RESEARCH ARTICLE

Mycobiology 40(1) : 35-41 (2012)
© The Korean Society of Mycology

http://dx.doi.org/10.5941/MYCO.2012.40.1.035
pISSN 1229-8093
eISSN 2092-9323

Study of Sugarcane Pieces as Yeast Supports for Ethanol Production from Sugarcane Juice and Molasses Using Newly Isolated Yeast from Toddy Sap

Neerupudi Kishore Babu[1]*, Botcha Satyanarayana[1], Kesavapillai Balakrishnan[2], Tamanam Raghava Rao[1] and Gudapaty Seshagiri Rao[1]

[1]*Department of Biochemistry, Andhra University, Visakhapatnam, Andhra Pradesh 530 003, India*
[2]*Biotechnology Group, BIT, Sathyamangalam, Tamil Nadu 638 401, India*

(Received November 1, 2011. Revised November 29, 2011. Accepted January 18, 2012)

A repeated batch fermentation system was used to produce ethanol using *Saccharomyces cerevisiae* strain (NCIM 3640) immobilized on sugarcane (*Saccharum officinarum* L.) pieces. For comparison free cells were also used to produce ethanol by repeated batch fermentation. Scanning electron microscopy evidently showed that cell immobilization resulted in firm adsorption of the yeast cells within subsurface cavities, capillary flow through the vessels of the vascular bundle structure, and attachment of the yeast to the surface of the sugarcane pieces. Repeated batch fermentations using sugarcane supported biocatalyst were successfully carried out for at least ten times without any significant loss in ethanol production from sugarcane juice and molasses. The number of cells attached to the support increased during the fermentation process, and fewer yeast cells leaked into fermentation broth. Ethanol concentrations (about 72.65~76.28 g/L in an average value) and ethanol productivities (about 2.27~2.36 g/L/hr in an average value) were high and stable, and residual sugar concentrations were low in all fermentations (0.9~3.25 g/L) with conversions ranging from 98.03~99.43%, showing efficiency 91.57~95.43 and operational stability of biocatalyst for ethanol fermentation. The results of the work pertaining to the use of sugarcane as immobilized yeast support could be promising for industrial fermentations.

KEYWORDS : Ethanol production, Immobilization, Repeated batch fermentation, *Saccharomyces cerevisiae*, Sugarcane pieces

Introduction

Because of the increasing demand for ethanol, there is a need to search for high-yielding strains and less expensive technology for production of ethanol, so that it can be made available at a cheaper rate [1, 2]. One such process is yeast cell immobilization because of its technical and economical advantages compared to free cell system, which facilitates faster fermentation rates by providing higher cell densities per unit fermentation volume. The in situ removal of cells further reduces the cost of recovery [3]. 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 [4]. Further, the reduction of costs, in bioprocesses involving immobilized cells systems are related to aspects such as the cost of raw materials, the use of cheap, abundant and stable immobilization supports, the high cell concentrations in the bioreactors, the simplicity and low cost of the immobilization techniques and the stability of the immobilized biocatalyst in operating conditions [5].

Various immobilization supports for variety of products have been reported such as alginates [4, 6], apple pieces [7-10], orange peel [11], gluten pellets [12], sugar cane pieces [13-15], guava piece and watermelon pieces [16, 17] and delignified cellulosic residues [18, 19]. Generally, the lignocellulose materials were delignified prior to application for the immobilization supports. Natural cellulose materials contained a large number of hydrophilic groups in the form of positive charge, ready for the absorption of negative charged cells. Through the delignification treatment, the lignin was removed partially and leading to exposing more hydrophilic groups, which increased the absorption [20]. The delignification treatment with sodium hydroxide solution increased the possibility cells going through the vesicles of cut pieces and so accommodated there for immobilization [18].

Immobilization techniques used in general can be grouped into four categories according to Tanaka and Kawamoto [21]. The first one includes those methods which involve the binding of the biocatalyst to a water-

*Corresponding author <E-mail : neerupudi@gmail.com>

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

insoluble support, by using ionic or covalent chemical links, bio specific coupling, or junctions due to adsorption phenomena. Natural polymers such as polysaccharides (cellulose, dextran, and agarose derivatives), proteins (gelatine and albumin), synthetic polymers (polystyrene derivatives and polyurethane) and inorganic material (sand, clay, ceramics, and magnetite) are commonly used for this purpose. A second category includes those methods using multifunctional compounds as glutaraldehyde, toluene or hexamethylene diisocyanate, to form Schiff's bases with functional groups in biocatalysts, thus producing water-insoluble agglomerate of catalysts [14].

The third category is constituted by those methods involving the trapping of the biocatalyst into a network formed by one or several polymers (polyacrylamide, alginate, carraginate, or synthetic resins), or those involving the embedding in membranes, encapsulating them inside microcapsules composed of synthetic polymers. A combination of the three former methods constitutes the last category. As we known, the adhesion of hydrophilic cells such as *Saccharomyces cerevisiae* is essentially dependent upon electrostatic interactions between the support and the normally negatively charged cell surface.

A strain of *S. cerevisiae* was isolated from toddy sap, which was found to produce significant amounts of ethanol, was deposited at National Collection of Industrial Microorganisms (NCIM), Pune, India and designated as NCIM 3640. In search of a biocatalyst that is suitable for industrial use, is cheap and abundant, and could be used a number of times, we thought of using sugarcane. Sugarcane is one of the abundant crops available in India. Incidentally, India is the second largest producer of sugarcane in the world (315.5 MT in 2008; FAO, USA). Its production is high during summer (April to July) because of its tropical nature and available throughout the year. Sugarcane is a natural, abundant, cheap material and it is a suitable substrate for cell growth, therefore, we decided to use sugarcane pieces as support for yeast immobilization and then utilize it for alcohol production from sugarcane juice and molasses by repeated batch fermentation and do not need special treatment before use. The aim of this study was to immobilize *S. cerevisiae* NCIM 3640 on sugarcane pieces and evaluated the efficiency and suitability of the immobilized *S.cereviseae* for ethanol production.

Materials and Methods

Materials, media and microorganism. Sugarcane was obtained from Mandal Agricultural Office, Jangareddygudem, West Godavari Distract, Andhra Pradesh. *S.cerevisiae* cell growth was carried out at 30°C in yeast extract peptone dextrose (YPD) liquid nutrient medium containing 20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone. Sugarcane juice containing a total sugar of 150 g/L was obtained by squeezing of sugarcane in mechanical mini crusher. Molasses was obtained from GMR distilleries, Sankili, Srikakulum District, Andhra Pradesh. All media were autoclaved at 121°C for 15 min.

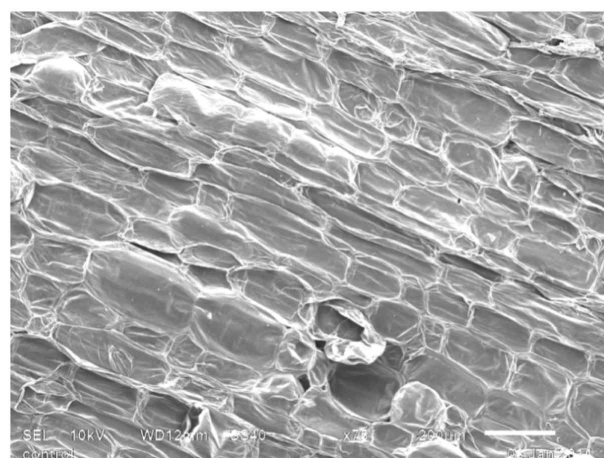
Preparation of supports by sugarcane pieces for yeast immobilization. First, sugarcane was defrosted after storage at −20°C. Sugarcane pieces were obtained by removal of the external part of the hard skin and cutting into small pieces of 1 cm length. Delignification was performed according to Bardi and Koutinas [18] and Kopsahelis *et al.* [19], by which the sugarcane pieces were mixed with 1% NaOH solution for 4 hr to remove the lignin present inside the material. After that the delignified pieces were washed well with water, and then sterilized at 121°C for 15 min. Cell immobilization on sugarcane pieces was carried out by suspending about 4×10^9 yeast cells in 200 mL of YPD medium, and mixed with 60 g of sterilized delignified sugarcane pieces. The mixture was allowed for culture about 16 hr. The fermented liquid was then decanted, the immobilized biocatalyst was washed twice with 200 mL of fresh YPD medium and sugarcane-supported biocatalyst was used for the following repeated batch fermentation [14].

Fermentation. An amount of 40 g (wet weight) of the sugarcane pieces containing yeast biocatalyst and 100 mL of sugarcane juice medium was added in a 500 mL flask and anaerobic repeated batch fermentation was successively carried out at 30°C by adding fresh medium at each cycle. The fermented liquid was decanted at the end of each fermentation batch, the biocatalyst was washed with 100 mL of the sugarcane juice medium and then fresh medium was added for the next fermentation batch. Samples of the fermented liquids were collected and analysed for the concentration of ethanol and residual sugar. Fermentations of diluted molasses were carried out in the same way at 30°C. All experiments were carried out in triplicate.

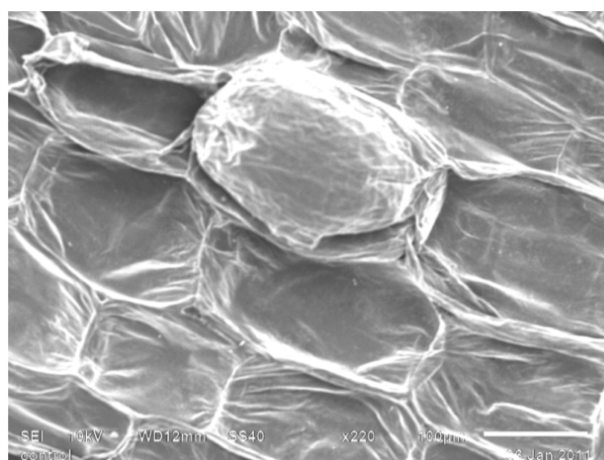
Analytical methods. The determinations were carried out for cell viability using the methylene blue method [22] and total reducing sugar in acid hydrolysate (1.2 M HCl for 10 min at 60°C), using the 3,5-dinitrosalicylic acid method [23]. The sugar concentration was calculated using standard curves and expressed as gram sugar per litre. For the biomass assays, cells were washed by vacuum filtration and dried 70°C until constant weight and expressed as grams per litre in the final medium. The samples of fermented broth derived at various intervals during the period of experimentation were analyzed for alcohol levels using gas chromatography (GC-2014; Shimadzu, Kyoto, Japan) with a flame ionization detector.

The samples were filtered through 0.2 μm micro-filters for ethanol concentration before injection (column: Rtx-5 Crossbond 5% diphenyl/95% dimethyl poly siloxane, column temperature 120°C, injector temperature 150°C, detector temperature 200°C, flow rate of carrier gas 5 μL/min). The ethanol yield (Y_{ps}) was calculated as the actual ethanol produced and expressed as g ethanol per g total sugar utilized (g/g). The volumetric ethanol productivity Q_p (g/L/hr) was calculated by ethanol concentration produced P (g/L) divided by fermentation time giving the highest ethanol concentration. Periodic samples were subjected to alcohol estimation after distilling the samples. Samples at every 48 hr interval were centrifuged (4,000 rpm, 10 min) and 2 mg of pellets were dispersed in 1 mL of distilled water and observed under microscope for cell viability using standard procedure of methylene blue staining. Average number of viable cells present in five microscopic fields was expressed as percentage of viability.

Scanning electron microscopy (SEM). For the microscopic studies, samples were transferred to vials and fixed with glutaraldehyde in 0.05 M/L phosphate buffer (pH 7.2) for 24 hr at 4°C and post-fixed in 2.5× aqueous osmium tetra oxide in the above mentioned buffer for 2 hr. After post-fixation, the samples were dehydrated in series of graded alcohol and dried to critical point of drying (CPD, Model K850; Emitech, Ashford, England) with an Electron Microscopy Science CPD unit. Later, the dried samples were mounted over the stubs with double-sided conductivity tape. Finally, a thin layer of platinum was passed through the sample using an automated sputter coater (JFC-1600; Jeol, Tokyo, Japan) for about 90 sec. Then samples were scanned under SEM (JSM 6610LV; Jeol) at various magnifications using 5 kV (accelerating voltage) at Advanced Analytical Laboratory (National Facility), Andhra University, Visakhapatnam, India [7, 8, 15].



A



B

Fig. 1. Scanning electron microscope image of inside the parenchyma of sugarcane pieces (A) control and (B) closer view of inside the parenchyma of sugarcane pieces (control).

Results and Discussion

Yeast immobilization was shown by the scanning electron micrographs. It was observed that high populations of yeast cells homogenously adhered to the surface of sugarcane pieces even after 10 repeated batch fermentations. Figs. 1~4 showing the proliferation of the yeast cells within matrix tissue structure. Immobilization did not affect the viability as immobilized yeast retained 90~95% of its viability. Also our results are in agreement with others in this regard that the application of low continuous stirring speeds during the immobilization could lead to better immobilization performance [15, 24].

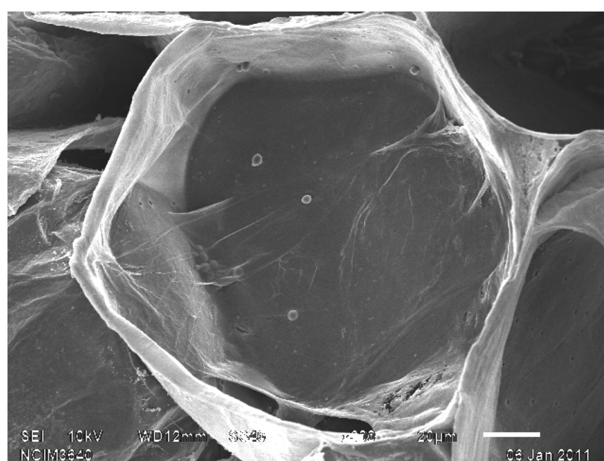


Fig. 2. Scanning electron micrograph of the middle part of the support after yeast immobilization. Cross sectional view showing absorbed yeast cells in parenchyma cells at first cycle.

Based on these immobilization techniques, the sugarcane pieces are believed to immobilize yeast cells as a result of natural entrapment into the porous structure of sugarcane materials and due to physical adsorption by electrostatic

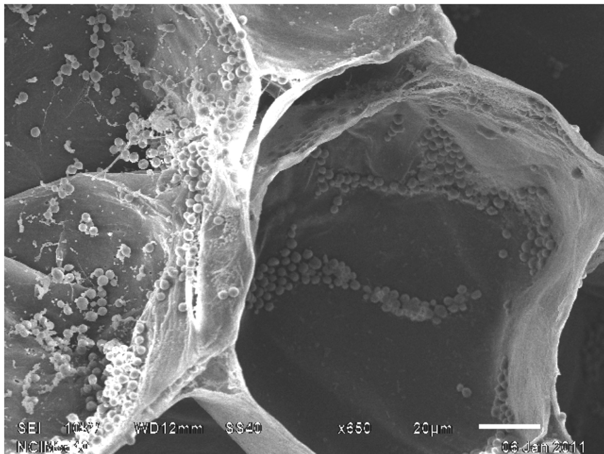


Fig. 3. Scanning electron microscope image of yeast (NCIM 3640) inside the parenchyma of sugarcane pieces after sixth cycle.

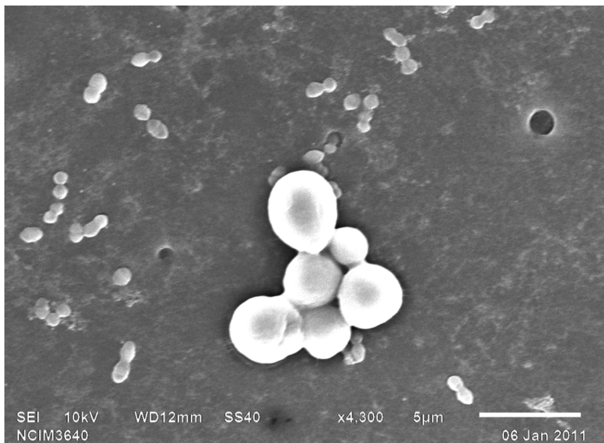


Fig. 4. Scanning electron microscope image of yeast (NCIM 3640) inside the parenchyma of sugarcane pieces after sixth cycle (closer view).

forces between the cell membrane and the carrier. Also, these observations indicate that cell retention is due to the action of capillary forces during the process of immobilization, which pull the cells to approach and keep close contact with the surface and through the channels where they can be entrapped or attached, and multiply. The flow of cells over a porous support causes a pressure differential within the vessels causing the cells to be taken into the vessels. High populations of immobilized yeast cells on the external surface of the support were observed after ten batches of fermentation. This can be explained by the greater nutrient availability (sugars) near the surface of the support. In addition, the greater nutrient availability (sugars) in the cavities of parenchyma cells is also an attraction to yeast and enable yeasts tend to migrate into the inner of parenchyma cells and budding in immobilization process [14].

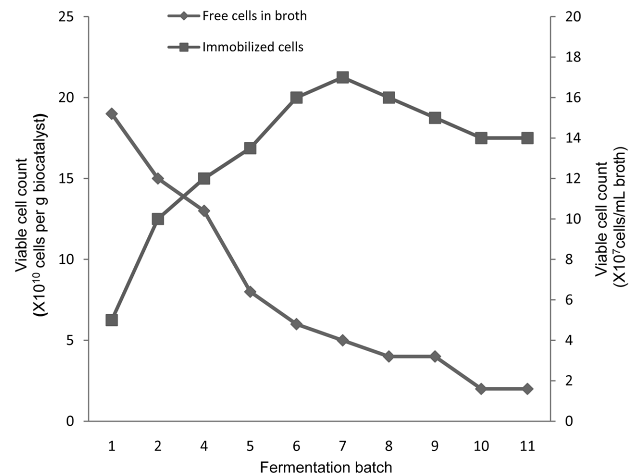


Fig. 5. Viable cell counts of free and immobilized cells during repeated batch fermentations of sugarcane juice (as $\times 10^{10}$ colony-forming unit [cfu]/g wet sugarcane or $\times 10^{7}$ cfu/mL fermentation broth).

Repeated fermentation batch. Yeast cell immobilization on the sugarcane and suitability of the immobilized biocatalysts for alcoholic fermentation was confirmed by satisfactory operational stability during repeated batch fermentations of sugarcane juice. The variation of viable cell was measured by the number of colony-forming units (cfu) in the fermentation broth (cell leakage) and on the biocatalyst (immobilized cells) during the repeated batch fermentation of sugarcane juice and molasses. According to Fig. 5, enumeration of immobilized viable cells on sugarcane immediately after immobilization indicated that the microbial populations were 10^{10} cells/g of biocatalysts. At the end of the third fermentation batch using sugarcane juice, the immobilized cells increased to 10^{11} cells/g of biocatalyst. It was observed that the number of cells attached to the support increased during the period, while fewer and fewer yeast cells leaked into fermentation broth. Therefore, the immobilization of the cells is believed to be a time depending process. This occurrence could be due to two main factors: cell multiplication and the formation of a strong and irreversible adhesion.

According to the immobilization theory Tanaka and Kawamoto [21], this time-dependence condition might be also influenced by interactions both Van der Waals forces and electrostatic forces. It was obvious that high densities of immobilized cells were achieved, which keep their viability at stable levels during successive fermentation batches, for comparison, fermentations of sugarcane juice were carried out using free yeast cells and the viable cells in the fermentation broth was only about $2{\sim}4 \times 10^{8}$ cells/mL, far lower than immobilized cells in our biocatalyst system. Effective immobilization was also established by the ability of the biocatalyst (after washing to remove free cells) to perform efficiently repeated batch fermentations

Table 1. Kinetic parameters of the anaerobic repeated batch fermentation of sugarcane juices and molasses with NCIM 3640 immobilized on sugarcane pieces at 30°C

Media	No. of cycles	t (hr)	Initial sugar (g/L)	Residual sugar (g/L)	Conversion (%)	P (g/L)	Q_p (g/L/hr)	Y_{ps} (g/g)	E_y (%)
Sugar cane juice	1	33	160.2	2.25	98.59	75.25	2.28	0.46	91.92
	2	32	160.6	2.85	98.22	75.15	2.34	0.46	91.57
	3	34	161.2	1.10	99.31	75.54	2.22	0.46	91.70
	4	34	160.6	0.90	99.43	75.60	2.22	0.46	92.12
	5	34	161.2	1.78	99.14	76.20	2.24	0.47	92.50
	6	34	161.6	1.00	99.38	76.28	2.24	0.47	92.37
Molasses	1	31	150.2	3.25	97.83	73.25	2.36	0.48	95.43
	2	32	150.6	1.85	98.77	73.51	2.29	0.48	95.52
	3	32	151.2	2.10	98.61	72.65	2.27	0.48	94.00
	4	31	150.6	2.50	98.33	73.00	2.35	0.48	94.85
	5	32	151.2	2.68	98.22	73.36	2.29	0.48	94.94
	6	32	151.6	2.98	98.03	73.62	2.30	0.48	95.03
Sugar cane juice	[a]	48	160.6	3.25	97.97	75.14	1.56	0.46	91.55
Molasses	[b]	48	150.2	4.20	97.20	72.12	1.50	0.48	93.96

t, fermentation time; P, ethanol concentration; Q_p, volumetric ethanol productivity; Y_{ps}, ethanol yield; E_y (%), fermentation efficiency.
[a]Sugarcane juice control (free cells).
[b]Molasses control (free cells).

of sugarcane juice and molasses in the production of bio ethanol.

The kinetic parameters obtained after repeated batch fermentations of sugarcane juice and molasses for ten cycles using our immobilization system were shown in Table 1 (the data showed only 6 cycles). These results showed that fermentation times for all the media investigated were lower than 34 hr and stable. Fermentation times were low even during the first batch, indicating that no significant period was needed for adaptation of the biocatalyst in the fermentation environment. Ethanol concentrations (about 73.25~76.28 g/L in average value), and ethanol productivities (about 2.24~2.36 g/L/hr in average value) were high and stable, and residual sugar concentrations were low in all fermentations (0.90~3.25 g/L) with conversions ranging from 97.67 to 99.80%, showing efficiency (91.57~95.55%) and operational stability of the biocatalyst for ethanol fermentation. However, as some of the carbon sources is used for biomass and volatile by-products generation, the actual ethanol yield is about 90~95% (0.46 g ethanol/g sugar) of the theoretical one [25, 26]. In most of the distilleries in Mexico, the yield of ethanol from molasses ranged from 0.33 up to 0.38 g ethanol/g reducing sugars. In the case of the fermentation of sugarcane juice (161.6 g of initial sugar/L) and molasses (151.6 g of initial sugar/L) by the biocatalyst that yeast immobilized on sugarcane pieces, the actual ethanol yields of this work were 0.47 g/g and 0.48 g/g, respectively, which are considered as acceptable values. Compared with those obtained with free cells, similar concentrations of ethanol were obtained, however, the main difference observed was the higher fermentation rate of immobilized cells, and higher ethanol productivity and fermentation efficiency obtained.

It is known that yeast cells can also be entrapped in calcium alginate and the resulting immobilized yeast can be used for fast paced fermentations. Numerous literature references describe this technique as it is applied usually in laboratory scale. Some efforts, however, have also been made to commercialize this technique. One of the best known is probably that of Kyowa Hakko in Japan [27], where growing cells of *S. cerevisiae* immobilized in calcium alginate gel beads were employed in fluidized bed reactors for continuous ethanol fermentation from cane molasses and other sugar sources. According to the report, the ethanol productivity was more than 50 g ethanol/L gel/hr and prolonged life stability for more than one-half year. Cell concentration in the carrier was estimated over 250 g dry cell/L gel. As a result, it was confirmed that 8~10% (v/v) ethanol-containing broth was continuously produced from non-sterilized diluted cane molasses for over one-half year. The productivity of ethanol was calculated as 0.6 L ethanol per L of reactor volume one day with a 95% conversion yield versus the maximum theoretical yield for the case of 8.5% (v/v) ethanol broth. The results of this work were generally comparable with these of the previous studies in the fermentation rates, yields and efficiencies, and contributed to an improved quality of the distillate due to the 10~11.5% (v/v) higher ethanol content [14].

On other hand, there are several weaknesses of alginate immobilized yeast for commercial scale operations. A major difficulty with alginate entrapment is the manner in which the particles are formed. A process plant that utilizes alginate entrapped yeast must have specially designed equipment just to produce these beads. Furthermore, there

is a potential risk for contaminating the yeast with wild microorganisms.

A second major difficulty for alginate beads used on a commercial scale is in the physical strength of the beads. The beads are soft and easily compressible. Operating large fermentation columns can be a problem and fast down flow process streams are difficult to handle. A third difficulty with entrapment is the diffusion limitations which slowdown the accessibility of the substrate in contact with the yeast inside the bead. Finally, if the system becomes contaminated or otherwise disturbed so that a continuous operation must be discontinued, the whole lot of column material (alginate together with the yeast) must be discarded. No reuse is possible. However, the spent sugarcane immobilized supports can be used as protein enriched (SCP production) animal feed for reusing. Therefore, in comparison with Ca-alginate entrapped yeast, this sugarcane immobilized biocatalysts is believed to be competitive for an industrial process [14]. The biocatalysts prepared by natural materials for the production of alcohol have also been extensively studied, such as apple pieces [7], orange peel [11], dried figs [28] and spent grains [19]. Most of the fermentation batches resulted in glucose consumption of 98.00~99.00% for the juice containing 134.00~187.00 g of sugar/L, and stable ethanol production with ethanol productivity ranging from 26.00 to 110.40 g/L/day at 30°C. Anyway, the results presented in this study, according to initial concentration of sugars in the must, showed that the sugarcane supported biocatalyst was equally efficient to that described in the literature for ethanol fermentation [14].

The results of this study mainly demonstrated the potential applications of the sugarcane as a matrix material for yeast immobilization and provided a good investigation in to the possibilities of immobilized yeast cells in ethanol production. Sugarcane pieces were found suitable as support for yeast cell immobilization in ethanol industry. The sugarcane immobilized biocatalysts showed high fermentation activity. Although ethanol fermentations have been only carried out on sterilized sugarcane juice, the fermentation technologies presented above could be applied in fresh juice as well. The immobilized yeast would dominate in the fermentation broth due to its high populations and lower fermentation time, therefore, the development of other wild yeast and bacteria that may exist in the fresh broth would be inhibited [8]. Owing to the low price of the support material and its abundance in nature, reuse availability make this biocatalyst attractive in the ethanol production as well as in wine making and beer production. Also, the particles form of the support give the possibility for fermentations using feed batch bioreactors and separation of biocatalyst employing centrifuge separators or separation after removal of the supernatant liquid. From this study, we can conclude that the sugar cane pieces are well suitable for yeast immobilization and can be serve as a good biocatalyst for ethanol fermentation. This method is very economical and easy method for ethanol production and suitable for ethanol industry. The sugarcane immobilized biocatalyst showed high fermentation activity. These results showed that fermentation time required for all the media investigated were lower than 35 hr and stable. Fermentation times were low even during the first batch, indicating that no significant period was needed for adaptation of the biocatalyst in the fermentation environment. Ethanol concentrations (about 76.28~73.25 g/L in average value), and ethanol productivities (about 2.24~2.36 g/L/hr in average value) were high and stable, and residual sugar concentrations were low in all fermentations (0.90~3.25 g/L) with conversions ranging from 97.67 to 99.80%, showing efficiency (91.57~95.55%) and operational stability of the biocatalyst for ethanol fermentation. The immobilized yeasts were dominating in the fermentation broth due to its high population and hence lower fermentation time. The development of other wild yeast and bacteria that may exist in the fresh broth was effectively inhibited. The low price of the support and its abundance in nature, reuse availability make this biocatalyst attractive in the ethanol production. Finally, this method is very economical, easy and rate of fermentation has also shortened when compared with other popular cell immobilization protocols.

Acknowledgements

One of the authors (Mr. N. Kishore Babu) is thankful to Rajiv Gandhi National Fellowship, New Delhi for providing research fellowship during this period.

References

1. Suresh K, Kiransree N, Rao LV. Production of ethanol by raw starch hydrolysis and fermentatation of damaged grains of wheat and sorghum. Bioprocess Eng 1999;21:165-8.
2. Suresh K, Sree NK, Rao LV. Utilization of damaged sorghum and rice grains for ethanol production by simultaneous saccharification and fermentation. Bioresour Technol 1999; 68:301-4.
3. Chaudhary AB, Chincholkar SB. Cell immobilization a critical approach to ethanol production by *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* Indian J Microbiol 1996;36:75-83.
4. Sree NK, Sridhar M, Suresh K, Banat IM, Rao LV. High alcohol production by repeated batch fermentation using an immobilized osmotolerant *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 2000;24:222-6.
5. Margaritis A, Merchant FJ. Advances in ethanol-production using immobilized cell systems. CRC Crit Rev Biotechnol 1984;1:339-93.
6. Cortón E, Piuri M, Battaglini F, Ruzal SM. Characterization of *Lactobacillus* carbohydrate fermentation activity using immobilized cells technique. Biotechnol Prog 2000;16:59-63.
7. Kourkoutas Y, Komaitis M, Koutinas AA, Kanellaki M.

Wine production using yeast immobilized on apple pieces at low and room temperatures. J Agric Food Chem 2001; 49:1417-25.

8. Kourkoutas Y, Kanellaki M, Koutinas AA. Apple pieces as immobilization support of various microorganisms. LWT-Food Sci Technol 2006:39:980-6.
9. Kourkoutas Y, McErlean C, Kanellaki M, Hack CJ, Marchant R, Banat IM, Koutinas AA. High-temperature wine making using the thermotolerant yeast strain *Kluyveromyces marxianus* IMB3. Appl Biochem Biotechnol 2004;112:25-35.
10. Kourkoutas Y, Xolias V, Kallis M, Bezirtzoglou E, Kanellaki M. *Lactobacillus casei* cell immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production. Process Biochem 2005;40:411-6.
11. Plessas S, Bekatorou A, Koutinas AA, Soupioni M, Banat IM, Marchant R. Use of *Saccharomyces cerevisiae* cells immobilized on orange peel as biocatalyst for alcoholic fermentation. Bioresour Technol 2007;98:860-5.
12. Chronopoulos G, Bekatorou A, Bezirtzoglou E, Kaliafas A, Koutinas AA, Marchant R, Banat IM. Lactic acid fermentation by *Lactobacillus casei* in free cell form and immobilised on gluten pellets. Biotechnol Lett 2002;24:1233-6.
13. de Vasconcelos JN, Lopes CE, de Franca FP. Continuous ethanol production using yeast immobilized on sugar-cane stalks. Braz J Chem Eng 2004;21:357-65.
14. Liang L, Zhang YP, Zhang L, Zhu MJ, Liang SZ, Huang YN. Study of sugarcane pieces as yeast supports for ethanol production from sugarcane juice and molasses. J Ind Microbiol Biotechnol 2008;35:1605-13.
15. Reddy LV, Reddy LP, Wee YJ, Reddy OV. Production and characterization of wine with sugarcane piece immobilized yeast biocatalyst. Food Bioprocess Technol 2011;4:142-8.
16. Reddy LV, Reddy YH, Reddy LP, Reddy OV. Wine production by novel yeast biocatalyst prepared by immobilization on watermelon (*Citrullus vulgaris*) rind pieces and characterization of volatile compounds. Process Biochem 2008;43:748-52.
17. Reddy LV, Reddy YH, Reddy OV. Wine production by guava piece immobilized yeast from Indian cultivar grapes and its volatile composition. Biotechnology 2006;5:449-54.
18. Bardi EP, Koutinas AA. Immobilization of yeast on delignified cellulosic material for room termperature and low-temperature wine making. J Agric Food Chem 1994;42:221-6.
19. Kopsahelis N, Agouridis N, Bekatorou A, Kanellaki M. Comparative study of spent grains and delignified spent grains as yeast supports for alcohol production from molasses. Bioresour Technol 2006;98:1440-7.
20. Sahin HT, Arslan MB. A study on physical and chemical properties of cellulose paper immersed in various solvent mixtures. Int J Mol Sci 2008:9:78-88.
21. Tanaka A, Kawamoto T. Cell and enzyme immobilization. In: Demain AL, Davies JE, Atlas RM, Cohen G, Hershberger CL, Hu WS, Sherman DH, Wilson RC, Wu JH, editors. Manual of industrial microbiology and biotechnology. 2nd ed. Washington, DC: ASM Press; 1999. p. 94-102.
22. Lee SS, Robinson FM, Wang HY. Rapid determination of yeast viability. Biotechnol Bioeng Symp 1981;11:641-9.
23. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426-8.
24. Peinado RA, Moreno JJ, Villalba JM, González-Reyes JA, Ortega JM, Mauricio JC. Yeast biocapsules: a new immobilization method and their applications. Enzyme Microb Technol 2006;40:79-84.
25. Sobočan G, Glavič P. Optimization of ethanol fermentation process design. Appl Therm Eng 2000;20:529-43.
26. Olguin EJ, Doelle HW, Mercado G. Resource recovery through recycling of sugar processing by-products and residuals. Resour Conserv Recycl 1995;15:85-94.
27. Nagashima M, Azuma M, Noguchi S, Inuzuka K, Samejima H. Continuous ethanol fermentation using immobilized yeast cells. Biotechnol Bioeng 1984;26:992-7.
28. Bekatorou A, Sarellas A, Ternan NG, Mallouchos A, Komaitis M, Koutinas AA, Kanellaki M. Low-temperature brewing using yeast immobilized on dried figs. J Agric Food Chem 2002; 50:7249-57.