The Feasibility of Lignocellulosic degradation from pineapple (*Ananas comosus*) wastes on batch ethanol fermentation by *Saccharomyces cerevisiae* TISTR 5013

By

Mr. Chakrit Choljararux

I.D. 431-8707

A special project submitted to the Faculty of Biotechnology, Assumption University in part fulfillment of the requirements for the degree Bachelor of Science in Biotechnology

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Faculty	:	Biotechnology
Academic Year	:	2004

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ABSTRACT

This research had studied about the effect of lignocellulosic degradation from pineapple (*Ananas comosus*) wastes on batch ethanol fermentation by *Saccharomyces cerevisiae* TISTR 5013 which concerned in the degradation of pineapple (*Ananas comosus*) wastes to glucose, and the production of ethanol. The pineapple (*Ananas comosus*) wastes were obtained from fresh market and were used as the raw materials for the ethanol fermentation. The objectives of this study were extracted glucose from pineapple waste and used this sugar solution as starter medium for yeast growth and ethanol fermentation. This objective could lead to reduce wastes and got value–added product from new source of raw material because Thailand is agricultural country. Some compositions (sugar and salt) of the degraded medium solution were determined. The obtained medium solution was used as starter medium on batch fermentation by

Saccharomyces cerevisiae TISTR 5013. The study of growth and ethanol fermentation on batch type fermentor was performed in order to investigate that pineapple wastes could use as raw material for ethanol fermentation which was the new source of raw materials, reduce the bio-waste and value-added product could be produced.

The results were shown that the percentage of waste almost around 49 – 52 % or half-half with edible part. The sugar analysis of neutralized solution was 31.25 grams per liters, and the salt analysis of neutralized solution was 9.243 %. After passed the separation process of salt from sugar, the sugar analysis was 30.20 grams per liters and salt analysis was 0.0975 %. Then this neutralized solution could be used as starter medium for study the yeast growth and ethanol fermentation in next part.

The study of yeast growth and ethanol fermentation on batch type fermentation was performed and the result was shown that the cell was increase while amount of glucose was reduced and at the termination of exponential phase of cell growth then the ethanol production was started to produce. So the final ethanol production, amount of glucose and cell dry weight were 2.4 %, 0.433 grams per liters, and 3.440 grams per liters, respectively.

According to the results of this study, degraded sugar that obtained from the pineapple (*Ananas comosus*) wastes could be used as raw material to produce ethanol on batch type fermentation. The utilization of this bio-waste could lead to a reduction of biodegradable wastes from the fresh market, and lead to get value-added product.

ACKNOWLEDGEMENT

This special project could be succeeded with many peoples so this is my occasion to express my appreciation to these following peoples.

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Chakrit Choljararux
June, 2004

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LIST OF ABBREVIATIONS

1. mm	abbreviates from	millimeter (s)
2. cm	abbreviates from	centimeter (s)
3. °C	abbreviates from	celsius degree (centigrade)
4. s	abbreviates from	second (s)
5. min	abbreviates from	minute (s)
6. h	abbreviates from	hour (s)
7. mg	abbreviates from	milligram (s)
8. g	abbreviates from	gram (s)
9. kg	abbreviates from	kilogram (s)
10. ml	abbreviates from	milliliter (s)
11. µl	abbreviates from	microliter (s)
12. pH	abbreviates from	the negative logarithm of the hydrogen in concentration
13. soln	abbreviates from	solution
14. v/v	abbreviates from	volume by volume
15. w/v	abbreviates from	weight by volume
16. w/w	abbreviates from	weight by weight
17. %	abbreviates from	percentage

CHAPTER I

INTRODUCTION

1.1 Statement of the problem

Nowadays, Petroleum was presented the main energy source of the world which always has the price problem. Thailand is the one of country that imported petroleum from abroad, especially, from OPEC (Organization of the Petroleum Exporting Countries) group. Every time that occur petroleum crisis, Thailand will gets the economic effect and following with many problems. So ethanol utilization as mixture with petroleum for car is the considerable way to solve problems. [54]

The ethanol production in industrial level for using as fuel instead of petroleum is more interested by the study of reduction of production cost, the development of production process, and the selection of low cost raw materials such as cassava, cassava powder, and molasses etc. [41] [53] which the cost for ethanol production from fermentation is not depend on petroleum price.

After the petroleum crisis in the Middle East War was terminated. The ethanol production was less interested because petroleum price had lower than petroleum that mixed with ethanol called Gasohol. At year 2000, Thailand was encountered with Baht problem and OPEC (Organization of the Petroleum Exporting Countries) group was reduced petroleum production which had affected the price in world market (higher price). So the ethanol production was more interested again.

In the past, Brazil was the one country that import petroleum from abroad and encountered with petroleum crisis same as Thailand. In 1975, Brazil government was set National Alcohol Program : PNA). The objective was reduced the need of petroleum and support the utilization of alcohol as energy source. In 1991, Brazil was the most in the world alcohol production and alcohol utilization [28]. Many countries interested in alcohol production for fuel such as United State of America, German, France, Australia, and Thailand etc. from the success.

From environmental part found that "car, that use gasohol, will release less carbonmonoxide than car, that use normal benzene as fuel, [28]. In addition, the addition of ethanol in benzene will be made octane rating higher. The increasing of octane rating was depended on octane rating and hydrocarbon composition of benzene. [24] [50]

Majority in Thailand, The ethanol production was used for beverage from many kinds of raw material. The production cost is quite high but low quality which is not worth-while for fuel production. So the development in ethanol production process should be set for higher quality than the present.

In the present day, the petroleum is high price which lead the chance to produce ethanol for higher fuel consumption combine with many kinds of raw material used in ethanol production are available in Thailand. [11]. Almost pineapples are contributed to canned industrial and fresh market and almost industrial will use all part of pineapple for minimize lost but in market will use only flesh, the skin and leaf will discard as waste. So all of this waste called "Biowaste" which can recycle in new form or value added this biowaste as ethanol production (use as

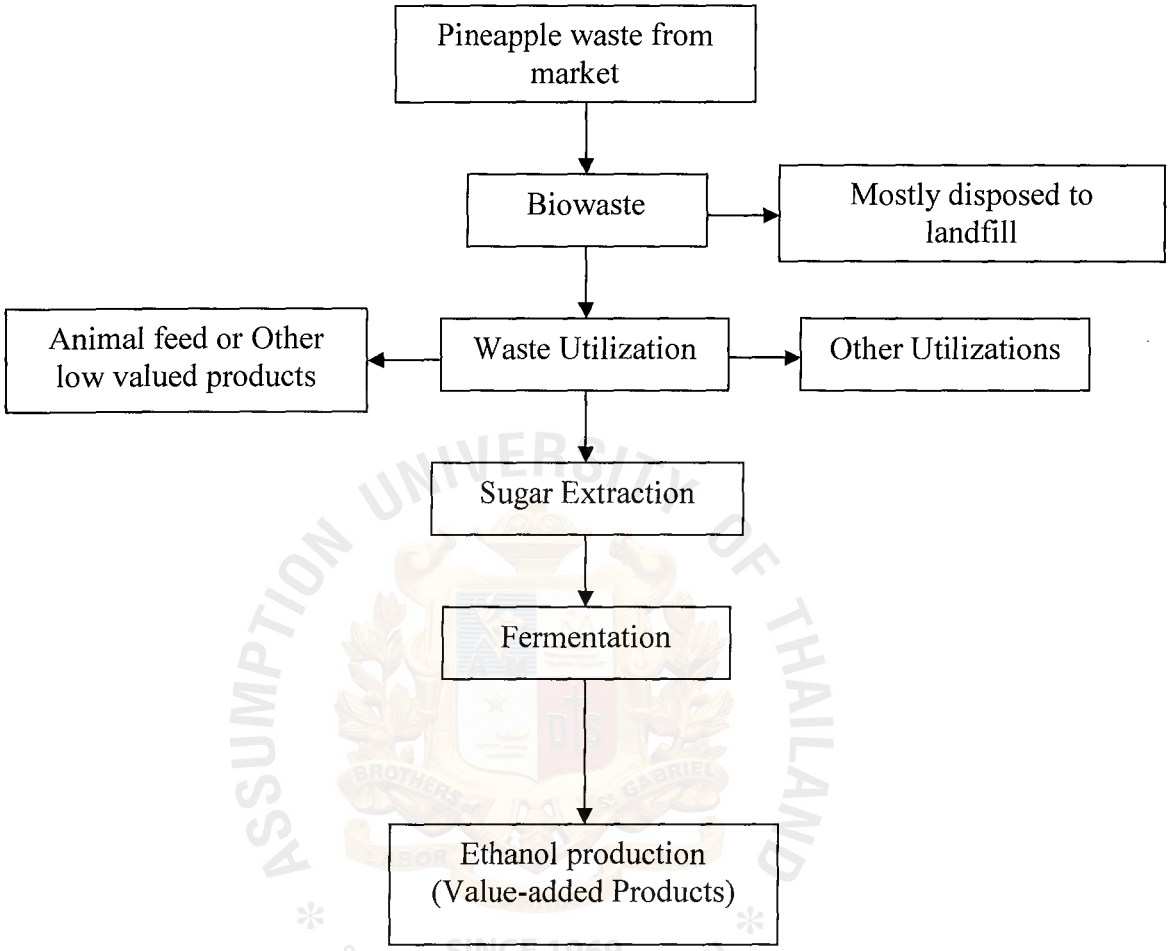
fuel). From this problem the pineapple waste can be utilized as raw material because leaf and skin of pineapple (waste part) composed of cellulose as composition and the small unit is glucose which is important substance in ethanol fermentation. [41] [53]. These will help in added value of pineapple waste, reduce environmental problem, include reduce of cost in ethanol production because raw material come from pineapple waste. If cost of ethanol production is lesser than normal benzene, it will be supported in using gasohol. Moreover, it will help to reduce import petroleum from abroad which led to reduce missing of trade balance with other countries.

As an alternative way to achieve value-added products from pineapple waste, this research was study probability in utilization of glucose from pineapple waste degrading with the direct acid hydrolysis in ethanol fermentation by *Saccharomyces cerevisiae* TISTR 5013 which is reported that have high fermentation potential in sugar from degraded cassava [41].

1.2 Objectives

The objectives of this study were to extract glucose from pineapple (*Ananus comosus*) waste obtained from fresh market (skin and leaf). The analysis of glucose and salt in medium that obtained from direct acid hydrolysis was examined. And also the percentage of producing ethanol from obtained glucose was examined.

1.3 Conceptual Framework



1.4 Scope

An experimental study on degradation of biowaste (Pineapple waste) to sugar and possibility of the degradation as material for the ethanol production was performed. Leaf and skin of pineapple (*Ananus comosus*) used in this study were obtained from a fresh market [Mod pineapple shop, Nongmon market] Chonburi province. The study was covered the percentage waste analysis, the cultivation of *Saccharomyces cerevisiae* TISTR 5013 from Freeze dried culture, the degradation step

via direct acid hydrolysis, the analysis of starter sugar and salt amount in medium. The separation of salt from medium which is cause of growth inhibition of culture, the ethanol production analysis was examined too.

1.5 Hypothesis

1.5.1 Direct acid hydrolysis could degrade pineapple waste (leaf and skin) to sugar which use as starter concentration of medium.

1.5.2 Extracted sugar obtained from pineapple waste (leaf and skin) could use as starter medium to produce ethanol.

1.5.3 Salt from medium can be extracted by selective precipitation process.

1.6 Anticipated Benefits

1.6.1 To increase the value-added of the waste residue from pineapple waste market in different way.

1.6.2 To increase fuel application.

1.6.3 To increase the use of biodegradable and recycle material.

1.6.4 To provide the new source of raw material in ethanol production using as fuel.

1.7 Definitions

1.7.1 **Amylopectin** : branch polymer that composed of glucose, in the linear part of glucose linked with alpha-1, 4- glucosidic linkage and the nranch part of glucose linked with alpha-1, 6-glucosidic linkage.

1.7.2 **Amylose** : linear polymer that composed of glucose around 2,000 units linked with alpha-1, 4-glucosidic linkage.

1.7.3 **Ashes** : The grayish-white to black powdery residue left when something is burned.

1.7.4 **Biodegradation** : Capable of being decomposed by biological agents, especially bacteria: *a biodegradable detergent.*

1.7.5 **Biotin** : A colorless crystalline vitamin, $C_{10}H_{16}N_2O_3S$, of the vitamin B complex, essential for the activity of many enzyme systems and found in large quantities in liver, egg yolk, milk, and yeast.

1.7.6 **Biowaste** : An unusable or unwanted substance or material produced during or as a result of a process, such as metabolism or manufacturing.

1.7.7 **By-Product** : Something produced in the making of something else.

1.7.8 **Catabolite repression** : Repression (inactivation) of certain sugar-metabolizing operons (eg lac) in favour of glucose utilization when glucose is the predominant carbon source in the environment of the cell.

1.7.9 **Cellulose** : A complex carbohydrate, $(C_6H_{10}O_5)_n$, that is composed of glucose units, forms the main constituent of the cell wall in most plants, and is important in the manufacture of

numerous products, such as paper, textiles, pharmaceuticals, and explosives.

1.7.10 **Cofactor** : A substance, such as a metallic ion or coenzyme, that must be associated with an enzyme for the enzyme to function.

1.7.11 **Decanter** : A vessel used for decanting, especially a decorative bottle used for serving wine.

1.7.12 **Decarboxylation** : Removal of a carboxyl group from a chemical compound, usually with hydrogen replacing it.

1.7.13 **Dextrin** : Any of various soluble polysaccharides obtained from starch by the application of heat or acids and used mainly as adhesives and thickening agents.

1.7.14 **Direct acid hydrolysis** : Decomposition of a chemical compound by reaction with water and acid such as the dissociation of a dissolved salt or the catalytic conversion of starch to glucose.

1.7.15 **Ergosterol** : A crystalline sterol, $C_{28}H_{43}OH$, synthesized by yeast from sugars or derived from ergot and converted to vitamin D_2 when exposed to ultraviolet radiation.

1.7.16 **Fermentation** : The anaerobic conversion of sugar to carbon dioxide and alcohol by yeast. Any of a group of chemical

reactions induced by living or nonliving ferments that split complex organic compounds into relatively simple substances.

1.7.17 **Fiber** : One of the elongated, thick-walled cells that give strength and support to plant tissue.

1.7.18 **Gasohol** : A fuel consisting of a blend of ethyl alcohol and unleaded gasoline, especially a blend of 10 percent ethanol and 90 percent gasoline.

1.7.19 **Glucosidic linkage** : the bond that combine between sugar and sugar.

1.7.20 **Lignocellulosic** : any of several combinations of lignin and hemicellulose, forming the essential part of woody tissue

1.7.21 **Liquefaction** : The process of liquefying or the state of being liquefied.

1.7.22 **Molasses** : A thick syrup produced in refining raw sugar and ranging from light to dark brown in color.

1.7.23 **Neutralize** : To make neutral.

1.7.24 **Oligosaccharide** : A carbohydrate that consists of a relatively small number of monosaccharides.

1.7.25 **Petroleum** : A thick, flammable, yellow-to-black mixture of gaseous, liquid, and solid hydrocarbons that occurs naturally

beneath the earth's surface, can be separated into fractions including natural gas, gasoline, naphtha, kerosene, fuel and lubricating oils, paraffin wax, and asphalt and is used as raw material for a wide variety of derivative products.

1.7.26 **Polymer** : Any of numerous natural and synthetic compounds of usually high molecular weight consisting of up to millions of repeated linked units, each a relatively light and simple molecule.

1.7.27 **Purine** : A double-ringed, crystalline organic base, $C_5H_4N_4$, not known to occur naturally, from which is derived the nitrogen bases adenine and guanine, as well as uric acid as a metabolic end product.

1.7.28 **Pyrimidine** : A single-ringed, crystalline organic base, $C_4H_4N_2$, that forms uracil, cytosine, or thymine and is the parent compound of many drugs, including the barbiturates.

1.7.29 **Saccharification** : To convert (starch, for example) into sugar.

1.7.30 **Thermostable** : Unaffected by relatively high temperatures, as certain ferments or toxins.

1.7.31 **Value-added** : Of or relating to the estimated value that is added to a product or material at each stage of its manufacture or distribution: *"Unlike the steel or aluminum industries, where heavier profits come from value-added fabrication, mining is the most lucrative stage of copper production" (Forbes).*

CHAPTER 2

LITERATURE REVIEW

Pineapple (*Ananus comosus*)

The scientific name of pineapple called "*Ananus comosus*" in Bromliceae family. The origin was in Latin-American and plant well in moist-tropical weather [35]. Department of Agricultural Extension (2000) reported that "Pineapple planting of Thailand in 1998-1999 (between May 1998 to April 2000) have planting are equal to 512,000 rai but almost famous planting in the West part and the East part of country only and the ratio was 60.60 % and 30.82 % respectively. All country pineapple production was 1,786,000 tons. The average pineapple production in the East part was equal to 5,347 kilograms per rai and in the West part was equal to 4,287 kilograms per rai. The pineapple production of both the West part and the East part were equal to 99.4 % of all country. [11]

The area, for planting pineapple in Thailand since After 1987 until now, has approximately 500,000 rai per year. The quantity of pineapple production per rai was approximately 3,800 kilograms but the purchasing price and cost production have higher trend since year 1996/97, especially, year 1998/99 the purchasing price was higher to 5.24 baht per kilograms which lead production cost to 9,558.6 million baht. However in the year

1987/88 until 1996/97, the pineapple price was quite low between 1.12 -2.83 baht per kilograms which is the one cause that farmer fear to make an investment and increase the planting area (Table 2-1). Majority pineapple production in Thailand was exported around 76.6 % and consumed in country only 23.4 % [5]. Anong 2549, suggested that the two proper ranges for planting which are beginning of rainy season (between March to May) and terminating of Winter season (between October to November). The harvesting of pineapple was performed in April to June and October to December [2]. Farmer will harvest pineapple for selling or transport to Canned pineapple industry which lead the pineapple was over-demanded in the market so the industry was find out the way to solve this problem by transform the pineapple to many product and increase production. [3]

Pineapple production of canned industrial factory is canned pineapple, pineapple juice and pineapple jams which are important export goods that make high income. The canned pineapple and pineapple juice have steps as follow: Fresh pineapple is cleaned and sorted. After that peeled the skin, terminal and head then sizing and de-core and cutting before packing. Residue from skin part and pineapple squeeze used as animal feed (Figure 2-2) but for market side, the residue from skin or unused part almost discard as garbage (Figure 2-1) so this waste can be recycled or transform to value added thing as ethanol production because in skin of pineapple contain cellulose (Table 2-3) which can degrade to sugar then can be transformed to ethanol by yeast.

Table 2-1 : The planting area, production, production per rai, price, and production cost of Thailand since 1987/88 - 1998/99

Area of planting = 1,000 rai

Production = 1,000 tons

Production per rai = kilograms

Price = Baht per kilograms

Production cost = million baht

Year of planting	Area of planting	Production	Production per rai	Price	Production cost
1987/88	395	1,510	3,819	1.99	3,004.9
1988/89	444	1,771	3,988	1.48	2,621.1
1989/90	486	2,005	4,122	1.12	2,245.6
1990/91	466	1,865	4,005	1.49	2,778.9
1991/92	498	1,931	3,876	2.59	5,001.3
1992/93	561	2,180	3,888	1.88	4,098.4
1993/94	624	2,589	4,146	1.18	3,044.0
1994/95	621	2,370	3,820	1.48	3,507.6
1995/96	566	2,088	3,691	2.01	4,196.9
1996/97	521	1,987	3,814	2.83	5,623.2
1997/98	529	2,083	3,936	3.33	5,936.4
1998/99	512	1,786	3,491	5.24	9,558.6

[12]

The export of pineapple of Thailand

The export of pineapple product of Thailand is, in form of canned pineapple, freeze-pineapple, pineapple juice and others since 1992 until now, exported as the first of the world. The four important markets are United States of America and Canada group, European countries group such as German and The Netherlands etc., Asia group such as Japan, Hong Kong, South Korea etc, and Middle East group such as Saudi Arabia, Arab, Emirate, Kuwait, and Bahrain. From the demand of pineapple production of the World market, in the present day, is around 11,200,000 tons per year. All production of Thailand is around 2,500,000 tons per year and for pineapple world market is around 22.3 % [12].

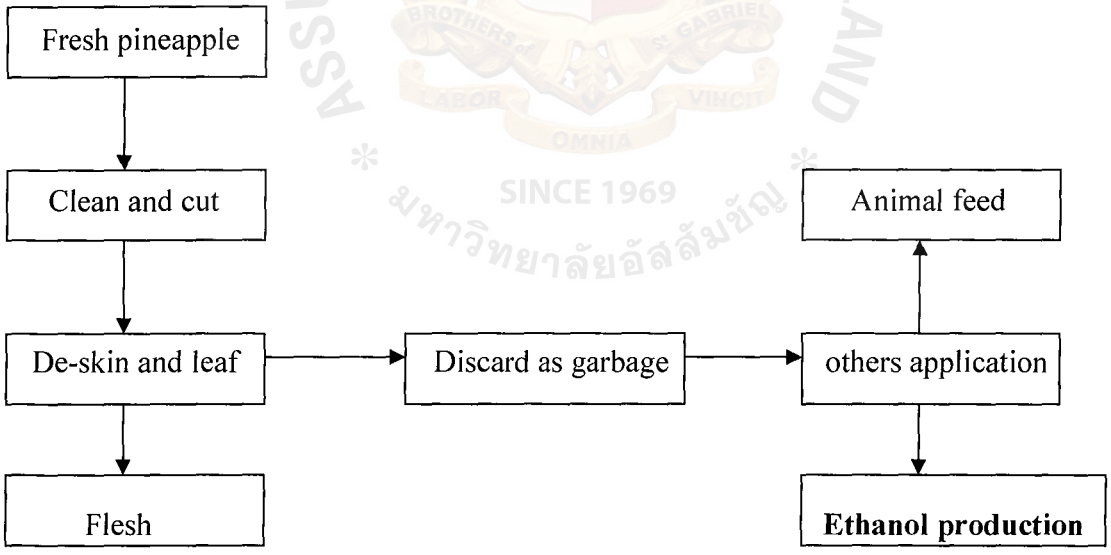


Figure 2-1 : The process of pineapple in market

2.1 Classification of Pineapple

The **Pineapple** (*Ananas comosus*) is a tropical plant and its fruit, native to Brazil, Bolivia, and Paraguay. The plant is a bromeliad (Family *Bromeliaceae*), a short, herbaceous perennial with thirty or more long, spined and pointed leaves surrounding a thick stem. The fruit was named "pineapple" because of its resemblance to a pine cone. The native Tupi word for the fruit was "anana", meaning "excellent fruit.". Southeast Asia dominates world production : in 1999 Thailand produced 2.331 million tonnes and the Philippines 1.495 million tonnes. Total world production in 1999 was 13.147 million tonnes. Pineapples are found abundantly in the West part and the East part of Thailand. (Figure 2-3)

Figure 2-3 : The pineapple [63]

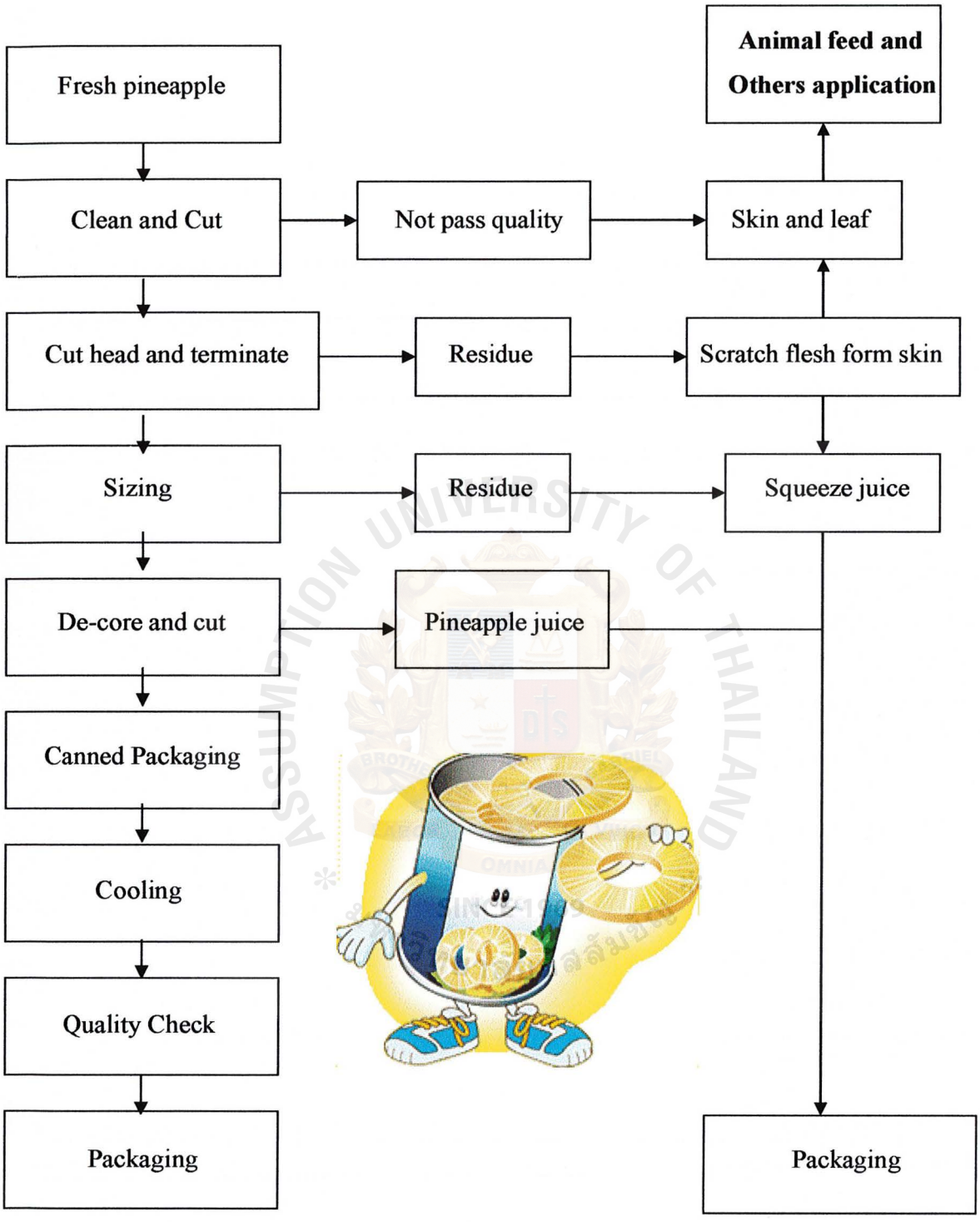


Figure 2-2 : The process of canned pineapple production

[3] [64]

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Liliopsida
Order	:	Poales
Family	:	Bromeliaceae
Genus	:	*Ananas*
Species	:	*A. comosus*
Common name	:	Pineapple, Ananas, Nanas, Pina.
Specific names	:	*Ananas comosus* Bromeliaceae
Reference	:	[63]

2.2 Structure and components of Pineapple (*Ananas comosus*)

Pineapple fruits are compound oval fruits 6 to 8 inches long with spiky, robust leaves at the top of the fruit. The tough, waxy rind is green, brown, and yellow in color with a scale-like appearance. The flesh of the pineapple is juicy and yellow to white in color as shown in figure 2-3 and figure 2-4.

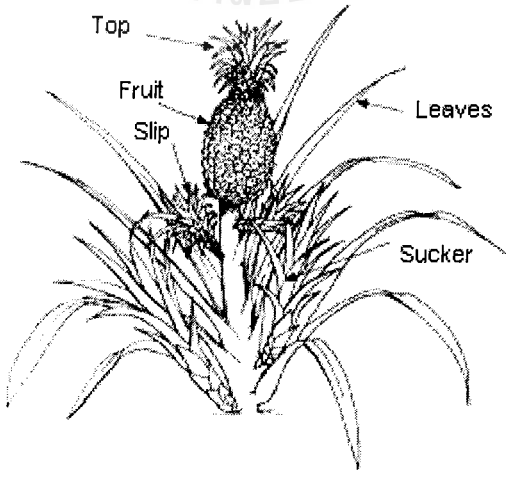


Figure 2-4 : The structure of pineapple [65]

Muller (1978) reported that "Residue of pineapple is by-product from remaining agricultural materials which, classified to three types, are residue from industrial factory, residue from harvesting, and residue from market. The ratio of all residues is approximately 56 %. The core and flesh have sugar approximately 74 %" (Table 2-2) [36]. Devendra (1998) reported that "Pineapple waste from industrial factory is around 40-50 %. In Thailand has pineapple waste from industrial factory more than 500,000 tons per year. [13]

Table 2-2 : Chemical nutrition of Pineapple waste (Percent of dried waste).

Composition of waste	**All ratio (%)**	**Combined Protein (%)**	**Lipid (%)**	**Fiber (%)**	**Nitrogen Free extract (%)**	**Ashes (%)**	**All sugar (%)**
Skin	56	6.4	0.02	16.7	71.88	4.1	42
Head	17	7.2	0.82	25.4	62.88	3.7	38
Termination	15	7.0	0.84	22.3	65.49	4.2	40
Core	5	7.1	0.96	19.7	64.49	2.3	73
Flesh residue	2	6.8	0.19	18.2	73.49	2.6	74
Residue	5	7.8	0.20	21.9	64.70	2.4	63

[36]

Dried pineapple waste has dried material 87 %, combined protein 4.6 %, fiber 20.9 % [38]. There are reported that "Pineapple waste have dried material 86.98 %, combined protein 3.05 %, carbohydrate 63.87 % and fiber

13.87 % [36]. Residue of pineapple is skin, head, termination, and core which all of these parts have different chemical nutrition. (Table 2-3).

Table 2-3 : The cellulose and hemi-cellulose analysis (Percentage of dried waste)

Composition of pineapple residue	Cellulose (%)	Hemi-cellulose (%)
Side skin	10.4	15.0
Head skin	15.2	21.7
Termination skin	17.6	26.5
Core	11.5	14.1
Flesh residue	5.2	8.7

[61]

2.3 The method for degrade Pineapple waste to Sugar

In the meaning of "The degradation of pineapple waste to sugar" is the degradation of remaining starch in pineapple waste or Starch saccharification

Cause of the starch characteristics from plant is glucose's polymer which composed of glucose unit link with glucosidic linkage. Starch composed of two kinds of glucose's polymer that is Linear polymer or Amylose and Branch polymer or Amylopectin in which amylase is main component around 75-85 % [53]

Amylose is linear polymer that composed of glucose around 2,000 units linked with alpha bond (1, 4) glucosidic [alpha-1, 4-glucosidic linkage] (Figure 2-5). The characteristics are long helix line, non-dissolve in water but spread as micelle, when combined with iodine will present dark blue color.

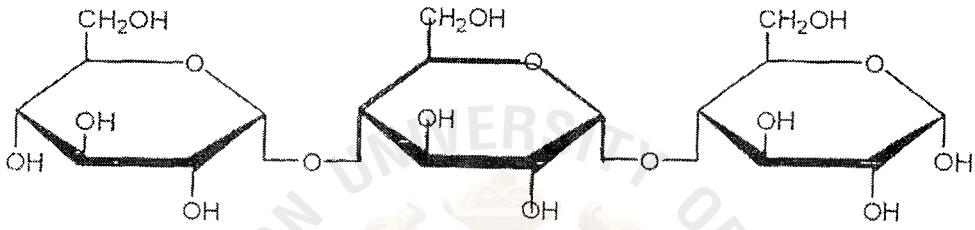


Figure 2-5 : Amylose's structure

Amylopectin is branch polymer that composed of glucose, in the linear part of glucose linked with alpha-1, 4-glucosidic linkage and the branch part of glucose linked with alpha-1, 6-glucosidic linkage. (Figure 2-6) Amylopectin in water will present colloidal solution, when combined with iodine will present purple-red color.

Because of normal yeast cannot use starch in Ethanol fermentation in one step so starch should be degraded to sugar in which there are two methods in starch degradation which are starch degradation via acid and starch degradation via enzyme.

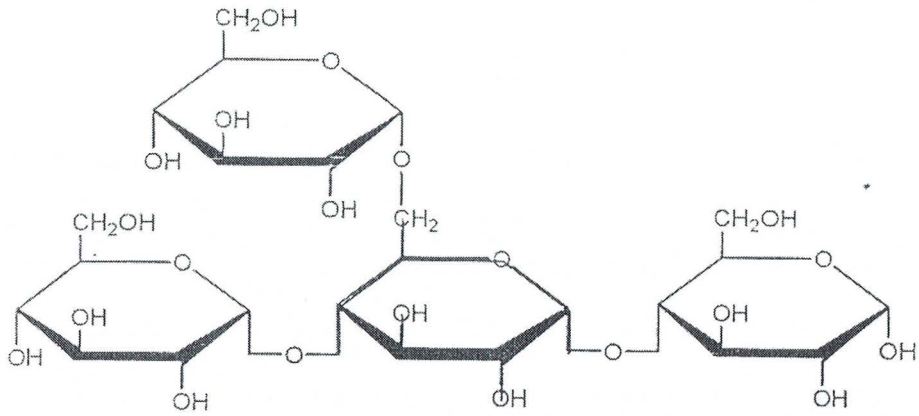


Figure 2-6 : Amylopectin's structure

2.3.1 Starch degradation via acid hydrolysis

Hydrolysis occurs in two stages to maximize sugar yields from the hemicellulose and cellulose fractions of biomass. The first stage is operated under milder conditions to hydrolyze hemicellulose, while the second stage is optimized to hydrolyze the more resistant cellulose fraction. Liquid hydrolyzates are recovered from each stage, neutralized, and fermented to ethanol.

Dilute acid hydrolysis of biomass is, by far, the oldest technology for converting biomass to ethanol. As indicated earlier, the first attempt at commercializing a process for ethanol from wood was done in Germany in 1898. It involved the use of dilute acid to hydrolyze the cellulose to glucose, and was able to produce 7.6 liters of ethanol per 100 kg of wood waste (18 gal per ton). The Germans soon developed an industrial process optimized for yields of around 50 gallons per ton of biomass. [19].

2.3.2 Starch degradation via enzyme

The enzyme that used is amylase group in which microorganisms produced and released out from cell [26]. The characteristics of enzyme processing in starch degradation can be classified to three groups [26] [34].

a) Exo-enzyme

Glucoamylase is enzyme that found in some molds such as *Aspergillus niger, Aspergillus oryzae,* and *Rhizopus spp.* . This enzyme classified to one kind of Alpha-amylase which is degrade both alpha 1,4 bond and alpha 1,6 bond from the terminal molecule (exo-hydrolase) by degrade alpha 1,4 bond faster than alpha 1,6 bond. This enzyme has no need cofactor.

Moreover, it still has Beta-amylase (exo-hydrolase), each two glucose molecules will get maltose as final product which affect to alpha 1,4 bond and when reach alpha 1,6 bond the activity of enzyme will stop. This enzyme need Calcium ion (Ca^{++}) as cofactor.

b) Endo-enzyme

Alpha-amylase is enzyme that degrade from inside molecule (endo-hydrolase) at alpha 1,4 bond but cannot degrade alpha 1,6 bond. The final product of this degradation is mixture of oligosaccharide, need calcium ion (Ca^{++}) as cofactor, can be found from microorganisms such as *Aspergillus*

oryzae, Bacillus subtilis, Bacillus licheniformis, and *Bacillus amyloliquefaciens* etc.

c) Debranching enzyme

Pullulanase is enzyme that degrade from outer molecule (exo-hydrolase) used to degrade alpha 1,6 bond around the branch part. It can be degrade until get about two to three units glucose line but cannot degrade to single glucose.

Isoamylase is enzyme that can degrade very well in the branch point of glycogen and amylopectin by no need cofactor.

2.4 The process in starch degradation to sugar via enzyme

Enzymes, in amylase group, have many types and different properties so to get maximum amount of glucose, the protocol will be separated to two steps [8] [34] as follows:

2.4.1 Liquefaction

The addition of alpha amylase enzyme will be made starch molecule shorter, lower viscousity. The example of commercial alpha-amylase is Termamyl. This enzyme got from *Bacillus licheniformis*. In the process will be used starch concentration around 30-40 %by weight, adjust pH to 6.0-6.5, used calcium ion in form of calcium chloride 20-80 mg/kg as cofactor. The

enzyme used about 0.5-0.6 mg/kg starch. Firstly start with temperature 100-105 degree celcius for five minutes for completely gelatinize, after that degrade at temperature 90-100 degree celcius for 1-2 hours then finish the process will be got small molecule of starch and dextrin.

Ejiofor and group (1996) [16] report about using of alpha amylase enzyme that resist to heat or thermostable from *Bacillus licheniformis* 0.3 g./kg. starch. In starch degradation, using 100 part per million concentrate calcium ion in form of calcium chloride as cofactor, adjust pH to 6.1-6.3, control temperature to 80 degree celcius for one hours. The final product is small starch and dextrin too. (Figure 2-7)

2.4.2 Saccharification

This step used glucoamylase enzyme in changing small starch to sugar. The example of commercial enzyme is Dextrozyme and AMG. After passed Liquefaction, the temperature will be reduced immediately to 60 degree celcius, adjust pH 4-4.5, add Glucoamylase 0.65-0.80 ml/kg. starch. The reaction occurred 24 hours and found that if more enzyme adding, the reaction will be faster.

In addition, it still has report in glucoamylase immobilized on porous diatomaceous earth in changing small starch molecule to sugar by the age of working of enzyme immobilized long up to three weeks.

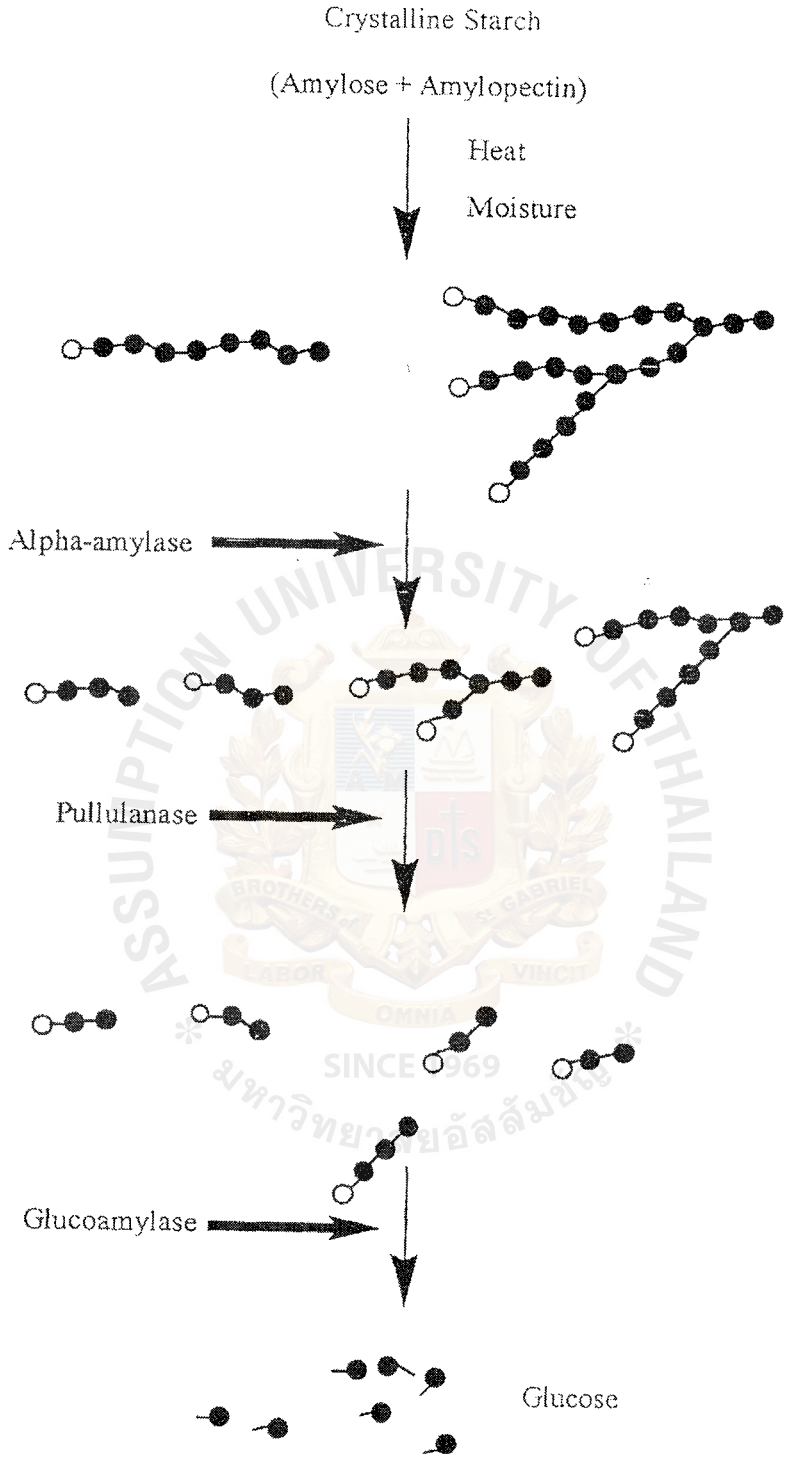


Figure 2-7 : The process of amylase group enzyme in starch degradation [8]

2.5 Microorganism (*Saccharomyces cerevisiae* TISTR 5013)

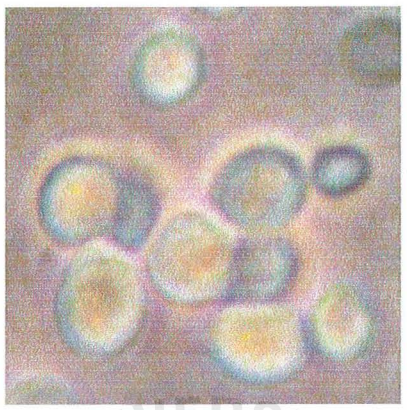


Figure 2-8 : *Saccharomyces cerevisiae* [66]

Yeasts such as *Saccharomyces cerevisiae* are single-celled fungi which that multiply by budding, or in some cases by division (fission), although some yeasts such as *Candida albicans* may grow as simple irregular filaments (mycelium). They may also reproduce sexually, forming asci which contain up to eight haploid ascospores. You can see examples of budding cells (arrow, left) (Figure 2-9) . *Saccharomyces cerevisiae* has thick-walled, oval cells, around 10 µm long by 5 µm wide. [66].

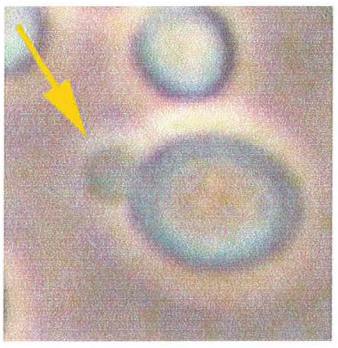


Figure 2-9 : Budding of *Saccharomyces cerevisiae* [66]

The study microorganism is *Saccharomyces cerevisiae* TISTR 5013 from the centre of conserve and collect the information of microorganism (Bangkok MIRCEN) which is reported that have high fermentation potential in sugar from degraded cassava [41] (Figure 2-10)

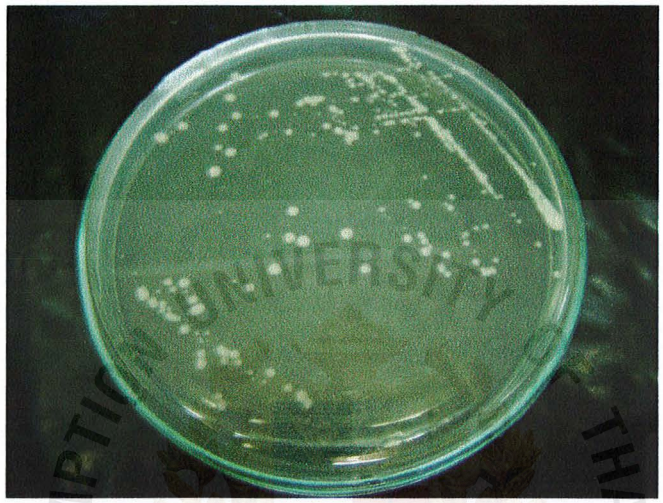


Figure 2-10 : The *Saccharomyces cerevisiae* TISTR 5013 in YM agar, incubate at 37°C for 48 hours.

Yeast extract is an important protein and vitamin source, obtain from cell yeast lysis via chemical method, physical method, exo-enzyme method or endo-enzyme inside yeast itself. After that separate debris and cell membrane out, separate all contaminated such as sugar. And concentrated or make its powder (Spray dryer). [55]

The important component of yeast extract

a) Protein

Crude protein of yeast can be found by Kjeldahl method. It can be calculate from N × 6.25 by value of N (Nitrogen) equal between 7.0-9.0 %. Protein efficiency ratio, PER, of bread yeast equal to 1.8 [42]. Amino acids, that is component of protein in yeast and yeast extract, have been shown quantity in Table 2-2

b) Vitamin

Yeast is the source of all Vitamin B. Vitamin B is derivative of purine and pyrimidine such as Vitamin B1, Vitamin B2, Vitamin B6, Niazine, and Folic acid. Vitamin B, that is complex compound of Phophyrinenucleotide, is Vitamin B12. All vitamin B have the important in activation of enzyme by derivative of many vitamin acts as Cofactor [42]. The quantity of Vitamin B in yeast and yeast extract shown in Table 2-4

Table 2-4 : The quantity of vitamin B in yeast.

Vitamin	**Dry yeast (µg/g.)**	**Yeast extract from Bread yeast (mg/g yeast extract)**
Thiamine	120.0	10-20
Riboflavin	40.0	50-100
Niazine	300.0	300-500
Pyridoxin	28.0	10.16
Pantotinic acid	70.0	*

Biotin	1.3	*
Folic acid	13.0	*
Vitamin B12	0.001	*

Remark : * : No data

From : [1] [42].

[2] [25].

Table 2-5 : The quantity of Amino acid in yeast.

Amino acid	The Quantity of Amino Acid (Percent of protein in yeast)							
	A[1]	B[1]	C[1]	D[2]	YE_1[3]	YE_2[3]	YE_1[4]	YA_2[4]
Lysine	9.4	8.1	8.2	7.4	*	*	6.92	6.51
Methionine	*	1.4	2.5	1.7	4.5	1.4	1.49	1.15
Tryptophan	1.2	*	1.2	*	1.7	0.6	*	-
Valine	7.4	5.5	5.5	4.7	6.0	5.6	6.93	6.00
Threonine	5.8	4.1	4.8	5.4	0.5	7.1	4.92	6.00
Leucine	9.0	6.6	7.9	7.7	7.0	6.2	9.16	7.66
Isoleucine	5.8	6.0	5.5	4.4	4.7	4.9	5.82	5.15
Histidine	3.5	2.8	4.0	2.2	2.3	1.8	1.89	1.39
Cysteine	1.8	*	1.6	1.3	less	less	less	less
Alanine	9.1	5.1	*	7.6	7.2	6.5	5.84	6.64
Glutamic acid	21.0	20.1	*	15.4	11.5	19.5	5.21	6.26
Aspartic acid	*	9.6	*	10.4	10.2	14.0	5.37	4.73
Proline	5.5	5.3	*	9.4	5.0	3.5	2.54	13.75

Serine	5.6	4.4	*	5.4	4.5	4.6	8.35	7.12
Phenylalanine	*	4.1	4.5	3.7	3.8	4.5	3.96	3.71
Aginine	*	8.3	5.0	4.7	1.7	3.8	4.64	1.57
Tyrosine	5.4	3.7	5.0	3.7	3.0	2.5	2.58	3.04
Glycine	5.8	4.9	*	5.4	5.5	4.4	3.21	3.31

Remark : A: *S. cerevisiae*, Univ. Food Assay

B: *S. cerevisiae*, Kockova-Kratochvilova

C: *S. cerevisiae*, Reed and Peppler, 1973

D: *Candida rugosa*

YE[1]: Yeast extract (Brewer's yeast)

YE[2]: Yeast extract (Brewer's yeast)

YA[1]: Yeast autolysate (*S. cerevisiae* RCM-Y-2465)

YA[2]: Yeast autolysate (*S. cerevisiae* RCM-Y-2465)

* : No data

From [1] [42] [2] [30]

[3] [25] [4] [6]

2.6 The process of ethanol fermentation

The process of ethanol fermentation is the process that occurred inside the cell using enzyme as accelerator of reaction by changing glucose to ethanol in anaerobic condition. The ethanol fermentation is processed by yeast by pass Glycolysis path-way or Embden-Meyerhof-Parnas path-way. Yeast will get a little energy from that path-way. From the theory, the result

of percentage ethanol after passed fermentation is 51.1 % and occurred Carbondioxide equal to 48.9 %.

In the process of fermentation, the sugar only 95 % can be transformed to ethanol, the left five percent yeast will utilize sugar for maintenance of cell and produce by-product. The by-product are Ethanol (48.4 %), Carbondioxide (46.5 %), Acetaldehyde (0-0.03 %), Acetic acid (0.05-0.25 %), Glyceride (2.5-3.6 %), Lactic acid (0-0.2 %), Succinic acid (0.5-0.77 %), Fusel oil (0.25-0.5 %) and Furfural (little bit).

2.6.1 The process of Ethanol fermentation by Bacteria.

The ethanol fermentation by bacteria almost found in anaerobic condition. The bacteria in *Clostridia* and *Sarcina* group produce ethanol by passed Glycolysis process. *Zymomonas mobilis* and *Leuconostoc mesenteroids* produce ethanol by passes Entner-Doudoroff (Figure 2-11) [20] [58].

Majority bacteria will utilize six carbon atoms sugar for Ethanol fermentation but some bacteria can produce ethanol from cellulose, eg. *Clostridium thermocellum* or produce ethanol from starch, eg. *Clostridium thermosaccharolyticum, Thermoaerobactor ethanolicus* [17].

Zymomonas mobilis is interesting and have many research because high rate in fermentation, high yield of ethanol product, resistance with high ethanol concentration, but easy to contaminate with others microorganisms.

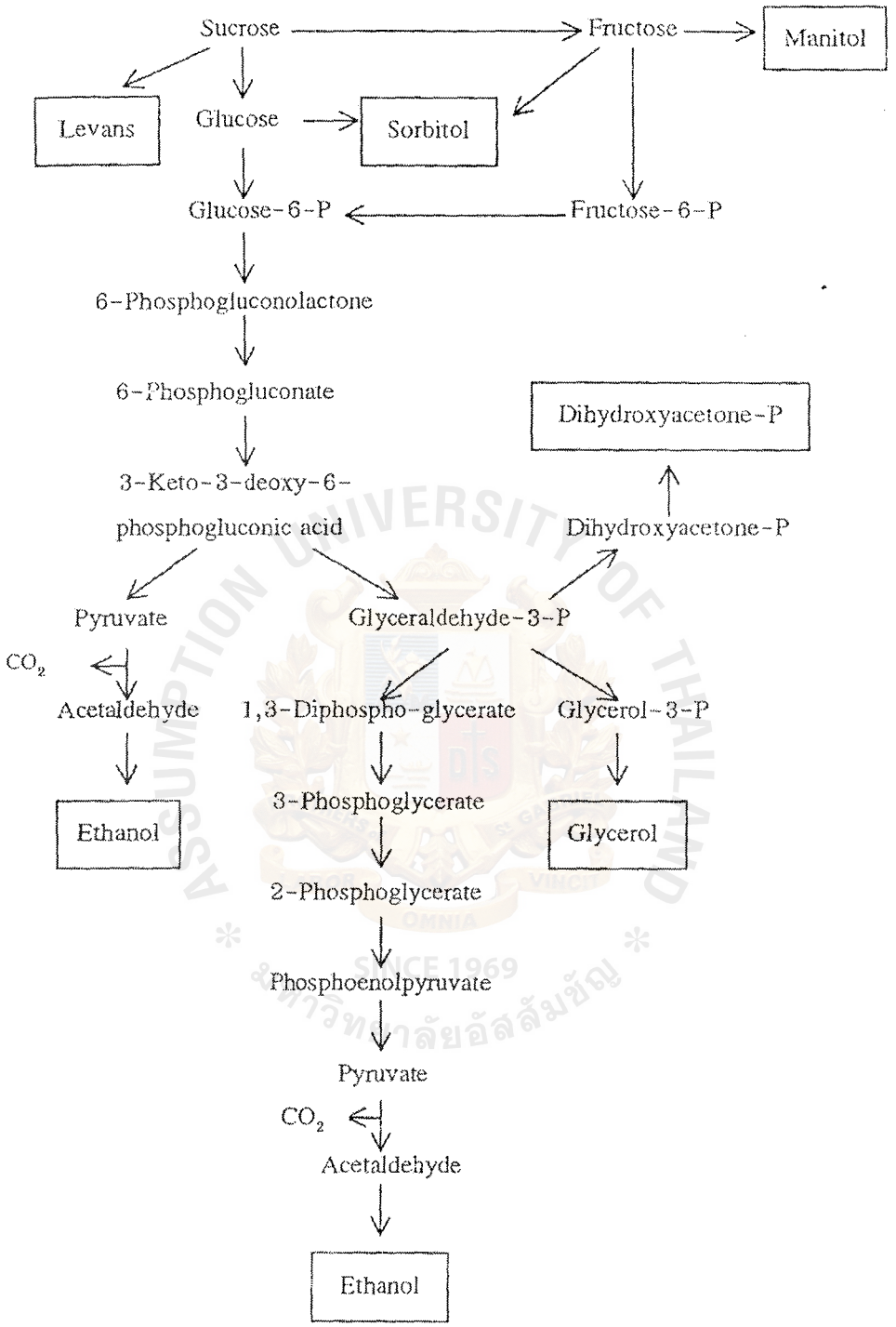


Figure 2-11 : The ethanol fermentation by *Zymomonas mobilis* by Entner-Doudoroff pathway.

2.6.2 The process of Ethanol fermentation by Yeast.

This process is occurred in the anaerobic condition by passed Glycolysis process and get Ethanol, Carbondioxide, and Energy in the form of ATP which is got less than energy that produce in aerobic condition.

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

In aerobic condition, yeast will utilize glucose in respiration process for their growth and increase cell number. The product is water, carbondioxide, and energy in form of ATP.

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6H_2O + 6CO_2 + 38ATP$$

Saccharomyces cerevisiae, Saccharomyces uvarum, Kluyveromyces fragilis can utilize six carbon atoms glucose (hexose) both single and complex form, eg. Glucose, Fructose, Galactose, Mannose, Sucrose, and Raffinose etc.

Moreover, some kind of yeast can utilize five carbon atoms (pentose). For example, *Candida utilis* can utilize Xylose. Normally, *Saccharomyces cerevisiae* cannot utilize starch and cellulose so both starch and cellulose should be passed degradation process to sugar before used in Ethanol fermentation process [27] [32].

Verma and group (2000) [59] studied the ethanol fermentation from starch in one step by compare between mix-culture process and single-

culture process. It's found that *Saccharomyces diastaticus* which is starch degradation yeast mix with *Saccharomyces cerevisiae 21*. The concentration of ethanol is 24.8 g/l or increasing 48 % when compare with *Saccharomyces diastaticus* only one strain (16.8 g/l). However, the efficiency is still lower than two steps process.

Birol and group (1998) [7] is developed *Saccharomyces cerevisiae* strain to grow and ferment from starch by genetic engineering process in which adding gene α-amylase from *Bacillus subtilis* and gluco-amylase from *Aspergillus awamori* to yeast.

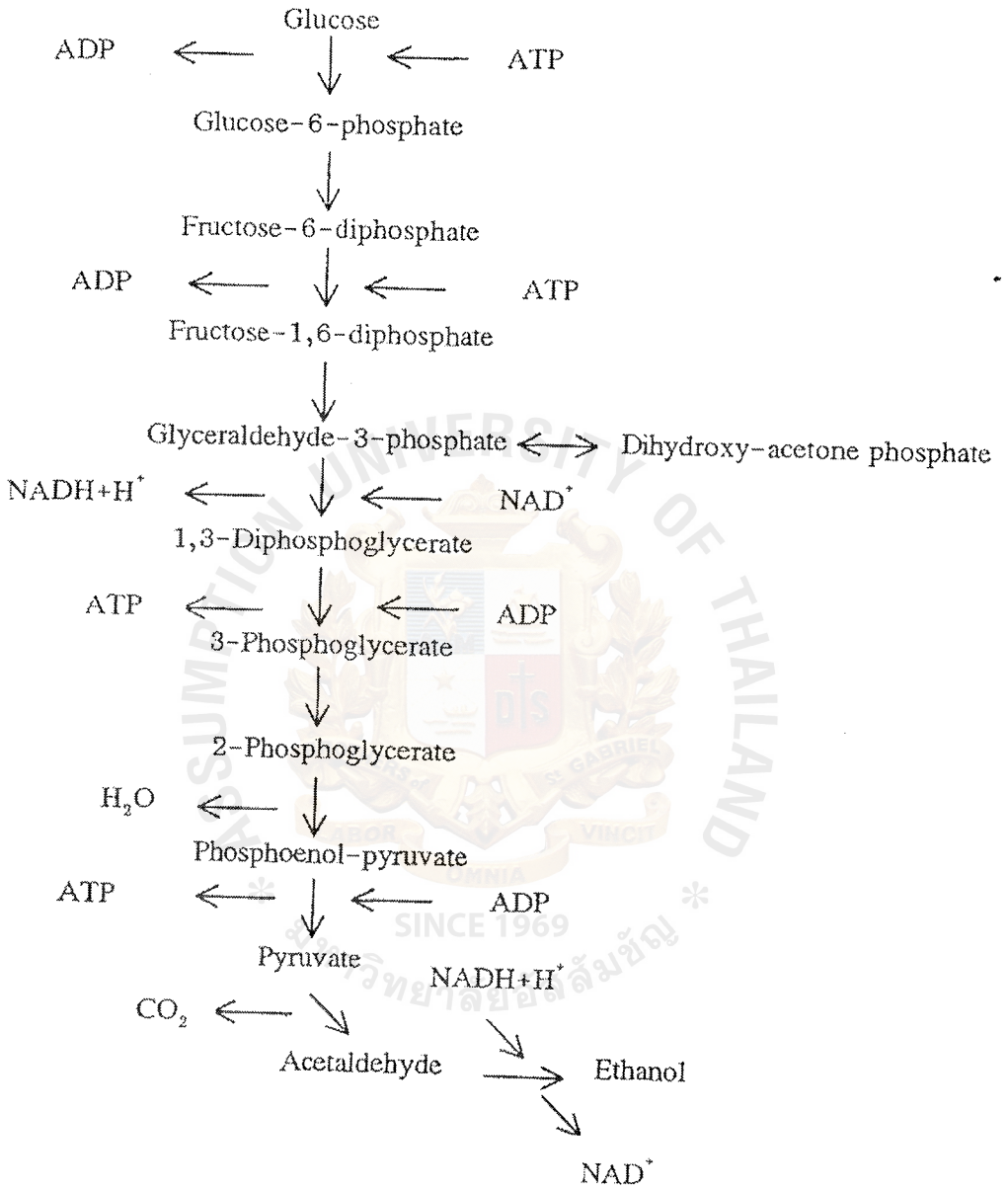


Figure 2-12 : The ethanol fermentation of yeast by Glycolysis pathway. [50]

2.7 The factors that effect to ethanol fermentation by yeast.

The high efficiency of ethanol fermentation can be set by well prepare and plan the proper process for getting high yield of ethanol. The factors that effect to ethanol fermentation are elements, feeds, ethanol concentration, amounts of culture, temperature, and pH value [23]

The preparation of inoculum and feed for fermentation needed similar food element. The different is food elements in inoculum will be more complete than feed because the important in increasing yield of culture.

Food elements for yeast can be classified as major food elements such as carbon, nitrogen, and phosphorus, etc. and minor food elements such as calcium, potassium, magnesium, and sulfur etc. Yeast needed high amount of major food elements compare with minor food elements but minor food elements is needed. Moreover, yeast needed some vitamin for their growth such as biotin, Panthotinic acid, inositol, thiamine, nicotinic acid, and pyridoxine in different amounts. [15]

2.7.1 Carbon source

Bread's yeast and yeast in ethanol fermentation can utilize glucose and fructose in same speed [51] [52] by both glucose and fructose is fermentable glucose but L-isomer sugar is non-fermentable sugar [18]. In addition, D-allulose is the important reducing sugar that can be found in molasses because yeast cannot utilize in fermentation [62].

Almost carbon source of yeast come from sugar in many form, eg. molasses, sugar cane, beet root, and whey, etc. include sugar from starch degradation, eg. corn starch, cassava starch, potato, and cellulose such as paper, rice straw, and sawdust.

2.7.2 Nitrogen source

Normally yeast can utilize nitrogen in both organic form such as amino acid, and others proteins etc and inorganic form such as ammonium salt (ammonium sulfate, ammonium phosphate). In addition, nitrogen in form of ammonia gas can be added. *Saccharomyces spp.* can utilize urea well similar to ammoniumsulfate in biotin present condition so urea is the most use as nitrogen source in case of use molasses as carbon source because molasses have high yield of biotin. For nitrate and nitrite, *Saccahromyces cerevisiae* cannot utilize as nitrogen source [43]

Saita and Slaughter (1984) [48] found that the addition of ammonia is not help in activation of ethanol fermentation directly but affect to growth of yeast and increase cell population then fermentation rate is increase also.

Somchai (2537) [53] found that using of sugar from cassava residue degradation in ethanol fermentation by *Saccharomyces cerivisiae* is not required yeast extract and ammoniumsulfate for their growth in ethanol fermentation but using of sugar from cassava residue degradation in ethanol fermentation needed the addition of yeast extract and ammoniumsulfate for their growth and ethanol fermentation. From the experiment is shown that

producing of cassava starch process have to get rid of many food elements that important for growth and ethanol fermentation of *Saccharomyces cerevisiae* which in cassava starch factory in the present day always set Decanter in the process for get rid of proteins and lipid out from starch. [26]

2.7.3 Phosphorus source

Normally phosphorus for yeast will be in form of phosphate such as diammonium hydrogen phosphate, potassium dihydrogen phosphate. Phosphate is the important element for ethanol fermentation process. Yeast will utilize phosphate in producing energy (ATP) process, nucleoprotein producing and others substances inside cells. The splitting of phosphate will be led to buffer condition which is maintenance stable pH. In case of lacking phosphate, cells will be weak. [46]

2.7.4 Sulfur source

Sulfur is the component of yeast cells. (approximately 0.4 % of dry weight) Methionine is well source of sulfur for yeast utilization and yeast still can use sulfate salt by reduce to methionine in cells in form of ammoniumsulfate which is cheap and have ammonium particle that yeast can utilize as nitrogen source. Normally in industrial factory can add sulfur by report that thiosulphate, sulphite, and selenate will inhibit the utilization of sulfate. For the utilization of sulfur amino acid will be inhibited by ethionine and methionine sulphone [44]

2.7.5 Magnesium and Calcium source

Magnesium is important for growth and ethanol fermentation process by particle of magnesium is cofactor of many enzymes in Glycolysis. For particle of calcium have effect with growth and ethanol fermentation too. At low concentration of sugar and particle of magnesium and calcium is low, it will not effect to rate of ethanol fermentation. [44]

2.7.6 Sugar concentration.

In the fermentation condition that have high sugar concentration can be reduced contamination well but high sugar concentration has the effect in inhibit of growth and ethanol fermentation. The one reason of inhibition come from osmosis pressure which lead to cell plasmolysis when cultivate in feed that contain more than 14 % sugar by weight. For sugar concentration, that can inhibit fermentation, is the specific characteristics of species. If sugar concentration more than 14 % by weight, fermentation rate will start to reduce but inhibition effect from high sugar concentration is less than ethanol and inhibition effect of high concentration of sugar and high concentration of ethanol are combine factor that lead to more inhibition of fermentation process. [49]

Thatipamala and group (1992) [56] studied the effect of substrate concentration to ethanol product in Batch fermentation with *Saccharomyces cerevisiae* and found that increasing of substrate concentration from 150 g/l

to 280 g/l, led to decreasing ethanol production from 0.45 g. ethanol/ g. substrate to 0.30 g. ethanol/ g. substrate.

2.7.7 Ethanol concentration.

The growth and ethanol fermentation can be inhibited by ethanol. Ethanol 1-2 % by weight effect to growth rate (decrease) and the growth will be stopped when ethanol reach 4.7-7.8 % by weight but still ferment until 14 % by weight. Yeast cannot grow then ethanol fermentation rate will be reduced because ethanol effect to enzyme and shape of cells [49].

The growth inhibition and ethanol fermentation may be occurred from the effect of alcohol dehydrogenase and hexokinase which is enzyme in Glycolysis pathway. The hypothesis had set that "Ethanol had effect to shape of cells by damage cell membrane and changing property".

Aiba and group (1968) [1] reported that ethanol effect to non-competitive Michaelis's Menten inhibition of yeast but the studied of Brown and group (1981) [9] said that growth inhibition is complicated and growth inhibition is not non-competitive Michaelis's Menten inhibition. The addition of ethanol in exponential phase lead to reduction of growth rate [21]

Luong (1985) [31] found that growth rate and ethanol fermentation will reduce when ethanol is produced and reduce in direct ratio with ethanol concentration. The most resistance with ethanol is *Saccharomyces cerevisiae.* There will be set hypothesis that "the resistance of ethanol is depends on component of phospholipids at cell membrane in which full of unsaturated

fat will support *Saccharomyces spp.* and *Kluyveromyces lactis* to more resist with ethanol.

Watson (1982) [60] reported that "*Saccharomyces cerevisiae*, that contain unsaturated fat as component in phospholipids of cells membrane, can resist with high ethanol concentration by cells, that contain low ergosterol but contain high unsaturated fat, can produce ethanol 13-15.5 % by weight. There are reported that "yeast that cultivate in feed with linoleic acid ($C_{18:2}$) will more resist to ethanol than cultivate in feed with oleic acid ($C_{18:1}$). It's expected that unsaturated fat help in excretion of ethanol from cells leading reduce toxic of ethanol in cells.

2.7.8 Yeast cells concentration.

Nishizawa and group (1983) [37] studied continuous ethanol fermentation by control cells density at 85 g/l with Hollow fibers and found that maximum ethanol producing rate is 27 g/l.hour.

Lee and Chang (1987) [29] studied Cell recycle ethanol fermentation by yeast density more than 40 g/l which high density will lead to growth and ethanol producing inhibition. This inhibition is direct ratio to cells concentration which maximum concentration that lead to growth and ferment inhibition are 225 and 640 g/l respectively. And so in the high density condition leading to higher ethanol fermentation rate but heat and carbondioxide are released by high density of cells including cells needed more air and food elements. So in the high cells density process should be thought especially about energy transfer and mass.

2.7.9 Temperature

Saccharomyces spp. is mesophilic strain and maximum growth rate at medium temperature. The growth will be stopped at 0 degree celcius and minimum temperature, that can grow, is 5-10 degree celcius. The resistance of cells to temperature will be increase when cells grow in completely medium. The suitable temperature for fermentation is more than growth temperature around 5-10 degree celcius [45]

Under the fermentation condition in bioreactor, the temperature will increase because releasing of energy (amount depends on kinds of substrate). So in industrial should control by cooling system for reduce temperature in the bioreactor.

2.7.10 pH value

The range of pH, that *Saccharomyces cerevisiae* can grow, is 2.4-8.6 but the suitable pH value is 4.5. The efficiency of ethanol fermentation from sugar will not change in range 3.5-6.0 pH. And ethanol fermentation from sucrose will more active to pH changing than glucose because invertase will change at lower pH (the efficiency in ethanol fermentation of cells will reduce when low pH).

Moreover, the ethanol fermentation at pH 4.5 will support fermentation and reduce contamination from others bacteria because almost bacteria can grow well in neutral pH.

2.7.11 Oxygen

Oxygen act as terminal electron acceptor in respiratory chain and act as Growth factor of yeast in which involved in synthesis process of unsaturated fatty acid, eg, oleic acid and linoleic acid etc., that lead to more ethanol resistance and support transfer of ethanol from cell to outer. So if have high quantity of unsaturated fatty acid in cell membrane, high quantity ethanol can be transferred to outer too then ethanol concentration in cell is reduce. For anaerobic condition, yeast cannot produce unsaturated fatty acid then the addition of unsaturated fatty acid is needed for survive.

In the culture preparation, oxygen is needed because of high growth in aerobic condition but ethanol fermentation rate is reduced. Oxygen supports Pasteur effect which leading to complete glucose oxidation and produce carbondioxide and water from TCA cycle (Tricarboxylic acid cycle) and respiratory chain. However respiratory of yeast still be controlled with carbohydrate concentration in feed medium that made ethanol fermentation from yeast although air addition. This condition can be called Reverse Pastuer effect or Counter-Pastuer effect or Crabtree effect which is one kind of Glucose effect. In the present day called Catabolite repression. This condition can be occurred by high carbohydrate in feed medium. From the experiment found that " if the concentration of glucose or sucrose is higher than 0.02-0.1 % by weight, the respiration was severe inhibited although the

air had present. The respiratory still occurred only 5-10 % of condition of glucose that is lower than 0.02 % by weight. [49]

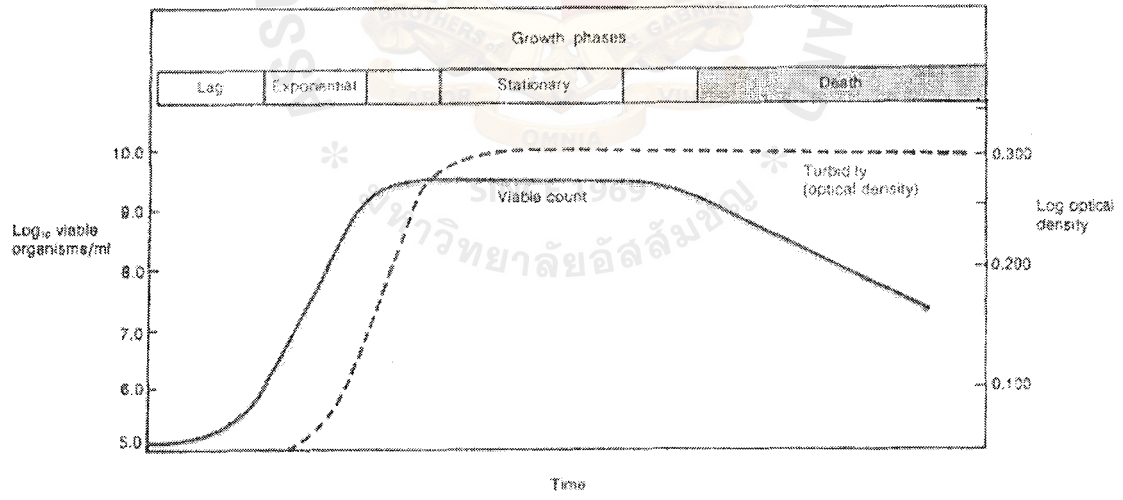
Normally yeast will use oxygen in respiratory for growth so in continuous fermentation should be add some air between fermentation for increase cells number instead of dead cells. In addition, in the experiment found that "The addition of little air lead to more utilization of glucose and yeast has higher capacity to ethanol resistance compare with no addition of air at all. [21] [47]

2.7.12 Carbondioxide

Carbondioxide affect growth of yeasts in both aerobic condition and anaerobic condition. At high pressure of carbondioxide will occur severe inhibition of growth and fermentation. Carbondioxide has effect to inhibit decarboxylation reaction and effect to cell membrane (transportation capacity change).

2.8 Batch culture

Batch culture is a closed culture system which contains an initial, limited amount of nutrient. The inoculated culture will pass through a number of phases, as illustrated in Figure 2-13. After inoculation there is a period during which it appears that no growth takes place; this period is referred to as the lag phase and may be considered as a time of adaptation. In a commercial process the length of the lag phase should be reduced as much as possible and this may be achieved by using a suitable inoculum. Following a period during which the growth rate of the cells gradually increases, the cells grow at a constant, maximum, rate and this period is known as the log, or exponential, phase. [40]



Typical growth curve for a bacterial population.

Figure 2-13 : Growth of a typical microbial culture in batch conditions. [71]

2.9 Relevant research

Chareonrat T. [57] had studied on the ethanol production from cassava as raw material including the proper formula for growth and ethanol fermentation of *Saccharomyces cerevisiae* TISTR 5013. The proper formula for growth and ethanol fermentation needed to add diammoniumhydrogenphosphate and yeast extract for support growth and ethanol fermentation. Yeast extract will support growth better than sugar residue because yeast extract can act as vitamin source and nitrogen source.

The study of ethanol fermentation (batch fermentation) of *Saccharomyces cerevisiae* TISTR 5013 in fermentor found that cassava that degraded to sugar via enzyme method can use as starter medium for growth and ethanol fermentation but via direct acid hydrolysis gave more production of sugar. So the direct acid hydrolysis gave more efficiency than enzyme method. Because in the small unit of cassava is glucose which large unit is cellulose.

W. Borzani and C.H. Jurkiewicz [70] had studied on variation of the ethanol yield during very rapid batch fermentation on sugar cane blackstrape molasses. During rapid ethanol fermentation (2-3 h) of sugar-cane blackstrap molasses, a significant increase in the ethanol yield was frequently observed as fermentation proceeded, eventually leading to yields higher than the theoretical value when the end of the process was approached. In order to explain the above facts, three assumptions were examined: 1. temporary ethanol accumulation within the yeast cells; 2. variation of the dry matter

content of the microorganism density during the fermentation; 3. transformation of sugars into undetectable extra-cellular fermentable compounds at the initial stages of the process. Based on the experimental results presented here, the third of the above assumptions seems to explain the observed increase in the ethanol yield.

CHAPTER 3

RESEARCH AND METHODOLOGY

This research was an experimental research to study the effect of lignocellulosic degradation (produced from pineapple (*Ananus comosus*) wastes obtained from market) on Batch ethanol fermentation by *Saccharomyces cerevisiae* TISTR 5013. In addition, the studies included, the waste analysis, the cultivation of *Saccharomyces cerevisiae* TISTR 5013, Conversion of cellulosic material to sugar via acid hydrolysis, Glucose analysis via DNS, Salt analysis via Mohr titration, Separating of Salt and Sugar, and Ethanol analysis. This will lead to waste recycle.

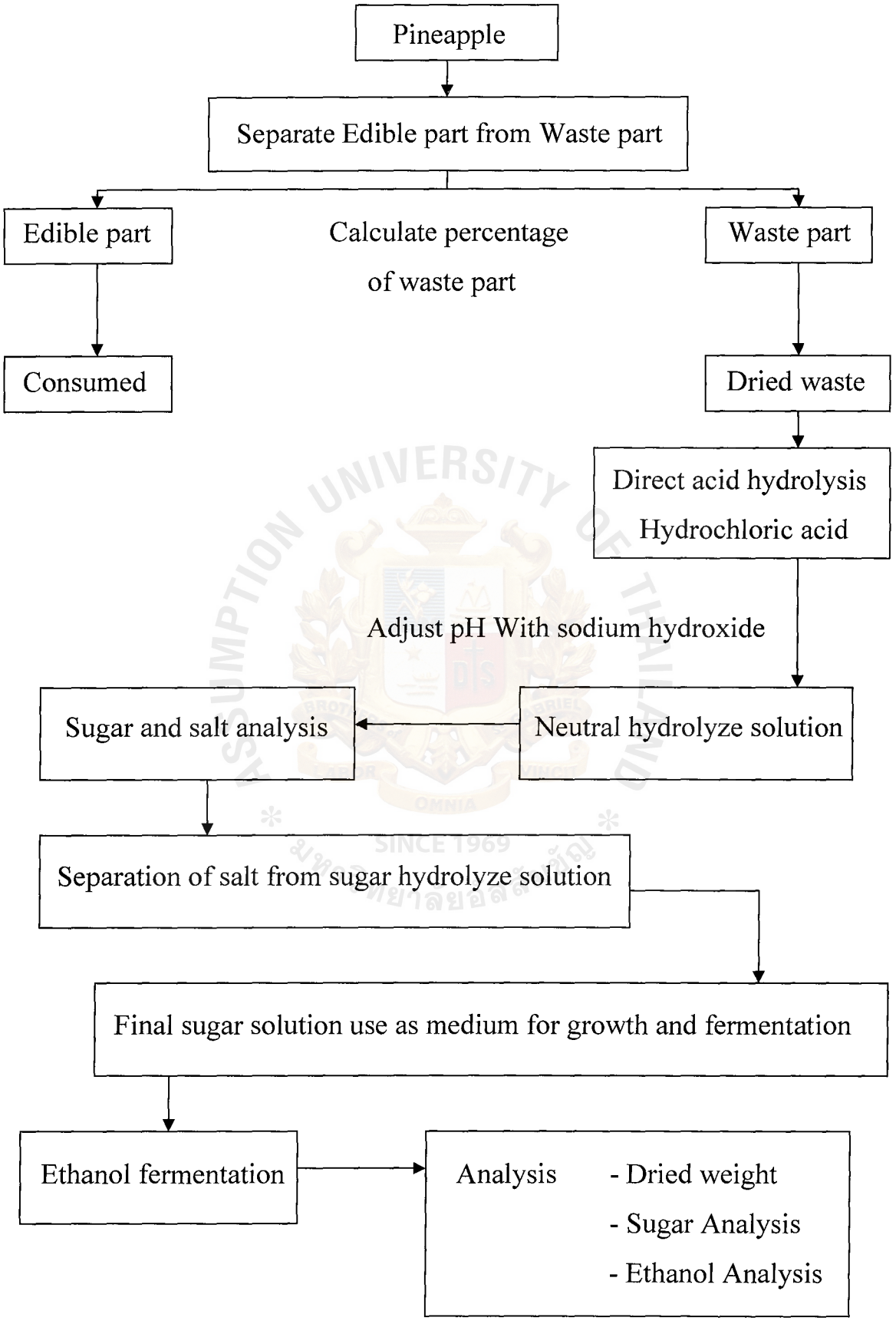


Figure 3-1 : Experimental Procedure Chart

3.1 Experimental locations

3.1.1 Raw material preparation (Pineapple waste) was conducted at Mod pineapple shop, Nongmon market, Chonburi province.

3.1.2 Waste acid hydrolysis and its chemical analysis were conducted at Laboratory, Faculty of Biotechnology, Assumption University.

3.1.3 *Saccharomyces cerevisiae* TISTR 5013 stock culture was taken from the centre of conserve and collect the information of microorganism (Bangkok MIRCEN).

3.1.4 Ethanol fermentation and its analysis were performed at Laboratory, Faculty of Biotechnology, Assumption University.

3.2 Chemical reagents and equipments

3.2.1 Chemical reagents

All chemical reagents used were reagents grade. For dilution of all chemical, distilled water was used

1) Hydrochloric acid (HCL); Conc.
2) Sodium hydroxide (NaOH); 10 % concentration
3) 3-5 Dinitrosalicyclic acid ($C_7H_4N_7O_7$) (Appendix A)
4) Sodium potassium tartrate
5) Silver nitrate ($AgNO_3$); 0.1 M (Appendix A)
6) Potassium chromate indicator (K_2CrO_4); 5 grams in 100 milliliters (Appendix A)

7) Diammoniumhydrogenphosphate; 1 grams per liters (Appendix C)
8) Yeast extract; 1 grams per liters (Appendix C)
9) Isopropanol.; Conc. (Appendix A)
10) Yeast extract malt extract agar slant (YM)
11) Yeast extract malt extract broth (YM broth)
12) Sugar extract medium from pineapple waste

3.2.2 Equipments

1) Hot air oven
2) Autoclave (Hirayama, Model HA 300MII)
4) Incubator (Jouan, EB 280)
5) pH meter
6) Electrical Balance
7) Hot plate
8) Centrifuge
9) Water Bath
10) Dessicator
11) Thermometer
12) Glassware
13) 10×10 cm. transparent glass plate
15) Stirring rod
16) Plastic bag
17) Titrimetric apparatus
18) Micropipette
19) Test tube
20) Circular block
24) Stop watch

25) Petri dish

26) Forcep

27) Loop

32) Parafilm

33) Fermentor.

34) Spectrophotometer.

35) Ebulometer.

36) Shaker.

37) Laminar flow.

38) Erlenmeyer flask

39) Volumetric flask

40) Cuvette

41) Burette

42) Beaker

43) Cylinder

3.2.3 Microorganism:

The study microorganism is *Saccharomyces cerevisiae* TISTR 5013 from the centre of conserve and collect the information of microorganism (Bangkok MIRCEN) which is reported that have high fermentation potential in sugar from degraded cassava. [41]

3.3 Raw Material

Pineapple wastes (*Ananas comosus*) for this study was supported by Mod pineapple shop; Nongmon market, Chonburi province. (Figure 3-2)

Figure 3-2 : Raw Pineapple from Mod pineapple shop; Nongmon market, Chonburi province.

3.4 Experimental Procedure.

This study could be divided into two main parts. First part was concerned in the percentage waste analysis, the degradation of pineapple waste (Skin and leaf part) to sugar units and its compositions analysis

(sugar and salt analysis), including the separation salt from sugar solution after neutralize with sodium hydroxide. While the second one was concerned in the ethanol fermentation from the obtained sugar solution and its analysis include, dried weight, sugar analysis, and ethanol analysis.

3.4.1 The percentage waste, degradation, and chemical analysis of pineapple waste and desalting

3.4.1.1 Percentage waste analysis

The whole pineapple was weighed on balance then the pineapple was peeled. The edible part and waste part was weighed and the percentage of useful part and waste part could be analyzed.

3.4.1.2 Digestion of pineapple waste using hydrochloric acid.

The pineapple waste degradation procedure was adapted from the protocol following alteration of AOAC (1990) and the study of Charoenrat T. [57]

Weigh pineapple waste around 14 g. in 250 ml. beaker and with 150 ml. distilled water and 25 ml. concentrate hydrochloric acid. The mixture was refluxed for two hours then leaving cool down. The hydrolyze solution was adjusted pH to neutral with sodium hydroxide, filtrated with filter and brought to find sugar quantity with DNS (dinitrosalicylic acid assay) analysis and salt analysis via MOHR titration.

The amount salt was separated out via selective precipitation methods then the feed sugar could be obtained in the final solution.

3.4.1.3 The analysis of sugar via the dinitrosalicyclic acid assay.

The analysis amount of glucose : the supernatant was diluted and analyze amount of glucose by use glucose as standard substance (Appendix A). The sample was pipette 0.2 ml. to test tube and add DNS solution two ml. After that dipped in boiling water for ten minutes and dipped in cooling water for two minutes. The solution was measured the optical density (OD) at 570 nanometers [57] via spectrophotometer then compare with sugar standard curve in range 1 mg./ml. (blank use water) [10].

Remark : The standard curve of sugar prepare following table 3-1

Table 3-1 : The preparation of sugar standard curve in range 1 mg./ml

Concentration = milligrams per milliliters
Stock sugar solution = milliliters
Deionized water = milliliters

Concentration	Stock sugar solution	Deionized water
0.0 (Blank)	0	5
0.2	1	4
0.4	2	3
0.6	3	2
0.8	4	1
1.0	5	0

3.4.1.4 The analysis of salt via the MOHR titration.

The analysis amount of salt : the supernatant was diluted and analyze amount of salt by titration (Appendix A). The dilution of brine was done by dilute 5 ml. of the brine to 250 ml. in a volumetric flask. And the titration step was done by pipette 25 ml. of the diluted brine into a 250 erlenmeyer flask and added 1 ml. of potassium chromate indicator. The titration was titrated with 0.1 M Silver nitrate solution until a distinct reddish-brown color appears and persists on brisk shaking. The analysis was repeated to obtained concordant results. [22]

3.4.1.5 The separation of Sugar and Salt.

The separation of Sugar and salt could be performed by the selective precipitation method. The solution was evaporated as much of the water as you could without precipitating one or the other compounds and slowly added a volatile organic solvent. A reasonable one might be acetone, but ethyl alcohol, or isopropanol, might also do. The salt should be much less soluble than the sugar in the blended solvent mixture and you may see “clounds” of salt form as you approach the solubility of salt in the mixed solvent. After that filter the precipitate then mixed solution was separated and analyzed the final solution again by Dinitrosalicyclic acid assay and MOHR titration. (Appendix A) [69]

3.4.2 The studying of growth and alcohol fermentation batch type fermentor.

3.4.2.1 Preparation of culture in flask for studying batch type fermentor.

Yeast was subcultured from YM slant one loop to 15 ml. YM broth in 250 ml Erlenmeyer flask size and cultivated on shaker 250 rpm, temperature 30°C for 24 hour, then subcultured in 250 ml media that proper for growth. in Erlenmeyer flask 500 ml. size. Shake on shaker 250 rpm., temperature 30°C for 24 hours.

3.4.2.2 The studying of growth and ethanol fermentation in batch type.

Yeast was subcultured from flask, 25 ml. to fermentor 125 ml. size which contain the media that proper for growth 100 ml., cultivate at 30°C. Cultivate for 72 hours, between cultivation collect the sample every 2-4 hours to analyze cell dry weight, glucose amount, and ethanol amount. (fermentor was performed 10 bottles ; each bottle used only once per one time check sample)

3.4.2.3 Cell dry weight

The sample in every period was spun in centrifuge, collect the supernatant to analyze amount of glucose and ethanol, the pellet part was cleaned with deionized water two times and dried at 105°C for 24 hours for finding cell dry weight. (Appendix A).

3.4.2.4 The analysis amount of glucose

The analysis amount of glucose : the supernatant was diluted and analyze amount of glucose by use glucose as standard substance (Appendix A). The sample was pipetted 0.2 ml. to test tube and add DNS solution two ml. After that dipped in boiling water for ten minutes and dipped in cooling water for two minutes. The solution was measured the optical density (OD) at 570 nanometers [57] via spectrophotometer then compare with sugar standard curve in range 1 mg./ml. (blank use water)

Remark : The standard curve of sugar prepare following table 3-1

3.4.2.5 The analysis amount of ethanol.

The supernatant was diluted and analysed amount of ethanol by use Ebulometer. (Appendix A).The boiling chamber of ebulliometer was rinsed (Figure 3-3) with some of the sample to be tested and drain through the outlet tap then fill the boiling chamber with approximately 50 ml of the sample, either using a pipette or the provided measuring cylinder. The condenser jacket was filled with cold water and inserted the special thermometer into the boiling chamber via the rubber stopper. The heat was applied as shown in Figure 3-3 and the temperature will naturally rise as indicated by the thermometer. When the temperature first remains steady for at least 30 seconds record the temperature as T_1. Repeat the above procedure using approximately 25 ml of distilled water instead of the 50 ml. of sample. Record this temperature as T_2. It is not necessary to have water in the condenser when measuring the boiling point of pure water (T_2). The alcohol concentration can be read by the ebulliometer chart and record. [67]

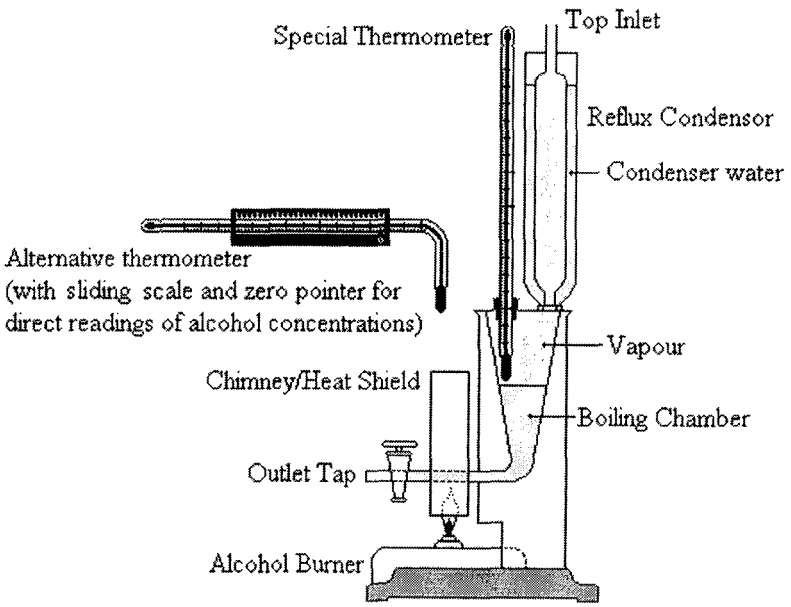


Figure 3-3 : The ebulliometer component [68]

3.5 The period in research.

Research start : March 30, 2004

Research end : October 15, 2004

Activities	Mar.	Apr.	May.	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Planing	↔									
Implement (Proposal preparation)	↔									
Buying things	↔	↔								
Waste analysis		↔	↔							
Waste degradation			↔	↔						
Chemical analysis of medium			↔	↔	↔					
Desalting				↔	↔					
Fermentation					↔	↔	↔	↔		
The study of growth, glucose, and ethanol					↔	↔	↔	↔		
Project book preparation		↔	↔	↔	↔	↔	↔	↔	↔	
Presentation									↔	↔

CHAPTER 4

RESULTS AND DISCUSSION

4.1 The percentage, degradation, and chemical analysis of pineapple waste and desalting.

4.1.1 The percentage of waste analysis

The whole pineapple was weighed on balance then the pineapple was peeled. The edible part and waste part was weighed and the percentage of useful part and waste part can be analyzed. And the result was performed as following table 4-1

Table 4-1 : The percentage of whole pineapple, edible part, and waste part analysis

Trial and average = Percentage

Pineapple	Trial			Average
	1	2	3	
Whole	100	100	100	100
Edible part	50.67	47.39	47.51	48.52
Waste part	49.33	52.61	52.49	51.48

(Appendix B)

From the table 4-1, the percentage of waste pineapple was analyzed by weight out waste from edible part and calculate the percentage from

whole weight as one hundred percent. The result got that almost percentage of pineapple waste is around 49 – 52 % but the average was around 51.48 % which quite similar to some of scientist that used to study the pineapple waste analysis. Muller (1987) [36] reported that "Residue of pineapple was by-product from remaining agricultural materials which, classified to three types, were residue from industrial factory, residue from harvesting, and residue from market. The ratio of all residues was approximately 56 %. So the waste from pineapple was around half-half with flesh or edible part which the half of waste was bio-waste that made the environmental problems. In each year pineapple was exported 1,500,000 to 2,589,000 tons [12] in which the half of this pineapple will be bio-waste. This bio-waste occurred a lot each year so that why this research try to find new way to get rid of these bio-waste or transform to advantage thing which is ethanol production, that is one way to solve this problem, because pineapple waste contain of many cellulose and hemicellose (Table 2-3) [61] that is large unit of glucose, the carbon source for yeast to grow and produce ethanol.

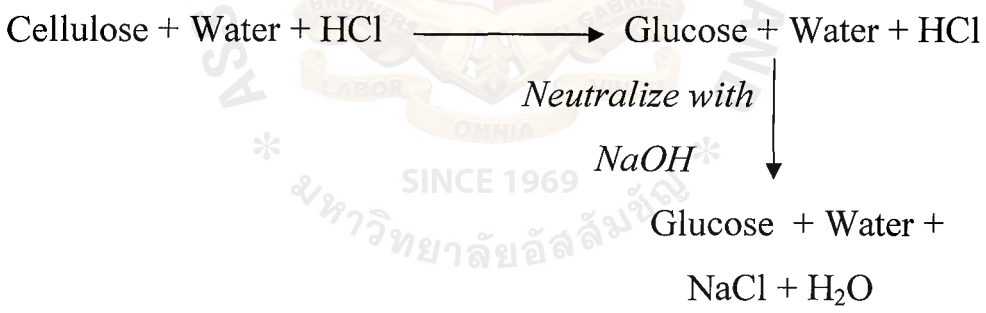
4.1.2 The degradation of pineapple waste using hydrochloric acid

The pineapple waste degradation procedure was adapted from the protocol following alteration of AOAC (1990) and the study of Charoenrat T. [57]

The experiment was conducted by weigh pineapple waste around 14 g. in 250 ml. beaker and with 150 ml. distilled water and 25 ml. concentrate hydrochloric acid. The mixture was refluxed for two hours then leaving cool down. The hydrolyze solution was adjusted pH to neutral with sodium hydroxide, filtrated with filter and brought to find

sugar quantity with DNS (dinitrosalicylic acid assay) analysis and salt analysis via MOHR titration. The amount salt was separated out via selective precipitation methods then the feed sugar can be obtained in the final solution.

The dried pineapple waste, 14 g., was added to 250 ml. beaker with 150 ml. distilled water and when added 25 ml. hydrochloric acid the solution was turn light yellow and the mixture was refluxed for two hours. The solution was turned brown and dark brown until nearly black. Then leave its cool down and the hydrolyze solution was neutralized by sodium hydroxide. The neutralize solution was filtered and the neutralize solution was analyzed sugar and salt because from the chemical equation hydrochloric acid plus sodium hydroxide will get sodium chloride and water as final product. (Figure 4-1)



Remark : *HCl* = *Hydrochloric acid*
NaCl = *Sodium chloride*
NaOH = *Sodium hydroxide*
H_2O = *Water*

Figure 4-1 : The chemical equation of direct acid hydrolysis of cellulose

From the equation in figure 4-1, the final neutralize solution got glucose as starter medium for yeast utilization as carbon source but we got sodium chloride also which sodium chloride is salt that can inhibit growth of yeast in fermentor if using as medium so this salt should be remove out from the medium solution. From the theory of osmotic pressure, Microbes contain approximately 80-90% water and if placed in a solution with a higher solute concentration will lose water which causes shrinkage of the cell (plasmolysis). However, some bacteria have adapted so well to high salt concentrations that they actually require them for growth. These bacteria are called halophiles (salt-loving) and are found in salterns or in areas such as the Dead Sea. But *Saccharomyces cerevisiae* TISTR 5013 is not halophile or halo-tolerant so it cannot grow in solution that contain high salt. So the neutralize solution should be analyze the salt and separate from medium solution.

4.1.3 The analysis of sugar via the dinitrosalicyclic acid assay.

The analysis amount of glucose : the supernatant was diluted and analyze amount of glucose by use glucose as standard substance (Appendix A). The sample was pipetted 0.2 ml. to test tube and add DNS solution two ml. After that dipped in boiling water for ten minutes and dipped in cooling water for two minutes. The solution was measured the optical density (OD) at 570 nanometers via spectrophotometer then compare with sugar standard curve in range 1 mg./ml. (blank use water)

Remark : The standard curve of sugar prepare following table 3-1

The standard curve was measured the OD_{570} (average) following table 4-2 and plotted curve following figure 4-2.

Table 4-2 : The Optical Density of standard curve of stock sugar concentration (1 mg./ml.)

Absolute amount = milligrams

Concentration = milligrams per milliliters

Optical density 570 (OD_{570}) = nanometers

Absolute amount	Concentration	Optical density 570 (Average)
0.00	0.0	0.0000
0.04	0.2	0.053
0.08	0.4	0.324
0.12	0.6	0.583
0.16	0.8	0.804
0.20	1.0	1.052

(Appendix B)

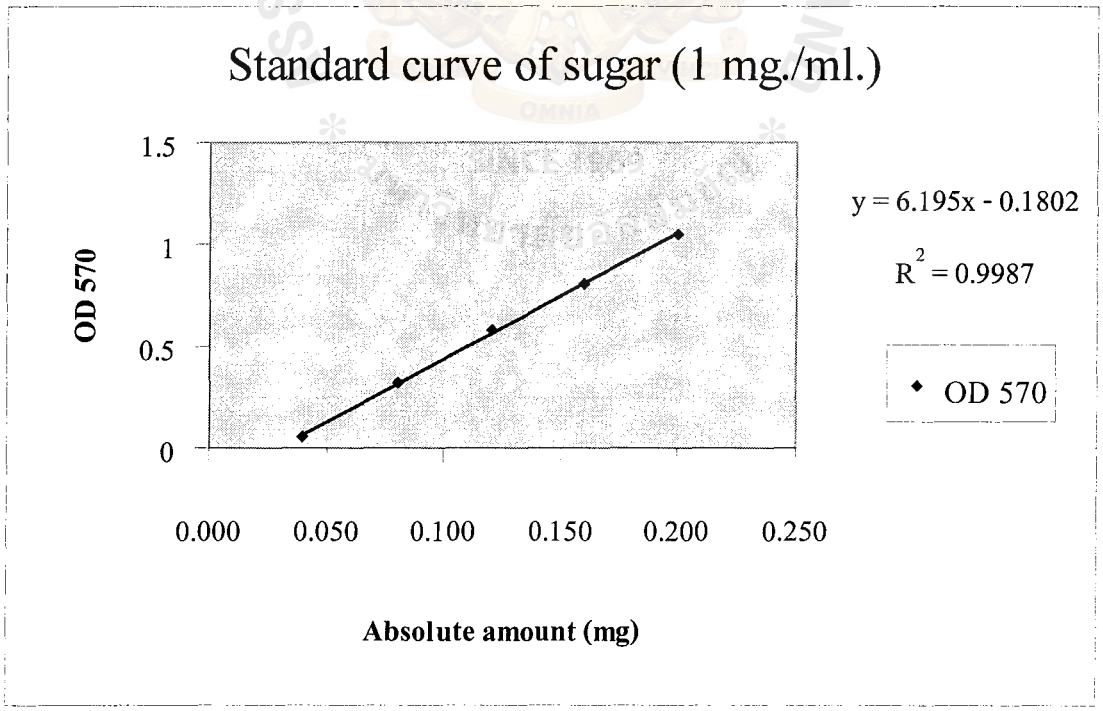


Figure 4-2 : The standard curve of stock sugar solution concentration (1 mg/ml.)

The stock sugar solution concentration (1 mg./ml.) can be measured OD_{570} and plotted curve as figure 4-2 which illustrated the equation

$$y = 6.195x - 0.1802$$

$y = OD_{570}$ (nanometers)

x = Absolute amount of sugar (milligrams)

From this standard curve, the equation could be interpreted the quantity of sugar unknown by measure the optical density at 570 nanometers and bring the OD_{570} value subscribe in y and calculate x from equation then the absolute amount milligrams glucose equivalent of unknown sugar will present. Because the sample was taken only 0.2 milliliters so compare back to one milliliter by divided by 0.2 milliliters after that times back dilution factor. The value is amount of glucose grams per liters. (Appendix A)

The R square is the coefficient of variation in y explained by variation in x which means optical density 570 nanometers is explained by absolute amount of glucose in milligrams. R is the coefficient of correlation which study on closeness of relationship between dependent and independent variables. The standard curve had correlation close to 1 (Figure 4-2) that means there's nearly perfect positive linear correlation or optical density 570 nanometers had relationship with absolute amount of sugar in milligrams.

Table 4-3 : The sugar analysis of neutralized solution.

Absolute amount of glucose = milligrams glucose equivalent
Compare to one milliliters = milligrams glucose equivalent
Amount of glucose = grams per liters

Sample	OD_{570} (Average)	Absolute amount of glucose	Compare to one ml.	Amount glucose
Sugar from pineapple waste at 10^{-2} dilution	0.207	0.06250	0.3125	31.25

(Appendix B)

From table 4-3, the result was shown that amount of glucose that extracted from pineapple waste equal to 31.25 grams per liters so the starter glucose in the medium for fermentation is 31.25 grams per liters. The dilution that was chosen to calculate is 10^{-2} dilution because from the (Appendix B) (Table B-3) is shown the optical density in each dilution from 10^{0} to 10^{-2} and the proper optical density value should be around 0.002 to 1.200.

4.1.4 The analysis of salt via the MOHR titration.

The analysis amount of salt : the supernatant was diluted and analyze amount of salt by titration (Appendix A). The dilution of brine was done by dilute 5 ml. of the brine to 250 ml. in a volumetric flask. And the titration step was done by pipette 25 ml. of the diluted brine into

a 250 erlenmeyer flask and added 1 ml. of potassium chromate indicator. The titration was titrated with 0.1 M Silver nitrate solution until a distinct reddish-brown color appears and persists on brisk shaking. The analysis was repeated to obtained concordant results. The percentage of salt in neutralized solution was shown in table 4-4.

Table 4-4 : The salt analysis in neutralized solution

Trial, Average salt analysis = Percentage

Sample	Trial			Average
	1	2	3	
Neutralized solution	9.243	9.243	9.243	9.243

(Appendix B)

From the table 4-4, the result was shown that in neutralized solution had salt 9.243 % (m/v) which correct with chemical equation (Figure 4-1) that salt would be form after add sodium hydroxide to hydrochloric acid. As the medium for yeast will inhibit growth and fermentation if the medium contain high salt then salt in this neutralized solution should be removed by selective precipitation method.

4.1.5 The separation of Sugar and Salt.

The separation of Sugar and salt can be performed by the selective precipitation method. The solution was evaporated as much of the water as you could without precipitating one or the other compounds and slowly added a volatile organic solvent. A reasonable one might be acetone, but ethyl alcohol, or isopropanol, might also do. The salt should

be much less soluble than the sugar in the blended solvent mixture and you may see "clounds" of salt form as you approach the solubility of salt in the mixed solvent. After that filter the precipitate then mixed solution was separated and analyzed the final solution again by Dinitrosalicyclic acid assay and MOHR titration (Appendix A)

Table 4-5 : The sugar analysis of neutralized solution after separation.

Absolute amount of glucose = milligrams glucose equivalent
Compare to one milliliters = milligrams glucose equivalent
Amount of glucose = grams per liters

Sample	OD_{570} (Average)	Absolute amount of glucose	Compare to one ml.	Amount glucose
Sugar from pineapple waste at 10^{-2} dilution	0.194	0.0604	0.302	30.20

(Appendix B)

Table 4-6 : The salt analysis in neutralized solution after separation

Trial, Average salt analysis = Percentage

Sample	Trial			Average
	1	2	3	
Neutralized solution	0.117	0.117	0.0585	0.0975

(Appendix B)

From the table 4-5 and 4-6 were shown that neutralized solution that contained both sugar and salt had change. The amount of sugar before separation was 31.25 grams per liters and after separation was 30.20 grams per liters which was lost 1.05 grams per liters. The weight lost of sugar, 1.05 grams per liters, might be occurred from not all sugar solution would dissolve to isopropanol but 1.05 grams per liters still not dissolve to isopropanol. Or the sugar, that got from evaporation of isopropanol, was attached to the wall of glassware. The amount of salt before separation was 9.243 % and after separation was 0.0975 % which means almost salt was removed out from neutralized solution because salt should be much less soluble than the sugar in the blended solvent mixture and you might see "clounds" of salt form as you approach the solubility of salt in the mixed solvent. Then salt was separated from neutralized solution which should have only sugar solution. So this final separation of neutralize solution will contain only glucose but from the studied of Theppunya (2544) [57] was shown that "the proper formula for growth and ethanol fermentation using cassava residue as raw materials was extracted sugar from cassava residue, diammoniumhydrogen phosphate 1.0 grams per liters and yeast extract 1.0 grams per liters. So the formula for growth and ethanol fermentation medium was extracted sugar from pineapple waste, diammoniumhydrogen phosphate 1.0 grams per liters, and yeast extract 1.0 grams per liters.

4.2 The studying of growth and alcohol fermentation batch type fermentor.

4.2.1 Preparation of culture in flask for studying batch type fermentor.

Yeast was subcultured from YM agar (Figure 4-3) to YM slant and one loop to 15 ml. YM broth in 250 ml Erlenmeyer flask size and cultivated on shaker 250 rpm, temperature 30°C for 24 hour, then subcultured in 250 ml media that proper for growth. in Erlenmeyer flask 500 ml. size. Shake on shaker 250 rpm., temperature 30°C for 24 hour

The characteristics of *Saccharomyces cerevisiae* TISTR 5013 is similar to normal *Saccharomyces cerevisiae* which is white color colonies, Gram negative, ellipse shape. (Figure 4-3) (Figure 2-8)

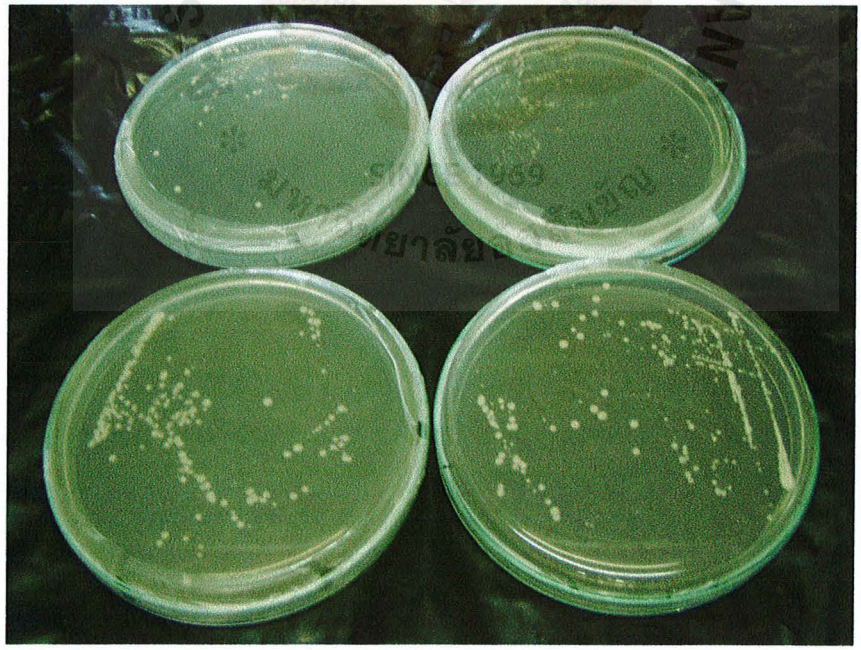


Figure 4-3 : The streak plate of *Saccharomyces cerevisiae* TISTR 5013, incubated at 37°C for 48 hours

4.2.2 The studying of growth and ethanol fermentation in batch type.

Yeast was subcultured from flask, 25 ml. to fermentor 125 ml. size which contained the media that proper for growth 100 ml., cultivated at 30°C. Cultivated for 72 hours, between cultivation collect the sample every 2-4 hours to analyze cell dry weight, glucose amount, and ethanol amount. (fermentor was performed 10 bottles ; each bottle used only once per one time check sample)

4.2.3 Cell dry weight

The sample in every period was spun in centrifuge, collect the supernatant to analyze amount of glucose and ethanol, the pellet part was cleaned with deionized water two times and dried at 105°C for 24 hours for finding cell dry weight.(Appendix A).

4.2.4 The analysis amount of glucose

The analysis amount of glucose : the supernatant was diluted and analyze amount of glucose by use glucose as standard substance (Appendix A). The sample was pipetted 0.2 ml. to test tube and add DNS solution two ml. After that dipped in boiling water for ten minutes and dipped in cooling water for two minutes. The solution was measured the optical density (OD) at 570 nanometers via spectrophotometer then compare with sugar standard curve in range 1 mg./ml. (blank use water)

Remark : The standard curve of sugar prepare following table 3-1

4.2.5 The analysis amount of ethanol.

The supernatant was diluted and analyse amount of ethanol by use Ebulometer. (Appendix A).The boiling chamber of ebulliometer was rinsed (Figure 3-3) with some of the sample to be tested and drain through the outlet tap then fill the boiling chamber with approximately 50 ml of the sample, either using a pipette or the provided measuring cylinder. The condenser jacket was filled with cold water and inserted the special thermometer into the boiling chamber via the rubber stopper. The heat was applied as shown in Figure 3-3 and the temperature will naturally rise as indicated by the thermometer. When the temperature first remains steady for at least 30 seconds record the temperature as T_1. Repeat the above procedure using approximately 25 ml of distilled water instead of the 50 ml. of sample. Record this temperature as T_2. It is not necessary to have water in the condenser when measuring the boiling point of pure water (T_2). The alcohol concentration can be read by the ebulliometer chart and record.

The following table was shown the result of growth analysis, sugar analysis and ethanol analysis in batch fermentation. (Table 4-7)

Table 4-7 : The cell growth, amount of glucose and ethanol production in batch fermentation.

Time = Hours

Cells = grams per liters

Glucose = grams per liters

Ethanol = percentage

Time	Cells	Glucose	Ethanol
0	0.700	30.202	0.0
2	1.280	28.829	0.1
4	1.850	20.839	0.3
6	2.530	13.012	0.9
8	3.130	9.404	1.3
10	3.310	4.941	1.6
12	3.440	2.423	1.9
14	3.450	0.586	2.1
16	3.520	0.433	2.3
18	3.440	0.433	2.4

(Appendix B)

From the table 4-7 can be plotted to graph as figure 4-4. The result was shown that when the time goes by if cell grow in proper medium and condition, cell will grow as the batch type following a period during which the growth rate of the cells gradually increases, the cells grow at a constant, maximum, rate and this period is known as the log, or exponential, phase. [40] (Figure 2-13) which relevant to glucose that should be reduce the amount down because cell will utilize glucose to

repair and grow themselves and utilize glucose as starter medium to ethanol production in anaerobic condition and also ethanol production should be increase due to reduction of glucose and at cell begin the terminate of log phase. From the figure 4-4 is shown that amount of glucose was reduced while number of cells and ethanol production was increase which is correct from theory. At 8 hours the cells is begin to enter stationery phase because cell number is quite no change which cause from no space available, no nutrient anymore, some toxic present, or no air present. In here, the case was no air present because ethanol production occurred under anaerobic condition.

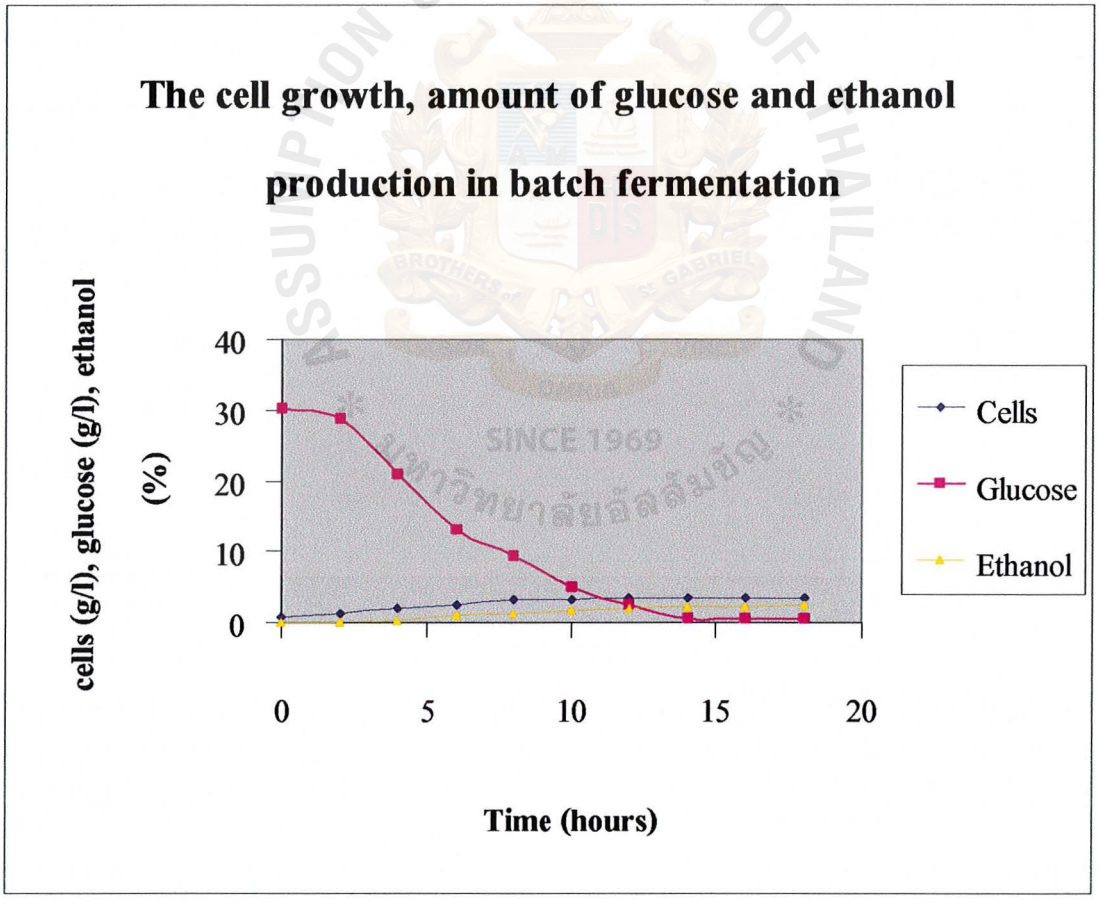


Figure 4-4 : The cell growth, amount of glucose and ethanol production in batch fermentation

For the amount of glucose was reduced due to cell utilization for their growth or utilization for ethanol production in anaerobic condition. The *Saccharomyce cerevisiae* TISTR 5013 utilize glucose for repair and multiply themselves and when no air present they will switch to anaerobic mode which can produce ethanol as product.

For the ethanol production was increased due to cell increase and reduction of glucose. So the final ethanol production, amount of glucose and cell dry weight were 2.4 %, 0.433 grams per liters, and 3.440 grams per liters, respectively.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the study had finished, the results and discussion from this study had led to this conclusion as follows :

5.1.1 The percentage of pineapple wastes (*Ananas comosus*) is around 49 – 52 % from the whole pineapple or it can be said that pineapple waste has half-half with edible part.

5.1.2 The pineapple waste is bio-waste which can use as raw materials for ethanol production because it composed of cellulose and hemicellulose that is large unit of glucose, the carbon source for yeast to grow and produce ethanol.

5.1.3 Cellulose can be degraded to glucose sub-unit by acid hydrolysis but when neutralize with sodium hydroxide, the final solution will composed of glucose, water, and salt.

5.1.4 Salt can inhibit growth and ethanol fermentation of *Saccharomyces cerevisiae* TISTR 5013 because *Saccharomyces cerevisiae* TISTR 5013 is not halophile or halo-tolerant that mean cannot grow in high salt solution.

5.1.5 The neutralized solution had amount of glucose equal to 31.25 grams per liters and salt equal to 9.243 % while after separation with selective precipitation method the amount of glucose equal to 30.20 grams per liters and salt equal to 0.0975 %.

5.1.6 The growth of cells in the experiment is grow following batch type. They had lag phase, log phase, and stationery phase.

5.1.7 The glucose in batch fermentation is reduced in contrast with cell number that increase and stable because cell utilize glucose for growth and produce ethanol when in anaerobic condition.

5.1.8 The ethanol production is 2.4 % from starter glucose 30.20 grams per liters and final glucose is 0.433 grams per liters and cells is 3.440 grams per liters.

5.2 Recommendations for further study

This study has done in the laboratory scale which the further studies are very important to improve the quantity of the products or to scale up as follows:

1. This study was only the laboratory scale which needed to enlarge the scale. The large scale may get more percentage of ethanol.
2. More raw materials in Thailand can be used as raw material for ethanol production because Thailand is agricultural country so differ source should be concerned.

3. The percentage of ethanol, that got, is quite low so further step, the distillation will be concerned to up purity scale and can be used as Biofuel.
4. The different way from using as fuel is distilled to get the percentage more than 70 % for steriled reagent.

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Appendix A

Analysis methods

1. The cell dry weight analysis. [57]

1.1 Prepare dry test tube and weigh their weight.

1.2 Add the 5 ml. samples in test tube, spun in centrifuge 3,000 rpm. for 20 minutes, collect the supernatant to analyse amount of glucose and ethanol.

1.3 Wash cell with 5 ml. deionized water and spun in centrifuge.

1.4 Bring the pellet to bake at 105°C for 24 hours and weigh the actual weight then calculate the dry cell weight from formula.

$$\text{Dry cell weight (g/l)} = \frac{\text{weight test tube and dry cell (g.)} - \text{weight test tube (g)}}{\text{Volume of sample (ml.)} \times 10^{-3}}$$

$$\text{Dry cell weight (g/l)} = \frac{46.9308 \text{ g.} - 46.9273 \text{ g.}}{5 \times 10^{-3}}$$

$$= 0.700 \text{ grams per liters}$$

2. The amount of glucose analysis by DNS method. [10]

Analyse the amount of reducing sugar by 3,5-dinitrosalicylic acid (DNS)

2.1 Chemical.

3,5-dinitrosalicylic acid (DNS) is prepared by weigh DNS 0.25 grams in deionized water 100 ml., add basic solution little by little (NaOH 4 grams dissolve in deionized water 50 ml.). Homogenize its and warm in water baht until clear and add K-Na tartrate little by little until

reach 75 grams, adjust the last volume to be 250 ml. and keep in brown color bottle at room temperature.

2.2 Analysis Protocol

2.2.1 Pipette sample solution 0.2 ml.

2.2.2 Add DNS solution 2 ml.

2.2.3 Dip in boiling water for 10 minutes.

2.2.4 Dip in cooling water for 2 minutes

2.2.5 Add deionized water 10 ml. in test tube, well mixing and measure at 570 nm. via spectrophotometer then compare with sugar standard curve in range 10 mg/ml. (blank use water).

2.3 Calculation protocol

The OD_{570} is 0.207 then subscribe in formula from standard curve (Figure 4-2)

$$y = 6.195x - 0.1802$$

$y = OD_{570}$ (nanometers)

x = Absolute amount of sugar (milligrams)

So x = 0.0625 milligrams glucose equivalent.

Compare to 1 ml. by

Sample from tube 0.2 ml have glucose 0.0625 milligrams

Sample from tube 1 ml. have glucose $\frac{0.0625 \times 1}{0.2}$ milligrams

So Sample 1 ml. have glucose = 0.3125 milligrams

Times by dilution factor (10^{-2}) is time 100

$0.3125 \times 100 = 31.25$ grams per liters

3. The amount of salt analysis via MOHR titration. [22]

Analyze the amount of salt

3.1 Chemical

0.1 M. Silver nitrate ($AgNO_3$) solution is prepared by dissolving 17 g. of $AgNo_3$ in distilled water and make up to 1 l. in volumetric flask. Keep it in a brown color bottle in the dark

Potassium chromate indicator, K_2CrO_4 is prepared by dissolve 5 g. K_2CrO_4 in distilled water and dilute to 100 ml.

3.2 Analysis Protocol

3.2.1 Dilution of brine : Dilute 5 ml. of the brine to 250 ml. in a volumetric flask.

2.2.2 Titration :

- Pipette 25 ml. of the diluted brine into a 250 erlenmeyer flask.
- Add 1 ml. of potassium chromate indicator.
- Titrate with 0.1 M Silver nitrate solution until a distinct reddish-brown color appears and persists on brisk shaking.
- Repeat to ontain concordant results.

3.3 Calculation Protocol

Calculation the sodium chloride content of original brine given that:

$$\% \text{ (m/v) Salt in brine} = 58.5 \times 0.1 \times T/5$$

where T = titrant of 0.1 M silver nitrate in ml.

titrant equal to 7.9 milliliters so percent salt in brine

$$\% \text{ (m/v) Salt in brine} = 58.5 \times 0.1 \times 7.9/5$$
$$= 9.243 \% \text{ (m/v)}$$

4. Separating Salt and Sugar [69]

The selective precipitation

4.1 Evaporate as much of the water as you can without precipitating one or the other compounds.

4.2 Slowly add a volatile organic solvent. A reasonable one might be acetone, but ethyl alcohol, or isopropanol, may also do. The salt should be much less soluble than the sugar in the blended solvent mixture and you may see "clounds" of salt form as you approach the solubility of salt in the mixed solvent.

4.3 Filter the precipitate.

5. The ethanol analysis [67]

5.1 Rinse the boiling chamber of the ebulliometer with some of the wine to be tested and drain through the outlet tap.

5.2 Fill the boiling chamber with approximately 50 ml of the wine, either using a pipette or the provided measuring cylinder.

5.3 Fill the condenser jacket with cold water.

5.4 Insert and seal the special thermometer into the boiling chamber via the rubber stopper.

5.5 Apply heat as shown in the diagram (figure 3-3).

5.6 The temperature will naturally rise as indicated by the thermometer.

5.7 When the temperature first remains steady for at least 30 seconds record the temperature as T_1.

5.8 Repeat the above procedure using approximately 20 ml of distilled water instead of the 50ml of wine. Record this temperature as T_2.

5.9 It is not necessary to have water in the condenser when measuring the boiling point of pure water (T_2).

5.10 Read the alcohol concentration from the ebulliometer chart

Appendix B

The Data from the experiment

1. The weight of pineapple.

Table B-1 : The weight data of pineapple (*Ananus comosus*)

Pineapple	Trial			Average
	1	2	3	
Whole	1500 g.	1141 g.	1216 g.	1285.67 g.
Edible part	760 g	600 g.	638 g.	666 g.
Waste part	740 g	541 g.	578 g.	619.67 g.

2. The analysis of sugar via the dinitrosalicyclic acid assay.

Table B-2 : The Optical Density of Standard curve of Sugar (1 mg./ml.)

Absolute amount (mg.)	Concentration (mg/ml)	OD_{570}		
		1	2	Average
0.00	0.0	0.000	0.000	0.0000
0.04	0.2	0.053	0.053	0.053
0.08	0.4	0.324	0.324	0.324
0.12	0.6	0.583	0.583	0.583
0.16	0.8	0.804	0.804	0.804
0.20	1.0	1.052	1.052	1.052

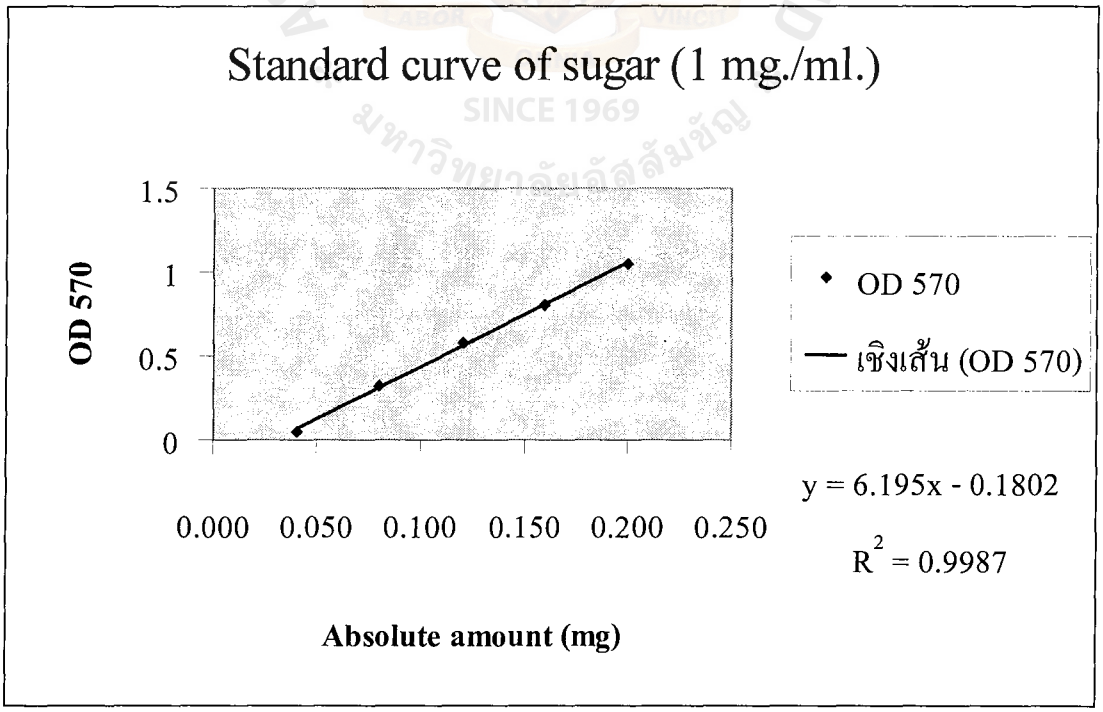

Figure B-1 : The graph of standard curve of sugar (1 milligrams per milliliters)

Table B-3 : The Optical Density of Sugar in medium before separation

Sample	OD_{570}			
	1	2	3	Average
Sugar from pineapple waste at 10^0 dilution	3.10	3.08	3.08	3.087
Sugar from pineapple waste at 10^{-1} dilution	2.82	2.82	2.77	2.803
Sugar from pineapple waste at 10^{-2} dilution	**0.204**	**0.196**	**0.221**	**0.207**
Sugar from pineapple waste at 10^{-3} dilution	0.002	0.005	0.035	0.014

Remark : The bolt alphabet : the selected range.

3. The analysis of salt via the MOHR titration.

Table B-4 : The volume of silver nitrate in MOHR titration

Sample	Volume of titrant (ml.)			
	Trial			Average
	1	2	3	
Medium	7.9	7.9	7.9	7.9

4. The analysis of sugar and salt after separation via selective precipitation

Table B-5 : The Optical Density of Sugar in medium after separate salt.

Sample	OD_{570}			
	1	2	3	Average
Sugar from pineapple waste at 10^0 dilution	3.08	3.07	3.08	3.077
Sugar from pineapple waste at 10^{-1} dilution	2.70	2.65	2.60	2.65
Sugar from pineapple waste at 10^{-2} dilution	**0.204**	**0.185**	**0.195**	**0.194**
Sugar from pineapple waste at 10^{-3} dilution	0.002	0.002	0.002	0.002

Remark : The bolt alphabet : the selected range.

Table B-6 : The volume of silver nitrate in MOHR titration after separate salt

Sample	Volume of titrant (ml.)			
	Trial			Average
	1	2	3	
Medium	0.1	0.1	0.05	0.083

5. The studying of growth and ethanol fermentation batch type fermentor.

Table B-7 : The data of cell dry weight

Empty plate (g.)	Empty plate + culture (g.)	Cell dry weight (g.)
46.9273	46.9308	0.00350
40.4070	40.4134	0.00640
43.4486	43.4579	0.00925
43.4506	43.4633	0.01265
45.2833	45.2990	0.01565
44.9190	44.9356	0.01655
43.7637	43.7809	0.01720
41.6112	41.6285	0.01725
41.1375	41.1551	0.01760
47.6262	47.6434	0.01720

Table B-8 : The Optical density of sugar in medium in each collecting sample at 10^{-2} dilution via batch fermentation

Time (hours)	OD_{570}			
	1	2	3	Average
0	**0.195**	**0.195**	**0.192**	**0.194**
2	**0.17**	**0.168**	**0.193**	**0.177**
4	**0.079**	**0.078**	**0.077**	**0.078**
6	0.002	0.002	0.002	0.002
8	0.001	0.001	0.001	0.001
10	0.000	0.000	0.000	0.000
12	0.000	0.000	0.000	0.000
14	0.000	0.000	0.000	0.000
16	0.000	0.000	0.000	0.000
18	0.000	0.000	0.000	0.000

Remark : The bolt alphabet : the selected range.

Table B-9 : The Optical density of sugar in medium in each collecting sample at 10^{-1} dilution via batch fermentation

Time (hours)	OD_{570}			
	1	2	3	Average
0	3.18	3.16	3.26	3.20
2	3.18	3.16	3.2	3.18
4	2.52	2.53	2.52	2.523
6	**1.432**	**1.433**	**1.431**	**1.432**
8	**0.985**	**0.986**	**0.984**	**0.985**
10	**0.431**	**0.435**	**0.43**	**0.432**
12	**0.13**	**0.14**	**0.09**	**0.12**
14	0.080	0.080	0.080	0.080
16	0.004	0.004	0.004	0.004
18	0.003	0.005	0.004	0.004

Remark : The bolt alphabet : the selected range.

Table B-10 : The Optical density of sugar in medium in each collecting sample at 10^{0} dilution via batch fermentation

Time (hours)	OD_{570}			
	1	2	3	Average
0	3.2	3.18	3.16	3.18
2	3.18	3.17	3.13	3.16
4	2.85	2.8	2.852	2.834
6	2.75	2.62	2.55	2.64
8	2	2.25	2.05	2.10
10	1.82	1.82	1.82	1.82
12	1.53	1.54	1.53	1.533
14	**0.546**	**0.547**	**0.545**	**0.546**
16	**0.357**	**0.356**	**0.355**	**0.356**
18	**0.356**	**0.356**	**0.356**	**0.356**

Remark : The bolt alphabet : the selected range.

Table B-11 : The Percentage ethanol

Time (hours)	Temp. of water (°C)	Temp. of sample (°C)	% Ethanol
0	99.95	99.95	0.0
2	99.95	99.8	0.1
4	99.95	99.6	0.3
6	99.95	99.05	0.9
8	99.95	98.65	1.3

10	99.95	98.4	1.6
12	99.95	98.1	1.9
14	99.95	97.9	2.1
16	99.95	97.75	2.3
18	99.95	97.65	2.4

Table B-12 : The cell growth, amount of glucose and ethanol production in batch fermentation.

Time = Hours

Cells = grams per liters

Glucose = grams per liters

Ethanol = percentage

Time	Cells	Glucose	Ethanol
0	0.700	30.202	0.0
2	1.280	28.829	0.1
4	1.850	20.839	0.3
6	2.530	13.012	0.9
8	3.130	9.404	1.3
10	3.310	4.941	1.6
12	3.440	2.423	1.9
14	3.450	0.586	2.1
16	3.520	0.433	2.3
18	3.440	0.433	2.4

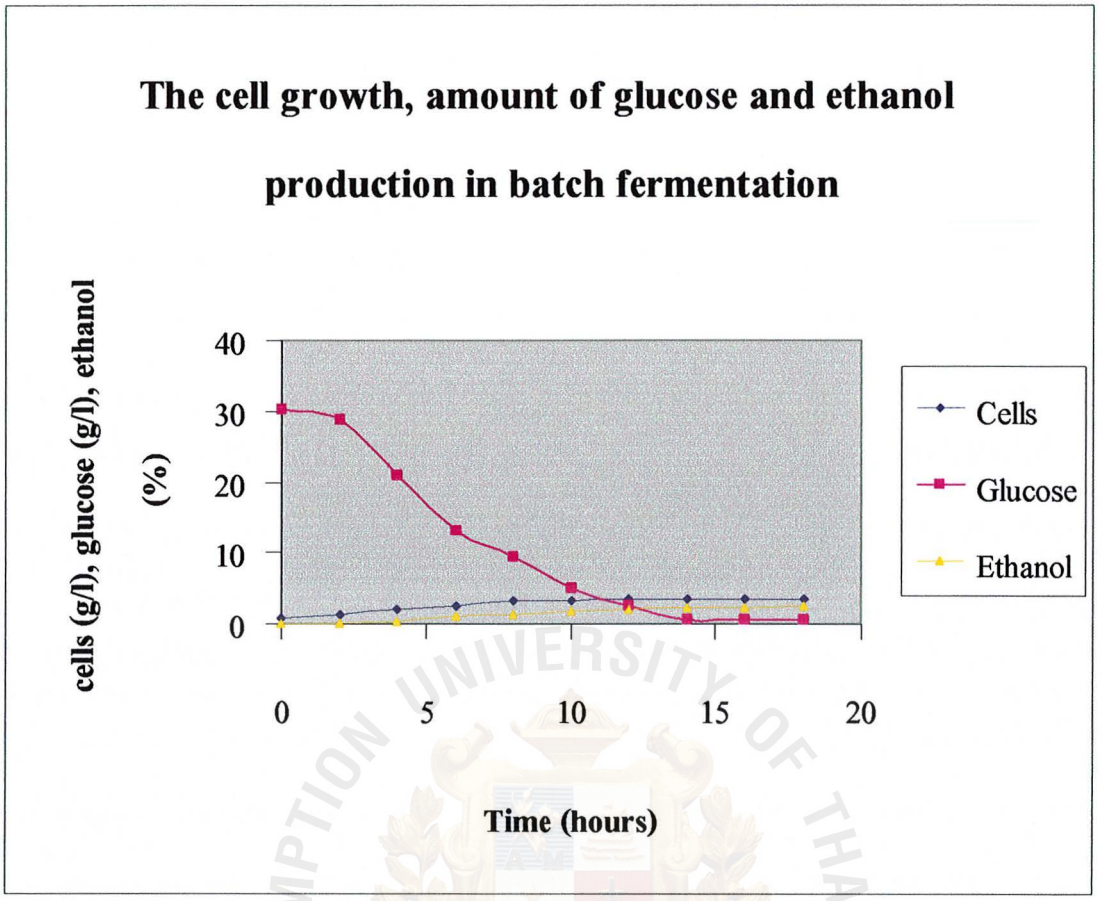

Figure B-2 : The graph of cell growth, amount of glucose and ethanol production in batch fermentation

Appendix C

Medium

1. Yeast extract Malt extract (YM) [57]

Peptone	5.0 grams
Yeast extract	3.0 grams
Malt extract	3.0 grams
Glucose	10.0 grams

All media components was dissolved with deionized water (in case of agar type, adding agar 15 grams) adjust volume to 1 liters, autoclave at 121°C pressure 15 pounds/inch^3 for 15 minutes.

1.1 Media for conservation use YM agar slant (Yeast extract Malt extract agar slant).

1.2 Media for preparation starting culture use YM broth (Yeast extract Malt extract broth).

2. Media that proper for growth and ethanol fermentation. [57]

Media for preparation culture in experiment use the formula that proper to the growth of strain of yeast in experiment which is got from studying of proper formula for their growth. The formula is sugar from degraded pineapple waste part, add diammoniumhydrogen phosphate 1.0 grams/liters and yeast extract 1.0 grams/liters. Autoclaving at 121°C for 15 minutes. (Theppunya, 2544)