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BIOETHANOL PRODUCTION FROM MOLASSES BY USING *SACCHAROMYCES CEREVISIAE* IMMOBILIZED IN CA-ALGINATE GEL BEADS

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ABSTRACT

On account of limited global supply of oil, ethanol has emerged as an alternative for petroleum based liquid fuels. Now a days, its use in automobiles as an alternative fuel has attracted worldwide attention for its production on a large scale while maintaining the economic status of a country. In present state of energy crises, efforts are being made to reduce the dependence upon nonrenewable energy sources, one of which is fuel alcohol produced by fermentation of agricultural/agro-industrial wastes and byproducts. In the present work, studies were carried out on the ethanol production by immobilized *Saccharomyces cerevisiae* under stationary culture. Cane molasses was used as sugar source for maximum conversion of reducing sugar into ethanol. The substrate was optimized after maintaining the different hrs i.e. 24, 48, 72, 96 etc., medium pH (5.5), incubation temperatures (25-30^{0}C), volume of fermentation medium (300 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 immobilized yeast cells. The results indicated that yeast on utilizing molasses at 48.3% sugar level with medium pH 5.5 at 30°C and 300 ml fermentation volume in 500ml Erlenmeyer flasks gave maximum ethanol production with the immobilized yeast cells. Maximum ethanol production by immobilized yeast cells was obtained at 96hr after which it declined markedly.

INTRODUCTION

Due to the diminishing fossil fuel reserves, alternative energy sources need to be renewable, sustainable, efficient, cost-effective, convenient and safe[1]. Bioethanol produced from renewable biomass has received considerable attention in current years. There has been an increasing interest in utilizing alternative sources of energy. Ethanol is a natural component of alcoholic beverages and its use has seen continued growth since the late 1970s, when it was used as a product extender due to gasoline shortages caused by the OPEC oil embargoes.

Alcoholic fermentation has been carried out using a number of sugary materials depending upon their availability and suitability in particular geographic situations. Various raw materials like sugarcane juice and molasses[2,3], sugar beet, beet molasses[3,4], sweet sorghum[5] and starchy materials like sweet potato[6], corn cobs and hulls[7,8], cellulosic materials like cocoa, pineapples and sugarcane waste[9] and milk/cheese/whey using lactose hydrolyzing fermenting strains[10,11] have been reported. Of these, simple sugar bearing materials are the easiest to process, since the yeast ferment these directly while other carbohydrates like starch/cellulose have to be first hydrolyzed to fermentable sugars using current commercial technologies (physio-chemical/enzymatic preparation) before they can be fermented to yield ethanol. Dabas *et al*[12] studied ethanol production from wheat starch. Hydrolyzed wheat starch was used as a substrate for ethanol production using two strains of *S.cerevisiae*. Wheat flour slurry (25% w/v) was gelatinized and conditions were standardized for saccharification and fermentation of wheat starch for ethanol production. Ethanol in India and other developing countries is mainly produced by fermentation of dilute molasses at ambient temperature of 25-35°C employing *Saccharomyces cerevisiae*[3,13]. Cane molasses is a complex mixture that varies in composition according to geographical sources, agricultural practices and sugar mill operations. Yadav *et al*[14] studied the effect of pretreatment of sugarcane molasses for ethanol production by yeast.

Recently, immobilized biomass activity has been given more attention, since it has been acknowledged to play a significant role in bioreactor performance[15]. Frequently, immobilized cells are subjected to limitations in the supply of nutrients to the cells. Thus, because of the presence of heterogeneous materials such as immobilized cells, there is no convective flow inside the beads and the cells can receive nutrients only by diffusion[16]. Immobilization of cells to a solid matrix is an alternative means of high biomass retention. The cells divide within and on the core of the matrix[17]. Immobilized cells exhibit many advantages over free cells, such as relative ease of product separation, reuse of biocatalysts, high volumetric productivity, improved process control and reduced susceptibility of cells to contamination[18]. Among the different cell immobilization techniques, entrapment in calcium alginate gel has been one of the most used matrices for whole cell entrapment due to its simplicity and non-toxic character. This simple and mild immobilization technique involves the drop-wise addition of cells suspended in sodium alginate onto a solution of calcium chloride whereon the cells are immobilized in precipitated calcium alginate gel in the form of beads[19]. Entrapment in calcium alginate gel beads has been applied for the immobilization of a large number of different cells such as bacteria, cyanobacteria, algae, fungi, yeast, plant protoplasts, and plant and animal cells[20]. Several studies have described continuous ethanol production using Ca-alginate immobilized yeasts with different bioreactor configurations[21,22]. In these studies, the most commonly used bioreactors are

the continuous flow stirred tank bioreactor, fluidized-bed bioreactor and packed-bed bioreactor. Packed-bed bioreactors have become very popular in recent years due to their low manufacturing and operating costs and also due to the ease of process automation in these reactors[23].
Alginate is widely used in food, pharmaceutical, textile, and paper products. The uses of alginate utilized in these products are for thickening, stabilizing, gel and film forming. Sodium alginate is a linear polysaccharide, normally isolated from many strains of marine brown seaweed and algae. Thus the name alginate, the copolymer consists of two uronic acids or polyuronic acid. It composed of primarily of D-mannuronic acid (M) and L-glucuronic acid (G). Alginic acid can be either water-soluble or non-water soluble depending on the type of the associated salt. Interchanging of sodium ions with calcium ions in the solution may follow solidification of sodium alginate in calcium chloride solution. The sodium salt, other alkaline metals and ammonia are soluble in water, whereas the polyvalent cations salts, e.g., calcium, are not water-soluble, except the magnesium ions. The immobilization of living cells facilitates the separation of cells from media as well as their recirculation. Immobilized yeast-cell technology has found various applications, particularly in ethanol production[24,25]. Immobilized *Saccharomyces cerevisiae* cells displayed an increase in ethanol productivity compared to free cells[26]. However, Senac and Hahn-Hagerdal found no improvement of the productivity after cell immobilization[27]. On the other hand, immobilization of *C.tropicalis* in agarose lowered ethanol yield and productivity, indicating that the physiology of immobilized cells differ from that of free cells[28]. The difference in behavior between immobilized and free cells is related to several factors. Nutrient limitations and microenvironment surrounding the cells are widely used to explain physiological and morphological changes of cells after immobilization[29].

The aim of the present study was to investigate continuous ethanol production from molasses by using *saccharomyces cerevisiae* immobilized in calcium alginate gel beads. The process was carried out in a vertical packed-bed bioreactor with beads of Ca-alginate in which *Saccharomyces cerevisiae* cells were immobilized.

MATERIALS AND METHOD

Microorganism and substrate

Compressed bakers' yeast, *Saccharomyces cerevisiae* (Pakmaya Yeast Co., Üzmir), was used throughout this investigation. The organisms were maintained in 250 ml Erlenmeyer flask containing 100 ml sterilized medium (MYGP: Malt extract, 0.5%; Yeast extract, 0.5%; Glucose, 2%; Peptone, 0.5%) and the pH was adjusted to 5.5 by dilute HCl or NaOH. It was cultured for 24 h at 30°C in an incubator.
Cane molasses used throughout the study was supplied by Sakti Sugars, Dhenkanal'. The molasses have the following composition: moisture, 23.5%; total sugar (glucose, fructose, sucrose and maltose), 48.3%; crude protein, 6.30; total ash, 10-12%; undetermined solids, 15-20%; and pH, 5.0-5.5.

Cell immobilization

To carry out immobilization, 2% of $CaCl_2$ solution was prepared and kept at 4^0C for chilling. 30-40ml of previously grown culture of *S.cerevisiae* was centrifuged at 500rpm for 15 minutes. The supernatant was discarded and the pellet washed with saline water. Again centrifugation was

carried out at 500rpm for five minutes to obtain the final pellet that was washed and then air-dried and weighed. The next step was to dissolve 2g of sodium alginate in hot water with constant stirring on magnetic stirrer. After cooling sodium alginate solution, 2g of yeast biomass was 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.

Cell growth and cell leakage

The immobilized cells, separated after fermentation, were reused for successive four batches. The biomass of immobilized cells in calcium alginate was determined by dissolving the gel beads in a 4% (w/v) EDTA solution, the beads were aseptically crushed by a sterile glass rod with sterile water. Finally the reading was taken at 550 nm against a suitable curve of absorbance versus dry weight[30]. The biomass of free cells was determined likewise. The cells leaked from the gel matrix were determined by plate count using Potato Dextrose Agar, incubated at 30ºC for 24 h. The fermentation kinetics was studied as per the formulae given by Bailey and Ollis[31].

Estimation of Total Sugar from Molasses

The amount of total soluble sugars present in the sample was estimated by Anthrone reagents. There is no need to hydrolyze the sample[32].

Analytical methods

At 24 h interval, fermented broths (in triplicate) were removed and the contents were analyzed for total sugar and ethanol. The ethanol content of the fermented broth was determined by measuring specific gravity of the distillate according to the procedure described by Amerine and Ough[33]. The total sugar was assayed by Anthrone method[32]. The pH was measured by a pH meter (Systronics, Ahmadabad, India) using glass electrode. The yeast cell population was counted using a haemocytometer.

Statistical analysis

The data of ethanol yield using immobilized cells were analyzed using one way ANOVA. Where significant difference in ANOVA ($p<0.05$) was detected by the Fisher's Least significant Difference (LSD) multiple comparison test which was applied to compare the factor level difference. The analysis was performed using MSTAT-C (version 2.0, Michigan State University, Michigan, USA).

RESULTS AND DISCUSSION

An upsurge of interest in cell immobilization for alcoholic beverages and potable alcohol production has been taking place recently[34]. This is mainly due to the numerous advantages that cell immobilization offers including enhanced substrate utilization and fermentation productivity, feasibility of continuous processing, lower cost of recovery, recycling and downstream processing[35,36]. Cell immobilization may also protect cells against shear force, less susceptible to the effect of inhibitory compounds and nutrient depletion[37]. A number of carrier materials (agar agar, Ca-alginate, k-carrageenan etc.) have been used for entrapping microbial cells for

production of various bio-products, i.e. ethanol[34,35,38], amino acids[39,40], enzymes[41,42] and organic acids[43,44]. Among these, entrapment in Ca-alginate beads is found most suitable in majority of studies as this matrix is cost effective, procedure is simple and easy to handle[37,45,46]. In the present study, immobilized cells of *Saccharomyces cerevisiae started* their growth in the log phase and maximum ethanol production was achieved during the stationary phase(96h)(Table-1).

Table-1: Showing the Bioconversion of sugar in to ethanol during the course of fermentation period

Time	Ethanol ml/50g	Ethanol ml/kg	Ethanol %	100% Ethanol ml/kg	100% Ethanol g/kg	Total Sugar g/kg
0						880
24	10.5	210	70	147	132.3	410
48	13	260	77.5	201.5	181.35	156
72	12.5	250	87.5	218.75	196.88	114
96	14	280	85	238	214.2	67.8

Fig-1: Showing simultaneous decrease in total sugar with increase in ethanol

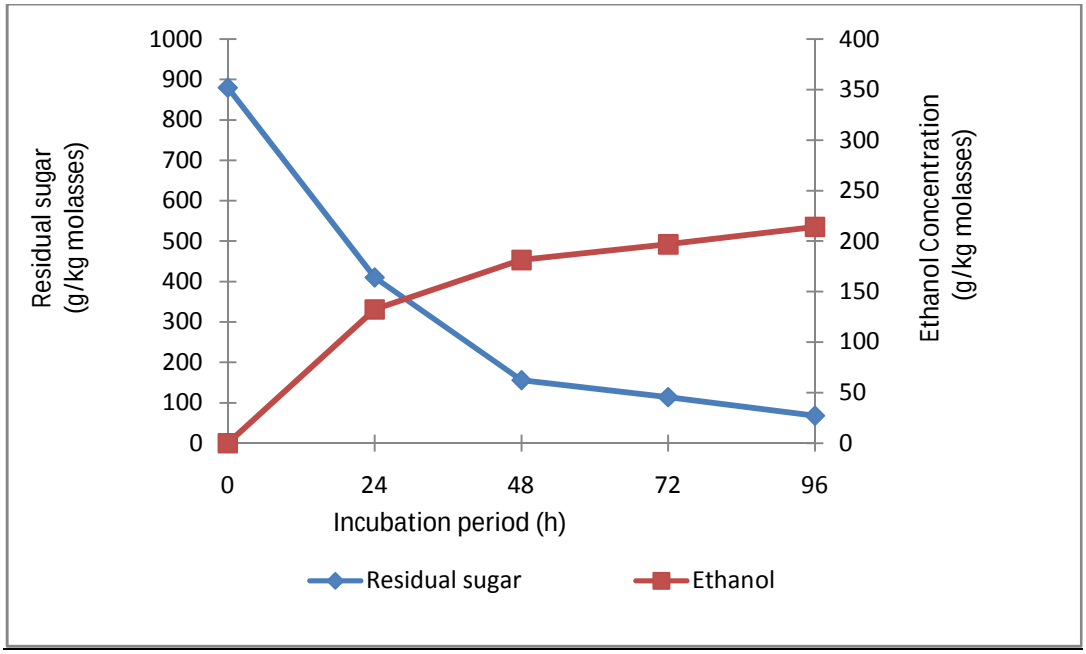

Table-2: Growth and fermentation kinetics of *Saccharomyces cerevisiae* cells immobilized in Ca alginate gel beads

Parameters	Kinetics
Ethanol yeild($Y_{p/s}gg^{-1}$)	0.264
Volumetric substrate up take ($Q_s gl^{-1}h^{-1}$)	1.41
Volumetric product productivity (Q_p, $gl^{-1}h^{-1}$)	0.372
Final ethanol (P,gl^{-1})	35.7
Conversion rate(%) in to ethanol	52.74
Specific Growth rate (μ,h^{-1})	0.098
Cell Yeild(Y×15, gg^{-1})	0.032
Final Biomass $conc^n$(X, gl^{-1})	4.35

Initially there was a fall of 53.40% in total sugar concentration over initial content with simultaneous production of 132.3g/kg ethanol up to 24 hr of fermentation using immobilized cells of *S.cerevisiae*. The decrease in sugar reserve might be also due to its utilization in part, for growth and metabolism of micro organism in addition to its conversion in to ethanol[47]. After 24hr, there was a gradual increase in ethanol concentration over the incubation period with simultaneous decrease in total sugar (Fig-1).

The results show that there was 52.74% sugar utilization at the end of 96 hr incubation period using immobilized yeast cells. 30kg of fermentable sugar (as glucose) yield 15-20kg of ethanol. Further, there was a statistically significant difference (Fischer's LSD test) found on ethanol yield after 96 hr using immobilized yeast cells. Swain *et al*[48] reported 6.3% increase in ethanol yield from molasses fermented with Ca-alginate entrapped yeast cells over free cells. In general, 10–20% increase in ethanol yield has been reported in immobilized cells (entrapped in matrices such as Ca-alginate, luffa sponge, agar agar, etc.) over free cell for various bio-products production[49,50]. In this study, the immobilized cells were further recycled for three more times limiting the duration of each fermentation cycle up to 96 hr as most of the sugars in molasses were consumed during this period. The cells not only survived but also active physiologically yielding ethanol 213.9, 211.2, 209.7g/kg molasses, respectively showing 0.27, 1.53 and 2.23% decrease in 2^{nd}, 3^{rd}, 4^{th} cycles over 1^{st} cycle of 96hr fermentation. This might be due to leakage of cells from Ca-alginate matrix. Further in the first cycle of operation the leakage of cells from immobilized support was negligible (<5%); hence, the observed ethanol production was presumed mainly because of the action of immobilized cells. So, the advantage of using

immobilized cells was that the cells survived and were active on the support used for immobilization for three cycles of fermentation, which could save considerable time and energy. Cell immobilization with Ca-alginate was found to be marginally superior (Fisher's LSD test $p<0.05$) than as per ethanol production is concerned. Although Ca-alginates do not rank among highly mechanically persistent matrices[37], the mechanical stability of Ca-alginate beads (prepared in 0.1 M $CaCl_2$ and hardened for 24 h) was good enough than that of others since no fragments of alginate beads were found in fermentation broth up to three cycles in our experiment. The other advantage is Ca-alginate gel forms rapidly in very mild conditions as compared to other methods[37]. The growth and fermentation kinetics of immobilized cells were also studied (Table-2).

The ethanol concentration (P) obtained with immobilized cells was 35.7g/l, where as the volumetric substrate uptake (Qs) was found to be 1.41g/lh. Likewise, the ethanol yield by immobilized cells was found to be $0.264gg^{-1}$. The volumetric product productivity (Qp) and final sugar to ethanol conversion rate (%) of ethanol with immobilized cells were found to be 0.372g/lh and 52.74% respectively. The final biomass concentration (X) in immobilized cells was $4.35gl^{-1}$. Bioethanol production from molasses is a very easy and inexpensive method, has an advantage over other sugar rich materials like mahula flowers as there is difficulty over collection of these flowers from the forests, its transportation charges and storage, which together account for higher cost of ethanol production[47,48]. So using molasses for the production of bioethanol is more preferred method. Similarly, the conversion of starchy biomass from cassava[30,51,52] and sweet potato[53,54] involves complicated steps such as liquefaction (conversion of starch to dextrin units) and saccharification (conversion of dextrin units to sugars)[35] before fermentation by alcohol producing yeast strains; these steps enhance production cost of ethanol (in terms of energy consumed and extra- time period taken) in comparison to ethanol production from sugar crops. Bioconversion of lingo-cellulosic biomass to sugars is a much more complicated process that require break down of lignin, cellulose and hemicellulose fractions by application of multi-enzyme (cellulase, ligninase, glucosidase) complex using a series of bio-reactors[35,55]. In this context, bio-ethanol from molasses can be highly economical in comparison with either starchy or lignocellulosic biomass.

CONCLUSION

The results indicated that the immobilization of *S.cerevisiae* possesses the capacity not only to utilize high concentration of sugar but also to yield higher ethanol productivities during the course of continuous fermentation. It is clear from the findings in the present investigation that there would be a potential application for utilizing the entrapment of *Sacchromyces cerevisiae* cells on Ca-alginate matrices is a promising method of cell immobilization for ethanol production from sugarcane molasses.

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