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Immobilization of *Saccharomyces cerevisiae* on Corn Cob Matrix for Ethanol Production

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ABSTRACT

Economical and technical advantages of immobilized yeast in alcoholic fermentation as compare to free cell system, has made the study of immobilization technique gain tremendous importance. This paper deals with immobilization of Saccharomyces cerevisiae strain (NCIM 3455) on grind corn cobs for alcoholic fermentation. Corn cobs are abundant, inexpensive, reusable, nontoxic, cellulosic biomaterial with high porosity, which permits easy flow of substrates and products between carrier and medium. In thisstudy, corn cobs were mechanically treated followed by drying, acidic hydrolysis and alkaline hydrolysis for delignification. Immobilization was carried out on both treated and untreated corn cobs by adsorption technique. Immobilized cells were found to have increased alcohol production rate and higher stability as compared to free cells. Alcohol production by immobilization of yeast was done using real substrate, sugarcane molasses. The immobilized cells showed a reusability of about 5 batches.

KEYWORDS: Immobilization; corn cobs; adsorption; Saccharomyces cerevisiae; ethanol

INTRODUCTION

The depleting reserves and competing industrial needs of petrochemical fuels, has led to emphasis on increasing production of alcohol fuels by fermentation process.The use of fuel ethanol can reduce the toxic exhaust emissions and greenhouse gases from vehicles.[1]Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrateand suitable process technology.[2] The fermentation feedstock has been mostly sugarcane molasses in tropical countries like India. Sugarcane molasses is black residue industrial waste obtained after sugar production from sugarcane juice. Itcontains about 45-50% total reducing sugar mainly inthe form of sucrose.There is a need of technology where it can be converted efficiently into ethanol.

One such technology is use of cell immobilization. Cell immobilization has various economic advantages compared to free cell system in many ways. Immobilized cell system can be reused which reduces the cost of recovery. It also helps in protecting cells from the toxic effects of low pH, temperature, osmotic, inhibitors, etc. and thereby increasing ethanol yield and reducing the costs required for inoculation development.[3]

One of the cell immobilization techniques is adsorption. In adsorption, the biocatalyst is adsorbed and bound on a solid water insoluble support. A variety of supports have been used for immobilization of yeast cells. Some inorganic supports for adsorption mentioned in literature are chrysotile,[4] clay aggregates, polyurethane foam,[5] porous silicate glass etc.[6]Adsorption of yeast cellshas also been reported on organic supports such as cellulosic biomass e.g. sugarcane pieces, corn stem ground tissue,[7,8] sweet sorghum,[1] wine making residues[9] and spent grains.[10] Supports such as bacterial cellulose membrane[11] have also been used for yeast cell immobilization.In one of the studies carried out by Lee S.E. et.al.[12]delignified corn cob grits have been used as a support matrix for immobilization of yeast cells.

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According to Mohammad A. et. al.[13]the ethanol production can be enhanced using strains tolerable to high sugar content. Hence, in present study the strain *Saccharomyces cerevisiae* NCIM no. 3455 having an application for molasses degradation on a support matrix of groundcorn cobs have been. Study of adsorption of this strain on corn cobs matrix is not reported earlier.

MATERIALS AND METHODS

Microorganism and its maintenance:

Saccharomyces cerevisiae strain used in the study ordered from National Collection of Industrial Microorganisms(NCIM), Pune, Maharashtra having NCIM no. 3455. The *Saccharomyces cerevisiae* was maintained at 4˚C on MGYP agar slants. The composition of MGYP agar is (g/L): malt extract 3, glucose 10, yeast extract 3, peptone 5, agar 20, (pH 6.4-6.8). The culture was maintained by sub-culturing after every 15 days and then preserving the agar slant at 4˚C.

Inoculum preparation:

The inoculum was prepared by culturing the microorganism in medium of following composition as stated by Ranulfo M.A. et. al.[4](g/L): Molasses 30, yeast extract 5, KH_2PO_4 5, NH_4Cl 1.5, $MgSO_4$ $7H_2O$ 0.7 and KCl 1.7.The Erlenmeyer flask was incubated at 30˚C in laboratory incubatorat 110 rpm. Optimum age of yeast cells for maximum reduction of molasses to ethanol was determined. Fermentation was carried out by inoculating yeast cells at different age as 3, 6, 7, 9, 11, 13, 14 days. It was observed that yeast cells were reducing maximum molasses at the age of 9^{th} to 11^{th} days. The liquid culture in this phase was further used as inoculum for further work.

Pretreatment of corncobs to be used as support for immobilization:

Corn cobs were obtained from local market of Pune. The corn cobs were grind and dried until constant weight at about 60˚C, the grind corn cobs were then sieved and cobs having particle size between 1-3 mm were used in the experimentation to maintain uniformity in size during the whole experimentation. Pretreatment of corn cobs was carried out as mentioned by Zlatina G. et al.[9]1g corn cobs were treated with 15ml dilute hydrochloric acid of concentration 3%, 5% and 7% (v/v) in a beaker. The beakers were kept for 2 hours in hot air ovenat 60˚C and then the corn cobs were washed with distilled water totill it shows pH of 6.5-6.8. Then 1g corn cobs were soaked in 10ml of 2% (w/v)sodium hydroxide solution for around 20-24 hours at 30˚C in shaking condition at about 90rpm. These treated corn cobs were again washedtillit attains pH of 6.5-6.8. Drying of these treated corn cobs was done to remove its water content and then used for immobilization.

Hydrophobicity measurement

According to Toshiyuki Nomura et. al. hydrophobicity on the surface of microbes in a medium is an important characteristic for immobilization on support matrix.Surface hydrophobicity of matrix and microbial cells was determined by microbial adhesion to hydrocarbon [MATH] assay.[14] Cells were collected by centrifugation and then were washed with distilled water. Cells were then suspended in PUMbuffer (K_2HPO_4 22.2g/L, KH_2PO_4 7.26g/L, Urea 1.8g/L, $MgSO_4.7H_2O$ 0.2g/L, pH 7.1). 4.8ml of this suspension was taken in a test tube to which 2.4ml of hydrocarbon (n- hexane) was added. This solution was then vortexed (REMI CM 101 Cyclomixer) for 2min and allowed to stand for about 15min till phase separation is achieved. Absorbance of the aqueous phase was measured at 400nm using UV spectrophotometer before and after hydrocarbon addition. Hydrophobicity of microbial cells and matrix was calculated using the following equation:

$F = (1 - At/A0) \times 100$ (1)

Where;

A0 is the initial absorbance of the microbial suspension before mixing, and

At is the absorbance after mixing.

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Immobilization of cells on support:

Pretreated and untreated corn cobs were used as support matrix for cell immobilization. The supports were first autoclaved (121°C, 15 min). 1 g corn cobs were soaked in 100 ml medium having the composition same as that of inoculum medium and 10ml of inoculum cell culture was added to it in a 250 ml Erlenmeyer flask. The cells were allowed to grow and naturally adsorb on support particles by incubating the flask for 44 hours in shaking incubator (90rpm, 30˚C). The cells immobilized on support were collected by decanting the liquid culture and gentle washing of the support with liquid media but devoid of sugars to remove any unbound cells. The cells immobilized on the support particles were further used for fermentation.

Fermentation:

Cell immobilized on corn cobs were applied for ethanol production by carrying out fermentation in media having composition (g/L): Molasses with initial sugar concentration in media is 20, yeast extract 1, $(NH_4)_2SO_4$ 0.5, $MgSO_4$ $7H_2O$ 0.025, and NaH_2PO_4 0.1386. The fermentations were carried out under static incubator (Hosp Corporations, Mumbai - 63) at 30˚C for 48 hours for both immobilized cells and free cells. After each batch broth was centrifuged for 10 minutes, 5000 rpm 4-10˚ C to collect the supernatant, ethanol produced was determined by potassium dichromate assay as given by Somdatta C. et. al.[15] Sugar remained in a broth was determined by dinitro salicylic assay.

For repeated batch fermentation, the fermented media was decanted, the supports were gently washed with media devoid of sugars and fresh media was added to the flask. The fermentations were carried out in 250 ml Erlenmeyer flasks containing 100ml media and 1g support having yeast cells immobilized on it. For free cell fermentation, the fermentation media was inoculated with 10 ml of inoculum culture.

Sugar estimation:

As the carbon source used in the work is molasses, the amount of sugar present in molasses before fermentation was determined. Acid hydrolysis of molasses was done using 6N HCl. Reducing sugar concentration in molasses was determined by 3,5-dinitrisalicylic acid methodas given in textbook ‘An Introduction to practical biochemistry’ by Plummer M.[16] The sugar concentration of molasses was calculated using standard calibration curve.

Ethanol estimation

The ethanol productivity (P) in the fermented media was determined spectroscopically using potassium dichromate method.The fermented culture is centrifuged. 1 ml of the supernatant is taken in a 25ml test tube and diluted with distilled water to a volume of 5 ml. 1ml of 10% Potassium dichromate solution is added followed by addition of4ml Concentrated sulfuric acid. The tubes were then cooled in ice bath and absorbance was measured at 660nm at room temperature. The blank test tube was carried out without supernatant. The ethanol yield (Y_p) as given by Brooks A.A. was calculated as the actual ethanol produced and expressed as g ethanol per g total sugar utilized (g/g). The fermentation efficiency (Ey) yield is expressed as the percentage of ratio of ethanol yield to the ethanol theoretically produced in the biochemical conversion of the sugar into ethanol.

$$\mathrm{Yp} = \frac{\text{Ethanol produced in grams}}{\text{Total sugar consumed in grams}}$$

$$\mathrm{Ey} = \frac{\text{Obtained ethanol yield}}{\text{Theoretica ethanol yield}}\ \%$$

Scanning electron microscopy

Immobilization of yeast cells on the corn cobs matrix was confirmed by scanning electron microscopy. For this yeast cell,immobilized corn cobs were first washed with sterile distilled water and then dried in hot air oven (60˚ C, 24 hours) This dried samples were fixed on a specimen holder and then sputtered with platinum;

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then micrographs were obtained using an analytical scanning electron microscope (JEOL JSM 6360A) at Savitribai Phule Pune University Pune, India. Each sample was examined at 1000X magnification.

RESULTS

Total reducing sugar estimation in molasses:

The total reducing sugar in molasses used for fermentation was determined periodically at regular intervals of 30days during the whole study and it was observed that there was no significant loss of sugars during storage of molasses in refrigerator at 4˚C. It was found to be in the range of 46-48% consistently preservation of molasses during for period of6 months.

Effect of pretreatment on corncobs:

The hydrophobicity of treated and untreated matrix was determined as the capacity tobind hydrocarbon. The hydrophobicity is expressed as MATH F % value as shown in tableno 1.

Table 1: Hydrophobicity of cells and support matrix measured by MATH method.

Sr. no.	Material	Absorbance before hydrocarbon addition 400 nm	Absorbance after hydrocarbon addition 400nm	MATH F %
1	Cells	0.319	0.235	26.33
2	Untreated matrix	0.485	0.439	9.48
3	Treated matrix with 3% HCl	0.041	0.041	0.0

Study of different concentration of acid from 3% to 7% was also done, however no significant effect was observed in higher concentrations, hence 3% HCl was used further. It is seen from the abovetable that MATH F% value for treated matrix is 0.0, which concludes that there are very less or no hydrophobic groups present on the surface of matrix.

Confirmation of immobilization by SEM

Immobilization of *S.cerevisiae* (NCIM 3455) on corn cob matrix was confirmed by scanning electron microscopy (SEM). Figure 1 shows the Scanning electron micrographs of the treated corn cobs before and after immobilization.

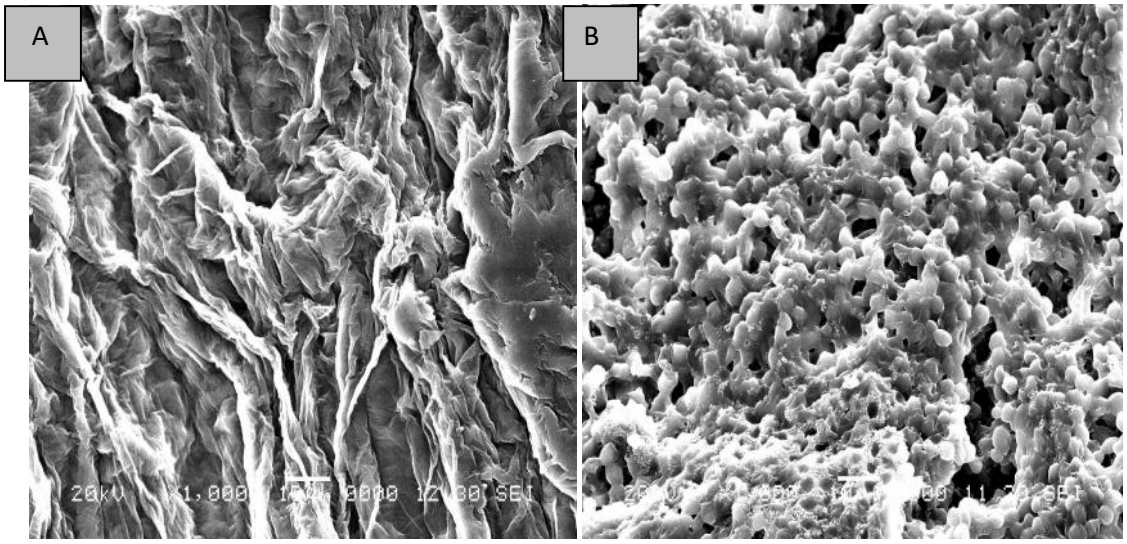

Figure 1: Scanning electron micrographs: (A) Treated matrix before immobilization (B) Cells immobilized on treated matrix.

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It can be also noticed from the micrographs that the corn cobs have a rough surface which provides a larger area for immobilization.

Repeated batch fermentation:

The reusability of the yeast cells immobilized on the corn cobs matrix was examined by carrying out repeated batch fermentation. Fermentation was carried out for both treated and untreated corn cobs up to 5 repeated batches. The results for first and fifth batch are shown in tableno 2.

Table 2: Fermentation parameters for repeated batch fermentation for untreated and treated matrix.

Batch no.	Time (t) min	Residual sugar g/L	Conversion (X) %	Ethanol Productivity (P) g/L	Ethanol yield (Y) g/g	Standard Deviation in ethanol production over 5 batches
Untreated corn cobs						
1	48	2.63	86.85	9.05	0.45	0.81
5	48	4.14	79.30	9.66	0.48	
Treated corn cobs						
1	48	2.70	86.5	8.05	0.40	0.74
5	48	4.11	79.45	9.24	0.46	
Free cells						
1	48	4.785	76.05	8.67	0.43	

Standard deviation in ethanol yield was calculated in Microsoft office excel considering all five batches of fermentation. It is observed in the above table that standard deviation in ethanol yield in case of treated as well as untreated matrix is less, which predicts that conversion of sugar to ethanol is consistent in all five repeated batches. Hence in both the systems it can be said that the immobilized biocatalyst is stable up to 5 batches.

DISCUSSION

In the present study, corn cobs matrix was pretreated with acid followed by alkali to reduce hydrophobicity of matrix. According to Zlatina G *et. al.,* 2011,pretreatment of matrix did not much improve the cell's adhesion to matrix, but work done here in this paper shows physical changes in the matrix. Treated matrix settled faster than untreated matrix which makes condition favorable for flocculating strains of yeast.Through this treatment, delignification of the corn cobs occur which has an effect on the hydrophobic characteristics of the matrix. Natural cellulose materials contained a large number of hydrophilic groups in the form of positive charge, ready for the absorption of negative charged cells.[3]

Zlatina *et al.* have reported reusability of immobilized *Saccharomyces cerevisiae* NP 01 cells on sweet sorghum stalks using sorghum juice as carbon source for about 8 batches. Vesna M.V. et.al.[17]used sugar beet pulp as a support for yeast immobilization where repeatability was seen upto seven batches but decrease in ethanol yield was observed after three batches. Whereas in this study, reusability of immobilized yeast cells on corn cobs matrix was observed upto 5 batches and ethanol yield was consistent.According to the Gay Lussac equation, theoretically, fermentation produces 511 g of ethanol and 489 g of CO2 from 1000 g of glucose. However, in industrial processes this fermentation yield is not achieved because yeast cells drive sugars to the production of cellular biomass and secondary Compounds.[18]The yield of ethanol obtained in this process is in the range of 0.47±0.02 (g/g) which is in relevance with the Gay Lussac equation. Similar range of ethanol yield was obtained by Zlatina G.*et al.*using another yeast strainandVesna M.V. et. al.using sugar beet pulp as matrix and molasses as carbon source. Immobilization of this strain *Saccharomyces cerevisiae* (NCIM 3455) on corn cobs matrix for ethanol production has not been reported earlier.

Comparing free yeast cell fermentation as shown in table no. 2 with the average performance of immobilized cell system using treated corn cobs it can be said that immobilized cell system is advantageous as the overall

ethanol productivity is higher comparing fermentation using free cells. Reusability of the microorganism is the major advantage of using immobilized cell system over free yeast cells.It ultimately impacts on the overall project cost at industrial level. As well as corn cobs is easily available source of immobilization matrix and does not require harsh treatments before use.

CONCLUSION

The results of this study reflect the potential of corn cobs as support for immobilization of yeast cells (*Saccharomyces cerevisiae* NCIM 3455). The cells immobilized on matrix showed reusability upto 5 batches. The system can be used on industrial scale, as the support matrix is agricultural waste and non-toxic. The yield of ethanol obtained after fermentation with immobilized cells was in the range of 0.45-0.49 g/g of sugar, which is in relevance with thereported value.

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