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High alcohol production by repeated batch fermentation using an immobilized osmotolerant *Saccharomyces cerevisiae*

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A repeated batch fermentation system was used to produce ethanol using an osmotolerant *Saccharomyces cerevisiae* (VS_3) immobilized in calcium alginate beads. For comparison free cells were also used to produce ethanol by repeated batch fermentation. Fermentation was carried for six cycles with 125, 250 or 500 beads using 150, 200 or 250 g glucose L^{-1} at 30°C. The maximum amount of ethanol produced by immobilized VS_3 using 150 g L^{-1} glucose was only 44 g L^{-1} after 48 h, while the amount of ethanol produced by free cells in the first cycle was 72 g L^{-1}. However in subsequent fed batch cultures more ethanol was produced by immobilized cells compared to free cells. The amount of ethanol produced by free cells decreased from 72 g L^{-1} to 25 g L^{-1} after the fourth cycle, while that of immobilized cells increased from 44 to 72 g L^{-1}. The maximum amount of ethanol produced by immobilized VS_3 cells using 150, 200 and 250 g glucose L^{-1} was 72.5, 93 and 87 g ethanol L^{-1} at 30°C. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 222–226.

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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 [13,14]. One such process is yeast cell immobilization which facilitates faster fermentation rates by providing higher cell densities per unit fermentation volume, the *in situ* removal of cells reduces the cost of recovery [2]. It also helps in increasing ethanol tolerance and thereby ethanol yield and reduces the costs required for inoculum development [6]. Immobilization of microbial cells in alginate systems provides an ideal means of biocatalyst recycling for use in fermentation systems concerned with conversion of soluble substrates to ethanol [3–5,11].

Yeast strains normally used in industrial processes have limited osmotolerance. For this reason alcohol fermentations are carried out at comparatively low sugar concentrations (usually ⩽20% w/v). High initial sugar concentration results in loss of sugar transport activity, producing less ethanol [12]. It is desirable to obtain a high ethanol concentration during fermentation which helps in decreasing distillation costs [1]. We have reported the production of ethanol by solid substrate fermentation by the VS_3 strain of *Saccharomyces cerevisiae* isolated in our laboratory [7,8].

In view of the need to produce high ethanol using inexpensive technology and the potential advantages of osmotolerance and immobilization in increasing ethanol yields, we have investigated the suitability of immobilized *S. cerevisiae* VS_3 for production of ethanol.

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Materials and methods

Microorganism

S. cerevisiae VS_3 was isolated from soil samples collected within the hot regions near the Kothagudem Thermal Power Plant located in Khammam Dist, AP India. The organism was identified as *S. cerevisiae* by Mayen Ex Hansen. It was maintained on yeast extract peptone dextrose medium (YEPD) and was transfered to two 500-ml conical flasks with 250 ml medium containing peptone 2%, yeast extract 1%, and dextrose 2% at pH 5.5. After incubation for 48 h on a shaker at 30°C, the cells were centrifuged at 500 × *g* for 15 min.

Immobilization in alginate

The pellet obtained after centrifugation was washed in phosphate buffer and recentrifuged. About 3 g of cells (wet weight) were added to 100 ml of 3% (w/v) sodium alginate. About 1200 beads of 5-mm diameter were obtained for every 100 ml of 3% sodium alginate. The suspension after mixing was taken up in a 10-ml syringe without a needle and added dropwise to 4% cold calcium chloride solution. After overnight incubation in calcium chloride, the beads were transferred to YEPD medium for activation. After incubation for 16 h in YEPD medium, about 125 beads (0.3125 g of yeast cells) were transferred to 100 ml yeast fermentation medium (YFM) containing 150, 200 or 250 g glucose L^{-1} at 30°C. Control experiments consisted of an equivalent amount of free cells inoculated into fermentation medium. In subsequent cycles immobilized cells were separated from the exhausted medium and replaced in fresh medium containing 150, 200 or 250 g glucose L^{-1}. For comparison the free cells after the first cycle of fermentation were recovered by centrifugation and re-fed with fresh medium containing 150 g glucose L^{-1}.

To study the effect of bead number (inoculum size) on production of ethanol, in subsequent experiments the num-

ber of beads was increased to 250 (0.625 g of yeast cells) in a sugar concentration of 150 or 200 g glucose L^{-1}. Repeated batch fermentations were carried out for six cycles except for 500-bead (1.25 g of yeast cells) experiments, which were conducted for only three cycles. All experiments were replicated twice and the average values are presented. A variation of about 5% was seen between the two experiments.

Analytical methods

Samples were withdrawn from the fermentation broth after 24 and 48 h of incubation for estimation of ethanol. Ethanol was estimated by High Pressure Gas Chromatography (HPGC, Hewlett Packard HP 4890D, Poropak Q, mesh size 80–100 μm) using nitrogen as carrier gas and hydrogen gas in the flame detectors, both at a flow rate of 32 ml min^{-1}. The amount of residual sugar was estimated by the DNS method [9] for calculating the theoretical yield.

Statistical analysis

Data obtained from all the experiments was subjected to statistical analysis using ANOVA to compare the significance of differences between cycles, time points, bead numbers and glucose concentrations.

Results and discussion

In the immobilization process productivity depends on such factors as size of inoculum, type of microorganisms, nature of the substrate and the type of carrier material used for immobilization. Alginate carriers enhance activities of yeasts compared to other carriers and hence alginate immobilization was used to study the production of ethanol by repeated batch fermentation [3].

S. cerevisiae VS_3 is thermotolerant and was found to be suitable for production of ethanol by solid substrate fermentation at a higher temperature [7,8]. When this strain was immobilized and used for ethanol production at higher temperatures it gave a lower ethanol yield (45 g ethanol L^{-1}) hence immobilization experiments were conducted at 30°C.

The amounts of ethanol present in 150, 200 or 250 g glucose L^{-1} after repeated batch fermentation for six cycles after 24 and 48 h using 125 beads are shown in Tables 1, 2 and 3. The time shown in the tables is for each cycle. These results show that less ethanol was produced during the first cycle using 125 beads. The maximum amount of ethanol produced in the first cycle after 48 h of incubation using 150, 200 or 250 g glucose L^{-1} was 34, 36 and 40 g ethanol L^{-1}, respectively.

This might be due to the decreased accessibility of substrate to the biocatalyst and lower substrate concentrations toward the center of the bead as opposed to any change in the metabolic rate of immobilized cells [11]. The amount of ethanol produced in subsequent cycles was greater than that produced in the first cycle. The maximum amount of ethanol produced after the sixth cycle was 62, 92 and 87 g ethanol L^{-1} from 150, 200 and 250 g glucose L^{-1}, respectively. Compared to immobilized cells the amount of ethanol produced by free cells in the first cycle was greater but decreased subsequently as shown in Figure 1. The amount of ethanol produced in the sixth cycle using 150 g L^{-1} glucose with 125 beads decreased from 62 g to 52 g which might be due to loss of vigour of the yeast cells because of aging and due to leakage of cells from beads.

The amount of ethanol produced by free cells in the first cycle using 150 g glucose L^{-1} was 72 g ethanol L^{-1} whereas immobilized cells gave only 34 g of ethanol L^{-1} using 125 beads. The amount of ethanol produced by free cells after the fourth cycle decreased to 25 g while in immobilized cells it increased to 62 g ethanol L^{-1}. This might be due to the fact that immobilized cells contain significantly higher percentages of saturated fatty acids compared to free cells and this increased saturation gradually leads to greater ethanol tolerance in the immobilized cells and hence greater survival and productivity in subsequent cycles compared to free cells [6].

Ethanol produced by immobilized cells using 250 g glucose was slightly less (87 g) compared to 200 g glucose (92 g). This might be due to a decreased rate of sugar transport activity at high sugar concentrations [12]. Hence in subsequent studies only 200 g glucose was used. In order to study the effect of inoculum size in repeated batch fermentations using immobilized cells, the number of beads was increased from 125 to 250 (0.3125 g of yeast cells to 0.625 g).

The amount of ethanol in the medium containing 150

Table 1 Ethanol production using alginate-immobilized cells using 150 g L^{-1} sugar and 125 beads by repeated batch fermentation

No. of cycles	Yield after					
	24 h			48 h		
	Practical yield	Theoretical yield	Fermentation efficiency %	Practical yield	Theoretical yield	Fermentation efficiency %
1	28	56.65	50	34	60.77	56
2	59	66.9	89	62	66.95	93
3	54	65.40	83	62	69.01	90
4	58	66.43	88	60	67.98	89
5	58	66.40	87	62	69.01	90
6	58	66.43	88	52	69.01	76

Table 2 Ethanol production using alginate-immobilized cells using 200 g L^{-1} sugar and 125 beads by repeated batch fermentation

No. of cycles	Yield after					
	24 h			48 h		
	Practical yield	Theoretical yield	Fermentation efficiency %	Practical yield	Theoretical yield	Fermentation efficiency %
1	26	97.90	28.04	36	81.37	44.20
2	63	92.70	67.96	69	95.79	72.0
3	57	92.18	61.83	66	93.73	70.40
4	68	95.27	71.37	78	99.90	78.0
5	80	97.85	81.75	92	100	90.1
6	80	97.85	81.75	92	102	90.1

Table 3 Ethanol production using Alginate-immobilized cells using 250 g L^{-1} sugar and 125 beads by repeated batch fermentation

No. of cycles	Yield after					
	24 h			48 h		
	Practical yield	Theoretical yield	Fermentation efficiency %	Practical yield	Theoretical yield	Fermentation efficiency
1	35	103.0	40.0	40	104.0	39.40
2	66	108.15	62.0	87	115.0	76.0
3	65	107.12	61.0	83	114.8	73.0
4	76	109.0	70.0	85	115.3	74.0
5	78	112.0	70.0	86	115.0	74.7
6	79	113.0	70.0	85	115.0	74.0

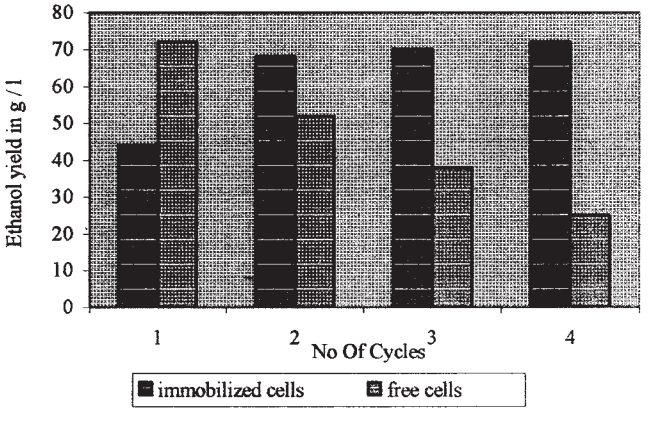

Figure 1 Comparison of ethanol production by immobilized and free cells by repeated batch fermentation using 150 g glucose L^{-1} after 48 h at 30°C.

and 200 g glucose after 24 and 48 h using 250 beads for six cycles is shown in Table 4. These results also indicate that the amount of ethanol produced in the first cycle was less compared to subsequent cycles. More ethanol was produced with 250 beads than 125 beads. The amount of ethanol produced with 150 g L^{-1} glucose using 125 beads in the first cycle after 24 h of fermentation was only 28 g compared to 38 g produced from 250 beads. The maximum amount of ethanol produced from 150 and 200 g glucose L^{-1} using 250 beads was 72 and 93 g after 48 h. About 90% of the ethanol was produced in 24 h of fermentation by increasing the bead number to 250. The amount of ethanol produced after 24 h from 150 and 200 g glucose L^{-1} using 125 beads was 59 and 80 g respectively compared to 63 and 88 g ethanol with 250 beads. This might be due to an increase in cell density which resulted in faster fermentation.

In the later experiments the bead number was increased from 250 to 500 (0.625 g of yeast cells to 1.25 g). Samples were collected after 8, 16, 24 and 48 h for estimation of ethanol for three cycles. The rate of fermentation increased but the amount of ethanol produced almost remained the same (as that of 250 beads) as shown in Table 5. Thus the maximum amount of ethanol produced from 150 g glucose using 500 beads was 35, 60, 68 and 72.5 g after 8, 16, 24 and 48 h. About 80% of ethanol was produced within 16 h using 500 beads.

The results of statistical analyses are shown in Table 6. These results indicate that ethanol production was significantly different with increasing cycles, time points, beads and sugar concentrations. The magnitude of differences for ethanol production between time and fermentation (beads and sugar concentrations) were more significant compared to cycles and fermentation. The differences in interactions between cycles and time points were not significant.

The results of statistical analyses of production by immobilization using 500 beads with 150 g L^{-1} glucose concentration at different time intervals are shown in Table 7.

Table 4 Ethanol production by repeated batch fermentation by alginate-immobilized cells using 250 beads with 150 and 200 g glucose L^{-1} at 30°C

No. of cycles	Yield at sugar concentration (g L^{-1})											
	150						200					
	24 h			48 h			24 h			48 h		
	PY	TY	FE	PY	TY	FE	PY	TY	FE	PY	TY	FE
1	38	60.7	63	44	63.3	70	54	82.4	66	60	86.5	70
2	60	71.0	85	68	74.1	92	85	98	87	90	100	90
3	62	71.5	87	68	73.6	93	88	99	89	92	101	91
4	60	70.5	85	70	75.1	94	87	99.3	88	93	102	91
5	62	71	88	72	76	95	88	99.6	89	93	102	91
6	63	72.1	88	72	76	95	88	99.3	89	93	102	91

PY = Practical yield. TY = Theoretical yield. FE = Fermentation efficiency %.

Table 5 Ethanol production by immobilized cells from 150 g glucose L^{-1} using 500 beads at 30°C

No. cycles	Practical yield after			
	8 h	16 h	24 h	48 h
1	30.0	36.0	42.0	50.0
2	35.0	59.5	64.0	70.5
3	35.0	60.0	68.0	72.5

Table 6 Anova table for ethanol production using 125 and 250 beads with different glucose concentrations

Source of variation	F ratios (variance ratios)	Degrees of freedom	Significance of F
Main effects			
Cycles	606.0	5, 60	0.001
Time	265.2	1, 60	0.002
Fermentation (Beads + sugar concentrations)	466.9	4, 60	0.003
2-way interactions			
Cycle *vs* time	1.59	5, 60	0.17
Cycle *vs* fermentation	17.8	20, 60	0.001
Time *vs* fermentation	8.39	4, 60	0.0001
3-way interactions			
Cycle, time & fermentation	2.77	20, 60	0.001

Table 7 Anova table for ethanol production using 500 beads with 150 g L^{-1} glucose at different time intervals

Source of variation	F ratios (variance ratios)	Degrees of freedom	Significance of F
Main effects			
Cycles	761.3	2, 12	0.0001
Time	131.8	1, 12	0.0001
Beads	62.5	1, 12	0.0001
2-way interactions			
Cycle *vs* time	1.1	2, 12	0.36
Cycle *vs* beads	1.6	2, 12	0.23
Time *vs* beads	0.2	1, 12	0.66
3-way interactions			
Cycle, time & beads	1.2	2, 12	0.31

There was a significant difference in ethanol production with increasing cycles, time points and beads. The differences between beads was greater than the difference between time points and cycles. Interactions between cycles and time points, cycles and beads, time and beads as well as among the three (cycles, time and beads) are not significant.

Nigam *et al* [10] reported maximum alcohol production of about 40.9, 63.4 and 68.6 g L^{-1} from 100, 150 and 200 g glucose L^{-1}, respectively, using *Kluyveromyces marxianus* IMB3 immobilized in mineral kissiris. But our strain gave maxima of 72 and 93 g ethanol L^{-1}. Though ethanol production by calcium alginate immobilization is frequently used, the high ethanol yield produced by *S. cerevisiae* VS_3 and viability of the system for more than 450 h makes the process novel and economical for ethanol production. These results indicate that *S. cerevisiae* VS_3 is suitable for producing high ethanol by alginate immobilization with less expensive technology.

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References

1 Bertolini MC, JR Ernandes and C Laluce. 1991. New yeast strains for alcoholic fermentation at higher sugar concentration. Biotechnol Lett 13: 197–202.
2 Chaudhary AB and SB Chincholkar. 1996. Cell immobilisation. A critical approach to ethanol production by *Saccharomyces cerevisiae* and *Schizo Saccharomyces pombe*. Ind J Microbiol 36: 75–83.
3 Ciesarova Z, Z Domeny, D Smogrovicova, J Patkova and E Sturdik. 1998. Comparision of ethanol tolerance of free and immobilised *Saccharomyces uvarum* yeasts. Folia Microbiologica 43: 55–58.
4 Gilson CD and A Thomas. 1995. Ethanol production by alginate

immobilized yeast in fluidized bed bioreactor. J Chem Technol Biotechnol 62: 38–45.
5 Kannan JR, G Sangili Yandi and P Gunasekharan. 1998. Improved ethanol production from sucrose by mutant *of Zymomonas mobilis* lacking sucrases in immobilised cell fermentation. Enzyme Microb Technol 22: 179–184.
6 Krisch J and B Szajani. 1997. Ethanol and acetic acid tolerance in free and immobilised cells of *Saccharomyces cerevisiae* and *Acetobacter aceti*. Biotechnol Lett 19: 525–528.
7 Kiran Sree N, M Sridhar, L Venkateswar Rao and A Pandey. 1999. Ethanol production. Solid substrate fermentation using thermotolerant yeast. Proc Biochem 34: 115–119.
8 Kiran Sree N, M Sridhar, K Suresh and L Venkateswar Rao. 1999. High alcohol production by solid substrate fermentation from starchy substrates using thermotolerant *Saccharomyces cerevisiae*. Bioproc Engin 20: 561–563.
9 Miller GL. 1959. Use of dinitro salicilic acid as reagent for determination of reducing sugars. Anal Chem 31: 426–428.
10 Nigam P, IM Banat, D Singh, AP McHale and R Merchant. 1997. Continuous ethanol production by thermotolerant *Kluyveromyces marxianus* IMB3 immobilized on mineral kissiris at 45°C. World J Microbiol Biotechnol 13: 283–288.
11 Nolan AM, N Brady Barron, T McAree, D Smith, L McHale and AP McHale. 1994. Ethanol production at 45°C by an alginate immobilized thermotolerant strain of *Kluyeromyces marxianus* following growth on glucose containing media. Biotechnol Lett 16: 849–852.
12 Salmon JM and JC Maurico. 1994. Relationship between sugar uptake kinetics and total sugar consumption in different industrial *Saccharomyces cerevisiae* strains during alcohol fermentation. Biotechnol Lett 16: 89–94.
13 Suresh K, N Kiran Sree and L Venkateswar Rao. 1999. Production of ethanol by raw starch hydrolysis and fermentation of damaged grains of wheat and sorghum. Biores Technol 8: 301–304.
14 Suresh K, N Kiran Sree and L Venkateswar Rao. 1999. Utilisation of damaged sorghum and rice grains for ethanol production by simultaneous saccharification and fermentation. Biores Technol 68: 301–304.