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The Influence of Inert Solids on Ethanol Production by *Saccharomyces cerevisiae**

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Abstract

The catalytic role of various inert solid supports on acceleration of alcohol fermentation by *Saccharomyces cerevisiae* was investigated. The enhanced rate of alcohol production was dependent on the nature of the support as well as on the amount used. Among all the tested supports, chitosan flakes showed the maximum yield of alcohol (93% of theoretical yield). This higher rate of alcohol production was associated with the twofold increase in the activity of alcohol dehydrogenase over control. Our results suggest that the addition of a small fraction of solids in submerged fermentations to facilitate cell anchorage for enhanced metabolic activity is easier and more economical compared to cell immobilization processes.

Index Entries: Alcohol dehydrogenase; ethanol; chitin; chitosan; river sand; sawdust; wheat bran; *Saccharomyces cerevisiae*.

Introduction

Surface-microbe interactions have been observed in biofilm formation as well as in the passive immobilization process. The promotive aspects of cell adhesion to the solid surface were discovered quite early *(1)*, well before cell immobilization technology was studied. Reports are also available on enhanced respiration rates of *Acrobacterium* sp. in which chitin was given as the support material *(2)*. With the present understanding of the physiology of immobilized cells, it is possible to offer meaningful explanations to

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earlier observations. Recently, it was reported that alcohol fermentation was accelerated by the addition of chitin *(3)*, zeolites *(4,5)*, ceramics *(6)*, and delignified sawdust *(7)*. The improved rate of fermentation has been speculated to the catalytic action of some of the enzymes involved in the fermentation pathway *(7)* and ion exchange and pH stabilization *(4,5)*. The exact reasons of promotive action of solid support material on alcohol fermentations have not been investigated.

In the present study, we used various supports for alcohol fermentation and studied their influence on alcohol production. The results indicated that alcohol production was improved to an extent of 84% in the presence of support material. We have attempted to explain the accelerated metabolic behavior of the yeast by estimating alcohol dehydrogenase activity in the cells, which were provided with the support and compared with the activities of the cells without support.

Materials and Methods

Chemicals

River sand (100- to 200-µm size granules), sawdust (500- to 750-µm size particles), and wheat bran flakes (0.5–1.0 cm) were locally procured. Chitin powder, chitosan flakes (0.5–1.0 cm), and titanium oxide (50-µ size particles) were obtained from Himedia, India. All other chemicals and reagents were obtained from Sigma.

Microorganism and Fermentation Conditions

Saccharomyces cerevisiae NCIM 3204 was obtained from the National Collection of Industrial Microorganisms, Pune, India. The inoculum was raised using MGYP Broth (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, and 3.0% glucose), pH 6.4, at 30 ± 2°C. The fermentations were carried out in 100-mL Erlenmeyer flasks using 100 mL of fermentation medium (1.0 g/L KH_2PO_4, 1.0 g/L $[NH_4]_2SO_4$, 5.0 g/L $MgSO_4 \cdot 7H_2O$, and 150 g/L glucose), pH 4.0, at 30°C by incubating under static conditions. Various solid supports (chitin, chitosan, delignified sawdust, delignified wheat bran, river sand, and TiO_2) at the 1% level, unless otherwise indicated, were added to the medium before autoclaving, and the 10% of the inoculum, having the absorbance of 1.0, was inoculated under sterilized conditions.

Delignification of Cellulosic Material

Sawdust and wheat bran delignification was performed according to Iconomou et al. *(7)* by heating 300 g of the material, at the boiling point of the mixture, in 3 L of 1% (w/v) sodium hydroxide solution for 3 h. The level of the mixture was kept constant by the addition of distilled water, during heating. Then the mixture was filtered using a Buchner funnel, and the residue was washed several times with hot water. This process was continued until the cellulosic material was completely delignified. The material

was then dried at 60°C overnight in an incubator, and the dried material was used for experiments.

Enzyme Extraction Procedure

After fermentation, the cells were harvested by centrifugation and washed twice with 0.1 *M* sodium phosphate buffer, pH 7.5. The pellet was suspended in 10 mL of this buffer, treated with 0.2% cetyl trimethyl ammonium bromide, and incubated at 37°C. After 10 min of incubation, the contents were centrifuged at 15,000*g* for 10 min, and the supernatant was used as the enzyme source.

Estimation of Protein

Protein content of the enzyme extract was measured according to the method of Lowry et al. *(8)*.

Estimation of Alcohol Dehydrogenase Activity

Alcohol dehydrogenase activity was determined based on the procedure described by Maitra and Lobo *(9)*. The reaction mixture contained 2.7 mL of phosphate buffer, 0.1 mL of ethanol (3.0 *M*), 0.1 mL of nicotinamide adenine dinucleotide (0.025 *M*), and 0.1 mL of enzyme source. Enzyme activity was measured by reading the absorbance of NADH conversion at 340 nm using a Beckman UV-VIS spectrophotometer. One unit of enzyme activity is defined as the amount that causes a change in absorbance of 0.001/min.

Estimation of Sugar and Alcohol

Glucose content in the medium was estimated by coupling with dinitrophenyl salicylic acid *(10)*, and alcohol content was measured based on the procedure described by Prema et al. *(11)*.

Results

To study the influence of solid supports on alcohol fermentation, experiments were carried out using various solid supports: chitin, chitosan, delignified sawdust, delignified wheat bran, river sand, and titanium oxide. Control experiments were conducted without addition of solids in the medium. Table 1 represents the fermentation pattern for a period of 60 h. Chitin, chitosan, and delignified sawdust at 1.0% (w/v) demonstrated considerable promoting action on alcohol fermentation in comparison to control. The addition of river sand, delignified wheat bran, and titanium oxide also exhibited a moderate improvement (up to 28%) on alcohol fermentation. Among all the material used, chitosan flakes had the maximum influence on alcohol fermentation. The maximum alcohol yield obtained was 70 g/L in 60 h of fermentation when 1.0% (w/v) chitosan was added to the medium containing 150 g/L of sugar. The volumetric productivity of

Table 1
Effect of Various Support Material on Alcohol Production[a]

S. No.	Support material	Alcohol yield (g/L)	Alcohol produced (%)	Rate of alcohol production (g/[L·h])
1	Control	37.96	100.0	0.63
2	Titanium oxide	42.70	112.0	0.71
3	Delignified wheat bran	44.53	117.3	0.74
4	River sand	48.91	128.8	0.81
5	Chitin	55.60	146.7	0.93
6	Delignified sawdust	56.21	148.1	0.94
7	Chitosan	70.08	184.6	1.17

[a]One percent (w/v) support material was used in all cases. The initial glucose concentration was 150 g/L. The inoculum size was 10% of the medium. Observation was made after 60 h of fermentation at 30 ± 2°C.

the alcohol fermentation was 1.16 g/(L·h), almost double that of control (0.63 g/[L·h]). Although chitin and chitosan belong to the same group of biopolymers, chitin's role as an inducer of alcohol fermentation was 26% lower than that of chitosan's. Similarly, the delignified cellulosic material, sawdust, and wheat bran showed significant variation in enhancement of alcohol production. The capacity of sugar fermentation of the yeast in the presence of delignified sawdust was 15% higher in comparison to wheat bran (0.74 g/[L·h]). These data suggest that the nature of the surface support material is the important in alcohol fermentations.

To investigate the optimum concentration of solids for effective alcohol production in the presence of supports, fermentation experiments were carried out by the addition of different concentrations of chitin and chitosan, using 150 g/L of glucose as substrate. It was observed that the alcohol fermentation rates increased with an increase in the concentration of support material during fermentation (Table 2). Alcohol productivity was enhanced from 0.66 to 1.17 g/(L·h) when the chitosan concentration, in the fermentation broth, was increased from 0.1 to 1% (w/v). A further increase in chitosan was not found to be favorable for alcohol fermentation. Similarly, the addition of chitin also showed the same trend in alcohol production (Table 2). However, the rate of alcohol production was different. Alcohol productivity was enhanced from 0.77 to 1.04 g/(L·h) with an increase in chitin concentration from 0.5 to 1.5% (w/v). The yield of alcohol was 65% higher than control at the end of 60 h of fermentation, when 1.5% (w/v) chitin was added to the medium. When experiments were conducted using 1.0% (w/v) of chitin and chitosan in the fermentation medium, a 40% higher yield of alcohol, at the end of 60 h, was observed in the case of chitosan.

The observed variations in enhancement of alcohol production by these materials, at 1.0%, may be attributed to their structural differences and physicochemical characteristics, which are involved in providing the

Table 2
Influence of Chitosan and Chitin
on Ethanol Production by *Saccharomyces cerevisiae*[a]

S. No.	Name of support	Concentration used (%)	Alcohol-produced improvement over control (%)	Theoretical yield (%)
1	Control	—	100.00	50.60
2	Chitosan	0.1	104.59	52.94
3	Chitosan	0.2	116.23	58.82
4	Chitosan	0.5	153.33	77.60
5	Chitosan	1.0	184.66	93.40
6	Chitosan	1.5	118.45	59.93
7	Chitin	0.5	122.85	62.17
8	Chitin	1.0	146.67	74.13
9	Chitin	1.5	165.72	83.87
10	Chitin	2.0	140.52	71.10

[a]One percent (w/v) support material was used in all cases. The initial glucose concentration was 150 g/L. The inoculum size was 10% of the medium. Observation was made after 60 h of fermentation at 30 ± 2°C.

support surface to the microbial cells. In fact, it is well known that chitosan is less dense than chitin, and the physical observation of chitin and chitosan in the fermentation broth indicated that chitin always settled at the bottom, whereas, chitosan circulated up and down. Moreover, morphologically, chitosan looked like flakes and chitin as course powder.

The alcohol production profile in the presence and absence of 1% chitin, chitosan, and delignified sawdust was studied over a period of 60 h to investigate the role of these materials from the beginning of the fermentation. The data (*see* Fig. 1) clearly suggest that these materials alter the alcohol production and fermentation rate from the beginning of the fermentation process. This can be evidenced from the fact that after 12 h of fermentation, the productivity of alcohol in the presence of these solid supports was almost double that of control. Thereafter, the fermentation process changed depending on the support supplement. In all support-added conditions, ethanol production was higher when compared to control. The fermentation rates were observed to be almost similar in the presence of delignified sawdust and chitin. This observation suggests that the enhanced alcohol productivity may be related to the surface nature of the material rather than the chemical nature.

These observations are similar to those observed with *Agrobacterium radiobacter (12)*. The alcohol dehydrogenase activity was measured, in the presence and absence of chitosan, to ascertain their role on metabolic activity of the microbial cells. The specific activity in chitosan-supplemented cultures after 12 h of fermentation was 46.21 U/mg of protein, whereas in the absence of chitosan, it was 23.93 U/mg of protein. A 93% increase of

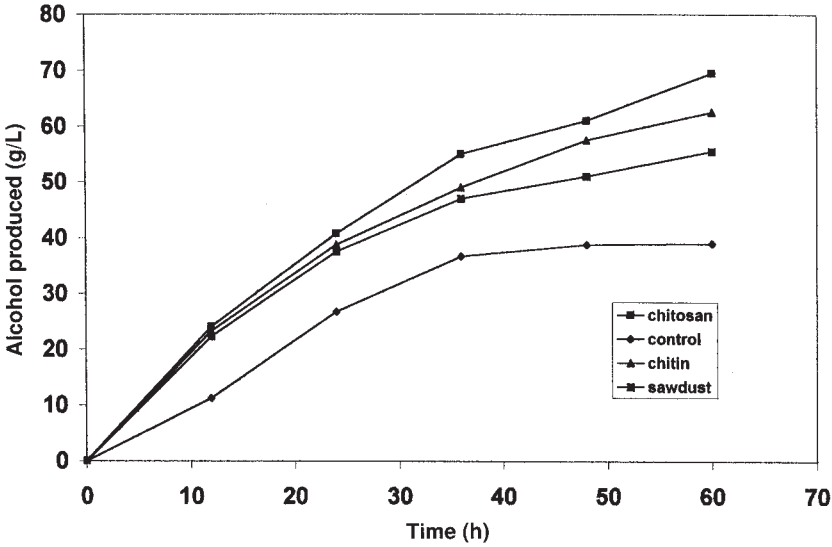

Fig. 1. Effect of solid supports on alcohol production.

enzyme activity in the presence of chitosan support compared to that of control indicates that the solid support in fermentation broth altered the metabolism of the yeast. Since the chitosan used in our study was not dissolved in broth, the observed enhanced biocatalytical activity of alcohol dehydrogenase can be related to the altered metabolic shift owing to anchorage.

Discussion

The influence of support particles, on cellular metabolism, has been studied by several researchers under various contexts. Solid-state fermentation, in which cells are proliferated on solid substrate, is a good example of cell-surface interactions. Similarly, the use of biofilms and induced adhesion on supports (passive immobilization methods) are a few of the known techniques in which cellular metabolism has been reported to be altered. Although the influence of supports on cell respiration was observed quite early, only after the development of cell immobilization techniques, the subject matter became the study of interest. The intensified bioactivity of immobilized cells has been attributed to the microenvironment created by the immobilization process. The influence of chemical, physical, and structural aspects of support matrices have been studied *(13)*.

In the present study, the addition of 1.0% (w/v) solids into the medium considerably increased the rate of production of alcohol since the support material added to the medium induced cell anchorage. At any point of time, the medium contained both, anchored and free cells, and the resultant metabolic activity was dependent on the fraction of the cells adhered to the supports. If a major portion of the cells adhered, the system resembled

a whole-cell immobilized system, and the productivities were very high. If the medium contained a very low fraction of anchored cells, the overall activity was nearer to that of the free-cell system.

From the results, it is obvious that the characteristics of the supports are important to derive the maximum benefit. Iconomou et al. *(7)* reported a higher activity of *S. cerevisiae* of 129% when 20% of solids were introduced into the fermentation. They speculated that the improved activity was owing to metabolic shifts of cells, whereas zeolite-mediated enhanced alcohol productivity was attributed to the neutralization associated with a better nutrient supply to the anchored cells *(7)*. Our results demonstrate the metabolic shifts of *S. cerevisiae*, as evidenced by higher alcohol dehydrogenase activity. Such observations are in accordance with the noticed changes in biomass as well as the product formation in celite-supported *Penicillium chrysogenum* fermentation *(14)*. Kim et al. *(14)* observed a 1.5- and 1.6-fold increase in cell mass and specific growth rate in the presence of celite with the antibiotic-producing fungus. This improved penicillin production was attributed to the increased mass transfer owing to reduced viscosity of the culture broth.

Although chitosan and chitin exhibited a maximum influence on alcohol production, because of economic reasons, it may not be possible to implement their use in a large-scale system. Similarly, the use of delignified sawdust or wheat bran is not feasible because of the processing cost involved. River sand (1.0%, w/v) addition to promote alcohol fermentation appears to be most promising, and one can achieve an enhanced rate of alcohol fermentation of 28–30% (Table 1).

The present investigation led us to postulate that incorporation of a small quantity of solids in submerged fermentations to promote cell anchorage will be advantageous over artificially prepared cell-immobilized systems. To the best of our knowledge, this is the first study on the use of sand for enhanced alcohol fermentation. We have also noticed such enhanced metabolic activity of *Escherichia coli* for the production of lipase (data not shown). Further studies are in progress regarding cell anchorage.

References

1. Douglas, S. R., Fleming, A., and Colebrook, M. B. (1917), *Lancet* **2,** 530–532.
2. Jannasch, H. W. and Pritchard, P. H. (1972), *Mem. Ist. Ital. Idrobiol.* **29(Suppl.),** 289–308.
3. Patil, S. G. and Patil, B. G. (1989), *Enzyme Microb. Technol.* **11,** 38–43.
4. Castellar, M. R., Aires-Barros, M. R., Cabral, M. S. J., and Iborra, J. L. (1998), *J. Chem. Technol. Biotechnol.* **73,** 377–384.
5. Roque-Malherbe, R., Delogado, R., Contreras, O., and Lago, A. (1987), *Biotechnol. Lett.* **9,** 640–642.
6. Demuyokor, B. and Ohta, Y. (1992), *Appl. Microbiol. Biotechnol.* **36,** 717–720.
7. Iconomou, L., Psarianos, C., and Koutinas, A. (1995), *J. Ferm. Bioeng.* **79,** 294–296.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. L. (1951), *J. Biol. Chem.* **193,** 265–275.
9. Maitra, P. K. and Lobo, Z. (1971), *J. Biol. Chem.* **246,** 475–477.
10. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.

11. Prema, P., Ramakrishna, S. V., and Madhusudana Rao, J. (1986), *Biotechnol. Lett.* **8,** 449, 450.
12. Stotzky, G. and Rem, L. T. (1966), *Can. J. Microbiol.* **12,** 547–563.
13. Rouxhet, P. G. and Mozes, M. (1990), in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., eds., Elsevier, Amsterdam, pp. 343–354.
14. Kim, J. H., Oh, D. K., Park, S. K., and Park, Y. H. (1986), *Biotechnol. Bioeng.* **28,** 1838–1842.