Isolation of wild-type yeasts from natural sources for bioethanol production, their characterization and the effect of mineral salts on ethanol productivity in molasses

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Dedicated to All the People that Matter

Declaration

I hereby solemnly declare that the research work exemplifying the results reported in this thesis paper entitled "***Isolation of wild-type yeasts from natural sources for bioethanol production, their characterization and the effect of mineral salts on ethanol productivity in molasses***", submitted by the signatory Wasif Kamal has been performed under the joint supervision of Prof. Dr. Naiyyum Chowdhury, Former Coordinator, Biotechnology and Microbiology Program and Dr. M. Mahboob Hossain, Associate Professor and Coordinator of Microbiology Program, Department of Mathematics and Natural Sciences. Furthermore, I also declare that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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November, 2015 ***(Wasif Kamal)***

Abstract

Ethanol, with the molecular formula of CH_3CH_2OH, is a highly useful hydrocarbon which is used extensively in major industrial and manufacturing processes. It is also used as a fuel, albeit on a much smaller scale. Ethanol is mainly sourced from the fermentation of carbon-based feedstock by microorganisms such as yeasts, which can be found almost everywhere in the environment. In this study wild-type yeast strains were derived from potential sugar-rich food sources commonly found in kitchen markets around Dhaka city, namely sugarcane juice, grapes, dates, honey and molasses. Once the desired microbes were isolated, they were identified as members of the ethanol producing *Saccharomyces spp*. after stress tolerance studies (thermo- and ethanol- tolerance) and biochemical characterization using Analytical Profile Index (API) ® 20C AUX and nitrate broth test. This study was conducted to analyze and compare ethanol yields obtained from untreated blackstrap molasses and molasses treated with mineral salts. To this end, the isolates were initially used to ferment molasses media at standard temperature of 30°C and pH 5.5 in order to establish their base ethanol productivity using Conway unit titration. Using the titrimetric results obtained as reference, the experiment was repeated with the molasses media treated with the mineral salts KH_2PO_4, $MgSO_4$, $MnSO_4$ and $FeSO_4$ respectively to study their effect on ethanol yield outputs. The results indicated an overall increase in yields upon the addition of the salts; maximum ethanol percentages for isolate S.C-1.0 obtained after a 48 hour incubation in KH_2PO_4 treated molasses was 8.78% in contrast to untreated media which yielded 7.31% after the same time period. Overall, all other isolates (S.C-1.1, D-H, HON, GRP-4 and MOL) displayed yield improvements with other salts as well.

Table of Contents

Title	Page No.
Abstract	I
List of Abbreviations	III
Chapter I: Introduction	2
Chapter II: Literature Review	7
Chapter III: Materials and Methods	18
Chapter IV: Results	29
Chapter V: Discussion	44
Chapter VI: References	48
Appendix	52

List of abbreviations

mm:	Millimeter
µg:	Microgram
µl:	Microliter
ml:	Milliliter
mg:	Milligram
gm:	Gram
Kg:	Kilogram
Kcal:	Kilocalories
KJ:	Kilojoules
e.g.:	For example
et al.:	And others
pH:	Negative logarithm of hydrogen ion concentration
spp.:	Species
%:	Percentage
°C:	Degree Celsius
API:	Analytical Profile Index ®
O.D:	Optical Density
D.F:	Dilution Factor

Chapter 1

Introduction

Introduction

1.1 Background

The failure of conventional fossil fuels in maintaining mankind's ever-increasing demand for energy in a sustainable and efficient manner is becoming more apparent with the passage of time. The issue of fast diminishing supplies of such fossil fuels as well as the negative impact they have on the quality of the environment has raised calls for alternative energy sources. Biotechnology has emerged to fill that role by providing a variety of innovative solutions to the problem. Some of the common criteria that are taken under consideration for development of such an alternative include the relative abundance of their source, their contribution in minimizing the negative impact on the environment and organic life, cost-effectiveness and renewability (Hánh-Hägerdal *et al.*, 2007). Most importantly, it must also be able to efficiently cater to the energy needs of a rapid growing worldwide population.

Bioethanol, ethanol that is derived from biological sources by the fermentative action of certain microorganisms, is one of many such alternatives and certainly the most promising of all. The use of ethanol in industry and manufacturing process however was not uncommon before the advent of fossil fuels, as it has been used in the production of many consumer products (Ragauskas *et. al.*, 2006; vanWyk, 2001). However, the inefficiency and slow production rates of those manufacturing processes in comparison to those using fossil fuels proved to be their undoing and they were eventually phased out. Recently however, the detrimental effects of fossil fuels on organic life and environment as well as impact of their limited supply on the world economy have become more apparent (Izmirlioglu and Demirci, 2012). Because of this and the technological improvements related to efficient usage of ethanol fuel, ethanol derived from biological sources has seen a resurgence in interest.

Ethanol from kitchen-waste is an attractive and sustainable energy source for transportation fuel to substitute gasoline; however it still requires a lot of development. Production techniques of bioethanol are categorized into two generation. The first generation methods, which is the current method of production, uses food crops such as sugar cane and corn and their by-products like molasses. On

the other hand second generation ethanol production, which is currently implemented on an experimental scale and is under development, utilizes cheaper and non-food feedstock like lignocelluloses or municipal solid waste. While the first generation methods yielded feasible result, production of ethanol is fairly low and its usage of potential food-crops has made it the subject of criticism (Babcock, 2011). Second generation methods aim to solve this by reducing the dependence of food crops and enable usage of cheaper and non-food feedstock like lignocelluloses or municipal solid waste/kitchen wastes, thereby making ethanol more competitive to fossil fuels (Matsakas and Christakopoulos, 2015). Furthermore, the processed substrates of bioethanol production from such biomasses can be used as organic fertilizer, thereby making it a truly sustainable energy resource.

1.1.1 Ethanol: "the fuel of the future"

Ethanol or ethyl alcohol is 2-carbon alcohol with the molecular formula CH_3CH_2OH. It is the principal type of alcohol found in alcoholic beverages, produced mainly by the fermentation of biomass – mainly sugar crops, e.g. cane and beet, and of grains by yeasts. It can also be produced synthetically from petrochemical derivatives by the acid-catalyzed hydration of ethane, but the process accounts for only 5% of global ethanol production (Licht, 2006). Besides being a key constituent of hard beverages ethanol is a very versatile compound, as seen in **Table 1.1**.

Compared to uses in other industries, ethanol finds the greatest use as a fuel, particularly as engine fuel and as an additive. Credited as the "fuel of the future" from as early as the late 1800s (Solomon et al., 2007) and also kick starting the Space Age with its implementation in World War II era V-2 rockets (Braeunig, 2008), ethanol has had a storied history. The advent of more efficient and cost-effective fossil fuels such as petroleum and its derivatives eventually led to it being shelved as a primary fuel. Concerns regarding the impact of fossil fuels on the environment and their overall diminishing supply, however, caused a resurgence in popularity of ethanol as fuel in the past decade. This is characterized by a huge increase in world ethanol production which tripled from 17 billion in 2000 to more than 52 billion liters in 2007 and a 1.7% increase from 3.7% in global usage as fuel in 2008 alone (UNEP, 2009). The United States and Brazil are the two major consumers of bioethanol; they are also the largest producers contributing to 85% of the total supply worldwide

(approximately 80 billion liters) in 2014 (RFA, 2015). Bioethanol is mainly used as a fuel mixture in most countries, with the exception of Brazil where flexible fuel vehicles compatible with pure ethanol have been introduced recently (ANFAVEA, 2012). Popular 'blends' (gasoline-ethanol mix) include E15, E85, and ED40 to name a few; E15 (15% ethanol gasoline) is especially notable since 70% of all automakers have approved the use of it in their latest vehicles (RFA, 2015).

Table 1.1: Uses of Ethanol

Industry/Sector	Application	References
Chemical Industry	Ethanol is a versatile product necessary for the establishment of a powerful chemical industry. It can be used to produce a long list of industrial chemical products and by-products. It is also used as a high performing solvent for agro industrial preparations.	Chandel *et. al.*, 2007
Medical	There is a market for ethanol in developing countries in the medical sector, which is normally imported from overseas. The ethanol finds use in hospitals, clinical operational needs and for equipment maintenance purposes. For example, bioethanol had undergone bioconversion by yeasts to produce probiotics and bio-therapeutic agents.	Demirbas, 2007
Alcoholic Beverages	Ethanol is used for the production of various types of liquors such as wine and beer.	Demirbas, 2007
Transportation	Substitute fuel in transportation sectors and use as a petrol additive. Besides using 100% ethanol as a gasoline substitute, the mixing of ethanol and gasoline can also be done. It has several advantages such that it can increase octane number, reduced toxic emissions and improving efficiency of spark ignition engines	Palmarola *et. al.*, 2005; Bon and Ferrara, 2007; Alam *et. al.*, 2007

Production of ethanol via fermentation can be carried out by both yeasts and bacteria, however yeast is more widely preferred because of high ethanol tolerance and production (Bansal and Singh, 2003). Modern processes implement a combination of the two organism classes because bacteria also allow the degradation of lignocellulosic biomass. Even so, these processes are still not considered to operate at 100% efficiency as it is difficult to maintain exact conditions of temperature, pH and nutrients at all steps of the process.

1.2 Objectives

The goal of the project is to establish a highly efficient microbial fermentation process by natural yeast isolates to produce ethanol as an energy source. To summarize, the following work has been performed:

- Isolation of ethanol producing wild type yeast strain from natural sources.
- Identification and characterization of isolates.
- Study of thermo-tolerance and ethanol-tolerance of the yeast strain.
- Determining base ethanol production yields of isolates grown in defined sugar media such as glucose and molasses
- Analysis of the impact of various inorganic salts on the yield of ethanol obtained from fermentation of molasses

Chapter 2

Literature Review

Literature Review

2.1 Overview

Fermentation is a metabolic process that breaks down large carbohydrates such as glucose and sucrose to smaller molecules in order to obtain energy to fuel cellular processes (Klein *et. al.*, 2006). An anaerobic process (organic reaction without oxygen), byproducts of this reaction include acids, gases or alcohols (Ibeto *et. al.*, 2011). Of the many different byproducts emanating from fermentation processes, ethanol is considered to be the most significant. The first studies on ethanol fermentation were conducted by Louis Pasteur in 1857 (Moreira, 1983).

The use of ethanol as a fuel has existed for many years before, albeit on a much smaller scale. Recently however, with the foreseeable depletion of fossil fuel sources and rising concerns regarding the quality of the environment, ethanol has re-emerged as the prime candidate to fill the need for a low-cost and clean alternative fuel. Not only are the raw-materials cheap and renewable, studies have also shown that fuel ethanol reduces greenhouse gas emissions by 86 to 90% (Isaias *et. al.*, 2004; Goettemoeller & Goettemoeller, 2007). Such enthusiasm has led to significant strides being made in this area of research. In spite of this, ethanol production from fermentation still remains below feasible levels. Emphasis is thus placed on research aimed at maximizing fermentation efficiency via optimized utilization of the resources and microbial action on the process (Lin-Tanaka *et. al.*, 2006). Many microorganisms can facilitate ethanol fermentation to some degree, but the most widely used and preferred are yeasts.

2.2 Microorganism: Yeast

The majority of the current ethanol fermentation processes are attributed to the action of a class of eukaryotic microorganisms known as yeasts. Belonging to the kingdom Fungi, yeast represent approximately 1% of all described species within its kingdom with approximately 1500 species currently described (Kurtzmann *et. al.*, 2006). Despite their small numbers, members display a high level of phylogenetic diversity, belonging to both Ascomycota and Basidiomycota phyla. Depending on species, yeasts reproduce asexually or sexually (Wayman-Parekh, 1990); asexual reproduction

by budding is the characteristic method of reproduction. As such, species which reproduce by the following method are known as 'true yeasts' and are classified in the order *Saccharomycetales* (SGD, 2005). The majority of the ethanol producing yeast species belongs to this order. Yeasts are chemo-organotrophs; they use organic compounds as energy source and the fermentation takes place anaerobically. The principle carbon sources are hexose sugars such as glucose, sucrose and maltose. Yeasts are found primarily in sugar rich environments such as fruits or flower nectar where they digest necessary nutrients externally and assimilate it afterwards. This ability to ferment sugars made them important for bioethanol production.

Yeasts are classified on the basis of the microscopic appearance of the cells, the mode of sexual reproduction, physiological (especially metabolic capabilities and nutritional requirements) and biochemical features. Physiological features that are generally analyzed include the range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can assimilate 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 osmo-tolerance) 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 Nikido, 1995). Besides microscopic observation of cells, colony growth characteristics on solid media are also used to distinguish between different yeast morphologies. While normally colorless, yeast colonies may appear white, cream colored, or tinged with brownish pigments on artificial media.

Notable examples of yeasts used in industrial scale fermentation processes (Matsushika *et al.*, 2008) include *Saccharomyces uvarum* (Detroy *et. al.*, 2004), *Schizosaccharomyces pombe* (Jong-Gubbels *et. al.*, 1996), *Kluyueromyces* spp. (Morikawa *et. al.*, 2004), *Candida Shehatae* and *Pichiastipitis*. However, the most widely used yeast species is *Saccharomyces cerevisiae* (Laplace *et. al.*, 1993), which is the subject of this research. *Saccharomyces cerevisiae* is considered to be the reference species in ethanol fermentation studies due to its high tolerance to ethanol (approximately ~18% concentration) (Morais *et. al.*, 1996). The low yield of by-product such as acetic acid is also an attractive feature. Yeasts have been used to generate electricity and ethanol for biofuel industries.

2.3 Sugar bioconversion to ethanol:

Ethanol fermentation is mainly attributed to anaerobic respiration in yeasts. This is facilitated by enzymes which convert sugars into bioethanol from a range of organic raw materials derived from biomass. Major biomass sources include wood from natural sources, industries or process mills, municipal solid wastes as well as agricultural residues and "energy crops"; plants that can be grown and maintained at a low cost with ease (Monique *et. al.*, 2003). The raw materials are themselves categorized into three groups (Jackman, 1987):

- **Simple sugars** (mono and di-saccharides) such as **glucose**, **fructose** and **sucrose**. These are mainly present in sugar beets, cane and molasses. Monosaccharide sugars like glucose are rarely found in their free state naturally; they are generally linked by a glycosidic bond to another monosaccharide to form disaccharide sugars like sucrose (a combination of glucose and fructose), also known as table sugar. This bond is broken by a class of enzymes called glycosidic amylases. Glucose is the main fuel for the metabolic process known as glycolysis, which eventually yields ethanol under anaerobic conditions.

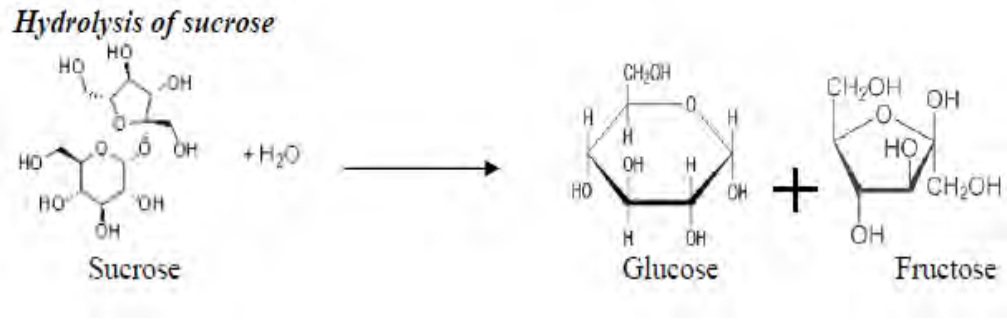


Figure 2.1: Hydrolysis of Sucrose to Glucose and Fructose

Substrates containing high concentrations of simple sugars can be fermented directly into ethanol with minimal processing. Thus, they remain the chief substrate for bioethanol production.

- **Starch**, a glucose-based polysaccharide which is produced by most green plants as an energy source. Derived from corn, potato and root crops, it is itself a combination of two different polysaccharides: the linear and helical polymer amylose which is composed of individual α-D-glucose units bound by (1-4)

glycosidic linkage, and the branched amylopectin which contains similar (1-4) linkages and additional α (1-6) branch points. Depending on the plant material, starch generally contains 20 to 25% amylose and 75 to 80% amylopectin by weight. The various glycosidic bonds linking the fermentable D-glucose together is hydrolyzed by several different glycosidic amylases like diastase and maltase for fermentation to proceed efficiently. These enzymes are either introduced in their purified form, or are secreted by microorganisms (Haissig *et. al.*, 2006).

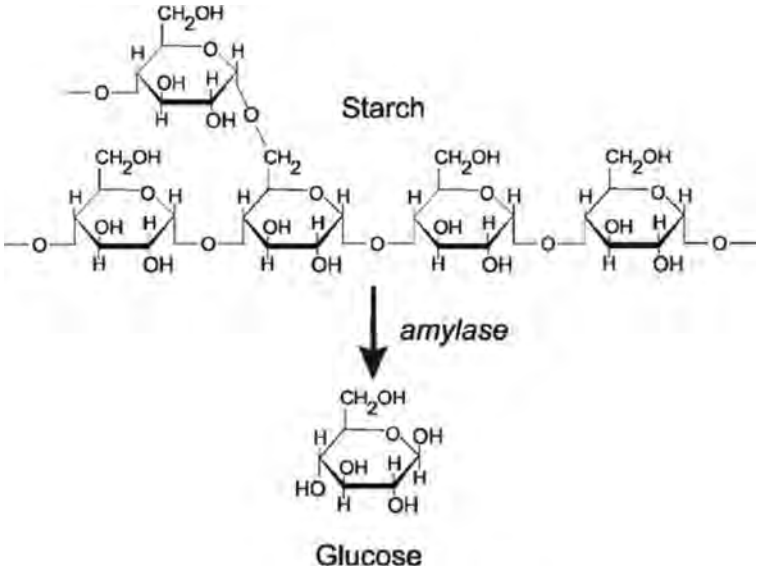


Figure 2.2: Breakdown of starch by amylases

- **Cellulose**, a complex polysaccharide composed of several hundreds to thousands of β (1→4) linked D-glucose molecules (Crawford, 1981). Major sources include wood, agricultural residues, vegetable peels and algae. Like starch, cellulose must be broken down for fermentation to occur efficiently, however the process is complex and requires extensive enzymatic action by **cellulase**. Cellulase is an enzyme used for the bioconversion of cellulosic and lignocellulosic residues. Cellulolytic activity is a multi-complex enzyme system and complete enzymatic hydrolysis of starch requires synergistic and sequential action of 3 enzymes: endo-•-glucanase (EC 3.2.1.4), exo-•-glucanase (EC 3.2.1.91) and •–glucosidase

(EC3.2.1.21) (Shankar and Isaiarasu, 2011). The overall catabolic pathway can be illustrated below:

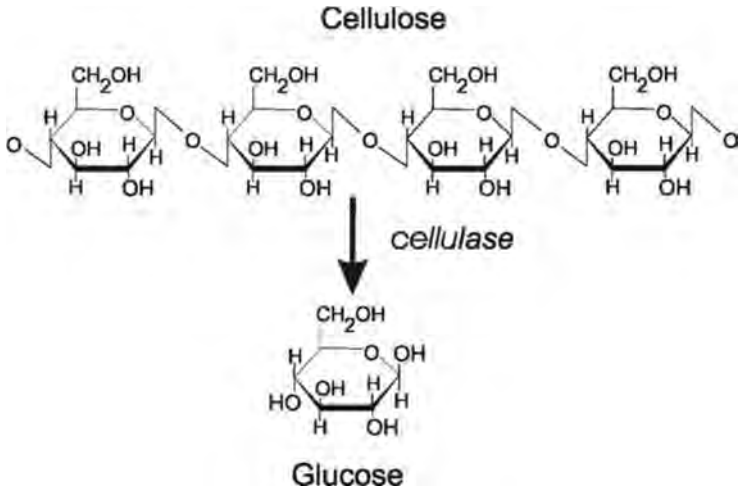


Figure 2.3: Cellulose Hydrolysis to Glucose

Owing to the complexity of the hydrolysis reactions and the processing costs involved, simple sugar containing substrates are chosen over starch and cellulose based raw materials in industrial scale production of ethanol.

2.4 Raw Materials: Molasses

Ethanol fermentation processes employ a wide variety of substrates, of which molasses is the most widely used. A by-product of the sugar refining industry, molasses is a viscous tar-like sugar syrup from which most of the sucrose has been crystallized out (Yansong *et. al.*, 2000). Molasses is classified into two categories based on crop source: cane molasses (sourced from sugarcane) and beet molasses (from sugar beet). Cane molasses is further classified as either refinery molasses, final molasses or blackstrap molasses in accordance to the purity of the sugarcane juice used in sugar production. Blackstrap molasses is available commercially, whereas refinery and final molasses are recycled for other purposes (Rao, 1997). Due to its commercial availability, blackstrap molasses is the main ethanol fermentation substrate. Besides this obvious implementation, blackstrap molasses can also be used to produce cattle feed, bakers' yeast and in the manufacture of the alcoholic beverage rum. Other products such as L-lysine, acetone-butanol, citric acid, lactic acid, glutamic acid are also possible, but the amount of end-product obtained is very low (Rao, 1997).

The composition of molasses from sugar cane varies with the sugar content of the cane, its variety and method of processing. It is slightly acidic, having a pH of 5.5 – 6.5. Sugars constitute the majority of its mass (60-75%), of which 14 – 25% are reducing sugars (glucose and fructose). The rest is sucrose. Unlike refined sugars, molasses also contains significant amounts of different vitamins and minerals such calcium, magnesium, iron, and manganese; one tablespoon provides up to 20% of the recommended daily value of each of those nutrients. It is also a good source of potassium. The chemical composition of cane blackstrap molasses is shown in **Table 2.1**.

Table 2.1: Nutritional Chart of Blackstrap Molasses

Nutrient	Unit	1 Value per 100 g
Proximates		
Water	g	21.87
Energy	kcal	290
Energy	kJ	1213
Protein	g	0.00
Total lipid (fat)	g	0.10
Ash	g	3.30
Carbohydrate, by difference	g	74.73
Fiber, total dietary	g	0.0
Sugars, total 1	g	74.72
Sucrose 1	g	29.40
Glucose (dextrose) 1	g	11.92
Fructose 1	g	12.79
Minerals		
Calcium, Ca	mg	205
Iron, Fe	mg	4.72
Magnesium, Mg	mg	242
Phosphorus, P	mg	31
Potassium, K	mg	1464
Sodium, Na	mg	37
Zinc, Zn	mg	0.29
Copper, Cu	mg	0.487
Manganese, Mn	mg	1.530
Selenium, Se	µg	17.8

2.5 Fermentation by yeast

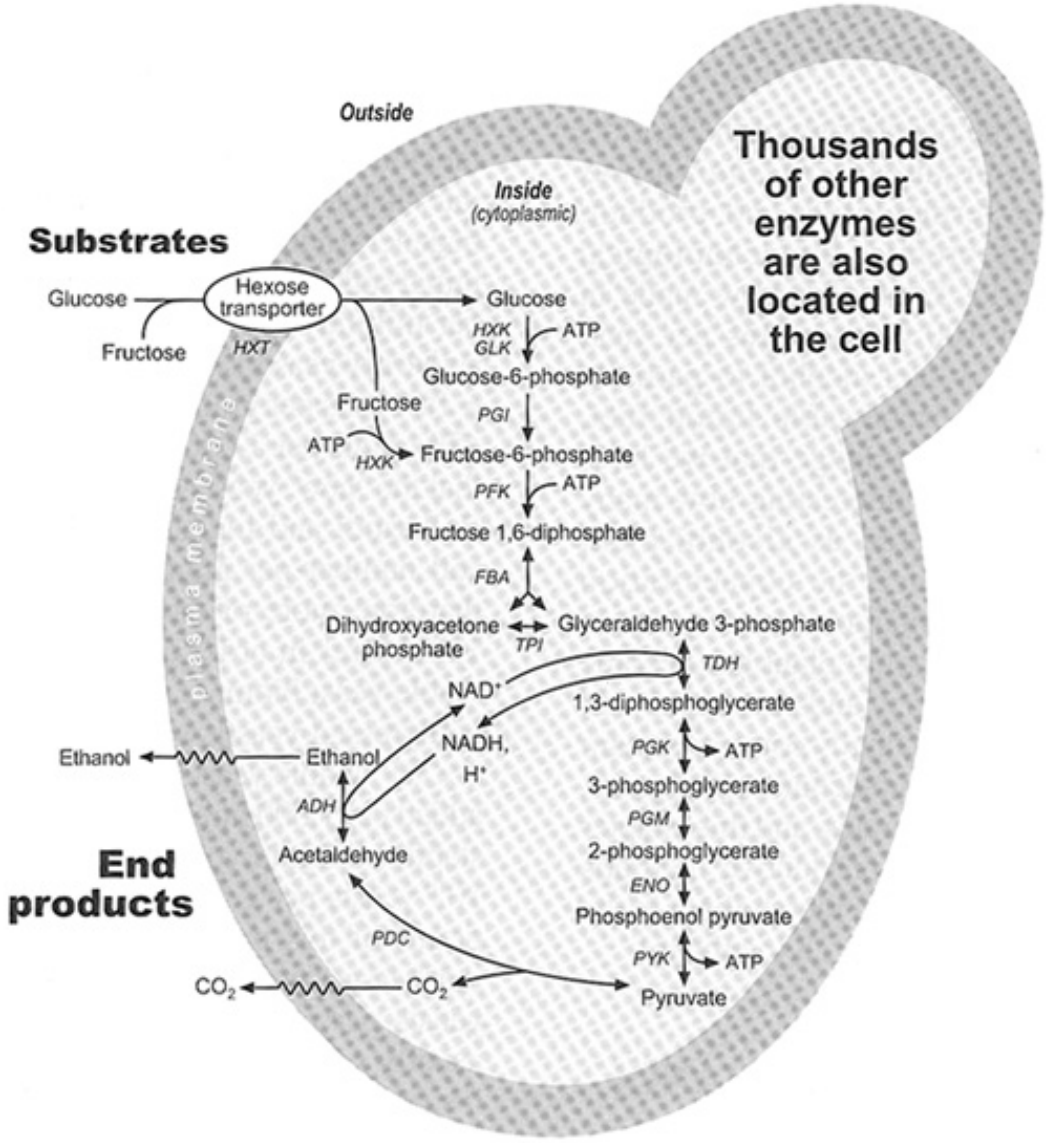


Figure 2.4: Glycolytic Pathway of yeast-cell

The fermentation process which yields ethanol from yeast (*Saccharomyces cerevisiae*) occurs due to the metabolism of simple sugars like glucose via the glycolytic pathway to produce energy. The reactions that take place within this pathway and the enzymes involved have been illustrated in **Figure 2.4**.

Because of the lack of oxygen, the end product of the glycolytic pathway (pyruvate) does not proceed to the Citric Acid cycle which completely breaks it down to CO_2 and

H_2O. Instead the pyruvate is broken down to CO_2 and ethanol via the reaction scheme illustrated in **Figure 2.5**. A smaller output of energy (in the form of ATP) is produced as a result.

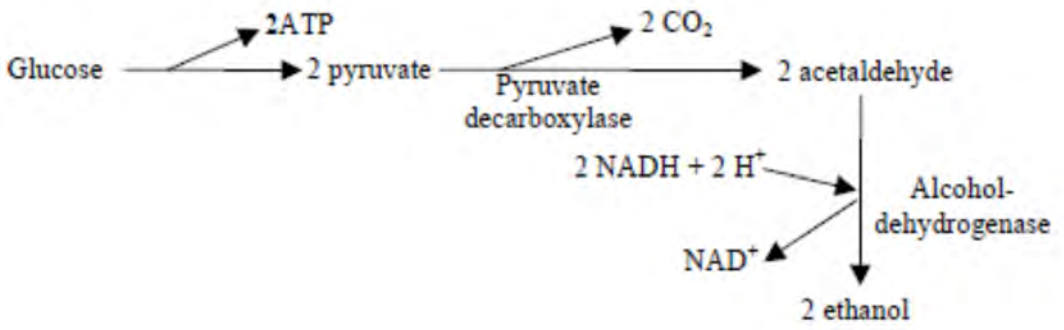


Figure 2.5: Conversion of glucose to ethanol in yeast cell in fermentation

2.6 Factors affecting fermentation:

Yeasts, especially *Saccharomyces cerevisiae*, are extensively used in batch or continuous fermentation of sugar substrates to ethanol to produce beverages and biofuels. However, due to limited understanding of physiological constraints that hamper the rate of glycolysis and ethanol production, product yield is generally low (Casey and Ingledew, 1986; Ingram and Buttke, 1985). While this is not an issue in the beverage industry, the biofuel industry requires any process to have maximum ethanol output in the most efficient and low cost manner to be feasible. Through identification of these limitations the overall process could be optimized via the development of improved organisms and process conditions, thereby achieving the goal (Dombek and Ingram, 1987).

Cellular tolerance studies of yeasts across a range of conditions have enabled us to pinpoint some of the factors which affect production (Olsson and Hägerdal, 1996). These studies have been used to assay the survival of the microorganism across a range of conditions that develop during large scale batch or continuous processes. Various parameters have been identified, some having a greater impact on production than others; these include fermentation temperature, media pH, ethanol tolerance and the presence of salts.

2.6.1 Temperature:

Temperature of the fermentation environment is an important factor as it directly affects the viability of the yeast cells. Although yeasts operate best at ambient temperatures of 25-30°C, maximum ethanol is obtained at higher temperatures around 38°C (Jones *et. al.*, 1981). A compromise has to be made between the two temperature ranges as heat emission from fermentation raise media temperature to above 40°C. This reduces yeast cell viability and survivability (Morimura *et. al.*, 1997). Furthermore, optimum temperature for growth and rate of ethanol formation were found to depend on medium composition and strain (Laluce *et. al.*, 1993).

2.6.2 Ethanol Concentration:

A major limiting factor in most bioprocess reactions is the accumulation of the end product in the producing organism cell which limits growth and viability. Ethanol is no different since it damages cell membrane and cause other physiological changes once a critical intracellular concentration is reached. Intracellular accumulation of ethanol is attributed resistance in excreting the product to its surroundings (Navarro, 1980). The degree of inhibition is also related to environmental factors, an example of which is the impact of high temperature. Because the overall ethanol production in the cell is increased by high temperatures in contrast to the amount of product excreted from cell, intracellular ethanol concentration builds up to critical levels. As a result, growth is inhibited (Navarro and Durand, 1978). Under optimum conditions and depending on the strain, certain yeasts can tolerate ethanol concentrations of up to 20%; these strains are also high yielding varieties. Analyses of the membrane structures have revealed a relation to this with unsaturated fatty acid and the fatty acyl compositions of the plasma membrane (Wayman and Parekh, 1990).

2.6.3 Effect of mineral salts:

The process of fermentation in yeast is a fairly complicated mechanism influenced by the changing conditions of the media being fermented. During this process the concentrations of various media components changes, which in turn causes the yeast cells to elicit an appropriate response. Examples of responses include changing membrane permeability to allow the entry of more nutrients and the exit of wastes, and the increased production of enzymes to name a few. These responses are coordinated with the assistance of metal ions. As such, even limited amounts of metal

ions in a media have a significant impact on the fermentation process (Walker & vanDijck, 2006).

While certain mineral salts have been observed to influence the fermentation performance by yeast (Birch *et al.*, 2003), four mineral ions are considered to be the most crucial. These include potassium (K^+), magnesium (Mg^{2+}), manganese (Mn^{2+}) and iron (Fe^{2+}) (Somda *et al.*, 2011). Potassium (K^+) ions are especially important; besides being an important constituent for cellular growth, they play an important role in maintaining the stability of the yeast cell by preventing the gradual breakdown of the membrane caused by accumulation of ethanol (Lam *et al.*, 2014). Magnesium has been found to be an essential ingredient for the production of enzymes and release of alcohol from the yeast cell (Jin-Bong *et al.*, 1990; Gawande *et al.*, 1998). It also acts an activator of certain enzymes such as phosphatidyl transferase and decarboxylase (Mori *et al.*, 1985). Fe^{2+} ions, on the other hand, have been observed to stimulate respiration and cellular multiplication in yeast cells (Shockey and Barta, 1991).

Chapter 3

Material and Methods

Materials and Methods:

3.1 Samples:

Yeast strains (wild type) sourced from fruits and fruit juices, namely sugarcane, grape and dates. These were purchased from different fruit vendors in Dhaka. Two different sugarcane juice samples were used; one sample was aged for three weeks whereas the other was aged for 2 to 3 days. In addition, honey and molasses were also selected as potential sources.

3.2 Growth Media and Inoculum Broth:

The experiment required the extensive use of only one type of media specifically for yeast proliferation. YEPD (Yeast Extract Peptone Dextrose) broth is composed of 1% yeast extract, 2% peptone, 2% glucose or dextrose and desired volume of distilled water. Solid media was prepared by addition of 2% v/w of bacteriological agar powder to the broth. Once prepared, both were sterilized by autoclaving at 121ºC for 15 minutes at 15 psi.

While the yeast isolates were directly sourced from the fruit juices, intact fruits were needed to be cultured in YEPD broth modified with malt extract. In this case, the basic procedure of preparation was similar to that of YEPD media except for the addition of 2-4% malt extract powder. The broth was sterilized by autoclaving at 121ºC for 15 minutes at 15 psi before use.

3.3 Fermentation Media:

Blackstrap molasses sourced from raw sugar cane served as the main substrate for fermentation/ethanol production studies. The base reducing sugar content of this substrate media is ~20% w/v. The fermentation media was prepared by diluting 250 ml of molasses to 1000 ml with tap water and boiling the resulting mixture. During the boiling process 0.10 gm urea and 0.30 ml concentrated sulphuric acid (~97%) was added. The boiling process was maintained in accordance to the final reducing sugar

concentration in the molasses required. Once the media was prepared, it was distributed into 250 ml Erlenmeyer flasks in 100 ml portions and autoclaved at 121ºC for 15 minutes at 15 psi pressure.

For testing the impact of inorganic salts on the fermentation process, blackstrap molasses was again used in the same proportions, but in this case the media was treated with 1g of a particular salt. The salt was added after the base molasses media was autoclaved. In all, four salts were used for each isolate which include:

- Potassium dihydrogen phosphate (KH_2PO_4)
- Magnesium sulphate ($MgSO_4$)
- Manganese sulphate ($MnSO_4$)
- Iron sulphate ($FeSO_4$)

For reference studies, glucose media of 10% concentrations was also used. They were prepared by dissolving the required amount of sugar in 100 ml distilled water. Sterilization was done by autoclaving at 121ºC for 15 minutes at 15 psi pressure after the media was transferred into conical flasks.

3.4 Reagents (for media preparation and other purposes)

- ✓ Dinitrosalicylic acid (DNS)
- ✓ 0.9% Sodium chloride solution (Normal Saline)
- ✓ Sulphuric acid
- ✓ Sodium hydroxide
- ✓ Sodium thiosulfate
- ✓ Potassium Iodide,
- ✓ Potassium dichromate,
- ✓ Soluble Starch
- ✓ Urea.

3.5 Equipment and Glassware:

- Spectrophotometer (Single Beam UV type)
- Incubator
- Shaking Incubator
- Autoclave

- pH Meter
- Microscope
- Glassware, Fractional distillation apparatus, Magnetic Stirrer, Petri dishes, Micro-pipettes, Bunsen burner, Hot plate, Balance, micro-burette, Laminar airflow cabinet etc.

3.6 Biochemical test kits and media:

Yeast specific API® identification kit manufactured by bioMérieux was primarily used. In addition, nitrate Broth, which consists of 0.005% w/v peptone, 0.003% w/v beef extract and 0.005% w/v potassium nitrate in desired volume of distilled water. The pH was adjusted to 7.0 before being distributed to screw capped test tubes. After this, the tubes were autoclaved.

3.7 Sample isolation and inoculum development:

Initially, the yeast sources (sugarcane juice, whole grapes and dates, honey and molasses) were left at room temperature for approximately 3-4 days (a second sugarcane juice sample was stored for 3 weeks instead). Solid samples were immersed in maltose containing YEPD broth, whereas liquid samples were left as it is. Liquid source samples were then extracted and serially diluted in sterile saline solution to around 100X D.F and inoculated onto separate YEPD agar plates in accordance to source by spread plating. These plates were incubated at 30°C for approximately 48 hours. Colonies from each agar plates were then inoculated into separate sterilized inoculation broth tubes, which were then incubated for 24 hours at 30°C and re-inoculated into fresh agar plates afterwards. By performing this technique, pure yeast cultures were eventually obtained.

3.7.1 Observation and culture maintenance

Culture maintenance was performed by sub-culturing. This was performed by selecting an isolated colony using a loop and streaking onto a fresh YEPD plate. The plates were incubated for 48 hours at 30ºC and subsequently preserved in a refrigerator at 4ºC, albeit temporarily.

3.8 Identification of the yeast:

Identification of yeast isolates were done on the basis of morphological (Kreger-Van Rij, 1984) and physiological characteristics:

3.8.1 Morphological characterization

For observing morphological characteristics of the isolates, single colonies from 48 hour old subcultures were selected and inoculated into fresh YEPD plates via streaking. After approximately 48 hours, the growth pattern of the colonies along with the texture, color and surface characteristics were noted. Further analyses of the colonies were performed at the cellular level using a compound microscope.

3.8.2 Physiological characterization

For ascertaining physiological characteristics of the isolates, biochemical tests were performed on each isolate. In all cases, an API® identification kit (manufactured by bioMérieux) was used. It consists of a strip containing different carbohydrates and their derivatives separated into individual cupules. These carbohydrates include glucose, glycerol, calcium 2-keto-gluconate, arabinose, xylose, adonitol, xylitol, galactose, inositol, sorbitol, methyl β-D-glucopyranoside, N-acetyl-glucosamine, cellobiose, lactose (bovine origin), maltose, sucrose, trehalose, melezitose and raffinose. Final results were obtained 72 hours after the strip was inoculated with API C-media inoculated with yeast cells. They are differentiated by turbidity changes of the media in each cupule in comparison to a blank cupule containing no carbohydrates. A description is given on the illustration in Figure 3.1:

Nitrate reduction test was performed to observe nitrate utilization by yeast. The methodology involved inoculating nitrate broth with a particular isolate and incubating the tubes for 24 hour. After incubation, two reagents (named A and B) were mixed carefully to the tubes. The result was obtained immediately afterwards and can be noted by a distinct color change.

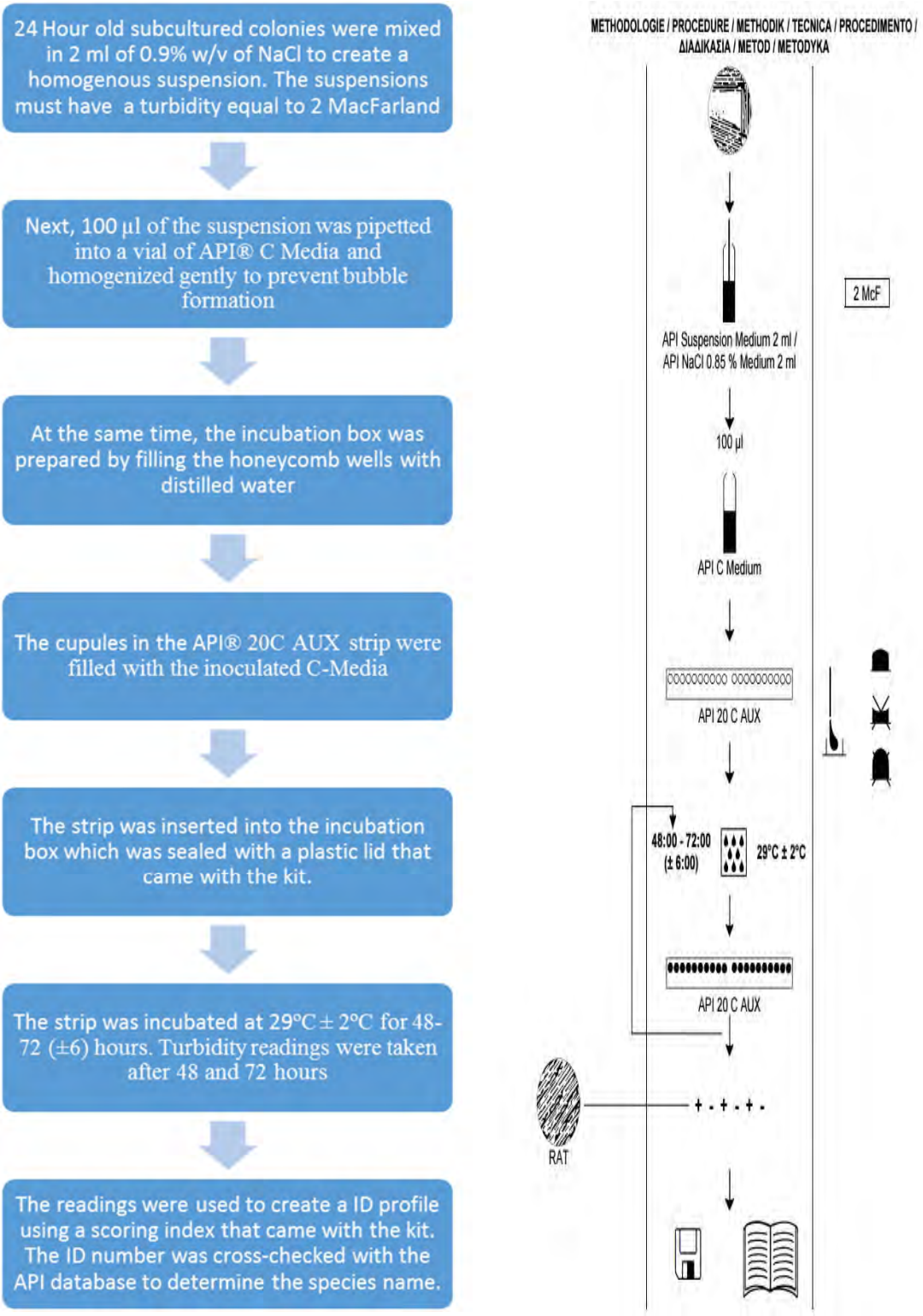


Figure 3.1: API ® 20C AUX kit usage

3.9 Stress tolerance test:

3.9.1 Detection of thermo-tolerance

In order to assay thermal tolerance of the isolates, 10 ml tubes of autoclaved YEPD broth were inoculated with 24-48 hour old yeast subcultures. As a control specimen, one of the test tubes was not inoculated. After inoculation, the initial optical densities of each tube were measured using the spectrophotometer at 600 nm against the medium as blank. The tubes were then incubated at different temperatures (25°C, 30°C, 37°C and 44°C) for 48 hours, with optical density readings being taken in 24 hour intervals

3.9.2 Detection of ethanol-tolerance

To assay ethanol tolerance of the isolates, the YEPD broth was slightly modified by addition of 1ml of varying concentrations (5%, 10%, 15% and 20%) of absolute ethanol. This was done after the base YEPD media was distributed as 10 ml portions in pre-marked test tubes and autoclaved. The marked tubes were inoculated with yeast subculture and a single tube is left un-inoculated to serve as a control. After inoculation, the initial optical densities of each tube were measured using the spectrophotometer at 600 nm against the medium as blank. The tubes were then incubated at 30°C for 48 hours, with optical density readings being taken in 24 hour intervals

3.10 Fermentation and Result Analysis:

Fermentation/ Ethanol production studies were carried out by inoculating yeast isolates in the prepared fermentation media. Analysis of ethanol production rates and overall efficiency of fermentation was performed on the basis of different parameters, but the overall process involved was similar in all cases. Media preparation was done in the sterilized environment of a laminar airflow bench.

3.10.1 Preparation of yeast cell suspensions

Yeast subcultures derived from 24-48 hours old streak plates were inoculated into 10 ml of 0.9% normal saline using a sterile loop. The suspension was made homogenous by vortexing the tubes after inoculation.

3.10.2 Fermentation of defined sugars

The fermentation media were inoculated with the yeast cell suspension prepared in the aforementioned step. The flasks were cotton plugged and incubated at 30ºC in shaking condition for 48 hours.

Figure 3.2: Glucose and Molasses fermentation media

3.10.3 Fermentation of molasses media treated with inorganic salts

The molasses media treated with the salts were inoculated with the yeast cell suspension after preparation. In this case however, the experiment was performed in separate phases; each isolate sample was inoculated into four flasks of molasses media treated with a different salt in each phase. In all cases, the flasks were cotton plugged and incubated at 30ºC in shaking condition for 48 hours.

Figure 3.3: Molasses media treated with salt

3.11 Estimation of ethanol:

Initial assay of ethanol production rates were performed by volumetric analysis in Conway units. Samples yielding feasible results were distilled and the ethanol percentages of the distillates were determined using an alcohol meter.

3.11.1 Experimental analysis: Conway method

The Conway method is based on a redox titration principle where the analyte, which interacts with the ethanol, is used to determine the percentage of ethanol. The analyte in this case is acidified potassium dichromate (0.05M) solution, which oxidizes vaporized ethanol to ethanoic acid. The unreacted dichromate is determined by adding potassium iodide (50% KI) solution which is also oxidized by the analyte to iodine. This iodine is titrated with a standard solution of sodium thiosulfate (0.1N). The titration reading is used to calculate the ethanol content after fermentation.

Figure 3.4: Conway unit titration apparatus

After incubating the inoculated fermentation media for a specific time period, 1ml portions were extracted and diluted up to 250x. Each of these samples was placed on the outer rim of a Conway unit. To serve as a control, a single Conway unit was loaded with distilled water. The centers of the Conway units were loaded with the dichromate reagent. Petroleum jelly was used to make them air-tight.

Overall, the procedure may be illustrated as follows:

- Distribution of 1ml 0.05M Potassium Dichromate solution to Conway unit center
- Addition of 1 ml of fermented sample in the outer rim of the Conway Unit
- Units sealed with paraffin wax coated glass plates and left for 24 hours
- 0.5ml 50% KI solution + 1-2 drop 1% starch solution added to dichromate
- Titration of reagent complex in the center with 0.1N Sodium Thiosulphate solution in microburette

3.12.2 Calculation of results:

Data obtained from titration readings were used to determine the percentage of ethanol (gm/100 ml) present in the sample using the following equation:

$$\text{Ethanol (\%)} = \frac{(\text{T.R of Control} - \text{T.R of F.S}) \times 11.6 \times 0.1 \times \text{D.F} \times 100}{(0.793 \times 1000)}$$

Where,

- ✓ Density of Ethanol: 0.793 g/ml
- ✓ D.F: Dilution Factor
- ✓ F.S: Fermented solution
- ✓ T.R: Titration Reading
- ✓ Volume of sodium thiosulfate used: 11.60 cm^3

Chapter 4

Results

Results:

4.1.1 Morphological characterization

The morphology of yeast cells were determined on the basis of growth in both liquid and solid YEPD media. Observation with the naked eye and microscopic examination was used to ascertain the presence of yeast cells.

4.1.1.1 Growth on YEPD agar plates and broth.

On agar plates, colonies of yeast grew rapidly and matured within two to three days. These colonies were smooth, flat, moist, opaque, and cream in color. Individual colonies had smooth outer edges. In liquid YEPD media, the yeasts formed heavy, dry climbing pellicles on the surface, with some settling to the bottom of the media.

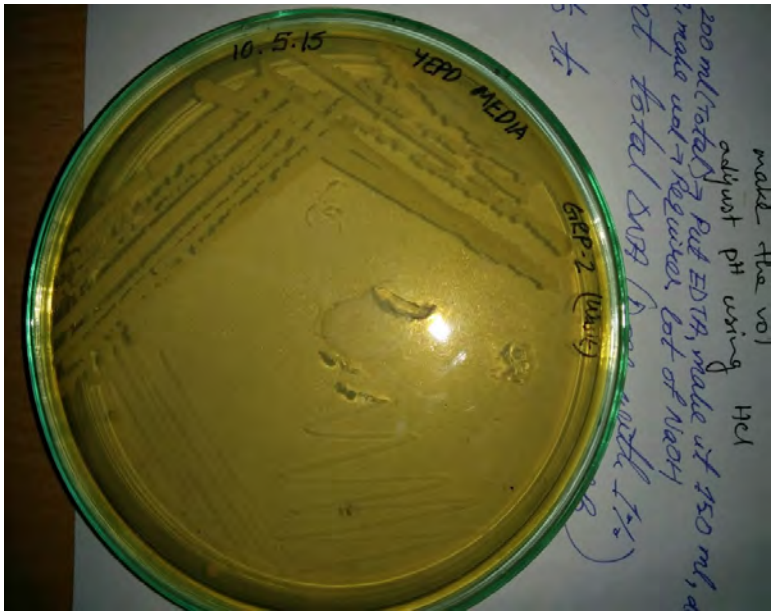


Figure 4.1: Yeast isolate colonies in YEPD agar medium after 24 hour incubation

4.1.1.2 Microscopic observation

Cellular morphology studies were carried out on all isolates using a compound microscope set to 40X magnification initially. Based on observations made in 24

hours and 48 hour intervals, unicellular, round cells were observed with a number of cells being ovoid in shape. These were noted to be vegetative cells undergoing reproduction via budding. Other key features noted were the absence of hyphae and pseudo-hyphae in all isolate cell samples.

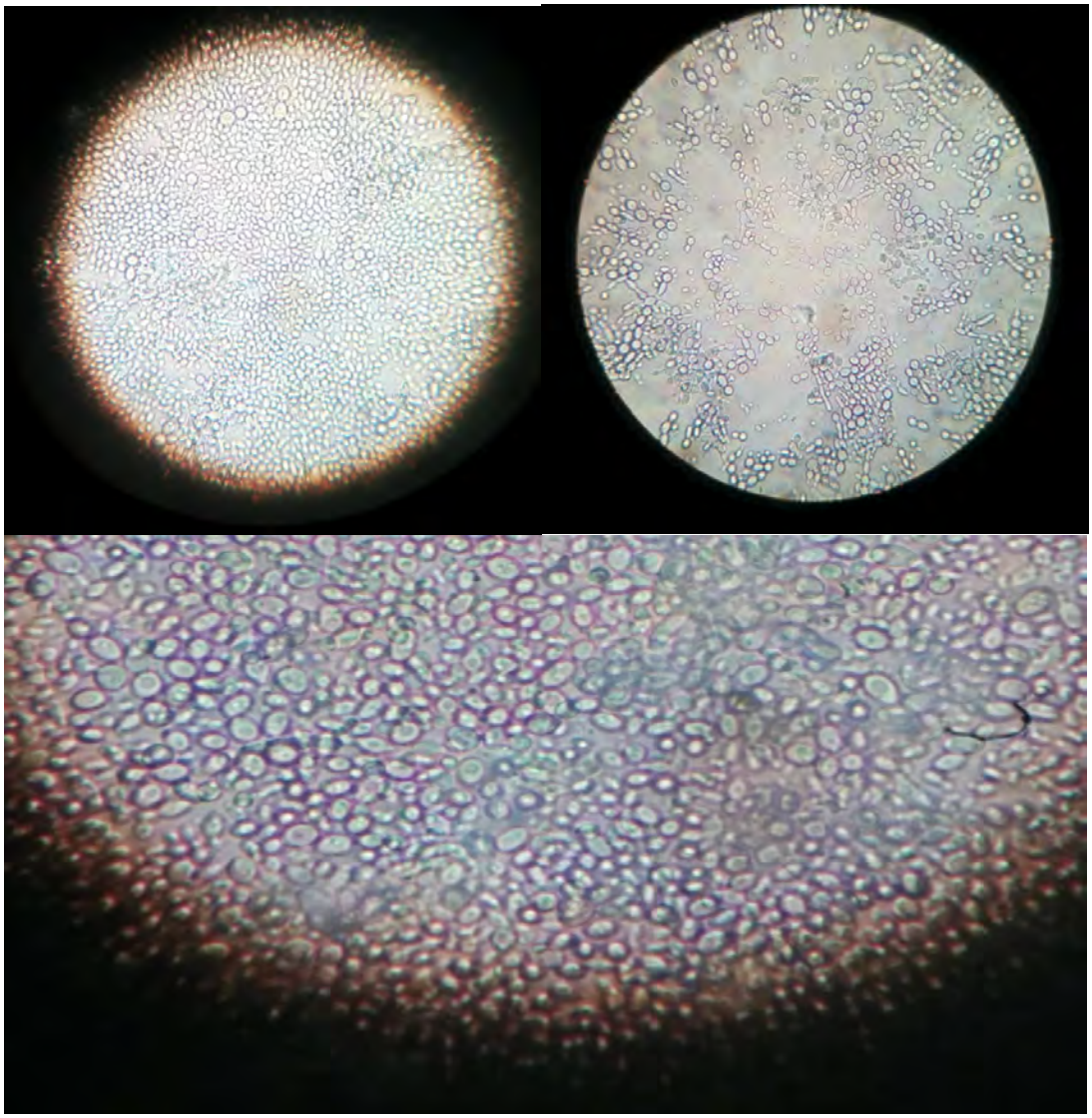


Figure 4.2: (Clockwise from top left) Cell morphologies of isolates SC-1.0, GRP-4 and D-H at 40X magnification

4.2 Biochemical and physiological characterization

4.2.1 Carbohydrate fermentation tests and API kit analysis

Carbohydrate assimilation tests were conducted using API® 20C test strips. Three isolates from the six sources (S.C-1.0, D-H and GRP-4) which showed the highest yields of ethanol were selected for this. In all cases positive results were obtained for glucose (GLU), methyl-αD-glucopyranoside (MDG), galactose (GAL), maltose (MAL), saccharose (SAC), melezitose (MLZ), trehalose (TRE) and raffinose (RAF). Unlike the other isolates however, GRP-4 displayed a positive reaction with cellibiose (CEL) which signified a split probability of the isolate being either of the species *Saccharomyces cerevisiae* or *Candida pelliculosa*. The results for SC-1.0, D-H and GRP-4 are illustrated in **Figures 4.5, 4.6** and **4.7** respectively.

4.2.2 Reduction of potassium nitrate

Following incubation of the isolates in nitrate media and treatment with reagent A and B after 24 hours, the following results were obtained as illustrated in **Table 4.1**.

Table 4.1: Nitrate reduction by yeast isolates (Positive: + +, Variable: + -, Negative: - -)

Isolate Name	Reaction
S.C-1.0	--
S.C-1.1	--
D-H	--
GRP-4	--
HON	--
MOL	--

Because there was no color change of the nitrate broth to pink/red, it was determined that no nitrate was reduced by the isolates. A visual representation of the results obtained from the experiment is illustrated in **Figure 4.3** and **4.4**.

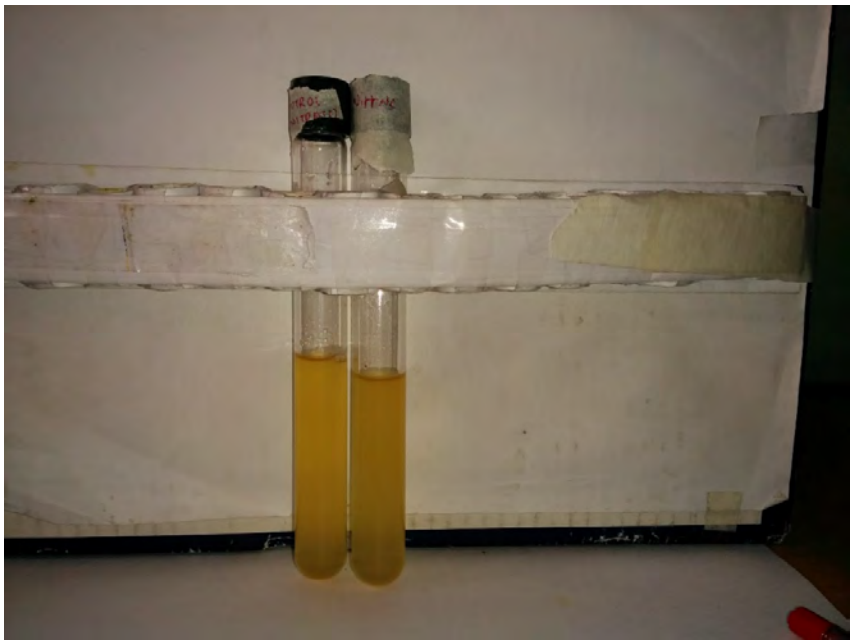


Figure 4.3: Result of Nitrate Reduction test against Control (Control to left)

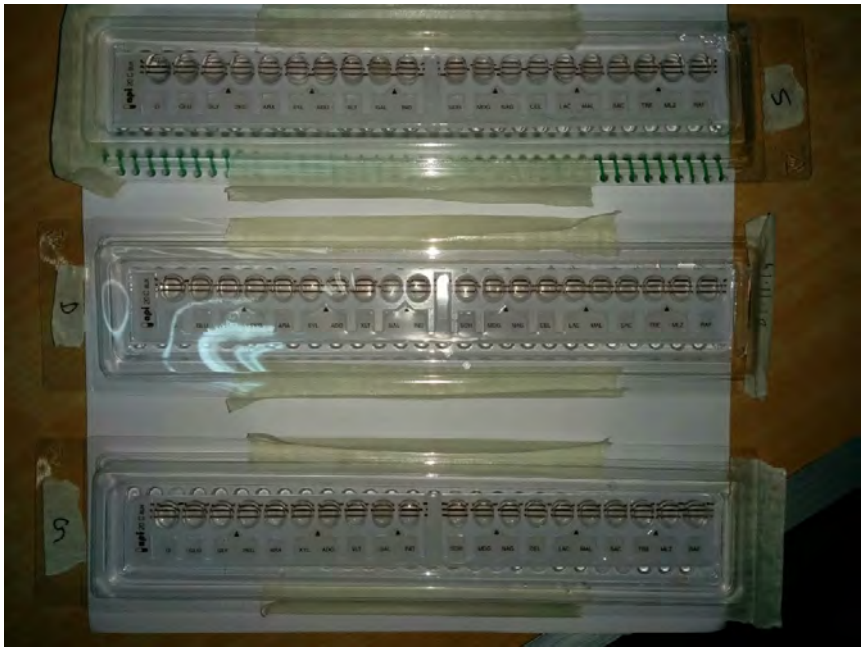
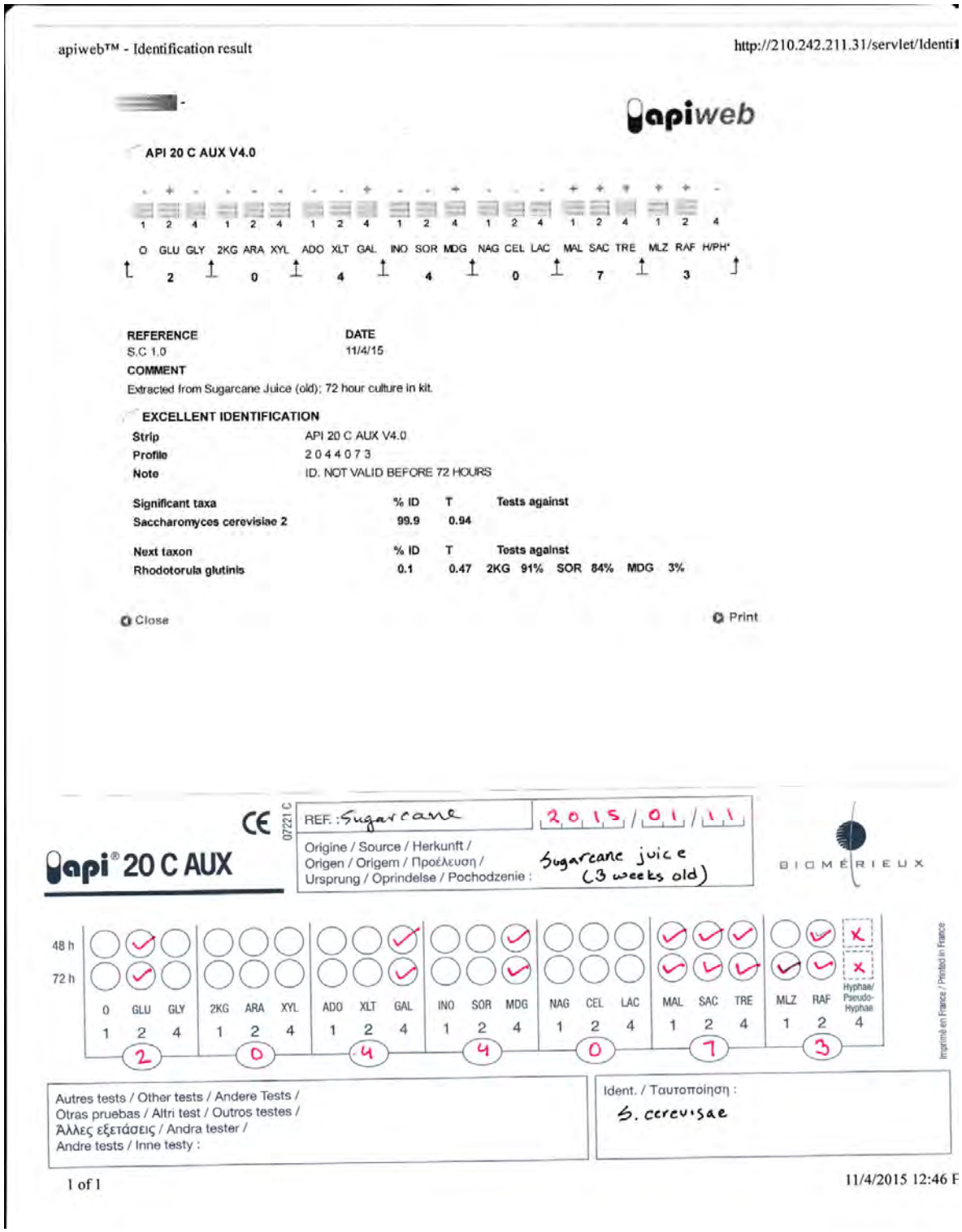


Figure 4.4 API® 20C AUX kit results after 48 hours



apiweb™ - Identification result

http://210.242.211.31/servlet/Identi

apiweb

API 20 C AUX V4.0

O	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	H/PH*
	2			0			4			4			0			7			3		

REFERENCE S.C 1.0

DATE 11/4/15

COMMENT

Extracted from Sugarcane Juice (old); 72 hour culture in kit.

EXCELLENT IDENTIFICATION

Strip	API 20 C AUX V4.0
Profile	2 0 4 4 0 7 3
Note	ID. NOT VALID BEFORE 72 HOURS

Significant taxa	**% ID**	**T**	**Tests against**
Saccharomyces cerevisiae 2	**99.9**	**0.94**	

Next taxon	**% ID**	**T**	**Tests against**					
Rhodotorula glutinis	**0.1**	**0.47**	**2KG**	**91%**	**SOR**	**84%**	**MDG**	**3%**

Close

Print

api® 20 C AUX

07221 C

REF. : Sugarcane

2015/01/11

Origine / Source / Herkunft / Origen / Origem / Προέλευση / Ursprung / Oprindelse / Pochodzenie : Sugarcane juice (3 weeks old)

BIOMÉRIEUX

	0	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	Hyphae/ Pseudo-Hyphae
48 h		✓							✓			✓				✓	✓	✓		✓	X
72 h		✓							✓			✓				✓	✓	✓	✓	✓	X
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
		2			0			4			4			0			7			3	

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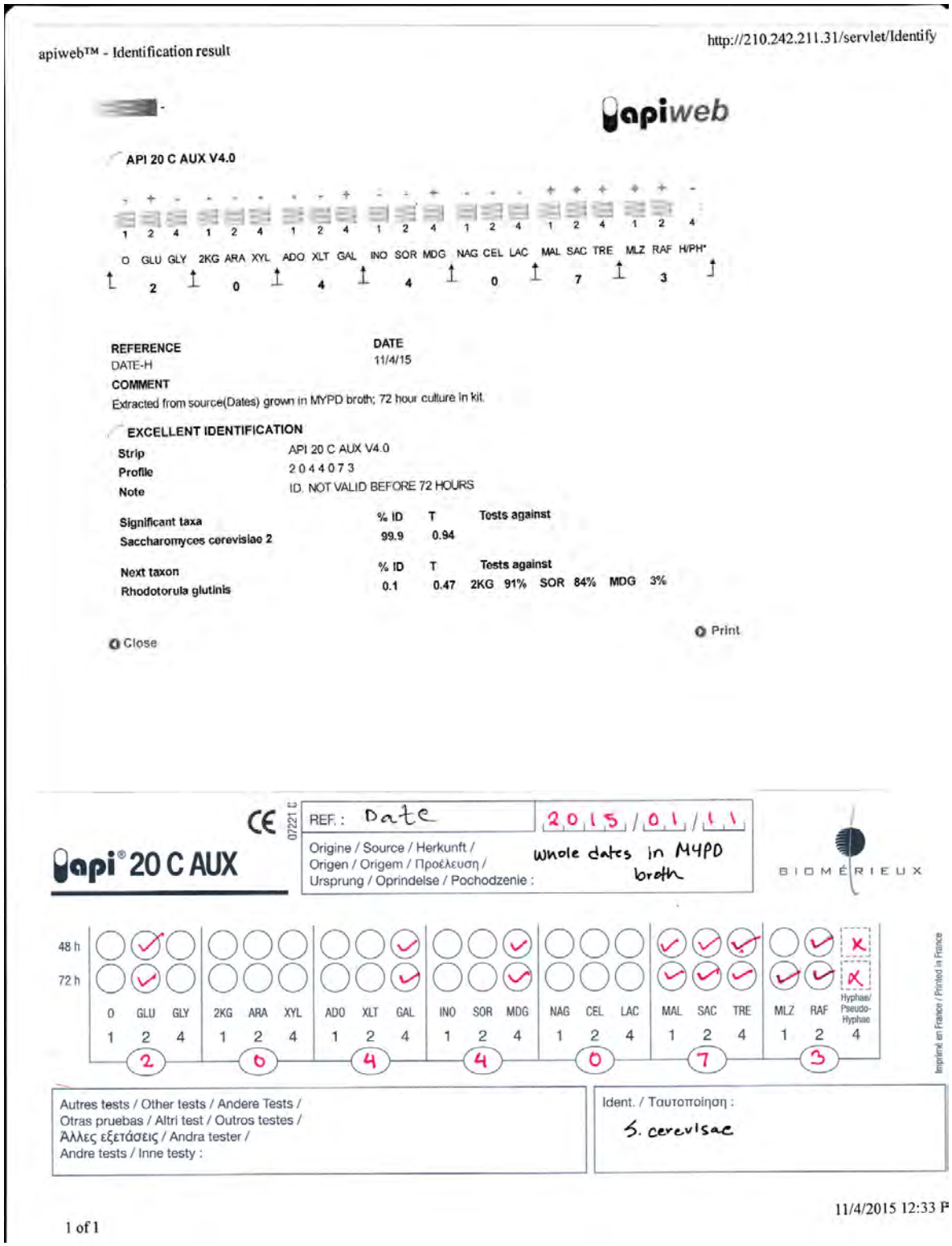
Autres tests / Other tests / Andere Tests / Otras pruebas / Altri test / Outros testes / Άλλες εξετάσεις / Andra tester / Andre tests / Inne testy :

Ident. / Ταυτοποίηση : S. cerevisae

1 of 1

11/4/2015 12:46 F

Figure 4.5: API kit analysis result for Sugarcane Juice sample



apiweb™ - Identification result

http://210.242.211.31/servlet/Identify

apiweb

API 20 C AUX V4.0

O GLU GLY 2KG ARA XYL ADO XLT GAL INO SOR MDG NAG CEL LAC MAL SAC TRE MLZ RAF H/PH*

2 0 4 0 4 4 0 7 3

REFERENCE
DATE-H

DATE
11/4/15

COMMENT
Extracted from source(Dates) grown in MYPD broth; 72 hour culture in kit.

EXCELLENT IDENTIFICATION

Strip API 20 C AUX V4.0
Profile 2044073
Note ID. NOT VALID BEFORE 72 HOURS

	% ID	T	Tests against
Significant taxa Saccharomyces cerevisiae 2	99.9	0.94	
Next taxon Rhodotorula glutinis	0.1	0.47	2KG 91% SOR 84% MDG 3%

Close

Print

api® 20 C AUX

CE 07221 EC

REF. : Date 2015/01/11

Origine / Source / Herkunft / Origen / Origem / Προέλευση / Ursprung / Oprindelse / Pochodzenie : whole dates in MYPD broth

BIOMÉRIEUX

	O	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	Hyphae/ Pseudo-Hyphae
48 h		✓							✓			✓				✓	✓	✓		✓	X
72 h		✓							✓			✓				✓	✓	✓		✓	X
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
		2			0			4			4			0			7			3	

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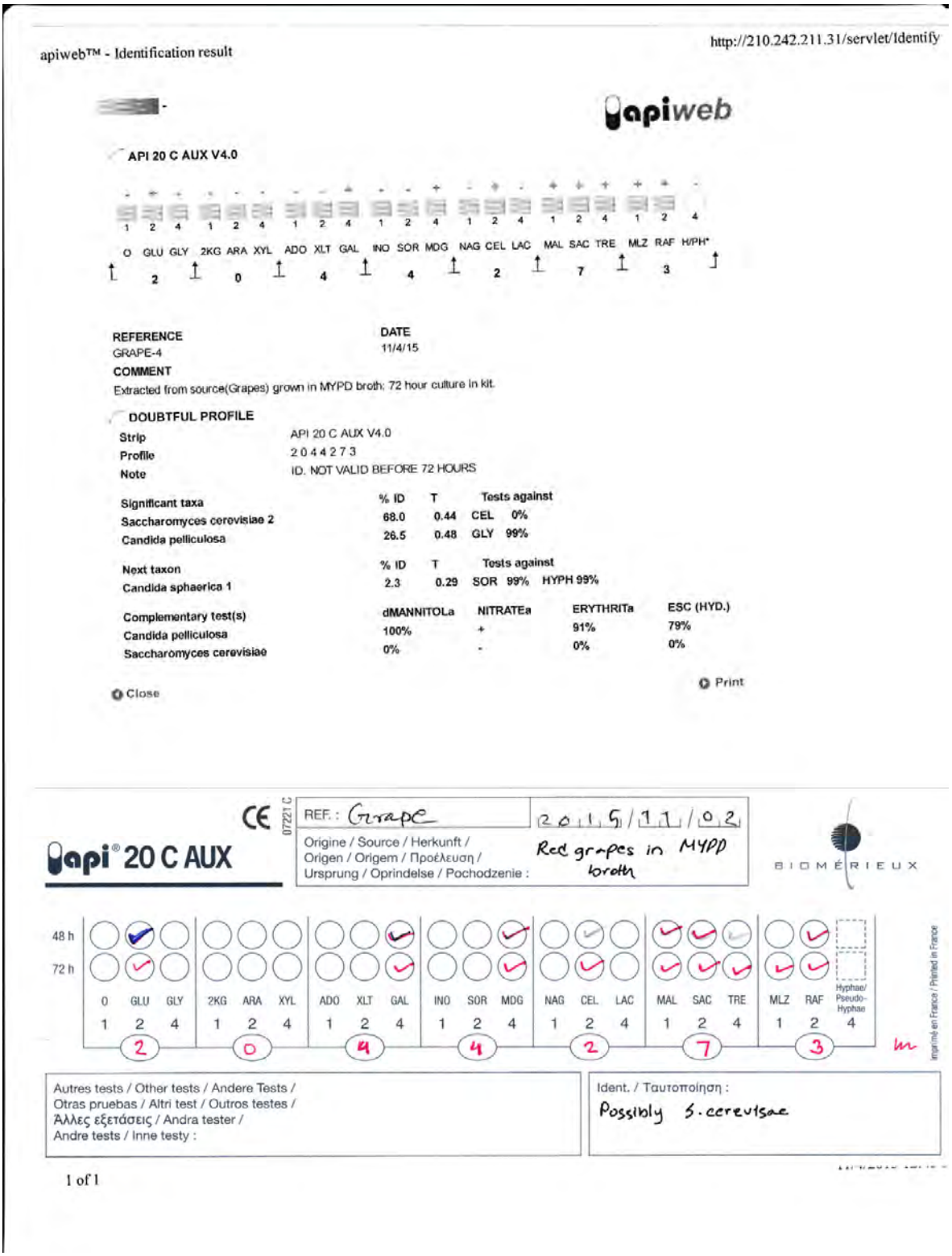
Autres tests / Other tests / Andere Tests / Otras pruebas / Altri test / Outros testes / Άλλες εξετάσεις / Andra tester / Andre tests / Inne testy :

Ident. / Ταυτοποίηση : S. cerevisae

11/4/2015 12:33 P

1 of 1

Figure 4.6: API kit analysis result for Date sample



apiweb™ - Identification result

http://210.242.211.31/servlet/Identify

apiweb

API 20 C AUX V4.0

O GLU GLY 2KG ARA XYL ADO XLT GAL INO SOR MDG NAG CEL LAC MAL SAC TRE MLZ RAF H/PH*

2 0 4 4 0 2 7 3

REFERENCE
GRAPE-4

DATE
11/4/15

COMMENT
Extracted from source(Grapes) grown in MYPD broth; 72 hour culture in kit.

DOUBTFUL PROFILE

Strip	API 20 C AUX V4.0			
Profile	2 0 4 4 2 7 3			
Note	ID. NOT VALID BEFORE 72 HOURS			
	% ID	T	Tests against	
Significant taxa				
Saccharomyces cerevisiae 2	68.0	0.44	CEL 0%	
Candida pelliculosa	26.5	0.48	GLY 99%	
	% ID	T	Tests against	
Next taxon				
Candida sphaerica 1	2.3	0.29	SOR 99%	HYPH 99%

Complementary test(s)	dMANNITOLa	NITRATEa	ERYTHRITa	ESC (HYD.)
Candida pelliculosa	100%	+	91%	79%
Saccharomyces cerevisiae	0%	-	0%	0%

Close

Print

CE 07221 C

api® 20 C AUX

REF. : Grape

2015/11/02

Origine / Source / Herkunft / Origen / Origem / Προέλευση / Ursprung / Oprindelse / Pochodzenie : Red grapes in MYPD broth

BIOMÉRIEUX

	0	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	Hyphae/ Pseudo-Hyphae
48 h		✓							✓			✓		✓		✓	✓			✓	
72 h		✓							✓			✓		✓		✓	✓			✓	
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
		2			0			4			4			2			7			3	

Autres tests / Other tests / Andere Tests / Otras pruebas / Altri test / Outros testes / Άλλες εξετάσεις / Andra tester / Andre tests / Inne testy :

Ident. / Ταυτοποίηση : Possibly S. cerevisae

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1 of 1

Figure 4.7: API kit analysis result for Grape sample

4.3 Results of stress tolerance studies

4.3.1 Thermotolerance test:

In the 4 different temperature ranges (25°C, 30°C, 37° and 44°C) selected for thermotolerance test, all isolates displayed optimum growth at 30°C in both 24 hour and 48 hour time intervals. Beyond that tolerance to temperature was subjected to decline after 48 hours of incubation for almost all strains, although at the extreme temperature of 44°C only a handful of isolates show a very minor level of growth (S.C-1.0 and GRP-4). At milder temperatures of 37°C, isolates SC-1.0 and GRP-4 demonstrated good growth throughout the total 48 hour incubation period, whereas SC-1.1 and D-H displayed good growth in the first 24 hours followed by a general decline. Isolates HON and MOL displayed comparatively slower growth during the whole incubation period, but much population levels were much higher than 44°C populations.

On the other end of the thermotolerance spectrum at 25°C, all isolates exhibited good growth during the first 24 hour interval, but the trend was maintained by isolates S.C-1.0 and D-H respectively. The table illustrates a summarized result of the spectrophotometer readings in a simplified format. **Table 4.2** illustrates the optical density changes observed for each isolates during 24 and 48 hour intervals at different incubation temperatures.

4.3.2 Ethanol tolerance test result

According to optical density readings taken after 24 hour and 48 hour intervals, the trend observed was a decrease in ethanol tolerance across all isolates. All six isolates (S.C-1.0, S.C-1.1, D-H, GRP-4, HON and MOL) showed positive growth at 5% and 10% ethanol concentrations throughout the entire 48 hour incubation period, except isolates HON and MOL whose growth slowed down after 24 hours. At 15% concentration, SC-1.0 and GRP-4 showed good growth after 24 hours, but eventually slowed down at 48 hours. On the other hand, the remaining isolates showed fairly low growth throughout the entire time period. At 20% concentration, growth was stopped almost completely for isolates SC-1.1, D-H and MOL but the other isolates showed growth but at a very minute rate. **Table 4.3** illustrates the optical density changes

observed for each isolates during 24 and 48 hour intervals at different ethanol percentages.

Table 4.2: Optical density measurements of yeast isolates to measure growth characteristics at different temperatures

Temperature	Isolate	Initial O.D readings	O.D readings after 24 hours	O.D readings after 48 hours	O.D change after 48 hours
25°C	S.C-1.0	0.465	1.214	2.327	1.862
	S.C-1.1	0.489	1.441	1.955	1.466
	D-H	0.398	1.093	2.076	1.678
	GRP-4	0.501	1.532	2.113	1.612
	HON	0.335	1.053	1.535	1.200
	MOL	0.373	1.146	1.574	1.201
30°C	S.C-1.0	0.503	2.078	3.177	2.674
	S.C-1.1	0.402	1.742	2.333	1.931
	D-H	0.458	1.959	2.459	2.001
	GRP-4	0.532	2.157	3.058	2.526
	HON	0.403	1.768	2.234	1.831
	MOL	0.338	1.674	2.122	1.784
37°C	S.C-1.0	0.533	1.633	2.459	1.926
	S.C-1.1	0.434	1.521	1.759	1.325
	D-H	0.575	1.598	2.135	1.560
	GRP-4	0.499	1.614	2.072	1.573
	HON	0.477	1.036	1.504	1.027
	MOL	0.452	1.118	1.331	0.879
44°C	S.C-1.0	0.342	0.465	0.471	0.129
	S.C-1.1	0.533	0.563	0.429	-0.104
	D-H	0.455	0.469	0.473	0.018
	GRP-4	0.602	0.693	0.711	0.109
	HON	0.513	0.525	0.557	0.044
	MOL	0.554	0.563	0.423	-0.131

Table 4.3: Optical density measurements of yeast isolates to test ethanol tolerance at different ethanol concentrations

Ethanol Percentage	Isolate	Initial O.D readings	O.D readings after 24 hours	O.D readings after 48 hours	O.D change after 48 hours
5%	S.C-1.0	0.464	1.855	2.740	2.276
	S.C-1.1	0.533	1.732	2.403	1.870
	D-H	0.465	1.599	2.459	1.994
	GRP-4	0.545	1.854	2.622	2.077
	HON	0.544	1.498	2.429	1.885
	MOL	0.412	1.378	2.205	1.793
10%	S.C-1.0	0.498	1.534	2.456	1.958
	S.C-1.1	0.353	1.330	1.858	1.505
	D-H	0.426	1.493	2.117	1.691
	GRP-4	0.375	1.442	2.270	1.895
	HON	0.316	1.569	1.857	1.541
	MOL	0.479	1.256	1.861	1.382
15%	S.C-1.0	0.374	1.125	1.356	0.982
	S.C-1.1	0.453	0.963	1.203	0.750
	D-H	0.395	0.757	0.858	0.463
	GRP-4	0.429	1.273	1.436	1.007
	HON	0.454	0.899	1.139	0.685
	MOL	0.319	0.642	0.813	0.494
20%	S.C-1.0	0.364	0.444	0.581	0.217
	S.C-1.1	0.231	0.247	0.345	0.114
	D-H	0.312	0.402	0.503	0.191
	GRP-4	0.276	0.437	0.531	0.255
	HON	0.373	0.415	0.475	0.102
	MOL	0.239	0.288	0.314	0.075

Figure 4.8: (Clockwise from left): Preparation of DNS Reagent + YEPD broth for Optical Density measurements; Result of ethanol tolerance tests; Yeast isolates grown at 30ºC

4.4 Ethanol fermentation

4.4.1 Ethanol production from defined sugar mediums

Ethanol yields from fermentation were measured in intervals of 24 and 48 hours, using the Conway unit titration method (Conway et al., 1994). On average yields were generally low for fermentations carried out in 10% glucose solution, with total alcohol percentages in 100 ml of substrate solution ranging between 2.99% to 3.96% after 48 hours. In this case, the isolates from old sugarcane juice (SC-1.0) and molasses (MOL) showed the highest (3.96%) and lowest yields (2.99%) respectively.

In the case of molasses fermentation media, the ethanol yields from all isolates were significantly higher than that obtained from 10% glucose solution. For 100 ml of molasses fermentation media, ethanol yield percentages were in the range of 5.49%

(HON) to 7.31% (SC-1.0) after complete fermentation. **Table 4.4** shows a full list of ethanol percentages obtained from all isolates in both 24 hour and 48 hour time periods.

Table 4.4: Titrimetric results of ethanol percentage in defined media (in %)

Fermentation Medium	Isolate	Percentage of ethanol after 24 hours	Percentage of ethanol after 48 hours
Glucose (10%)	S.C-1.0	3.67%	**3.96%**
	S.C-1.1	3.22%	3.35%
	D-H	2.98%	3.23%
	GRP-4	3.34%	3.41%
	HON	2.89%	3.11%
	MOL	2.74%	**2.99%**
Molasses (100ml)	S.C-1.0	6.57%	**7.31%**
	S.C-1.1	5.56%	4.58%
	D-H	6.19%	6.95%
	GRP-4	5.87%	6.58%
	HON	4.94%	5.85%
	MOL	5.07%	**5.49%**

Figure 4.9: Conway Units used for estimation of ethanol

4.4.2 Ethanol production from molasses with different salt supplements

In this instance, the assay was performed using the same tests as section 4.4.1, however in this case, 1mg of different salts were added to 100 ml of molasses before inoculation with different isolates. Compared to fermentation without salt additives, yields of ethanol obtained after addition of KH_2PO_4, $MgSO_4$, $MnSO_4$ and FeSO4 were generally higher in all cases. The highest yields of ethanol were obtained from isolates grown in KH_2PO_4 containing molasses media, with isolate SC-1.0 yielding the highest amount of ethanol (8.78%) after 48 hours. This was followed by $MnSO_4$ treated molasses media, then $MgSO_4$ treated molasses media and $FeSO_4$ treated molasses media. In all cases, the top three ethanol producing isolates were found to be S.C-1.0, D-H and GRP-4 respectively. Overall, the lowest yield was obtained from HON and MOL in media treated with $FeSO_4$ with the ethanol yield tied at 5.85%. **Table 4.5** shows the list of data obtained from the experiment in 24 hour and 48 hour time intervals.

Table 4.5: Titrimetric results of ethanol percentage in salt treated molasses medium (in %)

Salt supplement	Isolate	Percentage of ethanol after 24 hours	Percentage of ethanol after 48 hours
KH_2PO_4	S.C-1.0	7.31%	**8.78%**
	S.C-1.1	5.85%	6.95%
	D-H	6.58%	8.41%
	GRP-4	6.22%	8.05%
	HON	5.49%	6.58%
	MOL	5.85%	6.22%
$MgSO_4$	S.C-1.0	7.31%	8.05%
	S.C-1.1	5.49%	6.58%
	D-H	6.22%	6.95%
	GRP-4	6.95%	7.31%
	HON	6.58%	6.95%
	MOL	6.22%	6.58%
$MnSO_4$	S.C-1.0	7.68%	8.41%
	S.C-1.1	6.58%	6.95%
	D-H	6.95%	7.31%
	GRP-4	7.31%	7.68%
	HON	6.58%	7.31%
	MOL	5.85%	6.58%
$FeSO_4$	S.C-1.0	7.31%	7.68%
	S.C-1.1	6.22%	6.58%
	D-H	6.95%	7.31%
	GRP-4	5.85%	6.95%
	HON	5.12%	**5.85%**
	MOL	5.49%	**5.85%**

Chapter 5

Discussion

Discussion

Although yeast strains selected and genetically modified for use are available from various laboratory and industrial sources, the process of strain isolation is still an important facet of the ongoing research for bioethanol production. Derivation of yeast strains from local sources is one such aspect; the basis of this being that locally obtained strains are more adapted to the rigors of various environmental stresses of that particular location. As such, one of the keynotes of this study was the isolation of yeast strains from sugar-rich edible substrates commonly found in the local market. To that regard, six potential samples were selected which include dates, grapes, honey, sugarcane molasses and two sugarcane juices which were aged for different time periods. The isolates were selected after screening the samples using a variety of different physiological and biochemical parameters. Once the desired organism was obtained, these were used to ferment sugarcane molasses, a defined sugar substrate medium widely available in Bangladesh.

Morphological characterization studies of the six samples using and microscopic observation seemed to indicate that all of the samples contained yeasts that are members of *Saccharomyces spp.* This conclusion was reached on the basis of growth pattern studies in liquid and solid YEPD media; in all cases white and creamy colonies with butyrous colony texture were obtained after subculture (Kurtzmann, 1996). Microscopic observation further confirmed this, as oval and circular shaped individual cells which reproduced by polar budding were seen in all cases. Based on this, the isolates were named SC-1.0, SC-1.1 (from old and new sugarcane juice), D-H (from dates fermented in MYPD broth), GRP-4 (from grapes fermented in MYPD broth), HON (from honey) and MOL (from molasses).

The conclusion was further reinforced by biochemical tests performed using bioMérieux' API® 20C kits. Kit results for SC-1.0, D-H and GRP-4 indicated that all of the isolates, with the exception of GRP-4, are of the species *Saccharomyces cerevisiae.* This is in part due to their assimilation of the sugars glucose (GLU), methyl-αD-glucopyranoside (MDG), galactose (GAL), maltose (MAL), saccharose (SAC), melezitose (MLZ), trehalose (TRE) and raffinose (RAF) (Figures 4.5, 4.6 and

4.7); all of these are sugars normally utilized by *Saccharomyces cerevisiae*. However, as GRP-4 showed growth in cellibiose (CEL), its identity was not accurately determined although there was a 68% probability that the isolate was also *Saccharomyces cerevisiae.* As previous studies indicate that API® 20C kits have a statistical accuracy of 97% for common yeasts (Ramani *et al.*, 1998), the conclusions were assumed correct. Furthermore, nitrate assimilation tests for all isolates yielded negative results which confirm all isolates from the samples were *Saccharomyces cerevisiae* (Guilimares, 2006).

Thermotolerance tests also indicated that all isolates (SC-1.0, SC-1.1, D-H, GRP-4, HON and MOL) grew best at 30°C within a 48 hour incubation period; this is also the optimum growth temperature of *Saccharomyces cerevisiae* (Alexopoulos, 1962). However the isolates also showed good growth within a 5°C to 7°C temperature difference (at 25°C and 37°C) (Table 4.2). As for ethanol tolerance, the general trend observed was a decrease in tolerance of all isolates above 10% ethanol concentration signified by a slowdown in growth rate (Table 4.3), with a near growth stunt at 20% (S.C-1.0, GRP-4 and HON showed minor growth). Normally, members of *Saccharomyces spp.* can tolerate ethanol concentrations of up to 16.5 % (Teramoto *et al.*, 2005). However, since the isolates are wild-type *Saccharomyces* yeasts, an average maximum tolerance of 10% does not mean that they cannot be members of *Saccharomyces spp.*

The ethanol production rate was recorded from the fermentation of glucose and molasses after 24 and 48 hour time intervals. Additional parameters of fermentation included incubation at 30°C in shaking condition with a media pH of 5.0. In this study, the production rates of isolates in both glucose and molasses ranged from 2.53% to 7.31%. Isolate SC-1.0 grown in molasses had the highest yield of ethanol (7.31%), whereas the lowest yield was obtained from isolate MOL grown in glucose (2.58%). On average, ethanol yields from sugarcane molasses were found to be within the range of 7.8% (v/w) in earlier studies (Nofemele *et al.*, 2012). In the context of this study, the lowest yield obtain from molasses after 48 hours were obtained from isolate MOL (5.85%) which suggests that ethanol yields were more or less similar to previously observed level. In the local scenario, a study conducted using five yeast isolates named TY, BY, GY-1, RY and SY yielded ethanol concentrations of 12.0%, 5.90%, 5.80%, 6.70% and 5.80% under similar conditions (Khan *et al.*, 1989).

The primary goal of this study was to observe the impact of ethanol production from molasses upon the addition of various mineral salts. The parameters used were the same as the initial fermentation experiments with molasses media, but in this case molasses media were treated with KH_2PO_4, $MgSO_4$, $MnSO_4$ and $FeSO_4$ respectively. On average, the production yields were generally higher than that of non-treated media with yields ranging from 5.85% to 8.78%. The highest yield obtained was from isolate SC-1.0 grown in KH_2PO_4 treated media (8.78%) whereas the lowest yield was from isolates HON and MOL grown in $FeSO_4$ treated media (5.85%). Compared to other salt-treated molasses media, ethanol production was generally higher in KH_2PO_4 treated media across all isolates; isolate MOL outputted ethanol yields of 6.22% which was much higher than the initial 5.49% obtained from untreated media. On the other hand $FeSO_4$ treated media showed the least significant yield difference from the untreated molasses; isolate SC-1.0 yielded 7.68% (v/w) ethanol which is a slight increase from the 7.31% (v/w) ethanol obtained from untreated molasses. Overall, the results obtained alluded to a previous bioethanol production rate study using KH_2PO_4, $MgSO_4$, $MnSO_4$ and $FeSO_4$ salts, albeit using mango hydrolysate as a fermentation medium (Somda *et al.*, 2011). Although the ethanol yields were significantly higher than those obtained in the current study, the trend of yield increase in accordance to the salt used followed the same pattern.

In conclusion, the study managed to achieve its objective of strain isolation from local sources via successful isolation and identification of six yeast samples SC-1.0, SC-1.1, D-H, HON, MOL and GRP-4. Physiological and biochemical tests on the six isolates yielded the conclusion that the yeasts were of the species *Saccharomyces cerevisiae*. Analysis of the effect of mineral salts on ethanol fermentation also yielded the general result that the effect is largely positive and caused some improvements in ethanol yields. To this end, there are grounds for further studies to be conducted in areas such as improvement of the isolates, observing the effects of other salts on the fermentation as well as using other fermentation media. Scale-up studies could also be conducted, as the experiment was performed in laboratory settings.

Chapter 6

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Appendix

Appendix

Nitrate reagent:

- Solution A, Sulfanilic acid (1gm of sulfanilic acid was dissolved in 125 ml of 5N acetic acid).
- Solution B, Alpha-napthylamine (0.625 gm of α-napthaylamine dissolved in 120ml of 5N acetic acid.)

0.05M Acidified Potassium Dichromate solution:

Initially a 1M stock solution was prepared by addition of 2.9418 g of solid potassium dichromate to 10 ml of concentrated sulphuric acid (~97%) with stirring and cooling until the crystals were dissolved completely. This solution was diluted to 0.05M by the addition of 19 ml distilled water to 1ml of the stock solution.

Dinitrosalicylic acid (DNS):

About 1g of DNS was dissolved in 50ml of distilled water. To this solution, 30g of sodium potassium tartarate tetrahydrate was added. Then 20 ml of 2 N NaOH was added, which turns the solution to transparent orange yellow color. The final volume was made to 100 ml with the distilled water. This solution was stored in an amber colored bottle.

Sodium hydroxide solution:

Solution was made by adding 4 g of sodium hydroxide pellets in 50 ml of cold water and made up to 1 liter with water.

50% (w/v) potassium iodide solution:

Solution was prepared by addition of 8.3006 g solid potassium iodide in 10ml distilled water. The reagent was stored in a Durham bottle

0.1 M sodium thiosulfate solution:

To make the solution, 15.8 g of anhydrous sodium thiosulfate was dissolved in boiled deionized water and make the solution up to 1 liter using a volumetric flask