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Full Length Research Paper

Comparison of *Luffa cylindrica* L. sponge discs and Ca-alginate gel beads as immobilized matrices of *Saccharomyces cerevisiae* for bio-ethanol production from sugarcane molasses

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The production of bio-ethanol from cane molasses (diluted to 15% sugar, w/v) was studied, using the yeast, *Saccharomyces cerevisiae* immobilized in luffa (*Luffa cylindrica* L.) sponge discs and in Ca-alginate gel beads. At the end of 96 h fermentation, the ethanol yields were 64.67 ± 0.016 and 65.21 ± 0.030 g/l molasses, with luffa and Ca-alginate entrapped *S. cerevisiae* cells exhibiting 89.90 ± 0.008 and 91.86 ± 0.072% sugar conversion, respectively. There was no statistically significant difference [Fisher's least significance difference (LSD)] between the two immobilized systems in both sugar utilization (t = 0.254, p < 0.801) and in ethanol production (t = -0.663, p < 0.513).

Key words: Bio-ethanol, cell immobilization, fermentation, molasses, *Saccharomyces cerevisiae*.

INTRODUCTION

The use of bio-ethanol as an alternative source of fuel has raised considerable interest, due to high prices and environmental problems caused by fossil fuels (Khaw et al., 2007). Therefore, biotechnologists are always in a hunt to find cheaper substrates or novel technologies, in order to lower the overall production cost of bio-ethanol (Bothast and Schlicher, 2005). In general, commercial bio-ethanol production relies on the fermentation of sucrose from sugarcane molasses or from glucose, derived from starch-based crops such as corn, wheat and cassava (Yamashita et al., 2008).

Molasses is an agro-industrial by-product, derived from processing of sugarcane and sugar beet into sugar, often used in alcohol distilleries (Borzani et al., 2004). Due to the presence of high levels of fermentative sugars such as glucose, sucrose and fructose, molasses is an optimal carbon source for the microorganism metabolism. Sugarcane molasses is abundantly available in countries like Brazil, India, China and Cuba, and its low cost, 25 to 30 US dollars per ton in India, is an important factor for the economical viability of ethanol production by fermentation.

Bio-ethanol can be produced by using either free or immobilized cells. The use of immobilized cells offers a number of advantages: relative facility of cell recovery from fermentation broth, prevention of washout, protection of cells against toxic substances, and operational stability in comparison to suspended cells (Najafpour et al., 2004; Dursun and Tepe, 2005; Anisha and Prema, 2008; Ghorbani et al., 2011). Further more, compared to other immobilization methods, Ca-alginate

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Abbreviations: MYGP, Malt extract-yeast extract-glucose-peptone; **ANOVA,** analysis of variance; **LSD,** least significance difference.

immobilization seems to be more effective in reducing the inhibitory effect of metal ions or impurities on cell growth. On the other hand, luffa sponge, lignocellulosic matrices from *Luffa cylindrica* L., was found to be promising as cell carriers for ethanol production by flocculating cells (Ogbonna et al., 1997, 2001). The sponges are light, chemically stable, and are composed of interconnecting voids within an open network of fibers. Due to the random lattices of small cross sections of the sponges coupled with high porosity, the sponges are suitable for cell adhesion. Continuous production had been realized using yeast cells immobilized in luffa sponges, in a bubble column configuration (Ogbonna et al., 2001). In the present work, a comparative study was carried out on the production of ethanol from cane molasses by fermentation using, *Saccharomyces cerevisiae,* entrapped in Ca-alginate beads and in luffa sponge discs. Furthermore, the growth and fermentation kinetics between the two immobilization methods of yeast cell has been studied.

MATERIALS AND METHODS

Molasses

The molasses used for ethanol fermentation was brought from Sakthi Sugars Pvt. Limited, Dhenkanal, Odisha, India, during the month of October, 2010. The molasses had the following compositions in [(g/100 ml (w/v)]: water, 20 ± 0.055; sucrose, 36 ± 0.06; fructose, 11 ± 0.094; glucose, 9 ± 0.056; other reducing sugars, 4 ± 0.148; nitrogenous compounds, 3.5 ± 0.084; non-nitrogenous acids, 5 ± 0.195; ash, 7 ± 0.06 and others, 4.5 ± 0.07. The sugar content of molasses (60% w/v) was brought into 15% w/v by 1:3 dilutions with distilled water. Concentrated sulphuric 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; an upper shining black layer and a lower yellowish brown layer (due to the precipitates of trace metals). The clear supernatant (shiny layer) was used as fermentation medium with sugar content 15% w/v.

Microorganism and culture condition

The yeast *S. cerevisiae* (Central Tuber Crops Research Institute (CTCRI) strain) was earlier used for ethanol fermentation (Behera et al., 2010) maintained on malt-extract yeast-extract glucose-peptone (MYGP) medium [(g/l): malt extract, 3; yeast extract, 5; peptone, 5; glucose, 20 and agar, 15] and the pH was adjusted to 5.5. The culture was stored at 4 ± 0.5°C for further use.

Preparation of inoculum

The inoculum was prepared in 100 ml growth medium (as mentioned above but without agar) taken in sterilized (at 121°C for 20 min) 250 ml Erlenmeyer flask. The flask was inoculated with a loopful of the *S. cerevisiae* culture and incubated for 24 h at 30°C, at 120 rpm in an orbital shaker incubator (Remi Pvt, Limited, Bombay, India). 40 ml of the yeast inoculum [equivalent to 10% of the fermentation medium (400 ml)] was separately immobilized with Ca-alginate and luffa sponge discs, as described below.

Immobilization of whole cells in different matrices

Calcium alginate

40 ml of the yeast inoculum (prepared as described above) was centrifuged at 8000 rpm, for 20 min, in a refrigerated centrifuge (Model C-24, Remi Pvt., Limited, Bombay, India), washed, and then the pellets were suspended with 10 ml of deionized water. The cell suspension was used for cell immobilization. The *S. cerevisiae* cell suspension (1×10^5 CFU/ml) was added to 4% (w/v) sodium alginate solutions in 1:1 (v/v) ratio and mixed thoroughly. The cell-alginate mixture was then cast into beads by dropping from a hypodermic syringe, into cold sterile 0.1 M $CaCl_2$ solution. These beads had a diameter of approximately 3.0 mm and were hardened, by keeping n the dilute (0.1 M) $CaCl_2$ solution for 24 h, at 4°C, with gentle agitation (Behera et al., 2010). Finally, these beads were washed with sterile distilled water to remove excess Ca^{2+} ions and un-entrapped cells, before being used for the fermentation process.

Luffa sponge discs

The luffa sponge discs were obtained from mature dried fruits of *L. cylindrica*. The sponge was cut into discs of 2.5 cm diameter, 4 mm thick and washed in boiling water four times. The luffa sponge discs were then oven-dried at 70°C. 40 ml of the yeast inoculum was directly poured into six luffa sponge discs contained in 500 ml beaker. After the cells (inoculum) were trapped within the matrix of the sponge, these immobilized luffa discs were taken out and washed thoroughly with fresh sterilized MYGP broth, to remove the free cells, before being used for the fermentation process.

Fermentation medium

400 ml of treated cane molasses with 15% (w/v) sugar was taken into individual 500 ml Erlenmeyer flasks. The flasks were cotton plugged and steamed at 90°C in a water bath for 15 to 20 min. After cooling, $(NH_4)_2SO_4$ was added to the slurry (as a source of nitrogen) for growth of the yeast, at the rate of 1 g/l, and subsequently the pH was adjusted to 5.5. Then, the medium (400 ml) was inoculated with either immobilized luffa sponge discs (6 nos.) or Ca-alginate gel beads. The triplicate flasks (n = 3) for each treatment, were separately incubated for 96 h at the room temperature (30 ± 2°C).

Analytical methods

At 24 h time interval, fermented broths (in triplicate flasks) 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 (1984). By this procedure, the weight of a certain volume of an alcohol distillate was compared to the weight of exactly the same volume of distilled water. The ratio of the weights of the two (alcohol : water), gave the specific gravity of the distillate. The total sugar was assayed by Anthrone method (Mahadevan and Sridhar, 1999). The pH was measured by a pH meter (Systronics, Ahmadabad, India), using glass electrode. The immobilized yeast cells, separated after fermentation, were reused for successive three batches. The fermentation kinetics was studied following the formulae given by Bailey and Ollis (1986).

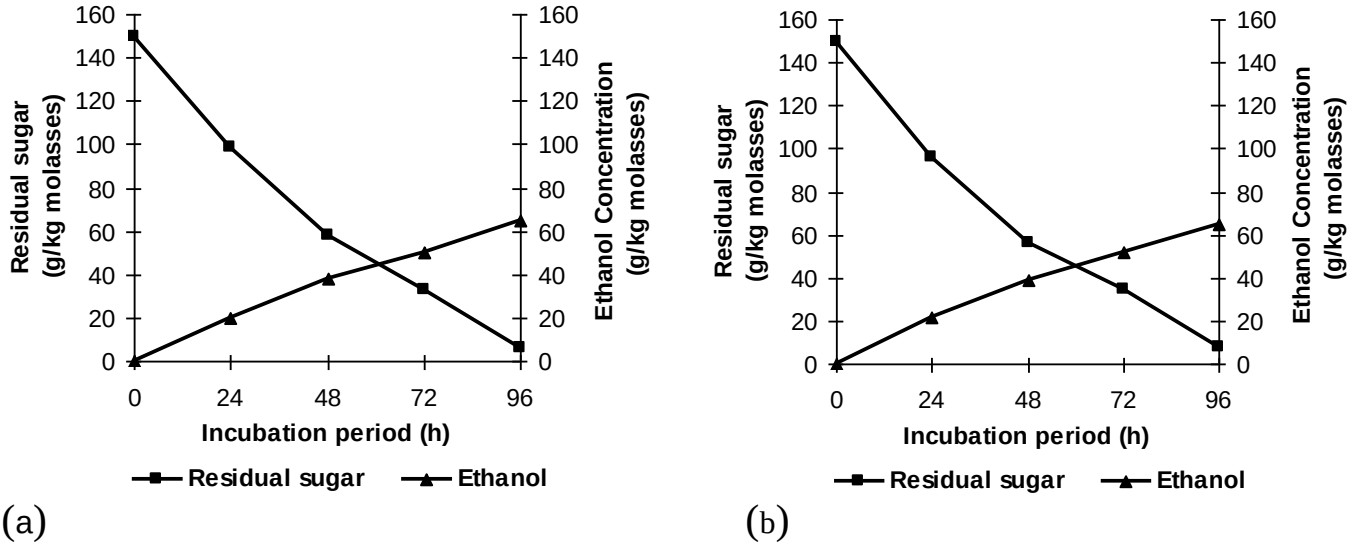

Figure 1. Study of ethanol production by luffa (a) and Ca-alginate immobilized cells (b) of *S. cerevisiae* on molasses.

Statistical analysis

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

RESULTS

Ethanol production and sugar utilization were compared during the fermentation of cane molasses by *S. cerevisiae* immobilized in luffa sponge disc and in Ca-alginate gel beads. The production of ethanol started in the log phase of the growth, and maximum ethanol yield was achieved during the stationary phase (96 h) (Figure 1a and b). After 24 h of fermentation, there was a fall of 34.2 and 36% in total sugar over the initial content, with simultaneous production of 19.72 ± 0.080 and 21.32 ± 0.050 g/l ethanol, with the cells immobilized in luffa sponge discs and in Ca-alginate gel beads, respectively. After 48, 72 and 96 h of fermentation with the luffa immobilized *S. cerevisiae* cells, the sugar concentrations were 58.13 ± 0.014, 33.07 ± 0.040 and 6.100 ± 0.074 g/l molasses, with simultaneous increase in ethanol concentration to 38.02 ± 0.038, 50.11 ± 0.104 and 64.67 ± 0.063 g/l molasses, respectively (Figure 1a). During the same period, sugar concentrations were 56.23 ± 0.089, 34.17 ± 0.068 and 8.030 ± 0.145 g/l molasses, with production of ethanol to 39.18 ± 0.610, 52.04 ± 0.047 and 65.21 ± 0.022 g/l molasses, respectively, when Ca-alginate immobilized yeast cells were used for fermentation (Figure 1b). Thus at the end of fermentation period (96 h), there was 89.90 ± 0.008 and 91.86 ± 0.072% sugar conversion into ethanol, with immobilized *S. cerevisiae* cells entrapped in luffa sponge discs and in Ca-alginate gel beads, respectively. Furthermore, there was no statistically significant difference (Fischer's LSD) in sugar utilization ($t = 0.254$, $p < 0.801$) and ethanol production ($t = -0.663$, $p < 0.513$) between the two immobilization matrices used.

The growth and fermentation kinetics of the immobilized yeast cells were also studied (Table 1). The ethanol concentration (P), obtained with Ca-alginate immobilized cells of *S. cerevisiae* (65.21 ± 0.022 g/l) was marginally higher (0.83%) than that of luffa immobilized cells (64.67 ± 0.063 g/l), whereas, the volumetric substrate uptake (Qs) was found to be 1.3% more in case of luffa (1.500 ± 0.029 g/l/h) than that of Ca-alginate (1.480 ± 0.083 g/l/h) immobilized cells. The volumetric product productivity (QP = 0.679 ± 0.015 g/l/h) and ethanol yield (Yp/s = 0.459 ± 0.010 g/g) obtained with cells immobilized in Ca-alginate matrix was found to be 0.74 and 2.18%, respectively, higher than that of Qp (0.674 ± 0.008 g/l/h) and Yp/s (0.449 ± 0.013 g/g) of *S. cerevisiae* immobilized in luffa sponge discs. Likewise, the final sugar to ethanol conversion rate (%) with Ca-alginate immobilized cells was 2.13% higher than with luffa immobilized cells.

The immobilized cells were further recycled for three more times, limiting the duration of each fermentation cycle up to 96 h as most of the sugars in molasses were consumed during these period. The cells not only survived but were also physiologically active in these three cycles of fermentation. The production of ethanol in

Table 1. Growth and fermentation kinetics of luffa and Ca-alginate immobilized cells of *S. cerevisiae* on molasses.

Parameter	Luffa immobilized cell	Ca-alginate Immobilized cell
Final ethanol (P, g L^{-1})	64.67±0.016	65.21±0.030
Ethanol yield (Yp/s, g g^{-1})	0.449±0.013	0.459±0.010
Volumetric substrate uptake (Qs, g L^{-1} h^{-1})	1.500±0.029	1.480±0.083
Volumetric product productivity (Qp, g L^{-1} h^{-1})	0.674±0.008	0.679±0.015
Conversion rate (%) into ethanol	89.90±0.008	91.86±0.072

Q_S, Substrate (sugar) uptake (g) per liter of hydrolysate per hour. Q_P, Product formed (g) per liter of hydrolysate per hour.

the 2nd, 3rd and 4th cycle with the luffa immobilized cells of *S. cerevisiae* was 62.37 ± 0.015, 59.96 ± 0.018 and 55.06 ± 0.030 g/l molasses, respectively, showing 3.56, 7.28 and 14.86% decrease over the 1st cycle (64.67 ± 0.016 g/l molasses). During the same period, the ethanol production was 63.53 ± 0.049, 61.06 ± 0.035 and 57.36 ± 0.075 g/l molasses, respectively with the Ca-alginate immobilized cells.

DISCUSSION

Preliminary studies (data not shown) have shown that 15% w/v sugar content in molasses was optimum for ethanol production by *S. cerevisiae,* which is in agreement with earlier studies (Amutha and Gunasekaran, 2001; Monte et al., 2003). In the present study, 89 to 92% sugar (150 g/l in the initial fermentation medium) conversion was attained with ethanol production of 64.7 to 65.2 g/l molasses during 96 h fermentation, using either of the two tested immobilized cell systems. Similar results regarding ethanol yield from molasses have been reported by other authors, using either free or immobilized yeast cells. Mariam et al. (2009) found maximum ethanol yield (52.9 g/l) by Ca-alginate immobilized yeast (*S. cerevisiae*) cells from cane molasses, after 120 h of incubation, with a maximum sugar consumption of 147.6 g/l. Also, Sheela et al. (2008) reported 7.9% ethanol production in the fermentation of molasses medium after 72 h of incubation with an initial sugar concentration of 15% using *S. cerevisiae* EP-17. Furthermore, Periyasamy et al. (2009) found 53% ethanol in the fermentation of sugar molasses using *S. cerevisiae* after 72 h of incubation period.

Several studies have shown that the superiority of immobilized cell systems over the free cells is reflected in the fermentation kinetics values (Amutha and Gunasekaran, 2001; Kourkoutas et al., 2004). Behera et al. (2010) reported 2.2 and 3.5% more ethanol yield in case of agar agar and Ca-alginate matrices over free cells, during fermentation of mahula flowers for ethanol production. Swain et al. (2007) reported 6.7% increase in ethanol yield from mahula flowers fermented with Ca-alginate entrapped *S. cerevisiae* cells over free cells. In the present study, there was no significant variation in ethanol yield by using the two cell immobilization systems. This was also reflected in the fermentation kinetics.

There are several reports to demonstrate the reusability of immobilized cells in bio-product production (Kar et al., 2009). In the present study, the immobilized cell systems were repetitively recycled for four cycles of fermentation. The decrease in ethanol concentration observed during the 2nd, 3rd and 4th cycle of fermentation might be due to marginal leakage of cells from the matrices. Similar results were obtained on ethanol production from cane molasses, using alginate-luffa as the carrier matrix for the immobilization of yeast cells (Phisalaphong et al., 2007). In that study, the ethanol production was the same during the 1st and 2nd cycles of operation (91.7 g/l cane molasses), with a marginal decrease (0.5%) in the 3rd cycle (90.6 g/l cane molasses). Behera et al. (2010) reported that the immobilized *S. cerevisiae* cells were physiologically active for three cycles 150.6, 148.5 and 146.5 g kg^{-1} (agar agar) and 152.8, 151.5 and 149.5 g kg^{-1} flowers (calcium alginate) for the first, second and third cycles, respectively, with a decrease of 10% on the ethanol yield. For example, repeated batch fermentations (up to 8th cycle) for α-amylase production by *Streptomyces erumpens* was reported with marginal (less than 10%) decrease in enzyme yield; only then the beads disintegrated (Kar et al., 2009)]. Similar finding is observed by Adinarayana et al. (2005) for production of alkaline protease by *Bacillus subtilis* PE-11, using various matrices that is, calcium alginate, *k*-carrageenan, ploy acrylamide, agar-agar and gelatin.

In this study, luffa matrix was found to be equally suitable as Ca-alginate matrix for cell immobilization of *S. cerevisiae* for ethanol production from cane molasses. Ogbonna et al. (1997, 2001) reported that luffa sponge alone could be used to achieve 99% immobilization of flocculating yeasts (*S. cerevisiae*) cells for ethanol production in a column bioreactor. On the other hand, luffa sponge was demonstrated as an excellent cell carrier for ethanol fermentation by flocculating cells, such as *S. cerevisiae* and non-flocculating cells, such as *Candida brassicae* (Ogbonna et al., 2001). Furthermore, the strength, abundance, low cost, biodegradability and

natural origin of luffa have gained the interest for cell immobilization. Also the utility of luffa sponge as an immobilizing matrix has been studied for other fermentative products. Luffa sponge was used as the carrier for yeast cell immobilization for the production of ethanol from sugar beet juice (Ogbonna et al., 2001) and cane molasses (Phisalaphong et al., 2007). Luffa sponge was also used as an ideal immobilization material for the production of pectinase (Slokoska and Angelova, 1998) and α-amylase (Kar et al., 2009). Likewise, luffa sponge was used as an excellent matrix for removal of heavy metal ions (Iqbal abd Edyvean, 2005).

Conclusion

Luffa sponge was found equally suitable as Ca-alginate matrix for yeast (*S. cerevisiae*) cell immobilization in ethanol production from cane molasses. Furthermore, both the immobilized cell systems could be re-used effectively for at least four cycles of batch fermentation.

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