CHARACTERIZATION OF AN ETHANOL PRODUCING YEAST ISOLATE AND OPTIMIZATION OF ETHANOL FERMENTATION.

November 2011

Dissertation submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

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CERTIFICATE

This is to certify that the thesis entitled "CHARACTERIZATION OF AN ETHANOL PRODUCING YEAST ISOLATE AND OPTIMIZATION OF ETHANOL FERMENTATION" submitted by Md. Abdul Quayum is a record of student's own work carried out by him under our joint supervision and guidance in the Bangladesh Council of Scientific and Industrial Research (BCSIR) and Department of Mathematics and Natural Sciences (MNS), BRAC University. It is further certified that the research work presented here is original, has not been submitted anywhere else for a degree and suitable for the partial fulfillment of the degree of Masters of Science in Biotechnology, BRAC University, Dhaka. The author and supervisor give permission to use this thesis for academic purposes only.

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TO

MY PARENTS AND TEACHERS

ACKNOWLEDGEMENT

First of all, I would like to thank my parents for sharing pain and pleasure during this work.

I would like to express my sincere thanks and gratitude to Prof. **Dr. SM Imamul Huq**, Chairman, Bangladesh Council of Scientific and industrial Research (BCSIR), for his kind permission to carry out this research at BCSIR. I sincerely express thanks and gratefulness to **Mr. Monzur Morshed Ahmed**, Senior Scientific Officer, Industrial Microbiology Section, Institute of Food Science & Technology (IFST), BCSIR, Dhaka, for his inspiring guidance, continues cooperation and constructive discussion in all phases of the study.

I take profound privileges to express my sincere thanks and heartfelt gratitude to my BRAC University supervisor **Prof. Naiyyum Choudhury**, Coordinator Biotechnology Program, MNS department for his encouragement, valuable advice and cooperation through the entire period of this work.

I also want to express my great sense of gratitude to **Md. Fakruddin**, Scientific officer, BCSIR, who was always involved in my research project.

I wish to express a very special thanks to **Dr. Mahboob Hossain**, MNS department for his constructive suggestions and continuous urging to finish my work in time.

I am thankful to all of my honorable teachers of the department of Mathematics and Natural Science for their contribution and inspiration to complete this paper. If pearls were words and flowers feelings, it would be easier to express my heartfelt gratitude and indebtedness to the all the PEOPLES and FRIENDS who HELPED me in any way throughout my life. May Almighty infuse me with the energy to fulfill their noble inspiration and expectations and further modify my competence. May Allah bless us all with long, happy and peaceful lives.

The Author

ABSTRACT

Bioethanol or biofuel as an alternative to fossil fuels has been expanded in the last few decades in the whole world. *Saccharomyces cerevisiae* is the choice of organism for ethanol production. Characterization of an ethanol producing ability and to optimization of ethanol production by a laboratory strain of yeast was attempted in the present study. Based on morphological and physiochemical characters, the yeast strain was identified to be *Saccharomyces cerevisiae.* The strain was moderately thermotolerant and ethanol tolerant as well as osmotolerant. It was resistant to cycloheximide at 0.0015g/100ml concentration but growth was inhibited in presence of Hydrogen peroxide. Ethanol producing capability of the *Saccharomyces cerevisiae* strain was studied using sugarcane molasses as substrate. The reducing sugar was estimated by DNS method and ethanol was estimated by redox titration. Fermentation was optimized with respect to temperature, reducing sugar concentration and pH. Analysis of fermentation characteristics under different substrate and environmental conditions, it was observed that reducing sugar concentration of 5%-6%, temperature of 30°Cand pH 6.0 were optimum for fermentation with maximum yield of ethanol. Maximum ethanol production was 11% by free cells using the initial reducing sugar concentration 5.50% at 48 hrs under shaking condition. Immobilized yeast cells showed significant improvement of ethanol production. Under shaking condition, 12% ethanol was produced by immobilized cells using the reducing sugar concentration 6% at 48 hrs. Ethanol production was higher in shaking condition than that in stationery non shaking condition using the same environmental condition. Influence of boron, chromium, copper, magnesium chloride was investigated on ethanol production. Only chromium was found to show stimulatory effect on ethanol production. This selected strain could be potential strain for ethanol production from cane molasses.

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LIST OF ABBREVIATIONS

BCSIR- Bangladesh Council of Scientific and Industrial Research.

IFST- Institute of Food Science and Technology

pH- Negative logarithm of hydrogen ion concentration

°C- Degree Centigrade

Temp- Temperature

Conc. - Concentration

DNS- 3, 5Dinitro salicylic acid

g or gm- Gram

hrs- Hours

fig. Figure

O.D- Optical density

µl- Micro liter

et.al.- And other people

Kg- Kilogram

L- Liter

mg- Milligram

ml- Milliliter

µm- Micrometer

Ppm- Parts per million

No. /no.-Number

V/v- Volume Per volume

W/v- Weight per volume

% - Percentage

/ - Per

α- Alpha

LIST OF ABBREVIATIONS

μ mol- Micro mole

ATP - Adenosine triphosphate

NADH - Nicotinamide adenine dinucleotide

Psi - Pound per square inch

Rpm - Round per minute

YPD - Yeast extract peptone dextrose

YMM- Yeast maintenance media

$CuSO_4$- Copper sulphate

$K_2Cr_2O_7$- Potassium dichromate

$MgCl_2$- Magnesium chloride

$CaCl_2$- Calcium chloride

CHAPTER: 1

INTRODUCTION

CHAPTER: 01
INTRODUCTION

1.1 Significance of the study

Bioethanol fuel is mainly produced by the sugar fermentation process, although it can also be manufactured by the chemical process of reacting ethylene with steam. Domestic production and use of ethanol for fuel can decrease dependence on foreign oil, reduce trade deficits, create jobs in rural areas, reduce air pollution, global climate change and carbon dioxide buildup (Ofoefule *et al.,* 2009).

Disadvantages of bioethanol include its lower energy density than that of gasoline, its corrosiveness, low flame luminosity, lower vapor pressure (making cold start difficult), miscibility with water and toxicity to ecosystems (Mustafa *et al.*, 2008).

The production of pure ethanol apparently began in the 12-14th century along with improvement in the art of distillation permitting the condensation of vapors of lower boiling liquids. During the middle ages, alcohol was not only mainly used for the production as a constituent of medical drugs, but also for the manufacture of painting pigments and other chemical industries. It was only in the 19th century that this trade became an industry with enormous production, due to economic improvements of the distilling process (Roehr, 2001). Now, ethanol is an important industrial chemical with emerging potential as a biofuel to replace vanishing fossil fuels (Alfenore *et al.*, 2002).

Ethanol is a group of chemical compounds whose molecules contain an OH group, bonded to a carbon atom. It melts at -114.1°C, boils at 78.5°C and has a density of 0.789 g/ml at 20°C (Kaur & Kocher, 2002).Ethanol is produced by fermentation when certain species of yeast (notably *Saccharomyces cerevisiae*) metabolize sugar in the absence of oxygen and produce ethanol and carbon dioxide. Ethanol is particularly useful in industrial applications because of its relatively high affinity for both water and organic compounds. The composition of other alcohols limits their flexibility as compared to ethanol (Anxo *et al.*, 2008)

Ethanol may be produced commercially by chemical synthesis or biosynthesis. Chemical synthesis is by hydration of ethylene (C_2H_4). For the biosynthesis, in the fermentation process, yeast uses monosaccharides as a carbon source and then converts these to ethanol via glycosis under anaerobic conditions.

The overall reaction can be summarized as follow:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + 2ADP + 2Pi \xrightarrow{\text{Yeast}} \underset{\text{Ethanol}}{2\,C_2H_5OH} + 2\,CO_2 + 2ATP + 2H_2O$$

At the beginning of the 20th century, several kinds of raw materials were exploited for ethanol production, such as molasses or agricultural and agroindustrial residues, and the possibility of hydrolyzing lignocellulosic materials was investigated (Roehr, 2001). Carbohydrate-rich raw materials suitable for ethanol production can be classified into three groups of agricultural products. The first raw material group, sugar refers to sugar-beet as well as sugarcane and molasses. The second group, starch from such crops as cassava, cereals and potatoes. The last group, lignocellulose, covers waste materials from the harvesting of agricultural crops such as rice and wheat straw, corn cob and sugarcane bagasse (Mogg, 2004). In general, industrial yeast strains are able to grow on free sugar and efficiently ferment ethanol at pH values of 3.5-6.0 and temperatures of 28-30° C, with efficiency dropping off rapidly at higher temperature. In the light of existing fuel crisis and absolute dependence on import, it would be very useful if some fermentation ethanol process can be developed using cheap raw materials available locally

1.2 Objectives of the present study

The objectives of the present study are as follows:

- Characterization of a laboratory yeast strain capable of ethanol production.
- Study of
  - thermotolerance and ethanol tolerance of laboratory yeast strain.
  - effects of physico-chemical parameters on ethanol production.
  - effects of immobilization on ethanol production.
  - effect of metals on ethanol production.
- Optimization of ethanol production

1.3 Research hypothesis

After detailed characterization and optimization of physico-chemical parameters for ethanol production of the selected yeast strain, it can be used as a potential strain for ethanol production industrially.

1.4 Scope and limitation of the study

An elected laboratory yeast strain was screened for thermotolerance and ethanol tolerance. The yeast strain was characterized using morphological and physiological studies. The optimization of some ethanol production conditions was also investigated.

1.5 Expected results

A highly efficient thermotolerant and ethanol tolerant yeast strain for ethanol production would be obtained. The yeast strain thus obtained could be useful for ethanol fermentation industry.

CHAPTER: 2

LITERATURE REVIEW

CHAPTER: 2

LITERATURE REVIEW

Fermentation is the slow decomposition by micro-organisms of large organic molecules (such as starch) into smaller molecules such as ethanol. Ethanol fermentation can be described as the biochemical process by which sugar such as glucose, fructose and sucrose are converted into partial cellular energy thereby producing ethanol and carbon dioxide as metabolic products. Yeasts carry out ethanol fermentation on sugar in the absence of oxygen. The fermentation is classified as anaerobic because the process does not require oxygen (Ibeto *et al.,* 2011).

Ethanol provides energy that is renewable and less carbon intensive than oil. Bioethanol reduces air pollution due to its cleaner emissions and also contributes to mitigate climate change by reducing greenhouse gas (GHG) emissions. In comparison to gasoline utilization, reduction of greenhouse gas emission occurs, since much carbon dioxide is taken up by the growing plants as is produced when the bioethanol is burnt, with a zero theoretical net contribution. Several studies have shown that sugarcane-based ethanol reduces greenhouse gases by 86 to 90% if there is no significant land use change (Isaias *et al*., 2004; Goettemoeller and Goettemoeller, 2007)

.Sugar cane also contains parts that cannot yet be profitably processed into ethanol. The bagasse is the name given to the biomass that remains from the sugar stalk after it has been crushed and the sugar and garapa (juices) have been extracted. Although, it is not yet commercially converted to ethanol, however research studies are on-going to profitably convert the bagasse into ethanol. The bagasse is also useful in the production process. Many sugar mills have utilized the bagasse for cogeneration of both heat and electric energy production to power the mills. Like other parts of the plant, the bagasse does not add to net atmospheric carbon dioxide, because any carbon dioxide released will be consumed by another cane plant. Bioethanol production from sugarcane was started in Brazil and the United States in the early 1970's (Chatanta *et al*., 2008)

2.1 Yeasts

Yeasts are ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission and form sexual states which are not enclosed in a fruiting body (Boekhout and Kurtzman, 1996). The yeast species are all characterized by a similar set of features, both morphological and physiological. This type of description, in which physiological characters are important, distinguishes yeast taxonomy from other fungal taxonomy (Kreger-van Rij, 1984).

Sugar assimilation and fermentation tests are commonly accomplished using glucose, galactose, maltose, sucrose, lactose, raffinose, trehalose and xylose. With regard to fermentation of these sugars, the anaerobic liberation of CO_2 into Durham tubes is not very accurate for detecting slowly fermenting yeast species (Scheffers ,1987). Ethanol production assays are deemed to be more appropriate determinants of sugar fermentation by yeasts (Walker, 1998).

Yeasts are used in many industrial processes, such as the production of alcoholic beverages, biomass and various metabolic products. The last category includes enzymes, vitamins, capsular polysaccharides, carotenoids, polyhydric alcohols, lipids, glycolipids, citric acid, ethanol, carbon dioxide and compounds synthesized by the introduction of recombinants DNA into yeasts. Some of these products are produced commercially while others are regarded as potentially valuable in biotechnology (Kurtzman and Fell, 1997). Some yeast species have potential to be used in food, beverage and fermentation industries (Jacobson and Jolly, 1989).

Some present and potential uses of yeasts in the food, beverage and fermentation industries are given table-1. (Jacobson and jolly, 1989)

Table-1: Application of yeasts in different industries

Yeast species	**Application**
Saccharomyces cerevisiae	Ale fermentation
S. cerevisiae, S. exiguus, S. rosei	Bread and dough leavening
Candida diddensiae	D- Arabitol (sweetener)
C. lipolytica	Emulsifier
S. cerevisiae	Ethanol fermentation
Phaffia rhodozyma	Fish and poultry feeds
C. utilis	Fodder and single cell protein
pseudotropicalis, Kluyveromyces fragilis	Lactose and milk fermentation *C.*

Yeast species	Application
S. carlsbergensis	Lager beer fermentation
Torulopsis manitofaciens	Mannitol (humectant)
Zygosaccharomyces rouxii	Shoyu, Miso
S. cerevisiae	Wine fermentation
T. candida	Xylitol
C. shehatae, Pachysolen tannophilus, Pichia stipis	D-Xylose fermentation

Yeasts occur widely in nature and have been recovered from widely differing terrestrial as well as marine sources. Certain yeasts are more or less ubiquitous while others appear to be restricted to very specific habitats. Yeasts seldom occur in the absence of either molds or bacteria (Kreger-van Rij, 1984).

2.1.1 Classification and identification of yeasts

Yeasts are classified on the basis of the microscopic appearance of the cells, the mode of sexual reproduction, certain physiological features (especially metabolic capabilities and nutritional requirements) and biochemical features. The physiological features, that distinguish different yeasts, include the range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can use as a source of carbon and energy under semi-anaerobic and aerobic condition, the relative ability to grow in the presence of 50-60% (w/v) D-glucose or 10% (w/v) sodium chloride plus 5% (w/v) glucose (a measure of osmotolerance) and the relative ability to hydrolyze and utilize lipids. These properties help investigators determine which yeast strains merit investigation for a particular application (Glazer and Nikaido, 1995).

Yeasts, which form one of the important subclasses of fungi, are rather more complex and usually larger than bacteria. They are distinguished from most fungi by their usual existence as single ovoid cells about 8 μm long and 5 μm in diameter, doubling every 1-3 hours in favorable media (Wayman and Parekh, 1990). Colonies of *S. cerevisiae* on agar plate are shown in fig-1:

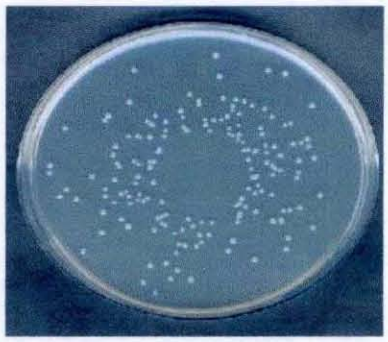

Fig-1: Colonies of *S. cerevisiae* on agar plate.

Individually yeast cells appear colorless, but when grown on artificial solid media they produce colonies which may be white, cream colored, or tinged with brownish pigments. Colony characteristics are useful in the taxonomy of yeasts, very difficult group to classify. Physiological characteristics are also used to a great extent in determining yeast species (Alexopoulos, 1962). Yeasts may reproduce asexually or sexually (Wayman and Parekh, 1990).

2.1.2 Asexual reproduction

Alexopoulos (1962) classified yeasts into the budding yeasts and the fission yeasts, depending on their types of asexual reproduction. The budding yeasts reproduce by budding. In this process the protoplasm of the cell, covered by a thin membrane, pushes out of the cell wall in the form of a bud and forms daughter cells. The bud enlarges until it is separated from the mother cell by a constriction at the base. The arrow signs indicate a bud and forms a daughter cell under microscopic observation as shown in fig-2.

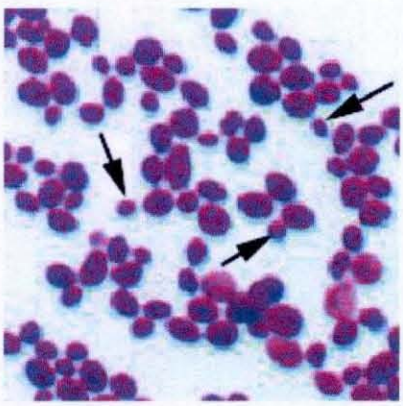

Fig-2: Microscopic observation of buds and daughter cells.

Under some conditions, buds do not separate from the mother cell and a branched chain of cells called a pseudomycelium forms. Pseudomycelium produced by yeasts during budding is shown in fig-3:

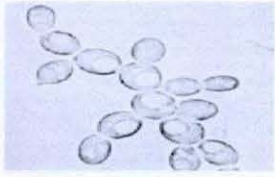

Fig-3: Chain of yeast cells (pseudomycelium) produced by budding

During the process of budding, the nucleus divides, one daughter nucleus passing into the bud, the other remaining in the mother cell. Most known yeasts reproduce by budding such as *S. cerevisiae.*

The fission yeasts reproduce by transverse division. The parent cell elongates, the nucleus divides, and a transverse wall (septum) is laid down somewhere near the middle, separating the mother cell into two uninucleate daughter cells. This septum is formed by annular growth beginning at the wall and proceeding in ward. The new wall thickens before the daughter cells separation (Conti and Naylor, 1959).

2.1.3 Sexual reproduction

Sexual union in the yeasts takes place either between two somatic cells or between two ascospores which assume the function of copulating gametangia, unite and form a zygote cell. Eventually an ascus forms which contains ascospores, their number depending on the number of nuclear divisions which take place and on the subsequent development of the nuclei. Four or eight ascospores per ascus are the usual number, but other numbers may also be encountered. The sexual reproduction of yeast usually proceeds by the formation of buds on the cell surface, but sexual reproduction can be induced under special condition. In the sexual cycle, a normal diploid cell divides by meiosis, and sporulation gives rise to asci, or spore cells, that usually contain four haploid ascospores. The ascospores are of two mating types; a and α. Each type can develop by budding into order haploid cells. The mating of a haploid cell and an α haploid cell yields a normal α diploid cell.

Haploid cells of the same sex also unite occasionally to form abnormal diploid cells (a/a or α/ α) that can reproduce only asexually, by budding in the usual way. The majority of industrial yeasts reproduce by budding (Glazer and Nikaido, 1995).The reproduction of yeast by sexual and asexual reproduction is shown in fig -4.

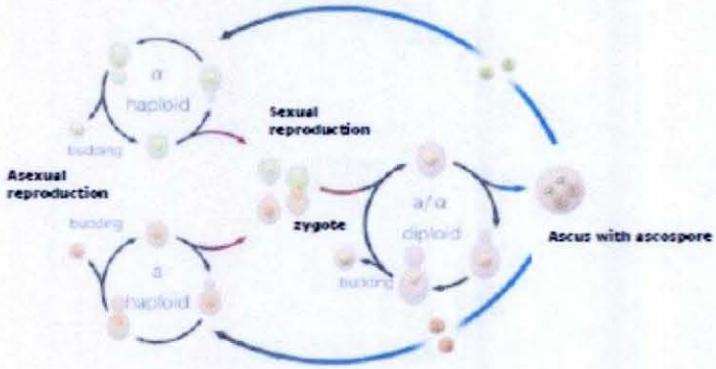

Fig-4: The reproduction of yeast by sexual and asexual reproduction.

Ascospores formed by yeasts are often globose or ovoid, as in *Debaryomyces, Saccharomyces, Schizosaccharomyces*, and *Saccharomycodes* . Other yeasts form different types of ascospores. Thus, in *Pichia* and some species of *Hansenula*, the ascospores are hat-shaped; in other species of *Hansenula* they may be hemispherical shaped like the planet Saturn. Release of ascospores may occur when the ascus wall deliquesces; this is the usual method of release in species with hat- or Saturn-shaped spores. In other species the germinating spores bud or form germ tubes, which results in bursting of the persistent ascus wall (Alexopolos *et al.*, 1996). Miller (1989) pointed out that yeast ascospores are much more durable than somatic cells and have the ability to withstand even snail gut enzyme, a distinct advantage in their natural environment. Ascospores of *Saccharomyces cerevisiae* are shown in fig-5.

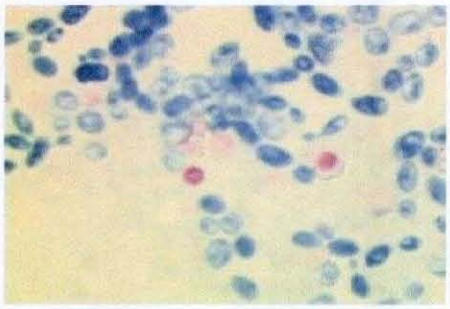

Fig-5: Ascospores of *Saccharomyces cerevisiae* (www. bmb.leeds.ac.uk)

However, morphological, physiological and biochemical tests have commonly been used for phenotypic characterization of yeast species. These methods are often unreliable, due to strain variability and, therefore, do not allow differentiation between yeast strains belonging to the same species. Genetic characterization using molecular techniques provides more powerful means of strain identification and differentiation among strains (Recek *et al*., 2002).

2.1.4 Criteria used for selection of yeast for ethanol production

Generally, to obtain high quality and yield of ethanol in ethanol industry, selection of fermentative yeast is very essential. Some desirable characteristics for selection of microbial culture include:

- ❖ Ability to utilize wide range of carbohydrates.
- ❖ Absence of metabolites other than ethanol.
- ❖ Low pH optimum and high optimum temperature and resistance to several physicochemical stresses.
- ❖ Rapid growth and fermentation rate.
- ❖ High osmotolerance and ethanol tolerance. (Spencer and Spencer, 1997)

In selecting yeasts for the efficient production of fuel ethanol (as opposed to potable ethanol), microbiologists have set out certain desirable characteristics of yeasts (Panchal *et al.,* 1981), as follows:

- ➢ Ethanol tolerant, osmotolerant, acid tolerant and thermo tolerant
- ➢ Genetically stable
- ➢ Rapid and efficient fermention

- Easy to propagate
- Able to utilize wide range of substrates
- Generate minimum heat during fermentation
- Possess flocculating or non flocculating characteristics depending upon the process requirements
- Possess "killer "activity, derepressed for di- or polysaccharide uptake in the presence of glucose
- Rresistant to certain toxic wastes.

It is safe to assume that there is no yeast strain used in the industry today that posses all the above characteristics and hence a research activities in this area have to be continued (Panchal *et al.,* 1981).

2.2 Ethanol fermentation

The fermentation of sugar to ethanol by yeast has an important procedure among the different processes that are used in industry. The yeasts, which are of primary interest to industrial operations, are *S. cerevisiae*, *S. uvarum* (*carlsbergensis*), *S. pombe*, and *Kluyveromyces* species. Yeasts metabolize glucose to ethanol by the glycolysis pathway. The overall net reaction involves the production of 2 moles each of ethanol, CO_2, and ATP per mole of glucose fermented. Therefore, on a weight basis, each gram of glucose can theoretically give rise to 51 % alcohol. The yield attained in practical fermentations, however, does not exceed 90-95% of the theoretical value. This is due to the requirement for some nutrients to be utilized in the synthesis of new biomass and cell maintenance-related reactions. Side reactions also occur in the fermentation (usually to glycerol) which many consume up to 4-5% of the total substrate. If these reactions could be eliminated, an additional 2.7% yield of ethanol from substrate would result (Roehr, 2001). The ethanol fermentation pathway results in the formation of ethanol and carbon dioxide (Norr *et al.,* 2003) is shown in fig-6:

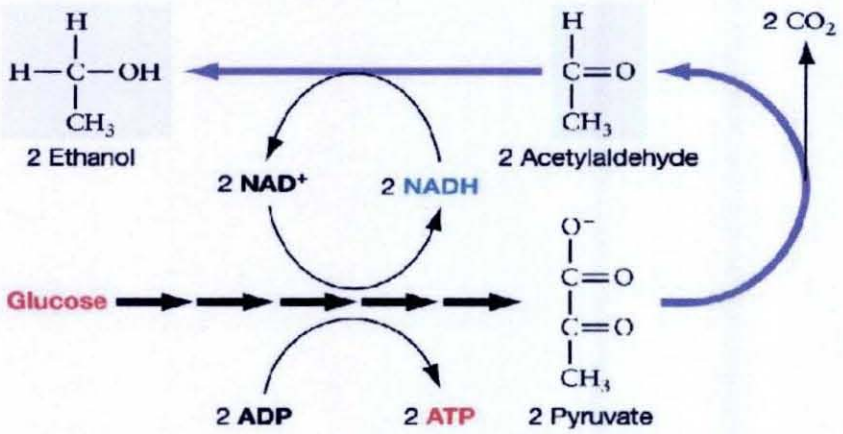

Fig-6: Fermentative pathway of ethanol.

The reducing power of NADH, produced by glycolysis, must be transferred to an electron acceptor to regenerate NAD+. In ethanol fermentation, it is not pyruvate but rather acetaldehyde, its decarboxylation product, which serves as the terminal electron acceptor. With respect to glycolysis, ethanol fermentation contains two additional enzymatic reactions, the first of which (catalyzed by pyruvate decarboxylase), decarboxylates pyruvic acid which have thiamine pyrophosphate (TPP) as cofactor (Ribéreau-Gayon *et al.*, 2000).

2.2.1 Principal methods of ethanol fermentation.

There are different types of fermentations among which the batch and continuous fermentation are the most common. The batch fermentation process is the oldest and conventional method used for the production of ethanol from molasses. In this process, several fomenters are usually operated in staggered intervals to provide a continuous feed to the distillation columns. As this method is not only laborious and time-consuming (36-72 hrs) but also less efficient (80%) and yields less alcohol and productivity (2.2 g/g/h) Continuous fermentation is among the several processes that have been developed for improvement. The inherent problems in the batch process are low cell density product and substrate inhibition. The productivity of continuous fermentation can be greatly enhanced by yeast cell recycle by the use of a centrifuge and the yeast cell density is increased to four-fold to 50% in a laboratory trial. The residence time for completion of conversion of 10% glucose feed was reduced to 1.6 hr with a corresponding

productivity of 30 g/g/h. Simple cell settling systems using flocculating yeasts with essentially no equipment (centrifuge) cost could be developed (Bassapa ,1989).

As a general solution to reduce production costs and improve process efficiency and ethanol yield; the continuous fermentation method is the most appropriate by using free cells, flocculent cells, or immobilized cells. When compared with processes using immobilized cells, alcoholic fermentation using free cells offers some advantages such as larger area of contact between cells and nutrient medium and management of current technology. However, disadvantages include the higher costs of microbial recycling and installation, high contamination risks, susceptibility to environmental variations, and the limitations of the dilution rate to reach steady state in continuous fermentation due to wash out problem (Vasconcelos *et. al.,* 2004).

2.3 Raw materials

Sugars from sugar cane, sugar beet, molasses, and fruits can be converted to ethanol directly.

The most widely used sugar for ethanol fermentation is blackstrap molasses which contains about 35 – 40 wt% sucrose, 15 – 20 wt% invert sugars such as glucose and fructose, and 28 – 35 wt% of non-sugar solids. (Osunkoya and Okwudinka, 2011)

The traditional method of starch hydrolysis to glucose (or fermentable sugars) is highly energy intensive as it involves the units operations of liquefaction by thermophilic alpha-amylase and subsequent saccharification by fungal gluco-amylase. The manufacture of ethanol or other feed stock from starchy material therefore is not economically feasible in comparison with directly fermentable sugars such as cane or molasses.(Highina *et al.*,2011)

Cassava contains 20-40% starch and about 70% moisture. However when dry, it contains 73% starch and gives a theoretical ethanol yield of 0.45 L kg^{-1}. Cassava starch can be easily hydrolyzed to sugars for production of fermentation-based products. Cassava starch costs 15-30% less to produce per acre than corn starch making cassava an attractive and strategic source of renewable energy (Hankoua and Besong, 2009)

Bioethanol can be manufactured from numerous sources. They can be produced from raw materials containing fermentable sugars such as sucrose-rich feedstock namely juices, sugarcane and beet etc. They can also be produced from some polysaccharides that can be hydrolyzed for obtaining sugars that can be converted to ethanol (Cardona and Sanchez, 2007).

Mainly grain (barley, wheat and rye) and some sugar beet have been used for bioethanol production (Enwald, 2007).

The nature of the substrate greatly affects the processes of the ethanol fermentation. Therefore, the raw materials selected for ethanol fermentation has great importance in the fermentation process (Baptista *et al.*, 2006)

Alcoholic fermentation has been carried out using a number of sugary materials depending upon their availability and suitability in particular geographic locations. Various raw materials like sugarcane juice and molasses (Morimura *et al.,* 1997 and Agrawal *et al.,* 1998), starchy materials like sweet potato (Sree*et al.,* 1999), and cellulosic materials like cocoa, pineapples and sugarcane waste (Othman *et al* 1992). Of these, simple sugar bearing materials are the easiest to process, since the yeast ferment these directly while other carbohydrates like starch/cellulose have to be first hydrolyzed to fermentable sugars using current commercial technologies (physio-chemical/enzymatic methods) before they can be fermented to yield ethanol.

Ethanol in India and other developing countries is mainly produced by fermentation of dilute molasses at ambient temperature of 25-35°C employing *Saccharomyces cerevisiae* (Sharma and Tauro 1986, Bulawayo *et al.,* 1996). Cane molasses is a complex mixture that varies in composition according to geographical sources, agricultural practices and sugar mill operations. Othman *et al.*(1992) showed that the removal of metal ions from molasses enhanced ethanol production.

The effect of pretreatment of molasses with H_2SO_4 and $K_4Fe(CN)_6$ on ethanol production by different yeast strain was studied in order to find an effective method to reduce the load of various inhibitory substances and to select a suitable yeast strain for fermentation of pretreated molasses(Yadav *et al.,* 1997). Pretreatment resulted in decreased level of inhibitory substances like Ca, Cu, and Fe in the molasses solution with improved ethanol production.

The inhibitory effect of these constituents was confirmed by supplementation of synthetic medium with residues from different pretreatments and the inhibitory level for various constituents was found to be Ca>0.5%, iron> 46ppm and Cu >5.4ppm.

The fermentable carbohydrates in molasses are sucrose and other sugars mainly glucose and fructose. The non-sugars may consist of nitrogenous substances like gums, polysaccharides, wax, sterols, pigments and salts of calcium, potassium and magnesium (Rao, 1983).

2.4 FACTORS AFFECTING FERMENTATION OF ETHANOL

A number of factors like high temperature, low ethanol and sugar tolerance of the yeast limit the industrial production of ethanol at low production costs. Physico-chemical and environmental factors such as inoculum type, moisture and water activity, pH, temperature, substrate, particle size, aeration and agitation, nutritional factors, and oxygen and carbon dioxide affecting fermentation (Krishna, 2005).

2.4.1. Effect of sugar concentration on ethanol fermentation

The profitability of ethanol production is dependent on favorable sugar cane molasses price and the quality of molasses (sugar %) (Arshad *et al.*, 2011).

Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. However high substrate concentrations are inhibitory to fermentation (Jones *et al.*, 1981) due to osmotic stress. Borzani *et al.* (1993) studied fermentation with various initial concentrations of sugar. They also demonstrated the logarithmic relationship between time of fermentation and initial concentrations of sugar. Bertolini *et al.* (1991) isolated yeast strains from sample collected from Brazilian alcohol factories. These strains were capable of fermenting upto 30% of sucrose efficiently. The efficiency of selected strains varied from 89% to 92% depending upon the utilization of total sugar available in the medium. A maximum amount of 19.7% (v/v) ethanol accumulated from fermentation of 30% sugar as compared to 2 reference strains, which produced 18.0(v/v) and 15.6 (v/v). A repeated batch fermentation system was used to produce ethanol using an osmotolerant *S.cerevisiae* (US3) immobilized on calcium alginate.

Fermentation was carried out with initial concentration of 150, 200, 250 g glucose per litre at 30°C .The maximum amount of ethanol produced by immobilization VS3 cells using 150, 200 and 250 g/l glucose was 72.5, 93 and 83 g ethanol per litre at 30°C after 48h. Maximum yield was obtained at initial sugar of 20% with fermentation efficiency of 90% (Sree *et al.*, 2000). Converti *et al.*(1998) studied the inhibition of the fermentation of oak hemicellulose acid hydrolysates by minor sugars. Synthetic xylose media and detoxified oak hemicellulose acid hydrolysates were fermented batchwise. Maximum productivity was calculated from the experimental data of ethanol concentration. The kinetic parameters calculated for the fermentation of both carbon sources indicate that a competitive inhibition is exerted by the minor sugars (arabinose, rhamnose and galactose) that are metabolized slowly or not at all.

2.4.2 Effect of temperature on ethanol fermentation

The fermentation process is always accompanied with evolution of heat that raises the temperature of the fermenter. As a result it becomes necessary to cool the large fermenters in the distilleries. This necessity often becomes a major operation and a cost factor in the production of ethanol. Temperature exerts a profound effect on growth, metabolism and survival of the fermenting organism. Fermentation in industries is usually carried out at ambient temperature of 25- 35°C but temperature exceeds 40°C during fermentation especially in northern regions which decreases the cell viability and productivity. Maintenance of high cell viability is a major characteristic of fermentation to get high ethanol yield. Fermentation at 35-40°C or above has advantages such as ethanol recovery and significant savings in operational costs of refrigeration control in distilleries for alcohol production. Therefore many studies have been carried out for the development of yeast to ferment at high temperature of upto 40-45°C.

Temperature is one of the major constraints that determine the ethanol production. To know the optimum temperature for ethanol fermentation, the fermentation media were kept at 25, 28, 30, 35 and 40°C. Two parameters were studied, the growth and the ethanol yield. The maximum ethanol production and biomass was obtained at 28-30°C (Osman *et al., 2011*).

Laluce *et al.*(1993) studied the effects of temperature on fermentation capacity of three strains 19G, 78I and baker's yeast in complete medium and sugarcane juice broth containing 15% total sugar. Complete conversion of total sugar to ethanol was observed after 12 hrs of fermentation at 39-40°C. Above 40°C a strong inhibitory effect of temperature on ethanol production in all classes was observed.

Further, optimum temperature for growth and rate of ethanol formation were found to depend on medium composition and strain. At high sugarcane syrup concentrations (20% w/v and above), a temperature of 35°C was found to be the best temperature for ethanol formation strain 78I.

Singh *et al.*(1998) further studied the ethanol production at elevated temperatures. They isolated a number of strains of *Kluyveromyces marxiaus* var. *marxianses* capable of growth at high temperatures coupled with production of high alcohol concentrations by fermentation of glucose and molasses.

Morimura *et al.*(1997) made an attempt to improve the salt tolerance of the thermotolerant flocculating yeast *Saccharomyces cerevisiae* strain KF-7 by maintaining a high concentration of

KCl in the molasses medium. Among selected strains, K211 had the highest cell viability and ethanol productivity in a molasses medium containing 25% (w/v) total sugar at 35°C. As a result of repeated batch fermentation tests with K211, stable ethanol production was achieved with an ethanol concentration of 92g/l and a productivity of 3.5 g/l-h at 33°C in 22% molasses medium. Even at higher temperature of 35°C, strain K211 gave stable ethanol concentration of 91 g/l and productivity of 2.7g/l-h.

2.4.3 Effect of ethanol on fermentation

A limitation of ethanol fermentation is the capacity of yeast to tolerate ethanol concentration, because ethanol inhibits alcoholic fermentation, which limits the concentration of ethanol which can be produced by a given strain of yeast. The maximum concentration of ethanol which can be produced by yeast varies with species up to 20% by volume. The degree of inhibition is also related to other environmental factors, in particular high sugar concentration and high temperature which cause the inhibition of ethanol fermentation. Ethanol, which is produced during fermentation, is more inhibitory to cell growth than that from an exogenous source (Wayman and Rarekh, 1990).

Navarro (1980) studied the high intracellular ethanol concentrations were a consequence of resistance to diffusion through the membrane to the outside. At elevated temperature, the rate of ethanol production increased faster than the rate of excretion.

Navarro and Durand (1978) also concluded that the effects of temperature on ethanol accumulation in *S. uvarum*. They found growth was arrested when a critical intracellular ethanol concentration had been reached, and this intracellular accumulation was greater at higher temperatures.

The toxic effect of ethanol has also been attributed to damaging the cell membrane or changing its properties. The extent of ethanol tolerance of yeasts is highly strain dependent and appears to be related to the unsaturated fatty acid and the fatty acyl composition of the plasma membrane (Wayman and Rarekh, 1990).

2.4.4 Effect of Nutrients on ethanol fermentation

Yeasts grow in simple media which contain fermentable carbohydrates to supply energy and carbon skeleton for biosynthesis, adequate nitrogen for protein synthesis, mineral salts and one or more growth factors. Sources of carbon included monosaccharides, disaccharides and trisaccharides (Priest and Campbell, 1996).

The metabolic activities of yeasts are greatly affected by the temperature at which they grow. Temperatures above the optimum lower the growth rate, oxygen solubility and also change the cellular composition of yeasts. It is known that under oxygen-limited conditions, yeasts require nutritional supplements for growth (Slapack *et al.*, 1987 and Thomas *et al.*, 2002). An increase in temperature does not inhibit substrate uptake nor does it significantly alter enzyme levels (Slapack *et al.*, 1987).

Helena da Cruz *et al.*(2003) concluded that nitrogen and carbon are the main nutrients in fermentation medium and this implies that the mutual interaction of these nutrients may play an important role in the metabolism of yeasts The supplementation of the growth media, containing maltose or glucose, with a more complex structural nitrogen source such as peptone induced higher biomass accumulation and ethanol production. In *S. diastaticus*,

Amore *et al.* (2002) reported by doubling the nutrient components in the medium, resulting in the production of 9.1% (w/v) ethanol. Increasing the fermentation temperature from 40 to 45 °C Most yeast grows well on a variety of amino acids, purines, and pyrimidines as the sole source of nitrogen. They require trace amounts of biotin, thiamine, pyridoxine, calcium pantothenate and inositol for the maximum growth and fermentation rate (Wayman and Parekh, 1990).

Amore *et al.*(2002) have also shown that role of magnesium in relieving the detrimental effect of high temperature may to some extent be related to the requirement of some of the glycolytic enzymes for this cation. In addition, extent of glucose utilization and ethanol production decreased at the higher temperature.

2.4.5. Effect of pH on ethanol fermentation

The rate of ethanol production by yeast cells is highly affected by the pH of the fermentation edium . *S. cerevistae* showed maximum growth under acidic conditions. More acidic and basic conditions, both retard the yeast metabolic pathways and hence the growth of cells (Willaert & Viktor, 2006)

Hydrogen ion concentration has a significant influence on industrial fermentation due as much to its importance in controlling bacterial contamination as its effect on yeast growth, fermentation rates and by-product formation. The best ethanol yields are generally obtained at pH 4.5-4.7. At higher pH, more glycerol and organic acids are formed at the expense of ethanol (Wayman and Parekh, 1990).

Under fermentation conditions, the intracellular pH of *S. cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 or between 5.9 and 6.75 when the external pH is varied between 6.0 and 10.0. As the gap between the extracellular pH and the intracellular pH widens, greater stress is placed on the cells and more energy is expended to maintain the intracellular pH within the range that permits growth and survival of the yeast. A greater proportion of glucose is converted to ethanol if the pH is adjusted to 4.5. This increased conversion is independent of the presence of nutrient supplements in the medium (Thomas *et al.*, 2002). If the pH is adjusted to 7 or above, acetic acid is produced from acetaldehyde due to the increased activity of aldehyde dehydrogenase due to glycerol production which inhibits ethanol fermentation (Wang *et al.*, 2001).

2.4.6 Inhibition of growth and fermentation by substrate

The production of high concentration of ethanol is frequently limited by the inhibitory effect on productivity of the fermenting microorganism exerted by the substrate, the concentration of which affects osmotic pressure (Van uden, 1989). With lower sugar concentrations start to ferment sooner, and the sugar is completely fermented. High sugar concentration inhibits fermentation by their high osmotic pressure, which draws water from the yeast cells (Rehm and Reed, 1995).

Direct substrate inhibition of fermentative ability becomes significant somewhere between 15-25% sugar concentrations (Van Uden, 1989). Values of specific ethanol production rate and specific uptake decrease almost linearly with the increase in sugar concentration. When the

substrates are introduced in several batches ethanol yields are higher (Casey and Ingledew, 1986; D,Amore and Stewart, 1987) and cell viability is close to 95% compared to 40% for single batch run(Casey and Ingledew, 1986).

2.4.7 Effect of Oxygen on ethanol fermentation

The microorganisms involved in ethanol fermentation are facultative microbes since they are able to grow with or without the utilization of oxygen. Thus, two of different path ways of pyruvate metabolism are available (Abbott, 2005). However for ethanol production, oxygen must be restricted from entering the fermenter. But, small concentration of oxygen must be provided to the fermenting yeast as it is necessary for the biosynthesis of polyunsaturated fats and lipids (Cysewaski and Wilke, 1977). According to Kosaric and Vardar-sukan (2001), typical amount of oxygen maintained in the broth is 0.05-0.10 mmHg. Any value higher than this will promote cell growth at the expense of ethanol productivity. The oxygen concentration which triggers aerobic or anaerobic growth processes, however, varies from culture to culture depending on substrate concentration and cell density (Munnecke, 1981).

2.4.8 Effect of Immobilization on ethanol fermentation

Immobilization is the restriction of cell mobility within a defined space. Immobilization provides high cell concentrations and cell reuse. It also eliminates washout problems at high dilution rates and the costly processes of cell recovery and cell recycle. High volumetric productivities can also be obtained with the combination of high cell concentrations and high flow rates. Immobilization may also improve genetic stability (Nicholas *et al.*, 2005).

The most significant advantages of immobilized yeast cell systems are the ability to operate with high productivity at dilution rates exceeding the maximum specific growth rate, the increase of ethanol yield and cellular stability and the decrease of process expenses due to the cell recovery and reutilization (Lin and Tanaka, 2006).

Perspective techniques for yeasts immobilization can be divided into four categories: attachment or adsorption to solid surfaces (wood chips, delignified brewer's spent grains, DEAE cellulose, and porous glass), entrapment within a porous matrix (calcium alginate, k-carrageenan, polyvinyl alcohol, agar, gelatine, chitosan, and polyacrilamide), mechanical retention behind a barrier

(microporous membrane filters, and microcapsules) and self-aggregation of the cells by flocculation. The application of these different immobilization methodologies and carriers, their impact in microbial growth and physiology, internal and external mass transfer limitations, product quality and consistency,bioreactor design, bioprocess engineering and economics have been largely discussed. (Verbelen *et al.*, 2006)

2.5 Uses of Ethanol

2.5.1 As a fuel

The largest single use of ethanol is as a motor fuel and fuel additive. The largest national fuel ethanol industries exist in Brazil and the United States. The Brazilian ethanol industry is based on sugarcane; Brazil produced 14 billion liters annually, enough to replace about 40% of its gasoline demand. Also as a result, they have become 80% independent from foreign oil. Most new cars sold in Brazil are flexible-fuel vehicles that can run on ethanol, gasoline, or any blend of the two. The United States fuel ethanol industry is based largely on corn. Thailand, India, China and Japan have now launched their national gasohol policies.

Ethanol with water content of 2% or less can be used as the alcohol in the production of biodiesel, replacing methanol, which is quite dangerous to work with.

2.5.2 Alcoholic beverages

Alcoholic beverages vary considerably in their ethanol content and in the foodstuffs from which they are produced. Most alcoholic beverages can be broadly classified as fermented beverages, beverages made by the action of yeast on sugary foodstuffs, or as distilled beverages, beverages whose preparation involves concentrating the ethanol in fermented beverages by distillation. The ethanol content of a beverage is usually measured in terms of the volume fraction of ethanol in the beverage, expressed either as a percentage or in alcoholic proof units. The proof of an alcohol beverage is equal to twice the percentage of alcohol contained therein.

Fermented beverages can be broadly classified by the foodstuff from which they are fermented. Beers are made from cereal grains or other starchy materials, wines and ciders from fruit juices, and meads from honey. Fermented beverages may contain up to 15–20% ethanol by volume, the upper limit being set by the yeast's tolerance for ethanol, or by the amount of sugar in the starting material.

2.5.3 Other uses

Ethanol is easily soluble in water in all proportions. Absolute ethanol and 95% ethanol are themselves good solvents, somewhat less polar than water and used in perfumes, paints and tinctures. Alcoholic drinks have a large variety of tastes because various flavor compounds are dissolved during brewing. Ethanol is used in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62%. Ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi, and many viruses, but is ineffective against bacterial spores. Wine with less than 16% ethanol cannot protect itself against bacteria. It is also used in preservation of biological specimens.

2.6 Current status of ethanol production in Bangladesh

Ethanol has emerged as an alternative for petroleum based liquid fuels. Now days, its use in automobiles as an alternative fuel has attracted many countries including Bangladesh. Currently in Bangladesh, there is only one sugar mill producing ethanol and few distilleries participating in downstream chemicals from ethanol. Among molasses driven products, ethanol takes the largest part, but its utilization must attract the attention of government policy makers in order to utilize it as a biofuel. With the present trend in sugar sector expansion and modernization in the country, implementation of different domestic measures for bioethanol production and utilization have to be taken into consideration. An efficient ethanol production requires four components: cheap fermentable carbohydrate, an efficient yeast strain, a few nutrients and simple culture conditions. Among the widely used substrates for ethanol production molasses of sugar cane and molasses of sugar beet are the most important sources. This is because they are ready for conversion with limited pre-treatments as compared with starchy or cellulosic materials (Yadav *et al.*, 1997)

Bangladesh through its potential in developing large scale sugar cane production can play a productive role in mitigating sugar, ethanol and electricity demands in the country. Molasses non-crystallizable residue remaining after crystallizing sucrose has additional advantage. It is relatively inexpensive, readily available and readily in, use for industrial ethanol production. Immobilization offers advantages of modern technique of continuous fermentation along with

low cost design and optimization of available expertise. So studies on isolation of new potent strain and improvement of the available strain to increase higher productivity are necessary for the existing and newly emerging ethanol industries of the country.

It is unfortunate that Bangladesh, a producer of sugar and sugar cane and also of molasses, the cheapest feed stock for ethanol has lagged far behind in exploiting this natural renewable resources. There is a wide scope of ethanol fuel production in Bangladesh. Bangladesh at present produces, on an average, about 80,000 tons molasses in 14 sugar mills under Bangladesh Sugar and Food Industries Corporate (BSFIC) against the molasses production capacity of about 1 lac tons.

Three to four more raw sugar processing factory will go on production within one to two years. Then molasses production in the country will be increased many folds which will result in sufficient feed stock available for ethanol, fuel production.

At present ethanol is produced in Carew's distillery under the management of BSFIC to meet the demand for beverage alcohol (foreign liqure). In the light of easy and sufficient availability of cane molasses in the country, it is worth trying use of the cheap material for bioethanol production.

CHAPTER: 3

MATERIALS AND METHODS

CHAPTER: 03
MATERIALS AND METHODS

3.1 Materials

- Molasses
- Industrial yeast strain
- DinitroSalicylic acid (DNS), Sulphuric acid, sodium-potassium tartarate (KNaC4H4O6.H2O), Sodium hydroxide, Indicators (methylene blue), YPD (yeast extract, peptone, dextrose) medium, Sodium-Metabisulfite, PHENOL, Potassium Sodium-Tartarate, NAOH, Potassium Iodide, Potassium dichromate, Solubole starch, sodium acetate, carbol-fuchsine etc.
- Spectrophotometer
- Incubator
- Autoclave
- Orbital Shaker
- Glasswares, Laboratory distillation apparatus, Neubauer counting chamber (Cell counter), Petri dishes, slants, micro-pipettes, Bunsen burner, hot plate, Desiccators, Balance micro-burette, etc.

3.2 Source of the strain

The yeast strain coded as "JY" was taken from the available stock culture of Industrial Microbiology Laboratory, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh.

3.2.1 Maintenance of culture

The culture of yeast was maintained by sub-culturing on slants using YMM (Yeast maintenance Media) media, incubating for 48 hrs at 30°C and thereafter storing in a refrigerator at 4°C for future use.

3.3 Characterization of the selected yeast strain JY

3.3.1 Morphological characterization:

According to the method of (Kreger-van Rij 1984) and (Kurtzman and Fell 1997), the morphology of the vegetative cells of yeast was grown in liquid and on solid media.

3.3.1.1 Growth on solid medium:

In the present study, morphology of cells of the selected strain and its appearance on YEPD agar media was examined. The medium was autoclaved at 121°C and 15 psi and poured on petridish and cooled. After cooling, the plates were inoculated by 48 hrs old yeast strain and incubated at 30 °C for 48 hrs. The following features of the appearance of cultures were recorded; texture, color and surface of colonies.

3.3.1.2 Growth in liquid medium:

Selected strain JY was cultured in YPD liquid medium. The medium was autoclaved at 121°C and 15 psi and cooled. 15 ml portion of the medium was distributed into McCartney bottle and after then inoculated with half loopful of 48 hrs old selected yeast strain and incubated at 30°C for 3 days. The culture was examined for the growth visually on the surface of YPD liquid medium and the shape of cells by compound microscope (Olympus, Japan).

According to Lorenz *et al.* (2000), the morphological changes in the culture yeast (*Saccharomyces cerevisiae*) leads to filament formation under unfavorable fermentation conditions. This causes scum formation and foaming. The budding yeast *S. cerevisiae*, starved for nitrogen, differentiates into a filamentous growth form.

3.3.1.3 Ascospore formation:

Selected yeast strain was examined for ascospore formation applied from (Kurtzman *et al.*, 2005). The culture was initially incubated for 2 days at 37 °C to facilitate growth and further incubated at 25 °C to induce ascospore formation. The culture was examined for ascospores at approximately weakly intervals for 3 weeks. Ascospore formation was detected by staining the heat-fixed preparation carbol-fuchsine and steamed gently for about 5 min. Slide was decolorized with 95% ethanol containing 1% concentrated hydrochloric acid. The slide was rinsed in water and counter stained with 1% methylene blue; the mature ascospores stain red and vegetative cells blue under microscope (Kreger-van Rij, 1984).

3.3.1.4 Pseudomycelium formation:

Following the method of Kreger-van Rij, (1984), the formation of pseudomycelium was investigated by slide culture technique. A Petri dish containing a U-shaped glass rod supporting of the glass slide was sterilized by dry heat at 180 °C for 2 hrs. YPD agar was melted and poured

into a second Petri dish. The glass slide was quickly removed from the glass rod with a flame-sterilized forcep and was dipped into the agar after which it was replaced on the glass rod support.
After solidification of the agar on the slide, the selected yeast was inoculated very lightly in two lines along slide and a sterile cover slip was placed over part of the lines. Some sterile water was poured into the Petri dish to prevent the agar from drying out. The culture was incubated at 25 °C for 5 days. For observation, the slide was taken out of Petri dish and the agar was wiped off the back of the slide. The edges of the streak under and around the cover slip were examined microscopically.

3.3.2 Physico-chemical characterization:

3.3.2.1 Fermentation of carbohydrates:

Yeast fermentation broth base with Durham tube was used for testing of yeasts for carbohydrates fermentation. Yeast fermentation broth media was used for identification yeasts based on fermentation of specific carbohydrates of fermentation pattern. The carbohydrates used were; glucose, dextrose, galactose, maltose, sucrose, lactose, trehalose, raffinose and xylose. Yeast fermentation broth was modification of media developed by Wickerham for the determination carbohydrate fermentation by yeasts, the color of the medium changed from blue to yellow due to the formation of acids and gas produced (Warren and shadomy, 1991).

Testing of selected strain for carbohydrate fermentation, the ability to ferment eight different carbohydrates was examined anaerobically.Capable of fermentation was assessed by looking for the formation of gas (CO_2) in Durham tube and colour change of the fermentation media. In addition, medium prepared from Tryptone 10g, NaCl 5g, Phenol red (to make colour), Carbohydrate 5g and make the volume upto 1000ml with distilled water. A volume of 15ml aliquots dispensed in different McCartney bottles and were autoclaved 121°c and 15 psi. After cooling, the tubes were inoculated with halfloopful of 48 hrs old selected yeast strain and incubated at 30°C for 48 hrs.

3.3.2.2 Detection of thermo tolerance:

YPD liquid medium was used for detecting thermotolerance and growth in liquid media of the selected yeast strain. The medium was autoclaved at 121 °C and 15 psi and cooled. 10 ml portion of the medium was distributed into McCartney bottles and then inoculated with halfloopful of 48 hrs old selected yeast strain. The initial optical density of each tube was recorded on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 25°C, 30°C, 37°C and 40°C for 2 days for observing thermo tolerance of yeast strain. The increase in

optical density in a tube was recorded as evidence of growth. Without it, growth on YEPD agar media at 25°C, 30°C, 37°C and 40°C was also observed to ensure thermotolerence of the strain.

3.3.2.3 Detection of ethanol tolerance:

The medium for the detection of ethanol tolerance of thermotolerant yeast was modified from (Osho, 2005). YPD liquid medium was used for detecting yeasts for ethanol tolerance. The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. One ml of various concentrations of absolute ethanol was varied from 5 to 25% (v/v), and then added to different flask of the same medium to constitute varying percentages of ethanol differing by 5% (v/v) from one flask to the others. Forty ml portion of the medium was distributed into 125 ml flask, and then inoculated with selected thermotolerant yeasts. The initial optical density of each flask was read off on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 40 °C for 5 days. The increase in optical density in a flask was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of yeasts.

In the present study, YPD broth was prepared containing 5%, 8%, 10%, 12%, 15%, 18% and 20% of absolute ethanol. Each McCartney contained 15 ml of YPD liquid media with appropriate concentration of ethanol and blank media was used as a control. Then each was inoculated by halfloopful of yeast cells and measured the initial optical density at 600 nm and incubated at 30°c for 48 hrs. After 48 hrs cell density was further recorded at 600 nm.

3.3.2.4 Growth at different pH in Liquid Media:

YEPD liquid medium was used for detecting the ability to grow at different pH. The medium was autoclaved at 121 °C and 15 psi and cooled. YEPD broth was prepared at pH 2-10. Each McCartney contained 15 ml of YEPD media with different pH and blank media was used as a control. Then each was inoculated by halfloopful of yeast cells and measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm for growth.

3.3.2.5 Osmotolerence observation:

YEPD broth was prepared containing 5%, 8%, 10%, 12%, 15%, 18% and 20% of NaCl. Each McCartney contained 15 ml of YEPD liquid media with appropriate concentration of salt and blank media was used as a control. Then each was inoculated by halfloopful of Yeast cells, measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm.

3.3.2.6 Cyclohexamide resistance test:

YEPD agar medium was used for detecting yeasts for Cycloheximide resistance. Cycloheximide antibiotic (0.0015g) was added into 100ml autoclaved YEPD agar media and poured into petridish. After cooling, plate was inoculated by yeast cells and kept it 48 hrs at 30°C for growing.

3.3.2.7 Growth on Hydrogen peroxide containing solid media:

Hydrogen peroxide can inhibit the growth of Yeast. At first petridish containing the solid YEPD agar media was inoculated by yeast cells. Then three discs containing 30 µl, 20 µl and 10 µl of hydrogen peroxide were placed on difference places on the plate. . Hydrogen peroxide containing plates were incubated at 30°C for 48 hours.

3.4 Pretreatment of Molasses for Ethanol Production:

Sulfuric acid is used to convert calcium to calcium sulfate salts. Calcium acts as an inhibitor agent during fermentation. Sulfuric acid decreases the fermentation medium pH that control bacterial contamination. Sulfuric acid in diluted molasses can precipitate calcium and reduce the scaling. Blackstrap molasses contains many nutrients for fermentation. Without it, it requires some nutrients elements. Urea is used as a nitrogen source in molasses fermentations for ethanol production, but it in alcoholic beverage it may produce carcinogenic ethylcarbamate, which is unacceptable. Diammonium phosphate may be added in Phosphorus deficient molasses. In our present study 0.30 ml concentrated Sulfuric acid and 0.10 gm urea was used to treat 250 gm molasses and diluted it with tap water.

3.5 Fermentation media preparation:

Sugarcane molasses was used as a fermentation media for the study. Molasses was collected from local market of Bangladesh at the district of Natore. It contains 15%-25%% of reducing sugar. In all experiments, reducing sugar concentration was estimated only.

Composition of Fermentation Media:

Molasses	250 gm
Urea	0 .10 gm
Conc. (H2SO4)	0 .30 ml

Tap water was added up to 1000 ml and the media was boiled. After boiling the media was autoclaved at 121° c and 15psi. The reducing sugar concentration was maintained by boiling the media.

3.6 Preparation of yeast cell suspension:

Autoclaved molasses fermentation media (10 ml) was added aseptically to a 48 hrs old slant culture of *S. cerevisiae* and the tube was shaken gently to form a homogeneous suspension.

3.7 Fermentation of molasses:

Fermentation was carried out in Erlenmeyer conical flasks. 250 ml fermentation media was taken into 500ml Erlenmeyer flasks and added the homogenous suspension of yeast was inoculated into the media in an aseptic condition. The flask was cotton plugged and incubated at different temperature to an incubator in non shaking and shaking condition.

3.8 Estimation of reducing sugars:

The reducing substance (sugar) obtained due to the enzymatic reaction was determined by DNS method (Miller *et al.*, 1959).

3.8.1 Procedure

The estimation of total reducing sugar was based on the dinitrosalycylic acid (DNS) method. A double beam UV scanning spectrophotometer was used for measuring absorbance. Reducing sugar contents before fermentation and after fermentation were determined by taking 1.0 ml diluted solution (1 ml sample in 100 ml distilled water) with 3.0 ml of DNS reagent in a test tube. Blank containing 1.0 ml of distilled water and 3.0 ml of DNS was run parallel. The tubes were heated in a boiling water bath for 15 min. After cooling the tubes at room temperature, 8 ml of distilled water was added in each and absorbance was noted at 540 nm using spectrophotometer. Reducing sugar concentration was determined from the standard curve of glucose and multiplied by dilution factor. A standard curve of glucose was prepared that shown in fig-7:

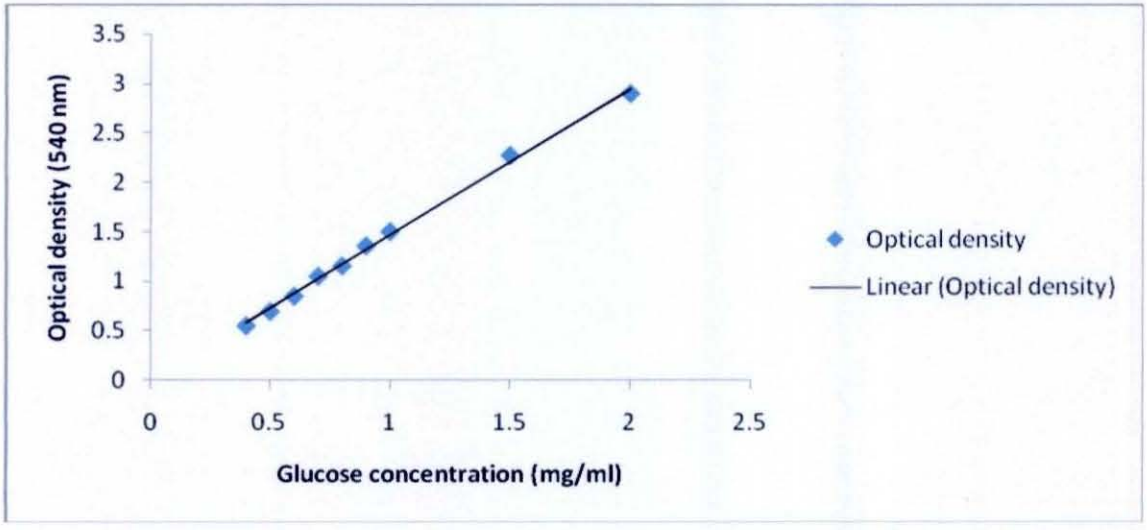

Fig-7: Glucose standard carve for reducing sugar estimation.

3.9 Estimation of Ethanol (Conway Method):

Ethanol was determined by Redox titration. In this method ethanol is oxidized to ethanoic acid when ethanol react with excess of potassium dichromate solution (0.05 N) and unreacted dichromate is then determined by adding potassium iodide (50% KI) solution which is oxidized by the potassium dichromate. Potassium iodide reacts with potassium dichromate and creates iodine. Then the iodine is titrated with a standard solution of sodium thiosulfate (0.1N). The titration reading is used to calculate the ethanol content after fermentation. One ml Fermented solution was diluted up to in 250 ml; 500 ml and 1000 ml distilled water and took each one ml diluted solution as a sample. A Conway unit was used for ethanol detection by this procedure. One ml potassium dichromate (0.05 N) was placed into the Conway unit center and sample was placed around the center. The Conway unit was covered by a glass plate for 24 hrs for reaction. The water and ethanol slowly evaporate, come in contact with Potassium dichromate and then oxidized. More ethanol evaporates until eventually all the ethanol from the fermented dilute solution has left the sample and reacted with the dichromate. One Conway unit was used as a blank and in that unit 1 ml distilled was used as a sample.

3.9.1 Alcohol Estimation (calculation):

Conway unit center (1 ml 0.05 N potassium dichromate solution)

↓

Round (1 ml sample)

↓

Reaction complete within 24 hours

↓

Conway unit center (50% KI solution 0.5 ml + 1-2 drop soluble starch)

↓

Microburatte (0.1 N sodium thiosulfate)

↓

Titration

↓

Colourless

$$\text{Percentage of Ethanol (\%)} = \frac{(\text{TR of blank sample- TR of FS}) \times 11.6 \times 0.1 \times \text{DF} \times 100}{0.793 \times 1000}$$

= Alcohol percentage gm/100 ml

Where,

Density of Ethanol 0.793 g/ml, DF-Dilution Factor, FS-Fermented solution ,TR-Titration Reading, Volume of sodium thiosulfate used: 11.60 cm^3

3.10 Optimization of fermentation process:

Fermentation process carried out by yeast is known to vary with respect to substrate concentration, temperature, N-source and inoculums size. It is therefore imperative to optimize the fermentation conditions for yeast cells, so that the production efficiency increases. Various factors were investigated affecting ethanol production from molasses.

3.10.1 Effect of sugar concentration:

To study the effect of sugar concentration on ethanol production by *S. cerevisiae*, the production media was prepared by diluting molasses to reducing sugar concentration 4.0, 4.30 , 5.50, 6.0, 6.50, 7, 9 and10 percent and fermentation was carried out in a volume of 250 ml media in a 500 ml conical flask. A fourty eight hrs old inoculum of yeast was added to the medium. Samples were withdrawn at different time and estimated for residual sugars (Miller, 1959) as well as ethanol content in the media.

3.10.2 Effect of pH

To study the effect of pH on ethanol production by *S. cerevisiae*, fermentation media with different reducing sugar concentration was used for the production of Ethanol. Fermentation was carried out at pH 5.3 and pH 6.0 in a volume of 250 ml media in a 500 ml conical flask. A fourty eight hour old inoculum of yeast was added to the medium. Samples were withdrawn at different time and estimated for residual sugars (Miller, 1959) as well as ethanol content in the media.

3.10.3 Effect of temperature on fermentation of molasses

Molasses diluted to different reducing sugars concentration and supplemented with nitrogen and phosphorus were used as production media and fermentation was carried out at different temperatures. To optimize the fermentation temperature, fermentation was carried out at 25, 30 and 37°C. The periodic samples were analyzed for reducing sugars and ethanol content.

3.11 Effect of immobilization

For carrying out immobilization, 2% of $CaCl_2$ solution was prepared and kept at 4°C for chilling. After that, 2 g of sodium alginate was dissolved in hot water with constant stirring with magnetic stirrer. After cooling sodium alginate solution, 2 g of yeast cells were added to the slurry under stirring conditions for even distribution. The slurry solution, with yeast biomass was dispersed drop wise into 2% chilled $CaCl_2$ solution. Spherical beads were formed which were washed with 0.2% chilled $CaCl_2$ solution and stored at 4°C for further use to carry out fermentation.
After accomplishment of immobilization of yeast cells in 2% sodium alginate, these were tested for ethanol production at 30^0C, pH 6 and reducing sugar concentration was maintained at 5.50%, 6.0% and 9% at different shaking condition.

CHAPTER: 4

RESULTS

CHAPTER: 04
RESULTS

4.1 Characterization of the selected yeast strain JY:

4.1.1 Morphological characterization

According to the method of Kreger-van Rij (1984 and Kurtzman and Fell (1997), the morphology of the vegetative cells of yeast grown in liquid and on solid media was studied.

4.1.1.1 Growth on solid medium

The yeast formed butyrous and smooth white raised colonies on YEPD agar medium. The budding stage of the yeast isolates was observed under microscope and confirmed them to be yeast.

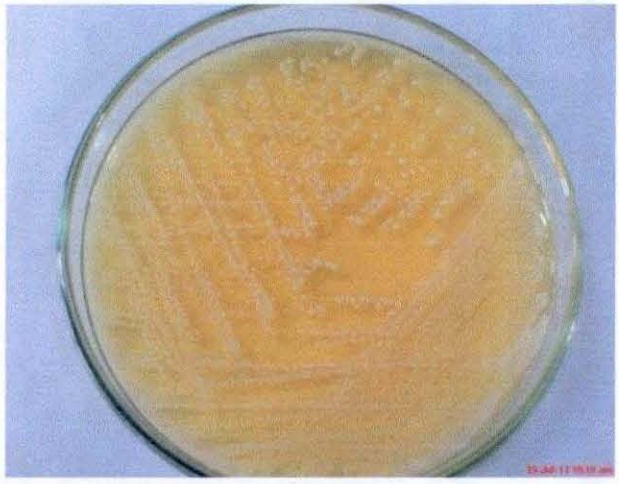

Fig-8: Colonies on YEPD agar medium after incubation of 48 hours at 30°C.

4.1.1.2 Growth in liquid medium

After 3 days of incubation at 30°C, heavy, dry climbing pellicles were formed on the surface of YPD broth medium.

Fig-9: Growth in liquid medium and surface film formation

4.1.1.3 Microscopic observation:

The cell morphology of the JY strain was observed under compound microscope and ovoidal to elongate cells with single, pairs, or triple budding were found. The selected strain reproduced vegetatively by budding.

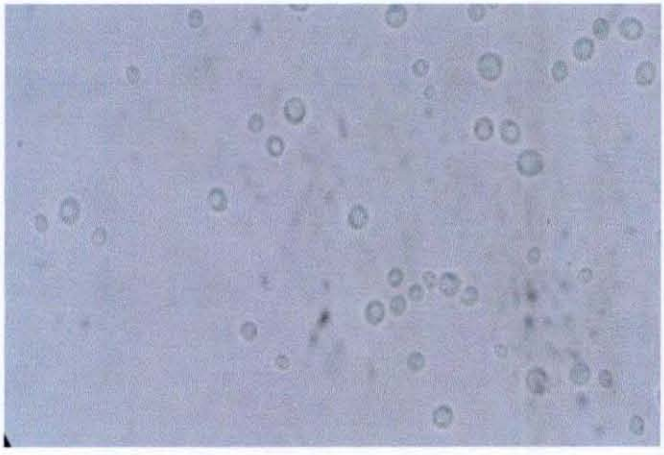

Fig-10: The cell morphology under compound microscope.

4.1.1.4 Ascospore formation:

Following the method of Kreger-van Rij (1984) and Kurtzman and Fell (1997), ascospores formation was detected for indication of the ascomycetous yeasts. Ascospore formed in ascospore forming media after incubating for 3 weeks at 25 °C. Mature ascospores were stain red and vegetative cells were blue.

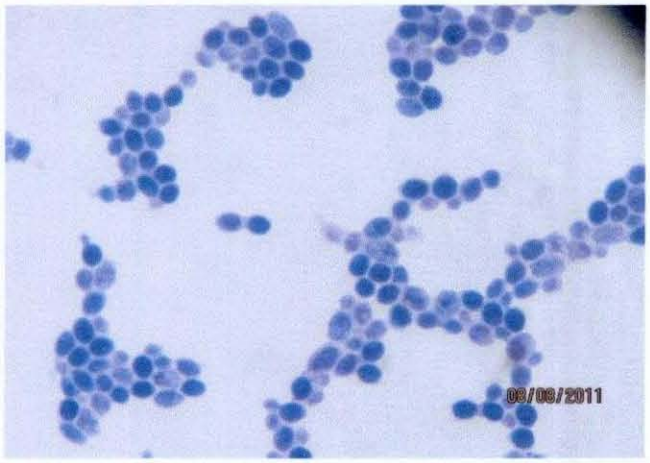

Fig-11: Ascospore formation observation under compound microscope.

(Mature ascospores stain red and vegetative cells blue.)

4.1.1.5 Pseudo mycelium formation:

Saccharomyces cerevisiae is dimorphic, existing either in a spherical, unicellular yeast-like morphology or in a filamentous form, termed pseudohyphae that results from elongated chains of cells that remain attached to one another (Gimeno *et al.*, 1992)

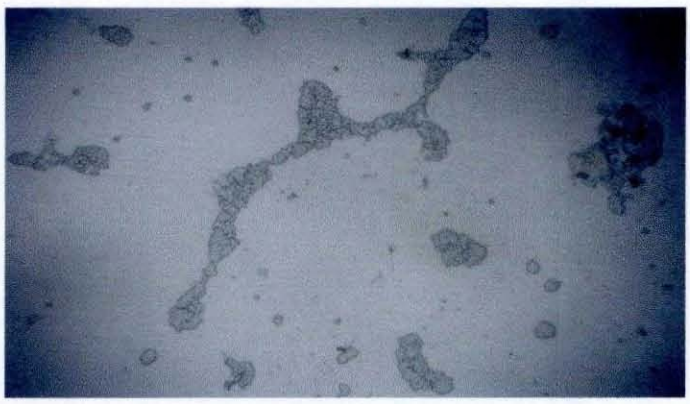

Fig-12: Elongated chain of *S. cerevisiae* Pseudo mycelium under microscope.

4.1.2 Physico-chemical Characterization

4.1.2.1 Fermentation of carbohydrates:

In this study, *Saccharomyces cerevisiae* showed variation of utilization of eight different sugars (Table-2). The strain utilized Glucose, sucrose maltose, Dextrose and Fructose but failed to ferment lactose and xylose, Rhamnose. After 48 hours the following results were shown in Table-2:

Table-2: Fermentation examination of different carbohydrates.

Carbohydrate	Before Fermentation	After Fermentation
Glucose	Pink color	+ (yellow)
Sucrose	Pink color	+(yellow)
Maltose	Pink color	+(yellow)
Lactose	Pink color	-(no color change)
Fructose	Pink color	+(yellow)
Xylose	Yellowish Pink	-(no color change)
Rhamnose	Pink	-(no color change)
Dextrose	pink	+(yellow)

Fig-13: Media Color change after Fermentation of Carbohydrate.

4.1.2.2 Thermotolerence of the selected strain (JY):

Five YEPD agar containing plates were stricking by yeast cell and incubated for 48 hours at 25°C, 30°C, 37°C, 40°C and 45°C. Yeast was able to grow at 25°C-40°C. To confirm the results obtained from solid media, thermo tolerance was repeated in liquid media. Growth at liquid media is shown in the table-3.

Table3- Growth observation at different temperature in YEPD liquid media.

Temperature	Initial optical density (O.D)	After 48 hours (O.D)	Concordant value
25°c	0.244	1.353	1.109
30°c	0.110	1.538	1.428
37°c	0.311	1.251	0.940
42°c	0.370	0.526	0.156

From the table-2, it was evidenced that the yeast strain was slightly thermo tolerant as it was able to grow up to 42 °c.

4.1.2.3 Ethanol tolerance of the strain JY:

The strain JY was selected for screening of yeasts ethanol tolerant (table-4) and able to grow up to 20% ethanol containing liquid YEPD media. Maximum growth was seen in 8% ethanol containing media. Growth were recorded at 5%, 8%, 10%, 12%, 15%, 18% and 20% of ethanol containing liquid media and O.D is given gradually :

Table-4: Growth in different concentration of ethanol containing liquid YEPD media.

Ethanol percentage (%)	Initial optical density (O.D)	After 72 hours (O.D)	Concordant value
5	0.105	1.478	1.373
8	0.170	1.553	1.383
10	0.263	0.486	0.223
12	0.126	0.203	0.077
15	0.098	0.158	0.060
18	0.117	0.133	0.016
20	0.076	0.110	0.034

Fig-14: Growth observation in ethanol containing liquid YEPD media.

4.1.2.4 pH sensitivity of the strain JY:

The strain JY was able to grow at wide range of pH. At pH 2, growth was decreased by highly acidic condition but can grow up to pH 10. Maximum growth was seen at pH 6. After 48 hrs cell density was recorded at 600 nm and given gradually (table-5) for evidence of growth:

Table-5: Growth in different pH containing YEPD liquid media

pH	Initial optical density (O.D)	After 48 hours (O.D)	Concordant value
2	0.187	0.067	-0.12
3	0.305	1.132	0.827
5	0.179	1.412	1.233
6	0.110	1.538	1.428
7	0.147	1.339	1.192
9	0.242	1.390	1.148
10	0.433	1.484	1.051

4.1.2.5 Osmotolerence of the selected strain JY:

The strain JY could successfully tolerate up to 10% sodium chloride salt concentration but at higher concentration growth was reduced.

Table-6: Growth in different concentration of salt containing YEPD liquid media.

NaCl (%)	Initial optical density (O.D)	After 48 hours (O.D)	Concordant value
5	0.212	0.933	0.721
8	0.242	0.774	0.532
10	0.206	0.496	0.29
12	0.237	0.186	-0.051
15	0.259	0.192	-0.067
18	0.187	0.141	-0.046
20	0.164	0.143	-0.021

4.1.2.6 Cycloheximide resistance capability of strain JY:

The selected yeast strain was resistant to cycloheximide at 0.0015g/100ml concentration. It showed very good growth pattern. Growth in cycloheximide containing media (Figure-15).

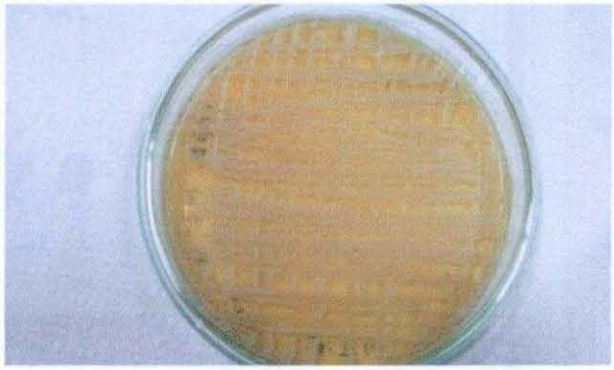

Fig-15: Growth on Cycloheximide containing solid YEPD agar media at 30°C.

4.1.2.7 Growth on Hydrogen peroxide containing solid media:

Hydrogen peroxide usually inhibits the growth of *S. cerevisiae* (Krems *et al.*, 1995). The yeast strain in this study was also inhibited by hydrogen peroxide (shown in figure-16).

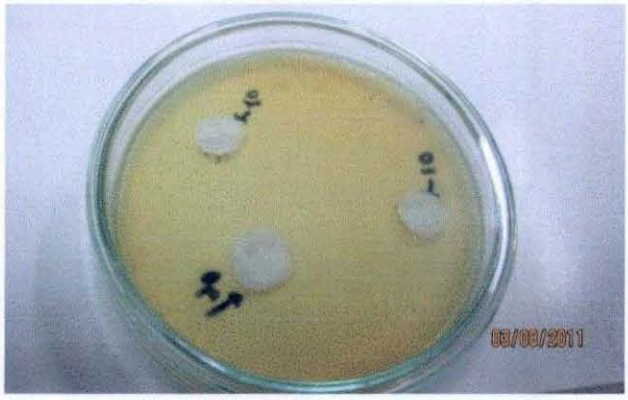

Fig-16: Growth on Hydrogen peroxide containing YEPD agar media at 30°C.

4.2 ETHANOL PRODUCTION

4.2.1 Ethanol production at room temperature using reducing sugar concentration 4.30%:

Under non shaking condition, a low ethanol was produced at 48 hrs without maintaining temperature. Using the initial reducing sugar concentration of the fermentation media 4.30% and pH 5.3, ethanol production was 3.65% at 48 hrs. During the summer temperature up and down between 25°C-35°C. Maximum production was 7.27% at 168 hrs. After 48 hrs of fermentation, the media pH changed from 5.3 to 5.2.

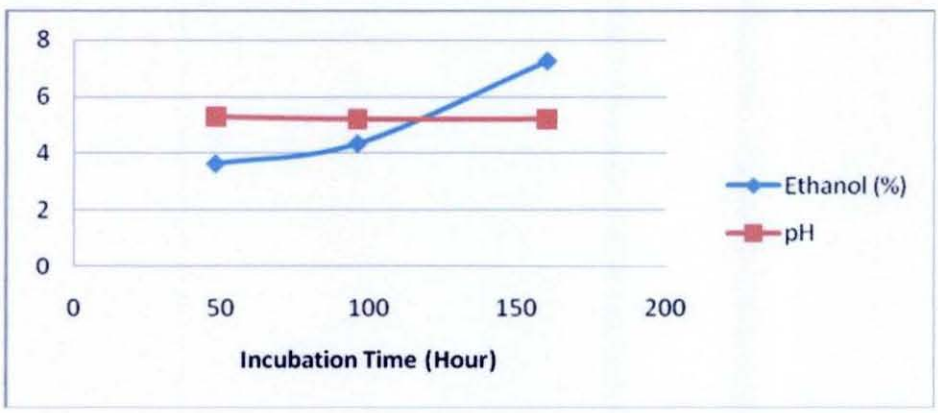

Fig-17: Production of ethanol and pH change at room temperature using reducing sugar concentration 4.30%.

Under non shaking condition, using the initial reducing sugar concentration of the fermentation media 4.30%, temperature 30°C and pH 5.3, ethanol production was increased by 3.65% to 5.85% at 48 hrs. At that condition maximum 7.65% ethanol was produced at 96 hrs. After 48 hrs of fermentation, the media pH changed from 5.3 to 5.2.

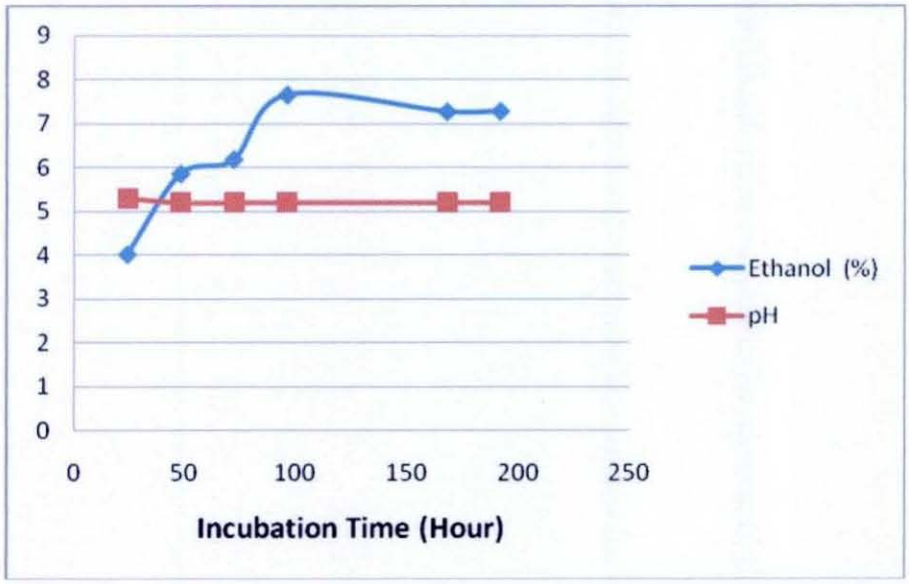

Fig-18: Production of ethanol and pH change at 30°C using reducing sugar concentration 4.30%.

4.2.3 Ethanol production at 30 °c and pH 6.0 using initial reducing sugar conc. 4.0%:

Under non shaking condition, at 30°C using Initial reducing sugar concentration of the fermentation media 4.0 % and pH 6.0, maximum ethanol production was 8.73% at 48 hrs. After 48 hrs of fermentation, the media pH changed from 6.0 to 5.5.

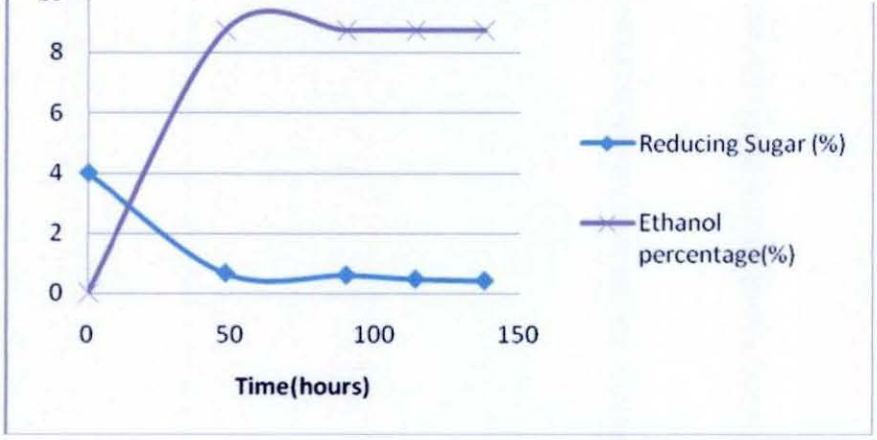

Fig-19: Production of ethanol using initial reducing sugar concentration 4.0% at 30°C under non shaking condition.

4.2.4 Ethanol production at 30 °C and pH 6.0 using initial reducing sugar conc. 5.30%:

Under non shaking condition, at 30°C using initial reducing sugar concentration of the fermentation media 5.30% and pH 6.0, maximum ethanol production was 10.18% at 48 hrs. After 48 hrs of fermentation, the media pH changed from 6.0 to 5.5.

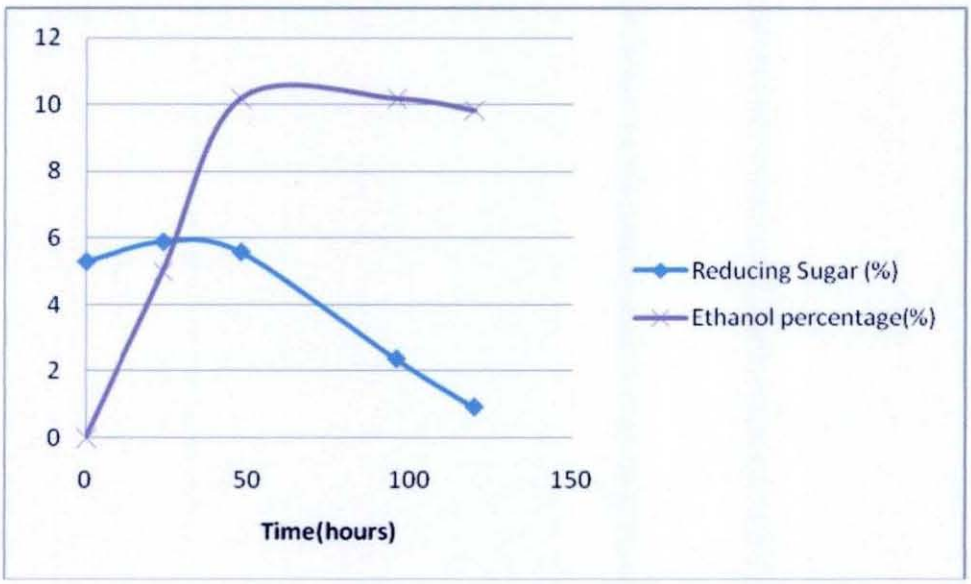

Fig-20: Production of ethanol using initial reducing sugar concentration 5.30% at 30°C under non shaking condition.

Under non shaking condition, at 30°C using initial reducing sugar concentration of the fermentation media 10% and pH 6.0, maximum ethanol productions was 10% at 48 hrs. After 48 hrs of fermentation, the media pH changed from 6.0 to 5.4.

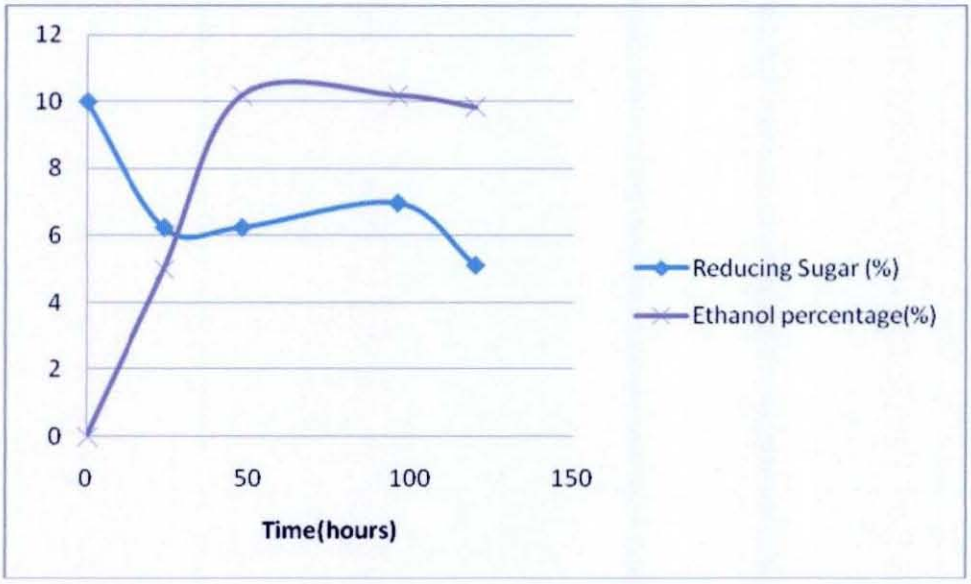

Fig-21: Production of ethanol using initial reducing sugar concentration 10% at 30°C under non shaking condition.

4.2.6 Ethanol production at 37 °C and pH 6.0 using initial reducing sugar conc. 6.50%:

Under non shaking condition, at 37°C using initial reducing sugar concentration of the fermentation media 6.50% and pH 6.0, ethanol production decreased. At that time a low ethanol was produced at 48 hrs. Only 4.36% ethanol produced during that time. After 48 hrs of fermentation, the media pH changed from 6.0 to 5.5.

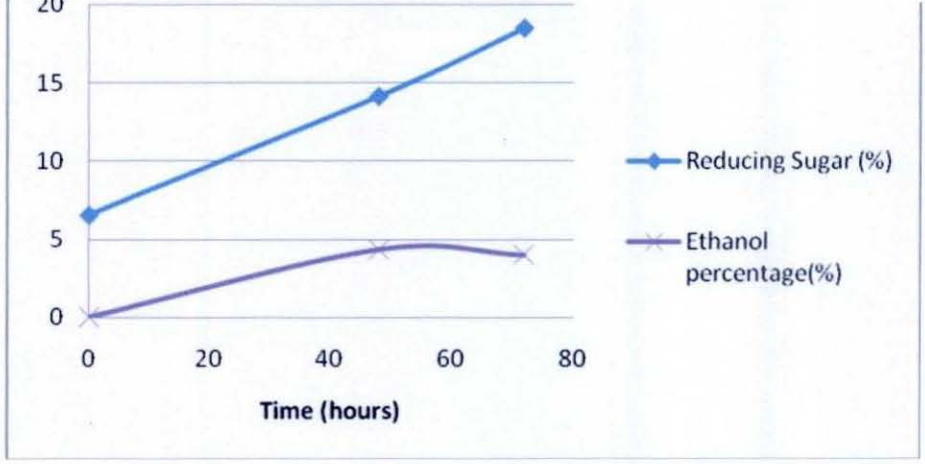

Fig-22: Production of ethanol using initial reducing sugar conc. 6.50% at 37°C under non shaking condition.

4.2.7 Ethanol production at 30 °C and pH 6.0 using initial reducing sugar Conc. 5.50% at 115 rpm:

Under shaking condition (115 rpm), at 30°C using initial reducing sugar concentration of the fermentation media 5.50% and pH 6.0, maximum ethanol production was 11% at 48 hrs. After 48 hrs of fermentation the media pH changed from 6.0 to 5.5.

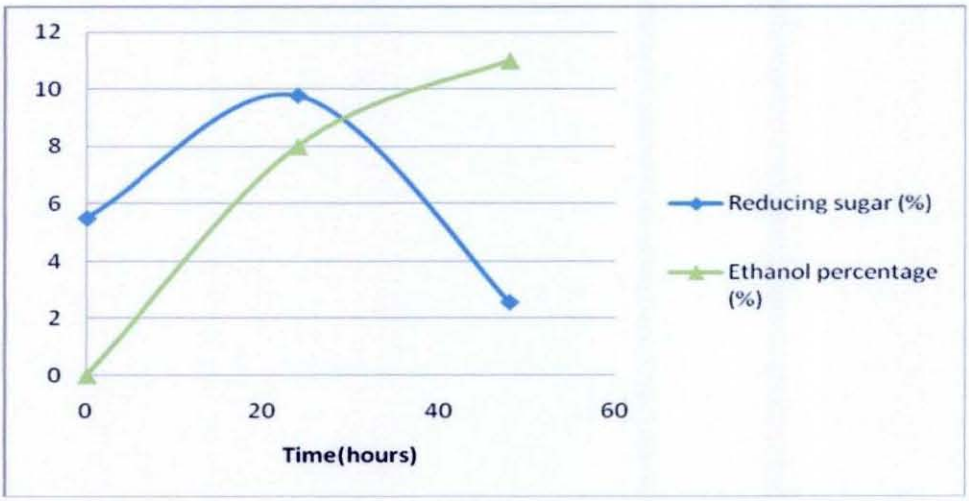

Fig-23: Production of ethanol using initial reducing sugar concentration 5.50% at 30°C under shaking condition (115rpm).

rpm:

Under shaking condition (115 rpm), at 30°C using initial reducing sugar concentration of the fermentation media 7% and pH 6.0, maximum ethanol production was 10% at 48 hrs. After 48 hrs of fermentation, the media pH changed from 6.0 to 5.5.

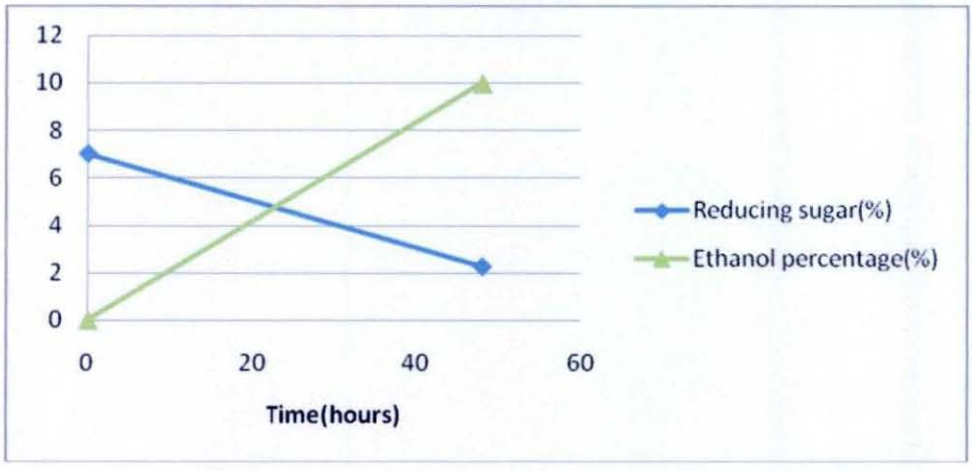

Fig-24: Production of ethanol using initial reducing sugar concentration 7% and pH 6 at 30°C under shaking condition (115rpm).

4.2.9 Ethanol production at 30 °C and pH 6.0 using initial reducing sugar Conc. 9.0% at 130 rpm:

Under shaking condition (130 rpm), at 30°C using initial reducing sugar concentration of the fermentation media 9.0% and pH 6.0, maximum ethanol production was 9.87% at 48 hrs. After 48 hrs of fermentation, the media pH changed from 6.0 to 5.5.

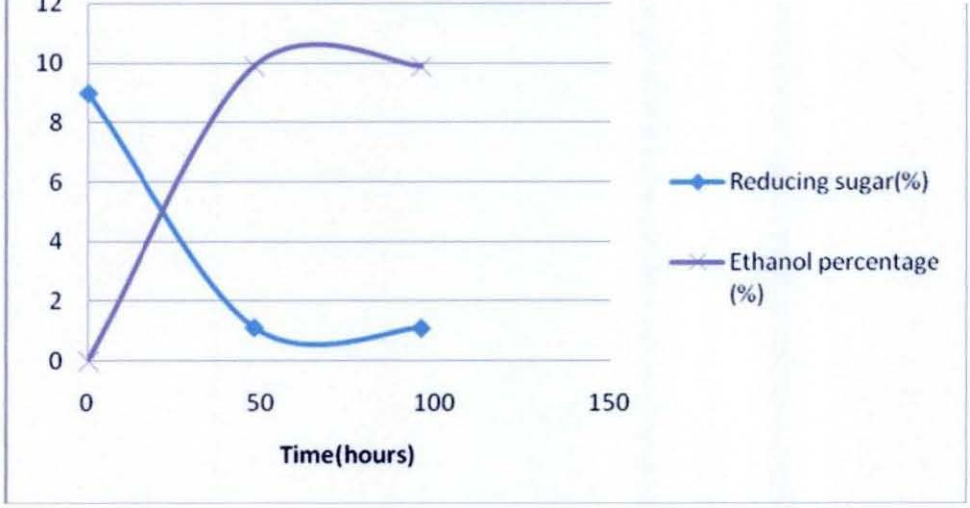

Fig-25: Production of ethanol using initial reducing sugar concentration 9.0% at 30°C under shaking condition (130 rpm).

4.2.10 Effect of immobilization on ethanol production:

Under non Shaking condition, at 30°C using initial reducing sugar concentration of the fermentation media 5.50% and pH 6.0, ethanol production was 7.27% at 48 hrs. After 48 hrs of fermentation, the media pH changed 6.0 to 5.5.Maximum ethanol produced at 72 hrs and during that time 8.73% ethanol was produced.

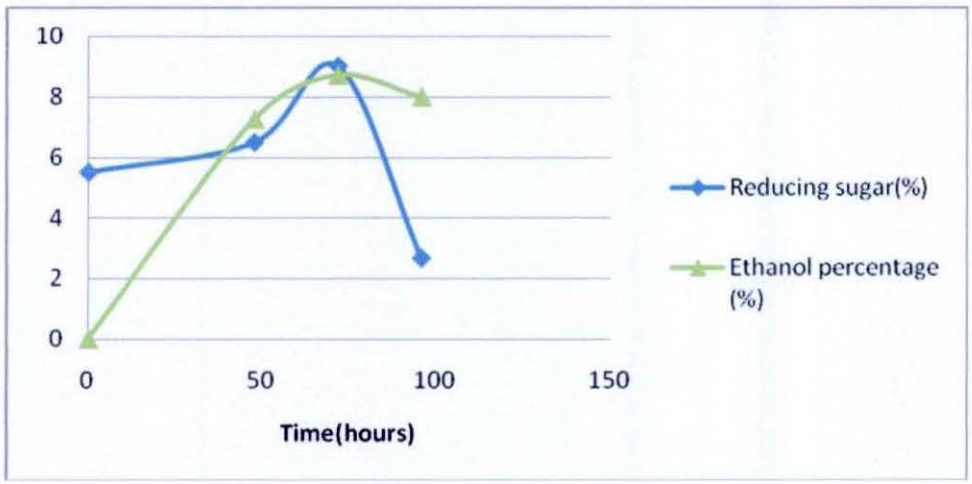

Fig-26: Production of ethanol using initial reducing sugar concentration 5.50% at 30°C under non shaking condition for immobilized cells.

Under shaking condition (115 rpm), at 30°C using initial reducing sugar concentration of the fermentation media 6.0% and pH 6.0, ethanol production was increased. Maximum 12% ethanol produced at 48 hrs. After 48 hrs of fermentation, the media pH changed from 6.0 to 5.5.

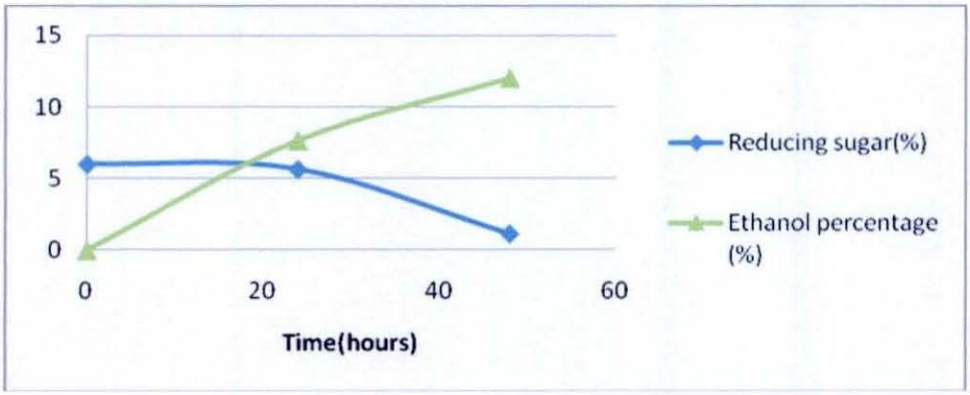

Fig-27: Production of ethanol using initial reducing sugar concentration 6.0% at 30°C under shaking condition (115rpm) for immobilized cells.

Under shaking condition (130 rpm), at 30°C using initial reducing sugar concentration of the fermentation media 9% and pH 6.0, ethanol production slightly increased. At that time 12.30% ethanol produced at 48 hrs and 13% ethanol was produced at 96 hrs by using immobilized yeast cells.

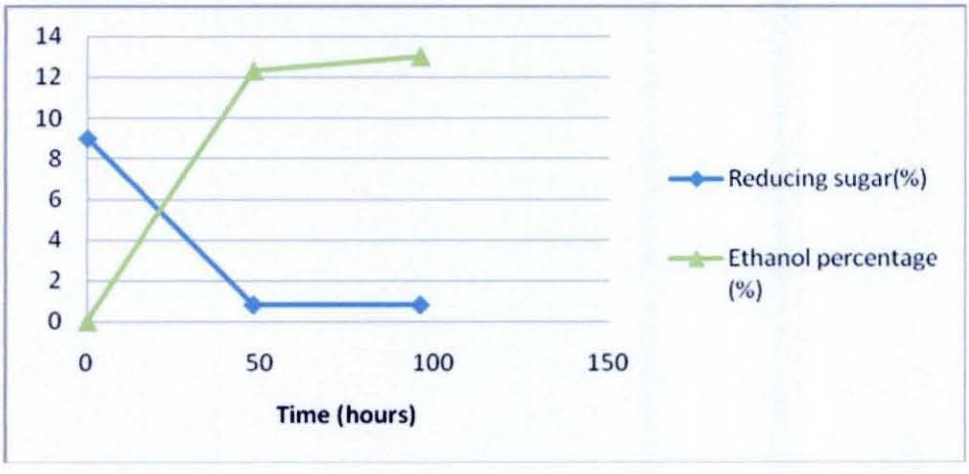

Fig-28: Production of ethanol using initial reducing sugar concentration 9.0% at 30°C under shaking condition (130rpm) for immobilized cells.

Table-7: Ethanol production by free yeast cells at different conditions.

Reducing Sugar concentration (%)	Temperature (°C)	Initial pH	Time(hrs)	pH falls after 48 hrs	Ethanol production (%)	RPM
4.30	Ambient	5.4	48	5.3	3.35	
4.30	30	5.4	48	5.2	5.85	
4.0	30	6.0	48	5.7	8.73	
5.30	30	6.0	48	5.5	10.18	
10	30	6.0	48	5.4	10.18	
6.50	37	6.0	48	5.5	4.36	
5.50	30	6.0	48	5.5	11	115
7	30	6.0	48	5.5	10	115
9.0	30	6.0	48	5.5	9.87	130

4.4 Comparison of ethanol production by immobilized yeast cells at different condition:

Table-8: Ethanol production by immobilized yeast cells at different condition.

Reducing Sugar concentration (%)	Temperature(°c)	Initial pH	Time(hrs)	pH falls after 48 hrs	Ethanol production (%)	RPM
5.50	30	6.0	48	5.5	7.27	
6.0	30	6.0	48	5.5	12	115
9.0	30	6.0	48	5.5	12.30	130

4.5 Effect of metals on ethanol production:

To determine the effect of metals on ethanol production cupper sulphate (II), potassium dichromate, and Boric acid and Magnesium chloride were added at different concentration in 250ml fermentation media and the fermentation was carried out in different 500 ml conical flasks in the presence of different metals. Magnesium chloride 1gm/250ml, copper sulphate (II) 0.10gm/250ml, potassium dichromate 0.10gm/250ml and Boric acid 0.10gm/250ml were added into the fermentation media. Ethanol production was observed at 30°c; pH6.0 and using the initial reducing sugar concentration 8.50% in shaking condition.

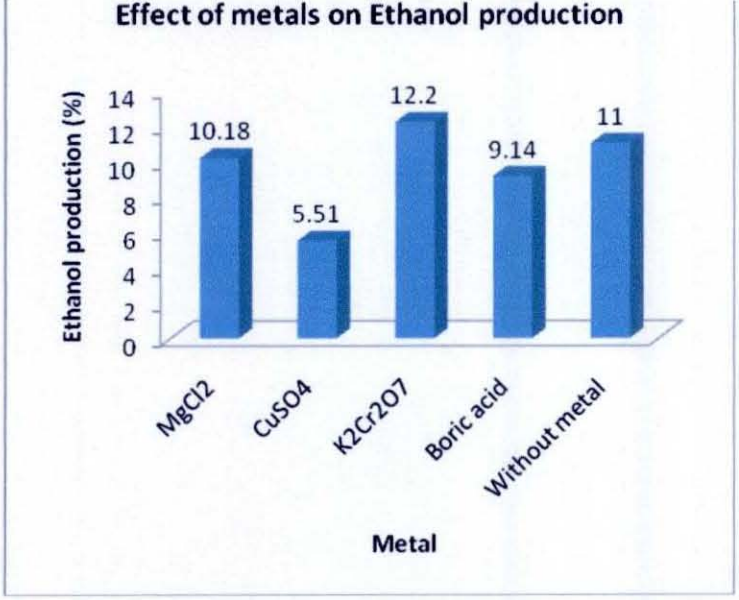

Fig-29: Production of ethanol at 30°c using the reducing sugar concentration 8.50% in presence of metals.

CHAPTER: 5
DISCUSSION

DISCUSSION

Based on the colony characteristics (white and creamy texture) ovoid microscope shape, the presence of ascospore and budding pattern (multipolar), the selected isolate was found to belong *Sacharomyces* type unicellular ascomycete according to (Lodder, 1971) and (Boekhout and Kurtzman ,1996) (Fig-10). The strain JY can also produce pseudomycelium.

The laboratory strain JY was tested for fermentation of carbohydrates and it was capable to ferment five sugars out of the eight sugars (Table-2). Glucose, sucrose maltose, Dextrose and Fructose were successfully fermented by the JY strain but it could not ferment lactose, xylose, and Rhamnose.

Sacchromyces yeasts are the most ethanol tolerant of the eukaryotic organisms and able to tolerate over 20% ethanol. In a previous study by (Casey and Ingledew, 1986), yeast strain *TGY2* could tolerate up to 16 %(v/v) ethanol. Almost Similar ethanol tolerance of 16.5 % (v/v) has been observed for *Saccharomyces cerevisiae* by (Teramoto *et al.*, 2005).

The selected strain JY was screened for ethanol tolerance and showed up to 20% ethanol tolerance in YEPD liquid growth media. A slow growth rate was observed at 10-20% ethanol containing media (Table-4).
The strain JY was resistant to antibiotic Cycloheximide. It was able grow Cycloheximide containing YEPD agar media but could not grow in the presence of Hydrogen peroxide (fig-15 and fig-16). The strain was also osmotolerant as it could tolerate and grew up to 10% NaCl containing media (Table-6).

At pH 3, no growth was observed and no ethanol was produced while pH 6 was the optimum for both biomass and ethanol production (Osman et al., 2011). The optimal pH range for growth of yeast can vary from pH 4.0 to 6.0, depending on temperature, the presence of oxygen and the strain of yeast. This likely is due to the optimum pH value for the activity of plasma membrane-bound proteins, including enzymes and transport proteins (Narendranath and Power, 2005).

pH 6.0 showed to be the optimum pH for it (Table-5). The strain grows comparatively well at low temperature and 30^{O}C is the optimum temperature.

A series of experiments had been conducted at different physio-chemical conditions to optimize ethanol production by the strain. To know the optimum temperature for ethanol fermentation, the solutions were kept at 25, 30 and 37°C with different initial sugar concentration. The molasses were diluted and fermentation was carried out in 500 ml flasks. Under non shaking condition, a low ethanol yield of 3.65% was observed at ambient temperature in 48 hrs and maximum ethanol was produced (7.27%) at 168 hrs using initial reducing sugar concentration 4.30% (Fig-17). So it took long time but the production was lower. But at 30°C using the same sugar concentration the yield of ethanol was increased from 3.65% to 5.85% at 48 hrs (Fig-18).The above two experiments were done at pH 5.3.

But at pH 6.0 and temperature 30°C the production of ethanol was increased, during that time 8.73% ethanol was produced at 48 hrs using 4.0% reducing sugar and maximum sugar was consumed by yeast after fermentation (Fig-19). By increasing reducing sugar concentration 4% to 5.30%, at pH 6.0 and 30°C ethanol production was 10.18% at 48 hrs (Fig-20). At 30°C using initial reducing sugar concentration 10% and pH 6 the same amount (10.18%) ethanol was produced at 48 hrs (Fig-21). If the temperature increased, the production of ethanol also decreased. At 37 °C only 4.36% ethanol was produced at 48 hrs using the reducing sugar concentration 6.50 % (Fig-22). At that condition, reducing sugar increased with time by the enzymatic activity of yeast due to conversion of sucrose to glucose and fructose at high temperature.

Shaking is a vital factor that influences ethanol fermentation. To determine the optimum shaking rate for ethanol production through incubating the fermentation media at different shaking rates (0, 50, 100, 150, 200, and 250 rpm) was investigated. The optimum shaking rate for ethanol production was at 200 rpm, while growth was increased by increasing the shaking rate (Osman et al., 2011).

and pH 6.0; the strain was able to produce 11% ethanol using the initial reducing sugar concentration 5.50% at 115 rpm (Fig-23). At pH 6.0 and 30°C using the reducing sugar concentration 7%, maximum ethanol production was 10% at 115 rpm (fig-24). Maintaining the temperature 30°C and pH 6.0, the strain was able to produce 9.87% ethanol at 48 hrs using the initial reducing sugar concentration 9% at 130 rpm (Fig-25). The results suggested that when the reducing sugar concentration was increased more than 5.50 - 6%, production of ethanol was decreased.

In a similar study in Bangladesh, five isolates produced alcohol by fermenting molasses at wide range of temperature (25-37°C). The production was maximal at 30°C after 48 hours of incubation. The isolates TY, BY, GY-1, RY and SY produced alcohol 12.0%, 5.90%, 5.80%, 6.70% and 5.80%, respectively at 30°C after 48 hrs of incubation. Using glucose as substrate in the fermented media varied between 2.3%-5.90%, the alcohol production rate were maximal up to 36 hours and stopped at 48 hrs (Khan *et al.,* 1989*).*

Immobilization of cells showed significantly affect of ethanol production, as ethanol production increased with immobilized cells. At 30°C and pH 6.0 using the reducing sugar concentration 5.50%, maximum ethanol production was 8.73% at 72 hrs under non shaking condition. But when reducing sugar concentration was increased to 6.0%, keeping pH and temperature unchanged, 12% ethanol was produced at 48 hrs at 115 rpm (Fig-27). By increasing reducing sugar concentration 6% to 9% and at pH 6.0 and 30°C, ethanol production was 12.30% at 48 hrs at 130 rpm (Fig-28).

Additions of very minute amount of metals also affect ethanol production. Of all the four metals, $CuSO_4$ dramatically decreased ethanol production in the fermentation media but $K_2Cr_2O_7$ increased ethanol production. Only 5.51% was produced by applying $CuSO_4$ while in the presence of $K_2Cr_2O_7$ 12.20% ethanol was produced at 48 hours using the reducing sugar concentration 8.50%, pH 6.0 and 30°c (Fig-29).

From the present results, it was concluded that a successful fermentation process depends on sugar concentration of the medium and nutritional parameters. The maximum amount of ethanol (11%) was obtained after 48 hrs of incubation at 115 rpm. Reducing Sugar (5.50%), initial pH (6.0), temperature (30°C) and volume of fermentation medium (250 ml in 500 ml Erlenmeyer flask) were optimized for free yeast cells.

CONCLUSION

CONCLUSION

The cell morphology of the yeast cells under microscope was ovoidal to elongate, single or in pairs. Budding cells were present and ascospores, pseudomycelia were developed. All the physiological and biochemical characters observed suggested that the strain is *Saccharomyces cerevisiae.*

The fermentation of molasses using *S. cerevisiae* (strain JY) under optimized conditions, such as reducing sugar concentration 5.50%-6%, temperature 30°C and pH 6.0 were suitable in fermentation efficiency for ethanol production by free cells and immobilized cells at 48 hrs in shaking condition. Immobilized cells were better in terms of ethanol production than free cells. Some metals such as Boron, Chromium etc. had stimulatory effect on ethanol production.

Pilot plant study of ethanol production by the strain with optimized conditions has to be done to make it industrially suitable strain. Productivity can also be improved by mutation through radiation or genetic manipulation. Metabolic pathway engineering to direct ethanol production may a promising way to improve productivity.

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APPENDIX

Appendix-A

Microbiological Media

Media used were prepared methods using appropriate compositions. Components used high grade. All media were sterilized by autoclaving for 20 minutes. The compositions used for different media have been shown below:

1) Composition of YMM media

Molasses	2 %
Agar	2%
Peptone	0.5%
Yeast extract	1%
Malt extract	1%

2) Composition YEPD agar media

Yeast extracts	3.0 g
Peptone	10.0 g
Dextrose	20.0 g
Distilled water	1.0 L
Agar	15.0g

3) Composition YEPD liquid media

Yeast extracts	3.0 g
Peptone	10.0 g
Dextrose	20.0 g
Distilled water	1.0 L

4) Composition: YPD liquid medium

Glucose	20 g
Peptone	20g
Yeast extracts	10g
Water	1 L

Reagents & Chemicals:

Reagents which were used in carrying out different methods together with their sources are mentioned below:

1) DNS (3, 5-Dinitro Salicylic acid) reagent:

Mix: 1. Distill water: 141.6 ml

2. NaOH (analytical grade):2.0gm

3. DNS: 1.06gm

Dissolved above then added: 4 K-Na-Tartarate: 30.6gm

5. Phenol: 760µl

6. Na-metabisulphite: 0.83gm

The solution was filtered and stored in room temperature in dark bottle.

2) 0.1 N Sodium thiosulfate solution:

Add 25 g Sodium thiosulfate and make the volume up to 1000 ml with Distilled water.

9.808 g dry Potassium Dichromate

↓

Pour in 500 ml 10 N sulfuric acid (No. 1-solution)

↓

62 ml no.1 solution dilute again in 500ml 10 N sulfuric acid

↓

0.05 N Potassium Dichromate(prepared)

4) 10 N sulfuric acid:

140 ml sulfuric acid and make the volume up to 500 ml with Distilled water.

5) 50% Potassium Iodide:

50 g KI and make the up to 100 ml with Distilled water.

APPENDIX-B

Instruments name	Model no.	Country
Autoclave	ALP	Japan
Autoclave	Model;Mc-40w,ALP Co. Ltd	Japan
Big weighting	XE-3100D, Denver Instrument CO.	U.S.A
Centrifuge machine	Mikro 120	Germany
Electronic microscope	Olympus BX 41	Japan
Eyela natural oven	NDO-600ND,Tokyo Rikakikai C.l	Japan
Electric balance	Denver Instrument Company,AA-160	
Freeze	Singer	
Incubator(28°C)	IRE-160	
Incubator	type;BE200,Memmert Gmbh+Co	KG
Oven	Memmert954,Schwabach	Germany
Hot water bath	Memmert	Germany
Haemocytometer	Neubauer Precidor, HBG	Germany
Incubator(37°C)	Memmert	England
Laminar air flow	Holten	Japan
Micro oven	Rangs, RMC-1BOK	
Magnetic Heating Stirrer	1100 hotplate &stirrer, Jenway	UK
Orbital shaker	GFL 3031	Germany
pH meter	Digital pH meter	Bangladesh
Refrigerator	General, ER-141F	Japan
Small weighting	AA-160, Dever Instrument CO	U.S.A
Spectro photometre	T60 UVVIS	
Vortex-1	Whirlimixer, Fisons	England
Water bath		

APPENDIX-C

SERIAL NO.	NAME OF THE GLASSS WARE AND OTHERS
1	Beaker
2	Conical flasks
3	Disposable micropipette
4	25 ml Erlenmeyer flask
5	Inoculating loop
6	Knife and forceps
7	Micro pipette 100µl
8	Micro pipette 1000µl
9	Micro pipette 5000µl
10	Measuring cylinder
11	Mackatny bottle
12	Pipette 10ml
13	Petri dishes , disposable
14	Screw cap bottle
15	Spreader
16	Slide
17	Screw cap bottle
18	Screw cap test tube
19	Cotton plug
20	Spatula
21	Scotted bottle