum, 5%.
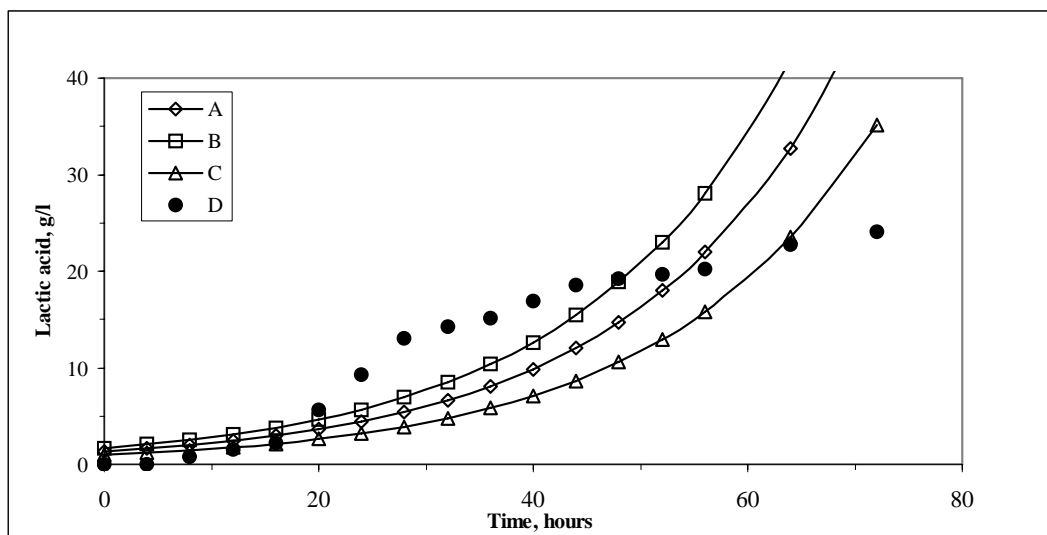
Figure 5.35: Production model parametric analysis on mixed sugar fermentation (55 g) with constant level of $k_2 = 0.02$. A) $k_1 = 8.5$, B) $k_1 = 11.0$, C) $k_1 = 6.0$ and D) observed data. Experimental conditions: pH, 6.0; T, 40°C; stirring speed, 50 rpm and inoculum, 5%.
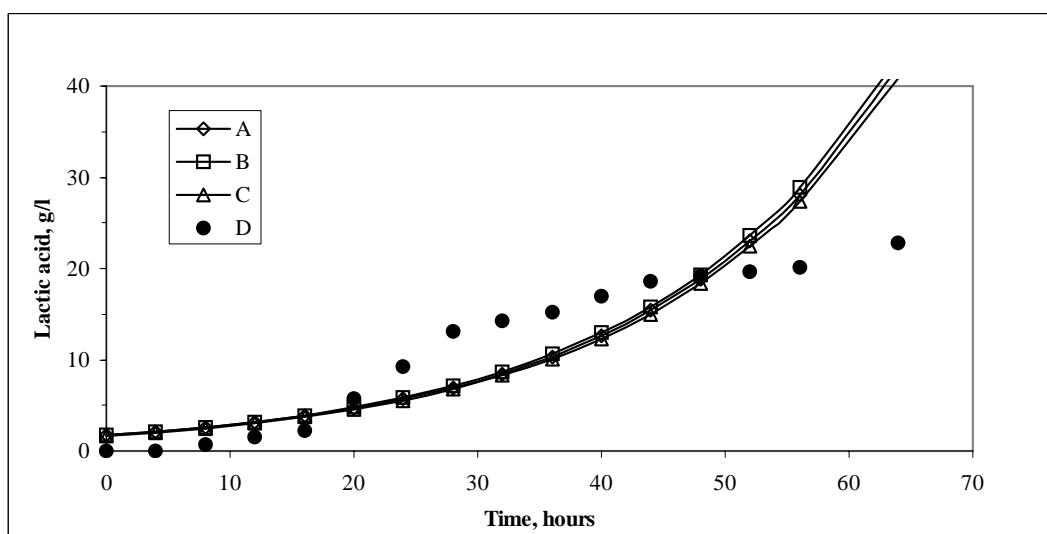
Figure 5.36: Production model parametric analysis on mixed sugar fermentation (55 g) with constant level of $k_1 = 8.5$. A) $k_2 = 0.02$, B) $k_2 = 0.035$, C) $k_2 = 0.005$ and D) observed data. Experimental conditions: pH, 6.0; T, 40°C; stirring speed, 50 rpm and inoculum, 5%.

CHAPTER VI

OPTIMISATION OF LACTIC ACID FERMENTATION

6.1 Introduction

The experiment required to solve any fermentation problem is expensive and time consuming. Each experiment involves preparation and the fermentation process needs several weeks. Thus it is important to minimise the number of experimental conditions which have to be investigated. The lengthy time between starting and experiment can be reduced to become a few experiments as possible. In order to optimise the operating conditions in lactic acid fermentation of pineapple waste by *L. delbrueckii,* the experimental design and response surface method were used in this study.

6.2 Experimental Design

The experimental design has been used by chemists and chemical engineers to minimise the number of experimental conditions which have to be investigated. An experimental design consists a set of experimental runs, with each run defined by a combination of factor levels (Murphy, 1977). Many factors may affect the lactic acid fermentation process as discussed in Section 4.4, which are temperature, time, concentration of substrate, concentration of nitrogen source and stirring speed, along

with the lactic acid yield as response variables. In order to get the greatest amount of information, several design aspects are presented in the following sub-sections.

6.2.1 2^{5-1} Fractional Factorial Design

The designs which are most used frequently for screening experiments are two level designs in which each factor is evaluated at a low setting and a high setting. In this experiment, two level factorial design were used because the ease of interpretation and effectiveness (Strange, 1990; Haaland, 1989).

Two levels, full factorial designs are very powerful tool because they provide information about all main effects and two factors interaction. To reduce the sample size, two level fractional factorial designs were used because they are very efficient (Haaaland, 1989; Box et al., 1987; and Karthikeyan et al., 1996).

There are several types of two level fractional factorial designs. In this study, one-half fraction was used because it is effective in searching the direction of the optimum domain (Carvalho et al., 1997; Karthikeyan, 1996; Liu and Tzeng, 1998). Therefore, the two factorial design used was $2^{(5-1)}$ design. This means that fractional design accommodating five variables, each one at two levels (-1/+1) where only $2^{(5-1)}$ =16 runs are employed as shown in Table 4.18 (Box et al., 1987; Haaland, 1989).

Table 6.1: 2^{5-1} fractional factorial designs.

No	Coded Variables				
	X_1	X_2	X_3	X_4	X_5
1	-1	-1	-1	-1	+1
2	+1	-1	-1	-1	-1
3	-1	+1	-1	-1	-1
4	+1	+1	-1	-1	+1
5	-1	-1	+1	-1	-1
6	+1	-1	+1	-1	+1
7	-1	+1	+1	-1	+1
8	+1	+1	+1	-1	-1
9	-1	-1	-1	+1	-1
10	+1	-1	-1	+1	+1
11	-1	+1	-1	+1	+1
12	+1	+1	-1	+1	-1
13	-1	-1	+1	+1	+1
14	+1	-1	+1	+1	-1
15	-1	+1	+1	+1	-1
16	+1	+1	+1	+1	+1

(- 1): Low level

(+1): High level

(0) : Centre point

6.2.2 Central Composite Design

Many researchers have used a central composite design to analyse the influence of variables. The central composite design allows us: 1) to show which variables significantly affect each response and 2) to optimise the values of variables that are found significantly in stage 1 (Monteagudo et al., 1993; Sung and Huang, 2000).

For example, the central composite designs with five experimental factors employ 32 experiments. This experiment consists of 16 runs at two levels (-1/+1), ten star points (-2/+2) and six replicates at the centre points (0) to allow estimation of the error and provide a check on linearity (Strange, 1990; Murphy, 1977; Hakkarainen et al., 1984). The proposed fractional factorial central composite experimental design used in this study is shown in Table 6.2.

Table 6.2: 2^{5-1} fractional factorial central composite experimental designs.

No	Coded Variables				
	X_1	X_2	X_3	X_4	X_5
1	-1	-1	-1	-1	+1
2	+1	-1	-1	-1	-1
3	-1	+1	-1	-1	-1
4	+1	+1	-1	-1	+1
5	-1	-1	+1	-1	-1
6	+1	-1	+1	-1	+1
7	-1	+1	+1	-1	+1
8	+1	+1	+1	-1	-1
9	-1	-1	-1	+1	-1
10	+1	-1	-1	+1	+1
11	-1	+1	-1	+1	+1
12	+1	+1	-1	+1	-1
13	-1	-1	+1	+1	+1

Table 6.2: 2^{5-1} fractional factorial central composite experimental designs (Continued).

No	Coded Variables				
	X_1	X_2	X_3	X_4	X_5
14	+1	-1	+1	+1	-1
15	-1	+1	+1	+1	-1
16	+1	+1	+1	+1	+1
17	-2	0	0	0	0
18	+2	0	0	0	0
19	0	-2	0	0	0
20	0	+2	0	0	0
21	0	0	-2	0	0
22	0	0	+2	0	0
23	0	0	0	-2	0
24	0	0	0	+2	0
25	0	0	0	0	-2
26	0	0	0	0	+2
27	0	0	0	0	0
28	0	0	0	0	0
29	0	0	0	0	0
30	0	0	0	0	0
31	0	0	0	0	0
32	0	0	0	0	0

(- 1): Low level

(+1): High level

 (0): Centre point

(- 2): Star point at low level

(+2): tar point at high level

The range and the levels of variables investigated in this study are given in Table 6.3. The central values (zero level) chosen for experimental design were: sugar concentration, 70 g/l; concentration of yeast extract, 15 g/l; fermentation time, 168 hours; temperature, 40°C; and stirring speed, 150 rpm. The experimental design needed in this study is given in Table 6.4.

Table 6.3: Experimental range and levels of independent variables.

X, Variables	Variable Levels					Step Change Value Δ X
	-2	-1	0	+1	+2	
X_1, Substrate (g/l)	40	55	70	85	100	15
X_2, Yeast Extract (g/l)	5	10	15	20	25	5
X_3, Time (h)	120	144	168	192	216	24
X_4, Temperature (°C)	30	35	40	45	50	5
X_5, Rpm (1/min)	50	100	150	200	250	50

Table 6.4: 2^{5-1} fractional factorial central composite design five variables.

Number of Experiments	Coded Variables				
	X_1	X_2	X_3	X_4	X_5
1	55	10	144	35	200
2	85	10	144	35	100
3	55	20	144	35	100
4	85	20	144	35	200
5	55	10	192	35	100
6	85	10	192	35	200
7	55	20	192	35	200
8	85	20	192	35	100
9	55	10	144	45	100
10	85	10	144	45	200
11	55	20	144	45	200
12	85	20	144	45	100

Table 6.4: 2^{5-1} fractional factorial central composite design five variables
(Continued).

Number of Experiments	Coded Variables				
	X_1	X_2	X_3	X_4	X_5
13	50	10	192	45	200
14	85	10	192	45	100
15	55	20	192	45	100
16	85	20	192	45	200
17	40	15	168	40	150
18	100	15	168	40	150
19	70	5	168	40	150
20	70	25	168	40	150
21	70	15	120	40	150
22	70	15	216	40	150
23	70	15	168	30	150
24	70	15	168	50	150
25	70	15	168	40	50
26	70	15	168	40	250
27	70	15	168	40	150
28	70	15	168	40	150
29	70	15	168	40	150
30	70	15	168	40	150
31	70	15	168	40	150
32	70	15	168	40	150

X_1 = Sugar concentration (g/l)
X_2 = Yeast extract concentration (g/l)
X_3 = Fermentation time (hour)
X_4 = Temperature (^{o}C)
X_5 = Stirring speed (rpm)

6.3 Model Mathematics

Since responses and factors are continuous in scale, it is useful to consider the factor response relationship in terms of a mathematical function or model. In order to interpret the experimental results effectively, it will be more economical by using mathematical model (Murphy, 1977).

6.3.1 Response Surface Models (RSM)

The first step in the experimental study of RSM is to decide a model which expresses the response as a function of independent variable in the process. Different types of models have been used to predict the optimal response such as first and second degree polynomial. However many literatures have reported that by using the quadratic model, the optimal response can be obtained accurately (Murphy, 1977; Vazquez and Martin, 1998). The form of second order (quadratic) polynomial model for five factors is given in the following equation:

$$
\begin{aligned}
Y = {} & bo + b_1X_1 + b_2X_2 + b_3\ X_3 + b_4X_4 + b_5\ X_5 + \ b_{12}\ X_1X_2 + \\
& b_{13}\ X_1X_3 + b_{14}\ X_1X_4 + \ b_{15}\ X_1X_5 + \ b_{23}\ X_2X_3 + b_{24}\ X_2X_4 + \\
& b_{25}\ X_2X_5 + b_{34}\ X_3X_4 + \ b_{25}X_3X_5 + b_{45}\ X_4X_5 + \ b_{11}\ X_1^2 + \\
& b_{22}X_2^2 + \ b_{33}\ X_3^2 + b_{44}X_4^2 + b_{55}\ X_5^2
\end{aligned}
\qquad \ldots..(6.1)
$$

`6.3.2 Model Development

To facilitate the estimation of the coefficients in the model (Equation 6.1), the variables in the model are expressed as coded variables. In developing the regression equation, the test variables were coded according to the equation:

$$
x_i = \frac{X_i - X_i^{*}}{\Delta X_i} \qquad \ldots\ldots(6.2)
$$

where x_i is the coded value of the ith independent variable, X_i is the uncoded value of the ith independent variable, X_i^{*} is the uncoded value of the ith independent variable at the centre point and ΔX_i is the step change value.

Substituting the tested variable of X_i (Equation 4.1) with coded variables of x_i (Equation 6.2), gives

$$Y = bo + b_1x_1 + b_2x_2 + b_3 x_3 + b_4 x_4 + b_5 x_5 + b_{12} x_1x_2 +$$
$$b_{13} x_1 x_3 + b_{14} x_1 x_4 + b_{15} x_1 x_5 + b_{23} x_2 x_3 +$$
$$b_{24} x_2 x_4 + b_{25} x_2 x_5 + b_{34} x_3 x_4 + b_{25} x_3 x_5 +$$
$$b_{45} x_4 x_5 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{44} x_4^2 + b_{55} x_5^2 \qquad \ldots\ldots(6.3)$$

where Y is the dependent variable, x_1 - x_5 are the dimensionless normalised independent variables and b_1 - b_5 are the coefficients obtained by multiple regression of the experimental data.

6.3.3 Evaluating The Model

The parameters of the mathematical model were estimated based on the data obtained using second order regression analysis which was carried out using Statistical Analysis System (SAS) software (Vazquez and Martin, 1998; Lee and Chen, 1997).

The statistical analysis began with the estimation of the effects of each experimental factor and their two factors interaction, and the estimation of the regression coefficient and standard error for each coefficient. The significance of each coefficient was determined using the student t-test and p-value. In the scientific literature, p values < 0.05 are generally considered to be significant, while p values < 0.0001 is very significant. The R-squared value was also estimated by Analysis of Variance to measure how the variability in the observed response values can be explained by the experimental factors and their interactions. The value of the R-squared is always between zero and one. A practical rule of thumb for evaluating the R-squared is that it should be at least 0.75 or greater. Values above 0.90 are considered to be very good (Haaland, 1989).

The response error can be estimated directly by replicated runs of centre point replication or factorial point replication (Murphy, 1977).

$$s = \sqrt{\frac{\sum (Y_i - Y_{avg})^2}{r - 1}} \qquad\qquad \dots\dots(6.4)$$

where s is the standard deviation , Y_i is the response value of run i from replicates r, Y_{avg} is the average response ($\sum Y_i$ / r) and (r-1) is the number degrees of freedom of the error estimated.

6.4   Optimisation Using Response Surface Methodology

In this study, the response surface methodology was used as optimisation tool. It consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and are measured responses, according to one or more selected criteria. The maximum values were taken as the response of the design experiments. The optimal concentrations of the factors were obtained by solving the regression equation and also by analysing the response surface contour plots (Liu and Tzeng, 1998; Sen, 1997).

6.4.1   Optimisation By Solving The Regression Equation

The lactic acid yield obtained from the experiments carried out according to the experimental design is given in Table 6.5. The application of the response surface methodology yielded the following regression equation which is an empirical relationship between lactic acid yield and the test variable in coded unit given in Equation (6.5).

$$Y = 81.32 - 3.0301\ x_1 + 0.7395\ x_2 + 2.0587\ x_3 + 8.0013\ x_4 +$$
$$2.5327\ x_5 - 0.4293\ x_1x_2 + 1.9650\ x_1\ x_3 + 1.2536\ x_1\ x_4 -$$
$$2.4741\ x_1\ x_5 - 1.0548\ x_2x_3 - 0.2575\ x_2\ x_4 + 2.2965\ x_2\ x_5 -$$
$$1.2460\ x_3\ x_4 + 1.8810\ x_3\ x_5 - 0.0268\ x_4\ x_5 - 2.9375\ x_1^2 -$$
$$0.3797\ x_2^2 - 2.6916\ x_3^2 - 11.5613\ x_4^2 - 6.0335\ x_5^2 \qquad \ldots\ldots(6.5)$$

The calculation of coefficient in second degree model using a central composite design according to Equation (6.5) were estimated using the formula given by Cornell (1991) and it can be found in Appendix E.

Table 6.5: 2^{5-1} fractional factorial central composite design five variables with the observed response.

Run	Sugar (g/l)	Yeast extract (g/l)	Time (hour)	Temperature (^{o}C)	Speed (rpm)	Yield (%)
1	55	10	144	35	200	52.48
2	85	10	144	35	100	42.72
3	55	20	144	35	100	55.19
4	85	20	144	35	200	37.90
5	55	10	192	35	100	52.50
6	85	10	192	35	200	43.14
7	55	20	192	35	200	62.60
8	85	20	192	35	100	42.29
9	55	10	144	45	100	73.78
10	85	10	144	45	200	53.94
11	55	20	144	45	200	79.55
12	85	20	144	45	100	64.02
13	50	10	192	45	200	71.14
14	85	10	192	45	100	66.84
15	55	20	192	45	100	61.30
16	85	20	192	45	200	64.30
17	40	15	168	40	150	72.79
18	100	15	168	40	150	65.83
19	70	5	168	40	150	77.90
20	70	25	168	40	150	81.47
21	70	15	120	40	150	59.22
22	70	15	216	40	150	81.66
23	70	15	168	30	150	23.40
24	70	15	168	50	150	46.52

Table 6.5: 2^{5-1} fractional factorial central composite design five variables with the observed response (Continued).

Run	Sugar (g/l)	Yeast extract (g/l)	Time (hour)	Temperature (°C)	Speed (rpm)	Yield (%)
25	70	15	168	40	50	43.48
26	70	15	168	40	250	70.66
27	70	15	168	40	150	82.82
28	70	15	168	40	150	80.41
29	70	15	168	40	150	80.22
30	70	15	168	40	150	81.98
31	70	15	168	40	150	80.47
32	70	15	168	40	150	82.28

X_1 = Sugar concentration (g/l)

X_2 = Yeast extract concentration (g/l)

X_3 = Fermentation time (hour)

X_4 = Temperature (°C)

X_5 = Stirring speed (rpm)

The fitness of the model can be checked by several criteria. The analysis of variance (ANOVA) for testing the significance of the coefficient is given in Table 6.6. The determination of coefficient R^2 = 0.98 indicates that only 2% of total variation were not explained by the model. The values of F and R^2 were obtained as follow,

$$F = \frac{MeanSquare\,\mathrm{Re}\,gression(MSR)}{MeanSquareError(MSR)} = \frac{SSR/(p-1)}{SSE/(N-p)} = \frac{7943.89/5}{1364.42/26} = 19.39$$

$$R^2 = \frac{SumSquare\,\mathrm{Re}\,gression(SSR)}{SumSquareTotal(SST)} = \frac{7943.89}{8058.60} = 0.9844$$

The values of F and R^2 were obtained by calculating the total sum square (SST), sum square regression (SSR) and sum square error or residual (SSR). These values are shown in Table 6.7.

To test the adequacy of the fitted model from Equation (6.5) using static F, the value of F is compared to the table value $F_{(p-1,N-p,\alpha)}$, which is the upper 100 α percent point of the F distribution with p-1 and N-p degrees of freedom, respectively. Since the value F = 19.39 exceeds the table value $F_{(5,26,.01)}$ = 3.8183, this indicates that the Fisher F test also demonstrates a high significance for the fitted regressions model.

Table 6.6: Analysis of variance lactic acid yields values.

Source	df	SS	MS	F	R^2
Regression (p-1)	5	7945.28	1589.056	19.39	0.9844
Residual (N-p)	26	1364.42	81.92		
Total (N-1)	31	8058.60			

Table 6.7: Calculation of the sum squares.

Run	Yo	Yp	$(Yo - \bar{Y})^2$	$(Yp - \bar{Y})^2$	$(Yo-Yp)^2$
1	52.48	51.91	123.38	136.36	0.32
2	42.72	40.83	435.45	517.90	3.57
3	55.90	59.53	59.10	16.46	13.18
4	37.19	35.46	696.83	791.16	2.99
5	52.50	45.51	122.93	326.80	48.86
6	43.14	54.82	418.10	76.87	136.42
7	62.60	61.96	0.98	2.65	0.41
8	42.29	49.25	453.58	205.56	48.44
9	73.78	62.49	103.89	1.20	127.46
10	53.94	69.22	93.07	31.73	233.48
11	79.55	75.36	254.80	138.59	17.56
12	64.02	61.28	0.19	5.32	7.51
13	71.14	66.71	57.04	9.75	19.62
14	66.84	71.26	10.58	58.87	19.54
15	61.30	50.01	5.23	184.35	127.46
16	64.30	78.15	0.51	212.07	191.82
17	72.79	61.69	84.69	3.60	123.21
18	65.83	74.94	5.03	128.88	82.99
19	77.90	77.21	204.85	185.57	0.48
20	81.47	82.46	319.78	356.17	0.98

| 21 | 59.22 | 65.31 | 19.08 | 2.97 | 37.09 |

Table 6.7: Calculation of the sum squares (Continued).

Run	Yo	Yp	$(Yo - \overline{Y})^2$	$(Yp - \overline{Y})^2$	$(Yo-Yp)^2$
22	81.66	73.56	326.62	99.45	65.61
23	23.40	19.96	1615.04	1903.36	11.83
24	46.52	51.96	291.30	135.20	29.59
25	43.48	42.82	404.31	431.29	0.44
26	70.66	73.34	50.02	95.11	7.18
27	82.82	81.32	369.89	314.44	2.25
28	80.41	81.32	283.00	314.44	0.83
29	80.22	81.32	276.64	314.44	1.21
30	81.98	81.32	338.28	314.44	0.44
31	80.47	81.32	285.02	314.44	0.72
32	82.28	81.32	349.41	314.44	0.92
$\sum Y_o$		$\overline{Y}$	SST	SSR	SSE
2032.80		63.525	8058.60	7943.89	1364.42

Each of the observed values Yo is compared with predicted value Yp calculated from the model given in Table 6.8 The comparison of the residual with residual variance (MS=52.47) denotes that none of the individual residual exceeds twice the square root at the residual variance. All these considerations indicate a good adequacy of the regression model.

Table 6.8: Observed responses and predicted values.

Run	Yo	Yp	Residual (Yo -Yp)
1	52.48	51.91	0.57
2	42.72	40.83	1.89
3	55.90	59.53	-3.63
4	37.19	35.46	1.73
5	52.50	45.51	6.99
6	43.14	54.82	-11.68
7	62.60	61.96	0.64
8	42.29	49.25	-6.96
9	73.78	62.49	11.29
10	53.94	69.22	-15.28
11	79.55	75.36	4.19
12	64.02	61.28	2.74
13	71.14	66.71	4.43
14	66.84	71.26	-4.42
15	61.30	50.01	11.29
16	64.30	78.15	-13.85
17	72.79	61.69	11.10
18	65.83	74.94	-9.11
19	77.90	77.21	0.69
20	81.47	82.46	-0.99
21	59.22	65.31	-6.09
22	81.66	73.56	8.10
23	23.40	19.96	3.44
24	46.52	51.96	-5.44
25	43.48	42.82	0.66
26	70.66	73.34	-2.68
27	82.82	81.32	1.50
28	80.41	81.32	-0.91
29	80.22	81.32	-1.10
30	81.98	81.32	0.66
31	80.47	81.32	-0.85
32	82.28	81.32	0.96

Yo: observed yield
Yp: predicted yield

The significance of each coefficient was determined using the student t-test and *p*- value as given in Table 6.9. The larger the magnitude of t- value is, the smaller the p value will be. This indicates a high significance of the corresponding coefficient. It can be seen that the variable with largest effect was squared term of temperature (X_4). This is followed by stirring speed (X_5) and concentration of sugar

(X_1). The factor temperature in squared term has largest t-value (-7.26) and seems to be very significant (p = 0.0001). The stirring speed has smaller t-value (-3.8676) but is still significant (p = 0.0003). The fermentation time and sugar concentration have very low t-values which are-1.90 and 1.88, respectively, and are not significant (p = 0.072 and p = 0.069). In linear term only temperature has largest t-value (5.128) and seems to be significant (p = 0.0003). The interaction affects all factors that have low t-values and seem to be insignificant (p > 0.1).

Error estimation was made for six experiments in the centre points from Equation (4.4). With a confidence level of 99%, the "t" of Student for five (r-1) degrees of freedom is 4.032. The calculated standard deviation was 1.56.

Table 6.9: Significance of regression coefficient.

Variables	Regression coefficient	Computed t value	Significance level, p value
Constant	81.3200	52.1282	-
x_1	-3.0300	-1.9423	0.0636
x_2	0.7395	0.4740	0.3522
x_3	2.0587	1.3192	0.1652
x_4	8.0113	5.1283	0.0003
x_5	2.5324	1.6234	0.0924
x_1*x_1	-2.9375	-1.8830	0.0698
x_2*x_2	-0.6560	-0.42051	0.3572
x_3*x_3	-2.9680	-1.9025	0.0722
x_4*x_4	-11.3380	-7.2679	0.0001
x_5*x_5	-6.0335	-3.8676	0.0007
x_1*x_2	-0.4293	-0.2751	0.3775
x_1*x_3	1.9650	1.2596	0.1772
x_1*x_4	1.2530	0.8032	0.2824

Table 6.9: Significance of regression coefficient (Continued).

Variables	Regression coefficient	Computed t value	Significance level, p value
x_1*x_5	-2.4740	-1.5858	0.1155
x_2*x_3	-1.0540	-0.6739	0.3060
x_2*x_4	-0.2293	-0.1475	0.3893
x_2*x_5	2.2930	1.4699	0.1396
x_3*x_4	-1.2460	-0.7987	0.2841
x_3*x_5	1.8800	1.205	0.1944
x_4*x_5	-0.0268	-0.017352	0.3920

Analytical optimisation method was used to solve the regression equation of (6.3). The optimal values of the tested variables in coded unit are as follows: $x_1 = -0.2759$, $x_2 = 0.1114$, $x_3 = 0.5935$, $x_4 = 0.3405$ and $x_5 = 0.06975$.

The optimal values of tested variables in uncoded unit can be obtained by Equation (6.2) and were found to be: sugar concentration (X_1), 65.87 g/l; yeast extract concentration (X_2), 15.35 g/l; fermentation time (X_3), 182.4 hours; temperature (X_4), 41.70; and stirring speed (X_5), 153.58 rpm. The model can predict the maximum of lactic acid yield which was 83.79 %.

6.4.2 Optimisation By Analysing The Response Surface Contour Plots

Optimum yield can also be predicted from the respective contour plots. Each contour curve represents an infinite number of two test variables with the other three maintained at their respective zero levels. The maximum predicted yield is indicated by the surface confined in the smallest ellipse in the contour diagram (Box et al., 1978; Cornell, 1990).

6.4.2.1 Effect Of Sugar And Yeast Extract Concentration On Yield

The sugar and yeast extract concentrations were studied in the range of 40-100 and 5-25 g/l, respectively. From the analysis of the response surface (Figure 6.1a), it can be concluded that there is no obvious optimal value within the investigation of the experimental range. Figure 6.1b illustrates that there is no maximum point of response but minimum point (saddle point) is recorded. Therefore the optimal value was estimated using method described by Cornell (1990). The optimal conditions were achieved at sugar concentration (X_1) of 50 g/l; and yeast extract concentration (X_2) of 11.44 g/l.

The prediction of factors and dependence variable can also be calculated by best explanatory equation: $Y_{12} = 51.962 + 0.751 X_1 - 0.649 X_2 - 0.007 X_1*X_1 - 0.007 X_1*X_2 + 0.04 3X_2*X_2$. This equation shows that effects of yeast extract and substrate concentration in linear term are moderate but the squared term and the interaction between factors are found to be insignificant.

By using analytical method, the optimal point was obtained at sugar concentration (X_1) of 47.91 g/l, and yeast extract concentration (X_2) of 11.44 g/l with maximum yield of 66.24 %.
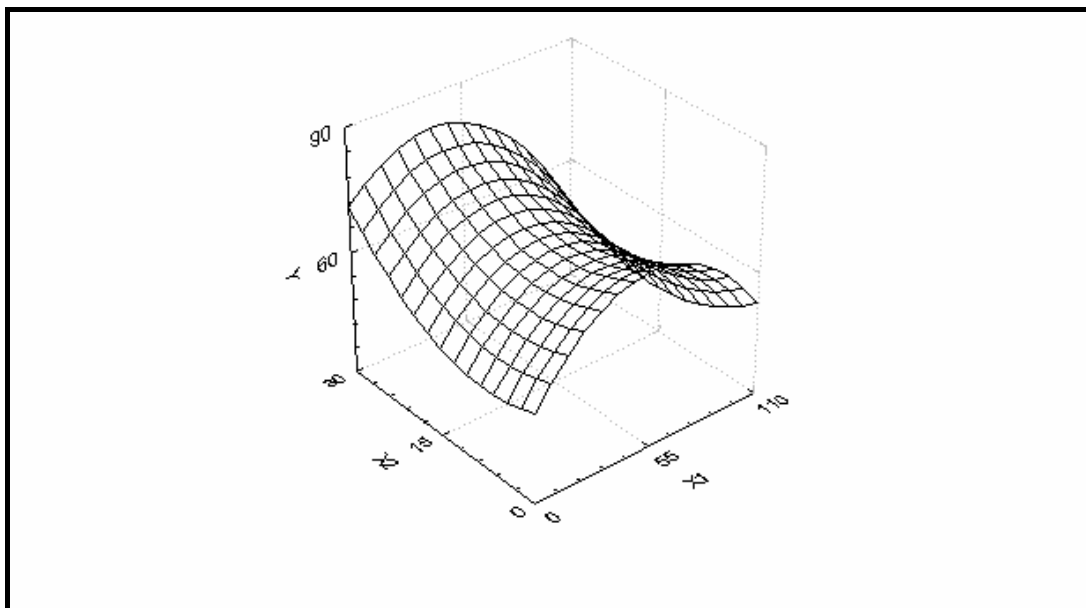
Figure 6.1a: The predicted surface response as a function of sugar and yeast extract
concentrations at constant levels of fermentation time,168 hours;
temperature, 40 ^{o}C and stirring speed, 150 rpm.
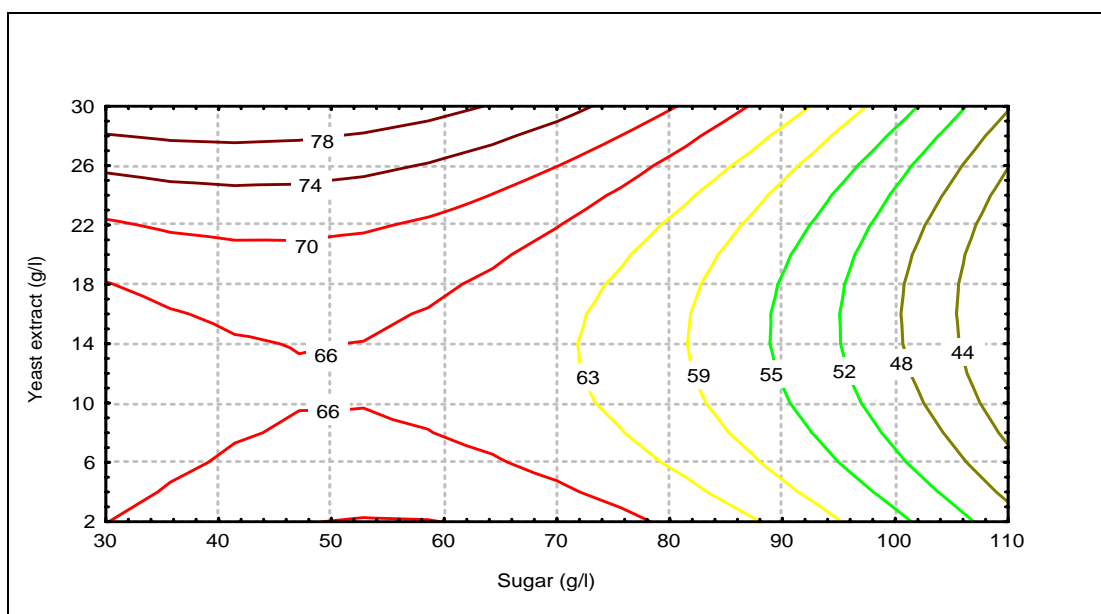
Figure 6.1b: The contour plot of the effect of sugar and yeast extract concentrations
on lactic acid yield at constant levels of fermentation time, 168 hours;
temperature, 40 ^{o}C; and stirring speed, 150 rpm.

6.4.2.2 Effect Of Sugar Concentration And Fermentation Time On Yield

Effects of sugar concentration and fermentation time on yield as given in Figure 6.2a, point out that with increasing fermentation time and sugar concentration, the yield also increases. If the concentration of substrate is greater than 70 g/l, the yield will decrease, which might be due to substrate (sugar) inhibition. By analysing contour plot in Figure 6.2b, the optimal condition obtained was 50 g/l of substrate and fermentation time of 168 hours.

The optimal yield were also calculated by best explanatory equation: $Y_{13} = 33.868 - 0.208 X_1 + 0.508 X_3 - 0.008 X_1*X_1 + 0.006 X_1*X_3 - 0.002 X_3*X_3$. This equation shows that effects of time and sugar concentration in linear term are moderate but the squared term and interaction between factors seem to be insignificant.

By using analytical method, the optimal point was at sugar concentration (X_1) of 79.14 g/l, and fermentation time (X_3) of 245.76 hours with maximum yield of 87.68 %.
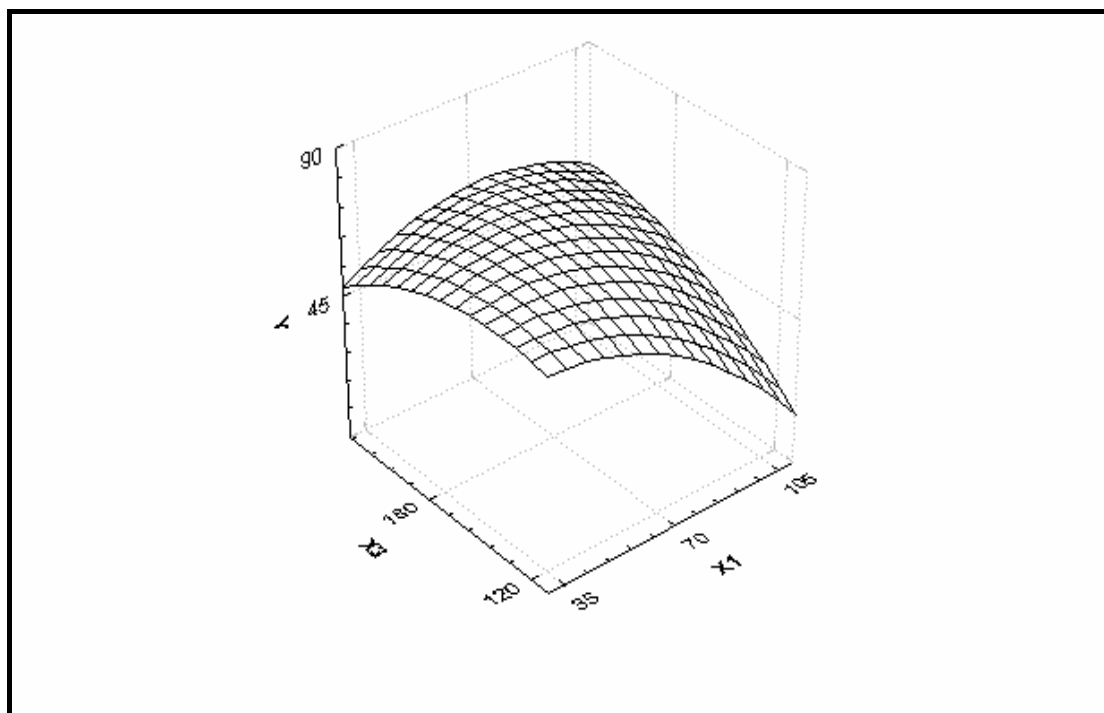
Figure 6.2a: The predicted surface response as a function of sugar concentration and fermentation time at constant levels of temperature, 40 ^{o}C; yeast extract concentration, 5 g/l and stirring speed, 150 rpm.
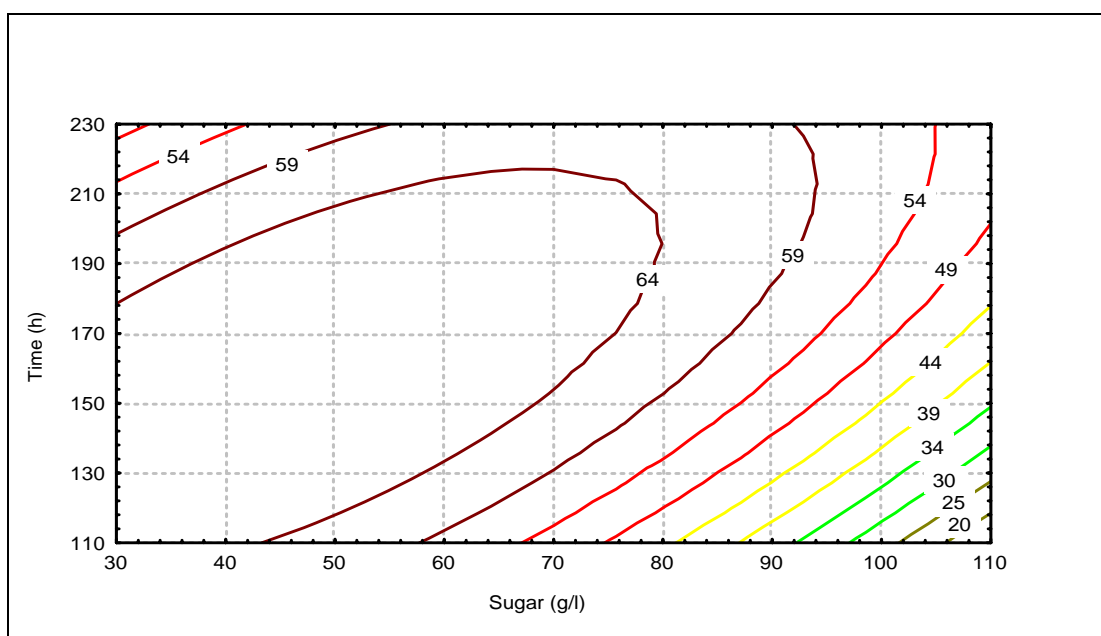
Figure 6.2b: The contour plot of the effect of sugar concentration and fermentation time on lactic acid yield at constant level of temperature, 40 ^{o}C; yeast extract concentration, 5 g/l and stirring speed, 150 rpm.

6.4.2.3 Effect Of Sugar Concentration And Temperature On Yield

The temperature studied was in the range of 30-50^{o}C, which was the optimal growth temperature for *Lactobacillus delbrueckii*. From the analysis of the response surface (Figure 6.3a), it can be seen that effects of temperature and substrate concentration on yield are significant. With increasing temperature and sugar concentration, the yield increases. However the yield decreases if temperature and substrate concentration are above 45 ^{o}C and 70 g/l, respectively. The optimal value obtained by analysing contour plot in Figure 6.3b was at sugar concentration (X_1) of 58 g/l; and temperature (X_4) of 42 ^{o}C.

The optimal yield was calculated by best explanatory equation: Y_{14} = -668.391 + 0.492 X_1+ 35.271 X_4 - 0.01 X_1*X_1+0.08 X_1*X_4 - 0.437 X_4*X_4. This equation shows that the effects of temperature are very significant but moderate for the substrate concentration, which are indicated by the values of regression coefficients at 35.27 and 0.491, respectively. The interactions between factors were found to be insignificant. By using analytical method, the optimal point was at sugar concentration (X_1) of 58.86 g/l, and temperature (X_4) of 41.56 ^{o}C with maximum yield of 77.31 %.
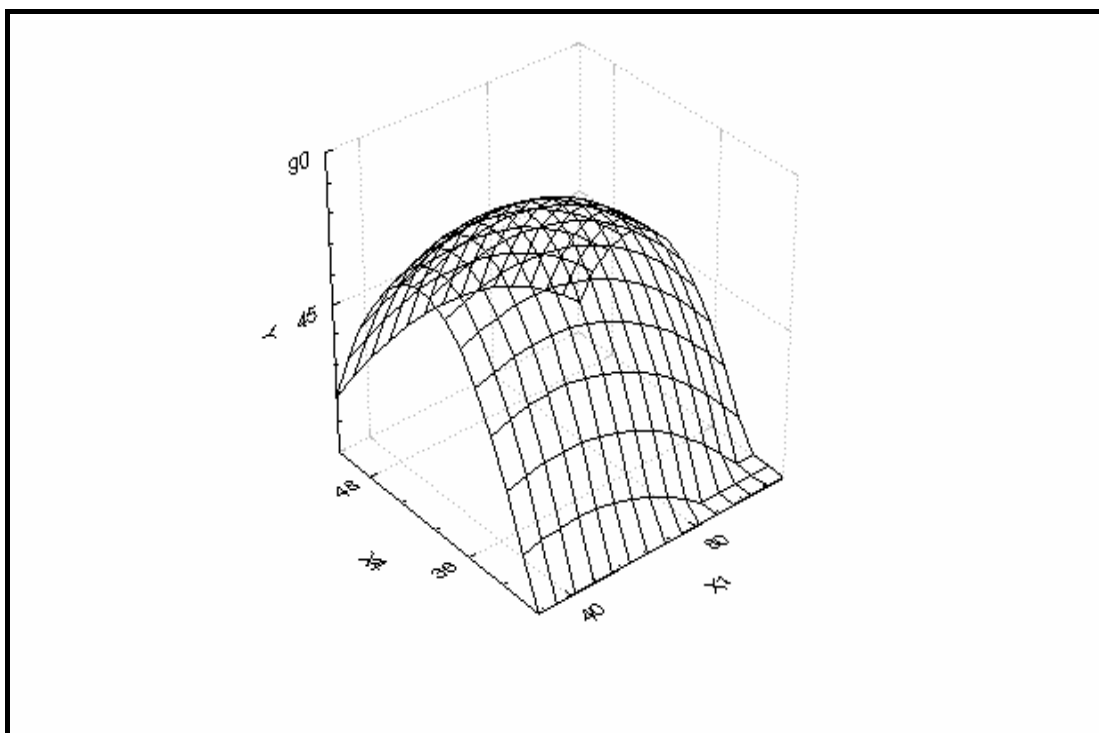
Figure 6.3a: The predicted surface response as a function of sugar concentration and temperature at constant levels of fermentation time, 168 hours; yeast extract concentration, 5 g/l; and stirring speed, 150 rpm.
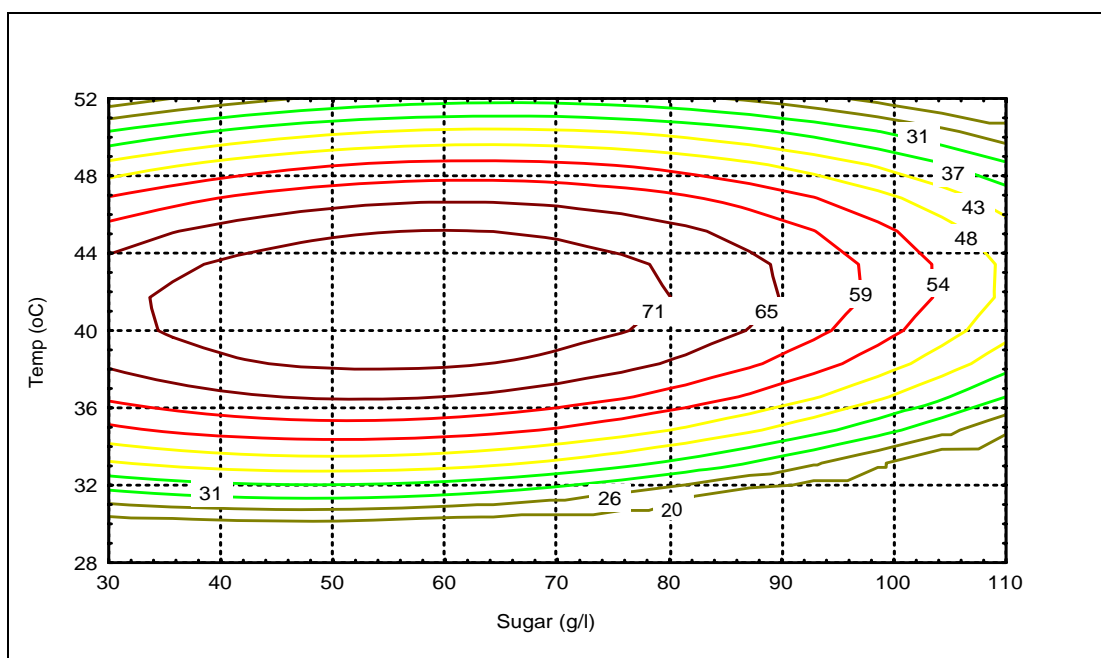
Figure 6.3b: The contour plot of the effect of sugar concentration and temperature on lactic acid yield at constant levels of fermentation time, 168 hours; yeast extract concentration, 5 g/l and stirring speed, 150 rpm.

6.4.2.4 Effect Of Sugar Concentration And Stirring Speed On Yield

Lactobacillus delbrueckii is an anaerobic bacterium. Therefore in order to maintain the fermentation in anaerobic condition, the agitation process was performed relatively at low speed. The agitation speed studied was in the range of 50-250 rpm. Effects of sugar concentration and stirring speed on the lactic acid yield are illustrated in Figure 6.4a. The yield increases with increasing sugar concentration and stirring speed. The yield decreases if sugar concentration and stirring speed are above 70 g/l and 200 rpm, respectively. Analysis of response surface (Figure 6.4.b) showed that effects of both factors are significant, and it gives maximum yield at operation conditions with sugar concentration of 48 g/l and stirring speed of 180 rpm.

The optimal yield was calculated by best explanatory equation: $Y_{13} = -39.091 + 1.394 X_1 + 0.879 X_5 - 0.009 X_1*X_1 + 0.003 X_1*X_5 - 0.002 X_5*X_5$. This
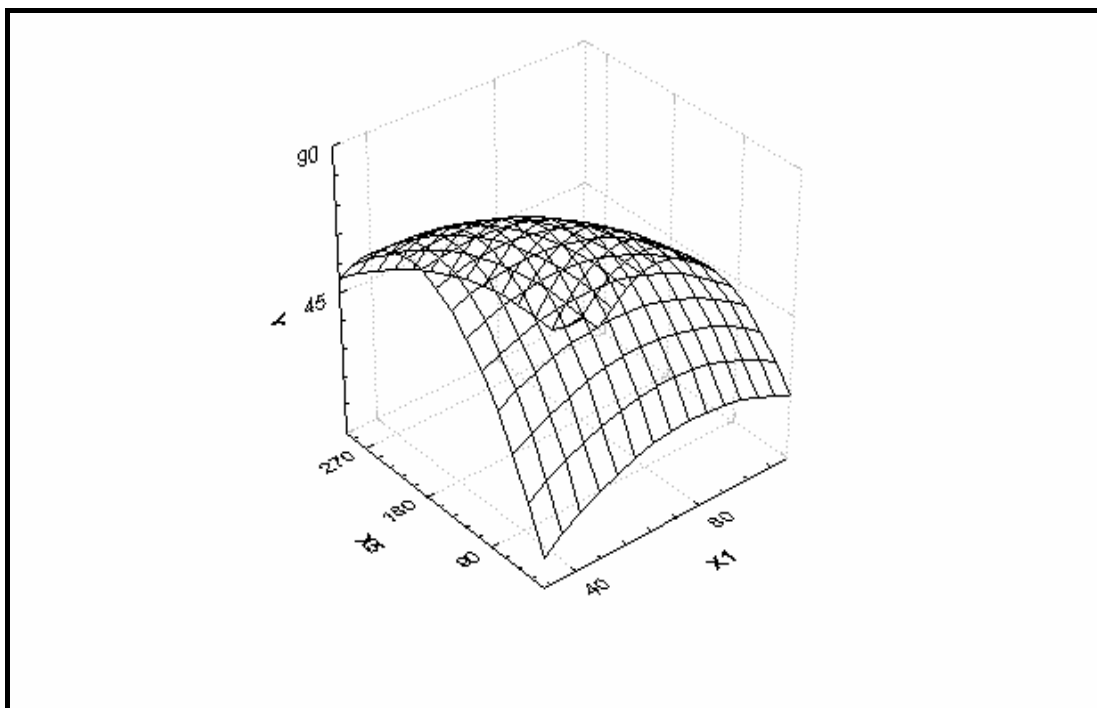
Figure 6. 4b: The contour plot of the effect of sugar concentration and stirring speed on lactic acid yield at constant levels of yeast extract concentration, 5 g/l; fermentation time, 168 hours; and temperature, 40 $^\circ$ C.

6.4.2.5 Effect Of Yeast Extract Concentration And Fermentation Time On Yield

Effects of yeast extract concentration and fermentation time on the lactic acid yield are demonstrated in Figure 6.5a. From the response surface analysis, it was found that there was no maximum response obtained, similar with the effect of yeast extract and sugar concentrations. The optimal values were estimated by analysing the contour plot as shown in Figure 6.5b. The yeast extract concentration and fermentation time obtained were 15 g/l and 190 hours, respectively.

The prediction of dependence variable was also calculated by best explanatory equation:$Y_{23} = -25.263 + 0.319 X_2 + 0.931X_3 + 0.043 X_2*X_2 - 0.009 X_2*X_3 - 0.002 X_3*X_3$. This equation designates that effects of yeast extract and fermentation time in linear term were moderate but insignificant in squared term. The interaction between factors was also found to have insignificant impact.

By using analytical method, the optimal point obtained was found to be yeast extract concentration and fermentation time of 16.70 g/l and 195.18 hours, respectively with the optimal yield of 68.14 %.
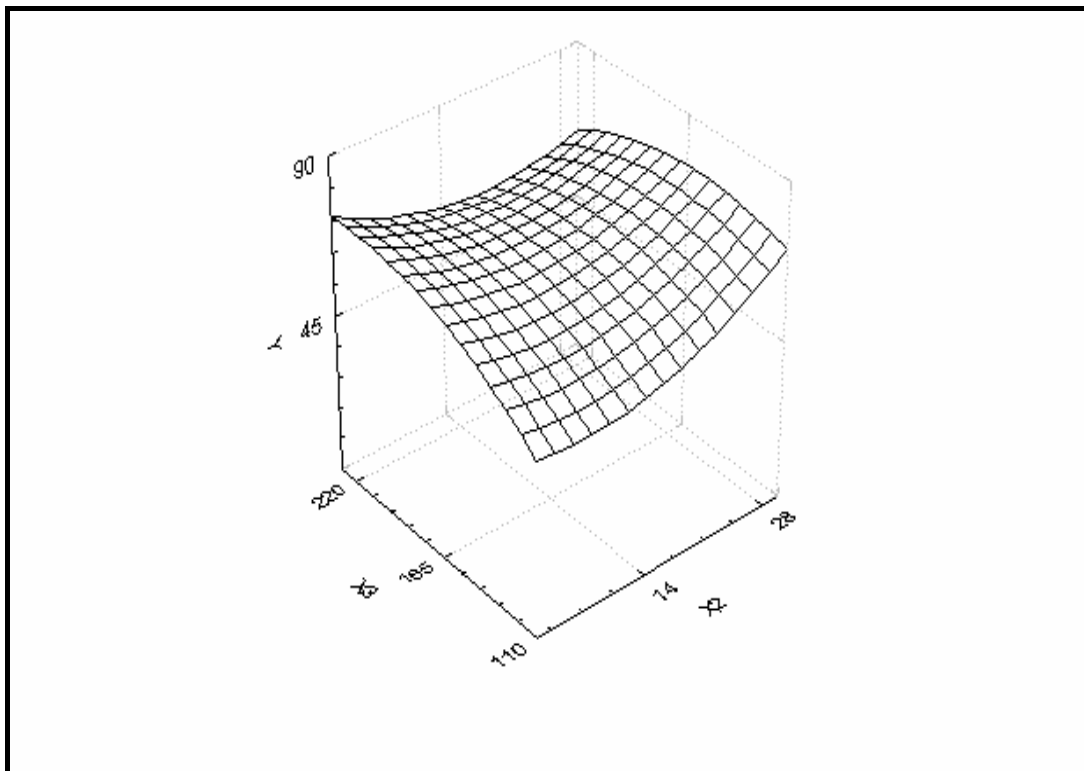
Figure 6.5a: The predicted surface response as a function of yeast extract concentration and fermentation time at constant levels of sugar concentration 70 g/l; temperature, 40 °C; and stirring speed, 150 rpm.
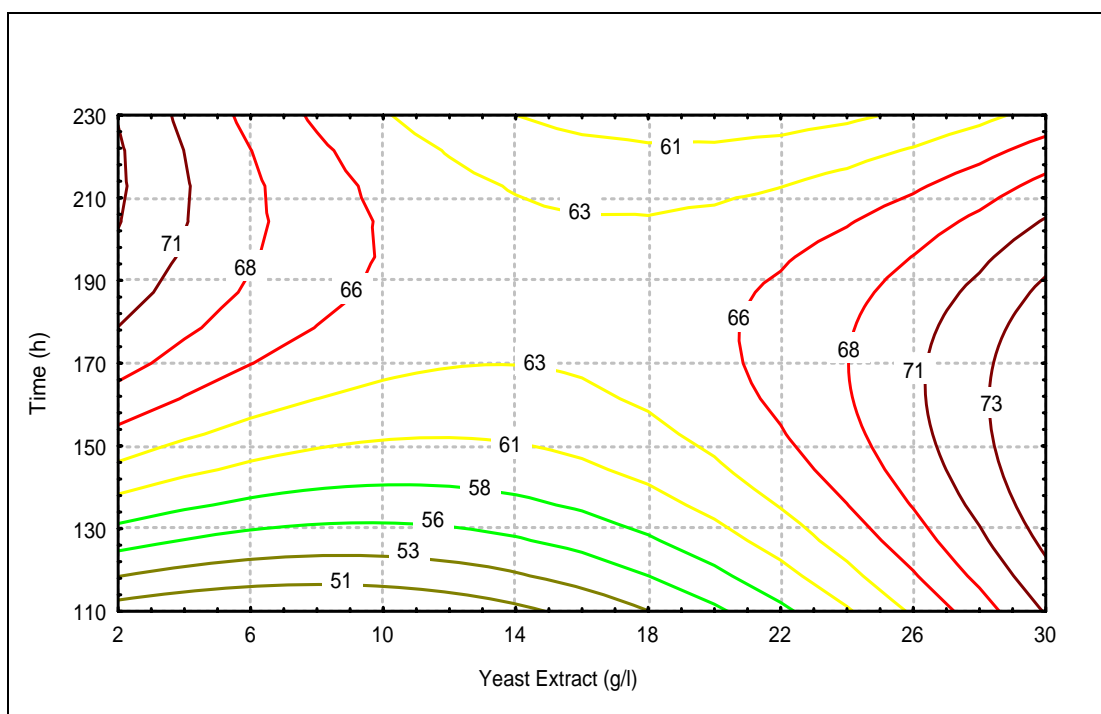
Figure 6. 5b: The contour plot of the effect of yeast extract concentration and fermentation time on lactic acid yield at constant levels of sugar concentration, 70 g/l; temperature, 40 ^{o}C; and stirring speed, 150 rpm.

6.4.2.6 Effect Of Yeast Extract Concentration And Temperature On Yield

Effects of yeast extract concentration and temperature on the yield are shown in Figure 6.6a. Effect of temperature on the yield is significant, contrary to the effect on yeast extract concentration. It was apparent that increase in yeast extract concentration (5 to 25 g/l) would not significantly increase the yield. No clear optimal value was found within experimental range under investigation (Figure 6.6b). Similar effect was also acquired for yeast extract concentration and substrate concentration, as well as yeast extract concentration and fermentation time.

The prediction of independence variable can be calculated by best explanatory equation:$Y_{24} = -682,997 - 0.03 X_2 + 36.068 X_4 + 0.043 X_2*X_2 - 0.009 X_2*X_4 - 0.002 X_4*X_4$. This clearly shows that the effect of temperature is significant but the effect of yeast extract concentration and the interaction between factors were

insignificant. Figure 6.6b shows that the maximum yield is at operational conditions of temperature, 42 $^{\circ}$C and yeast extract concentration, 15 g/l.

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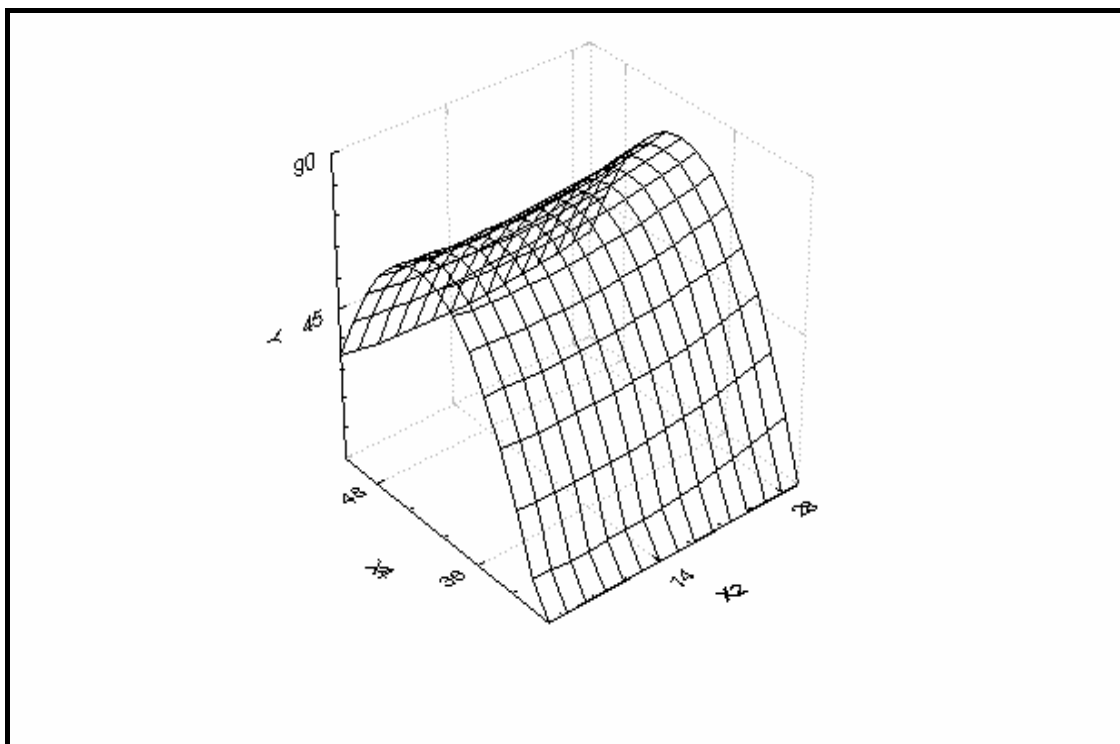
Figure 6.6a: The predicted surface response as a function of yeast extract
concentration and temperature at constant levels of sugar concentration,
70 g/l; fermentation time, 168 hours; and stirring speed, 150 rpm.
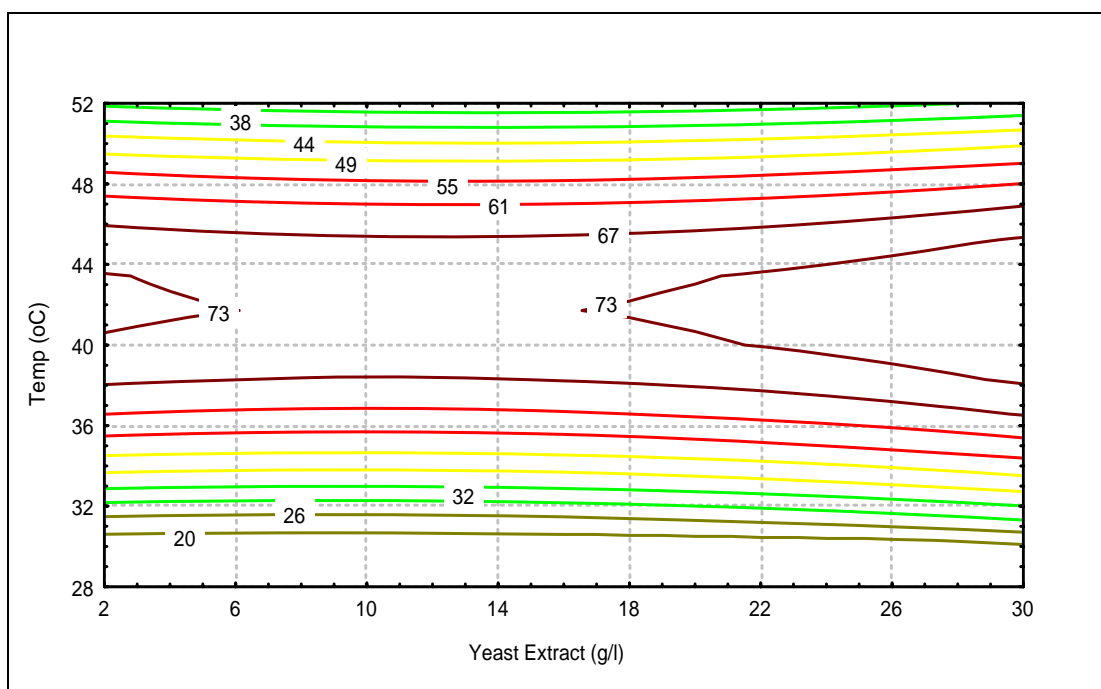
Figure 6.6b: The contour plot of the effect of yeast extract concentration and temperature on lactic acid yield at constant levels of sugar concentration, 70 g/l; fermentation time, 168 hours; and stirring speed, 150 rpm.

6.4.2.7 Effect Of Yeast Extract Concentration And Stirring Speed On Yield

Effects of yeast extract concentration and stirring speed on yield are given in Figure 6.7a. Effects of yeast extract concentration and stirring speed on yield are considerable. From the analysis of the response surface, it can be seen that there is no maximum point of response within the experimental range investigation. Therefore the optimal values were estimated by using analysing contour plot (Figure 6.7b). The optimum conditions obtained were at yeast extract concentration of 13 g/l and stirring speed of 164 rpm, respectively.

This is analogous with the effects of substrate concentration and yeast extract, fermentation time and yeast extract, along with temperature and yeast extract on yield which indicate that no maximum point or yield is obtained within experimental range under investigation.

The prediction of dependence variable was also calculated using best explanatory equation: $Y_{25} = 41.149 - 2.193 X_2 + 0.494 X_5 + 0.034 X_2*X_2 + 0.009 X_2*X_5 - 0.002 X_5*X_5$. Effects of both factors in linear term were found to be significant, contrary to square term and interaction between factors, giving optimal yield of 65.028 % at yeast extract concentration and stirring speed at 12.25 g/l and 151.09 rpm, respectively.
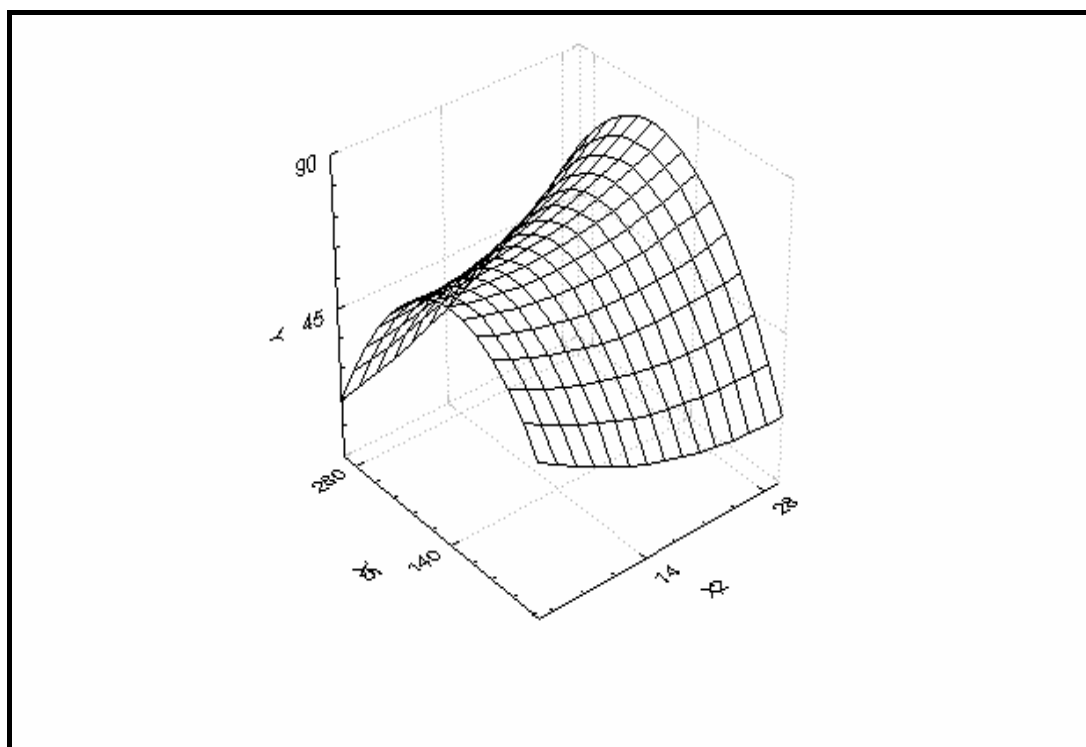
Figure 6.7a: The predicted surface response as a function of yeast extract concentration and stirring speed at constant levels of sugar concentration, 70 g/l; fermentation time, 168 hours; and temperature, 40 °C.
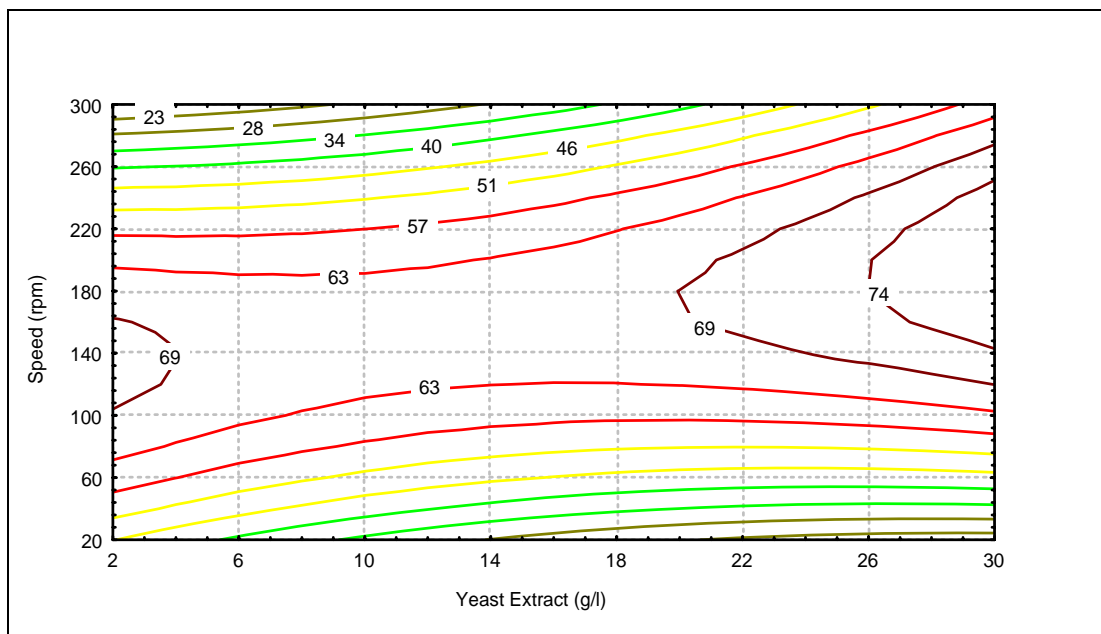
Figure 6.7b: The contour plot of the effect of yeast extract concentration and stirring speed on lactic acid yield at constant levels of sugar concentration, 70 g/l; fermentation time, 168 hours; and temperature, 40° C.

6.4.2.8 Effect Of Fermentation Time And Temperature On Yield

Figure 6.8a illustrates the effects of fermentation time and temperature on yield. It can be observed that the effects of temperature and time are noteworthy. The yield increases with increasing temperature but above 45 $^{\circ}$C the yield decreases, which is alike with fermentation time but above certain time the yield becomes constant.

The maximum response was obtained by analysing contour plot in Figure 6.8b. Optimal conditions were achieved at fermentation time and temperature of 178 hours and 42 $^{\circ}$C, respectively.

The prediction of dependence variables were also calculated by best explanatory equation: $Y_{34} = -871.282 + 1.681 X_3 + 38.204 X4 - 0.004 X_3*X_3 - 0.01 X_3*X_4 - 0.436 X_4*X_4$. Herein the effect of temperature is more significant than that

of fermentation time but effects of both variables in square term and interaction between factors were minor. Using this equation, the optimal yield was achieved at 63.564 %, with operational conditions at fermentation time of 157 hours and temperature of 42 °C, respectively. By analysing the contour plots in Figures 6.2b, 6.5b, 6.8b and 6.9b, the optimal fermentation time in this experiment is around 178 - 190 hours.
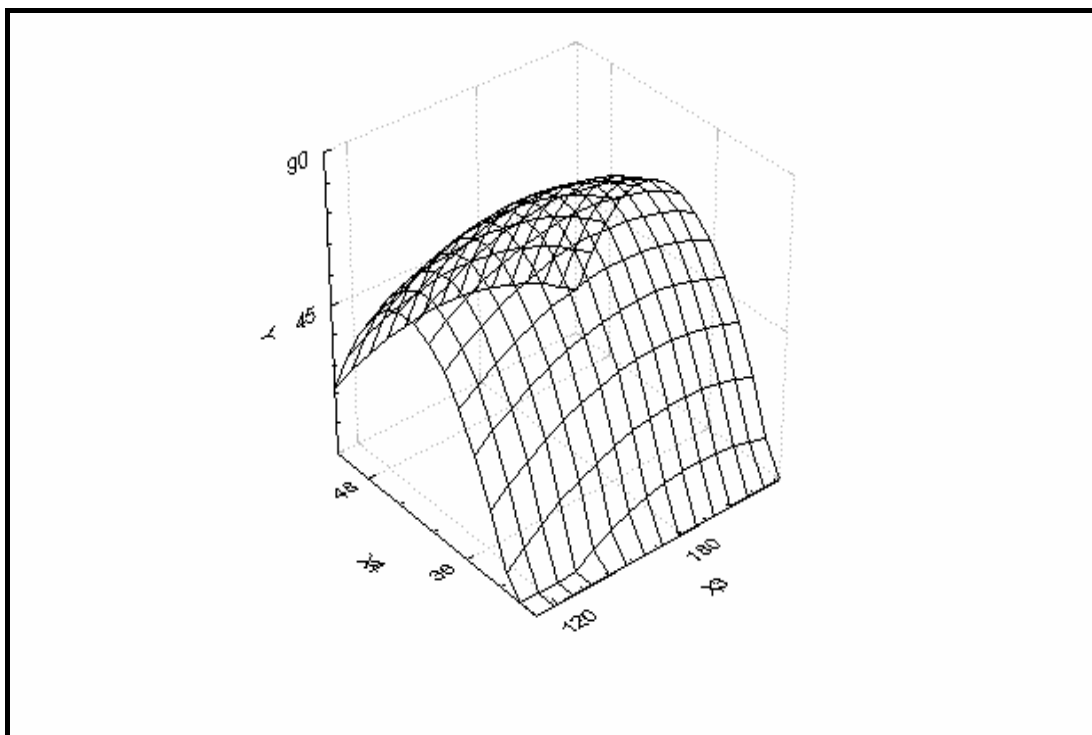
Figure 6.8a: The predicted surface response as a function fermentation time and temperature at constant levels of sugar concentration, 70 g/l; yeast extract concentration, 5 g/l; and stirring speed, 150 rpm.
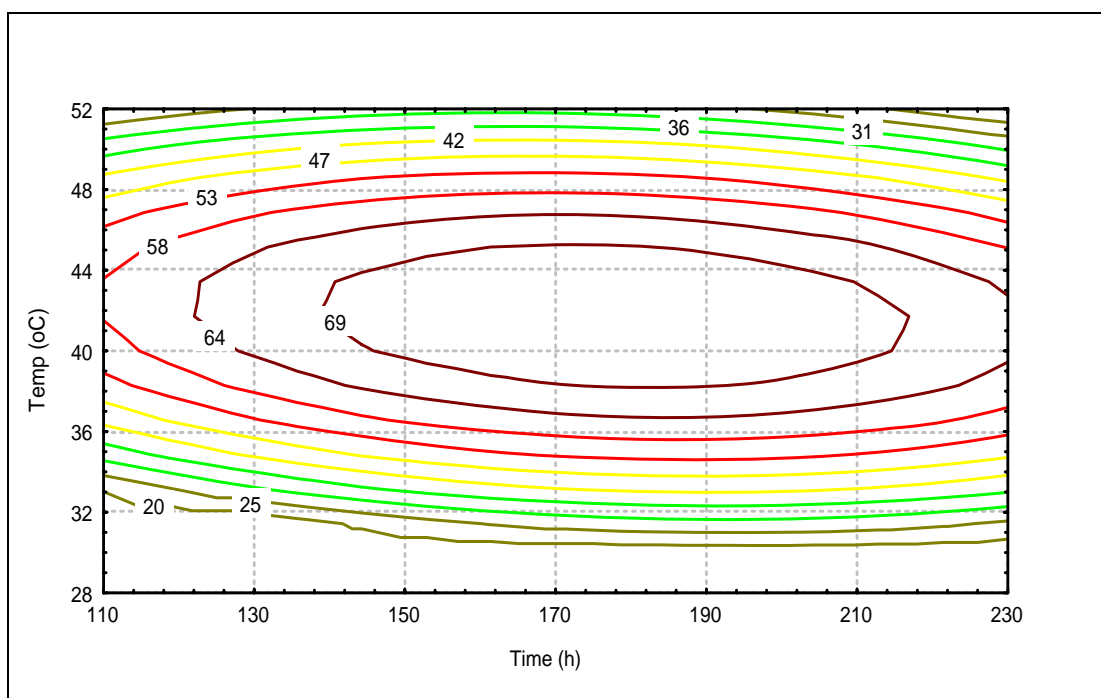
Figure 6.8b: The contour plot of the effect of fermentation time and temperature on lactic acid yield at constant levels of sugar concentration, 70 g/l; yeast extract concentration, 5 g/l; and stirring speed, 150 rpm.

6.4.2.9 Effect Of Fermentation Time And Stirring Speed On Yield

The fermentation time was studied at range 120 to 216 hours. Assumption was made that the time of fermentation is enough to produce maximum lactic acid production. The response surface was obtained (Figure 6.9a) showing that the lactic acid yield increases with increasing fermentation time, but after certain time, the yield becomes constant. The yield also increases with escalating stirring speed, but at 170 rpm, the yield declines. From the counter plot (Figure 6.9b), it can be seen that the maximum lactic acid yield was obtained at stirring speed of 164 rpm and fermentation time of 186 hours.

Effects of fermentation time and speed on the yield can be predicted by best explanatory equation: $Y_{35} = -36.425 + 0.789 X_3 + 0.370 X_5 - 0.003 X_3*X_3 + 0.002 X_3*X_5 - 0.004 X_5*X_5$. The linear main effects of factors studied are moderate but the

interaction between factors was found to be negligible, so was the quadratic main effect of factors that was indicated by low the regression coefficient.

The calculated conditions for fermentation time and the stirring speed were found to be 194 hours and 189.86 rpm, respectively, with the optimal yield at 75.55 %. By analysing the contour plots (Figures 6.2b, 6.6b, 6.9b and 6.10b), the optimal fermentation time is found to be around 164 -190 hours.
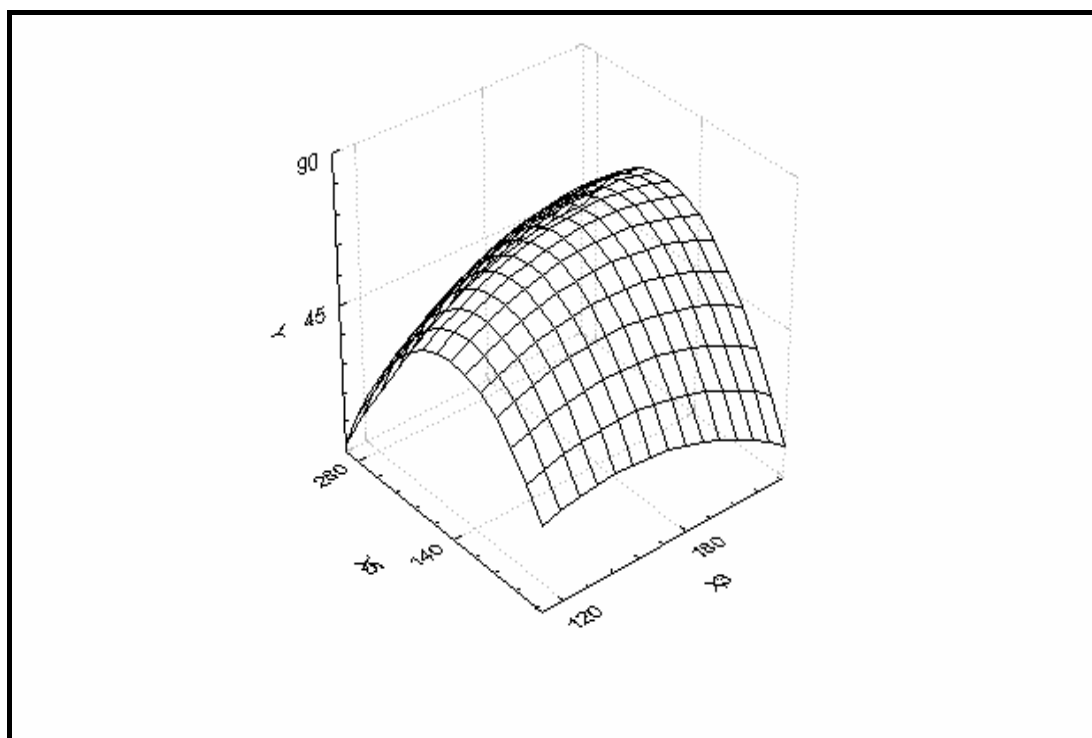
Figure 6.9a: The predicted surface response as a function of fermentation time and stirring speed at constant levels of sugar concentration,70 g/l; yeast extract concentration, 5 g/l; and temperature, 40 $^{\circ}$C.
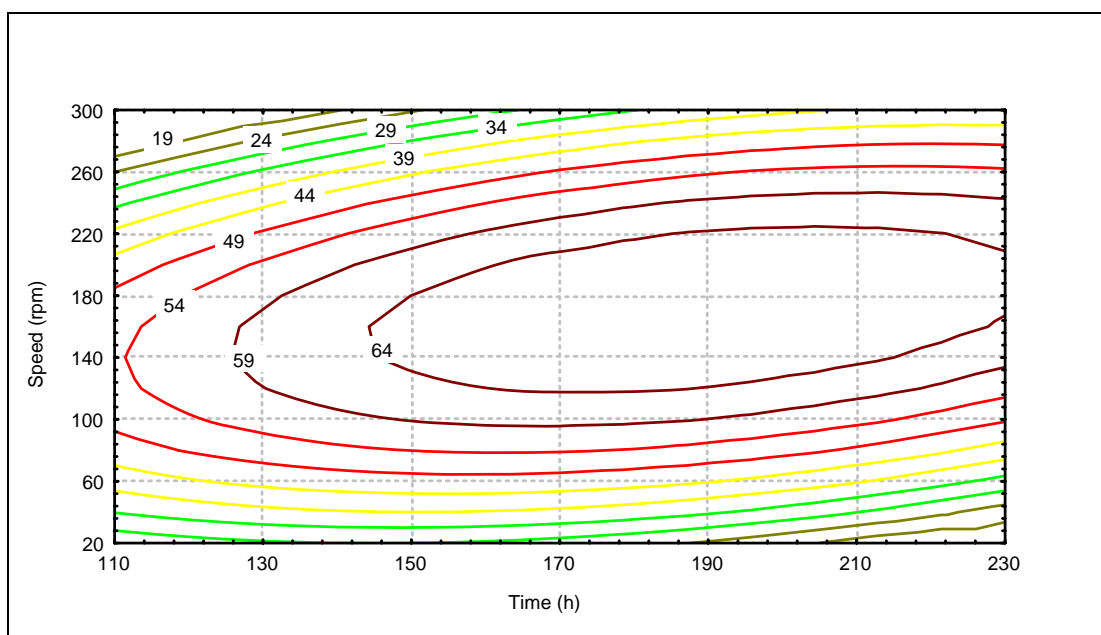
Figure 6.9b: The contour plot of the effect of fermentation time and stirring speed on lactic acid yield at constant levels of sugar concentration, 70 g/l; yeast extract concentration, 5 g/l; and temperature, 40° C.

6.4.2.10 Effect Of Temperature And Stirring Speed On Yield

Figure 6.10a demonstrates the effects of temperature and stirring speed on yield. The yield increases with increasing temperature and stirring speed, but at particular conditions, the yield decreases. The maximum response can be obtained from Figure 6.10b. The optimal value was obtained at temperature, 42 °C and stirring speed, 168 rpm.

The maximum response was also predicted by the best explanatory equation: $Y_{45} = -756.756 + 37.185 X_4 + 0.712 X_5 - 0.445 X_4*X_4 - 0.00 X_4*X_5 - 0.002 X_5*X_5$. The optimal condition was achieved at temperature of 42 °C and stirring speed of 178 rpm with the maximum yield at 83.43%. The effect of temperature was more significant than stirring speed in both square and linear term which was denoted by the values of regression coefficient, but there was no interaction between factors (regression coefficient = 0). The quadratic main effect for temperature was

significant but opposite for the stirring speed, indicated by the low value of coefficient (0.002).

By analysing the contour plots (Figures 6.3b, 6.5b, 6.8b, 6.10b and 6.4b, 6.7b, 6. 9b, 6.10b), optimal temperature is found at 42 ^{o}C, and stirring speed at around 164-180 rpm.
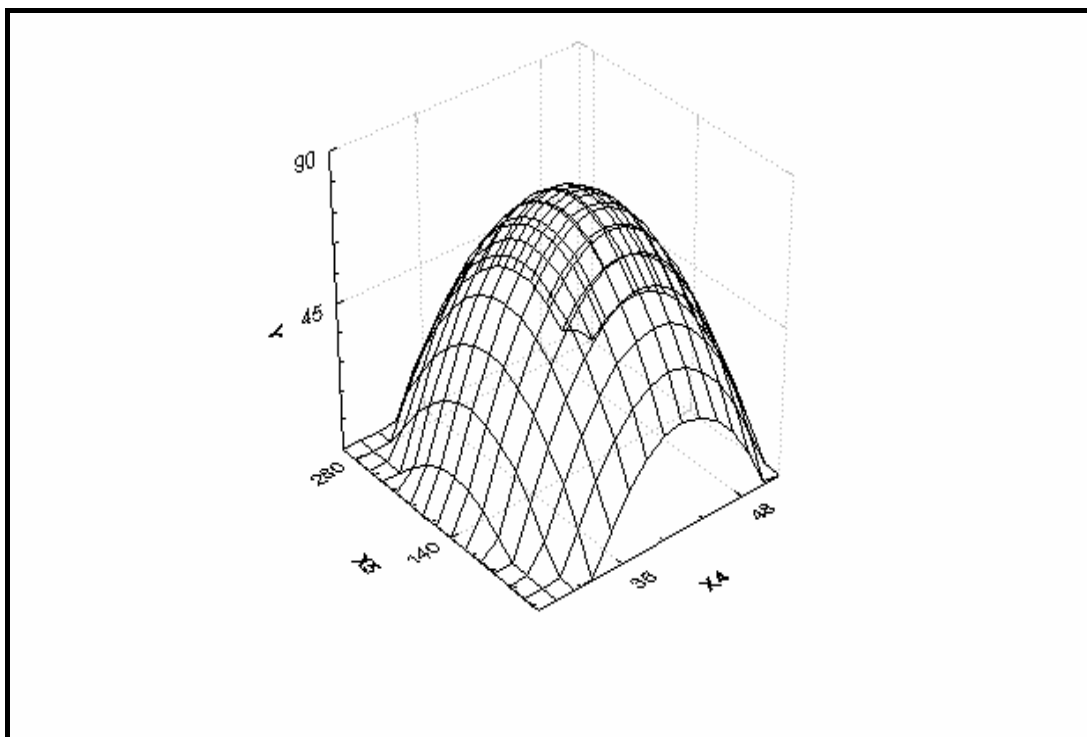
Figure 6.10a: The predicted surface response as a function of temperature and stirring speed at constant levels of sugar concentration, 70 g/l; yeast extract concentration, 5 g/l; and fermentation time, 168 hours.
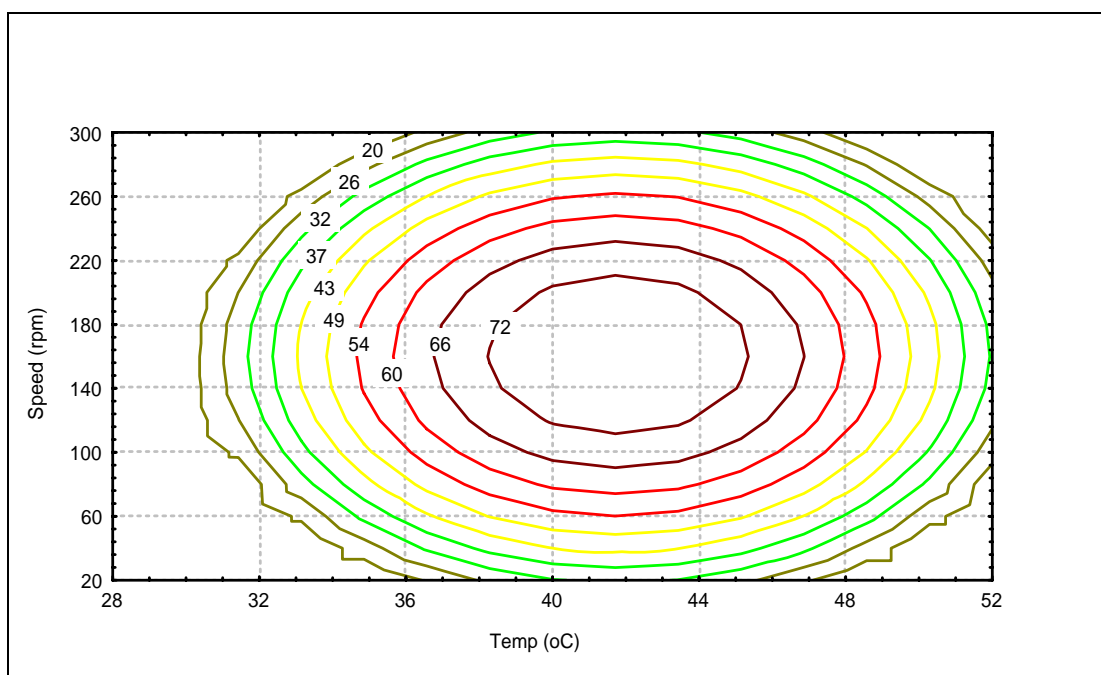
Figure 6.10b: The contour plot of the effect of temperature and stirring speed on
lactic acid yield at constant levels of sugar concentration, 70 g/l; yeast
extract concentration, 5 g/l; and fermentation time, 168 hours.

6.5   Effect Of Different Sizes And Types Of Fermentor

6.5.1   Introduction

As reported in the literatures, improvements in fermentation performance
may be achieved by a better understanding of fluid dynamics in the fermentor. To
obtain enhanced productivity and product quality, fermentation processes can be
carried out by changing the geometric of the fermentor and impeller such as tank
diameter (T), impeller diameter (D), impeller clearance and the liquid height (H)
(Amanullah et al., 1998).

In the commercial size, the configurations are often altered. For example, the
ratio of the liquid height to tank diameter (H/T) may increase from 0.5 to 2.0
whereas the ratio of the impeller diameter to the tank diameter (D/T) may decrease
from 0.5 to 0.3 for process and economic reasons (Von Essen, 1987).

In order to increase the productivity in lactic acid fermentation of pineapple waste by *L. delbrueckii*, effects of different sizes and types of fermentor were studied. The lactic acid fermentation was carried out in shake flask (100 ml) and culture vessel (1 and 5 litre) using stirrer shaft with two 6-bladed disc impellers (Figure 3.2). The fermentor and impeller geometric are shown in Table.6.10

Table 6.10: The fermentor and impeller geometric with different sizes of fermentor.

Conditions	Culture Vessel (BIOSTAT B)	
	Type B2 (3 litres)	Type B5 (6.6 litres)
Working volume (ml)	1,000	5,000
Liquid height (cm) (H)	24.60	10.60
Tank diameter (cm) (T)	19.70	16.30
Impeller diameter (cm) (D)	6.40	5.30
Impeller clearance (cm) (C)	4.40	3.60

6.5.2   Shake Flask (100 ml)

Shake flask fermentation was carried out under optimal conditions of tested variables affecting shake flask fermentation for lactic acid production in optimisation process. The optimal conditions are: sugar concentration, 65.0 g/l; yeast extract concentration, 15.0 g/l; fermentation time, 192 hours; temperature, 42.0 ^{o}C and stirring speed, 150 rpm. With the above conditions, shake flask experiment produced lactic acid yield of 83.07%. The result was almost similar with the predicted lactic acid yield which was 83.79 %. Therefore it can be concluded that the model is valid to predict the lactic acid production of pineapple waste fermentation at the range of the experimental conditions understudied.

6.5.3 Culture Vessel (1 and 5 litres)

The fermentation was carried out in 3-litre fermentor (Biostat B Model) with working volume of 1 litre. The pH, temperature and stirring speed were manipulated at 6.0, 42 ^{o}C and 75 rpm. The concentrations of sugar and yeast extract are 65 g/l and 15 g/l respectively. The fermentor containing 950 ml substrate was first sterilised at 121^{o}C for 15 minutes. 50 ml of inocolum was sterilised separately and added aseptically to the fermentor. Anaerobic conditions were created by sparging the fermentor using nitrogen at flow rate of 6.5 ml/minute. The fermentation was also carried out in 6.6-litre fermentor with working volume of 5 litres using the same conditions.

The growths of *L. delbrueckii* in the stirring batch reactor with different working volumes are shown in Figure 6.11. The growth curve represented by dry cell weight (biomass) is to follow the same pattern. Growth was started with a short lag phase at 4 hours, followed by the exponential growth at 28 hours and finally the bacteria entered a slow growth phase at 44 until 50 hours. The short stationary phase which occurred between 56 to 80 hours of incubation and then followed by decreasing dry cell weight was caused by cell lysis.

During the lag phase (4 hours), the concentrations of glucose and fructose increased while the concentration of sucrose decreased (Figure 6.12). Hydrolysis of sucrose to glucose and fructose made their concentrations grow. During the exponential growth, the concentrations of glucose and fructose accumulated in the fermentation medium until 28 and 32 hours, with the maximum concentrations of 30 and 29 g/l, respectively. Although the growth associated with lactic acid production, the concentrations of glucose and fructose continued to increase. This might be due to sucrose hydrolysis was faster than conversion of both sugars to lactic acid.
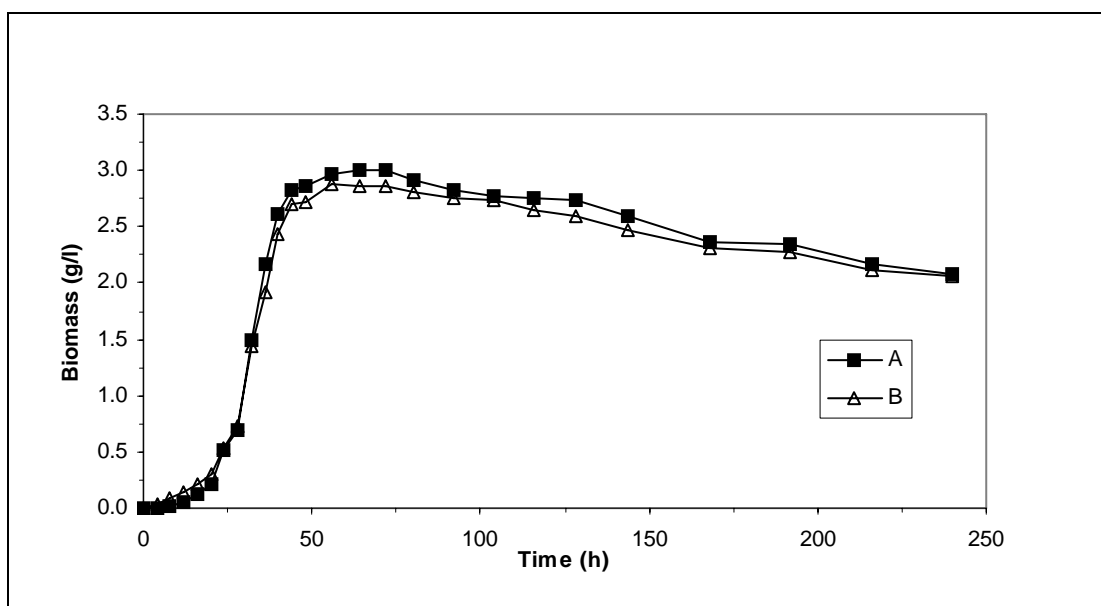
Figure 6.11: Effect of different sizes and types of fermentor on *L. delbrueckii* growth of lactic acid fermentation of pineapple waste: A) working volume, 5 litres and B) working volume, 1 litre. Experimental conditions: pH, 6.0; temperature, 42 ^{o}C; inoculum, 5%; and stirring speed, 75 rpm.

Figure 6.12 shows that the substrate utilisations on different working volumes follow an identical pattern. The sucrose was completely utilised at 24 hours, followed by glucose at 144 hours whereas fructose was not totally consumed even after 240 hours. By examining individual sugar concentrations during fermentation, it was obvious that sucrose was hydrolysed to glucose and fructose, and the rate of hydrolysis was faster than the conversion of these substrates to lactic acid. Therefore the concentrations of glucose and fructose increased after 4 hours of fermentation contrary to sucrose concentration.
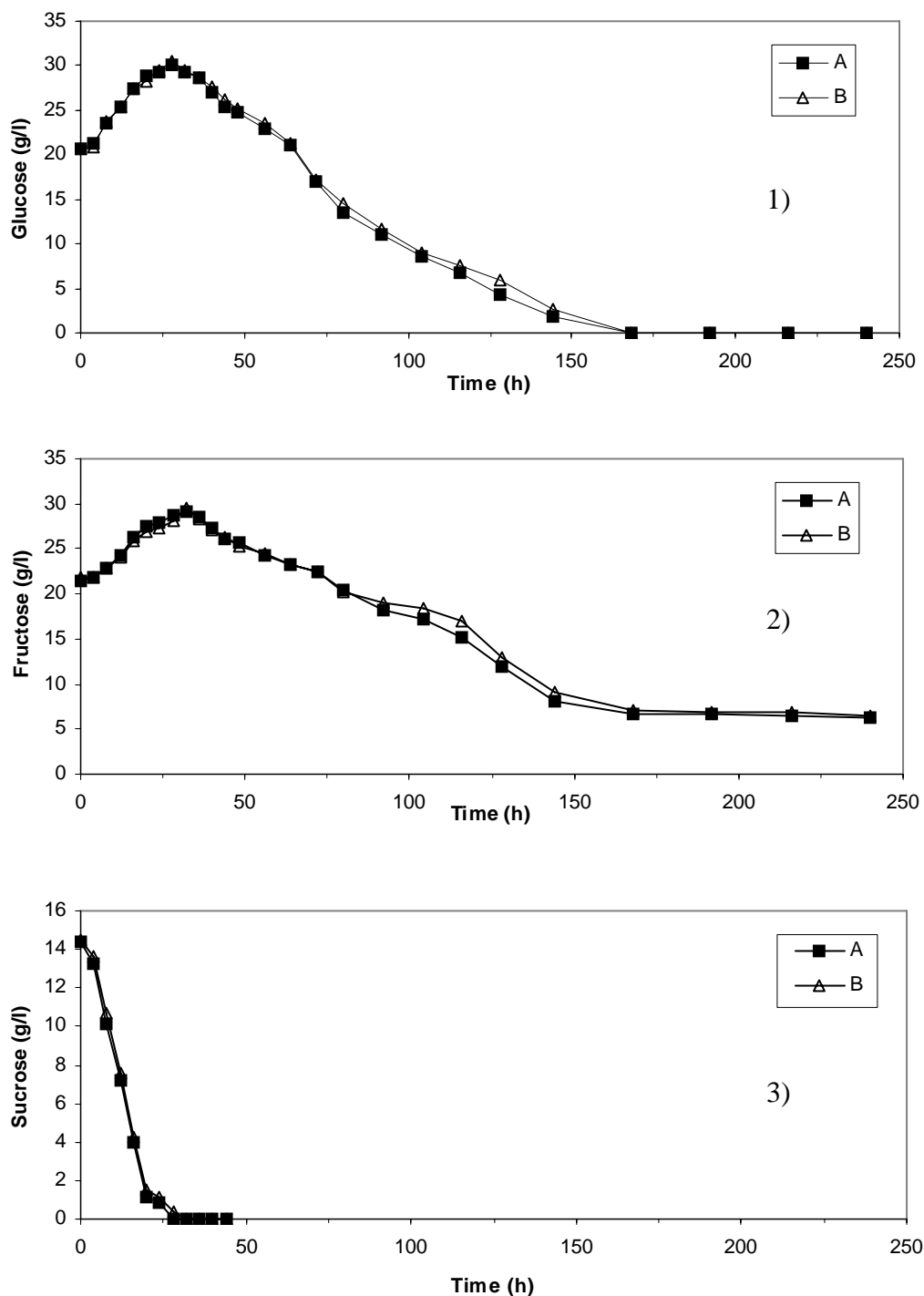
Figure 6.12: 1) Glucose, 2) fructose and 3) sucrose consumption on effect of different sizes and types of fermentor of lactic acid fermentation of pineapple waste: A) working volume, 5 litres and B) working volume, 1 litre. Experimental conditions: pH, 6.0; temperature, 40^o C; inoculum, 5% and stirring speed, 75 rpm.

The lactic acid production on different working volumes is given in Figure 6.13. According to the bacterial growth, the lactic acid productions in large (5 litres) and small volumes (1 litre) at similar fermentation conditions is almost parallel, which were 56.73 and 54.68 g/l, respectively. The minor discrepancy was possibly caused by the fermentation process that was directly influenced by fluid dynamics of the system. The fluid or shear rate affects the heat and mass transfer processes in the fermentation system. Therefore the results were slightly different on the bacterial growth and lactic acid production.

The lactic acid production in shake flask experiment (83 %) is lower than larger scale (86 %). This indicates that the mixing process carried out in incubator shaker and fermentor system is different in which mass transfer and heat transfer in the fermentation medium were affected.

The effect of different sizes and types of fermentor on the performance of lactic acid fermentation using pineapple waste by *L. delbrueckii* is summarised in Table 6.11.
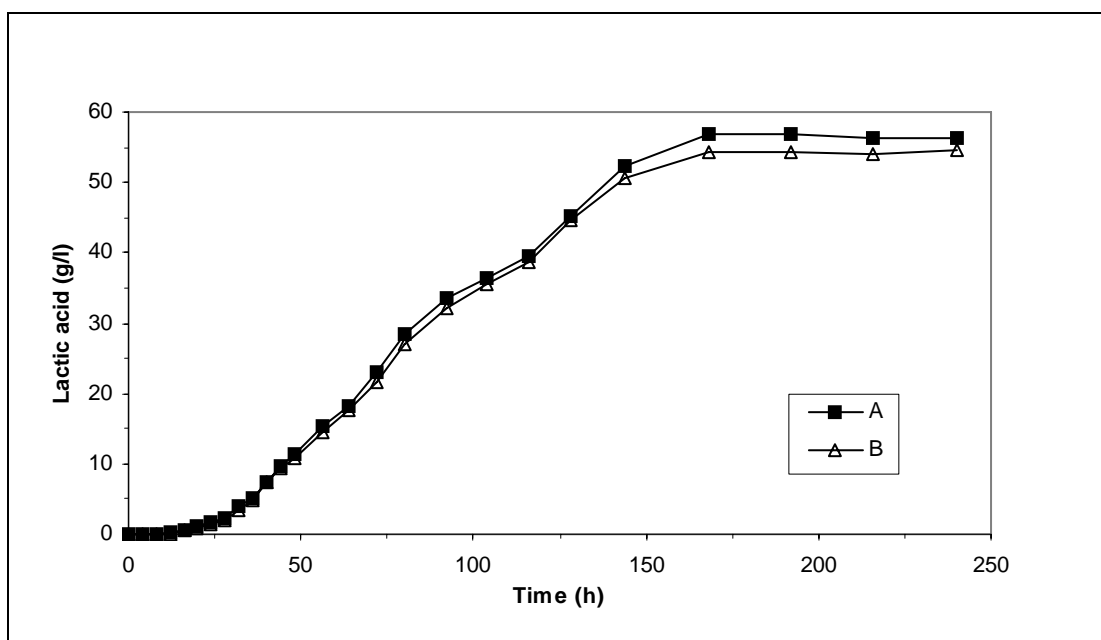
Figure 6.13: The effect of different sizes and types of fermentor on lactic acid

production of lactic acid fermentation of pineapple:

A) working volume, 5 litres and B) working volume, 1 litre.

Experimental conditions: pH, 6.0; temperature, 42 $^{\circ}$C; inoculum, 5%

; and stirring speed, 75 rpm.

Table 6.11: The summary of fermentation results for effect of different sizes and

types of fermentor.

Size/Type of Fermentor	D/T	C/T	H/T	X_m	P_m	Yield (%)	Productivity (g/l.h)
Shake Flask (100ml)	-	-		-	53.9	83.0	0.321
Culture Vessel (1,000ml)	0.32	0.22	0.65	2.87	54.4	83.7	0.324
Culture Vessel (5,000ml)	0.32	0.22	1.24	3.02	56.8	87.0	0.364

CHAPTER VII

A TECHNO-ECONOMIC EVALUATION

7.1   Introduction

Lactic acid was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881. The first successful use of lactic acid in the leather and textile industries began in 1894 and the production levels were about 5.0 tonnes per year. In 1942, about half of 2,700 tonnes of lactic acid produced per year in the US was used by leather industry, and an emerging use is in food production. During the World War II, US production peaked at 4,100 tonnes per year (Vickroy, 1983). In 1982 world wide production of lactic acid was 26 tonnes per year. More than 50 % of lactic acid produced are used in food as an acidulant and a preservative. Fermentation is presently used to make about half of the world's total production of lactic acid (Vickroy, 1983; Blanch and Clark, 1997).

The annual world production of lactic acid has a total volume of nearly 30,000 ton and about half of this production use synthetic chemical as raw material, and it is rapidly becoming regarded as a commodity chemical. With increasing price of petroleum, production of lactic acid by fermentation becomes popular because the process is cheap. This has created an increasing interest of study to improve fermentation and recovery processes (Van Ness, 1991; Blanch and Clark, 1997). In economic terms primary parameters to consider for production are cost and production volume. The world production of lactic acid is relatively small compare to other biotechnological products. However in terms of price per tones, it is relatively high-priced (Hacking *et al*., 1987).

Lactic acid is a colourless, sour taste, odourless, highly hygroscopes and syrup liquid which is commercially available at different grades (qualities) (Paturau, 1982). There are four grades of lactic acid where their applications and prices are given in Table 7.1.

Table 7.1: Prices and applications of various grades of lactic acid (Paturau, 1982).

Lactic Acid Grades	Concentration (%)	Price / Kg ($)	Application
Technical	50	0.99	Textiles, solvents and plasticizer
	80	1.69	Polymer, varnish impregnating agent
Food	50	1.01	Beverages, essence and syrups
	88	1.75	Pharmaceutical industry

The production of lactic acid has attracted a great deal of interest due to its potential use as a raw material in the production of biodegradable polymer such as poly lactic acid. The world production of lactic acid is approximately 50.000 tonnes per year and the commercial price of lactic acid ranges from US$1.40 /kg for 50% to US$ 1.90/kg for 88% of food grade lactic acid (Akerberg and Zacchi, 2000)

7.2   Lactic Acid Process

The choice of the medium in lactic acid fermentation has great effect on the process as a whole. It is known that the recovery process of lactic acid from the fermentation broth is more difficult than the fermentation itself. The more complex the medium is, the more difficult a pure product is produced. Thus the cheapest raw material such as beet molasses can cause the most problem in recovery while the most expensive (such as sucrose) give the least problem (Milson, 1987).

7.2.1 Lactic Acid Industry

Industrial lactic acid is prepared by the fermentation of glucose, maltose, lactose and sucrose. In the US, corn sugar, glucose, enzyme hydrolysed starch, molasses and whey are the main raw materials commonly used as substrates. In Europe, potato starch, beet molasses and sucrose have been employed. Other suggested substrates are sorghum and cellulose (Paturau, 1982).

Nowadays lactic acid is mainly produced technically by fermentation. Homolactic acid bacteria such *as L. delbrueckii*, *L. bulgaricus* and *L. leichmanii* are used. The requirement of vitamins and growth factors can be supplied by yeast extract. Large amount of bacteria have to be added to the fermentation solution in order to carry out the fermentation safely and rapidly on a technical scale. These are made by propagation of pure culture and procedure as given in Figure 7.1. (Holten, 1971; Buchta, 1983).

Batch fermentation of starch or glucose was carried out in close metal tanks with a capacity of 20.000-100.000 litres. 1000 litres of inoculum was added to the fermentation broth (inoculum size of 5%). The fermentation completed after 2-8 days, depending on the substrate used. During this time the pH and the sugar concentration were controlled continuously. Calcium carbonate was added to maintain the pH of 6.0 (Holten, 1971; Atkinson and Mavituna, 1991).

Recovery of lactic acid from the fermentation broth requires consideration of the corrosive nature of lactic acid. Usually 316 stainless steel vessels are employed (Blanch and Clark, 1997). The fermentation liquid is heated to 80-100 $^{\circ}$C to kill the bacteria and to dissolve all the calcium lactate. The fermentation broth is then filtered and the crude lactic acid is treated by sulphuric acid. The calcium lactate precipitated is removed by filtration and the lactic acid solution is bleached with activated carbon and evaporated to a particular concentration depending on the final use (Blanch and Clark, 1997; Kascak, 1996).

In continuous fermentation process, considerably higher productivity can be achieved. Therefore continuous fermentation processes have been performed in various forms by many researchers. The earliest report of continuous lactic acid fermentation was presented by Childs and Welsby (1966) using cell suspension where they achieved lactic acid productivity of 3.7g litre^{-1} h^{-1}. Vickroy (1983) also reported that with cell recycle the productivity was much higher, which was 76 g litre^{-1} h^{-1}. Major and Bull (1985) also studied a process without cell recycle and the maximum productivity obtained was 8.93 g litre^{-1} h^{-1}. A number of investigations have been made into the possibility for lactic acid production using immobilised cell. As far as it is known, no immobilised system or continuous process have yet been operated commercially (Kascak *et al*. 1996).

Earlier economical studies of the fermentative lactic acid production have resulted in a range of production cost of the final product (Paturau, 1982). Akerberg and Zacchi (2000) also studied the production cost of 70% lactic acid from whole white flour. They only focused on the optimisation of the process to reduce the total lactic acid production cost while the details of economic evaluation have not been investigated.

7.2.2   Lactic Acid Fermentation From Pineapple Waste

Liquid pineapple waste contains mainly fructose, glucose and sucrose, which is made up of 20, 20 and 15 g/l each of the sugars in general. Batch fermentations were studied in 3-litre stirred fermentor (Biostat B Model) with working volume of1 litre. Effects of some parameters such as temperature, pH, inoculum size, sugar concentration and nitrogen source were studied. The highest yield was achieved at temperature: 40^{o}C, pH: 6.0, inoculum size: 5 %, sugar concentration: 70 g/l, and yeast extract as nitrogen source with the maximum lactic acid production and yield obtained at 54.97 g/l and 79 %, respectively (Moch-Busairi and Mat, 2000).

Optimisation studies were also carried out for selected parameters in the Erlenmeyer flask containing 100 ml of production medium. The maximum yield of lactic acid was obtained by the response surface methodology. The optimal values were found to be: sugar concentration, 65.87 g/l; yeast extract concentration, 15.35 g/l; fermentation time, 182.4 hours; temperature, 41.70^{o}C and stirring speed, 153.58 rpm with the lactic acid yield of 83.79 %. Scale up fermentation was also studied on working volume of 5 litres with the lactic acid yield of 86.15% (Moch-Busairi and Mat, 2001).

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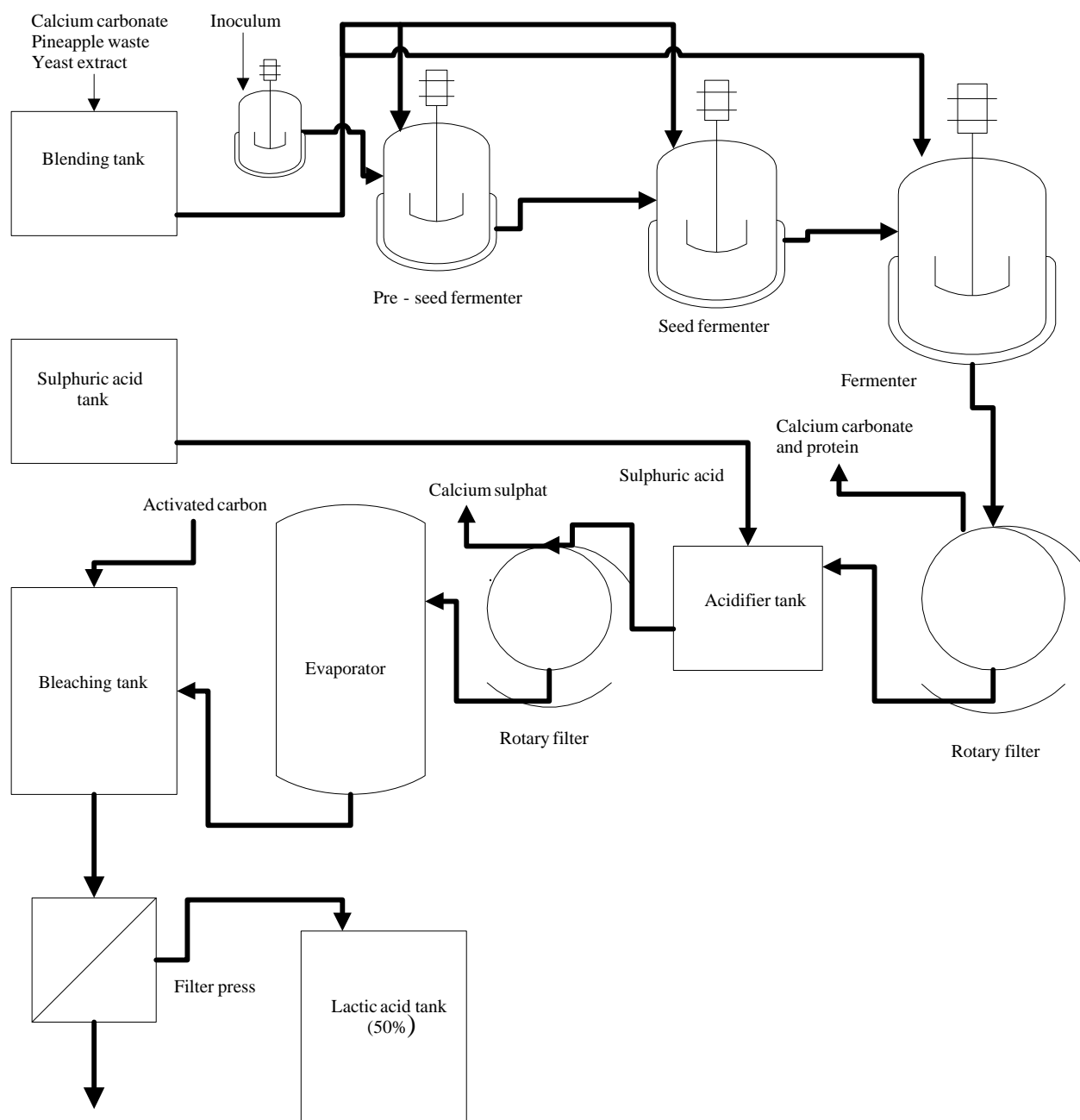
Figure 7.1: Schematic flow sheet of lactic acid production

7.3 Method Evaluation

7.3.1 Model Of Lactic Acid Plant

The economic analysis of lactic acid production using pineapple waste was evaluated. The lactic acid production capacity at 20.000 tonnes/year and operating time at 8000 hrs/year were assumed. The batch process was used to produce 50% of lactic acid (technical grade) and the biological information was given as follows:

Batch fermentation time   : 160 hours (including turnaround)

Initial sugar concentration   : 70 g/l

Lactic acid yield   **: 0.8 g lactic acid/ g sugar**

Temperature   : 42^{o}C

pH   : 6.0

Inoculum ratio   : 5 %

Based on desired annual production capacity and stream plant operating time with the basis of production of 1000 ton/yr, material and energy requirements for the process were calculated in order to obtain the equipment size.

Broth volume per year = $(0.5) \times (1.000 \text{ tonnes} \times 10^{6} \text{ g/tonnes}):(0.8 \text{ g/g} \times 70 \text{ g/l})$

                     = 8,928,571 litres

Total number of batch = (8000 hrs/yr) : 160 hrs/batch

                     = 50 batch/yr

Total volume of batch = (8,928,571) : 50 = 178,571 litres

Number of fermentors = 4 (arbitrary)

Liquid volume each   = 44,642 litres

The process steps (Figure 7.1) and assumptions made to obtain the equipment size are presented in the following sections.

7.3.1.1 Raw Material

The liquid pineapple waste consisting 70 g/l of total sugar was mixed in blending tank with calcium carbonate (3% w/v) to control the pH at 6.0. Yeast extract (0.05 %) was also added as a nitrogen source (Moch-Busairi and Mat, 2001).

7.3.1.2 Fermentor

In developing the process flow sheet, holding tanks were required to be considered. For the size of fermentation equipment such as fermentor and tanks, a working volume of 80% of the total tank capacity was assumed (Akerberg and Zacchi, 2000).

From the material balance, the volume of each fermentor is found to be 44,642 litres and thus the actual volume = 44,642 litres : 0.8 (working/actual volume) = 55,803 litres.

With 5 % of inoculum ratio, the estimation of the inoculum media for three fermentors which are:

a) The seed fermentor volume = 55,803 litres × 0.05 volume ratio = 2,790 litres, and the actual volume = 3,487 litres

b) The pre seed fermentor volume = 2,790 litres × 0.05 volume ratio = 139 litres, and the actual volume = 173 litres

c) The inoculum development fermentor = 139 litres × 0.05 volume ratio = 7 litres, and the actual volume = 9 litres

7.3.1.3 Rotary Filter I

The fermentation broth is then filtered to separate the calcium carbonate and protein sediment using Rotary Drum Vacuum Filter I. The filtrate was assumed consisting of 1% of protein and 3% of calcium carbonate. The area of drum vacuum was assumed to be 350 litres/m^2 hr (Blanch and Clark, 1997). If the filtration was completed in 4 hours, the filter area required = (44,462 litres : 4 hr) : 350 litres/m^2 hr = 31.75m^2 = 310 ft^2.

7.3.1.4 Acidifier Tank

The crude product was then reacted using 78% of sulphuric acid resulted in lactic acid and calcium sulphates (Kascak, 1996). Recovery of lactic acid from the fermentation broth required consideration of the corrosive nature of lactic acid and usually 316 stainless steel vessels were employed. Based on stoichiometry, each batch needed 1,750 kg of sulphuric acid (78%). The working volume of acidifier tank = 44,642 + 1,750 = 46,212 litres. The actual volume of acidifier tank = 46,212 litres : 0.8 = 57,765 litres.= 15,281gal.

7.3.1.5 Rotary Filter II

The resultant calcium sulphate precipitate is filtered by Rotary Drum Vacuum Filter II. The suspension contains 4% of calcium sulphate (stoichiometric). The area of drum vacuum was assumed to be 350 litres/m^2 hr (Blanch and Clark, 1997). If the filtration was completed in 4 hours, the filter area required = (44,462 litres : 4 hr) : 350 litres/m^2 hr = 31.75m^2 = 310 ft^2.

7.3.1.6 Evaporator

The bleached lactic acid is then concentrated by vacuum evaporator to give a food grade material containing about 50% of lactic acid. Multi-effect evaporators were used to minimise the energy utilisation. The heat transfer coefficient assumed for the three evaporators were 4,800, 3,000 and 900 kcal / m^2 ^{o}C.h, respectively (Brocklebank, 1990; Akerberg and Zacchi, 2000). The evaporation was assumed to perform co-currently in three steps below atmospheric pressure due to the polymerisation property of lactic acid. The evaporator temperatures for three steps were assumed to be 90, 75 and 55 ^{o}C, respectively and the increase of water boiling point due to the presence of lactic acid could be calculated using Equation (7.1) (Akerberg and Zacchi, 2000).

$$\Delta T = 5.32w \qquad\qquad\qquad\qquad …..(7.1)$$

where ΔT is the increase of boiling point (K) and w is the mass fraction of lactic acid (g lactic acid/g solution).

7.3.1.7 Bleaching Tank

After the calcium sulphate was filtered, the free lactic acid is bleached by activated carbon. The addition of carbon active to the solution was assumed of 0.05% w/v (Paturau, 1982) and therefore the requirement of activated carbon = 0.001 × 44,462 = 22.23 kg. The working volume of bleaching tank = 44,462 + 0.4 (44,462) + 22.23 = 42,705 litres = 11,291 gal. The actual volume of bleaching tank = 11,291 : 0.8 = 14,114 gal.

7.3.1.8 Filter Press

After bleaching, the solution is then filtered to remove the activated carbon using Plate and Frame Filter Press. Two filters were used with the volume of filtrate

per batch 750 ft^3. The operating time and pressure drop were assumed at 4 hours and 40 psi, respectively (Peters and Timmerhaus, 1981). The total area of filtering surface required was approximately 180 ft^2.

7.3.1.9 Sulphuric Acid Tank

The sulphuric acid was presumed to be supplied for 2 months and hence, therefore the requirement of sulphuric acid tank = 1.75 tonnes/batch × 4 × 50 batch : 6 = 58.333 litres = 15.430 gal. The actual volume of sulphuric acid tank = 15.430 : 0.8 = 20.000 gal.

7.3.1.10   Lactic Acid Tank

The product was assumed to be stored in the plant for two months. If the lactic acid production/year was 1.000 tonnes, the requirement of lactic acid tank = 166 tonnes = 43,915 gal. The actual volume of lactic acid tank = 43, 915 : 0.8 = 54,890 gal.

7.3.2   Process Economic Evaluation

7.3.2.1 Components Of The Cost Estimation

a)   The Capital Investment

The categories for capital investment include the costs for the installed purchased equipment, land, building construction and engineering design; start-up costs, reflecting modification that may be required when the plant is commissioned;

and the working capital used to provide the inventory of raw materials, supplies and cash to pay salaries and vendors. The sum of the fixed and working capital is the total capital investment. In this problem, the capital investment will be determined by method describe by Petter and Timmerhaus (1981) and the elements cost are summarised in Table 7.3.

b) Total Production Cost

The total production cost is divided into manufacturing cost and general expenses. The manufacturing cost includes elements that contribute directly to the cost of production such as operating cost, fixed cost and plant overhead cost. The direct operating cost includes the raw materials, utilities and supplies; these costs will generally scale with the plant size. The fixed cost is related to the physical plant and does not change with productivity level. The plant overhead cost includes charges for services that are not directly attributed to the cost of production. Meanwhile general expenses include those charges for marketing, research and development. Total production cost can be broken into components as listed in Table 3.5 that are described by Blance and Clark (1997), Peters and Timmerhaus (1981) and Swartz (1986).

7.3.2.2 Cost Estimation For Future Year

The value of money will change because of inflation and deflation. Hence cost data is accurate only at the time when they are obtained and soon it will go out of date. Data from cost records of equipment and projects purchased in the past may be converted to present day values by means of cost index (Perry, 1984). If the equipment cost and cost index at present time were Cp and Ip, the original cost and the index cost at time of original cost obtained would be Co and Io, respectively and thus the equivalent cost at the present time (Cp) can be determined by

$$C_P = C_O \left(\frac{Ip}{Io} \right) \qquad \qquad \text{….(7.2)}$$

The cost indices are obtained from The Chemical Engineering Plant Cost Index and Marshall and Swift equipment cost index are given in each issue of the publication of Chemical Engineering. For examples, the plant cost indices for 1980 and 1997 were found to be 660 and 1480, respectively.

7.3.2.3 Cost Estimation By Scaling

If the cost date of a piece of equipment are unavailable for the particular size of operational capacity involved, it can be estimated by using capacity ratio exponent based on existing data of a company or drawn from published correlations (Perry, 1984). If the cost of equipment with capacity q_1 is C_1, then the cost of similar equipment with capacity q_2 can be calculated from

$$C_2 = C_1 \left(\frac{q_2}{q_1} \right)^n \qquad \qquad \text{…..(7.3)}$$

where the value of the exponent n is depended on the type of the equipment

7.3.3 Profitability Analysis

There are number of methods which are used to determine whether the proposed process will be profitable. One common approach is to calculate the rate of return on investment (ROR) determined before or after taxes. The rate of return is ordinarily on an annual percentage basis and obtained by dividing the annual profit with the total investment.

$$ROR(\%) = \frac{\text{Annual profit (before and after taxes)}}{\text{Total capital investment}} \qquad \text{....(7.4)}$$

The second method is payout time or payout period which is the minimum length of time theoretically necessary to recover the original capital investment in the form of cash flow of the project based on the total income minus all cost except depreciation (Peters and Timmerhouse, 1981).

$$POT\ (\text{year}) = \frac{\text{depreciable fixed capital investment}}{\text{profit /year} + \text{depreciation}} \qquad \text{....(7.5)}$$

Other methods include the cash flow analysis which relates the net cash in (profits plus depreciation) to the cash out (initial investment), net present worth, internal rate of return, discounted cash flow and capitalised cost (Blanch and Clark, 1997; Peters and Timmerhaus, 1981).

7.4   Results

7.4.1   Cost Estimation

Based on the flow sheet in Figure 7.1, the complete equipment list for lactic acid production plant is summarised in Table 7.2. The cost of equipment was obtained from Plant Design and Economics for Chemical Engineers (Peters and Timmerhaus, 1981). The cost of the equipment at 1999 was estimated using Equation (7.2) by using The Chemical Engineering Plant Cost Index

Table 7.2: The summary of equipment list for lactic acid production.

Equipment (specification)	Size	Unit	Quantity	Cost ($1,000) (1979)	Cost ($1,000) (1999)
Blending tank (agitated, stainless steel)	14,743.00	gal	1	30.00	67.20
Development inoculum (jacketed, agitated, stainless steel)	2.50	gal	2	0.95	2.13
Pre-seed fermenters (jacketed, agitated, stainless steel)	46.00	gal	2	4.00	8.96
Seed fermenters (jacketed, agitated, stainless steel)	921.00	gal	2	18.00	40.32
Production fermenters (jacketed, agitated, stainless steel)	14,743.00	gal	4	30.00	67.20
Rotary drum filters (continuous, stainless steel)	325.00	ft^2	2	56.00	125.44
Acidifier tanks (agitated, stainless steel)	15,281.00	gal	2	30.00	67.20
Evaporator (triple effect, vacuum)	225.00	ft^2	1	110.00	246.40
Bleaching tanks (agitated, stainless steel)	14,446.00	gal	2	30.00	67.20
Filter press (plate and frame, stainless steel)	180.00	ft^2	2	22.00	49.28
Sulphuric acid storage tank (stainless steel)	20,000.00	gal	1	23.00	51.52
Lactic acid storage tank (stainless steel)	27,000.00	gal	2	27.00	60.48

For scale-up equipment cost, Equation (7.3) was used. Based on the descriptions above, the list of equipment cost in the plant is given in Table 7.3

Table 7.3: The list of equipment cost for lactic acid production plant.

Equipment	Cost of small size	Exponent (n)	Cost of large size	Quantity	Total cost ($1,000)
Blending tank	67.20	0.50	300.53	1	300.53
Development inoculum	2.13	0.57	11.74	2	23.47
Pre-seed fermenters	8.96	0.57	49.42	2	98.84
Seed fermenters	40.32	0.57	222.39	2	444.77
Production fermenters	67.20	0.57	370.64	4	1,482.57
Rotary drum filters	125.44	0.48	528.36	2	1,056.72
Acidifier tanks	67.20	0.50	300.53	2	601.06
Evaporator	246.40	0.48	1,037.85	1	1,037.85
Bleaching tanks	67.20	0.50	300.53	2	601.06
Filter press	49.28	0.57	271.80	2	543.61
Sulphuric acid tank	51.52	0.30	126.56	1	126.56
Lactic acid tank	60.48	0.30	148.57	2	297.13
Purchase equipment					**6,614.61**

For preliminary study estimates, the estimation of fixed capital investment by percentage of delivered equipment cost was employed (Peters and Timmerhaus, 1981). The fixed capital investment was broken into the components listed in Table 7.4

Table 7.4: The fixed capital investment estimation by percentage of delivered
equipment cost (Peters and Timmerhaus, 1981).

Components	% PE	Cost ($)
Purchased equipment (delivered), (PE)	100	6,614,600.00
Purchased equipment (installation)	39	2,579,694.00
Instrumentation (installed)	28	1,852,088.00
Piping (installed)	31	2,050,526.00
Electrical (installed)	10	661,460.00
Buildings (including service)	22	1,455,212.00
Yard improvement	10	661,460.00
Service facilities	55	3,638,030.00
Land	6	396,876.00
Total direct plant cost (D)		**19,909,964.00**
Engineering and supervision	32	2,116,672.00
Construction expenses	34	2,248,964.00
Total direct and indirect cost (D+I)		**24,275,582.00**
Contractor fee, 5% (D+I)		1,213,779.10
Contingency, 10% (D+I)		2,427,558.20
Fixed capital investment		**27,916,919.30**
Working capital (10% FCI)		2,791,691.93
Total capital Investment		**30,708,611.23**

The raw material is the element of the total production cost. The cost of the
raw materials was found in Malaysia Imports (Statistics Department of Malaysia,

1995). The raw material requirements and their cost for production process are shown in Table 7.5.

Table 7.5: The raw material requirements and their cost.

Raw Material	Quantity	Units	Cost/unit ($)	Annual Cost ($)
Liquid pineapple waste	178,500.00	tonnes	0.00	0.00
Calcium carbonate	5,500.00	tonnes	110.00	605,000.00
Sulphuric acid (78%)	6,900.00	tonnes	124.00	855,600.00
Yeast extract	900.00	tonnes	2,688.00	2,419,200.00
Activated carbon	90.00	tonnes	676.00	60,840.00
Total				5,007,040.00

The estimation of direct production cost, fixed cost, plant overhead cost and general expenses were adapted from Peter and Timmerhause (1981), Blanch and Clark (1997), and Swartz (1986). The total production cost was summarised in Table 7.6

7.4.2 Economic Analysis

7.4.2.1 The Break Even Point (BEP)

BEP is the point of production rate (capacity) where the total production cost equals the total income. The determination of BEP recommends the production rate and operating schedules that will give the best economic results (Peter and Timmerhause, 1981). If the production rate is n tonnes/year, the direct production cost is $366.79/tonnes and the sum of fixed cost, overhead cost and general expenses

is $7,062,981$/year while the product sells at $1,400/tonnes, the break even point in ton/year will be:

$$366.79\,n + 7,062,981 = 1,400\,n$$

$$1033.20\,n = 7,062,981 \rightarrow n = 6,836 \text{ ton/year}$$

or

Table 7.6: Total production cost.

Components	Annual cost ($)	Annual cost ($)
I. Direct Operating Cost (DOC)		
A. Raw materials and supplies		
1.Raw materials	5,007,040.00	5,007,040.00
2.Supplies (2% DOC)	0.0198 X-141,259	146,776.92
B. Labor and supervision (10% MC)	0.10 X	1,454,726.90
C. Utilities (5% MC)	0.05 X	727,363.45
Total	**4,865,780+0.169 X**	**7,335,905.26**
II. Fixed cost		
A. Depreciation (10% FCI)	3,070,861	3,070,861.00
B. Interest (10% FCI)	3,070,861	3,070,861.00
C. Taxes (2% FCI)	614,172	614,172.00
D. Insurance (1% FCI)	307,086	307,086.00
Total	**7,062,981**	**7,062,981.00**
III. Plant overhead (10% labor)	**0.01 X**	**145,472.69**
IV. Manufacturing cost	**X**	**14,547,269.51**
V. General Expenses		
A. Administration (2% TPC)	0.02 Y	316,244.98
B. Distribution and selling (3% TPC)	0.03 Y	474,367.47
C. Research and development (3% TPC)	0.03 Y	474,367.47
Total	**0.08Y**	**1,265,699.92**
VI. Total Product Cost = IV + V	**Y**	**15,812,249.47**

$$X = 4,865,780 + 0.1698 X + 7,062,981 + 0.01 X = \$14,547,269.51$$

$$Y = 0.08 Y + X = 0.08 Y + \$14,547,269.51 = \$15,812,249.47$$

$$\frac{6,836}{20,000} \times 100\% = 34.18\% \text{ (full capacity)}$$

It was observed that the BEP was achieved at 34.18% of the present plant operating capacity. Figure 7.2 shows a graphical analysis of the effect on costs and profits when the rate of production varies. This figure also indicates that the fixed cost remains constant and the total production cost increases if the rate of production is increased. The plant has to be operated above the minimum economic capacity (the production rate at break even point) which are 6,800 ton/year Under this condition, an ideal production rate for this lactic acid industry would be approximately 18.000 tonnes/year or 90% of maximum capacity.
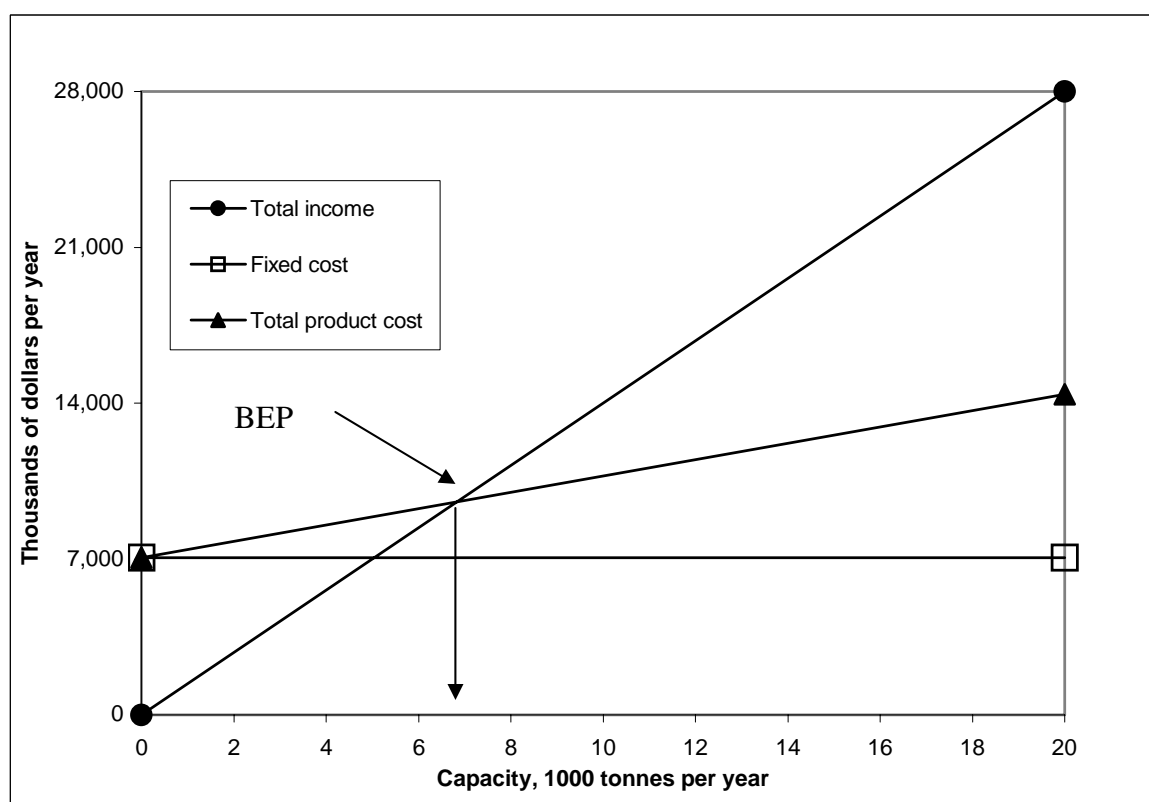
Figure 7.2: Break even chart for lactic acid processing plant.

7.4.2.2 Annual Profit Before And After Income Taxes

a)   Annual profit before income taxes   = total annual sell - total annual product cost

$$= \$1,400 \times 20,000 - \$15,812,249$$

$$= \mathbf{\$12,187,751}$$

b)   Annual profit after income taxes

The income taxes amount is assumed as 35% of all pre taxes profit. Therefore,

Annual profit after income taxes $= \$12,187,751 \times 0.65 = \mathbf{\$7,922,038}$

7.4.3   Process Profitability

7.4.3.1 Rate Of Return On Investment (ROR)

The rates of return before and after taxes were determined by Equation (7.4).

a) $\text{ROR.(\%).before taxes} = \dfrac{12,187,751}{30,708,611} \times 100 = 39.68\%$

b) $\text{ROR (\%) after taxes} = \dfrac{7,922,038}{30,708,611} \times 100 = 25.79\%$

According to the literature, a 20% ROR before income taxes would be the minimum acceptable return for any types of business proposition (Peters and Timmerhouse, 1981). Therefore, in economic term, the utilisation of liquid pineapple waste is feasible for lactic acid production using *L. delbrueckii.*

7.4.3.2 Payout Time (POT)

The payout time or payout period was determined by Equation (7.5).

$$POT\ (years) = \frac{30,708,611}{12,187,751 + 3,070,861} = 2.01\ years$$

This result indicates that the fixed capital investment is recovered only after 2 years. In general, a 5-year recover before income taxes would be the maximum acceptable recover for chemical industry.

7.5   Comparison To Other Substrates

The comparisons of economic evaluations on different substrates used in lactic acid fermentation plant are given in Table 7.6. The ROR value of Plant C was higher than both other plants which denotes that the return on investment on Plant C was higher than Plant B and A with ROR of 76.80, 51.12 and 39.68 % for before taxes and 49.92, 32.71 and 25.97 % for after taxes, respectively. The highest ROR value indicates that Plant C was most profitable than others. This also indicates that the capital recovery of Plant C was faster than Plant B and A with the values of payout time for each plant which are 1.15, 1.6 and 2 years, respectively.

However the cost of substrate at Plant A was nil but it was observed that the raw material cost of Plant A contributed about 31.10 % to the total product cost, followed by Plant B and C which were 27.61 and 18.42 %, respectively. The high production cost affects the annual profit and thus decreases the return on investment or increases the recovery of the capital.

Table 7.7: The comparison of profitability on lactic acid plant with different substrates.

Conditions	Plant A	Plant B	Plant C
	This Work, 2002	Akerberg, 2000	Paturau, 1982
Substrate	Pineapple waste	Wheat flour	Molasses
Technical grade	50 %	70%	80%
Capacity (tonnes/year)	20.000	30.000	1000
Substrate cost ($ / ton)	0.00	91.50	80.00
Lactic acid price ($ / kg)	1.40	1.66	1.69
Total production cost ($/kg)	0.79	0.83	0.76
Ratio of raw material and total production cost (%)	31.10	27.61	18.42
ROR before taxes (%)	39.68	51.12	76.80
ROR after taxes (%)	25.79	32.71	49.92
POT (years)	2.00	1.60	1.15

7.6   Conclusion

The feasibility study of lactic acid production from liquid pineapple waste using *L. delbrueckii* was investigated. The estimation of total capital investment and production cost was evaluated. The profits before and after taxes were calculated and the profitability was also evaluated. These results can be concluded as follow:

1. The raw material cost contributed to 30% of total production cost, which resulted in a production cost of 0.79 US$/kg product. It was lower than those reported by

Akerberg and Zacchi (2000) which were 0.833 US$/kg product. It was likely due to the difference of substrate used and the purity of lactic acid produced.

2. The minimum economic capacity on lactic acid plant was achieved at production rate of 6,800 tonnes per year (break even point). Under this condition, this lactic acid plant has to be operated at production rate above 7,000 tonnes per year and ideal production rate would be approximately 18,000 tonnes per year or 90% of full capacity to obtain the maximum profit.

3. The returns on investment before and after taxes obtained were 39.68 and 25.79%, respectively. According to the literature, a 20% ROR before income taxes would be the minimum acceptable return for any types of business proposition. Therefore, in economic term, the utilisation of liquid pineapple waste is feasible for lactic acid production using *L. delbrueckii.*

4. The profitability was also evaluated using payout time and result obtained was 2.0 years which indicates that the fixed capital investment is recovered after 2 years. In general, a 5-year recover before income taxes would be the maximum acceptable recover for chemical industry.

5. However the profitability of lactic acid plant using pineapple waste as substrate was lower than the utilisation of molasses and wheat flour, but it is still potential to be produced at industrial scale.

CHAPTER VIII

CONCLUSIONS AND RECOMMENDATIONS

This final chapter will summarise all the results and discussions of the data presented in Chapters IV, V, VI and VII, as well as recommend for further study in order to obtain a better understanding on lactic acid fermentation using pineapple waste, and subsequently to improve the lactic acid productivity.

8.1   Summary

8.1.1   The Chemical And Physical Properties Of Pineapple Waste

The liquid pineapple waste contains sugar mainly fructose, glucose and sucrose along with little protein, which vary depending on area, season and process used in canning industry. The solid waste contains 20 % (w/w) of fructose and glucose. Both solid and liquid wastes contain minerals and other nutrients such as citric acid, which are needed for growth of *Lactobaciilae*. The solid and liquid wastes appear to be potential substrates for lactic acid fermentation. Fermentation of solid waste gives the highest yield compared with liquid waste or liquid waste extract.

8.1.2   Parameters Influencing In Lactic Acid Fermentation

The batch fermentations were carried out under anaerobic condition with flushing to the fermentor using nitrogen gas. Effects of various parameters such as temperature, pH, inoculum size, initial substrate concentration and nitrogen source were studied. Effect of controlled pH has advantages in term of higher yield and rate of production compared with uncontrolled pH. If the fermentation process were carried out in shake flask, the addition of calcium carbonate (3 % w/v) in the fermentation medium would be very effective in controlling pH value at about 6.0. Effects of various parameters on the yield show that the maximum lactic acid production and yield obtained were 54.97 g/l and 79 %, respectively. The optimal conditions achieved were at pH: 6.0; temperature: 40 ^{o}C; inoculum size: 5 %; yeast extract as nitrogen source: 0.5 %; and initial sugar concentration of 70 g/l. Effect of initial sugar concentration was also carried out in shake flask fermentation by diluting the pineapple waste, and the optimal yield was found to be 52.50 g/l.

The effects of types and concentrations of sugar on lactic acid fermentation were also studied. Glucose, fructose and sucrose were used, and the yields were found to be 93% for all types of sugar at concentration of 20 g/l. By using mixed sugar at same concentration, the yield was found to be 93%. If the concentration was increased similar to that of sugar in pineapple waste (55 g/l), only 87 % of yield was obtained. The sugar utilisation on lactic acid fermentation for pure sugar or mixed sugar at concentration of 20 g/l shows that all sugars are completely utilised, with sucrose as the first sugar to be utilised followed by glucose and fructose. If the sugar concentration was increased to 55 g/l, only fructose was not completely utilised. This is different with the case where fermentation was carried out using pineapple waste in which only sucrose was completely utilised. It was found that fed batch fermentation was more efficient since it produced higher lactic acid concentration or lactic acid yield, and the productivity of 2.5 fold of batch fermentation was obtained.

8.1.3 Modelling And Kinetics Parameters Estimation In Lactic Acid Fermentation

A simplified unstructured kinetic model for lactic acid fermentation of liquid pineapple was developed. The model development was based on assumption that the rate of increase in biomass was a function of biomass concentration and one substrate (limiting substrate) only. The model took account of three responses, which were cell growth, total sugar utilisation and lactic acid production.

It was found that maximum specific growth rate (μ_{max}) in the lactic acid fermentation was affected by the types of substrates used. In batch fermentation of 20 g/l of various types of sugar such as: glucose, fructose, sucrose and mixed sugar, it can be found that the glucose medium gave the highest of maximum specific growth rate. When the concentration of the sugar was increased to 55 g/l, the value of μ_{max} increased as well. This indicates that the growth rate for 55 g/l was faster than for 20 g/l. The μ_{max} for 55 g/l was higher for lactic acid fermentation of liquid pineapple waste at all variables understudied. Other kinetic parameters such as saturation constant (K_s) were not significantly affected by the types of sugar used and process variables such as pH, temperature and inoculum size. Using sensitivity analysis, the growth kinetics of lactic acid fermentation appeared to be controlled mainly by maximum specific growth rate during the whole batch. The values of growth associated product formation constant (k_1) did not affected by process variables understudied, but the non growth associated product formation constant (k_2) was significantly affected by the pH. The lactic acid production by *L. delbrueckii* is favoured by fermentation at controlled pH of 6.0. The variation of k_1 is very sensitive to lactic acid production than k_2. Thus the lactic acid production is controlled by parameter of k_1 which is almost totally dependent of the growth rate.

The models were found to be in good agreement with the fermentation data. The kinetic model of the microbial growth, sugar utilisation and lactic acid production from pure sugar, mixed sugar (glucose, fructose and sucrose) and pineapple waste are similar and can be expressed by same type of mathematical model.

The simple unstructured model for growth in fed-batch fermentation was also developed. The model development was based on several assumptions, which are the growth rate is a function of concentrations of biomass and limiting nutrient only; single stage chemostat and no recycle. It was found that the kinetic parameters such as specific growth rate (μ) and biomass yield ($Y_{x/s}$), k_1 and k_2 were affected by sugar feeding concentration. The highest μ and $Y_{x/s}$ were obtained at 90 g/l sugar feeding concentration. The values of k_1 were higher than k_2. This indicates that the lactic acid production is to be controlled by parameter k_1.

8.1.4 Optimisation Of Conditions In Lactic Acid Fermentation

Many variables may affect the lactic acid fermentation process. The screening of these variables identified five variables such as sugar concentration, yeast extract concentration, fermentation time, temperature and stirring speed which have significant effect on fermentation system. 2^{5-1} fractional factorial central composite design (FFCCD) was used to determine the optimal values of the process variables. Response surface methodology (RSM) permitted in formulating two second-order polynomial empirical models relating to the responses and the significant variables. From these models it was possible to determine the values of the variables giving the maximum yield of lactic acid production. The optimal values of tested variables were found to be: sugar concentration, 65.87 g/l; yeast extract concentration, 15.35 g/l; fermentation time, 182.4 hours; temperature, $41.70^{o}C$ and stirring speed, 153.58 rpm. The maximum of lactic acid yield predicted by the model was 83.79 %. Based on optimum conditions obtained, shake flask fermentation (100 ml) was carried out. The lactic acid yield obtained was 83.07 %, while in scale up fermentation (5 litres) the lactic acid yield obtained was 86.15 %.

8.1.5   A Techno-Economic Evaluation Of Lactic Acid Production

The raw material cost contributed to 30% of total production cost, which resulted in a production cost of 0.79 US\$/kg product. It was lower than those reported by Akerberg and Zacchi (2000) which was 0.833 US\$/kg product. It might be due to the different substrates used and different purity of lactic acid produced. The minimum economic capacity on lactic acid plant was achieved at production rate of 6,800 tonnes per year (break even point). Under this condition, this lactic acid plant has to be operated at production rate above 7,000 tonnes per year and ideal production rate would be approximately 18,000 tonnes per year or 90% of full capacity to obtain the maximum profit.

The returns on investment before and after taxes obtained were 39.68 and 25.79%, respectively. According to the literature, a 20% ROR before income taxes would be the minimum acceptable return for any types of business proposition. Therefore, in economic term, the utilisation of liquid pineapple waste is feasible for lactic acid production using *L. delbrueckii*. The profitability was also evaluated using payout time and result obtained was 2.0 years. This shows that the fixed capital investment is recovered after 2 years. In general, a-5 year recover before income taxes would be the maximum acceptable recover for chemical industry. However the profitability of lactic acid plant using pineapple waste as substrate was lower than using molasses and wheat flour. However it is still prospective to be produced at industrial scale.

8.2   Recommendations For Future Study

The lactic acid production depends on the growth of the lactic acid bacteria in the medium like liquid pineapple waste. Growth of *L. delbrueckii* requires a wide range of growth factors including amino acids, vitamins, organic acids and minerals. Therefore the characterisation study of pineapple waste should not focus only on

elements like sugar, mineral and organic acid but further study is needed to determine vitamins especially B complex and amino acids in the pineapple waste.

Batch fermentation is used industrially in lactic acid production by *L. delbrueckii*. The substrate used was glucose (15 %) with the maximal yield and productivity of 93 % and 2 g l^{-1} h^{-1}, respectively. In continuous stirred tank reactor, the productivity increased to 76 g l^{-1} h^{-1} with initial sugar concentration of 7 %. By immobilised *L. delbrueckii* in calcium alginate beads, the productivity increased to 100 g l^{-1} h^{-1} with the maximum yield of 97 % and initial sugar concentration of 5 %. Therefore in order to increase the lactic acid productivity, further study is desired to include immobilised cell as well as the use of continuous bioreactors. In addition, up to now, studies on lactic acid production by immobilised cell have been mainly focused on using whey, starch, molasses, and synthetic medium containing glucose and lactose. Study on the use of complex substrate like pineapple waste by immobilised cell is yet to be reported in literatures.

In this work, for fed-batch fermentation, the productivity obtained was only 0.441 g l^{-1} h^{-1}. Further study is needed to increase the productivity by application of cell recycle using cross flow filtration. Further more, study on extractive fermentation system in which lactic acid can be extracted using Alamine 336 should be carried out to obtain higher productivity.

Among the various complex nitrogen sources, yeast extract is the best choice for lactobacillus growth and lactic acid production. However, the high cost of yeast extract has a negative impact on the techno-economics of the process. In the economic analysis for lactic acid production, as reported in the literature, material cost of yeast extract was estimated to contribute over 30 % to the total production cost. Therefore, in order to reduce the cost, cheaper nitrogen sources must be searched to replace the yeast extract.

In kinetic study the unstructured model was developed by assuming that the biomass composition was constant during all operation conditions. Even though this model is sufficient to simulate the current experimental results obtained, more generalised model for lactic acid fermentation which can be simulated at widely

different operating conditions is needed. Therefore it is very important to further study the structure model of lactic acid fermentation. This model is expected to give better description on transient operating conditions and it contains a small number of parameters which can easily be estimated on the basis of steady state experiments. Furthermore, optimisation study of batch and fed-batch lactic acid fermentation is also requisite to obtain the optimal productivity using development model.

Initial study indicated that solid pineapple waste is also potential to be used as substrate for lactic acid production carried out by submerged and semi-solid fermentation. Further study on solid state fermentation of solid pineapple waste is very encouraging by utilising the existing cheap raw materials as well as to obtain higher yield at lower cost.

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APPENDIX A

DATA 0F LACTIC ACID FERMENTATION USING PINEAPPLE WASTES

Table A1: Time dependence of biomass, glucose, fructose and lactic acid concentration during fermentation of solid pineapple waste. Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Glucose (g/l)	Fructose (g/l)	Lactic acid (g/l)
0	6.83	8.89	0.00
24	7.49	10.75	1.24
48	5.21	8.85	5.35
72	3.90	5.77	10.85
96	1.12	4.34	14.77
120	0.56	3.91	16.18
144	0.00	2.82	17.25
168	0.00	0.05	19.71
192	0.00	0.18	22.51
216	0.00	0.00	22.37
240	0.00	0.00	22.14

Table A2: Time dependence of biomass, glucose, fructose and lactic acid
concentration during fermentation of liquid pineapple waste extract. Experimental
conditions, T: 40^{o}C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Biomass (g/l)	Glucose (g/l)	Fructose (g/l)	Lactic acid (g/l)
0	0.000	10.260	12.840	0.000
4	0.003	10.140	12.680	0.000
8	0.020	10.050	12.560	0.000
12	0.138	9.810	12.390	0.070
16	0.375	9.700	12.240	0.133
20	0.531	9.632	12.130	0.355
24	0.914	9.510	12.050	0.682
28	1.279	9.430	11.920	0.971
32	1.321	9.140	11.760	1.200
36	1.329	8.870	11.530	1.740
40	1.327	8.010	11.280	2.070
44	1.322	7.860	11.010	2.370
48	1.294	7.790	10.960	2.580
56	1.278	7.060	10.830	2.940
68	1.245	6.940	10.640	3.200
80	1.167	6.510	10.360	3.520
92	1.076	5.850	10.120	4.360
104	0.650	4.050	9.330	6.730
116	0.319	3.190	7.260	8.650
128	0.296	2.380	6.540	10.360
144	0.281	1.240	4.450	13.108
168	0.246	1.220	4.310	13.376
192	0.237	1.130	4.230	13.054
216	0.220	1.100	3.990	12.960
240	0.181	1.030	3.960	12.830

Table A3: Time dependence biomass concentration during fermentation of
pineapple waste with controlled and uncontrolled pH. Experimental
conditions,T : 40^{o}C, inoculum: 5% and stirring speed: 50 rpm.

Time	Biomass (g/l)	
(h)	Controlled pH	Uncontrolled pH
0	0.000	0.000
4	0.013	0.003
8	0.126	0.007
12	0.215	0.112
16	0.463	0.241
20	0.857	0.334
24	0.965	0.337
28	1.004	0.359
32	1.103	0.407
36	1.218	0.440
40	1.346	0.493
44	1.543	0.577
48	1.616	0.654
52	1.602	0.793
56	1.484	0.817
64	1.412	0.615
72	1.384	0.437
80	0.997	0.330
92	0.790	0.322
104	0.643	0.319
116	0.585	0.332
128	0.463	0.334
144	0.444	0.341
168	0.355	0.333
192	0.280	0.331
216	0.124	0.329
240	0.096	0.327

Table A4: Time dependence of glucose concentration during fermentation of pineapple waste with controlled and uncontrolled pH. Experimental conditions, T: 40 ^{o}C, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Glucose concentration (g/l)	
	Controlled pH	Uncontrolled pH
0	19.560	19.840
4	21.290	21.770
8	22.690	22.510
12	23.900	23.160
16	24.880	24.270
20	25.610	25.420
24	26.240	25.850
28	26.870	26.240
32	27.540	26.760
36	28.360	27.100
40	28.900	27.820
44	29.170	28.160
48	30.960	29.270
52	29.480	29.830
56	28.130	30.420
64	27.280	31.850
72	26.180	30.060
80	24.540	29.100
92	23.080	28.650
104	20.260	28.530
116	16.160	27.860
128	11.320	27.600
144	7.440	26.330
168	3.250	26.310
192	3.070	25.780
216	2.920	25.610
240	2.570	25.450

Table A5: Time dependence of fructose concentration during fermentation of
pineapple waste with controlled and uncontrolled pH. Experimental
conditions, T: 40 ^{o}C, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Fructose concentration (g/l)	
	Controlled pH	Uncontrolled pH
0	20.270	20.240
4	20.430	20.770
8	22.360	21.640
12	23.940	22.680
16	24.510	23.710
20	26.580	24.070
24	27.030	24.610
28	29.620	24.840
32	30.140	25.190
36	29.320	25.460
40	29.040	25.870
44	28.910	26.760
48	28.480	27.830
52	28.320	28.760
56	28.150	28.990
64	27.830	29.140
72	27.623	30.810
80	27.440	30.440
92	26.540	30.010
104	22.450	29.260
116	18.290	29.420
128	15.580	28.980
144	13.700	28.850
168	9.880	28.660
192	9.420	28.330
216	9.230	28.560
240	9.110	28.190

Table A6: Time dependence of sucrose concentration during fermentation of
pineapple waste with controlled and uncontrolled pH. Experimental
conditions, T: 40°C, inoculum: 5% and stirring speed: 50 rpm.

Time	Sucrose concentration (g/l)	
(h)	Controlled pH	Uncontrolled pH
0	15.530	14.960
4	13.840	13.480
8	11.450	11.290
12	9.340	9.050
16	7.590	8.160
20	6.420	6.240
24	5.150	5.770
28	4.360	4.960
32	3.120	4.180
36	2.280	3.620
40	1.050	2.880
44	0.460	1.750
48	0.000	0.840
56	0.000	0.000
64	0.000	0.000
72	0.000	0.000

Table A7: Time dependence lactic concentration during fermentation of
pineapple waste with controlled and uncontrolled pH. Experimental
conditions, T: 40°C, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Uncontrolled pH		Controlled pH (6.0)
	Lactic acid (g/l)	pH	Lactic acid (g/l)
0	0.000	6.00	0.000
4	0.000	6.00	0.000
8	0.000	6.00	0.220
12	0.480	5.95	0.760
16	0.820	5.90	0.950
20	1.110	5.85	1.680
24	1.470	5.80	2.390
28	1.730	5.75	3.150
32	2.220	5.70	3.820
36	2.980	5.65	4.380
40	3.160	5.60	5.410
44	3.720	5.60	6.700
48	4.220	5.50	7.950
52	4.520	5.50	9.240
56	5.850	5.50	10.410
64	6.480	5.45	13.060
72	7.260	5.40	13.920
80	7.920	5.40	15.240
92	8.760	5.40	17.860
104	10.110	5.30	22.970
116	10.710	5.30	29.630
128	11.630	5.30	36.280
144	12.930	5.25	47.790
168	13.870	5.20	54.970
192	13.520	5.10	54.610
216	13.270	5.10	54.130
240	13.250	5.10	53.680

Table A8: Time dependence of biomass concentration during fermentation of pineapple waste with different pH. Experimental conditions, T: 40^{o}C, inoculum: 5% and stirring speed: 50 rpm.

Time	Biomass concentration (g/l)		
(h)	pH=6	pH=6.5	pH=5.5
0	0.000	0.000	0.000
4	0.013	0.000	0.000
8	0.026	0.000	0.000
12	0.215	0.000	0.000
16	0.463	0.000	0.000
20	0.857	0.000	0.000
24	0.965	0.000	0.000
28	1.004	0.070	0.090
32	1.103	0.120	0.100
36	1.218	0.140	0.110
40	1.346	0.386	0.112
44	1.543	0.605	0.113
48	1.616	0.705	0.125
52	1.602	0.718	0.132
56	1.484	0.730	0.137
64	1.412	0.765	0.149
72	1.384	0.810	0.164
80	1.198	0.708	0.187
92	0.997	0.493	0.206
104	0.790	0.477	0.329
116	0.643	0.466	0.446
128	0.585	0.482	0.474
144	0.463	0.403	0.526
168	0.444	0.384	0.491
192	0.355	0.323	0.366
216	0.124	0.301	0.343
240	0.096	0.288	0.326

Table A9: Time dependence of glucose concentration during fermentation of pineapple waste with different pH. Experimental conditions, T: 40°C, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Glucose concentration (g/l)		
	pH = 6.0	pH = 6.5	pH = 5.5
0	19.560	19.840	19.600
4	21.290	20.480	20.420
8	22.690	21.860	21.340
12	23.900	23.270	21.650
16	24.880	24.080	22.080
20	25.610	25.100	22.780
24	26.240	25.850	23.140
28	26.870	26.760	23.650
32	27.540	27.580	24.320
36	28.360	29.410	24.930
40	28.900	31.850	25.310
44	29.170	32.330	25.960
48	30.960	32.470	26.520
52	29.480	32.290	26.864
56	28.130	31.960	27.360
64	27.280	29.730	28.580
72	26.180	29.326	29.640
80	24.540	28.890	30.480
92	23.080	27.580	31.140
104	20.260	25.100	32.660
116	16.160	24.730	31.850
128	11.320	23.660	31.370
144	7.440	22.720	30.980
168	3.250	21.180	27.600
192	3.070	19.760	26.280
216	2.920	19.460	25.390
240	2.570	19.110	25.750

Table A10: Time dependence of fructose concentration during fermentation of
pineapple waste with different pH. Experimental conditions, T: 40^{o}C,
inoculum: 5% and stirring speed: 50 rpm.

Time	Fructose concentration (g/l)		
(h)	pH = 6.0	pH = 6.5	pH = 5.5
0	20.270	20.340	20.140
4	20.430	20.560	20.880
8	22.360	20.910	21.090
12	23.940	21.730	21.610
16	24.510	22.120	21.980
20	26.580	22.570	22.180
24	27.030	23.580	22.470
28	29.620	24.100	22.850
32	30.140	25.390	23.280
36	29.320	26.240	23.710
40	29.040	28.070	24.080
44	28.910	29.260	24.760
48	28.480	30.810	25.490
52	28.320	30.740	26.150
56	28.150	30.620	26.880
64	27.830	29.490	27.630
72	27.623	29.320	28.700
80	27.440	29.250	30.510
92	26.540	28.220	31.540
104	22.450	27.840	32.830
116	18.290	27.560	32.210
128	15.580	27.170	31.940
144	13.700	26.830	31.760
168	9.880	25.670	31.550
192	9.420	25.780	31.270
216	9.230	25.560	30.930
240	9.110	25.540	30.770

Table A11: Time dependence of sucrose concentration during fermentation of pineapple waste with different pH. Experimental conditions, T: 40°C, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Sucrose concentration (g/l)		
	pH = 6.0	pH = 6.5	pH = 5.5
0	15.53	15.16	15.39
4	13.84	14.87	15.02
8	11.45	13.53	14.79
12	9.34	12.61	14.25
16	7.59	11.28	13.94
20	6.42	10.47	13.63
24	5.15	8.94	12.86
28	4.36	7.60	12.07
32	3.12	4.75	11.01
36	2.28	3.45	10.74
40	1.05	1.18	9.96
44	0.46	0.78	9.30
48	0.00	0.42	8.86
52	0.00	0.18	8.59
56	0.00	0.00	8.34
64	0.00	0.00	7.72
72	0.00	0.00	7.04
80	0.00	0.00	6.65
92	0.00	0.00	5.19
104	0.00	0.00	3.57
116	0.00	0.00	2.24
128	0.00	0.00	0.86
144	0.00	0.00	0.00
168	0.00	0.00	0.00
192	0.00	0.00	0.00
216	0.00	0.00	0.00
240	0.00	0.00	0.00

Table A12: Time dependence of lactic acid concentration during fermentation of pineapple waste with different pH. Experimental conditions, T: 40°C, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Lactic acid concentration (g/l)		
	pH = 6.0	pH = 6.5	pH = 5.5
0	0.000	0.000	0.000
4	0.000	0.000	0.000
8	0.220	0.000	0.000
12	0.760	0.000	0.000
16	0.950	0.000	0.000
20	1.680	0.000	0.000
24	2.390	0.370	0.000
28	3.150	0.560	0.000
32	3.820	0.830	0.000
36	4.380	1.290	0.000
40	5.410	2.930	0.000
44	6.700	3.740	0.000
48	7.950	4.260	0.180
52	9.120	4.590	0.397
56	10.410	5.230	0.630
64	12.260	6.110	1.170
72	13.920	8.290	1.640
80	15.240	9.360	2.030
92	17.860	10.790	2.570
104	22.970	12.170	3.210
116	29.630	13.780	4.470
128	36.280	14.610	5.150
144	47.790	16.950	6.520
168	54.970	19.820	9.040
192	54.610	20.740	10.220
216	54.130	21.880	11.590
240	53.680	21.390	11.180

Table A13: Time dependence of biomass concentration during fermentation of pineapple waste with different temperature. Experimental conditions, T: 40^oC, inoculum: 5% and stirring speed: 50 rpm.

Time	Biomass concentration (g/l)		
(h)	T= 50 ^{o}C	T= 45 ^{o}C	T= 40 ^{o}C
0	0.000	0.000	0.000
4	0.000	0.011	0.013
8	0.010	0.020	0.126
12	0.020	0.137	0.215
16	0.030	0.259	0.463
20	0.050	0.316	0.857
24	0.173	0.361	0.965
28	0.326	0.458	1.004
32	0.377	0.567	1.103
36	0.413	0.654	1.218
40	0.452	0.766	1.346
44	0.495	0.832	1.543
48	0.513	0.926	1.616
52	0.522	1.012	1.602
56	0.536	1.085	1.484
64	0.648	1.136	1.412
72	0.814	1.227	1.337
80	0.992	1.227	1.198
92	1.208	1.380	0.997
104	1.314	1.394	0.790
116	1.297	1.199	0.643
128	1.251	0.973	0.585
144	0.983	0.836	0.463
168	0.711	0.548	0.355
192	0.441	0.317	0.280
216	0.345	0.228	0.124
240	0.142	0.130	0.096

Table A14: Time dependence of glucose concentration during fermentation of pineapple waste with different temperature. Experimental conditions, T: 40°C, inoculum: 5% and stirring speed: 50 rpm.

Time	Glucose concentration (g/l)		
(h)	T= 50 $^{\circ}$C	T= 45 $^{\circ}$C	T= 40 $^{\circ}$C
0	20.080	20.130	19.560
4	20.360	20.310	21.290
8	20.930	21.680	22.690
12	22.050	23.750	23.900
16	23.760	24.560	24.880
20	24.510	26.740	25.610
24	26.140	28.540	26.240
28	28.780	29.080	26.870
32	30.970	30.740	27.540
36	32.330	31.430	28.360
40	33.510	31.020	28.900
44	32.140	30.640	29.170
48	31.830	29.930	30.960
52	31.180	29.670	29.480
56	30.480	29.440	28.130
64	29.970	27.720	27.280
72	28.740	25.850	26.180
80	26.900	24.190	24.540
92	25.620	23.640	23.080
104	25.180	22.980	20.260
116	23.780	20.520	16.160
128	22.280	15.680	11.320
144	20.250	8.080	7.440
168	19.440	7.530	3.250
192	15.390	4.140	3.070
216	15.080	3.980	2.920
240	14.960	3.670	2.570

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Table A15: Time dependence of fructose concentration during fermentation of
pineapple waste with different temperature. Experimental
conditions: pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time	Fructose concentration (g/l)		
(h)	T= 50 °C	T= 45 °C	T= 40 °C
0	19.860	20.060	20.270
4	20.410	20.470	20.430
8	20.840	21.130	22.360
12	21.620	22.430	23.940
16	22.480	23.290	24.510
20	23.620	25.970	26.580
24	25.310	26.580	27.030
28	27.890	27.930	29.620
32	28.330	28.560	30.140
36	30.710	29.910	29.320
40	32.860	30.110	29.040
44	32.450	29.530	28.910
48	32.420	29.360	28.480
52	32.260	29.190	28.320
56	32.060	29.040	28.150
64	31.830	28.210	27.830
72	31.641	27.726	27.623
80	31.510	27.390	27.440
92	31.080	26.480	26.540
104	30.570	26.150	22.450
116	30.260	25.960	18.290
128	30.030	24.180	15.580
144	29.900	20.960	13.700
168	29.300	16.710	9.880
192	27.340	13.260	9.420
216	27.260	12.780	9.230
240	26.840	12.510	9.110

Table A16: Time dependence of sucrose concentration during fermentation of pineapple waste with different temperature. Experimental conditions, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time	Sucrose concentration (g/l)		
(h)	T= 50 °C	T= 45 °C	T= 40 °C
0	14.970	15.260	15.530
4	14.020	13.220	12.230
8	13.260	10.380	9.280
12	11.190	8.510	7.370
16	10.320	7.440	5.900
20	9.070	6.250	5.110
24	8.760	5.270	4.720
28	7.890	4.130	3.460
32	5.260	2.830	1.710
36	4.190	1.200	0.750
40	2.620	0.640	0.000
44	1.380	0.000	0.000
48	0.530	0.000	0.000
52	0.000	0.000	0.000
56	0.000	0.000	0.000
64	0.000	0.000	0.000
72	0.000	0.000	0.000
80	0.000	0.000	0.000

Table A17: Time dependence of lactic acid concentration during fermentation of pineapple waste with different temperature. Experimental conditions, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Lactic acid concentration (g/l)		
	T= 50 °C	T= 45 °C	T= 40 °C
0	0.000	0.000	0.000
4	0.000	0.000	0.000
8	0.000	0.000	0.220
12	0.000	0.280	0.760
16	0.000	0.560	0.950
20	0.120	0.740	1.680
24	0.520	1.050	2.390
28	1.030	1.750	3.150
32	1.450	2.160	3.820
36	2.170	2.890	4.380
40	2.830	3.210	5.410
44	3.190	4.160	6.700
48	3.860	5.320	7.950
52	4.160	6.570	9.120
56	4.450	7.650	10.410
64	5.390	10.280	12.260
72	5.980	11.860	13.920
80	6.610	13.310	15.240
92	8.300	15.110	17.860
104	9.430	16.890	22.970
116	10.160	18.650	29.630
128	11.350	21.930	36.280
144	13.790	30.560	47.790
168	17.240	43.220	54.970
192	24.710	53.610	54.610
216	25.140	53.130	54.130
240	24.560	53.680	54.530

Table A18: Time dependence of biomass concentration during fermentation of
pineapple waste with different nitrogen source. Experimental
conditions, T: 40^{o}C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time	Biomass concentration (g/l)				
(h)	Yeast extract	Urea	Corn steep liquor	Malt sprout	Ammonium sulphates
0	0.000	0.000	0.000	0.000	0.000
4	0.013	0.000	0.000	0.000	0.000
8	0.126	0.000	0.000	0.000	0.000
12	0.215	0.000	0.000	0.036	0.052
16	0.463	0.000	0.000	0.076	0.071
20	0.857	0.000	0.000	0.094	0.095
24	0.965	0.033	0.036	0.126	0.109
28	1.004	0.086	0.098	0.168	0.147
32	1.103	0.104	0.113	0.204	0.184
36	1.218	0.138	0.155	0.249	0.216
40	1.346	0.186	0.238	0.301	0.259
44	1.543	0.226	0.292	0.372	0.285
48	1.616	0.261	0.349	0.416	0.317
52	1.602	0.386	0.415	0.446	0.346
56	1.484	0.496	0.471	0.482	0.381
64	1.412	0.678	0.654	0.594	0.460
72	1.384	0.706	0.698	0.572	0.458
80	1.198	0.763	0.732	0.551	0.458
92	0.997	0.889	0.825	0.463	0.449
104	0.790	0.731	0.893	0.46	0.331
116	0.643	0.576	0.753	0.456	0.288
128	0.585	0.473	0.624	0.452	0.263
144	0.463	0.392	0.512	0.450	0.249
168	0.444	0.252	0.411	0.436	0.225
192	0.355	0.145	0.358	0.386	0.198
216	0.124	0.044	0.310	0.253	0.186
240	0.096	0.009	0.065	0.076	0.021

Table A19: Time dependence of glucose concentration during fermentation of pineapple waste with different nitrogen source. Experimental conditions, T: 40^{o}C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time	Glucose concentration (g/l)				
(h)	Yeast extract	Urea	Corn steep liquor	Malt sprout	Ammonium sulphates
0	19.560	19.450	20.130	19.790	19.560
4	21.290	20.180	20.480	20.060	19.940
8	22.690	21.440	21.650	21.160	20.350
12	23.900	22.370	22.190	21.730	20.820
16	24.880	23.240	22.720	22.240	22.200
20	25.610	24.890	23.370	22.900	22.590
24	26.240	26.120	23.800	23.210	22.910
28	26.870	27.280	24.150	23.940	23.880
32	27.540	28.490	26.410	24.830	25.260
36	28.360	29.200	28.250	26.610	26.750
40	28.900	30.120	29.220	28.170	27.620
44	29.170	30.580	31.650	29.630	28.950
48	30.960	31.660	32.640	30.910	29.740
52	29.480	31.972	33.020	31.620	30.240
56	28.130	32.350	33.260	32.150	30.880
64	27.280	32.720	33.940	32.850	31.240
72	26.180	33.170	34.580	33.120	31.730
80	24.540	32.610	34.790	33.340	32.060
92	23.080	32.240	34.910	32.910	32.480
104	20.260	31.900	33.520	32.520	32.910
116	16.160	31.240	33.240	31.250	31.850
128	11.320	31.020	32.020	30.660	31.340
144	7.440	30.820	31.650	29.780	30.810
168	3.250	29.990	31.180	27.120	29.890
192	3.070	27.940	30.390	27.050	28.740
216	2.920	27.360	28.700	26.850	28.590
240	2.570	22.490	25.660	26.850	28.320

Table A20: Time dependence of fructose concentration during fermentation of pineapple waste with different nitrogen source. Experimental conditions, T: 40^{o}C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Fructose concentration (g/l)				
	Yeast extract	Urea	Corn steep liquor	Malt sprout	Ammonium sulphates
0	20.270	20.480	20.180	20.120	20.650
4	20.430	20.970	20.370	20.350	20.870
8	22.360	21.300	20.810	20.550	20.980
12	23.940	21.680	20.980	20.670	21.360
16	24.510	22.800	21.260	20.890	21.660
20	26.580	24.120	21.650	21.050	21.760
24	27.030	25.540	21.960	21.330	22.180
28	29.620	25.920	22.260	21.520	22.320
32	30.140	26.680	22.770	21.890	22.550
36	29.320	27.490	24.280	22.920	22.830
40	29.040	28.250	25.540	23.480	23.250
44	28.910	29.630	27.320	25.420	24.320
48	28.480	30.180	28.210	26.940	26.040
52	28.260	30.370	28.940	27.280	26.530
56	28.150	30.670	29.350	27.680	27.120
64	27.830	31.680	31.940	29.260	28.360
72	27.440	31.900	31.650	29.870	28.550
80	26.540	31.620	31.480	30.130	29.870
92	22.450	30.240	31.260	31.260	30.640
104	18.290	30.070	31.020	31.400	31.440
116	15.580	29.850	30.960	31.310	31.280
128	13.700	29.420	30.730	31.180	31.120
144	11.460	29.220	30.180	30.020	30.960
168	9.880	28.870	29.850	30.250	30.730
192	9.420	27.930	28.910	29.940	30.260
216	9.230	27.110	28.660	29.340	30.140
240	9.110	27.340	27.790	30.540	30.350

Table A21: Time dependence of sucrose concentration during fermentation of pineapple waste with different nitrogen source. Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Sucrose concentration (g/l)				
	Yeast extract	Urea	Corn steep liquor	Malt sprout	Ammonium sulphates
0	15.530	15.360	15.150	15.160	15.480
4	13.840	14.580	14.550	14.620	15.240
8	11.450	13.420	14.270	14.310	14.920
12	9.340	12.150	14.080	13.610	14.680
16	7.590	10.960	13.890	13.420	14.120
20	6.420	9.360	13.420	13.330	13.760
24	5.150	8.280	13.330	13.070	13.160
28	4.360	7.050	13.070	11.150	12.380
32	3.120	6.310	11.150	10.140	10.960
36	2.280	5.920	9.220	8.300	9.280
40	1.050	5.240	7.300	7.540	8.520
44	0.460	4.700	6.390	5.580	6.640
48	0.000	3.440	4.520	4.080	4.950
52	0.000	2.290	3.790	3.160	4.250
56	0.000	1.280	3.180	2.230	3.120
64	0.000	0.000	1.030	1.210	2.040
72	0.000	0.000	0.000	0.850	1.260
80	0.000	0.000	0.000	0.000	0.790
92	0.000	0.000	0.000	0.000	0.000
104	0.000	0.000	0.000	0.000	0.000

Table A22: Time dependence of lactic acid concentration during fermentation of
pineapple waste with different nitrogen source. Experimental
conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time	Lactic acid concentration (g/l)				
(h)	Yeast extract	Urea	Corn steep liquor	Malt sprout	Ammonium sulphates
0	0.000	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000	0.000
8	0.220	0.000	0.000	0.000	0.000
12	0.760	0.000	0.000	0.000	0.000
16	0.950	0.040	0.000	0.050	0.000
20	1.680	0.150	0.240	0.270	0.030
24	2.390	0.320	0.780	0.680	0.510
28	3.150	0.470	0.930	1.050	0.790
32	3.820	0.680	1.120	1.390	0.940
36	4.380	0.790	1.380	1.800	1.250
40	5.410	0.850	1.540	2.110	1.470
44	6.700	0.920	1.690	2.540	1.650
48	7.950	1.180	1.800	2.990	1.890
52	9.120	1.540	1.920	3.320	1.960
56	10.410	1.760	2.060	3.740	2.050
64	12.260	2.490	2.120	4.510	2.170
72	13.920	3.120	2.280	4.960	2.380
80	15.240	3.770	2.460	5.220	2.640
92	17.860	4.370	2.840	5.670	2.970
104	22.970	5.020	3.370	6.140	3.290
116	29.630	6.320	3.980	6.650	3.310
128	36.280	7.800	4.420	7.260	3.440
144	47.790	8.150	4.870	8.050	3.720
168	54.970	8.430	5.260	8.770	3.980
192	54.610	10.240	6.290	9.870	3.720
216	54.130	14.450	9.970	9.630	3.650
240	53.680	18.340	13.250	9.320	3.590

Table A23: Time dependence of biomass concentration during fermentation of
pineapple waste with different inoculum size. Experimental conditions,
T: 40°C, pH: 6.0 and stirring speed: 50 rpm.

Time	Biomass concentration (g/l)		
(h)	Inoculum= 5%	Inoculum= 10%	Inoculum= 15%
0	0.000	0.000	0.000
4	0.013	0.000	0.000
8	0.126	0.000	0.000
12	0.215	0.123	0.000
16	0.463	0.346	0.000
20	0.857	0.403	0.146
24	0.965	0.528	0.378
28	1.004	0.600	0.528
32	1.103	0.781	0.624
36	1.218	0.806	0.79
40	1.346	0.905	0.825
44	1.543	1.004	0.936
48	1.616	1.202	1.065
52	1.602	1.339	1.061
56	1.484	1.476	1.053
64	1.412	1.617	1.04
72	1.337	1.597	0.962
80	1.198	1.325	0.902
92	0.997	1.124	0.826
104	0.790	0.923	0.715
116	0.643	0.748	0.643
128	0.585	0.591	0.512
144	0.463	0.424	0.469
168	0.444	0.375	0.359
192	0.355	0.282	0.276
216	0.124	0.158	0.148
240	0.096	0.148	0.121

Table A24: Time dependence of glucose concentration during fermentation of
pineapple waste with different inoculum size. Experimental conditions,
T: 40^{o}C, pH: 6.0 and stirring speed: 50 rpm.

Time (h)	Glucose concentration (g/l)		
	Inoculum= 5%	Inoculum= 10%	Inoculum =15%
0	19.560	19.350	19.240
4	21.290	20.650	20.370
8	22.690	21.750	21.180
12	23.900	22.140	21.720
16	24.880	22.860	22.580
20	25.610	23.300	23.150
24	26.240	24.380	23.860
28	26.870	24.920	24.280
32	27.540	25.160	24.730
36	28.360	25.870	25.160
40	28.900	26.350	25.750
44	29.170	27.220	26.050
48	30.960	28.350	26.440
52	29.43	28.740	27.110
56	28.130	29.210	27.790
64	27.280	30.560	29.420
72	26.180	31.370	30.380
80	24.540	29.640	31.580
92	23.080	27.920	29.870
104	20.260	26.480	27.190
116	16.160	22.650	26.620
128	11.320	19.260	23.440
144	7.440	12.290	17.380
168	3.250	5.650	11.940
192	3.070	4.780	6.380
216	2.920	4.510	6.200
240	2.570	4.940	5.930

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Table A25: Time dependence of fructose concentration during fermentation of
pineapple waste with different inoculum size. Experimental conditions,
T: 40°C, pH: 6.0 and stirring speed: 50 rpm.

Time	Fructose concentration (g/l)		
(h)	Inoculum= 5%	Inoculum= 10%	Inoculum= 15%
0	20.970	20.370	20.480
4	21.830	20.580	20.820
8	22.510	21.200	21.360
12	23.480	21.750	21.450
16	24.560	22.200	22.110
20	24.930	23.610	22.950
24	25.160	24.170	23.180
28	25.420	24.570	23.560
32	25.890	24.340	24.280
36	26.150	25.760	24.530
40	26.860	26.880	24.970
44	27.290	26.510	25.320
48	28.380	27.410	26.360
52	27.790	27.120	26.980
56	27.420	26.740	27.580
64	26.240	26.150	28.640
72	25.680	25.590	27.190
80	24.290	24.820	26.530
92	23.380	23.350	25.840
104	22.880	22.500	24.350
116	20.200	20.280	23.260
128	16.640	17.360	21.620
144	11.150	13.480	20.780
168	9.650	10.660	18.050
192	9.420	10.380	17.520
216	9.220	10.720	17.610
240	8.790	10.390	16.140

Table A26: Time dependence of sucrose concentration during fermentation of
pineapple waste with different inoculum size. Experimental conditions,
T: 40°C, pH: 6.0 and stirring speed: 50 rpm.

Time (h)	Sucrose concentration (g/l)		
	Inoculum= 5%	Inoculum= 10%	Inoculum =15%
0	15.530	15.030	14.960
4	13.840	13.920	14.650
8	11.450	11.730	12.430
12	9.340	10.540	11.430
16	7.590	9.160	9.860
20	6.420	7.140	7.240
24	5.150	5.420	5.690
28	4.360	4.800	5.150
32	3.120	3.590	4.820
36	2.280	2.920	3.260
40	1.050	1.460	2.180
44	0.460	0.890	1.370
48	0.000	0.260	0.820
52	0.000	0.120	0.450
56	0.000	0.000	0.240
64	0.000	0.000	0.000
72	0.000	0.000	0.000

Table A27: Time dependence of lactic acid concentration during fermentation of
pineapple waste with different inoculum size. Experimental conditions,
T: 40^{o}C, pH: 6.0 and stirring speed: 50 rpm.

Time	Lactic acid concentration (g/l)		
(h)	Inoculum= 5%	Inoculum= 10%	Inoculum= 15%
0	0.000	0.000	0.000
4	0.000	0.000	0.000
8	0.220	0.000	0.000
12	0.760	0.120	0.000
16	0.950	0.550	0.000
20	1.680	0.960	0.000
24	2.390	1.530	0.350
28	3.150	2.360	0.720
32	3.820	2.710	1.360
36	4.380	4.000	1.480
40	5.410	4.320	1.940
44	6.700	5.290	2.060
48	7.950	6.060	2.680
52	9.120	7.260	3.690
56	10.410	8.320	4.800
64	12.260	10.650	6.820
72	13.920	11.430	8.410
80	15.240	13.720	10.530
92	17.860	15.460	12.040
104	22.970	18.110	16.160
116	29.630	24.350	20.770
128	36.280	30.110	25.960
144	47.790	42.580	32.580
168	54.970	51.800	39.380
192	54.610	51.670	44.840
216	54.130	51.360	44.560
240	53.680	51.240	44.750

Table A28: Time dependence of biomass concentration during fermentation of pineapple waste with different initial sugar concentration. Experimental conditions, T: 40^{o}C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Pineapple waste (g/l)	Pineapple waste + 25 g sugar (g/l)	Pineapple waste + 65 g sugar (g/l)
0	0.000	0.000	0.000
4	0.013	0.007	0.017
8	0.126	0.020	0.050
12	0.215	0.037	0.103
16	0.463	0.126	0.223
20	0.857	0.239	0.331
24	0.965	0.405	0.478
28	1.004	0.532	0.707
32	1.103	0.635	0.768
36	1.218	0.779	0.813
40	1.346	0.805	0.886
44	1.543	0.808	0.978
48	1.616	0.814	1.021
52	1.602	0.880	1.076
56	1.484	0.980	1.131
64	1.412	1.114	1.179
72	1.337	1.228	1.284
80	1.198	1.343	1.389
92	0.997	1.924	2.014
104	0.790	2.064	2.286
116	0.643	2.103	2.502
128	0.585	2.147	2.435
144	0.463	2.216	2.416
168	0.444	2.336	2.373
192	0.355	2.273	2.355
216	0.124	2.254	2.356
240	0.096	2.246	2.349

Table A29: Time dependence of glucose concentration during fermentation of
pineapple waste with different initial sugar concentration.
Experimental conditions, T: 40^{o}C, pH: 6.0, inoculum: 5% and
Stirring speed: 50 rpm.

Time (h)	Pineapple waste (g/l)	Pineapple waste + 25g sugar (g/l)	Pineapple waste + 65g sugar (g/l)
0	19.560	30.980	43.020
4	21.290	34.750	43.890
8	22.690	35.800	52.540
12	23.900	37.340	55.620
16	24.880	38.110	58.590
20	25.610	39.250	66.270
24	26.240	43.370	67.830
28	26.870	44.260	67.940
32	27.540	44.420	67.220
36	28.360	44.130	64.180
40	28.900	43.790	62.570
44	29.170	43.080	61.480
48	30.960	42.640	60.390
52	29.430	41.930	59.420
56	28.130	41.220	58.790
64	27.280	39.500	58.145
72	26.180	37.210	57.562
80	24.540	34.340	56.830
92	23.080	31.170	49.250
104	20.260	29.530	48.630
116	16.160	27.930	48.410
128	11.320	26.270	47.860
144	7.440	24.510	47.580
168	3.250	16.930	47.380
192	3.070	11.800	46.870
216	2.920	10.630	47.340
240	2.570	10.510	46.390

Table A30: Time dependence of fructose concentration during fermentation of
pineapple waste with different initial sugar concentration.
Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and
stirring speed: 50 rpm.

Time (h)	Pineapple waste (g/l)	Pineapple waste + 25g sugar (g/l)	Pineapple waste + 65g sugar (g/l)
0	20.970	31.240	43.930
4	21.830	34.750	43.760
8	22.510	35.350	46.880
12	23.480	37.110	50.730
16	24.560	38.270	52.680
20	24.930	39.530	53.410
24	25.160	39.190	53.050
28	25.420	38.640	52.780
32	25.890	38.420	52.970
36	26.150	38.350	52.810
40	26.860	37.910	52.530
44	27.290	37.190	52.640
48	28.380	36.210	52.680
52	27.790	35.310	52.610
56	27.420	34.260	52.570
64	26.240	33.250	52.490
72	25.680	32.640	52.630
80	24.290	32.090	52.340
92	23.380	31.820	52.710
104	22.880	30.370	51.430
116	20.200	27.370	51.210
128	16.640	28.140	50.820
144	11.150	25.390	50.460
168	9.650	24.870	49.720
192	9.420	22.870	50.640
216	9.220	22.350	50.330
240	8.790	22.060	49.880

Table A31: Time dependence of sucrose concentration during fermentation of pineapple waste with different initial sugar concentration. Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Pineapple waste (g/l)	Pineapple waste + 25g sugar (g/l)	Pineapple waste + 65g sugar (g/l)
0	15.530	21.700	33.310
4	13.840	18.460	31.090
8	11.450	16.790	29.670
12	9.340	15.230	28.650
16	7.590	14.180	26.950
20	6.420	13.260	25.210
24	5.150	11.380	24.470
28	4.360	9.720	23.310
32	3.120	6.890	21.680
36	2.280	5.470	19.420
40	1.050	3.530	15.060
44	0.460	1.690	13.620
48	0.000	1.450	12.320
52	0.000	1.130	12.040
56	0.000	0.980	11.910
64	0.000	0.000	11.740
72	0.000	0.000	11.320
80	0.000	0.000	11.210
92	0.000	0.000	11.020
104	0.000	0.000	10.500
116	0.000	0.000	10.290
128	0.000	0.000	10.380
144	0.000	0.000	10.660
168	0.000	0.000	10.460
192	0.000	0.000	10.550
216	0.000	0.000	10.610
240	0.000	0.000	10.340

Table A32: Time dependence of lactic acid concentration during fermentation of
pineapple waste with different initial sugar concentration.
Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and
stirring speed: 50 rpm.

Time (h)	Pineapple waste (g/l)	Pineapple waste + 25g sugar (g/l)	Pineapple waste + 65g sugar (g/l)
0	0.000	0.000	0.000
4	0.000	0.000	0.000
8	0.220	0.000	0.860
12	0.760	0.586	1.430
16	0.950	1.235	2.200
20	1.680	1.411	2.970
24	2.390	1.823	4.040
28	3.150	2.040	4.970
32	3.820	3.260	5.160
36	4.380	4.780	5.340
40	5.410	5.950	6.650
44	6.700	6.150	7.480
48	7.950	7.240	8.850
52	9.120	8.780	9.520
56	10.410	10.560	11.600
64	12.260	11.230	12.690
72	13.920	12.040	14.360
80	15.240	15.120	18.520
92	17.860	17.240	23.460
104	22.970	18.100	26.650
116	29.630	18.560	29.850
128	36.280	18.880	31.810
144	47.790	19.760	35.100
168	54.970	19.470	42.230
192	54.610	18.870	48.890
216	54.130	18.660	51.530
240	53.680	18.420	50.870

APPENDIX B

DATA 0F LACTIC ACID FERMENTATION USING PURE SUGAR

Table B1: Time dependence of biomass, glucose and lactic acid concentration during fermentation of glucose. Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Biomass (g/l)	Glucose (g/l)	Lactic acid (g/l)
0	0.000	19.750	0.000
2	0.000	19.460	0.000
4	0.060	19.030	0.430
8	0.150	18.860	0.940
12	0.220	17.640	1.740
16	0.390	17.280	2.590
20	0.520	15.730	3.820
24	0.690	13.950	4.920
28	0.785	11.740	6.510
32	0.821	9.660	8.290
36	0.920	8.860	9.720
40	0.989	7.290	10.840
44	1.060	6.380	11.650
48	1.120	5.620	12.480
52	1.270	4.480	13.750
56	1.490	3.640	14.320
60	1.860	2.960	15.890
64	2.190	1.330	17.590
68	2.280	0.000	18.160
72	2.260	0.000	18.380
80	2.220	0.000	18.200

Table B2: Time dependence of biomass, fructose and lactic acid concentration during fermentation of fructose. Experimental conditions, $40^{\circ}C$, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Biomass (g/l)	Fructose (g/l)	Lactic acid (g/l)
0	0.000	19.640	0.000
2	0.000	19.020	0.000
4	0.050	18.850	0.058
8	0.069	18.560	0.670
12	0.095	18.120	0.970
16	0.121	17.970	1.180
20	0.176	16.860	2.270
24	0.221	15.140	3.960
28	0.292	14.730	4.870
32	0.373	13.160	5.450
36	0.412	12.350	6.180
40	0.499	11.440	7.630
44	0.571	10.910	8.340
48	0.698	9.390	9.150
52	0.726	8.160	10.420
56	0.846	7.720	10.760
60	0.884	7.240	11.160
64	0.937	6.720	11.540
68	0.978	5.880	12.330
72	1.026	4.520	13.850
80	1.083	2.860	15.470
92	1.145	0.940	17.360
104	1.205	0.460	18.320
116	1.228	0.000	18.250
128	1.267	0.000	18.240
144	1.237	0.000	18.020

Table B3: Time dependence of biomass, glucose, fructose, sucrose and lactic acid concentration during fermentation of sucrose. Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Biomass (g/l)	Glucose (g/l)	Fructose (g/l)	Sucrose (g/l)	Lactic Acid (g/l)
0	0.000	0.530	0.000	19.440	0.000
2	0.000	0.780	0.160	19.060	0.000
4	0.016	1.120	0.950	17.240	0.000
8	0.028	2.180	1.560	15.350	0.360
12	0.030	2.720	1.870	14.560	0.540
16	0.033	3.210	2.280	13.490	0.790
20	0.052	3.960	2.470	11.050	1.160
24	0.069	4.480	2.880	9.250	1.920
28	0.118	4.610	3.640	7.110	2.560
32	0.194	4.840	4.330	6.380	3.610
36	0.237	5.050	4.640	5.310	4.420
40	0.275	5.820	4.750	3.320	5.230
44	0.280	5.540	4.920	2.680	6.450
48	0.339	5.380	5.070	1.250	7.060
52	0.387	5.320	5.280	0.280	7.890
56	0.459	4.960	4.920	0.000	8.960
60	0.592	4.550	4.580	0.000	9.580
64	0.728	3.970	4.220	0.000	10.220
68	0.876	3.140	3.860	0.000	11.560
72	1.054	2.180	3.330	0.000	12.880
80	1.253	0.580	2.970	0.000	14.890
92	1.297	0.000	1.240	0.000	16.460
104	1.357	0.000	0.360	0.000	18.020
116	1.409	0.000	0.000	0.000	18.310
128	1.432	0.000	0.000	0.000	18.410
144	1.467	0.000	0.000	0.000	17.860

Table B4: Time dependence of biomass, glucose, fructose, sucrose and lactic acid concentration during fermentation of mixed sugar (20g/l). Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Biomass (g/l)	Glucose (g/l)	Fructose (g/l)	Sucrose (g/l)	Lactic Acid (g/l)
0	0.000	7.560	7.280	5.360	0.000
2	0.000	7.750	7.410	4.860	0.000
4	0.090	8.250	7.620	3.590	0.430
8	0.132	8.480	7.841	2.860	0.940
12	0.162	8.180	7.520	2.240	1.740
16	0.320	7.920	7.330	1.730	2.590
20	0.470	7.720	7.160	0.860	3.820
24	0.620	7.540	6.860	0.000	4.920
28	0.744	6.090	6.750	0.000	6.510
32	0.816	5.150	6.410	0.000	8.290
36	0.886	3.620	5.860	0.000	9.720
40	0.905	2.770	5.620		10.840
44	1.065	2.510	5.350		11.650
48	1.142	1.490	5.160		12.480
52	1.236	0.380	4.980		13.750
56	1.428	0.000	4.580		14.320
60	1.657	0.000	3.280		15.890
64	1.814	0.000	1.140		17.590
68	1.960	0.000	0.370		18.160
72	2.150	0.000	0.000		18.380
80	2.180	0.000	0.000		18.200
104	2.160	0.000	0.000		18.200

Table B5: Time dependence of biomass, glucose, fructose, sucrose and lactic acid concentration during fermentation of mixed sugar (55 g/l). Experimental conditions, T: 40^{o}C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Biomass (g/l)	Glucose (g/l)	Fructose (g/l)	Sucrose (g/l)	Lactic Acid (g/l)
0	0.000	19.870	19.360	14.880	0.000
4	0.054	20.280	19.720	14.370	0.000
8	0.088	20.410	20.370	14.260	0.760
12	0.285	22.370	21.730	10.410	1.560
16	0.592	24.360	23.140	5.560	2.210
20	0.974	23.570	23.780	3.420	5.680
24	1.145	20.650	23.460	1.540	9.240
28	1.257	18.680	22.930	0.000	13.090
32	1.394	17.640	22.210	0.000	14.230
36	1.456	16.960	21.330	0.000	15.170
40	1.512	15.100	20.250	0.000	16.930
44	1.685	14.430	19.360	0.000	18.550
48	1.826	13.790	18.770	0.000	19.220
52	1.849	13.480	18.560	0.000	19.690
56	1.736	13.200	18.340	0.000	20.170
60	1.708	13.060	18.164	0.000	21.453
64	1.693	12.880	18.060	0.000	22.770
72	1.656	11.670	17.620	0.000	24.080
80	1.611	11.240	17.130	0.000	25.510
92	1.584	10.700	16.580	0.000	27.980
104	1.482	7.280	15.260	0.000	31.090
116	1.423	4.130	14.050	0.000	36.100
128	1.322	0.000	12.810	0.000	38.450
144	1.315	0.000	11.500	0.000	40.620
168	1.302	0.000	5.810	0.000	47.560
192	1.289	0.000	4.870	0.000	47.720
216	1.268	0.000	4.860	0.000	48.110
240	1.265	0.000	4.170	0.000	47.860

APPENDIX C

DATA 0F FED-BATCH FERMENTATION

Table C1: Time dependence of biomass concentration during fed-batch fermentation of pineapple waste. Experimental conditions, T: 40^{o}C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Biomass concentration (g/l)		
	65 g/l	90 g/l	115 g/l
0	0.000	0.000	0.000
4	0.008	0.007	0.005
8	0.010	0.009	0.008
12	0.083	0.041	0.044
16	0.153	0.145	0.147
20	0.337	0.379	0.398
24	0.604	0.568	0.497
28	0.649	0.708	0.524
32	0.797	0.950	0.609
36	1.031	1.287	0.698
40	1.282	1.649	0.835
44	1.516	1.949	0.965
48	1.629	2.339	1.106
52	1.736	2.462	1.274
56	1.894	2.550	1.482
64	1.883	2.588	1.698
72	1.837	2.504	2.100
80	1.531	2.418	2.237
92	1.289	2.373	2.448
104	0.960	2.346	2.522
116	0.809	2.331	2.513
128	0.790	2.310	2.498
144	0.780	2.256	2.462
168	0.710	2.238	2.424
192	0.654	2.226	2.410
216	0.613	2.215	2.380
240	0.586	2.203	2.310

Table C2: Time dependence of sugar concentration during fed batch fermentation of pineapple waste. Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time (h)	Sugar (g/l)		
	65 g/l	90 g/l	115 g/l
0	65.480	65.230	65.780
4	64.510	64.290	64.600
8	64.120	64.020	63.920
12	63.090	63.180	63.050
16	62.450	62.250	62.370
20	61.380	61.420	61.240
24	59.540	59.380	59.170
28	57.080	57.920	58.940
32	53.740	54.650	59.290
36	50.560	52.480	58.930
40	49.530	50.640	58.780
44	48.420	49.280	58.150
48	47.360	48.610	58.150
52	46.180	48.050	58.220
56	45.240	47.750	58.340
64	42.510	46.160	58.690
72	40.550	45.730	58.800
80	37.920	45.190	58.560
92	35.310	43.870	57.520
104	32.470	38.320	56.270
116	28.850	37.330	54.150
128	25.590	34.540	51.790
144	19.940	32.210	49.280
168	13.310	27.59	47.520
192	11.910	23.190	43.180
216	12.560	24.260	39.360
240	13.230	23.950	41.770

Table C3: Time dependence of lactic acid concentration during fed batch
fermentation of pineapple waste. Experimental conditions, T: 40^{o}C, pH:
6.0, inoculum: 5% and stirring speed: 50 rpm.

Time	Lactic acid concentration (g/l)		
(h)	65 g/l	90 g/l	115 g/l
0	0.000	0.000	0.000
4	0.080	0.160	0.210
8	0.480	0.570	0.640
12	1.240	1.750	1.590
16	2.010	2.380	2.440
20	3.080	3.860	4.250
24	6.870	6.330	6.400
28	7.060	8.10	7.980
32	9.570	10.940	8.670
36	12.260	13.20	10.500
40	14.08	15.440	12.210
44	15.560	18.690	14.760
48	16.790	21.230	17.440
52	17.510	22.390	18.560
56	18.340	23.410	19.770
64	20.490	26.020	22.140
72	22.950	28.520	24.510
80	25.080	29.410	26.290
92	27.690	32.430	28.830
104	30.530	37.210	32.980
116	33.150	39.540	36.870
128	36.410	42.160	40.360
144	41.520	44.370	43.710
168	47.690	50.980	47.320
192	51.240	57.760	53.190
216	47.720	53.590	54.750
240	43.340	49.680	49.84

Table C4: Time dependence of productivity during fed batch fermentation of pineapple waste. Experimental conditions, T: 40°C, pH: 6.0, inoculum: 5% and stirring speed: 50 rpm.

Time	Productivity (g / l.h)		
(h)	65 g/l	90 g/l	115 g/l
0	0.000	0.000	0.000
4	0.020	0.040	0.053
8	0.060	0.070	0.080
12	0.103	0.150	0.133
16	0.126	0.150	0.153
20	0.154	0.190	0.213
24	0.286	0.260	0.267
28	0.252	0.290	0.285
32	0.299	0.340	0.271
36	0.341	0.370	0.292
40	0.352	0.390	0.305
44	0.354	0.420	0.335
48	0.350	0.440	0.363
52	0.336	0.430	0.356
56	0.328	0.420	0.353
64	0.320	0.410	0.346
72	0.319	0.400	0.340
80	0.314	0.370	0.329
92	0.301	0.350	0.313
104	0.294	0.360	0.317
116	0.286	0.340	0.318
128	0.284	0.330	0.315
144	0.288	0.310	0.304
168	0.284	0.303	0.282
192	0.267	0.300	0.277
216	0.221	0.250	0.276
240	0.181	0.210	0.228

APPENDIX D

DATA 0F LACTIC ACID FERMENTATION WITH DIFFERENT SIZE OF FERMENTOR

Table D1: Time dependence of biomass, sugar and lactic acid concentration during fermentation scale-up of pineapple waste (5 l). Experimental conditions, T: 42°C, pH: 6.0, inoculum: 5% and stirring speed: 75 rpm.

Time (h)	Biomass (g/l)	Glucose (g/l)	Fructose (g/l)	Sucrose (g/l)	Lactic acid (g/l)
0	0.000	20.650	21.510	14.369	0.000
4	0.007	21.220	21.860	13.270	0.000
8	0.012	23.450	22.940	10.150	0.000
12	0.054	25.380	24.190	7.180	0.280
16	0.132	27.450	26.310	3.970	0.670
20	0.215	28.780	27.550	1.160	1.190
24	0.509	29.340	28.010	0.840	1.650
28	0.701	30.170	28.760	0.000	2.340
32	1.497	29.200	29.200	0.000	3.960
36	2.175	28.560	28.450	0.000	5.220
40	2.614	27.100	27.360	0.000	7.410
44	2.817	26.420	26.030	0.000	9.680
48	2.852	25.390	25.790	0.000	11.390
52	2.904	24.520	25.1800	0.000	13.220
56	2.975	23.910	24.230	0.000	15.450
64	2.997	21.120	23.180	0.000	18.120
72	3.002	17.950	22.50	0.000	23.040
80	2.918	14.560	20.490	0.000	28.370
92	2.822	11.120	18.160	0.000	33.690
104	2.773	9.650	17.20	0.000	36.420

Table D1: Time dependence of biomass, sugar and lactic acid concentration during fermentation scale-up of pineapple waste (5 l). Experimental conditions, T: 42°C, pH: 6.0, inoculum: 5% and stirring speed: 75 rpm (Continued).

Time (h)	Biomass (g/l)	Glucose (g/l)	Fructose (g/l)	Sucrose (g/l)	Lactic acid (g/l)
116	2.750	7.840	16.150	0.000	39.550
128	2.742	5.210	12.900	0.000	45.130
144	2.594	2.490	8.150	0.000	52.320
168	2.367	0.000	6.740	0.000	56.890
192	2.348	0.000	6.670	0.000	56.730
216	2.175	0.000	6.540	0.000	56.40
240	2.078	0.000	6.330	0.000	56.250

Table D2: Time dependence of biomass, sugar and lactic acid concentration during fermentation scale-up of pineapple waste(1 l). Experimental conditions, T: 42°C, pH: 6.0, inoculum: 5% and stirring speed: 75 rpm.

Time (h)	Biomass (g/l)	Glucose (g/l)	Fructose g/l	Sucrose (g/l)	Lactic acid (g/l)
0	0.000	20.730	21.850	14.472	0.000
4	0.041	20.960	21.940	13.590	0.000
8	0.088	23.710	22.850	10.680	0.000
12	0.147	25.360	24.040	7.620	0.000
16	0.214	27.430	25.960	4.220	0.480
20	0.306	28.180	26.840	1.510	0.870
24	0.535	29.530	27.250	1.170	1.395
28	0.723	30.500	28.120	0.380	2.062
32	1.435	29.480	29.550	0.000	3.480
36	1.921	28.590	28.380	0.000	4.930
40	2.436	27.630	27.130	0.000	7.520
44	2.697	26.170	26.380	0.000	9.360
48	2.712	25.210	25.260	0.000	10.940
52	2.752	24.320	24.810	0.000	12.660
56	2.874	23.450	24.440	0.000	14.420
64	2.866	21.320	23.350	0.000	17.590
72	2.852	17.280	22.420	0.000	21.580
80	2.802	14.630	20.280	0.000	27.140
92	2.752	11.570	19.060	0.000	32.172
104	2.729	9.100	18.420	0.000	35.460
116	2.654	7.520	16.940	0.000	38.800
128	2.588	5.970	12.880	0.000	44.690
144	2.463	2.680	9.040	0.000	50.520
168	2.304	0.000	7.110	0.000	54.440
192	2.273	0.000	6.930	0.000	54.210
216	2.108	0.000	6.840	0.000	54.120
240	2.062	0.000	6.390	0.000	54.080

APPENDIX E

FORMULAS FOR CALCULATING THE COEFFICIENT IN A SECOND DEGREE MODEL

The second-degree model in k variables fitted to data collected at the points of a central composite design is

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j}^{k} \beta_{ij} x_i x_j + \varepsilon$$

In order to estimate the values of b_o, b_i, b_{ii} and b_{ij} of the coefficients β_o, β_i, β_{ii}, and β_{ij}, by the following equation (Cornell, 1991).

$$b_0 = \frac{2(1 - k / \sqrt{M})}{D} \sum_{n=1}^{Mn} Y_u + \frac{(k - \sqrt{M})}{D} \sum_{u=M+1}^{M+2k} Y_u + \frac{(k+2)}{D} \sum_{u=M+2k+1}^{N} Y_u$$

$$b_i = \frac{1}{H} \sum_{u=1}^{M} x_{ui} Y_u + \frac{\sqrt[4]{M}}{H} (Y_{M+2i} - Y_{M+2i-1}) \qquad 1 \le i \le k$$

$$b_{ii} = \frac{(N - M - 2\sqrt{M})}{MD} \sum_{u=1}^{M} Y_u + \frac{(M + 2\sqrt{M} - N)}{2\sqrt{M} D} \sum_{u=M+1}^{M+2k} Y_u$$

$$- \left(\frac{1 + 2 / \sqrt{M}}{D}\right) \sum_{u = M + 2k + 1}^{N} Y_u \quad + \quad \frac{1}{2\sqrt{M}} (Y_{M + 2i} + Y_{M + 2i - 1})$$

$$b_{ij} \; = \; \frac{1}{M} \sum_{u = 1}^{M} x_{ui} x_{uj} Y_u \qquad\qquad 1 \le i < j \le k$$

where, $D \; = \; N(k + 2) - \left(2 + \sqrt{M}\right)^2$ and

$H \; = \; N(k + 2) - \left(2 + \sqrt{M}\right)^2$

$M \;\; = \;\; r2^k$ and $N \;\; = \;\; M + 2k + n_o$, M is number of observation at the factorial, 2k is the star points, n_o is total of center points, N is total number of observation and r is replicate observation.