PRODUCTION OF ETHANOL FROM ENZYME PRETREATED OIL CAKES

Submitted by
SHRAVYA SHETTY
(4NM09BT040)

MONISHA
(4NM09BT021)

SWATHI M S
(4NM09BT044)

DHANYASHREE
(4NM09BT011)

Under the guidance:
Dr. C. Vaman Rao
Professor & Head,
Dept. of Biotechnology Engineering,

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DEPARTMENT OF BIOTECHNOLOGY ENGINEERING
NMAM INSTITUTE OF TECHNOLOGY
NITTE – 574 110
KARKALA TQ, UDUPI DISTRICT

1. **Name of College/Place:** NMAM Institute of Technology, Nitte, Karkala Tq.

2. **Name of the SPP Co-ordinator:** Dr. Peter Fernandes

   Professor, Dept. of Mechanical Engineering

   NMAMIT, Nitte

3. **Project title:** Production of Ethanol from Enzyme Pretreated Oil Cakes.

4. **Department:** Biotechnology Engineering

5. **Name of guide:** **Dr. C. Vaman Rao**

   Professor & Head,

   Dept. of Biotechnology Engineering,

   NMAMIT, Nitte-574110,(Udupi Dist)

   Email: vaman.rao@gmail.com

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1. INTRODUCTION

FUEL, it is a substance that is consumed to produce energy. This energy can be used to do different types of work. In the present fast developing world, fuel can be referred to as one of our basic need that is used in every move of our life. World devoid of fuel is very difficult, even to just imagine.

With the ever increasing demand for energy and fast depleting petroleum resources, globally there is an increased interest in alternative fuel especially liquid transportation fuels. Energy crisis is one of the most serious threats towards the sustainability of human mankind and civilization. Although industrial revolution has changed the world to its sophisticated edge, excessive dependence on fossil fuels as the main source of energy has lead to the diminishing of this non-renewable supply. Furthermore, demand for petroleum derived fossil fuels is not slowing down but instead increasing substantially over the past few decades.

In 1925, Henry Ford has quoted ethyl alcohol, i.e. ethanol, as **"fuel of the future".** Today Henry ford's futuristic vision significance can be easily understood. Ethanol is a two-hydrocarbon with a hydroxyl group. It is a clear, flammable, colourless liquid that is miscible with water, as its hydrophilic hydroxyl group is capable of hydrogen bonding with water molecules.

ETHANOL: CH_3CH_2OH

Bio ethanol refers to ethanol that is produced from biomass. It is a sustainable energy resource that offers environmental and long-term economic advantages over fossil fuels (gasoline). Biomass is derived from the plants which use the light energy from the sun to convert water and carbon dioxide to sugars that can be stored through a process called Photosynthesis. Organic waste is also considered as biomass because it began as plant matter. Bio ethanol from lignocellulosic biomass and starchy sources are the important alternatives being considered due to the easy adaptability of this fuel to the existing engines and because this is a cleaner fuel with higher octane rating than gasoline.

Bio ethanol can be classified under three broad sub headings:

1. First generation bio ethanol (FGB): bio ethanol derived from edible sources such as corn, sugarcane is called first generation bio ethanol. But the drawback of FGB is that here we utilize edible sources which may lead to its depletion.

2. Second generation bio ethanol (SGB): bio ethanol derived from non-edible sources such as grasses, sugarcane leaves and bagasse, oil seeds etc is called second generation bio ethanol. SGB offers great promise to replace fossil fuels without causing harm to food-fuel supply.

3. Third generation bio ethanol: bio ethanol derived from algal biomass is called as third generation bio ethanol.

A. LIGNOCELLULOSIC BIOMASS:

Lignocellulosic biomass is considered as the foreseeable, feasible and sustainable resource for renewable fuel. Inexpensive, abundant and renewable non- food lignocellulosic feedstock has high potential for producing ethanol via fermentation by microorganisms. Corn is currently the predominant feedstock for ethanol production in the US. However, corn- based ethanol production is not economically or environmentally sustainable because it not only competes with food and feed production for limited agricultural land, but has also been associated with some substantial environmental problems such as nutrient pollution and soil erosion. Lignocellulose-to-ethanol conversion is regarded as a sustainable technology to supplement corn- based ethanol production due to the abundance of lignocellulosic biomass and diverse raw materials available .

Specific feed stocks under considerations include:

- **Agriculture Residues :**

Eg : Left over materials from crops, such as the stalks, leaves , husks of corn plants, chopped trunks , dead fronds, empty fruit palm bunches, shell and fibres etc.

- **Forest Residues:**

Eg: Woods and straws from pulp and paper industries, dead trees, chips and sawdust from lumber mills.

- **Municipal Solid :**

Eg: Household garbage, waste generated from residential, commercial and municipal services.

- **Energy Crops :**

Eg: Fast growing trees and grasses developed just for this purpose.

The main components of these types of biomass are cellulose, hemicelluloses and lignin. Cellulose and hemicelluloses are polysaccharides that can be used for ethanol production, while lignin is a complex aromatic polymer that stiffens and surrounds the fibres of polysaccharides. the extensive interaction between these 3 components render a recalcitrant structure of lignocelluloses which necessitates a pre-treatment step thus making celluloses and hemicelluloses more accessible to hydrolytic enzymes for fermentable sugar production.

The production of Bio ethanol from lignocelluloses biomass involves following 3 important steps

1. **Pre-treatment of Raw Biomass:** The purpose is to improve the enzymatic digestibility of lignocelluloses by removing lignin, hemicelluloses, reduce cellulose crystlanity and increase the surface area of the material through physical, chemical or biological methods.
2. **Hydrolysis for fermentable sugar production**: Is a chemical reaction that converts the complex polysaccharides in the raw feedstock to simple sugar. In the biomass-to-bio ethanol process, acid and enzymes are used to catalyse this reaction.
3. **Fermentation:** It is a series of chemical reaction that convert sugar to ethanol. The fermentation reaction is caused by yeast or bacteria, which feed on the sugar .Ethanol and carbon dioxide are produced as the sugar is consumed.

The simplified fermentation reaction equation for the 6-carbon sugar, glucose is:

$$C_6H_{12}O_6 \text{----------} \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$$

Glucose Ethanol Carbon dioxide

There are many lignocellulosic biomass used for bio ethanol production. Some of them are sugarcane bagasse, rice straw, wheat straw, oat straw, barley straw, rice hulls, tobacco stalk, apple pomace, groundnut shell, coconut husk, Bermuda grass, switch grass etc. [11,39,40].

B. STARCHY SOURCES:

Starch is a high yield feedstock for ethanol production. It is a very important and abundant natural solid substrate. Starch is a homopolysaccharide of alpha-glucose residues linked through glycosidic bond. Essentially starch is composed of two related polymers in different proportions according to its source: amylase (16-30%) and amylopectin (65-85%). Amylase is a polymer of glucose linked by α-1, 4 bonds, mainly in linear chain. Amylopectin is a large highly branched polymer of glucose including also α- 1,6 bonds at the branch point. To produce bio ethanol from starch its hydrolysis is required. I.e. it is necessary to break down the chains of carbohydrates for obtaining glucose syrup, which then can be converted into bio ethanol by yeast. No pre-treatment is required as starch is an amorphous compound which can be easily hydrolysed by enzymes and acids. Starch was traditionally hydrolyzed by acids, but the specificity of enzymes, their inherent mild reaction condition and the absence of secondary reaction have made amylases to be the catalyst generally used for this process. α-amylases are generally produced by themoresistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis.*

Starch is much easier to hydrolyse to glucose than cellulose as starch contains only glucose, whereas biomass substrates contain significant levels of other sugars, most notably xylose. Thus starch based process does not require cellulolytic enzyme production or pentose fermentation to achieve high conversion yields. Another difference is that solids handling requirements are typically higher for biomass substrates, compared to starchy sources.

Some of the example for starchy sources is corn, maize starch, tubers like dioscorea, colocasia, cassava, residual seeds like jackfruit seeds. [24,10,31].

1.1 ADVANTAGES OF BIOETHANOL:

Increasing industrial activity and population growth has resulted in a rising concentration of ‘greenhouse gases ‘in the atmosphere that contribute to the ‘Greenhouse Effect’. These gases include carbon dioxide, methane and nitrous oxide. The term ‘Greenhouse Effect” refers to the Earth’s trapping of the sun’s incoming solar radiation, causing warming of the Earth’s atmosphere. With the threat of global warming and energy cries in today‘s environment, need for clean, “green” fuels is quickly becoming necessity.

Bio ethanol is a replacement of fossil fuels because it is a renewable agricultural source and it does not add net carbon dioxide- a greenhouse gas - to the atmosphere. The carbon dioxide released by the combustion of ethanol is absorbed by agricultural crops, which will eventually be converted to ethanol and thus a carbon dioxide balance is maintained in the atmosphere. Ethanol has a higher octane number (99) than petrol (80-100). As a result, pre ignition’ does not occur when ethanol is used. Higher octane rating of ethanol allows the compression ratio of the engines to be increased. This results in increased production of power. Ethanol is burnt more completely so that hydrocarbon emission is drastically lower as compared to that in case of petrol. Ethanol is much less likely to catch and explode in cases of fuel leakage. Moreover it even degrades quickly. The flashpoint of bio ethanol is much higher than of petrol. Ethanol

has a much higher latent heat of vaporizations (855 MJ/kg) than petrol (293KJ/kg). As a result, the fuel mixture entering the cylinder is much cooler and hence denser in case of ethanol than in the case of petrol. Generally it is mixed with petrol. This increases the octane rating of petrol.

Ethanol is made from agriculture crops, which "breathe" carbon dioxide and give off oxygen. This maintains the balance of carbon dioxide in the atmosphere. Increased use of renewable fuels like ethanol will help counter the pollution and global warming effects of burning gasoline. Under current conditions, use of ethanol blended fuel as E10 reduces greenhouse gases by 2.4-2.9%. Ethanol blended fuel as E10 blend reductions are lower because a smaller fraction of the blend is ethanol. With improved technologies and use of ethanol made from cellulose, these reductions in emissions will increase.

Carbon monoxide(that causes respiratory problems), formed by the incomplete combustion of fossil fuels, is produced most readily from petroleum fuels, which contains no oxygen in their molecular structure .since bio ethanol and other oxygenated compounds contain oxygen, their combustion in automobile engines is more complete. The result is a sustainable reduction in carbon monoxide emission.

Because of its effect in reducing hydrocarbons and carbon monoxide in exhaust, adding ethanol to gasoline results in an overall reduction in exhaust ozone-forming potential. Adding ethanol to gasoline can potentially increase the volatility of gasoline. Adding of ethanol to gasoline does create slightly greater amounts of aldehydes during fuel combustion. Yet the resulting concentrations are extremely small and are effectively reduced by the three-way catalytic converters in the exhaust system of all recent-model cars.

Furthermore, the investigation of secondary by-products associated with ethanol production, which would be effective in medicinal and pharmaceutical fields, is of greater importance. In addition to their extreme usefulness, these by-products will reduce the production cost of the main target process. Optically active compounds, such as methyl-diols and secondary alcohol derivatives were mentioned as by-products produced by yeasts. For example, cholorozotocin is a cytostatic agent that is used in the investigational treatment of cancers of stomach, large intestine, pancreas, lungs, melanoma and multiple myeloma. This compound when it is produced from yeast during fermentation could explore a new important trend in fermentation biotechnology.

1.2 LITERATURE REVIEW:

A) SUGARCANE LEAVES (LIGNOCELLULOSIC BIOMASS):

Sugarcane *(saccharum officinarum)* is an economically important plant of several countries including Thailand. It is cultivated in about 10.7 thousand million square –meters in Thailand. Harvesting of sugarcane is difficult because the leaves are sharp. Therefore, removal of the leaves by burning before cutting the sugarcane shoot is a popular practice, but this causes

serious air pollution. Moreover, the price of the resultant sugarcane shoot is reduced. Value addition to sugarcane leaves would be likely to stop or to reduce the practice of leaf burning as well as to improve the environmental and economic efficiency of sugarcane agriculture. Each year sugarcane cultivations generate 18 million tons of leaves, which are currently a waste product. As a lignocellulosic biomass, sugarcane leaves are potential source of bio fuels including production of bio ethanol. Hydrolysis of the cellulose and hemicelluloses components results in the formation of the fermentable monosaccharides, glucose and xylose. However, prior to the ability to saccharify the cellulose content of the sugar cane leaves, in order to use it as a raw material for ethanol production, the cellulose must first be unshielded from the hemicelluloses and lignin, and so made accessible to the cellulose enzyme. Such pre-treatment processing of lignocellulosic biomass can be achieved from physical methods, such as heat or pressure treatment, and by chemical methods, such as acid or alkaline treatment. After the leaves were pre-treated with sodium hydroxide or with alkaline hydrogen peroxide, the pre-treated residues were subjected to enzymatic hydrolysis. The obtained hydrolysate was then supplemented with nutrients prior to fermentation to ethanol. This pre-treated and hydrolysed sugarcane leaves have been used as a substrate for ethanol production by the simultaneous saccharification and fermentation method using *Saccharomyces cerevisiae* and also sometimes the thermo tolerant yeast *Kluyveromyces fragilis. [36]*.

B) POTATO STARCH (STARCHY SOURCE):

Potato starch residue stream produced during chips manufacturing was used as an economical source for biomass and bio ethanol production. Residue streams from starch processing industries, obtained after the separation of the high-value gluten and the main starch fraction, can provide a low-cost substrate for fuel ethanol production. Many industries all over the world utilize potato as raw material. Chips manufacturing industry is one of the most industries, which depend completely on potato. During chips processing, huge quantity of residue stream is drained. This stream contains some dissolved minerals and solids. Here the main constituent of the drained solids is starch. As we know starch is a most important and abundant natural solid substrate. Potato starch requires a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Typically, starch is mixed with water to form slurry; this is then stirred and heated to rupture the cells walls. Specific enzymes that will break the chemical bonds are added at various times during the heating cycle [2]. Starchy grains and effluent from starch generating unit are cheap substrates and could used as potential raw material for ethanol fermentation. *Saccharomyces cerevisiae* is the microorganism of choice used for the fermentation here. Since *S. cerevisiae* lacks α-amylase and glucoamylase, it is not able to hydrolyze starch but requires previous hydrolysed starch for fermentation efficiency. [24].

C) SACCHAROMYCES CEREVISIAE:

Sacchromyces cerevisiae is one of the most relevant microorganism (yeast) considered for the fermentation of lignocellulosic biomass and starchy sources via consolidated bio processing. It is the microorganism of choice and currently enjoys the monopoly fuel ethanol industry as it is cheaply and readily available. It could produce ethanol to a concentration as high as 18% of the fermentation broth and is proven to be quite robust and less sensitive to inhibitors. But there are some drawbacks regarding this species in the fermentation process.

In case of lignocellulosic biomass:

This yeast is unable to hydrolyze cellulose. It cannot depolymerise cellulose or ferment ethanol directly from cellulose. Hence a consortium of enzymes (endoglucanase, exoglucanase, and β-glycosidase) is needs to break down cellulose into fermentable glucose monomers. In present days, in order to overcome this problem, many successful attempts have been made to express cellulose gene. Cellulose have been expressed either as proteins attached to the surface of yeast cells or secreted into culture media. Although enzyme produced mainly by the fungus in *S. cerevisiae Trichoderma reesei* are commercially available, their high cost is a major limitation to economical utility due to large amount of cellulose required.

Unlike hexose, pentoses cannot be fermented by ordinary *saccharomyces cerevisiae*. In order to improve the economic viability of lignocelluloses-to-ethanol conversion, hemicelluloses hydrolysates containing high proportion of pentose should also be utilized for ethanol production. Based on principles of breeding and natural selection, Attfield and Bell (2006) developed strains of *S. cerevisiae* that can double in less than 6 hrs using xylose as a sole source. This represented an important an important step in developing pentose-utilizing *S.cerevisiae* strains for ethanol production. Development of genetically engineered microorganism capable of metabolizing both hexodes and pentose's is another way to make the most of biomass-derived sugars. Some industrial pentose-fermenting *S.cerevisiae* strains have been developed to address this challenge. [11].

In case of starchy materials, ethanol production has been commercially dominated by yeast S. *cerevisiae.* Two strains of *S.cerevisiae* are commonly used. They are Y-1646 and another commercial one. The strain Y-1646 gives maximum yield of ethanol at 35®c after 36hrs in the presence of $Zncl_2$. Zinc seems to be one of the most effective element in yeast's metabolic pathway. So its addition has good impact on its growth and ethanol production. One of the drawbacks here is since *S.cerevisiae* lacks α-amylase and glucoamylase, it is not able to hydrolyze starch. Hence it requires previous hydrolyzed starch for fermentation effiency.

D) ENHANCED PRODUCTION OF BIOETHANOL:

As we all are aware of the interest in ethanol production from biomass feed stocks as an alternative energy source for petroleum, has grown as the price of petroleum has generally increased over the last few years. Current research on bio ethanol is driven by the need to reduce the cost of ethanol production and as focused on improving feedstock pre-treatment methods, enzymes and fermentation. Ethanol is produced by four main types of industrial

operation, including batch, fed-batch, continuous, and semi-continuous operation. However most ethanol produced today is prepared by batch operations because batch fermentation has the advantage of low investment costs, simple controls, and operation that do not require specialized labour, complete sterilization and management of feedstock. Another advantage of batch fermentation is that the methodology of immobilized yeast can be applied here.

Yeast immobilized on alginate beads produced a higher ethanol yield more rapidly than did free yeast cells, under the same batch fermentation conditions. The optimal fermentation conditions were 30®C, ph 5.0, and 10% initial glucose concentration with 2% sodium alginate beads. All bead samples resulted in nearly 100% ethanol yield where as the free cells resulted in an 88% yield.

Some of the advantages of using immobilized yeast cells for fermentation during bio ethanol production are:

- Ability to separate immobilized yeast from the ethanol product.
- Smaller reactor size required.
- Higher ethanol tolerance and cell concentration.
- Shorter fermentation time.
- Increased period of aging time.
- Long-term preservation.
- Greater cell activity.
- Greater stability.
- Enhanced fermentation productivity.
- Greater feasibility of continuous processing.

Kourkoutas discussed the properties of carriers and immobilized cells. Immobilization technique can be divided into four main categories based on the physical mechanism employed:

1. Attachment or adsorption on solid carrier surfaces.
2. Entrapment within a porous matrix.
3. Self aggregation by flocculation or with cross-linking agents.
4. Cell containment behind barriers.

Various immobilization substrates have been used, including alumina beads and membranes, porous glass beads, diatomaceous earth, DEAE-cellulose, calcium alginate beads, k-carrageenan, wood chips, silicon carbide and spent grains.

Sodium alginate is a water soluble polymer that produces highly viscous solutions, a characteristic that contributes to stabilizing the suspension of microorganisms in the alginate matrix. Sodium alginate solutions form gels in the presence of cations such as Ca^{2+} (Draget al., 1997). Immobilizing cells in alginate is simple, cheap and non-toxic. Therefore, alginate is frequently used for immobilization. Moreover, calcium alginate is the most widely used gel matrix in laboratory, pilot-plant and industrial-scale fermentation projects. [16].

1.3 PRODUCTION OF BIOETHANOL FROM LIGNOCELLULOSIC BIOMASS:

1.3.1 PRETREATMENT:

Although Lignocellulosic is the most abundant plant material resource, its susceptibility has been curtailed by its rigid structure. Lignocellulosic biomass in its natural form is a tough feedstock for hydrolysis due to the crystallinity of cellulose and due to the compact packing of cellulose, hemicellulose and lignin in the plant material. As the result effective pretreatment is needed. The purpose of pretreatment is to improve the enzymatic digestibility of Lignocellulosic by removing lignin/hemicelluloses, reduce cellulose crystallinity, increase the surface area of the material through physical, chemical or biological methods and render it accessible for a subsequent hydrolysis step. By far, most of the pretreatments are done through physical and chemical means. Now a day's biological pretreatment is also in use. In order to achieve higher efficiency both physical and chemical pretreatment are required. Physical pretreatment is often called size reduction to reduce biomass physical size. Chemical pretreatment is to remove chemical barriers so that the enzymes can have access to cellulose for microbial destruction. Biological pretreatment is to breakdown the tough lignin structure. Hence pretreatment is believed to have great potential for efficiency improvement and cost lowering through research and development (Mosier et al., 2005) and a number of technologies have been investigated for the effective pretreatment.

a) Physical pretreatment:

There are two categories of physical pretreatments, mechanical and non mechanical (53).The first class includes those procedures like milling and grinding in which the reduction in the substrate size is accomplished by an increase of the surface-to-volume ratio thus making cellulose more accessible to hydrolysis. Although mechanical pretreatment methods increase cellulose reactivity towards enzymatic hydrolysis, they are unattractive due to their high energy and capital costs. The non mechanical pretreatments provide a combination of mechanical forces with powerful external forces. This class includes pretreatments like irradiation and high pressure streaming, which decompose lignocellulose. Although this treatment ensures an appreciable degree of hydrolysis, it requires along the treatment time, a significant energy input, for this reason it s not economically viable.

b) Chemical pretreatment:

Chemical pretreatments employ different chemical agents such as acids, alkalis, peroxide, organic solvents and ozone. Acid and alkaline prehydrolysis are the most intensively studied chemical methods in pretreatment of Lignocellulosic biomass. Chemical pretreatment helps to liberate the hemicelluloses, sugars or lignin, which are susceptible to enzymatic hydrolysis. Acid pretreatment results in disruptions of covalent bonds, hydrogen bonds and Vander Waals forces that hold together the biomass components, which consequently causes solubilization of hemicelluloses and reduction of cellulose crystallinity (Li et al., 2010).Inorganic acids as H_2SO_4 and HCl have been preferably used for biomass pretreatment. Mild acid treatment will

result in hydrolysis of cellulose which is recovered in the liquid fraction while lignin and cellulose is recovered as solid fraction which can then be hydrolyzed using enzyme. Generally hydrolysis with dilute sulphuric acid has been successfully developed given that high reaction rates can be achieved improving significantly the subsequent process of cellulose hydrolysis. Although diluted acid is less toxic, it some times proves to be hazardous and corrosive to reactors (Sun and Cheng 2002).In contrast, alkaline pretreatment cause's delignification of biomass and make the lignocelluloses swollen through saponification reactions (Xu et al.2010a).

Unlike acid pretreatment, alkaline pretreatment has been prove effective within a wide temperature range at various chemical concentrations (Xu et al., 2010a, b).Sodium hydroxide(NaOH) and lime($Ca(OH)_2$) are the two alkaline reagents that have attracted most attention. In case of alkali pretreatment, lignin component is dissolved in alkali and removed in liquid fraction, while the hemicelluloses and cellulose fractions are recovered together in the solid fraction. Alkaline pretreatment is based on the effects of the addition of dilute bases on the biomass: increase of internal surface by swelling, decrease of polymerization degree and crystallinity, destruction of link between lignin and other polymers and break down of lignin. The effectiveness of this method depends on the lignin content of biomass (Sun and Cheng 2002).

Other chemical techniques investigated include ozonolysis, phosphoric acid-acetone pretreatment and enzyme pretreatment. Ozone pretreatment effectively removes lignin, slightly attacks hemicelluloses, while hardly degrades cellulose (Sun and cheng, 2002).It does not produce toxic byproducts that will be inhibitory to the downstream processes. Another innovative pretreatment technology involves the application of the combination of a nonvolatile cellulose solvent (phosphoric acid) and a volatile organic solvent (acetone), which effectively separates the lignocelluloses components at moderate temperature, with both solvents easily recycled after pretreatment (Zhang et al., 2007).

c) Physico-Chemical pretreatment:

Physical-chemical pretreatment methods are considerably more effective than physical. The steam explosion and ammonia fiber explosion (AFEX) is the most investigated physico-chemical techniques.

In the AFEX process, lignocellulosic biomass is treated with liquid ammonia at moderate temperature and under high pressure for 10 to 15 min, followed by an explosive pressure release (Reshamwala et al. 1995).Instantly releasing the pressure disrupts the fibrous structure of biomass and increases the accessible surface area, thus improving the digestibility of biomass (Teymouri et al., 2004).AFEX pretreatment also reduces lignin content, decrystallize cellulose, and prehydrolyze hemicelluloses (Teymouri et al. 2005).

Steam explosion s another most effective and costly physico-chemical pretreat for biomass. The process provides the use of saturated water steam at high temperature and pressure to cleave the chemical bonds between lignin, cellulose and hemicelluloses. Biomass s chopped to an appropriate size and fed into a high pressure reactor where t remains for a pre-selected time.

At the end of the selected time, the treated biomass s expelled through a valve and the material literally explodes into flash tank. The exploded biomass and volatile steam are recovered.

d) Biological pretreatment:

Biological pretreatment employs microorganisms and their enzyme systems to break down lignin structure of the Lignocellulosic materials. White- rot fungi, which belong to Basidiomycetes, are the most affective and intensively studied organisms for lignocellulose pretreatment. Free radicals n the aromatic moieties generated by oxidative enzymes from the fungi, along with catalysts, result n degradation of aromatic compounds (Anderson and Akin, 2008).

Although biological pretreatment avoid high energy requirements for heating and other chemical expenses, they have not been studied as extensively as other chemical or physical methods due to the low reaction rates.

1.3.2 HYROLYSIS:

After pretreatment there are two types of processes to hydrolyze the feed stocks into monomeric sugar constituents required for fermentation into ethanol. The hydrolysis methods most commonly used are acid (dilute or concentrated) and enzymatic. To improve the enzymatic hydrolytic efficiency, the lignin-hemicelluloses net work has to be loosened for the better amenability of cellulose to residual carbohydrate fraction for sugar recovery. Dilute acid treatment is employed for the degradation of hemicellulose leaving lignin and cellulose network in the substrate. Other treatments are alkaline hydrolysis or microbial pretreatment with white-rot fungi (Phaenerochate chrysosporium, Cyathus stercoreus, Cyathus bulleri and pycnoporous cinnabarinus etc.) preferably act upon lignin leaving cellulose and hemicellulose network in the residual portion. However during both treatment processes, a considerable amount of carbohydrates are also degraded, hence the carbohydrate recovery is not satisfactory for ethanol production.

a) Acid hydrolysis:

There are two types of acid hydrolysis process commonly used – dilute and concentrated acid hydrolysis. The dilute acid process is conducted under high temperature and pressure and has reaction time in the range of seconds or minutes. The concentrated acid process uses relatively mild temperatures, but at high concentration of sulfuric acid and a minimum pressure involved, which only creates by pumping the materials from vessel to vessel. Reaction times are typically much longer than for dilute acid process.

i. Dilute acid hydrolysis:

In dilute acid hydrolysis, the hemicellulose fraction is de-polymerized at lower temperature than the cellulosic fraction. Dilute sulfuric acid is mixed with biomass to hydrolyze hemicellulose to xylose and other sugars. Dilute acid s interacted with the biomass and slurry

is held at temperature ranging from 120-220°C for a short period of time. Thus hemicellulose fraction of plant cell wall s de-polymerized and will lead to the enhancement of cellulose digestibility n the residual solids (40,6,29,37).Dilute acid hydrolysis has some limitations. If higher temperatures (or longer residence time) are applied, the hemicellulosic derived monosaccharide's will degrade and give rise to fermentation inhibitors like furan compounds, weak carboxylic acids and phenolic compounds. These fermentation inhibitors are know to affect the ethanol production performance of fermenting microorganisms (Chandel et al., 2006b).In order to remove the inhibitors and increase the hydrolysates fermentibilty, several chemicals and biological methods have been used. These methods include over liming, charcoal adsorption (Chandel et al., 2006b), on exchange, detoxification with laccase (Martin et al., 2002; chandel et al., 2006b), and biological detoxification. The detoxification of acid hydrolysates has been shown to improve their fermentibilty; however, the cost s often higher than the benefits achieved. Dilute acid hydrolysis is carried out in two stages: **first stage and two stage.**

In first stage the lignocellulosic material is first contacted with dilute sulfuric acid (.75%) and heated to approximately 50°C followed by raising the temperature to 190°C. Approximately, 80% of the hemicelluloses and 29% of cellulose are hydrolyzed in the first reactor. The hydrolysate is further incubated at a lower temperature for a residence time of 2 hour to hydrolyze most of the oligosaccharides into monosaccharide followed by the separation of solid and liquid fractions. The solid material again washed with plentiful of water to maximize sugar recovery. The separated solid material is sent to second stage acid hydrolysis reactor.

In a two stage dilute acid hydrolysis process, first, biomass is treated with dilute acid at relatively mild conditions during which the hemicelluloses fraction is hydrolyzed and the second stage is normally carried out at higher temperature for de-polymerization of cellulose into glucose. The liquid phase, containing the monomeric sugars is removed between the treatments, thereby avoiding degradation of monosaccharide formed. It is very important to avoid monosaccharide degradation products for improving the ethanol yield. The highest yield of hemicelluloses derived sugars were found at a temperature of 190°C, and a reaction time of 5-10 min, whereas in second stage hydrolysis considerably higher temperature (230°C) was found for hydrolysis of remaining fraction of cellulose.

ii. Concentrated acid hydrolysis:

This method uses concentrated sulfuric acid followed by a dilution with water to dissolve and hydrolyze the substrate into sugar constituents. This process provides complete and rapid conversion of cellulose to glucose and hemicelluloses to xylose with a little degradation. The concentrated acid process uses 70% sulfuric acid at 40-50°C for 2 to 4 hr in a reactor. The low temperatures and pressure will lead to minimize the sugar degradation. The hydrolyzed material is then washed to recover the sugars. In the next step, the cellulosic fraction has to be de-polymerized. The solid residue from first stage is de-watered and soaked in 30-40% sulfuric acid for 50 min, at 100°C for further cellulose hydrolysis. The resulting slurry mixture is pressed to obtain second acid-sugar stream (approximately 18% sugar and 30% acid). Both the

sugar steams from two hydrolysis steps are combined and may be used for subsequent ethanol production.

The primary advantage of the concentrated acid process is the potential for high sugar recovery efficiency, about 90% of both hemicelluloses and cellulose fraction gets de-polymerized into their monomeric fractions. The acid and sugar syrup are separated via ion exchange and then acid is re-concentrated through multiple effect evaporators. The remaining lignin rich solids are collected and optionally palletized for fuel generation.

b) Enzymatic Hydrolysis:

Enzymatic hydrolysis of the cellulosic component of pretreated biomass is the key step in lignocellulosic biomass to ethanol technology. The yield of sugars from a pretreated feedstock is largely dependent on the type of enzymes and their activities. These features will largely determine the enzyme loading and duration of hydrolysis which in turn determines the overall process economics (Sukumaran and Pandey, 2009a). Compared with conventional acid or alkaline hydrolysis, enzymatic hydrolysis requires less utility cost as it is normally carried out at mild conditions (about 50°C and pH 4.8) (Duff and Murray, 1996). The enzymatic cocktails are usually mixtures of several hydrolytic enzymes comprising of celluloses, xylanases, hemicellulases and mannanases. In last decade, new cellulases and hemicellulases from bacterial and fungal sources have continued been isolated and regular efforts have been made for the improved production of enzymatic titers. However, the cellulases were produced at concentration too low and too slow to be formed although the process is slower. For fermentation of lignocellulosic materials, cellulose should be degraded into glucose (saccharification). Cellulose is hydrolyzed by cellulases which can be generated by various microorganism including bacteria, fungi such as clostridium, cellulomonas, Tricho-derma, Penicillum, Neurospora, Fusarium, Aspergillus etc. Fungal cellulases are the most promising cellulytic enzymes to release reducing sugars for ethanol production on a commercial scale. Cellulase hydrolyzes the β-1, 4 D-glucan linkages in cellulose and produces as primary products glucose, celloboise and cello oligosaccharides.

Three major types of Cellulase enzyme are involved in the hydrolysis of native cellulose namely endo-β-1,4-glucanase (EG), cellobiohydrolase (CBH) and β-glucosidase (Schulin 1988). Endoglucanases produces nicks in the cellulose polymer exposing reducing and non reducing ends, to liberate cello-oligosaccharides and cellobiose units, and β-glucosidase cleaves the cellobiose to liberate glucose completing the hydrolysis. This complete cellulase system comprising CBH, EG, and BG components, thus acts synergistically to convert crystalline cellulose to glucose (Bguin and Aubert, 1994; Henrissat 1994). The final step in cellulase mediated hydrolysis catalyzed by β-glucosidase is much of relevance, since the substrate of this enzyme-cellobiose which is generated by action of cellobiohydrolase is very potent inhibitor of the CBH and EG enzymes at higher concentrations. Celloboise can decrease the rate of cellulose hydrolysis by CBH and EG as much as 50% at a concentration of 3g/l (White and hindle, 2000). Cellobiose accumulation has been a major problem in enzymatic hydrolysis because the commercially used cellulase producing microbes make very little β-

glucosidase compared to the other enzyme classes. For this reason β-glucosidase from other source needs to be added in order to complement the action of cellulases.

1.3.3 FERMENTATION:

Fermentation is the process of extracting energy from the oxidation of organic compounds, such as, carbohydrates, using an endogenous electron acceptor, which is usually an organic compound. Fermentation of sugars generated from enzymatic hydrolysis of biomass is another important step where a lot of technical advances are needed to make lignocelulosic ethanol technology feasible. Yeasts, bacteria and fungi can ferment ethanol based on lignocellulosic hydrolysate. The most microbes for ethanol production are *Sacchromyces cerevisiae*. It could ethanol concentration as high as 18% of the fermentation broth and is proven to be quite robust and less sensitive to inhibitors. (Varga et al.2004; Lin and tanaka 2006). Bur *S. cerevisiae* is capable of converting only hexose sugar to ethanol. The most promising yeast that have ability to use both C_5 and C_6 sugars are Pichia *stepitis, Candida shehatae* and *Pachysolan tannophilus*. But their ethanol production rate from glucose is at least 5 times less than that observed by *S.cerevisiae*. Moreover, they require oxygen and ethanol tolerance is 2-4 times lower.

Researchers are now focusing on developing recombinant yeast, which can greatly improve the ethanol production yield by metabolizing all forms of sugars, and reduce the cost of operation. In this contention the researches have made efforts by following two approaches. The first approach has been to genetically modify the yeast and other natural ethanologens, additionally pentose metabolic pathway. The second approach is to improve ethanol yields by genetic engineering in microorganism that have ability to ferment both hexoses and pentoses (40, 18), (katharia et al; 2006).

The classic configuration employed for fermenting biomass hydrolyzates involves a sequential process where the hydrolysis of cellulose and the fermentation are carried out in different units. This configuration is known as separate hydrolysis and fermentation (SHF). In the alternative variant, the simultaneous saccharification and fermentation (SSF), where the hydrolysis and fermentation are performed in a single unit. During convectional SHF, the increased glucose content in hydrolyzate is inhibitory to both cellulose and cellobiase (xiae et al; 2004). In order to avoid end-product inhibition, the approach of SSF has been developed, in which the fermentable sugars generated during enzymatic hydrolysis is consumed immediately by yeast for ethanol fermentation.

a) Separate hydrolysis and fermentation (SHF):

One of the main feature of SHF process is that each step can be performed at its optimal operating conditions. The separation of hydrolysis and fermentation offers various processing advantage and opportunities. It enables enzymes to operate at high temperature for increased performance and fermentation organisms to operate at moderate temperatures optimizing the utilization of sugars. Here cellulose is first hydrolyzed into C_6 sugars, which are inturn fermented into ethanol. The most important factors to be taken into account for saccharification

step are reaction time, temperature, pH, enzyme dosage and substrate load. However, the inhibitory effect of glucose on microorganism activity and the disadvantage of operating with two bioreactors should be considered.

b) Simultanoeus saccharification and fermentation (SSF):

The SSF process shows more attractive index than the SHF as the higher ethanol yield and lees energetic consumption. In this case, the cellulases and microorganisms are added to the same process unit allowing that the glucose formed during the enzymatic hydrolysis of cellulose be immediately consumed by microbial cells converting it into ethanol. Thus, the inhibition effect caused by the sugars over the cellulases is neutralized. The primary advantage is the increase in the hydrolysis rate due to decreased product inhibition, as cellulase is inhibited by glucose and cellobiose. Besides, the lower cost resulting from the reduction in the number of reactor vessels needed means this process is cheaper than SHF. The major technological issue to be solved lies in finding the proper combination between the optimal operational condition, pH, and temperature values for both the enzymes and the microorganisms. In addition, this process operates at non-optimal conditions for hydrolysis and requires higher enzyme dosage, which positively influences on substrate conversion, but negatively on process costs. Considering that enzymes account for an important part of production costs, it is necessary to find methods for reducing the cellulases doses to be utilized.

1.4 PRODUCTION OF BIOETHANOL FROM STARCHY SOURCES:

1.4.1 HYDROLYSIS:

Starch is a high yield feedstock for ethanol production but its hydrolysis is required to produce ethanol by fermentation. The starchy material is peeled off manually and is boiled to kill the inhibitors if present in it. The boiled materials are subjected to hydrolysis by acids or enzymes. In acid hydrolysis, sulphuric acid cleaves the glycosidic bond to yield reducing sugars. The mixture of starchy materials and sulphuric acid, after hydrolysis is filtered to obtain reducing sugar solution which is used for fermentation. In enzymatic hydrolysis, the enzyme amylase acts on the glycosidc bonds and yields reducing sugars which can be fermented to ethanol. No pre-treatment is required as starch is an amorphous compound which can be easily hydrolysed by enzymes and acids.

Starch was traditionally hydrolyzed by acids, but the specificity of enzymes, their inherent mild reaction conditions and the absence of secondary reactions have made the amylases to be the catalysts generally used for this process. However, the high cost of initial investment and enzymes, as well as the requirements for specialized labour and sophisticated laboratories are factors limiting the use of enzymes. For amylase to attack starch, these suspensions should be brought to high temperatures (90-110°C) for the breakdown of starch kernels. The product of this first step, called liquefaction, is a starch solution containing dextrines and small amount of

glucose. Further, the liquefied starch is subjected to saccharification at lower temperature (60-70°C).

1.4.2 FERMENTATION:

The saccharified substrate is subjected to the process of alcohol fermentation generally by employing yeast species. *Sacchromyces cerevisiae* is the most commonly used microorganism in case of fermentation of starchy sources to ethanol. Fermentation period can be 48-72 hrs.

2. MATERIALS AND METHODOLOGY

2.1 MATERIALS

LIGNOCELLULOSIC BIOMASS

- Sugarcane leaves using Saccharomyces cerevisiae TISTR 5596
- Saccharomyces cerevisiae TISTR 5596
- Cellulase

STARCHY SOURCES

- Potato starch residue stream
- Saccharomyces cerevisiae y-1646
- Amylase

ENCHANCED IMMOBILIZED TECHNIQUE

- Sodium alginates
- Saccharomyces cerevisiae KCTC 7906
- Calcium chloride.

2.2 METHODOLOGY

2.2.1 ETHANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

- **SUGARCANE LEAVES**

Green sugarcane leaves were collected from cultivation sites. These leaves were cut, dried at 60°C. Hammer milled and then sieved to a 20-40 mesh size. Their major component was found cellulose38.5%,hemicellulose23% and lignin15.6%weight for weight(w/w) on dry basis

weight(DS).The composition results agrees well with Krishnan et al[13] who reported that sugarcane leaves were composed of glycan 35.3%,xylan 23% and lignin19.6%(w/w,DS).

- **MICROORGANISM**

Saccharomyces cerevisae TISTR 5596 was obtained from the Thailand Institute of Scientific and Technological Research(TISTR).A single colony of S.cerevisae grown on yeast extract peptone dextrose (YPD) agar (yeast extract 10g/l,peptone 20g/l,glucose 20g/l,agar15g/l,pH4.5)at 30°C with shaking (200rpm)for 24h,and the culture was transferred at 1% volume by volume (v/v) to the same medium and incubated under same condition for either 6 or 12h.The resulting 6 or 12h old yeast culture was then used as starter inoculum at 10%(v/v).In some experiments the culture suspension was first centrifuged(11,857×g,10min)at 4°C to precipate the yeast cell and the cell were resuspended in fresh YPD broth without glucose at original volume of separated supernatant,and used as the inoculum.

- **PRETREATMENT AND SACCHARIFICATION OF SUGARCANE LEAVES**

a) Sulphuric acid and lime treatment:

Ground dried sugarcane leaves were pretreated by suspending at 3%(w/w,DS) in either dilute sulphuric acid or lime solution(50ml in 250ml flask) and autoclaved at 121°C,15lb/in^2 for 30min .Optimization of pretreatment condition was performed by varying concentration of sulphuric acid (2%(w/v)) lime solution ,autoclaving period(5-60min) and the amount of sugarcane leaves loaded and pretreated sugarcane leaves were examined for their susceptibility of cellulose hydrolysis.

b) Determination of pretreatment efficacy on improvement of susceptibility of cellulose:

The pretreatment slurry, which contained pretreated sugarcane leaves suspended in pretreatment hydrolysate was filtered through a 0.88mm stainless steel sieve .The resultant pretreated sugarcane leaves and filtrate(pretreatment hydrolysate) were separately centrifuged(4°C,11,857×g for 10min)to completely remove the pretreated hydrolysate and residual lime powder respectively. The pretreated sugarcane leaves(pellet) were suspended in 0.5 M citrate buffer(pH 5.0) to the same volume as separated pretreated hydrolysate.Cellulase GC220(71.3FPU/μl;Gencor International,Inc.,USA) was added at 10 FPU/g(DS) and incubated at 40°C with shaking (125rpm) for 72h.The susceptibility of the pretreated sugarcane leaves to cellulase hydrolysis was determined from amount of reducing sugar liberated.

c) Analysis of sugar and pretreatment by-product:

Sugarcane leaves were pretreated by autoclaving at 121°C (15lb/in^2) with either 1.5%(w/v) sulfuric acid for 30min or with 2% (w/v) lime for 15min, and pretreated slurry was centrifuged at 4°C,11,857×g for 20min.The supernant was harvested and the pH adjusted to

7.0 prior to analysis of the levels of glucose ,xylose and five pretreatment by-product (furfural, hydroxymethylfurfural, 4-hydroxybenzaldehyde, syringaldehyde and vanillin).

- **ETHANOL FERMENTATION**

a. ***Ethanol production from the dilute acid pretreated sugarcane leaves saccharified in buffer solution :***

Sugarcane leaves at 6%(w/v,DS) in 1.5%(w/v) sulfuric acid(50ml in250ml flask) were pretreated by autoclaving at 121°C (15lb/in^2)for 30min and then the resultant pretreatment slurry was centrifuged at 4°C,11,857×g for 20min to separate the pretreated sugarcane leaves (pellet) from the pretreatment hydrolysate(supernant).The pellet was resuspended in 0.5M citrate buffer (pH 5.0) to same volume was separated pretreatment hyrolysate,and then saccharified by adding Accellerase™1000 at 160FPU/g,DS. After incubation for 6h, the suspension was centrifuged (11,857×g;20min,4°C),the supernant was harvestedand adjusted to pH 4.5 before being sterilized (110°C,20lb/in^2,10min) and used medium for ethanol fermentation by S.cerevisiae at 10%(v/v) inoculum. Fermentation was performed at 30°C, pH4.5 under oxygen limited condition for 72h.The oxygen limited condition was performed by fermanting 40ml of inoculated medium in 50ml flask without shaking. The flask is capped with a cotton plug and covered tightly with parafilm.The effect of inoculum age (6-and 12h-old) and fermentation period (0-96h) on the ethanol with parafilm.The effect of inoculum age (6-and 12h-old) and fermentation period (0-96h) on the ethanol yield obtained was determined.

b. Ethanol production from the dilute acid pretreated sugarcane leaves when saccharified in the pretreatment hydrolysate:

Sugarcane leaves were pretreated in 1.5% (w/v) sulfuric acid (50 ml in 250 ml flask) at 121 °C (15 lb/in^2) for 30 min, and then the pretreatment slurry containing the dilute acid treated leaves and the hydrolysate was adjusted to pH 5.0 and saccharified by adding Accellerase™ 1000 at 160 FPU/g, DS. After incubation for 6 h, it was centrifuged (11,857×g; 20 min, 4°C) and the resulting supernatant was harvested, adjusted to pH 4.5, sterilized at 110 _C and 10 lb/in2 for 10 min, and then used as the medium for ethanol fermentation by the 6-h-old S.cerevisiae culture at a 10% (v/v) inoculum.Fermentation conditions were 30°C, pH 4.5 under oxygen limited conditions (see Section 2.3.1) for 72 h. The effect of the fermentation period (0-96 h) under these conditions on the ethanol yield was determined.

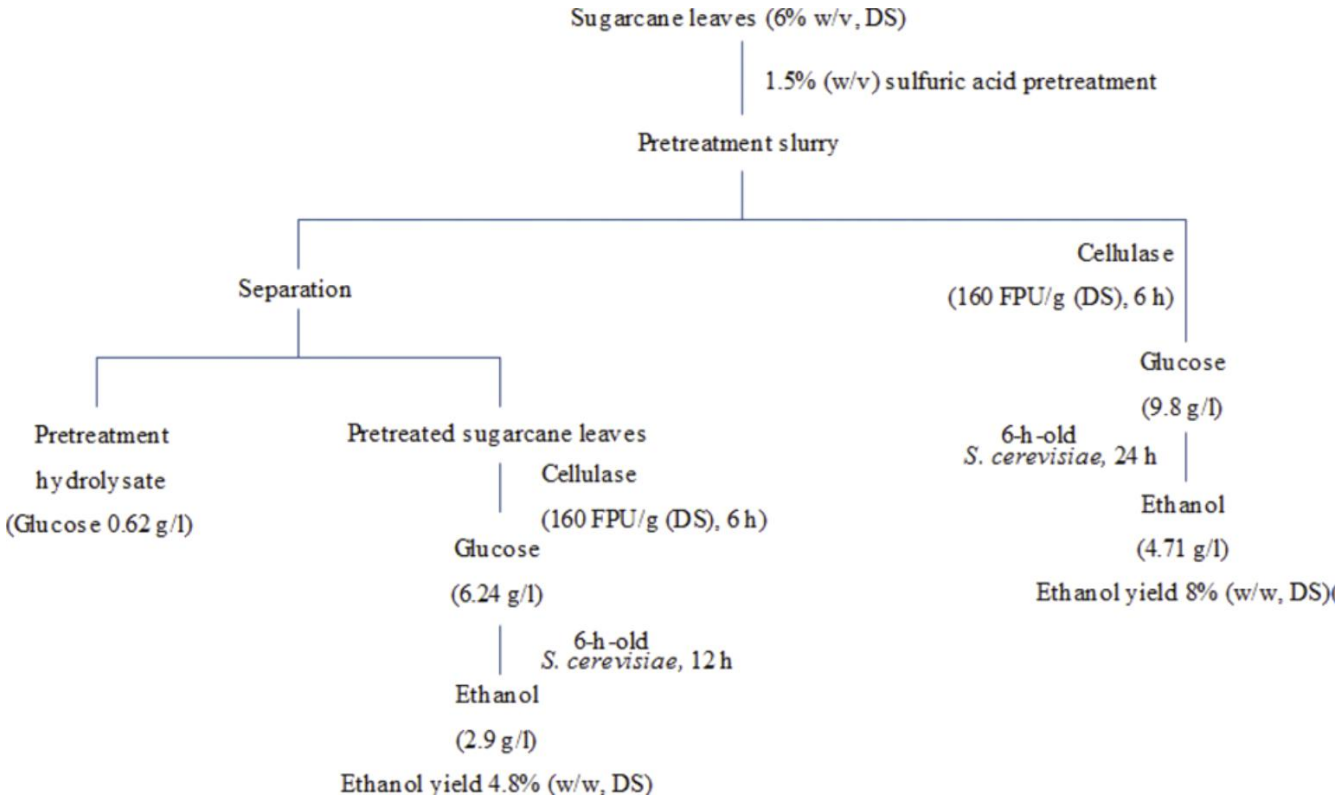

Fig. 6 Schematic summary of the two saccharification approaches showing the glucose and subsequent ethanol yields obtained in each approach.

2.2.2 ETHANOL PRODUCTION FROM STARCHY SOURCES

- **POTATO STARCH RESIDUE STREAM :**

The potato starch residue stream was collected from a Chips Factory for Food Industries, Assiut, Egypt. Samples were transferred to the laboratory in an icebox, and then kept frozen until use. Concentration of starch in the residue stream samples was 10-20 g L^{-1}.

- **ACID HYDROLYSIS:**

Cold and hot acids (HCl and H_2SO_4) were applied to hydrolyse the starch contained in potato starch residue stream. Serial concentrations of cold acids were added to the samples (1–5% v/v), and incubated at room temperature for 15 min with hand shaking intervals. Effect of hot acid and time of heating on hydrolysis of starch were studied by adding 1% of the acid separately and boiling for different periods. Five milliliters of starch solution were drown at constant intervals (0, 20, 40, 60 and 80 min) and subjected to glucose estimation. Glucose resulting from hydrolysis was estimated using dinitrosalicylic acid method [22]. The resulted hydrolyzed starch solution was neutralized with NaOH and prepared as a growth or fermentation medium for the yeast.

- **YEAST STRAIN:**

S. cerevisiae y-1646 was obtained from South Africa (Department of Microbiological, Biochemical and Food Biotechnology, Faculty of Natural and Agricultural Sciences, University of the Free State) and S. cerevisiae (commercial) was obtained from public market in Assiut, Egypt. Yeast strains were propagated and stored on yeast extract–malt extract agar

(YMA) slants (3 g L^{-1} of yeast extract, 3 g $L^{-1}$of malt extract, 5 gL^{-1} of peptone and 10 g $L^{-1}$of glucose) at 4°C. Active cultures for inoculation were prepared by growing the yeast in YM broth on a rotary shaker at 150 rpm for 16 h at 25°C (initial pH: 3.8–4.5).

- **BIOMASS AND FERMENTATION OF YEAST UNDER AEROBIC AND SEMI ANAEROBIC CONDITION:**

Repeated batch cultures were carried out in triplicate using a medium contained potato starch residue stream hydrolysed with 1%, v/v H_2SO_4 at pH7.0 to estimate the biomass and ethanol production by both yeast strains aerobically. The prepared medium was sterilized at 121°C for 20 min. Experiments were initiated by transferring prepared cell suspension with 10 ml (1.2×106 cell ml^{-1}) into 150 ml of the medium in 250 ml Erlenmeyer flasks, and shaken in the incubator at 150 rpm at 30°C for 96 h. The experiments were monitored by taking 5ml samples under sterilized conditions every 12h for biomass determination (by absorbance at 500 nm) and ethanol analyses. The experiment was repeated under semi-anaerobic conditions by replacing conical flasks with 250 bottles fitted with rubber plugs and incubated under the same conditions described above for 60 h. Incubation temperature was adjusted at the desirable degree (30, 35, 37, 40°C) in case of S. cerevisiae y-1646 only. By the end of the incubation time, samples were taken and subjected to analysis of associated secondary byproducts. Different concentrations of $ZnCl_2.7H_2O$ (0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 0.7, 0.8, 0.9%) and NH_4NO_2 (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5) were added separately to the hydrolysed potato starch residue stream for testing their effect on biomass production and fermentation process by S. cerevisiae y-1646.

- **STATISTICAL ANALYSIS:**

All experiments were carried out in a completely randomized design. The results were subjected to analysis of variance (one-way ANOVA), and the treatment means were compared using the least significant difference (LSD) values at a significance level of $P < 0.05$.

2.2.3 ENHANCHED ETHANOL PRODUCTION FROM YEAST IMMOBILIZED CALCIUM ALGINATE BEADS

- **PREPARATION OF CULTURE MEDUIM:**

Saccharomyces cerevisiae KCTC 7906 (Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) cells were grown in media containing 5 g glucose, 1 g peptone, 1 g yeast extract, 0.1 g $MgSO_4$, and 0.1 g K_2HPO_4 100 ml deionized water. The media was sterilized at 121°C for 20 min. At 30°C, 0.6 g of yeast powder was added under sterile conditions. A seed culture of S. cerevisiae was grown in a shaking incubator at 30°C and 300 rpm for 24h. The yeast cell seed culture was transferred to prepared fermentation-culture medium or used for making calcium alginate beads. Fermentation-culture medium was prepared using 100 g glucose for 10% (150 g glucose for15%), 5 g peptone, 5 g yeast extract, 1 g $MgSO_4$, 1 g K_2PO_4 1 litre distilled water.

- **PREPARATION OF CALCIUM ALIGNATE BEADS:**

A 24-h culture was harvested at the exponential growth phase and mixed with a sodium alginate solution. To prepare the calcium alginate beads, 100 ml of yeast seed culture was added to a sodium alginate solution prepared by dissolving 8 g of powder for 2% sodium alginate (Spectrum Chemical, Gardena, CA, USA) (10 g for 2.5% sodium alginate) in 300 ml of

deionized water (Najafpour et al., 2004). The mixture of sodium alginate and seed culture was dropped through syringes into a 0.1 M $CaCl_2$ solution. The beads were stored after washing with deionized water to remove residual $CaCl_2$. The 3.8-mm beads were uniformly packed and stored in deionized water at 4°C for 3 days before fermentation. Beads were collected by filtration, added to the fermenter (Bioflo110 Fermenter/ Bioreactor, New Brunswick Scientific, Edison, NJ, USA) with 1 litre of fermentation medium at 300 rpm and varying temperatures, pHs, sodium alginate concentrations, and initial glucose concentrations for each experiment. Samples were collected every 2 or
3 h to determine the amount of residual glucose and the ethanol
concentration.

- **GLUCOSE AND ETHANOL CONCENTRATION**

Glucose concentration was measured using the dinitrosalicylic acid method (Miller, 1959). Ethanol concentrations were measured by gas chromatography (GC-8A, Shimadzu, Tokyo, Japan) equipped with a 20% carbowax column. Isopropanol was used as an internal standard to measure ethanol content from each fermented sample. The column temperatures at the injector and detector were 130°C and 110°C, respectively.

3. RESULT AND DISCUSSION

3.1 SUGARCANE LEAVES (RESULTS)

- ***DILUTE SULFURIC ACID PRETREATMENT:***

When autoclaved at 121°C, 15 lb/in^2 for 30 min, sugarcane leaves at 3% (w/v, DS) were most susceptible to cellulose hydrolysis when suspended in 1.5% (w/v) sulfuric acid with a lower yield at either 1 or 2% (w/v) sulfuric acid (Fig.1a). Increasing the autoclave period of the 3% (w/v) ground leaf suspension in 1.5% (w/v) sulfuric acid to 45 min, and especially to 60 min, significantly decreased the susceptibility of the pretreated sugarcane leaves to cellulase hydrolysis (Fig. 1b).Finally, in the 1.5% (w/v) sulfuric acid and autoclaving as
above but for 30 min, increasing the amount of sugarcane leaves loaded in the pretreatment process from 3 to 6% (w/v,DS) had no significant affect but increasing it further up to 8%(w/v, DS) decreased the susceptibility to cellulase hydrolysis of the pretreated sugarcane leaves some 1.16-fold (Fig. 1c).
A high substrate loading in the pretreatment process may well block the heat transfer in the reaction. Under the above partially optimized pretreatment condition of autoclaving 6% (w/v, DS) sugarcane leaves in 1.5% (w/v) sulfuric acid for 30 min, then the subsequent hydrolysis with cellulase yielded 0.34 g/g DS reducing sugars or 0.057 g/g DS glucose. There was a direct relationship between the amount of reducing sugars released in the pretreatment hydrolysate and the susceptibility of dilute sulfuric acid pretreated sugarcane to cellulase hydrolysis.

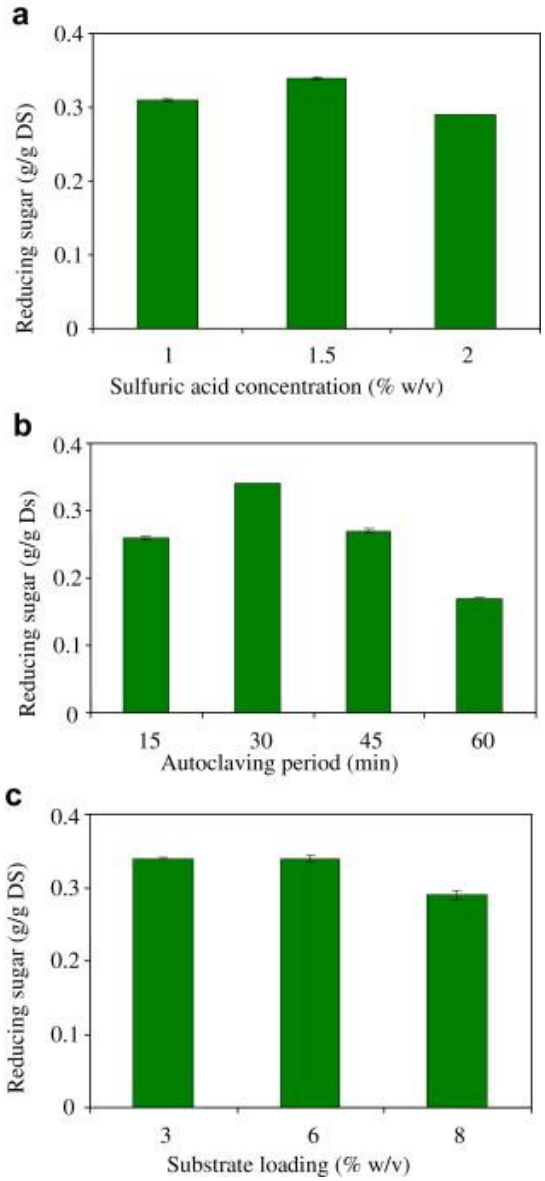

Fig. 1 e Effect of the (a) sulfuric acid concentration, (b) autoclaving time and (c) substrate loading levels during the pretreatment of sugarcane leaves on the susceptibility to subsequent cellulase hydrolysis (measured as the amount of released reducing sugar). The data are displayed as the mean ± 1SD, and are derived from triplicate experiments.

- ***LIME PRETREATMENT:***

Pretreatment with lime (calcium hydroxide) was evaluated in a similar manner to sulfuric acid above. With a ground sugarcane leaf suspension at 3% (w/v, DS) in the calcium hydroxide (0.1e3% (w/v)) being autoclaved at 121°C, 15 lb/in^2 for 30 min, they were most susceptible to subsequent cellulase hydrolysis when the calcium hydroxide concentration was 1% (w/v) calcium hydroxide (0.33 g calcium hydroxide/g, DS) (Fig. 2a). Maintaining the calcium hydroxide at this optimal 1% (w/v) and then decreasing the autoclave period of the 3% (w/v, DS) leaf suspension to 15 min resulted in a slight but significant increased susceptibility to cellulose (Fig. 2b). Lime pretreatment of 6% (w/v, DS) sugarcane leaves suspended in 2% (w/v) calcium hydroxide (0.33 g/g, DS) and autoclaved (121°C, 15 lb/in^2) for 15 min, then the subsequent hydrolysis with cellulase yielded 0.107 g/g DS reducing sugars or 0.039 g/g DS

glucose. Thus, comparing the two pretreatments that with the 1.5% (w/v) sulfuric acid pretreatment resulted in a greater digestibility (susceptibility to hydrolysis) of the pretreated sugarcane leaves to cellulase than that obtained with the lime pretreated leaves. The level of liberated reducing sugars and glucose from the optimized sulfuric acid and the calcium hydroxide treated ground leaves were 3.2- fold (34% vs. 10.7% (w/w, DS)) and 1.46-fold (5.7% vs. 3.9% (w/w, DS)) higher, respectively. This result agrees well with the previous report that dilute sulfuric acid pretreated sugarcane leaves gave a higher ethanol yield upon subsequent fermentationthan alkaline peroxide pretreated sugarcane leaves. Therefore, sugarcane leaves at 6% (w/v, DS) in 1.5% (w/v) sulfuric acid were autoclaved at 121 °C, 15 lb/in^2 for 30 min as the pretreatment in all the following experiments. [4].

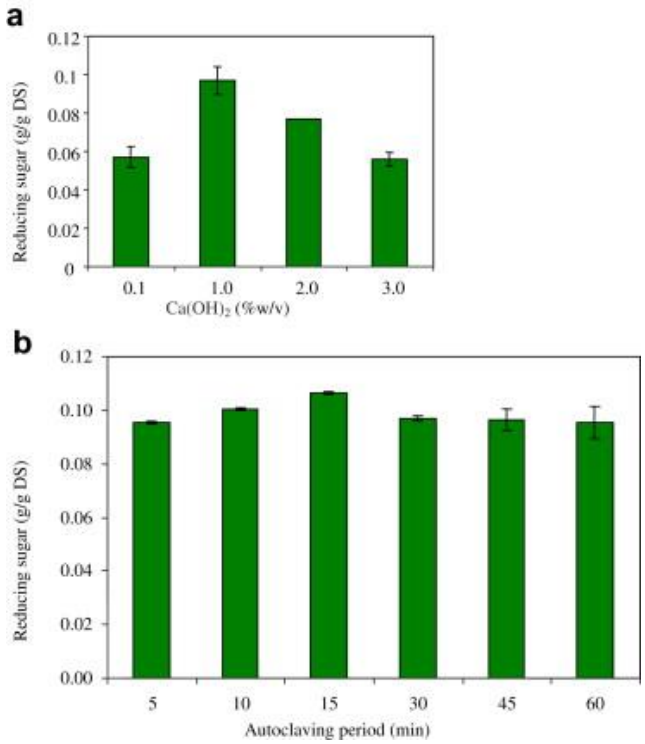

Fig. 2 e Effect of the (a) calcium hydroxide concentration, (b)autoclaving time during the pretreatment of sugarcane leaves on the susceptibility to subsequent cellulose hydrolysis (measured as the amount of released reducing sugar). The data are displayed as the mean ± 1SD, and are derived from triplicate experiments.

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- **SUGARS AND BY-PRODUCTS IN PRETREATMENT HYDROLYSATE**

The 1.5% (w/v) sulfuric acid pretreated hydrolysate contained a small amount of glucose (0.62 g/l) and a higher level of xylose (9.0 g/l). All five assayed pretreatment by-products (furfural, hydroxymethylfurfural, 4-hy-roxybenzaldehyde, syringaldehyde and vanillin) were at detectable concentrations in the dilute sulfuric acid pretreated samples but two of them (furfural and hydroxymethylfurfural) were below detectable limits in the lime pretreatment hydrolysates. However, they were all at concentrations below their reported toxic levels for ethanol fermentation and growth of S. cerevisiae. Therefore, detoxification of the pretreatment hydrolysate before ethanol fermentation by S. cerevisiae was not necessary.

- **ETHANOL FERMENTATION**

i. Ethanol production from the dilute acid pretreated sugarcane leaves saccharified in 0.5 M citrate buffer:

Sugarcane leaves at 6% (w/v, DS) were pretreated with 1.5% (w/v) sulfuric acid, and then separated from the pretreatment hydrolysate, which was found to contain 0.62 g/l glucose and 9.0 g/l xylose, suspended in 0.5Mcitrate buffer pH 5.0 and then further hydrolysed by 160 FPU/g (DS) of Accellerase™ 1000, as

Out lined in Section 2.4.1. After cellulase hydrolysis and clarification by centrifugation, the cellulase hydrolysate, found to contain 6.24 g/l glucose, was sterilized (110°C, 10 lb/in^2 for10 min) and used as a medium for ethanol fermentation. A 6-or 12-hold S. cerevisiae culture was inoculated at 10% (v/v),

whereupon after 72 h the yield of ethanol was 2.76 and 1.76 g/l, respectively. Therefore, the time course of ethanol production by S. cerevisiae from the 6-h-old culture as the inoculum was followed and found to be maximal at 2.9 g/l or 4.8% (w/w, DS) after 12 h of incubation, remaining at or slightly below this level thereafter (Fig. 4).

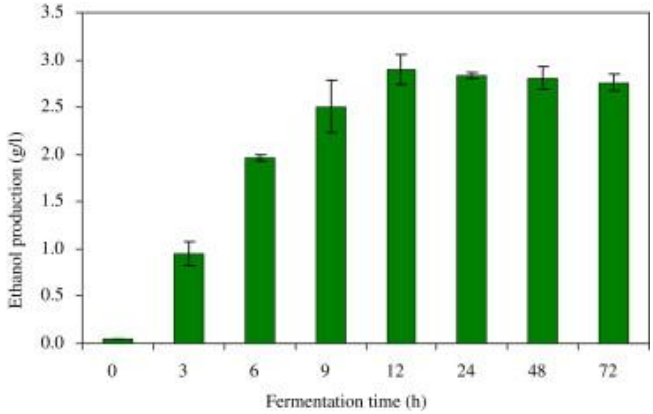

Fig. 4 e Time course of ethanol production from the acid pretreated leaves (glucose) by a 6-h-old culture of S. cerevisiae as the inoculum. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments.

ii. Ethanol production from the dilute acid pretreated sugarcane leaves saccharified in the pretreatment hydrolysate

Sugarcane leaves at 6% (w/v, DS) were pretreated by autoclaving in 1.5% (w/v) sulfuric acid (as in Section 3.5.1), and then the pretreatment slurry obtained was adjusted to pH 5.0 and hydrolysed by Accellerase™ 1000 (160 FPU/g, DS) for 6 h, as outlined in Section 2.4.2. The resulting cellulase hydrolysate, after removal of the particulate matter by centrifugation, was found to contain 9.8 g/l glucose, which is 1.57-fold higher than that of the corresponding cellulase hydrolysate obtained from saccharification of the dilute sulfuric acid pretreated leaves in 0.5 M citrate buffer. The increase in the level of glucose in the cellulase hydrolysate was the result of the pretreatment hydrolysate saccharification. After sterilization (110 °C, 10 lb/in^2 for 10 min) the cellulase hydrolysate (9.8 g/l glucose) was fermented to ethanol by the addition of a 10% (v/v) inoculum of a 6-h-old culture of S. cerevisiae. The ethanol yield reached 4.15 g/l or 7% (w/w, DS) after 12 h, and the ethanol yield was maximal (4.71 g/l or 8% (w/w, DS)) after 24 h of incubation declining slightly thereafter (Fig. 5).

Under the above conditions, except that the 6-h-old S. cerevisiae culture inoculum was inoculated directly into the cellulase hydrolysate (9.8 g/l glucose) without any sugar or nutrient supplementation instead of YPD without glucose, the ethanol yield (after 24 h incubation) was reduced some 2.5 fold to 3.91 g/l or 6.5% (w/w, DS). The addition of (NH4)2SO4 to 0.45 mM

did not improve the ethanol yield (data not shown), but it could be restored to 4.71 g/l or 8% (w/w, DS) by prolonging the incubation period to 72 Hr.

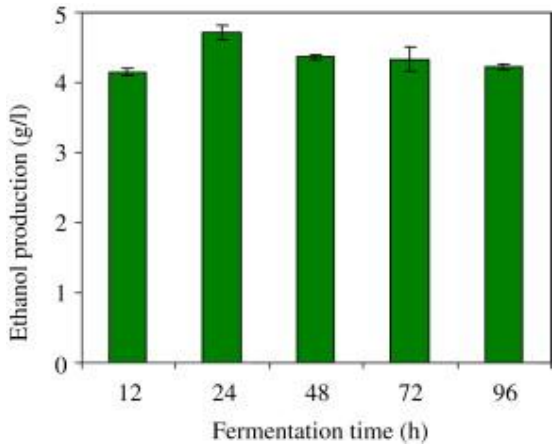

Fig. 5 e Time course of ethanol production from the combined acid-pretreated leaves and hydrolysate (glucose/ xylose mixture) by a 10% (v/v) inoculums of a 6-h-old culture of S. cerevisiae. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments.

DISCUSSION:

Sugarcane leaves pretreated with dilute sulfuric acid were more susceptible to cellulase hydrolysis than those pretreated with lime. The optimally pretreated sugarcane leaves, at 6% (w/v, DS) in 1.5% (w/v) sulfuric acid and autoclaved at 121°C, 15 lb/in2 for 30 min, were hydrolysed with cellulase (160 FPU/g, DS) while suspended in the pretreatment hydrolysate. The hydrolysate (9.8 g/l glucose) obtained was then further fermented to ethanol by S. cerevisiae to give a maximal ethanol yield of 4.71 g/l or 8% (w/w, DS) after 24 h of incubation. Based on this study, dilute (1.5% (w/v)) sulfuric acid pretreated sugarcane leaves are recommended for hydrolysis with cellulase while suspended in the pretreatment hydrolysate for maximal ethanol yield. It was found that The acid pretreated samples released more glucose than the lime treated ones (5.7% and 3.9% weight by weight (w/w, DS), respectively). Accellerase 1000 hydrolysis (160 FPU/g, DS) of the dilute sulfuric acid pretreated ground sugarcane leaves suspended in 0.5 M citrate buffer, or in pretreatment hydrolysate yielded 0.104 and 0.163 g glucose/g (DS) of leaves after 6 h, respectively. Fermentation of the two above obtained glucose sources by Saccharomyces cerevisiae for 12 and 24 h, respectively, yielded ethanol at 4.8% and 8.0% (w/w, DS), respectively.

3.2 POTATO STARCH RESIDUE (RESULTS)

- ***EFFECT OF ACID PRETREATMENT ON POTATO STARCH RESIDUE STREAM HYDROLYSIS:***

Fig. 1 shows that cold HCl was more efficient than H_2SO_4 in hydrolysis of potato starch residue stream. The increase in concentration of both acids, the increase in hydrolysis rate of potato starch residue stream which was expressed in glucose concentration. Addition of HCl in 4% or

5% achieved the maximum hydrolysis, and there was no significant difference ($P < 0.05$) between the two concentrations (15.66 and 15.34 g L^{-1}, respectively). This indicates that at 4% of the cold acid, all hydrolysable materials were hydrolyzed and maximum achievable sugars were produced. Our result could be supported by Tasic´ et al. [42] who investigated the hydrolysis of starch from fresh potato tubers by HCl and H_2SO_4 and concluded that the rate of hydrolysis and the maximal dextrose equivalent (DE) increased with increasing acid concentration, probably due to the increase in the activity of hydrogen ions participating in the reaction as catalyst. The same trend was observed when the acid hydrolysis of sweet potato was studied [12]. Tasic´ et al. [42] almost obtained the same reaction rates and a DE of about 80% after 60 min, when they used these acids.

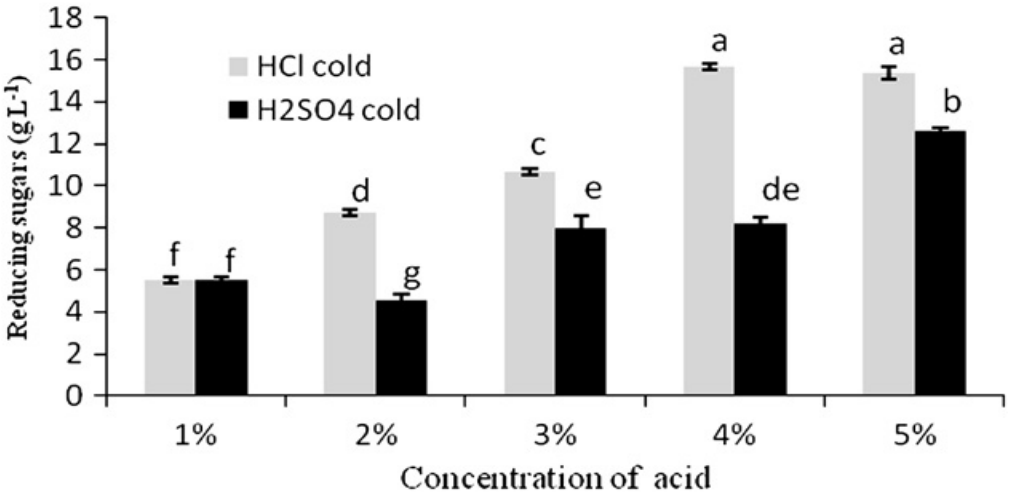

Fig. 1 – Effect of cold HCl and H_2SO_4 on potato starch residue stream hydrolysis. Columns followed by the same letter(s) are not significantly different at LSD P<0.05.

When hot acids were applied, a large amount of sugar(15.93–16.2%) was produced at low concentration of the two acids (1%) and there was no significant difference ($P < 0.05$)between efficiency of both acids at this concentration (Fig. 2).By increasing the concentration of the acids at constant time of heating (60 min.), a slight increase in sugar production was obtained. Effect of heating time on acid hydrolysis of starch was studied using the lowest acid concentration (1%). Results indicated that by increasing the time of heating, glucose yield was increased in case of HCl and H_2SO_4 until 60 min., where the maximum yield of sugars was achieved, however, after this time there was a slight but not significant increase in hydrolysis (18.9%). This indicates that a complete hydrolysis of starch in the residue can be achieved by using 1% H_2SO_4 after 60 min at 100°C (Fig. 3). This concentration was selected to hydrolyze the residue during all experiments of growth and fermentation of yeast.

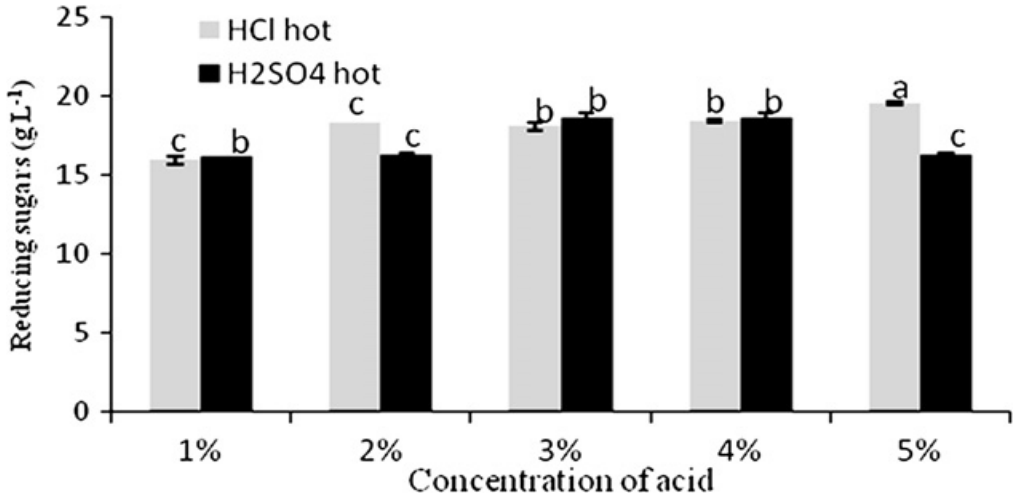

Fig. 2 – Effect of hot HCl and H_2SO_4 on potato starch residue stream hydrolysis. Columns followed by the same letter are not significantly different at LSD P<0.05.

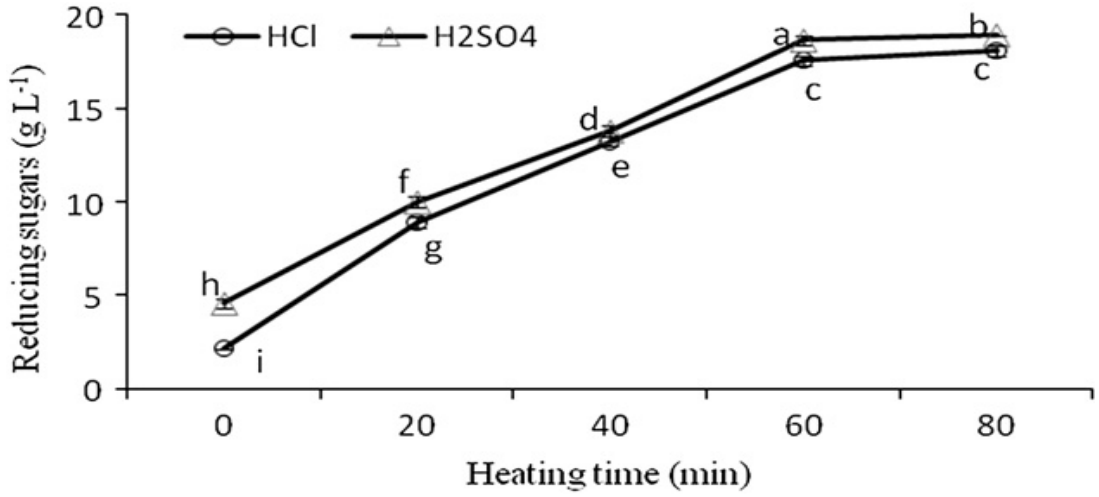

Fig. 3 – Effect of heating time of 1% of HCl and H_2SO_4hydrolysis efficiency of potato starch residue stream. Values followed by the same letter are not significantly different at LSD P<0.05

- ***BIOMASS AND FERMENTATION OF POTATO STARCH RESIDUE STREAM HYDROLYZATE BY S. CEREVISIAE***

Experiments were conducted to measure the growth rate of two yeast strains (S. cerevisiae y-1646 and S. cerevisiae commercial). At 30°C, growth curves (measured by OD500 nm) of both yeasts were nearly the same. They reached the stationary phase at about the same time (Fig. 4). Growth rate began to decline after 48 h (Fig. 4).

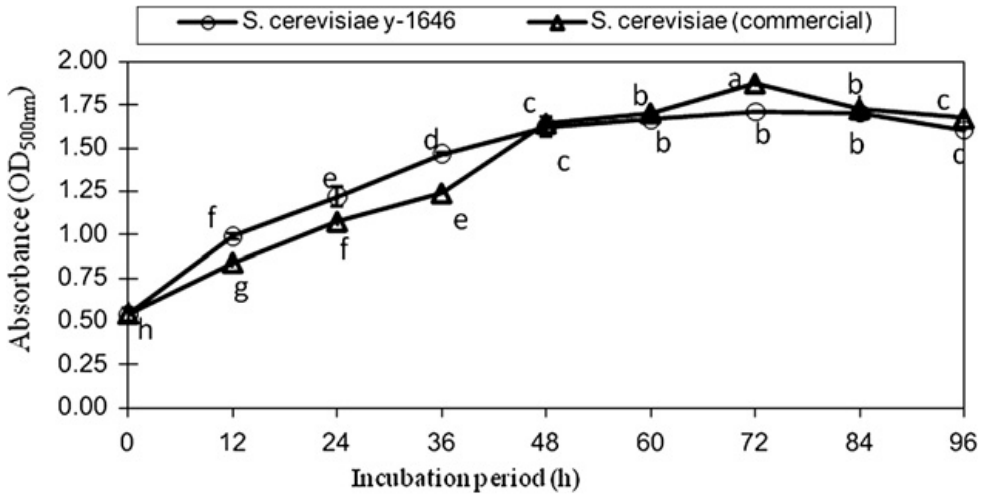

Fig. 4 – Growth pattern of yeasts on pretreated starch residue stream measured by absorbance (OD_{500} nm) under aerobic conditions at 30°C. Values followed by the same letter are not significantly different at LSD P<0.05

During the aerobic growth of the two strains, samples were taken at different intervals and analyzed for their content of ethanol (Fig. 5). Data showed that, the maximum yield of ethanol under aerobic growth, were achieved after 24 h (3.61–3.52 g L^{-1}). Ethanol content was still constant until 36 h, and then began to decline. When we compare the reduction in ethanol production rate after 36 h with the growth rate of the yeast strains, which was increasing until 48 h, we could assume that after consumption of utilizable sugars in growth media, yeasts potentially utilized the produced ethanol as a carbon source under aerobic conditions.

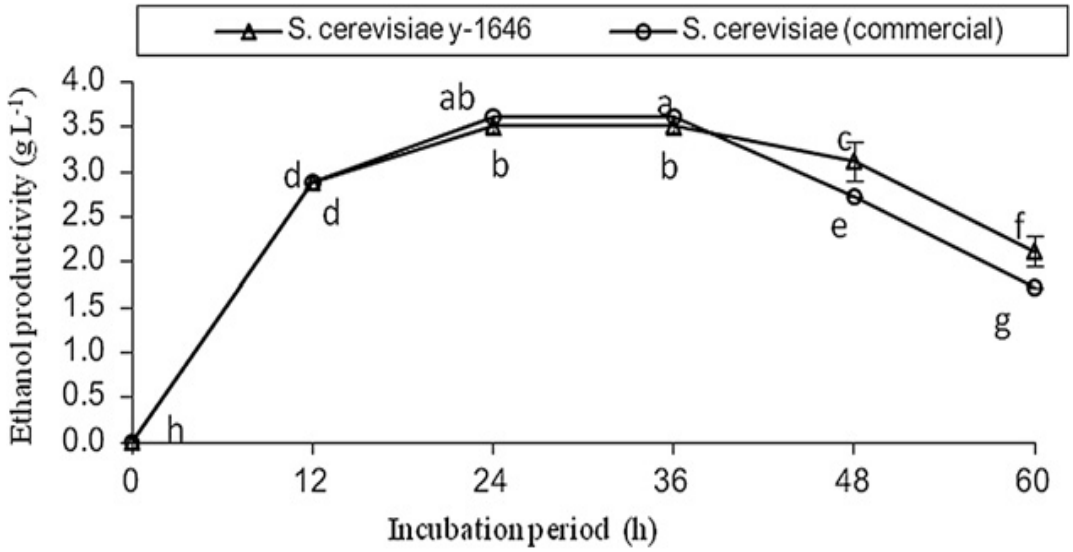

Fig. 5 – Ethanol productivity (g L^{-1}) by yeasts grown in pretreated potato starch residue stream under aerobic conditions at 30°C. Values followed by the same letter(s) are not significantly different at LSD P<0.05.

Growth pattern and ethanol production by the two yeast strains were studied under semi-anaerobic conditions and data were presented in Figs. 6 and 7. There was a close similarity in growth pattern of the two yeasts. The growth peak of the two yeasts was noticed at 24 h. It was estimated as 2.55 and 2.59 (OD500) for S. cerevisiae y-1646 and S. cerevisiae commercial, respectively. After that, growth slightly decreased to 1.74 and 1.65 after 36 h. Fig. 7 shows ethanol productivity of both yeast strains at 30°C under semi-anaerobic conditions. The results indicate that the maximum ethanol yield from the two strains (3.47–3.62 g L^{-1}) was achieved after 24 h and still constant 12 h later. After 36 h of incubation, the yield began to decline until the end of the experiment. After 36 h, it was noticed that the decrease in ethanol production rate was accomplished by stability in growth rate. This observation confirms the hypothesis that yeasts were able to utilize ethanol as a carbon source when sugar is being consumed to survive. We could assume that after 24 h of fermentation of the residue, the batch should be terminated and ethanol should be separated. Growth and ethanol production curve are supposed to be greatly dependant on availability and concentration of fermentable sugars[17-28].

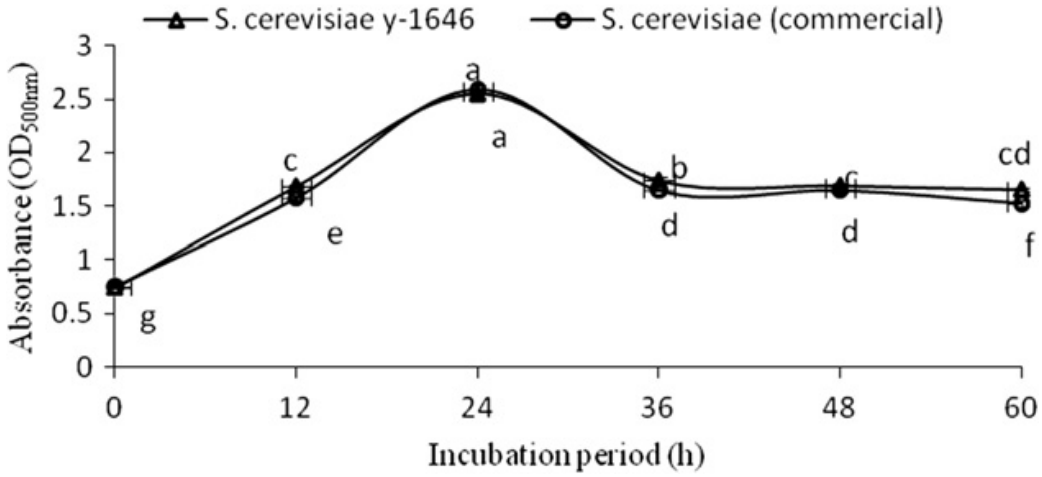

Fig. 6 – Growth pattern of yeasts on pretreated potato starch residue stream measured by absorbance (OD_{500} nm) under semi-anaerobic conditions at 30°C. Values followed by the same letter(s) are not significantly different at LSD P<0.05.

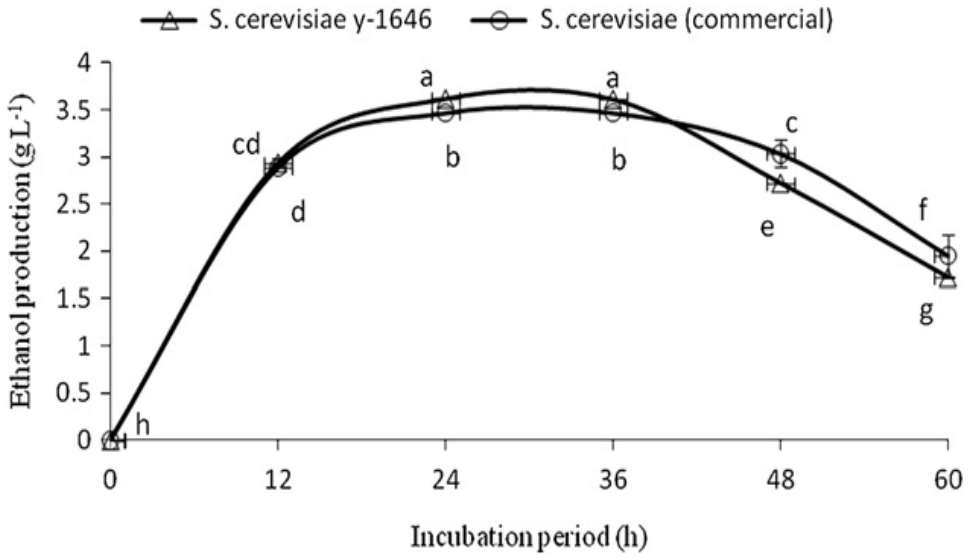

Fig. 7 – Ethanol productivity (g L^{-1}) by yeasts grown on pretreated potato starch residue stream under semi-anaerobic conditions at 30 8C. Values followed by thesame letter(s) are not significantly different at LSDP<0.05.

- ***EFFECT OF TEMPERATURE ON BIOMASS AND ETHANOL PRODUCTIVITY***

Effect of temperature on growth of S. cerevisiae y-1646 and ethanol production was studied. Data proved that, 30°C was the most appropriate temperature for yeast growth. At this temperature biomass was estimated as 2.47 (OD500 nm), while, 35 °C and 37°C reduced the growth significantly (Fig. 8). Conversely, production of ethanol by S. cerevisiae y-1646 was favoured by temperature of 35°C and reached its maximum (5.29 g L^{-1}) after 36 h. At 37° C, ethanol production was reduced to 4.38 g L^{-1}. Both low and high temperature (30 and40°C) had great negative impact on ethanol production and reduced it to 3.52 and 3.07 g L^{-1}, respectively (Fig. 9). Thermostability of a yeast strain is more likely genetically controlled. Variation in thermal requirements for biomass and ethanol production stimulate the suggestion that enzymes involved ethanol fermentation vary in their thermal optima than those are involved in biomass synthesis

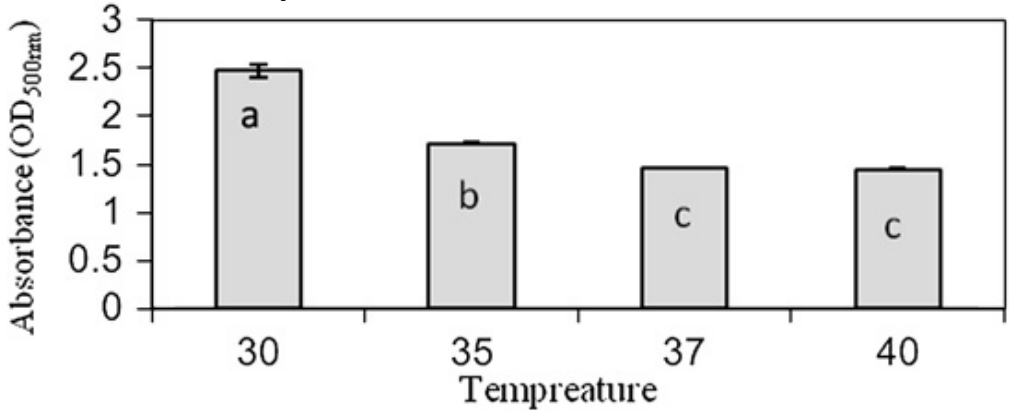

Fig. 8 – Effect of temperature on growth of S. cerevisiae y-1646 on pretreated potato starch residue stream under semi-anaerobic conditions. Columns followed by the same letter are not significantly different at LSD P<0.05.

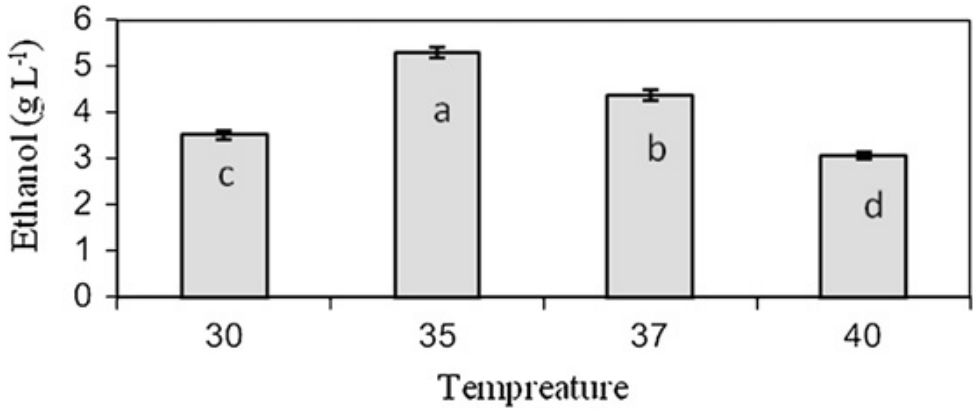

Fig. 9 – Effect of temperature on ethanol production by S. cerevisiae y-1646 on pretreated potato starch residue stream under semi-anaerobic conditions. Columns followed by the same letter are not significantly different at LSD P<0.05.

DISCUSSION:

Our results conclude the possibility of using potato starch residue stream as an economical source for yeast biomass and bioethanol production for fuel after appropriate hyrolysis. This substance do not need any other additional organic additives, but some minerals such Zn in small concentrations could have a good impact on ethanol yield. A very important medicinal compound ‘‘chlorozotocin’’ was found to be synthesized during ethanol production from the target source. Production of such medicinal active compounds should be confirmed in further research and its optimal conditions should be optimized. Results demonstrated that 1% H2SO4 at 100 _C for 1 h was enough to hydrolyze all starch contained in the residue stream. Two strains of S. cerevisiae (y-1646 and commercial one) were able to utilize and ferment the acid-treated residue stream under both aerobic and semi-anaerobic conditions. The maximum yield of ethanol (5.52 g/L) was achieved at 35°C by S. cerevisiae y-1646 after 36 h when ZnCl2 (0.4 g/L) was added.

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3.3 YEAST IMMOBILIZED CALCUIM ALGINATE BEADS (RESULTS)

- ***EFFECT OF SODIUM ALGINATE CONCENTRATION ON GLUCOSE CONSUMPTION:***

To determine the effect of sodium alginate concentration on fermentation yield, the residual glucose concentration after fermentation with 2% and 2.5% calcium alginate beads or free cells (no immobilized yeasts) was determined under the same fermentation conditions (10% initial glucose, 30° C, and pH of 5.0) (Fig. 1A). The results showed that batch fermentation with yeast immobilized in calcium alginate beads required less fermentation time to convert glucose to ethanol than did cells in batch fermentation. Fermentation time to consume the glucose by free cells was about 23 h, whereas immobilized cells with 2% and 2.5% calcium alginate beads required 20 and 22 h, respectively. No physical breakage was observed with the 2% and 2.5% calcium alginate beads during fermentation. Higher sodium alginate concentrations resulted in

delayed glucose consumption because the surfaces of the calcium alginate beads were expected to hold more strongly and be more dense at higher concentrations, thus making it more difficult for glucose to percolate into the cell pores. The results showed that the 2% alginate concentration provided the best balance between porosity and strength.

Ethanol productivity of immobilized cells was equal to or lower than that of free cells. Roukas (1994) reported that both free and immobilized S. cerevisiae cells produce the same maximum ethanol concentration under the same fermentation conditions as non sterilized carob pod extract in a batch-fed culture. Singh et al. (1998) also concluded that the concentrations of ethanol produced in both free and immobilized batch systems (Ca-alginate) were relatively similar. Mariam et al. (2009) reported that the ethanol yield obtained by free cells was 7.3% higher than the yield obtained from immobilized cells. However, the more common finding is that under the same fermentation conditions, immobilized yeast cells yield more ethanol than do free cells. Yu et al. (2007) reported that ethanol productivity of immobilized cells in sorghum bagasse was 2.24 times higher than that of free cells. However, Holcberg and Margalith (1981) reported that the rate of ethanol production by entrapped cells with agar, j-carrageenan, alginate, and polyacrylamide gels was higher than that with free cells. Norton et al. (1995) reported a significant increase in yeast resistance against ethanol for immobilized cells compared with free cells. The results reported by Roukas (1994) and Mariam et al. (2009) are different from the results shown in Fig. 1A, but the results by Yu et al. (2007), Holcberg and Margalith (1981), and Norton et al. (1995) are similar to ours.

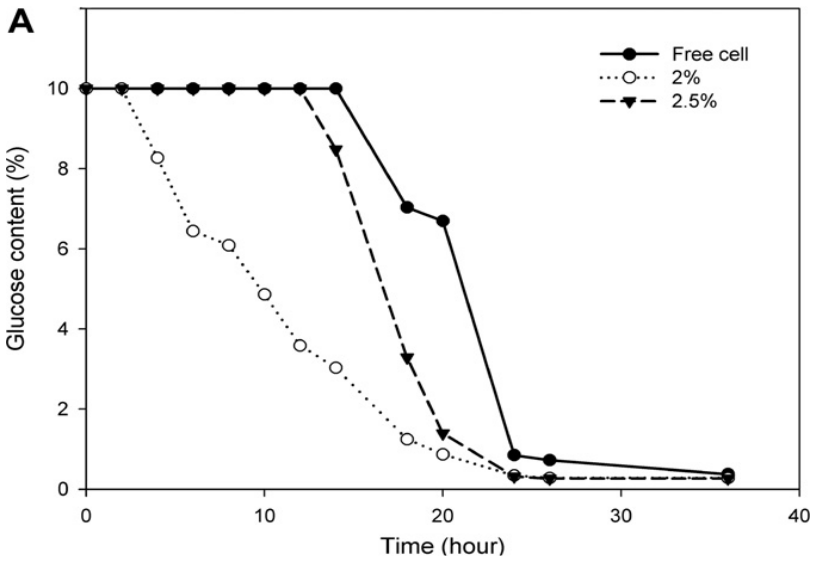


Fig. 1. Comparison of glucose consumption: yeast immobilized in 2% and 2.5% alginate beads vs. free yeast cells with 10% initial glucose concentration (A)

- ***EFFECT OF TEMPERATURE***

Four different temperatures (30, 32.5, 35, and 37.5 °C) were tested to determine the optimal temperature for batch fermentation with yeast immobilized in 2% alginate beads at pH 5.0 (Fig. 2). The results showed that an increase in temperature from 30 to 37.5 °C had a fluctuating effect on glucose conversion time: 19.5, 20.5, 19, and 23 h, respectively, were needed to reach 90% ethanol conversion. Temperature values from 30 to 37.5 °C, except 27.5 °C, showed very similar retention times for glucose consumption, but 30 °C at the same fermentation conditions was the optimal temperature using immobilized yeast under batch fermentation. Notably, this

low-temperature fermentation reduced energy consumption. The influence of fermentation temperature (from 15 to 35 °C) on a mixed-strain population was studied by Torija et al. (2003). After 4 days of initial fermentation, the cell population increased at high temperatures compared with low temperatures. The usual growth curve, with a series of short lags and exponential, stationary, and decline phases, was observed at 25 and 30 °C, whereas at 35 °C, many of the yeast cells died. A similar result was found when the effect of incubation temperature on ethanol production was examined in free and immobilized yeast at 15% initial sugar concentration and pH 4.5 (Mariam et al., 2009). They estimated ethanol production at different incubation temperatures (25–40 °C) and found that 30 °C supported maximum ethanol production by free yeast cells after 120 h of incubation. Although immobilizedcells showed lower ethanol yield and sugar consumption compared ith free cells, the overall optimal temperature of fermentation was 30°C. Another study reported
that the ethanol yield increased from 75.79% to 89.89% as fermentation temperature increased from 28 to 37°C (Liu and Shen, 2008). The highest ethanol yield was observed at a fermentation temperature of 37°C. The optimum temperature of free S. cerevisiae fermentation is usuallyabout 30°C (Torija et al., 2003). Liu and Shen (2008) found that the optimum temperature of fermentation with immobilized yeastis higher than that with free yeast. As shown in Fig. 2 and 30°C was the optimal fermentation temperature without noticeable retention time.

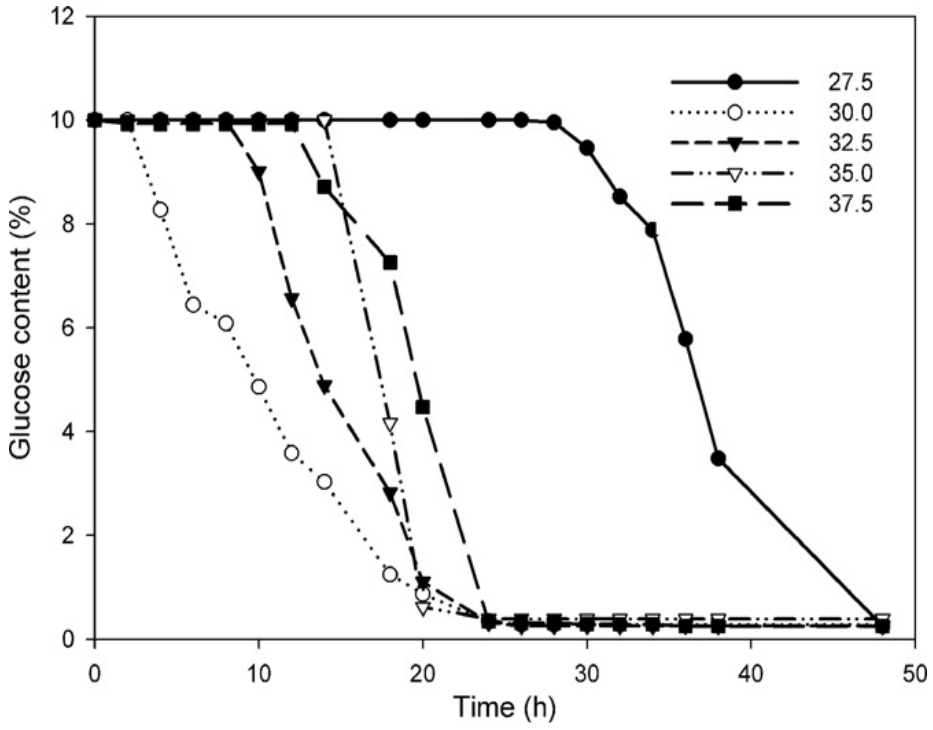


Fig. 2. Comparison of glucose consumption by temperature, ranging from 27.5 to 37.5°C, with a 10% initial glucose concentration and pH 5.0.

- ***EFFECTS OF pH***

pH changes were measured in unregulated culture medium during fermentation (Fig. 3A). To find the pH effect on fermentation, we studied fermentation at pH 4.5, 5.0, and 5.5 and compared the results with those from a natural (unregulated) pH (Fig. 3B).Fig. 3A shows that

the pH values decreased to 3.85 from 6.4 under unregulated conditions. Fig. 3B shows that regulated conditions are better than unregulated in terms of fermentation time. Among regulated samples, pH 5.0 was the best, with >90% of glucose consumed in 19.5 h. By decreasing the external pH from 7.0 to 2.2, a progressive reduction in the intracellular pH from 7.1 to 5.1 was observed in exponentially grown cells. However, stationary cells maintained constant intracellular pH at around 6.1 when the external pH was in the range of 7.0–5.5, and an intracellular pH of 5.5 was maintained by stationary cells even with pH values decreasing from 5.0 to 2.2.

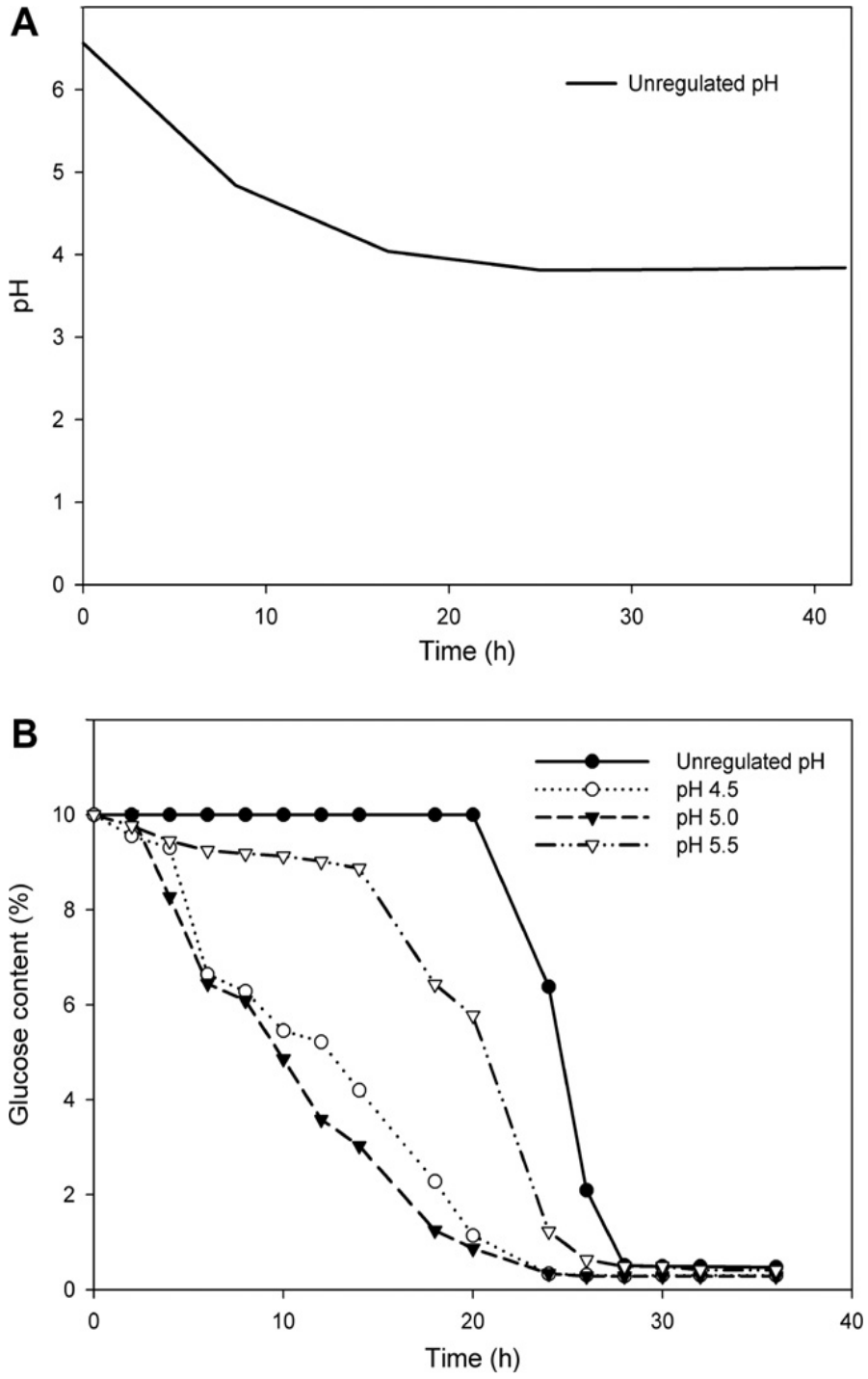
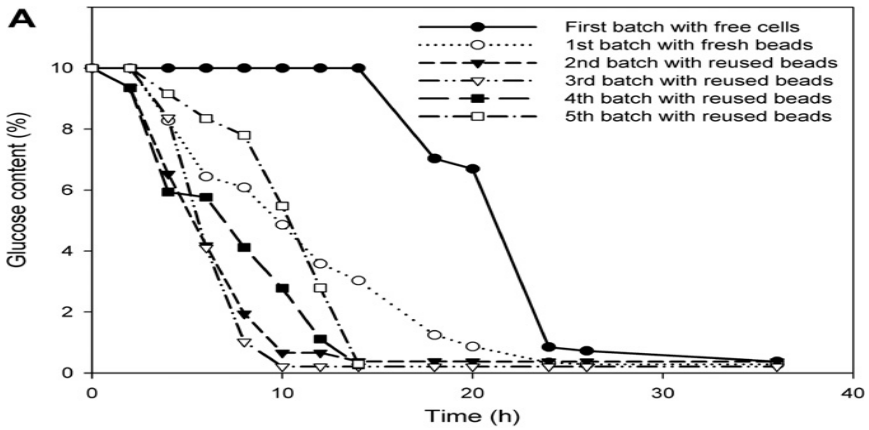


Fig. 3. pH change vs. unregulated fermentation (A); Comparison of glucoseconsumption by pH, ranging from 4.5 to 5.5, with a 10% initial glucose concentration at 30°C (B)

- ***ETHANOL PRODUCTION BY FRESH AND REUSED BEADS:***

Glucose consumption and ethanol production were studied as a function of time with free cells and reused beads at 30 °C, pH 5.0 (Fig. 4). Reused beads provided overall better performance compared with free cells and fresh beads. Among the reused cases, the three-time reused beads produced the best results considering both fermentation time (10 h) and ethanol yield. Fermentation times with two, four, and five-time reused beads were also favorable:14 h compared to 24 h with fresh beads and 36 h for free cells (Fig. 4A). For ethanol production, fresh or reused beads produced close to a theoretical ethanol yield of 100% (0.51 g ethanol/g glucose), but free cells produced a lower yield (88%) (Fig. 4B). Mariam et al. (2009) reported that during six consecutive batch runs with immobilized yeast cells, ethanol yield increased up to the third batch run, and maximum ethanol yield was obtained in the fourth batch run. Additionally, both sugar consumption rate and ethanol yield decreased sharply in the fifth and sixth batch runs. The best ethanol yield from the fourth batch run reported by Mariam et al. (2009) was approximately 86%, whereas all runs with reused cases were close to 100% in the present study. It is also important to note that their fermentation time was significantly longer than ours, at an average of 120 h compared to 10–14 h. The reason for this marked difference in performance is that Mariam and colleagues ran their fermentation with a 15% initial glucose solution at pH 4.5 and 30 °C. A comparison of our results with those from the literature is shown in Table 1. This Table shows that the previous fermentation time was >24 h, but our results required 10–14 h. Therefore, ethanol productivity in the present study was about 1.7-fold higher than previous results.



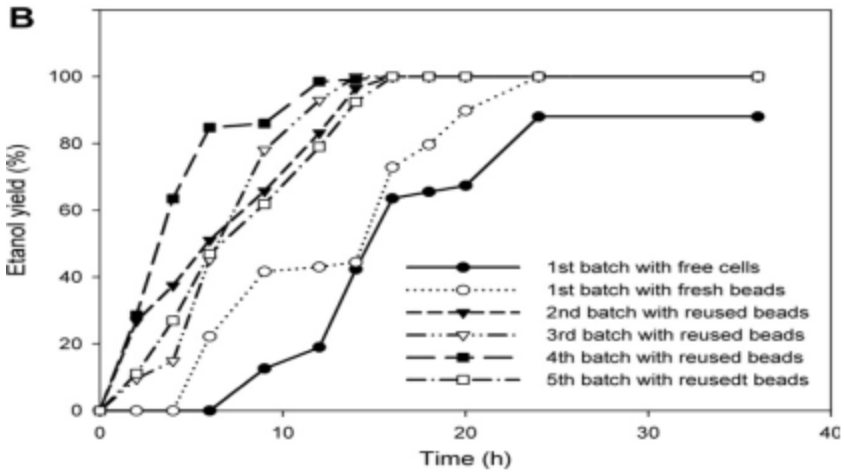


fig. 4. Comparison of glucose consumption vs. time (A) and ethanol production vs. time (B) among free cells, fresh 2% alginate beads, and 2% reused alginate beads at 30°C, pH 5.0.

DISCUSSION:

Results with all immobilized yeast produced 100% ethanol yields, whereas the free cells yielded 88%. The optimal fermentation conditions were 30 _C, pH 5.0, and 10% initial glucose concentration with 2% sodium alginate beads. The fermentation time using reused alginate beads was 10–14 h, whereas fresh beads took 24 h, and free cells took 36 h. All bead samples resulted in nearly a 100% ethanol yield, whereas the free cells resulted in an 88% yield. Transmission electron microscopy (TEM) showed that the shortened time and higher yield with the reused beads was due to a higher yeast population per bead as well as a higher porosity.

4. METHODOLGY ADOPTED FOR OUR PROJECT:

1. PREHEATING OF OIL CAKES

- The preparation of 5% oil seed cake, which are obtained from different sources.
- It is pre-cooked in water for 20 min to separate the inhibitory enzymes and oil if present.

2. HYDROLYSIS

- The samples prepared are taken in different test tubes in constant volume.
- Variable concentration of amylase is added to all the tubes, to undergo enzymatic hydrolysis.
- Hydrolysis is carried out in varying time intervals.

3. FILTRATION

- The samples are filtered.
- Undigested sample is separated, which can further be treated by sulfuric acid so that lignin is separated and remaining residue can be fermented for ethanol production.

4. ESTIMATION OF GLUCOSE CONCENTRATION

- Reducing sugar concentration is estimated by DNSA method.

- Optimization of substrate concentration can be done using Michellous-menton method.

5. FERMENTATION

- Measure the total quantity of hydrolysate and divide it into equal proportion and take not more than 150 ml in 500ml conical flask.
- Fermentation of hydrolysate is carried out using two different types of Yeast (commercial yeast and termite gut yeast).

6. DISTILLATION

- The fermented liquor is subject to distillation.

7. ESTIMATION OF ALCOHOL

- Estimate the alcohol content in the fermented sample using CAN reagent in a colorimeter.

5. CONCLUSION:

With the ever increasing demand for transportation fuels and rapidly depleting petroleum resources, India has to develop alternative fuels, especially for the transportation sector. Currently, biomass seems to be the only feasible resource for renewable fuel, but the lack of cost-effective technologies for biomass conversion to fuel hinders progress in this direction. The government's decision for mandatory blending of ethanol at a 5% level in gasoline in 11 states and three union territories has created an increased demand for fuel-grade ethanol., which at present can be met by the current production capacity; however, the demand will exceed current production capacity once the blending has been implemented nationwide or if the blending ratio is increased. The availability, variability and sustainability of feed stock for second generation bio fuel production are important issues to be addressed by R&D personnel as well as policy makers.

Statistics indicate that India has ample biomass resources in the form of agro-residues to support production of lignocellulosic bio fuels, though this potential is restricted by current uses of the residues as the cattle fodder, fertilizer and products. The most likely hot spots for second generation bio fuel production based on rice straw will be the states of west Bengal, Andhra Pradesh, Uttar Pradesh and Punjab, while wheat straw based second generation biofuel production is most likely to be located in the states of Uttar Pradesh, Punjab, Haryana, Madhya Pradesh and Rajasthan. All these states have the required infrastructure and production plants can be located near the area of raw material availability. Sugar cane bagasse enjoys a rather centralized availability near major sugar factories and distilleries, and the most likely [producers for bio fuel from this raw material are the existing distilleries themselves, since the incorporation of lignocellulosic bio fuel production into their existing infrastructure would be easier than starting new. However, there would be entirely new plants based on biomass likely to be concentrated in the sugar producing areas of the states of Uttar Pradesh, Maharashtra, Tamilnadu and Karnataka. India lacks mature technologies for second generating bio fuel

production from lignocellulosic biomass, which is an abundant source of renewable energy that may be exploited in the most parts of the country. Though biomass itself is cheap, the costs of its processing are relatively high.

Technologies for biomass to bio fuel conversion are also under various stages of development. The government should positive steps towards promoting the use of ethanol as fuel by providing tax exemptions at least in the initial; stages. Establishing markets for bio fuel and increasing subsidies can stimulate R&D activities in this field and help spur growth of investment in technology development and unemployment. It is important to bring fuel-ethanol programmes to the attention of big investors, especially the petroleum companies, since this can hasten the commercialization of second generation bio-fuel technology. Private investors should be encouraged to invest in bio fuel programs an government policies should be conducive to their participation.

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