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ENHANCED PRODUCTION OF ETHANOL FROM FREE AND IMMOBILIZED *SACCHAROMYCES CEREVISIAE* UNDER STATIONARY CULTURE

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Abstract

In the present work, studies were carried out on the ethanol production by free and immobilized *Saccharomyces cerevisiae* GC-IIB31 under stationary culture. Cane molasses in different concentration was used as sugar source for maximum conversion of reducing sugar into ethanol. The substrate was optimized after maintaining different levels of sugar concentrations (12-21%), medium pH (4.0-5.5), incubation temperatures (25-30°C), volume of fermentation medium (200-350 ml) and reuse of immobilized yeast cells. Immobilized yeast cells gave significant results up to four consecutive batches. Rate of ethanol production was maximal with the free cells. The results indicated that 2 g vegetative cells of yeast on utilizing molasses at 15% sugar level with medium pH 4.5 at 30°C and 300 ml fermentation volume in 500ml Erlenmeyer flasks gave maximum ethanol production with both free and immobilized yeast cells. Maximum ethanol production by immobilized yeast cells was obtained in the 4th batch after which it declined markedly. The optimal results are highly significant ($p \leq 0.05$, LSD 3.962).

Introduction

Ethanol is one of the most advanced liquid fuels because it is environmental friendly. It is a clear, colorless liquid with a characteristic, agreeable odor. In dilute aqueous solution, it has a sweet flavor, but in more concentrated solutions it has a burning taste (Patil 1991). It is an alcohol, 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, they 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). It is usually sold as industrial methylated spirits which is ethanol with small quantity (5-10%) of methanol added and possibly with some color. It is a bio-fuel, which is produced from biomass and wastes. Bio-fuels provide an alternative to fossil fuel dependency and emit fewer pollutants (Carvalho *et al*., 1993). Various processes have been developed for ethanol production but world wide demand of ethanol is generally satisfied by biotechnological fermentation process. A number of organisms including fungi, yeast and bacteria have been screened for ethanol fermentation. Extensive studies have been carried out on the fermentation process of ethanol by these organisms, especially through yeast cells (Bajaj *et al*., 2001). However, *S. cerevisiae* remained the organism of choice, which is the same species used for bread making and some wines or beers (Walker *et al*., 1990; Converti *et al*., 2003; Moreira *et al*., 2005). In pure and mixed cultures, *S. cerevisiae* presents almost same

yield and productivity. Other organisms of primary interest include *S. uvarum, S. pombe, S. vini, S. acldodevoratus* and *Kluyveromyces* sp., (Tao *et al*., 2005; Haq & Ali, 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 (Prescott & Dunn, 1987; Baptista *et al*., 2006). Hydrolyzed enzymes ferment the complex sugars to reducing sugars and then to high concentrations of ethanol. It is also being made from a variety of agricultural bye-products such as grain, fruit juices, fruit extracts, whey, sulfite waste liquor and molasses (Nigam *et al*., 1998). The molasses is obtained from different sources such as cane, beet and citrus etc. It is a syrupy material left after the removal of sugar from the mother syrup. The viscous material is composed of sucrose, glucose and fructose at total carbohydrate concentration of 45-60% (w/v). The molasses is of three types, the black strap, refinery and invert or high test molasses. Cane molasses has less sucrose and more invert sugar, and lower content of nitrogen and raffinose, more intense color and more buffer capacity (Wang *et al*., 1985; Borzani *et al*., 1993; Borzani 2001). Work is needed to enhance ethanol production by free and immobilized *Saccharomyces cerevisiae* GC-IIB31 under stationary culture. Pre-treated sugar cane molasses was used as a basal fermentation medium.

Materials and Methods

Materials: Instruments used in the present study are incubator (Model; MIR -153 SANYO, Japan), rotary shaking incubator (Model; 10X 400.XX2C, SANYO, Gallankamp PLC, UK), cold cabinet (Model: MPR1410, SANYO Japan). All the chemicals, including dinitrosalicylic acid (DNS), potassium dichromate, sodium alginate, agar, sodium potassium tartarate, $CaCl_2$, sulfuric acid, potassium dichromate were of analytical grades and purchased directly from Sigma (USA), E-Merck (Germany), Acros (Belgium).

Organism and culture maintenance: The strain *Saccharomyces cervisiae* GC-IIB31 maintained on yeast extract peptone glucose (YPG) agar medium (pH 4.5), containing yeast extract (3.0 g/l), peptone (5.0 g/l), glucose (10.0 g/l), agar (20.0 g/l), was obtained from the available stock culture of Institute of Industrial Biotechnology, GC University Lahore, Pakistan. The slants were incubated at 30°C for 1-2 days for maximum growth.

Preparation of yeast cell suspension: Sterilized distilled water (10 ml) was added to a 24-36 h old slant culture of *S. cerevisiae*. The cells were scratched with a sterilized inoculating needle and the tubes were shaken gently to form a homogeneous suspension. The cell count was made using a Haemocytometer.

Development of inoculum: Fifty milliliter of YPG medium was transferred to the individual 250ml Erlenmeyer flasks. The flasks were cotton plugged, autoclaved and allowed to cool at room temperature. One milliliter of cell suspension (2.74×10^6 CFU) was added to each flask aseptically. The flasks were incubated in a rotary shaker (160 rpm) at 30°C for 24h. Pre-grown culture of *S. cerevisiae* was centrifuged at 6000rpm for 15 min and yeast cells were separated out. The supernatant was discarded and the pellet was washed with saline water. It was re-centrifuged for another 5 min to obtain the final pellet that was washed and then air-dried and weighed. These were the free cells.

Immobilization of yeast cells: To carry out immobilization, 2% of $CaCl_2$ solution was prepared and kept at 4°C for chilling. The next step was to dissolve 2 g of sodium alginate in hot water with constant stirring on magnetic stirrer. After cooling sodium alginate solution, 2 g of yeast cells were added to the slurry under stirring conditions for even dispersal. 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.

Pretreatment of molasses: The industrial by- product' cane molasses' obtained from Pattoki sugar mills' District Qasur (Pakistan)'was used in the present study. Initially the sugar contents of molasses were about 48%, which were maintained to 30% (w/v) by dilution. Concentrated Sulfuric acid (0.5% v/v) was added to the molasses medium and heated to 80°C for 30 min and left overnight. Two layers were formed, upper shining black, while lower yellowish brown (due to the precipitates of trace metals).the clear supernatant (shiny layer) was used as fermentation medium with 15% sugar contents.

Fermentation procedure and critical phases: Three hundred milliliters of treated cane molasses with 15% (w/v) sugar (initial pH 4.5) was taken into individual 300ml Erlenmeyer flasks. The flasks were cotton plugged and steamed at 90°C in a water bath for 15-20 min. After cooling to an ambient temperature, 2 g yeast cells were added to one flask and to other flask added immobilized yeast cells with same cell mass and placed in an incubator at 30°C for 120h. After the required incubation period, the cells and beads were separated out and beads were stored for use of more experiments. The fermented medium was used for estimation of ethanol and residual sugar contents.

Assay methods: The estimation of total reducing sugar was based on the dinitrosalicylic acid (DNS) method (Miller, 1959). A double beam UV/VIS-scanning spectrophotometer was used for measuring absorbance. Sugar contents in the supernatant were determined by taking 1.0 ml of supernatant along with 2.0 ml of DNS reagent in a test tube. Blank containing 1.0 ml distilled water and 2.0 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, added 8 ml of distilled water in each and absorbance was noted at 546nm using spectrophotometer. Sugar concentration was determined from the standard curve of glucose.

Ethanol estimation

Distillation method: The known volume of fermented mash was distilled. Fermented solution was heated to force the lowest boiling material into the vapor phase. The vapors were passed over the bulb of a thermometer at which point vapor was determined (El-diwany *et al*., 1992). The vapor was condensed to a liquid in the horizontal condenser that was cooled with a flow of cold water. The distillate was collected in a receiver. The volume of the distillate was measured and 0.0-110% of the alcohol was determined by alcoholmeter, the alcohol-meter was calibrated using ethanol solution of known concentration

Dichromate method: Ethanol was also determined with good precision by oxidation with acid dichromate solution (Kiransree *et al*., 2000). The ethanol in the known masses of the solution was oxidized to acetic acid using a known mass of standard potassium dichromate (0.1N) in the presence of sulfuric acid.

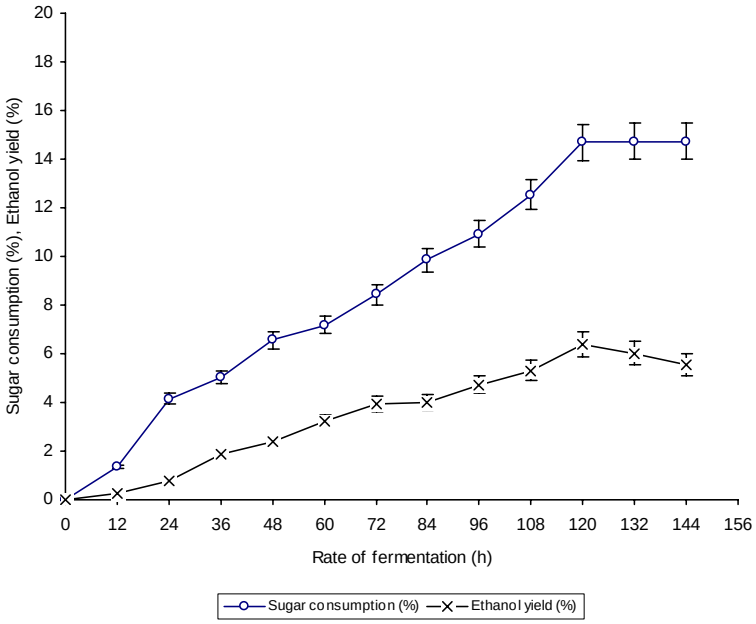
Results

Rate of ethanol production by free and immobilized *S. cerevisiae* cells: Rate of ethanol production by free and immobilized *Saccharomyces cerevisiae* (GC-IIB31) was investigated. The rate was studied from 12-144 h after inoculation. The time course profiles are shown in Fig. 1. The sugar consumed by free cells after 12 h was 1.36% and ethanol yield was 0.75%, which was very low. However, the sugar consumed by immobilized cells after 12 h was 0.92% and ethanol yield was 0.39%. Sugar consumption and ethanol yield was improved with the increase in time period. Maximum ethanol yield (6.38%) by free yeast cells was obtained after 120 h of incubation with a maximum sugar consumption of 14.69%. However, immobilized yeast cells gave maximum ethanol (5.29%) with sugar consumption of 14.76%. The ethanol yield obtained by free cells was thus 7.3% higher than ethanol yield by the immobilized cells.

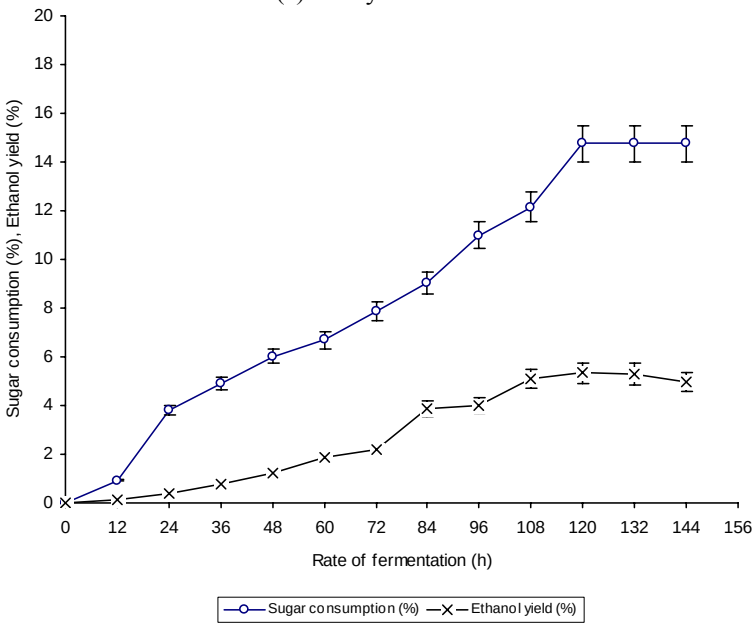
Reuse of immobilized yeast cells for ethanol production by free and immobilized yeast cells: The rate of ethanol production by immobilized *S. cerevisiae* cells (2.0% $CaCl_2$) was investigated by reusing the cells up to six consecutive batches (Fig. 2). The rate was studied from 24-144 h. Samples were drawn every 24 h. The sugar consumption and ethanol yield were noted. In the 1st batch, ethanol yield was found to be 5.38% while sugar consumption was 11.95% with immobilized yeast cells. The ethanol yield increased in next batches (up to 3rd batch) and was found maximal (7.56%) in the 4th batch with a sugar consumption of 14.89%. Both the sugar consumption rate and ethanol yield decreased sharply in the 5th and 6th batches. The ethanol yield in the 4th batch was 1.18 fold higher than the free cells. The net enhancement was 7.41% over to the batch with free cells. As ethanol yield was encouraging in the 4th batch with the immobilized yeast cells, therefore further studies were carried out to compare the variables with the free cells.

Effect of pH optima on ethanol production: Fig. 3 highlights ethanol production by free and immobilized *S. cerevisiae* at different initial pH. The pH ranged from 4.0-5.5 and each fermentation was run from 24-120 h after inoculation. Ethanol yield obtained at pH 4.0 with free cells was 5.5% with a sugar consumption rate of 12.98%. The immobilized yeast cells gave 3.27% ethanol with a maximum sugar consumption of 14.09%. At pH 4.5 with free cells ethanol was 6.39% while sugar consumption was noted to be 13.59%. However, the maximum results were obtained with the immobilized cells which gave 5.69% ethanol with sugar consumption of 14.09% when the initial pH was adjusted to 4.5. At pH 5.0, ethanol production decreased by both the free and immobilized cells. Free yeast cells gave 6.05% ethanol with a sugar consumption of 14.97% while with immobilized cells it was 5.34% and sugar consumption was recorded to be 12.09%. At pH 5.5, again low alcohol levels were obtained.

Effect of incubation temperature: In Fig. 4 is depicted the effect of different incubation temperature on ethanol production with free and immobilized yeast cells. The temperature ranged from 25-40°C and course of fermentation was studied from 24-120 h. Ethanol yield with free cells after 120 h was 5.38% with maximal sugar consumption (14.62%). However, immobilized yeast cells gave ethanol yield of 4.18% with sugar consumption of 11.71%. The optimal alcohol production was obtained at 30°C; free cells gave ethanol yield 6.42% with 14.79% sugar consumption. However, immobilized cells gave 5.83% with 14.02% sugar consumption. Among the different temperatures compared, 30°C supported maximum ethanol production by free yeast cells, 120 h after incubation under the optimal conditions.

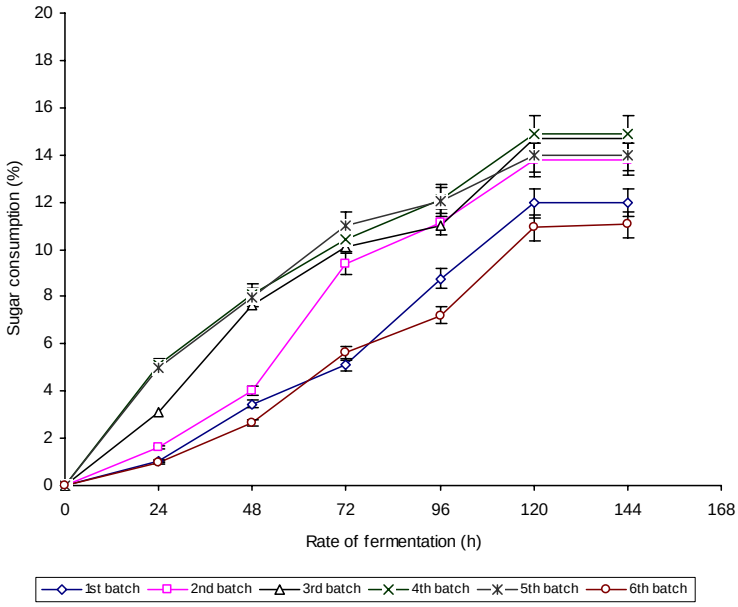


(a) Free yeast cells

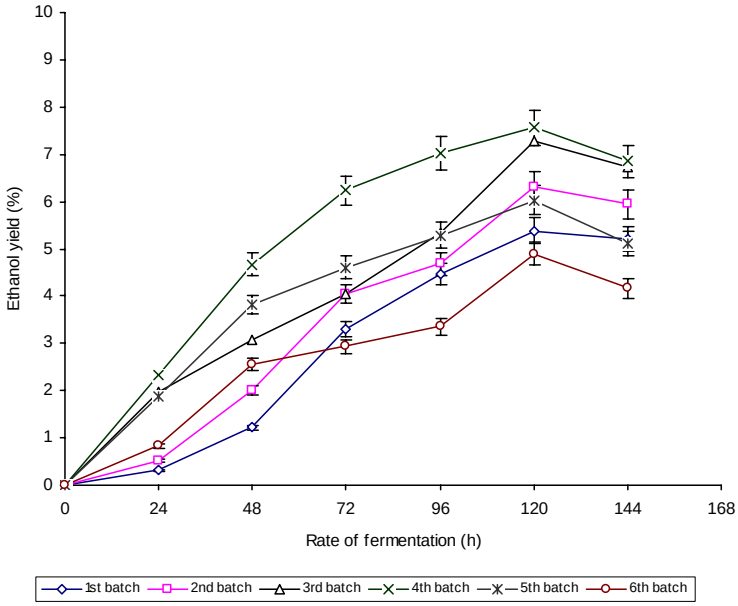


(b) Immobilized yeast cells

Fig. 1. Rate of ethanol production by free and immobilized *S. cerevisiae* GC-IIB31 under stationary culture. Sugar conc. 15%, incubation temperature 30°C, initial pH 4.5. The standard error bars indicate the standard deviation (±sd) among the three parallel replicates calculated at 5.0% level.



(a) Rate of sugar consumption



(b) Rate of ethanol yield

Fig. 2. Reuse of immobilized *S. cerevisiae* GC-IIB31 for ethanol production under stationary culture. Sugar conc. 15%, incubation temperature 30°C, initial pH 4.5. The standard error bars indicate the standard deviation (±sd) among the three parallel replicates calculated at 5.0% level.

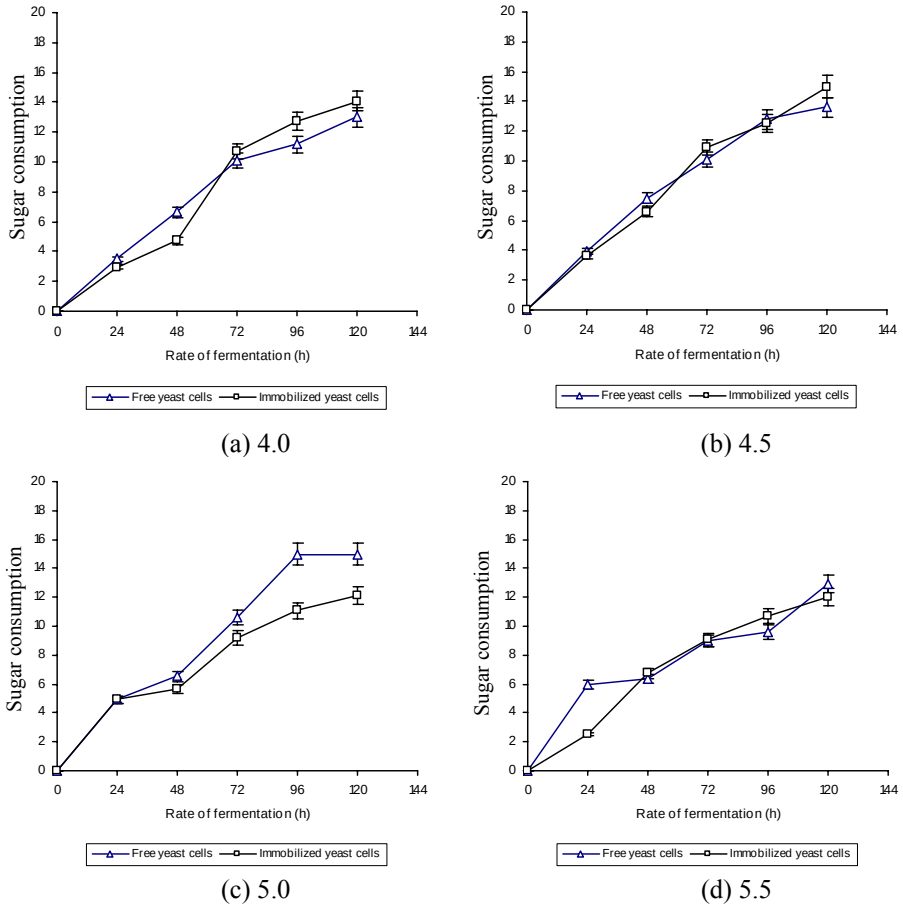


Fig. 3. Effect of initial pH of cane molasses on ethanol production by free and immobilized *S. cerevisiae* GC-IIB31 under stationary culture.
Sugar conc. 15%, incubation temperature 30°C. The standard error bars indicate the standard deviation (±sd) among the three parallel replicates calculated at 5.0% level.

Effect of initial sugar concentration: Effect of different initial sugar concentrations on ethanol production was investigated (Fig. 5). Sugar concentration ranged from 12-21% and fermentation period was ranged from 24-120 h. At 12% initial sugar concentration, free cells gave 2.34% ethanol with sugar consumption of 8.08%. However, immobilized cells gave 4.13% ethanol with 11.04% sugar consumption. Maximum production was obtained at 15% sugar level by both free and immobilized cells. Free cells gave 6.49% ethanol with 14.92% sugar consumption while with the immobilized it was 5.85% (sugar consumption 14.90%). Ethanol production with free cells was 4.29% higher than immobilized yeast cells, while at other sugar levels ethanol production was extremely low.

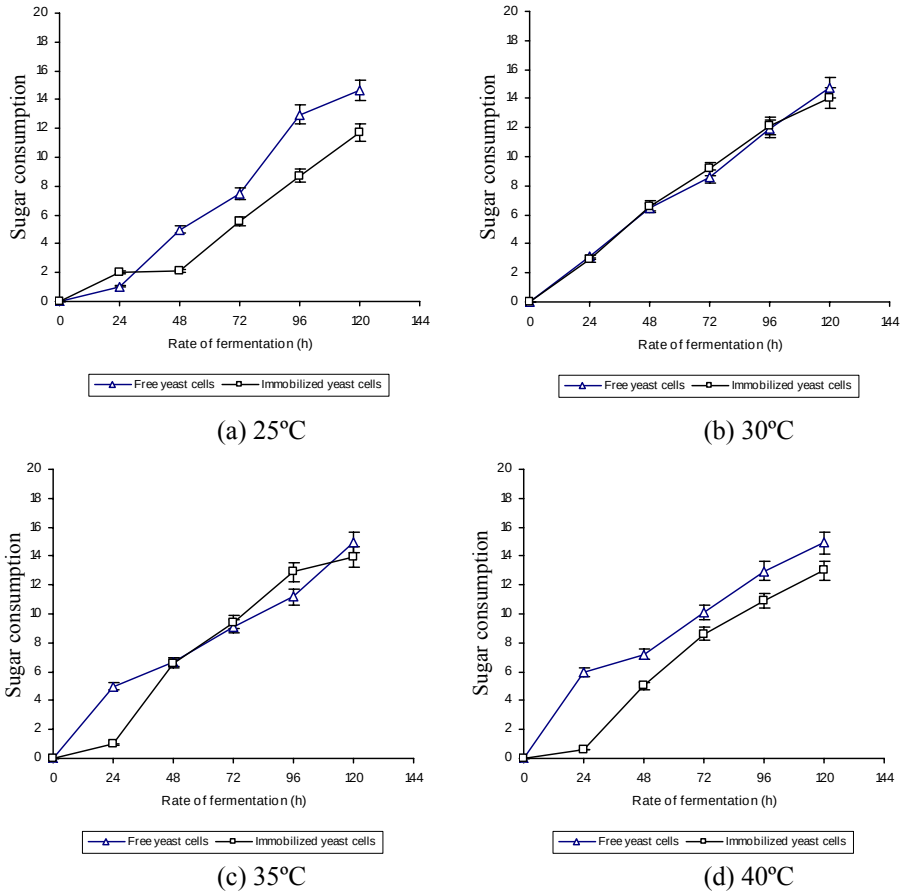


Fig. 4. Effect of incubation temperature on ethanol production by free and immobilized *S. cerevisiae* GC-IIB31 under stationary culture.
Sugar conc. 15 %, initial pH 4.5. The standard error bars indicate the standard deviation (±sd) among the three parallel replicates calculated at 5.0 % level.

Effect of different volume of fermentation: In Fig. 6 is shown the effect of different volume of fermentation medium (200, 250, 300 & 350 ml) on ethanol production by free and immobilized yeast cells. The microbial fermentations were carried out from 24 to 120 h after the inoculation. At 200 ml volume (in 500 ml Erlenmeyer flask), the free cells gave 4.29% ethanol with 12.92% sugar consumption. However, immobilized cells gave 2.24% ethanol with 13.01% sugar consumption. The maximum alcohol production was obtained at 300 ml fermentation medium in the stationary flask with both free and immobilized *S. cerevisiae.* Free cells gave 6.95% ethanol with a sugar consumption rate of 13.99%. The immobilized cells however, gave 6.21% ethanol with sugar consumption of 14.49%. At 250 and 350 ml no appreciable results were obtained.

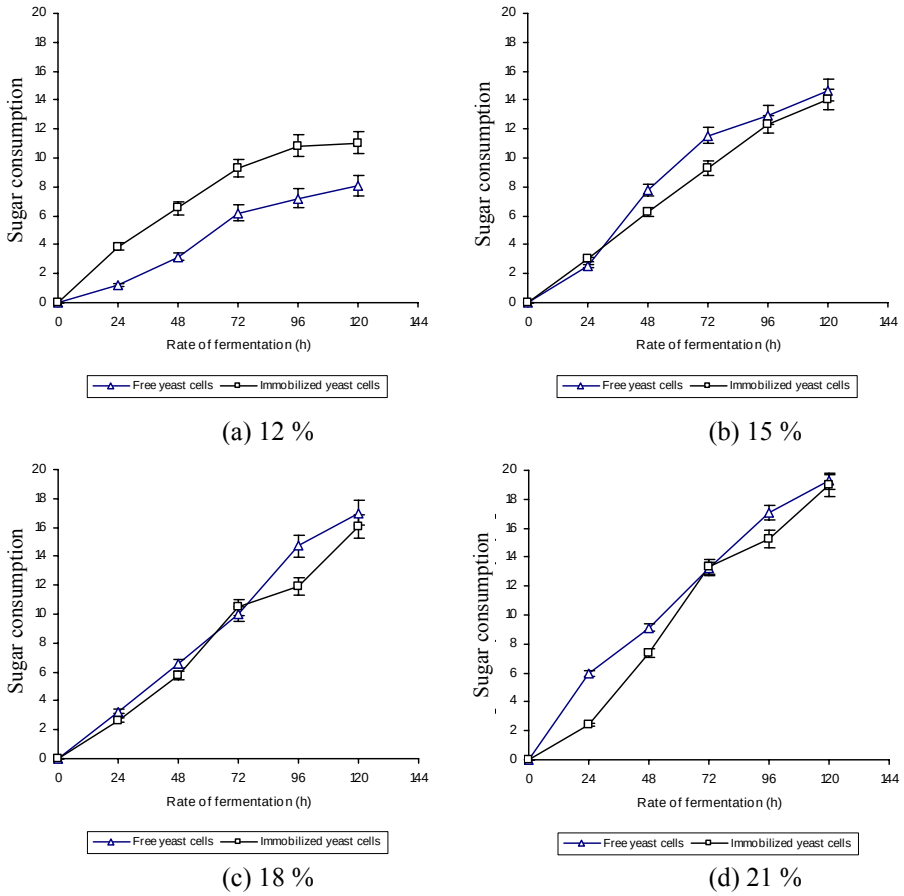


Fig. 5. Effect of different initial sugar conc. on ethanol production by free and immobilized *S. cerevisiae* GC-IIB31 under stationary culture.
Incubation temperature 30°C, initial pH 4.5. The standard error bars indicate the standard deviation (±sd) among the three parallel replicates calculated at 5.0 % level.

Discussion

In the present study, *Saccharomyces cerevisiae* GC-IIB31 was used as an organism of choice for nutritional studies. The parameters used during the course of study were incubation period with free and immobilized *S. cerevisiae*, reuse of immobilized yeast cells, initial pH, incubation temperature, initial sugar concentration, initial volume of fermentation medium. Reports have been published on the production of ethanol under stationary conditions (Tyagi & Ghose, 1982; Roukas 1996). In the present study, cane molasses was used as the basal fermentation medium. Cachot and Marie-Noelle (1991) treated cane molasses with sulfuric acid and got encouraging results. Other workers have also reported cane molasses as the best raw material for enhanced and consistent yields of ethanol (Hamdy *et al*., 1992; Kiss *et al*., 1999). A number of reports have been published on the production of ethanol submerged fermentation techniques using different strains of yeast. Cultural conditions for ethanol production vary from strain to strain and also depend

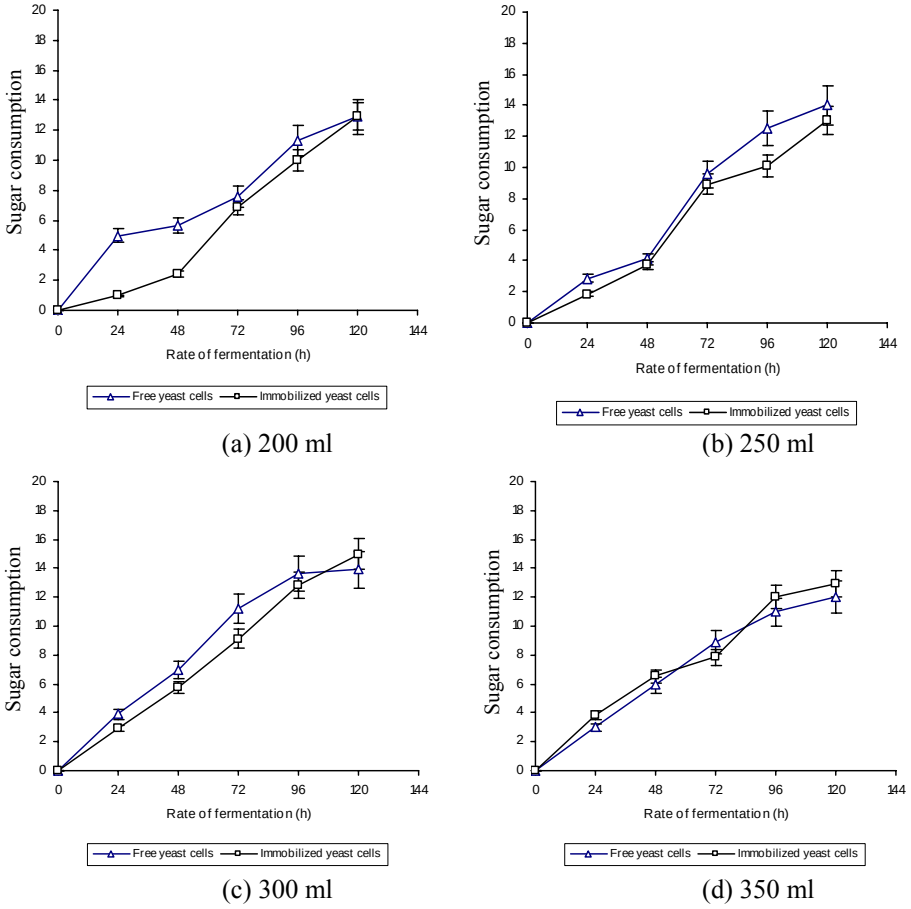


Fig. 6. Effect of different volume of fermentation medium on ethanol production by free and immobilized *S. cerevisiae* GC-IIB31 under stationary culture.
Incubation temperature 30°C, initial pH 4.5, sugar conc. 15%. The standard error bars indicate the standard deviation (±sd) among the three parallel replicates calculated at 5.0% level.

on the type of process adopted. Among all the yeast *S. cerevisiae* was proved more successful for ethanol production as compared to other species (Ergun & Ferda, 2000). This is due to the fact that some species adopt different metabolic pathways by having special genes or special enzymes such as invertase genes and invertase enzymes respectively for the conversion of sugars to ethanol or other metabolites (Fregonesi *et al*., 2007).

Sugar concentration is also critical to this fermentation and influencing the rate of production and the final yield in addition to physiological growth of yeast. Initial sugar concentration has also been found to determine the amount of alcohol. In the present study, maximum ethanol production was obtained in the medium containing 15% sugar contents by both free and immobilized yeast *S. cerevisiae*. Free cells gave ethanol yield 6.49%, while immobilized cells gave 5.85% ethanol yield. The reduction in the ethanol fermentation was observed with the increase in sugar concentration. It might be due to the fact that medium viscosity was increased because of higher sugar concentration, which

resulted in the decreased metabolism, hence reduction in the ethanol production. Thus, level of 15% sugar contents was found to be suitable for ethanol production as an agreement with the work reported earlier (Amutha & paramasamy, 2001; Monte *et al*., 2003).

Immobilized yeast cells were used in the consecutive six batches. Maximum ethanol yield (7.50%) was obtained in the fourth batch. Ethanol yield then decreased in the fifth and sixth batch. It might be due to fact that immobilized cells were stable for four batches, thereafter, the ethanol productivity was decreased with the extent of cell leakage and gel beads become fragile and deformed in shape. Repeated batch fermentation has advantage of improving ethanol productivity reducing the tine of inoculum preparation (Carvalho *et al*., 1993; Kourkoutas *et al*., 2004; Haq *et al*., 2005). 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 rate of ethanol production by yeast cells is highly affected by the pH of the fermentation medium. *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). Results showed that the maximum rate of sugar conversion to ethanol by the free and immobilized cells was achieved with medium pH 4.5. Productivity was decreased by increase and decrease in pH due to the lower metabolic rate of the yeast cells. It may also be due to the growth of other microbes with the increase in pH, as the fermentation was carried out without sterilization (Amutha & Paramasamy, 2001; Kourkoutas *et al*., 2004). In addition, pH of the surrounding medium change the configuration and permeability of the cell membrane thus reduced the rate of sugar fermented enzymes.

In the present study, the optimal temperature for growth and ethanol productivity was found to be 30ºC. However, at slightly higher temperature growth rate, yield of ethanol and the death rate may be adversely affected. Some strains of *S. cerevisiae* and *Kluyveromyces marxiamus* have also been reported, capable of growing and fermenting cane molasses at 40=45 c under batch conditions (Wang *et al*., 1985; Cachot & Marie-Noelle, 1991; Amutha & Paramasamy, 2001). The mechanism of cell inhibition by ethanol and sugar probably depend upon temperature. The relationship of initial sugar concentration with yeast cells at different incubation time has been investigated (Sritrakul1 *et al*., 2007). In the present study, on the basis of ethanol yield 120 h was found to be optimal for maximal production by both free and immobilized yeast cells. It night be due to the fact that the time necessary to complete batch fermentation of sugar cane molasses to ethanol is correlated with the initial sugar concentration and yeast cells (Kaur & Kocher, 2002; Willaert & Viktor, 2006). Different volumes of fermentation medium were taken in 500 ml flask. Fermentation medium with 300 ml volume gave maximum ethanol, while others gave low results. It may be due to availability of oxygen in the vacant space between the mouth of bottle and fermentation medium.

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 (7.50%) was obtained after 120 h of incubation. Sugar (15%), initial pH (4.5), temperature (30°C) and volume of fermentation medium (300 ml in 500 ml Erlenmeyer flask) were also optimized. Immobilized yeast cells were used up to six batches and maximum results were obtained in the 4^{th} batch. However, further work is still needed on the recovery of ethanol from the fermented broth and to improve the substrate consumption rate by the organism at higher substrate levels.

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