Sudan University for Sciences and Technology

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Optimum Production of Ethanol by Fermentation of Sugar Cane

الإنتاج الأمثل للكحول الإيثيلي من تخمير سكر القصب

A thesis submitted in partial fulfillment of the degree of Master Degree in Chemistry

By

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إستهلال

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿ فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ ۗ وَلَا تَعْجَلْ بِالْقُرْآنِ مِن قَبْلِ أَن يُقْضَىٰ إِلَيْكَ وَحْيُهُ ۖ وَقُل رَّبِّ زِدْنِي عِلْمًا ﴾

سورة طه : (114)

Dedication

I dedicate this work

To my family, who always supports me.

To my teachers, with great respect for all

Acknowledgement

I would like to convey my sincere gratitude to all those who gave me the opportunity to complete this thesis. Special thanks go to my supervisor Dr. Kamal Mohammed Saeed, who dedicated a lot of his precious time to give me valuable assistance and guidance throughout the whole work and for continuous support and guidance throughout this study. Also, I'm grateful and indebted to staff members in department of chemistry at faculty of science.

Abstract

This study aimed to determine the optimum conditions for production of ethanol using sugar cane under enzymatic catalysis of favorable kinetic parameters; duration, temperature, pH, enzyme concentration have been studied.

Fibers were manually removed ready sugar cane juice. The juice obtained was sterilized by heating at temperature of 90ºC for 40 minutes, and then cooled. Octapol powder (3g), was added to (1 L) of juice. Then the sample was filtered. Polarimeter device was used to determine sugar percentage. Three grams of sodium metabisulphite ($Na_2S_2O_4$) were added to prevent the growth of undesirable microorganisms. The yeast was added to the substrate and was properly mixed and shaked, then left for 10 minutes. The results showed that maximum rate of alcohol produced was (2.42) after 90 minutes of fermentation, the optimal temperature was 34 C°, the optimal substrate concentration was (85.7%) at pH 5, and one gram of yeast.

المستخلص

هدفت هذه الدراسة إلى تحديد الظروف المثلى لإنتاج الإيثانول باستخدام قصب السكر تحت التحفيز الأنزيمي المعلمات الحركية الملائمة ؛ تم دراسة المدة ودرجة الحرارة ودرجة الحموضة وتركيز الإنزيم.

تمت إزالة الألياف يدويًا من عصير قصب السكر الجاهز. تم تعقيم العصير الناتج عن طريق التسخين عند درجة حرارة 90 درجة مئوية لمدة 40 دقيقة ، ثم تبريده. تمت اضافة 3جرام من مسحوق (Octapol) إلى (1 لتر) من العصير، ثم تمت تصفية العينة، وتحديد نسبة السكر باستخدام جهاز (Polarimeter). تمت إضافة ثلاثة جرامات من بيروكبريتيت الصوديوم (Na_2S2O_4) لمنع نمو الكائنات الدقيقة غير المرغوب فيها.

تمت إضافة الخميرة إلى العينة وخلطها ورجها بشكل جيَّد. وتركت لمدة دقائق 10 لتنشيط التفاعل. أظهرت النتائج أن أقصى معدل للكحول المنتج كان (2.42) جم/لتر بعد مرور 90 دقيقة من التخمير، وعند درجة الحرارة 34 درجة مئوية، وتركيز كان (85.7٪) بقيمة أس هيدروجيني تساوي 5، وكمية واحد جرام من الخميرة.

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Introduction and Literature Review

1. 1 Introduction

1.1.1 Background

Two main fermentation modes have been frequently reported for bioethanol production: separate hydrolysis and fermentation (SHF) and simultaneous scarification and fermentation (SSF). In the SHF process, the pretreated material is hydrolyzed to simple sugars and subsequently undergoes fermentation. A major advantage of this process is it allows independent optimization of the enzymatic and fermentation phase to maximize sugar and ethanol yield respectively however, there is high cost to separating the solid and liquid fractions of the hydrolysate, particularly at large scale. Several studies have highlighted the SSF system as a potential solution. In this strategy, the hydrolysis and fermentation occur in the same reactor, thus negating the need for a separation stage. This technique also has significant drawbacks such as specialized equipment requirements, high concentration of inhibitor formation and no reusability of the yeast due to lignin separation (Carrillo, 2017, page2).

However, the main drawback is the different optimum temperatures required for the enzyme (usually cellulose) and the fermenting microorganism, usually 50 and 30 °C respectively. Ultimately, preference is given to the microorganism resulting in a sub-optimal scarification process. Another potential solution is to remove the separation stage from the SHF process. There is a dearth of knowledge on the effect of filtered and unfiltered enzymatic

hydrolysate on fermentation process kinetics. Moreover, the solid waste residue from the SHF process effluent could be an attractive additional revenue stream for animal feed since the plant material has been delignified to enhance digestibility. Furthermore, there could be an increase in protein content due to the yeast cell biomass (Zadrazil,1995).

With increasing interest in the commercial applications of batch bioethanol processes, several kinetics models have been proposed which describe microbial growth, product formation and substrate consumption. These models are extremely useful in the process development of bioethanol production, since they assist in predicting fermentation performance in response to changes in various factor (Manikandan, 2008).

1.1.2 Statement of the problem

Alcohol is being widely studied as an alternative fuel, and its production is increasing. Fuel markets are hundreds of times greater than the traditional ethanol markets which the existing industry supplies. To make a material contribution to fuel supply, fermentation ethanol must be treated as a major chemical and produced in large-volume, highly efficient plants. Such plants must be assured of a continuous supply of low-cost raw materials for which suitable processes have been developed and commercially proven. Sugar cane in the tropics and grains in some temperate countries meet these requirements; cellulosics do not qualify at present, nor will they in the foreseeable future, without major breakthroughs. The research problem can be stated in the following

question; "***how to get optimum production of ethanol from the fermentation of cane sugar using Saccharomyces cerevisiae as acatalyst?***. Cerevisiae is superior to bacteria and other yeasts in its various physiological characteristics for ethanol production at industrial level. It can tolerate wide range of pH with acidic pH as optimum, which protects contamination. Temperature greatly affects the enzymatic activity of yeast cells and yeasts which are active and tolerant at high temperature are ideal for industrial bioethanol production. S. cerevisiae.

1.1.3 Justification

In all of the required kinetic in formation for industrial production is lacking the direction of reaction as well as the specific activity of the enzyme. This study will try to explore the fermentation process using Saccharomyces cerevisiae ITV-01 yeast strain in a batch system at different glucose and ethanol concentration to determine the optimum fermentation conditions.

1.1.4 Objectives

A. General Objectives

To determine the optimum conditions of ethanol production using Saccharomyces cerevisiae from cane sugar.

B. Specific objectives

i. To investigate fermentation of cane sugar as substrate by Saccharomyces cerevisiae (enzyme) to obtain certain useful kinetic parameters for ethanol production (duration (min) temperature, pH, yeast cane)

ii. To determine the effect of temperature, pH, substrate and yeast (enzyme) concentration on the rate of fermentation.

1.2 Literature Review

In first generation ethanol production technology, Saccharomyces cerevisiae is the most widely used microorganism for the fermentation of sucrose available in the juice or molasses into ethanol. This yeast can also be used for 2G ethanol production from glucose solution obtained by pretreatment of cellulosic fraction of SB . (Buratti,,2018)

For the economic ethanol production from SB, it is equally important to consider hemi cellulosic fraction along with cellulosic part of cell wall. Hemicellulose represents about one-third of the carbohydrate fraction available in SB. This macromolecular fraction is rich in pentose residues, mainly xylose, which are not fermented by native S. cerevisiae. However, there are some microorganisms able to ferment xylose to ethanol or other products. (Alvira, 2010)

The use of xylose metabolizing microorganism will increase in the global yield of ethanol in sugarcane based biorefineries.

Dilute acid hydrolysis is an efficient process for the hemicellulose depolymerization into variety of priority pentose sugars such as arabinose and mainly xylose. The remaining solid fraction is known as cellulignin which can be hydrolyzed into glucose from the cellulose fraction by cellulase enzymes .(Alves, 1998)

Hemicellulose depolymerization by dilute acid hydrolysis yields primarily xylose and other sugar monomers, although some other

byproducts considering inhibitors to microbial metabolism, such as furans, 5-hydroxymethylfurfurals, phenolics, and weak acids . Therefore, it is necessary to reduce the concentration of these inhibitors prior to using the hemi cellulosic hydrolysate into ethanol via microbial fermentation. Calcium oxide mediated neutralization of hydrolysate followed by activated charcoal treatment efficiently removes the inhibitors. (Chandel, 2012)

For the production of hemi cellulosic ethanol, Scheffersomyces shehatae (Syn. Candida shehatae) has been considered a promising microorganism which provides high ethanol productivities.

However, a balanced nutrient supplementation is required for the optimal growth of S. shehatae for the production of ethanol with desired yield and productivities. In this work, ethanol production from sugarcane bagasse hemi cellulosic hydrolysate was evaluated, using the yeast S. shehatae UFMG-HM 52.2 in different fermentation medium (Martiniano, 2014).

1.2.1 Fermentation,

Below are some definitions of fermentation. They range from informal, general usages to more scientific definitions.

i. Preservation methods for food via microorganisms (general use).
ii. Any large-scale microbial process occurring with or without air (common definition used in industry).
iii. Any process that produces alcoholic beverages or acidic dairy products (general use).

iv. Any energy-releasing metabolic process that takes place only under anaerobic conditions (somewhat scientific).

v. Any metabolic process that releases energy from a sugar or other organic molecule, does not require oxygen or an electron transport system, and uses an organic molecule as the final electron acceptor (most scientific. (Tortora, 2010)

Fermentation is a chemical process by which molecules such as glucose are broken down. More generally, fermentation is the foaming that occurs during the manufacture of wine and beer, a process at least 10,000 years old. The frothing results from the evolution of carbon dioxide gas, though this was not recognized until the 17th century. French chemist and microbiologist Louis Pasteur in the 19th century used the term fermentation in a narrow sense to describe the changes brought about by yeasts and other microorganisms growing in the absence of air ; he also recognized that ethyl alcohol and carbon dioxide are not the only products of fermentation. The term fermentation now denotes the enzyme-catalyzed, energy-yielding pathway in cells involving the anaerobic breakdown of molecules such as glucose. In most cells the enzymes occur in the soluble portion of the cytoplasm. The reactions leading to the formation of ATP and pyruvate thus are common to sugar transformation in muscle, yeasts, some bacteria, and plants. (Bowen, 2018)

A. Industrial application of fermentation

Industrial fermentation processes begin with suitable microorganisms and specified conditions, such as careful adjustment of nutrient concentration. The products are of many types: alcohol, glycerol, and carbon dioxide from yeast

fermentation of various sugars; butyl alcohol, acetone, lactic acid, monosodium glutamate, and acetic acid from various bacteria; and citric acid, gluconic acid, and small amounts of antibiotics, vitamin B_{12}, and riboflavin (vitamin B_2) from mold fermentation. Ethyl alcohol produced via the fermentation of starch or sugar is an important source of liquid biofuel.

B. Methods of fermentation

i. Lactic Acid Fermentation

Lactic acid is formed from pyruvate produced in glycolysis. NAD+ is generated from NADH. Enzyme lactate dehydrogenase catalyses this reaction. Lactobacillus bacteria prepare curd from milk via this type of fermentation. During intense exercise when oxygen supply is inadequate, muscles derive energy by producing lactic acid, which gets accumulated in the cells causing fatigue.

ii. Alcohol Fermentation

This is used in the industrial production of wine, beer, biofuel, etc. The end product is alcohol and CO_2. Pyruvic acid breaks down into acetaldehyde and CO_2 is released. In the next step, ethanol is formed from acetaldehyde. NAD+ is also formed from NADH, utilized in glycolysis. Yeast and some bacteria carry out this type of fermentation. Enzyme pyruvic acid decarboxylase and alcohol dehydrogenase catalyse these reactions.

iii. Acetic acid Fermentation

Vinegar is produced by this process. This is a two-step process. The first step is the formation of ethyl alcohol from sugar anaerobically using yeast. In the second step, ethyl alcohol is further oxidized to

form acetic acid using acetobacter bacteria. Microbial oxidation of alcohol to acid is an aerobic process.

iv. Butyric acid Fermentation

This type of fermentation is characteristic of obligate anaerobic bacteria of genus clostridium. This occurs in retting of jute fibre, rancid butter, tobacco processing and tanning of leather. Butyric acid is produced in the human colon as a product of dietary fibre fermentation. It is an important source of energy for colorectal epithelium. Sugar is first oxidized to pyruvate by the process of glycolysis and then pyruvate is further oxidized to form acetyl-CoA by the oxidoreductase enzyme system with the production of H_2 and CO_2. Acetyl-CoA is further reduced to form butyric acid. This type of fermentation leads to a relatively higher yield of energy.

v. Advantages and disadvantages of fermentation

1. Fermentation is suitable for all kinds of environments. It is one of the oldest metabolic processes which is common to prokaryotes and eukaryotes. Fermentation is widely used in various industries.

Using suitable microorganisms and specified conditions different kinds of products are formed namely: wine, beer, biofuels, yoghur, pickles, bread, sour foods containing lactic acid, certain antibiotics and vitamins.

Fermentation can make food nutritious, digestible and flavoured. There are many benefits of consuming fermented food.

- It improves digestion and helps to maintain intestinal bacteria

- It has an anti-cancer effect.
- Improves immune system
- Reduces lactose intolerance

Other than the food industry, there are many other areas where the fermentation process is used. Methane is produced by fermentation in the sewage treatment plants and freshwater sediments.(Bowen, 2018).

1.2.2 Yeast

Saccharomyces cerevisiae yeast is unicellular fungi that divide asexually by budding or fission and whose individual cell size with a large diameter of 5-10^m and a small diameter of 1-7^m. The cells of S. cerevisiae are pigmented, where cream color may be visualized in surface-grown colonies. Yeast cell is completely deferred than bacterial cell in both structure and function. (Walker and White, 2011)

Yeast performs fermentation to obtain energy by converting sugar into alcohol. Fermentation processes were spontaneously carried out before the biochemical process was fully understood. Yeasts can carry out their metabolism and fermentation activity satisfactorily as they have the necessary nutrients and substrates.

On a nutritional level, yeasts are not particularly demanding compared to other microorganisms such as lactic acid bacteria. However, their growth is supported by the existence of basic compounds such as fermentable sugars, amino acids, vitamins, minerals and also oxygen. Upon a morphological point of view,

yeasts present a high morphological divergence, with round, ellipsoidal and oval shapes being the most common. In fact, in the identification processes, microscopic evaluation is the first resource followed by other more discriminatory tests such as microbiological and biochemical ones. In a next stage, the classical classification includes other more laborious tests such as those of sugar fermentation and amino acid assimilation. The production and tolerance to ethanol, organic acids and SO are also important tools to differentiate among species. The reproduction of yeasts is mainly by budding, which results in a new and genetically identical cell. Budding is the most common type of asexual reproduction although cell fission is a characteristic of yeasts belonging to the genus Schizosaccharomyces. Growing conditions that lead to nutrient starvation, such as lack of amino acids, induce sporulation, which is a mechanism used by yeasts to survive in adverse conditions. As a result of sporulation, yeast cells suffer from genetic variability. In industrial fermentation processes, the asexual reproduction of yeasts is advisable to ensure the preservation of the genotype and to maintain stable fermentation behaviour that does not derive from it for as long as possible. At the metabolic level, yeasts are characterized by their capacity to ferment a high spectrum of sugars, among which glucose, fructose, sucrose, maltose and maltotriose predominate, found both in ripe fruit and in processed cereals. In addition, yeasts tolerate acidic environments with pH values around 3.5 or even less.(Pando, 2012)

Most commercial products contain a mixture of varying proportions

of live and dead S. cerevisiae cells. Those with a predominance of live cells are sold as live yeasts, while others containing more dead cells and the growth medium are sold as yeast cultures.

In addition to its use in food processing, S. cerevisiae is widely used for the production of macromolecular cellular components such as lipids, proteins, enzymes, and vitamins (Bigelis, 1985; Stewart and Russell, 1985). S. cerevisiae has been regarded having GRAS status by FDA. Furthermore, the National Institutes of Health in its Guidelines for Research Involving Recombinant DNA Molecules considers S. cerevisiae a safe organism.

The abundance of information on S. cerevisiae, derived from its role in industrial applications, has positioned S. cerevisiae as a primary model for the genetic manipulation. (Callaway, 1997)

1.2.3 Sugar Cane

Cane sugar is processed into raw sugar by a sequence of operations: harvesting, cutting, crushing, extraction of juice, clarification, evaporation, crystallization, centrifugation and refining.

Beet sugar processing involves harvesting, slicing, extraction of juice, carbonization, evaporation, crystallization and refining.

The heating process used for these processes and the resulting low water activity of the end products greatly decreases the initial microflora with the remaining organisms consisting primarily of heat-resistant spores (Owen, 1977) such as those

from Bacillus and Clostridium species. While spores of Clostridium botulinum have been detected in sugar, they are of little relevance due to the low water activity of the confectionery products. For

high fructose corn syrup, which utilizes enzymatic processing, the primary organisms of concern are spoilage organisms such as osmophilic yeast ike Zygosaccharomyces rouxii and Saccharomyces cerevisiae, which are not relevant to hard candy confectionery processing (Godshall, 2003).

A. Types of sugar cane

- **Granulated Sugar**

Granulated sugar is the white sugar that you rely upon every day for stirring into your coffee or mixing up a batch of shortbread. Made from sugarcane, this sugar is sometimes known as white, table or refined sugar. (Kim, 2010)

- Powdered Sugar powdered sugar is sometimes referred to as icing or confectioners' sugar. This sugar is used frequently in glazes and frostings since it dissolves so easily. Powdered sugar is just very fine white sugar with a bit of added corn starch to prevent it from becoming cakey.

- Superfine Sugar

Somewhere between granulated and powdered is superfine sugar (sometimes called caster sugar). It has smaller granules than regular white sugar, but isn't quite ground into fine powder. This sugar variety dissolves quickly making it perfect for meringues (you can get more meringue tips here).

- **Brown Sugar**

brown sugar is another pantry staple you can use morning, noon and night. Both these sugars are granulated sugar with an added

touch of molasses. While dark brown sugar does have a bit more molasses than its lighter counterpart, both these sugars can be used interchangeably in recipes to add a subtle caramel or toffee flavor.

- **Sanding Sugar**

Sanding sugar isn't typically mixed into any recipe, rather it's used for decoration (and you can find it in a wide array of colors).

- **Turbinado Sugar**

Turbinado sugar is derived from sugar cane and is minimally processed. This variety has larger sugar crystals than it's granulated counterparts and is also darker in color—it looks very similar to a light brown sugar. The difference here between turbinado and brown sugars is that molasses is not added. Instead, turbinado sugar retains a very (very!) light caramel flavor due to its light processing. In fact, turbinado sugar is a better substitute for white sugar than brown.

- **Demerara Sugar**

Demerara sugar is similar to turbinado sugar since it is also minimally processed and has a coarser grain. However, demerara sugar is darker and has more of a molasses flavor, making it a better substitute for brown sugar.

- **Muscovado Sugar**

Muscovado is a type of unrefined cane sugar. Sometimes called Barbados sugar, this variety is dark brown and packed with the sugar's natural molasses. You can substitute muscovado for

traditional brown sugar, but be aware that the granules are larger and the molasses flavor much stronger.

B. Contents of sugar cane

Chemical composition of sugar cane bagasse was determined to be 42% cellulose, 25% hemicellulose, and 20% lignin, and that of energy cane was 43% cellulose, 24% hemicellulose, and 22% lignin. Sweet sorghum was 45% cellulose, 27% hemicellulose, and 21% lignin. Theoretical ethanol yields would be 3,609 kg per ha from sugar cane, 12,938 kg per ha from energy cane, and 5,804 kg per ha from sweet sorghum. (Kim, 2010)

1.2.4 Saccharomyces cerevidsiae

Saccharomyces cerevisiae is a species of yeast . The species has been instrumental in winemaking, baking, and brewing since ancient times. It is believed to have been originally isolated from the skin of grapes .

It is one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like Escherichia coli as the model bacterium. It is the microorganism behind the most common type of fermentation. S. cerevisiae cells are round to ovoid, 5-10 gm in diameter. It reproduces by budding.

Saccharomyces cerevisiae is one of the types of yeast extensively found in naturally occurring ecosystems, but it is mainly cultivated for use in the food and wine industries. Live S. cerevisiae cultures have been identified as potentially conferring health advantages on the host when administered in adequate quantities. S. cerevisiae has

long been exploited as a functional food and dietary supplement. Biologically active secondary metabolites of S. cerevisiae including naringenin, reticuline, artemisinin, and other pigments have been shown to exhibit physiological activities and health effects in humans.

Cerevisiae has been used traditionally as a neuroprotective, antioxidant, antidiabetic, antiinflammatory, immune booster, antimalarial, and antitumoral.

The use of S. cerevisiae in different clinical trials has shown promising results against different diarrheal diseases. Treatment with S. cerevisiae is effective in immune-compromised patients and those with serious or general intestinal diseases, of which most cases are affected by the presence of a central venous catheter (De Llanos etal. 2006).

In the prevention of antibiotic-associated diarrhea (AAD), S. cerevisiae has been comprehensively evaluated and yeast has been potentially effective in adults and children in decreasing AAD (Kotowska etal., 2005; Surawicz etal., 2000). A reduction of 60% was seen in disease manifestation in patients with mild to moderate ulcerative colitis by using yeast in traditional therapies (Guslandi etal., 2013).

1.2.5 Factors affecting fermentation process :

Reduction of the non-soluble solids content to below 0.5% prior to white wine fermentation can result in nutrient deficiencies. Too high a level may cause fermentation rates to proceed too quickly. Fermentation in contact with bentonite is occasionally done to help

obtain white wine protein stability. Bentonite additions in the fermenter can reduce must N and should only be done in conjunction with supplemental nutrient additions

i. Temperature

Temperature affects the rate of fermentation yielding slower fermentation rates and longer fermentation. The maximum fermentation rate increases also with temperature.

Maintaining an ideal temperature range during the fermentation process is a difficult task that all ethanol plant operators face, especially in the hotter months. The optimum temperature range for yeast fermentation is between 9O·F-95·F (32·C-35·C). Every degree above this range depresses fermentation. While elevated temperature is problematic in all phases of ethanol production, it is specifically hazardous during the later stages of fermentation. As ethanol accumulates, the optimum temperature range to maximize ethanol growth becomes more narrow . Ethanol fermentation at relatively high temperature is an important target for effective ethanol production in tropical countries where average day-time temperatures are usually high throughout the year. The advantages of rapid fermentation at high temperature are not only to decrease the risk of contamination but also to reduce the cooling costs (Eiadpum et al., 2012). To achieve high temperature fermentation it is necessary to use an efficient yeast strain that can tolerate high temperature (Limtong et al., 2017)

ii. Concentration

It is likely that the initial concentrations of glucose and fructose

(main grape sugars) will selectively influence the species and strains of yeast present during fermentation**.**

The increase in sugar concentration led to an increase in ethanol concentration and the highest ethanol yield was obtained at a sugar concentration of 20.8% (w.v-)

iii. Duration:

For large scale fermentations, in particular, the proportion of time during which a fermenter is producing under these high rate conditions should be large relative to the total fermenter operating time to minimize operating costs. That is, the time period, BC, during which maximum production is observed, should be large relative to the total of the remaining intervals that the fermenter is occupied**.**

In this expression, the interval DA corresponds to the turnaround time, or time required to empty the fermenter, clean and sterilize it, refill it with fresh sterile substrate, and introduce a 'starter' of the appropriate organism. If this situation does in fact hold, then batch operation of the fermentation process is generally quite commercially acceptable**.**

iv. Yeast

This yeast converts hexose sugars to ethanol, CO2, and a variety of compounds including alcohols, esters, aldehydes, and acids, that contribute to the sensory attributes of the food and beverage**.**

Among several yeasts, Saccharomyces cerevisiae and S. bayanus var. uvarum are the most important species present during the fermentation process (Pretorius, 2000; Querol and Fleet, 2016).

Gonzalez et al. (2016) described wine yeast hybrids between the

species S. cerevisiae X Saccharomyces kudriavzevii and S. cerevisiae X S. bayanus

v. PH

The effect of pH on regulation of these pathways is understood in broad terms, but there are unexplained deviations. For example, itis unknown whether product spectrum is dominated by phylogenetic (microbial community) or physiological (chemical) factors. When pH is changed incrementally and progressively, the culture gradually adapts to a new pH and is acclimatized. This adaptation results in a gradual change in product mix as pH changes, with particular shifts to propionate and/or ethanol at high and low pH values. The alternative is to set a reference pH in advance and then adjust to another pH (reset).

1.2.6 Ethanol

Ethanol is an organic chemical compound. It is a simple alcohol with the chemical formula $C_2 H_6O$. Its formula can be also written as CH_3--CH_2-OH or C_2H_5OH (an ethyl group linked to a hydroxyl group), and is often abbreviated as EtOH. Ethanol is a volatile, flammable, colorless liquid with a slight characteristic odor. It is a psychoactive substance, recreational drug, and the active ingredient in alcoholic drinks.

Ethanol is naturally produced by the fermentation of sugars by yeasts or via petrochemical processes such as ethylene hydration. It has medical applications as an antiseptic and disinfectant.

It is used as a chemical solvent and in the synthesis of organic compounds. Ethanol is an alternative fuel source. (The PubChem,

2008)

A. Chemical properties of Ethanol

Ethanol is a 2-carbon alcohol. Its molecular formula is CH3CH20H. An alternative notation is CH3-CH2-OH, which indicates that the carbon of a methyl group (CH3-) is attached to the carbon of a methylene group (-CH2-), which is attached to the oxygen of a hydroxyl group (-OH). It is a constitutional isomer of dimethyl ether. Ethanol is sometimes abbreviated as EtOH, using the common organic chemistry notation of representing the ethyl group (C2H5-) with Et. (Windholz M ,1976)

B. Physical properties of Ethanol

Ethanol is a volatile, colorless liquid that has a slight odor. It burns with a smokeless blue flame that is not always visible in normal light. The physical properties of ethanol stem primarily from the presence of its hydroxyl group and the shortness of its carbon chain. Ethanol's hydroxyl group is able to participate in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of similar molecular weight, such as propane. Ethanol is slightly more refractive than water, having a refractive index of 1.36242 (at 1=589.3 nm and 18.35 °C or 65.03 °F). The triple point for ethanol is 150 K at a pressure of 4.3 X 10^{-4} Pa. (Windholz M, 1976).

1.2.7 Theory of Fermentation Kinetics

The bioconversion of starch into ethanol is a two-step process. The first step is saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as glucoamylase and a-amylase. The second step is fermentation, where

sugar is converted into ethanol using Saccharomyces cerevisiae .(Inlow, 1988)

The use of amylolytic yeasts for the direct fermentation of starch is an alternative to the conventional multistage process which offers poor economic feasibility. Although there are over 150 amylolytic yeast species, their industrial use is limited because of their low ethanol tolerance. Therefore, most research is focused on the development of genetically engineered amylolytic strains of S. cerevisiae, and in these strains, heterologous genes encoding a-amylase and glucoamylase from various organisms have been expressed and their products excreted .(Shigechi, 2004**)**

Several studies have pointed out the potential of utilizing respiration-deficient nuclear petites for the commercial production of ethanol. Despite the vast number of strategies adopted for the construction of amylolytic strains of S. cerevisiae, there have been no reports about the application of respiration-deficient nuclear petites for the production of ethanol from starch. Hence, we were interested in determining the extent of improvement that this mutation would bring to starch-utilizing ethanol fermentation processes.

It is reported for the first time the development of a respiration - deficient nuclear petite S. cerevisiae strain excreting a bifunctional fusion protein that contains both Bacillus subtilis a-amylase and Aspergillus awamori glucoamylase activities. (Panoutsopoulou, 2001)

1.2.8 Previous related studies

The fermentation of cane sugar as substrate by Saccharomyces cerevisiae (enzyme) was critically investigated to obtain certain useful kinetic parameters and to determine the effect of temperature, pH, and substrate and yeast (enzyme) concentration on the rate of fermentation. Felix (2014) in his research reported that the rate of fermentation (measured as rate of production of CO_2) increased in proportion with temperature (optimum 32°C - 36°C), pH (optimum 5.5) substrate (optimum 50 v/v%) and yeast concentration (optimum 3.5 - 4.5 w/v%) up to a limit and subsisted either as a plateau and/or, decreases as the case may be. This suggests that the reaction takes place in two steps. The kinetic parameters examined are maximum rate of reaction V_{max} (2.0 X 10^2 M-min^{-1}), catalytic constant, k_2 (1.81 X 10^{-1} min^{-1}), overall rate constant, k (1.53 X 10_1 min^{-1}), order of initial reaction (approx. first order), dissociation constant of enzyme-substrate complex, k_s (2.74 X 10_3), Michaelis constant, k_m (2.74 X 10_3 M), and the specific activity of enzyme on substrate concentration (1 X 10^{-1} w/v%). The result of this study showed that the equilibrium step involving k_{-1}/k_1 is the limiting step deciding the direction of reaction as well as the specific activity of the enzyme till confined to the laboratory. Therefore, a comprehensive economic and process analysis is required to develop an industrially suitable production strategy that will solve our energy crisis by producing more ethanol in a stable way. (Felix, 2014)

Another study conducted in USA reported that one of the strategies to reduce production costs and to make ethanol fuel economically competitive with fossil fuels could be the use of wild yeast with

osmotolerance, ethanol resistance and low nutritional requirements. The aim of this work was to investigate the kinetics of ethanol fermentation using Saccharomyces cerevisiae ITV-01 yeast strain in a batch system at different glucose and ethanol concentrations, pH values and temperature in order to determine the optimum fermentation conditions. The study results found strain showed osmotolerance (its specific growth rate (^max) remained unchanged at glucose concentrations between 100 and 200 g L-1) as well as ethanol resistance (it was able to grow at 10% v/v ethanol). Activation energy (Ea) and Q10 values calculated at temperatures between 27 and 39 °C, pH 3.5, was 15.6 kcal mol-1 (with a pre-exponential factor of 3.8 X 1012 h-1 (R2 = 0.94)) and 3.93 respectively, indicating that this system is biologically limited. The optimal conditions for ethanol production were pH 3.5, 30 °C and initial glucose concentration 150 g L-1. In this case, a maximum ethanol concentration of 58.4 g L-1, ethanol productivity of 1.8 g L 1 h 1 and ethanol yield of 0.41 g, 1g were obtained (Ortiz-Muniz, 2010)

Sugarcane bagasse (SB) and sugarcane straw (SS) are the attractive second-generation renewable feedstock available in several countries like Brazil. This feedstock if used judiciously may provide the sustainable supply of drop-in ethanol, industrial enzymes, organics acids, single cell proteins, and so forth. However, a significant fraction of this biomass goes to industries for steam and electricity generation. The remaining fraction represents the ideal feedstock for the generation high-value commodities. Last three decades of vigorous developments in pretreatment technologies, microbial biotechnology, and downstream processing have made it reality to harness the sugarcane residues for the production of many products of commercial significance at large scale without jeopardizing the food/feed

requirements. Biomass recalcitrance is a main challenge toward the successful exploitation of these residues. To overcome the biomass recalcitrance, pretreatment is an inevitable process to ameliorate the accessibility of carbohydrate for the subsequent enzymatic hydrolysis reaction to generate fermentable sugars. There are several robust pretreatment methods available; however, the ultimate choice for the selection of pretreatment process depends upon the effective delignification or hemicellulose removal, minimum generation of inhibitors, low sugar loss, time savings, being economic and causing less environmental pollution. The released sugars after enzymatic hydrolysis and hemicellulose de-polymerization are converted into ethanol by the suitable ethnologic strain. In order to get desired ethanol yields, the ethnologic strains should have ability to utilize pentose and hexose sugars, inhibitor resistance, and high osmotolerance. The following ten requirements are pivotal in order to establish a long-term sustainable second-generation ethanol production process from sugarcane residues:

(1) Fullest utilization of SB and SS generated in the country for the better management.

(2) Selection of right pretreatment and detoxification strategy.

(3) In-house cellulase production and development of cellulolytic strains and ethanol producing strains from pentose and hexose sugars showing inhibitor resistance, ethanol tolerance, and faster sugar conversion rates.

(4) Process intensification: hydrolysis and fermentation together in one place.

(5) Cheap, fast, and effective ethanol distillation.

(6) Integration of bioethanol producing units with sugar/distilleries for the coutilization of machinery, reactors, and other equipment.

(7) Maximum by products utilization (lignin, furans, and yeast cell mass).

(8) Environmental protection. (Canilha et al. 2012)

A study conducted in Malaysia concluded that optimum conditions for highest bioethanol production using molasses are at 35 °C, pH 4.5, and 125rpm. Under this optimum operating condition the maximum of 9.31% of ethanol was produced and 34.77% of sugars were converted into ethanol. The production is acceptable theoretically as maximum ethanol production by wild type industrial yeasts can only achieves maximum up 10% v/v since greater amount of ethanol produced in the system will halt the growth of S.cerevisae. The data was validated and it was proven that the model is well fits and the findings are reliable with R2 = 99.95%. The objectives of the study were successfully achieved where the process parameters were optimized and validated (Nassereldeen et al., 2012)

In Korea a repeated batch fermentation system was used to produce ethanol using Saccharomyces cerevisiae strain (NCIM 3640) immobilized on sugarcane (Saccharum officinarum L.) pieces. For comparison free cells were also used to produce ethanol by repeated batch fermentation. Scanning electron microscopy evidently showed that cell immobilization resulted in firm adsorption of the yeast cells within subsurface cavities, capillary flow through the vessels of the vascular bundle structure, and attachment of the yeast to the surface of the sugarcane pieces. Repeated batch fermentations using sugarcane supported biocatalyst were successfully carried out for at least ten times without any significant loss in ethanol production from sugarcane juice and molasses. The number of cells attached to the support increased during the fermentation process, and fewer yeast cells leaked into fermentation broth. Ethanol concentrations (about 72.65~76.28 g/L in an average value) and ethanol productivities (about 2.27~2.36 g/L/hr in an average value) were high and

stable, and residual sugar concentrations were low in all fermentations (0.9~3.25 g/L) with conversions ranging from 98.03~99.43%, showing efficiency 91.57~95.43 and operational stability of biocatalyst for ethanol fermentation. The results of the work pertaining to the use of sugarcane as immobilized yeast support could be promising for industrial fermentations.(Babu et al. 2012)

Two hundred and thirty-four yeast isolates from Greater Mekong Subregion (GMS) countries, i.e., Thailand, The Lao People's Democratic Republic (Lao PDR) and Vietnam were obtained. Five thermotolerant yeasts, designated Saccharomyces cerevisiae KKU-VN8, KKU-VN20, and KKU-VN27, Pichia kudriavzevii KKU-TH33 and P. kudriavzevii KKU-TH43, demonstrated high temperature and ethanol tolerance levels up to 45 ◦C and 13% (v/v), respectively. All five strains produced higher ethanol concentrations and exhibited greater productivities and yields than the industrial strain S. cerevisiae TISTR5606 during high-temperature fermentation at 40 ◦C and 43 ◦C. S. cerevisiae KKU-VN8 demonstrated the best performance for ethanol production from glucose at 37 ◦C with an ethanol concentration of 72.69 g/L, a productivity of 1.59 g/L/h and a theoretical ethanol yield of 86.27%. The optimal conditions for ethanol production of S. cerevisiae KKU-VN8 from sweet sorghum juice (SSJ) at 40 ◦C were achieved using the Box–Behnken experimental design (BBD). The maximal ethanol concentration obtained during fermentation was 89.32 g/L, with a productivity of 2.48 g/L/h and a theoretical ethanol yield of 96.32%. Thus, the newly isolated thermotolerant S. cerevisiae KKU-VN8 exhibits a great potential for commercial-scale ethanol production in the future (Techaparin et al, 2017)

Chapter Two

Materials and Methods

2. Materials and Method

To produce ethanol from sugarcane, sugarcane obtained freshly from different sources and areas. pH meter yeast (Saccharomyces cerevisiae) was used as received. Fermentation vessels, vials were also used for the experiments.

2.1 Materials

2.1.1 Chemicals

1. Sugar Cane
2. Yeast
3. Sodium Metabisulphite ($Na_2S_2O_4$) taken 3g
4. Distil water
5. Octapol powder
6. Sulfuric acid
7. Sodium hydroxide

2.1.2 Glassware and devices

Pipettes, flasks, beakers, droppers, laboratory balance, heater, pH meter, centrifugal, ethanol measuring device, filter paper, test tubes, stop watch.

2.2 Methods

2.2.1 Sugarcane preparation:

The ready sugarcane juice was taken, then fibers then manually removed by squeezing out the juice from it by hand. The juice obtained was sterilized by heating in an aluminum cane at temperature of 90ºC for 40 minutes, then cooled. After cooling, 1liter of the juice, was taken for the determination of the different parameters (test solution). 3g of the octapol powder was added to the test solution. The sample strained and filtered. Polarimeter device was used to determine sugar percentage. Three grams of Sodium metabisulphite ($Na_2S_2O_4$) was added to prevent the growth of undesirable microorganisms.

The yeast was added to the substrate and were properly mixed and shacked, then left to activate for 10 minutes.

2.2.2 Determination of fermentation period (duration):

- Fermentation durations were determined at 7 periods: (30, 60, 90, 120, 150, 180, 210 minutes).
- Each period has (7) replicates (flask tubes 50 ml) filled with 43 ml of substrate of (85% conc.) and pH. (3) at room temperature. 1 g of yeast was added to each flask and gently shacked.
- Then the samples were centrifuged using centrifuging device for three minutes to make solution homogenous and remove the yeast residuals.
- Then the sucrose was measured.
- Also, the corresponding ethanol was measured.

2.2.3 Determination of the effect of temperature:

The effect of temperature on fermentation was determined by keeping other factors, such as substrate concentration, pH of the juice, yeast concentration, and fermentation time constant. The temperature was varied between 28ºC - 30ºC - 32ºC - 34ºC – 36ºC by using incubator.

The sample was centrifuged, and ethanol was measured for each temperature mentioned above.

2.2.4 Determination of pH effect:

Substrate concentration, temperature time and yeast concentration were kept constant in determining the effect of pH on fermentation kinetics. The pH meter was standardized with a buffer 4 solution. The pH was varied by the introduction of 0.1 M H_2SO_4 or (0.1 M) NaOH solution to the required pH value and measured by a pH meter. The pH of the juice was varied between 3, 3.5, 4, 5, 6. (pH). The sample was centrifuged, sucrose was measured, and ethanol was measured for each pH mentioned above.

2.2.5 Determination of the effect of substrate concentration:

In determining the effect of substrate concentration on fermentation kinetics, all other factors such as temperature, pH, yeast concentration, and fermentation time were kept constant. The substrate concentration was varied between 40 – 85.7 (v/v%). The sample was centrifuged, sucrose was measured, and ethanol was measured for each substrate mentioned above.

2.2.6 Determination of the effect of yeast concentration:

The effect of yeast concentration on fermentation was determined by varying yeast concentration between 1, 1.5, 2, 2.5, 3, 3.5g. The sample was centrifuged, sucrose was measured, and ethanol was measured for each yeast mentioned above.

2.3 Data analysis and presentation

The collected data was processed and analyzed by using computer software (Excel) and (SPSS-version 24), the results presented in forms of frequency tables and charts.

Chapter Three

Results and Discussion

3.1 Results and Discussion

The study results showed that maximum rate of alcohol produced was (2.42) after 90 minutes of fermentation table (1) and figure (1). Concerning effect of temperature maximum rate of alcohol was (2.82) obtained at 34 (C°) as show in table (2) and figure (2). With regard to the effect of substrate concentration, maximum rate of alcohol production was obtained at (+50 distilled water) of concentration percent (85.7%), table (3) and figure (3) show this result. The results also showed that maximum rate of alcohol production was obtained at PH value equal 5 , as shown in table (4) and figure (5). These results are similar to that found by Felix (2014) who found that the rate of fermentation (measured as rate of production of CO_2) increased in proportion with temperature (optimum 32°C-36°C), pH (optimum 5.5), substrate (optimum 50 v/v%). In the present study test the effect of yeast concentration revealed that maximum yield of ethanol (2.49) was corresponding to use of one gram of yeast, see table (5) and figure (6), this result unlike to that found by Fleix, who reported that optimum yeast concentration (3.5 - 4.5 w/v%). Also differ from that concluded by Bušić et al (2018) that the optimal conditions for ethanol production were pH 3.5, 30 °C and initial glucose concentration 150 g L^{-1}. This study results is partially agree with that reached by Ortiz-Muniz (2010) who investigated the kinetics of ethanol fermentation using Saccharomyces cerevisiae ITV-01 yeast strain in a batch system at different glucose and ethanol concentrations, pH values and temperature in order to determine the optimum fermentation

conditions, he found the optimal conditions for ethanol production were pH 3.5, 30 °C and initial glucose concentration 150 g /L.

Table (3.1): Alcohol production at different durations

Tubes	duration(min)	Alcohol produced (g//l)	ethanol%
1	30	1.95	4.56
2	60	2.02	4.72
3	90	2.42	5.65
4	120	1.03	2.41
5	150	0.45	1.05
6	180	0.4	0.93
7	210	0.2	0.47

Source: The researcher data analysis (SPSS & Excel)- 2021

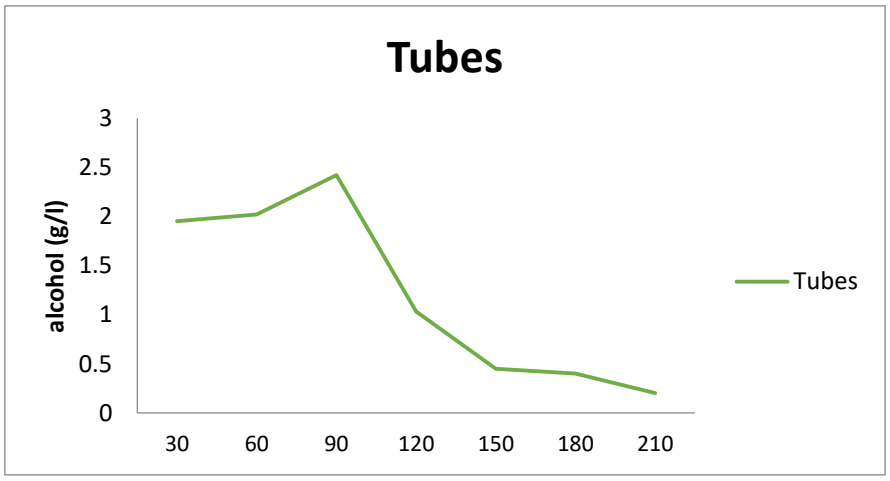

Figure (3.1): Alcohol production at different durations

Table (3.2): Alcohol production at different level of temperature

Temperatue(C°)	duration (min)	Alcohol produced (g//l)	ethanol%
28	90	2.42	5.7%
30	90	2.42	5.7%
32	**90**	2.01	4.7%
34	**90**	**2.82**	6.6%
36	90	1.01	2.4%

At constant (time, PH, yesat conc.)
Source: The researcher data analysis (SPSS & Excel)- 2021

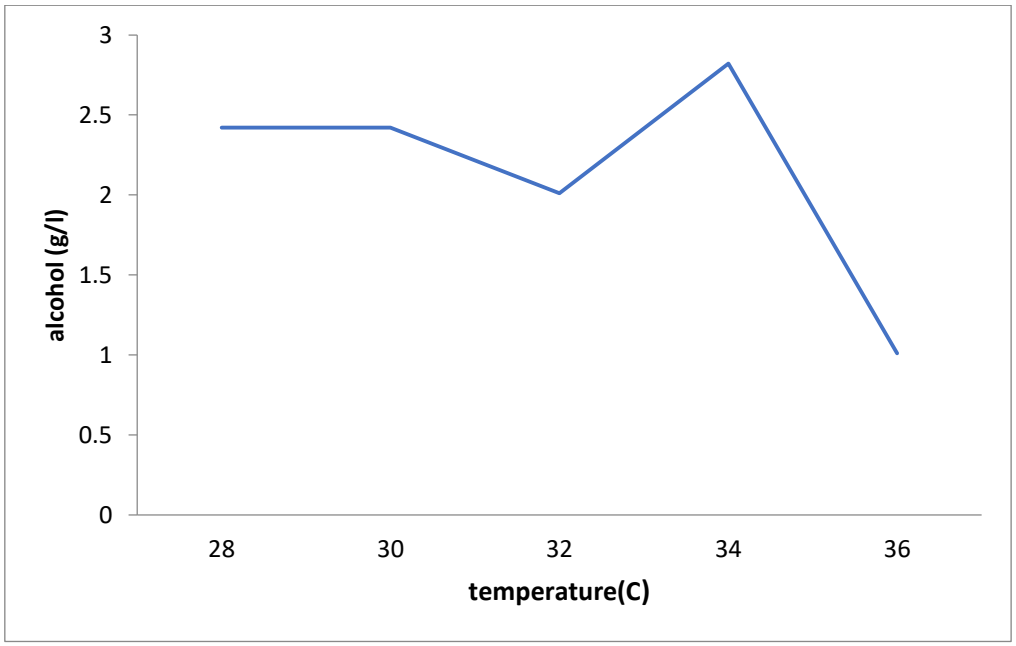

Figure (3.2): Alcohol production at different Temperature

Table (3.3): Alcohol production at different level of substrate concentrations

Substrate concentration (%)	duration (min)	Temp. (C°)	Alcohol Produced (g/l)	Ethanol (%)
85.7%	90	34	2.00	4.7%
75%	90	34	1.89	4.4%
66.7%	90	34	1.03	2.4%
60%	90	34	0.39	0.9%
40%	90	34	0.258	0.6%

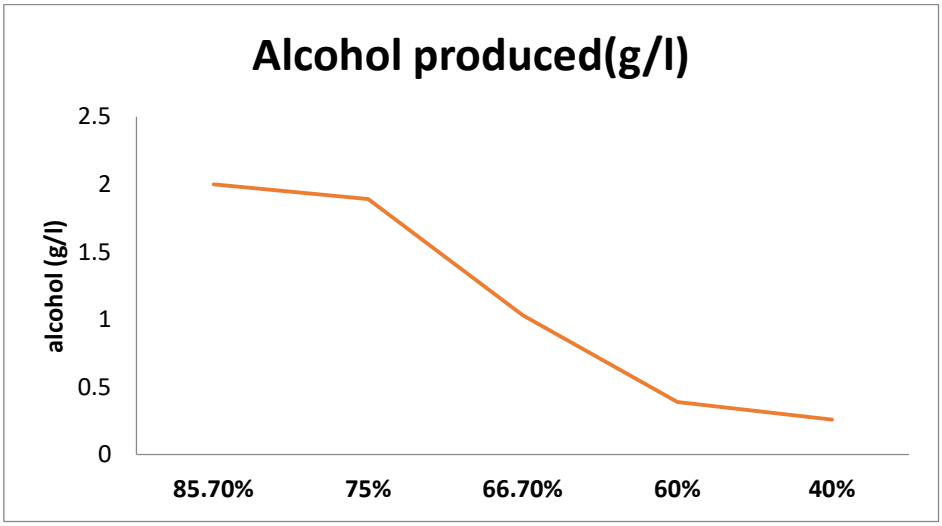

Figure (3.3): Alcohol production at different level of substrate concentrations

Table (3.4): Alcohol production at different levels of pH

PH	duration (min)	Temperature (C°)	Alcohol produced (g//l)	Ethanol %
3.5	90	34	1.05	2.5%
4	90	34	1.05	2.5%
5	**90**	**34**	**2.41**	5.6%
6	90	34	0.45	1.1%

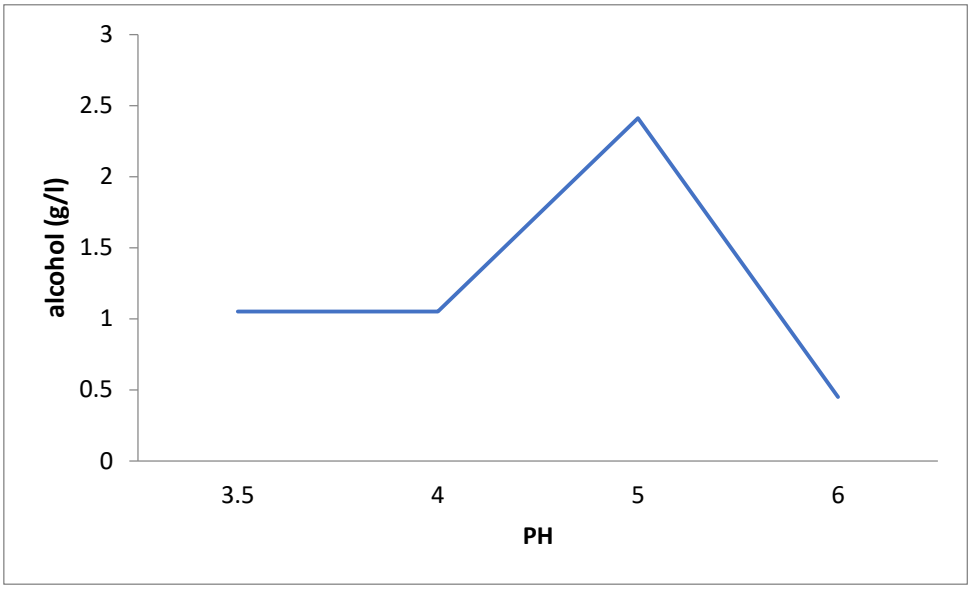

Figure (3.4): Alcohol production at different levels of pH

Table (3.5): Alcohol production at different yeast conc.

Yeast Conc.	PH	duration (min)	Temperature (C°)	Alcohol Produced (g/l)	Alcohol Produced %
0.25	5	90	34	0.72	1.7%
0.5	5	90	34	1.60	3.7%
1	5	90	34	2.49	5.8%
1.5	5	90	34	0.89	2.1%
2	5	90	34	0.62	1.4%
2.5	5	90	34	0.4	0.9%

At constant (time, Temperature, substrate conc. and PH)

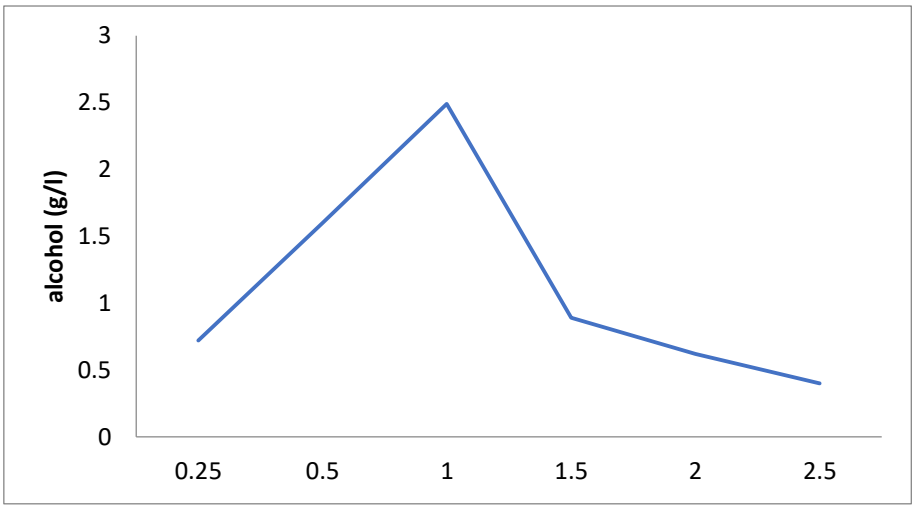

Figure (3.5): Maximum rate of alcohol production was obtained at yeast conc

Table (3.6): Substrate concentration vs angle of rotation (θ)

Substrate Conc.(%)	Angle of rotation (θ) dgree
85.7	21.9
75	20.3
66.7	18.6
60	17.5
40	15

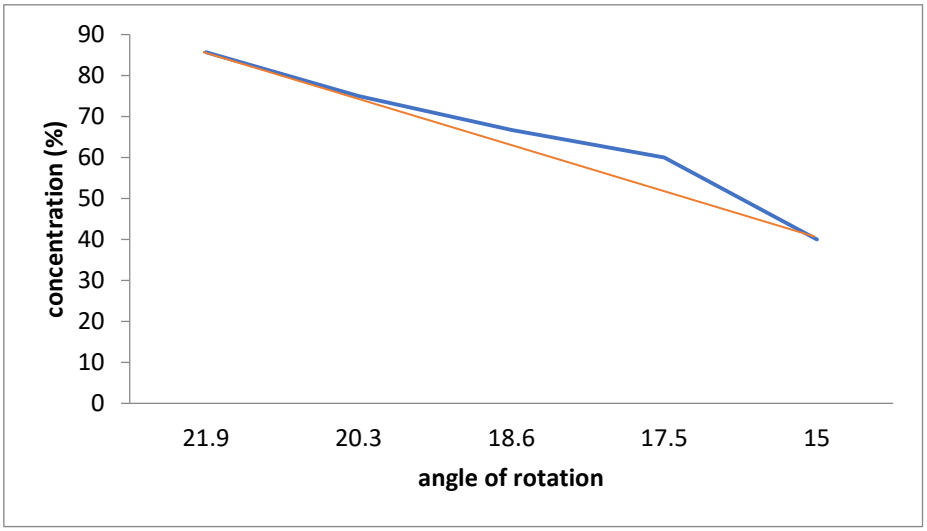

Figure (3.6): Substrate concentration versus angle of rotation

Figure (3.3) also showed linear relationship between the production of alcohol and the substrate concentration at low concentrations. Since production of alcohol is proportional to the substrate concentration, The rate of the reaction is zero order at very high concentration. On lowering the concentration of the substrate. The order increases, until in dilute solution the rates become proportional to the substrate concentration which is in agreement with the Michaelis- Menten (Latham and Burgess, 1977).

The Michaelis- Menten mechanism:

$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES; ES \xrightarrow{k_2} E + products$

The Michaelis- Menten mechanism: rate $= \frac{k_2 \times [E] \times \{S]}{K_m + \{S]}$

Where, [E] denotes the concentration of the enzyme, [S] denotes the concentration of the substrate and K_m denotes the Michaelis constant. At very low concentration of the substrate, ($K_m >> \{S]$), $\{S]$can be neglected in the denominator and the rate is directly proportion to the substrate concentration while at very low concentration ($K_m << \{S]$), Km can be neglected in the denominator and the rate is independent of the substrate concentration.

3.2 Conclusion;

Based on the presented data, it is obvious that ethanol can be produced in large industry by using the most optimal conditions to reduce the cost; the biggest challenge remains how to reduce the production cost of bioethanol. This study concluded that maximum rate of ethanol production by Fermentation of Cane Sugar using Saccharomyces Cerevisiae can be obtained at duration of 90 minutes, temperature of 34° C, and sugar cane juice of concentration 85.7%, pH of 5 and yeast of one gram.

3.3 Recommendations

The researcher recommended further kinetic studies about fermentation using other sugar substrate and different fermentation agent.

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Appendixes

Microscope used to determine yeast living cells

Device used to determine polysaccharose %

Ph-meter

Ethanol measuring device