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Comparative Study on Ethanol Production by Repeated Batch Fermentation Using an Immobilized Yeast Strain, Isolated from Toddy Sap

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ABSTRACT

Sodium-alginate immobilized yeast strain (NCIM3640) was employed to produce ethanol continuously using cane molasses as a carbon source in repeated batch fermentation system. For comparison free cells were also used to produce ethanol by repeated batch fermentation. Fermentation was carried out six cycles with 200, 400 or 600 beads using 150, 200 or 250 g sugar in molasses/L^{-1}. The maximum amount of ethanol produced byimmobilized NCIM 3640 strain suing 150 g glucose was only after 36 hr, while the amount of ethanol produced by free cells in the first cycle was 71.92 g ethanol L^{-1}. However in subsequent fed batch cultures more ethanol was produced by immobilized cells compared to free cell. The amount of ethanol produced by free cells decreased from 71.38 to 48.10 gL^{-1}after the fourth cycle, while that of immobilized cells increased from 37.21 to 71.12g L^{-1}. Among the three strains such as NCIM3, the maximum amount of ethanol produced by immobilized NCIM 3640 cells using 150, 200 and 250 g molasses/L^{-1} was 61, 86.2 and 80.2 g ethanol L^{-1} respectively at 30^0C.

Keywords: Ethanol production; Immobilization; Repeated batch fermentation; *Saccharomyces cerevisiae;* Molasses

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INTRODUCTION

As the demand for ethanol is showing a progressive rise over the years, there is an imminent requirement of high yielding production strains and economically viable process realization for the production of ethanol [1]. 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, 3]. It also helps in increasing ethanol tolerance and thereby ethanol yield and reduces the cost required for inoculums development [4]. Immobilization of microbial cells in alginate systems provides an ideal means of biocatalysts recycling for use in fermentation systems concerned with conversion of soluble substrates to ethanol. Yeast strains normally used in industrial processes have limited osmotolerance [5-7]. For this reason alcohol fermentations are carried out at comparatively low sugar concentrations. High initial sugar concentration results in loss of sugar transport activity, producing less ethanol [8]. It is desirable to obtain a high ethanol concentration during fermentation which helps in decreasing distillation costs [9].

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* for production of ethanol.

MATERIAL AND METHODS

S.cereviseae NCIM3640 was isolated from toddy sap, Visakhapatnam, Andhra Pradesh, India. It was maintained on yeast extract peptone dextrose medium (YEPD) and was transferred 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 36 h on a shaker at 30^0C, the cells were centrifuged at 500x g for 15 min.

The seed culture obtained after centrifugation was washed in phosphate buffer and re-centrifuged. About 3 g of cells (wet weight) were added to 100 ml of 4% (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 drop wise 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 200 beads, equivalent to 0.3125 g of yeast cells were transferred to 100 ml yeast fermentation medium (YFM) containing 150, 200 or 250 g molasses 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 molasses 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 molasses L^{-1}. To study the effect of bead number (inoculum size) on production of ethanol, in subsequent experiments the number of beads was increased to 400 (0.625 g of yeast cells) in a sugar concentration of 150 or 200 g glucoseL^{-1}. Repeated batch fermentations were carried out for six cycles except for 600-bead (1.25 g of yeast cells)

experiments, which were conducted for only three cycles. All experiments were carried out in triplicate and the average values are presented. A variation of about 5% was seen between the three experiments.

Analytical methods

The determinations were cell viability using the methylene blue method [10]. Total reducing sugar in acid hydrolysate (1.2M HCL for 10 min at 60^0C), using the 3,5-dinitrosalicylic acid method [11]. 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 70^0C 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 (SHIMADZU, GC-2014, Kyoto, Japan) with a flame ionization detector was used for ethanol analysis of the samples. The samples were filtered through 0.2µm micro filters for ethanol concentration before injection. Column: Rtx^R-5 Crossbond^{R}5% diphenyl/95% dimethyl poly siloxane, Column Temperature - 120^0C, Injector Temperature- 150^0C, Detector Temperature - 200^0C, Flow rate of carrier gas- 50ml\minute. The ethanol yield (Yps) was calculated as the actual ethanol produced and expressed as g ethanol per g total sugar utilized (gg^{-1}). The volumetric ethanol productivity Qp ($gl^{-1}h^{-1}$) was calculated by ethanol concentration produced P (gl^{-1}) 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 (4000 rpm, 10 minutes) 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.

Determinants of ethanol concentration – a statistical analysis

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

To identify the determinants of Ethanol concentration levels the following linear regression model in its modified form has been used.

$Y = f(\alpha+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+U)$

Where Y stands for Levels of Ethanol concentration

X_1 stands for Number of Beads

X_2 stands for Number of Cycles

X_3 stands for Time

X_4 stands for Sugar concentration

β_1 to β_4 are the coefficients of the independent variables(X_1,X_2,X_3,X_4)

U stands for error term

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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 [5]. Sree*et al.,*used immobilized cells of *S.cerevisiae*that could ferment upto 25% glucose [2]. Yadav*et al.,*studied high ethanol productivity in an immobilized cell reactor. Maximum ethanol productivity (40 g l^{-1} h^{-1}) was achieved at 30ºC, pH 4.5 at a dilution rate 0.20h^{-1}. The maximum amount of cells that could be immobilized in 1.5% Ca alginate gel was found to be 50% (wet wt basis) but the gel beads containing 30% (w/v) cells resulted in maximum productivity. In order to maintain cells viability and metabolic activity, the best tolerant strains were used in alginate immobilization (4%) [12]. Immobilization of cells is a method to increase the cell mass concentration in the fermenter to increase the process productivity and minimize the production costs, while offering advantages over free cell fermentation operations [13]. George and Colin (1991) reported in a review is to assess the merits of viable cell immobilization in the light of published literature and to elucidate the underlined mechanisms. Particular attention is paid to the generally unanticipated, but widely observed enhanced stability of immobilized cell fermentation processes [14]. The strain of *S. cerevisiae*(NCIM 3640), used in this work, was a good alcohol producer isolated from toddy sap. Other Industrial and commercial strains of *S. pombe*and *S.cerevisiae*(NCIM3570) were also used with this strain using diluted sugar cane molasses as the source of carbon. The amounts of ethanol present in 150, 200 or 250 g molassesL^{-1}after repeated batch fermentation for six cycles after 18 and 36 h using 200 beads are shown in Tables 1, 2& 3.

Table 1. Ethanol production using alginate immobilized cells with 200 beads and free cells using 150 g/sugar by repeated batch fermentatione

No of cycles	NCIM3640 strain yield after					
	18			36		
	$P(gl^{-1})$	Y_{ps} (gg^{-1})	E_y(%)	$P(gl^{-1})$	$Y_{ps}(gg^{-1})$	E_y(%)
1	24.06	0.16	31.3	36.21	0.24	47.8
2	52.14	0.34	66.7	60.52	0.39	77.9
3	51.14	0.33	66.4	61.24	0.40	79.8
4	52.18	0.34	67.6	61.62	0.40	79.8
5	51.68	0.34	66.5	60.01	0.39	77.2
6	49.51	0.33	64.0	57.14	0.38	74.5
	Free cell yield after 24 hr			48 hr		
1	50.56	0.33	65.5	71.38	0.46	91.5
2	48.51	0.32	62.7	66.51	0.43	85.2
3	48.14	0.31	62.3	54.86	0.36	71.0
4	46.98	0.31	60.8	48.10	0.31	62.1

P: ethanol concentration; $Y_{ps:}$ ethanol yield, Ey (%) ;fermentation efficiency

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Table 2. Ethanol production using alginate immobilized cells using 200 g/sugar and 200beads by repeated batch fermentation

No of cycles	NCIM3640 strain yield after					
	18			36		
	$P(gl^{-1})$	Y_{ps} (gg^{-1})	$E_y(\%)$	$P(gl^{-1})$	$Y_{ps}(gg^{-1})$	$E_y(\%)$
1	28.06	0.14	27.4	38.29	0.19	37.3
2	57.14	0.27	53.7	68.52	0.34	66.9
3	56.14	0.27	54.6	70.24	0.34	68.4
4	57.18	0.28	55.9	83.12	0.41	81.2
5	72.68	0.36	71.0	86.21	0.43	84.3
6	73.51	0.36	71.7	86.14	0.43	84.1

P: ethanol concentration; $Y_{ps:}$ ethanol yield, Ey (%) ;fermentation efficiency

Table 3. Ethanol production using alginate immobilized cells using 250 g/sugar and 200beads by repeated batch fermentation

No of cycles	NCIM3640 strain yield after					
	18			36		
	$P(gl^{-1})$	Y_{ps} (gg^{-1})	$E_y(\%)$	$P(gl^{-1})$	$Y_{ps}(gg^{-1})$	$E_y(\%)$
1	28.05	0.11	21.9	40.10	0.16	31.4
2	57.14	0.22	44.7	68.52	0.27	53.6
3	56.14	0.22	43.9	72.24	0.28	56.5
4	64.18	0.25	50.2	79.18	0.31	61.9
5	71.68	0.28	56.1	80.25	0.32	62.8
6	70.51	0.28	56.2	80.17	0.32	62.7

The amount of ethanol present in 150 g molasses L^{-1} after repeated batch fermentation for six cycles after 18 and 36 hr using 200 beads are shown in Table 1. The time shown in the tables for each cycle.These results show that less ethanol was produced during the first cycle using 200 beads. The maximum amount of ethanol produced in the first cycle after 36 hr of incubation using 150 g molasses L^{-1} was 36.21 g ethanol L^{-1}. The amount of ethanol produced after the sixth cycle was greater than that produced in the first cycle. The maximum amount of ethanol produced after sixth cycle was 61.62 g ethanol L^{-1}. Compared to immobilization cells the amount of ethanol produced by free cells in the first cycle was greater but decreased subsequently. The amount of ethanol produced by free cells in the first cycle was 68.25 g ethanol L^{-1} whereas immobilized cells gave only 36.21 g ethanol L^{-1}. The amount of ethanol produced by free cells after the fourth cycle decreased to 18 g while in immobilized cells it increased to 61.62g ethanol L^{-1}.

The amount of ethanol present in 200 g molasses L^{-1} after repeated batch fermentation for six cycles after 18 and 36 hr using 200 beads are shown in Table 2. These results also indicate that the amount of ethanol was produced in the first cycle was less compared to subsequent cycles. The maximum amount of ethanol produced in the first cycle after 36 hr of incubation using 200 g molasses L^{-1} was 38 g ethanol L^{-1}. The amount of ethanol produced after the sixth cycle was greater than that produced in the first cycle. The maximum amount of ethanol produced after sixth cycle was 86 g ethanol L^{-1}.

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The microorganisms when entrapped with the spongy matrices led to decreased medium viscosity and enhanced oxygen and nutrient transfer which eventually showed more substrate consumption with faster rates yielding ethanol within less incubation times [15]. Ganguly*et al*., also observed the lactic acid production reached its maximum level (80.75 g/L) after 48 h of incubation with immobilized cells while, the maximum production level (86.13 g/L) with free cells was obtained after 72 h of incubation. These systems, therefore, can be used for developing the repeated batch and continuous processes for economizing the production process [16]. Recently, Chandal*et al.,* reported that free cells showed maximal ethanol production (19.45 ± 0.55 g/L) at 48 h of incubation and remained constant till 72 h. A maximum of 88 ± 0.37% sugars were utilized during the fermentation. The left over sugars were mainly pentoses (6.46 ± 0.05 g/L). Interestingly, immobilized *S. cerevisiae VS3* cells showed maximum ethanol produced (21.66 ± 0.62 g/L) at 36th h of incubation and thereafter remained constant till 72 h. The maximum ethanol yield (0.434 ± 0.021 g/g) was observed with the productivity of 0.601 ± 0.022 g/L/h with the sugar utilization efficiency of 92.60 ± 0.68%. The left over sugars were mainly pentoses (4.01 ± 0.03 g/L) [17].

The amount of ethanol present in 250 g molasses L^{-1} after repeated batch fermentation for six cycles after 18 and 36 hr using 200 beads are shown in Table 3. These results also indicate that the amount of ethanol was produced in the first cycle was less compared to subsequent cycles. The maximum amount of ethanol produced in the first cycle after 36 hr of incubation using 250 g molasses L^{-1} was 40.1 g ethanol L^{-1}. The amount of ethanol produced after the sixth cycle was greater than that produced in the first cycle. The maximum amount of ethanol produced after sixth cycle was 80.5 g ethanol $L^{-1.}$ Ethanol produced by immobilized cells using 250 g glucose was slightly less (80.5) compared to 200 g glucose (86.25). These results show that less ethanol was produced during the first cycle using 200 beads. The maximum amount of ethanol produced in the first cycle after 36 h of incubation using 150, 200 or 250 g molasses L^{-1} was 36, 38 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 [18]. 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 61, 86.2 and 80.2 g ethanol L^{-1} from 150, 200 and 250 g molassesL^{-1}, respectively. The amount of ethanol produced in the sixth cycle using 150 g L^{-1}molasses with beads decreased from 61 g to 57 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 molasses L^{-1} was 61 g ethanol L^{-1}whereas immobilized cells gave only 36 g of ethanol L^{-1} using 200 beads. The amount of ethanol produced by free cells after the fourth cycle decreased to 18.4 g while in immobilized cells it increased to 61 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 [18]. Ethanol produced by

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immobilized cells using 250 g glucose was slightly less (80.2 g) compared to 200 g molasses (86.2 g). This might be due to a decreased rate of sugar transport activity at high sugar concentrations [8]. Hence in subsequent studies only 200 g molasses 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 200 to 400 (0.5 g of yeast cells to 1 g). The amount of ethanol in the medium containing 150 and 200 g molasses after 24 and 48 h using 400 beads for six cycles is shown in Table 4&5. 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 400 beads than 200 beads. The amount of ethanol produced with 150 g L^{-1} molasses using 200 beads in the first cycle after 18 h of fermentation was only 24 g compared to 37 g produced from 400 beads. The maximum amount of ethanol produced from 150 and 200 g molasses L^{-1} using 400 beads was 71 and 87 g after 36 h. 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. About 90% of the ethanol was produced in 24 h of fermentation by increasing the bead number to 400. The amount of ethanol produced after 18 h from 150 and 200 g molasses L^{-1} using 200 beads was 52 and 73 g respectively compared to 62 and 75 g ethanol with 400 beads. This might be due to an increase in cell density which resulted in faster fermentation.

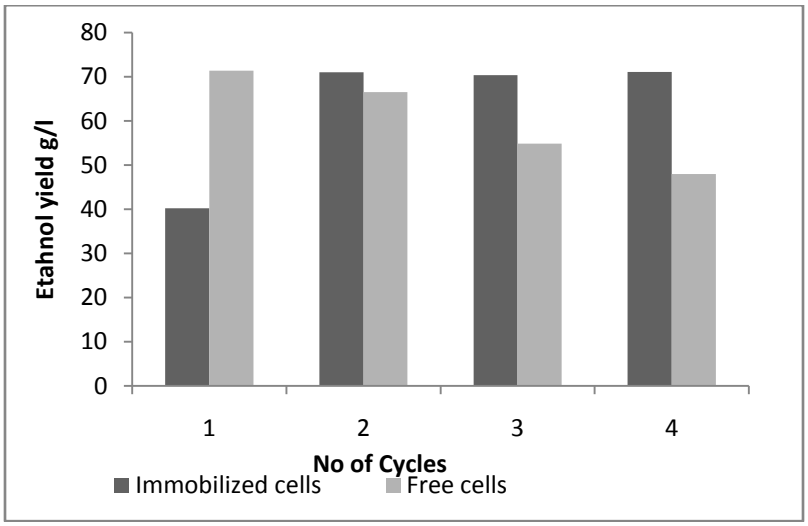

Figure 1 Comparison of ethanol production by immobilized and free cells by repeated batch fermentation using 150g molasses L^{-1} after 36 and 48h at30^{0}C

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Table 4. Ethanol production by repeated batch fermentation by alginate-immobilized cells using 400 beads with 150g glucose L^{-1} at 30^0C

No of cycles	NCIM3640 strain yield after					
	18			36		
	P(gl^{-1})	Y_{ps} (gg^{-1})	E_y(%)	P(gl^{-1})	Y_{ps}(gg^{-1})	E_y(%)
1	37.21	0.24	48.3	40.21	0.26	52.3
2	62.12	0.40	80.0	71.01	0.46	91.5
3	54.14	0.35	69.8	70.35	0.46	90.7
4	68.14	0.34	67.6	71.12	0.46	91.3
5	62.68	0.41	81.2	66.81	0.44	86.6
6	64.51	0.42	83.3	70.50	0.46	91.0

P: ethanol concentration; $Y_{ps:}$ ethanol yield, Ey (%) ;fermentation efficiency

Table 5. Ethanol production by repeated batch fermentation by alginate-immobilizedcells using 400 beads with 200 g glucose L^{-1} at 30^0C

No of cycles	NCIM3640 strain yield after					
	18			36		
	P(gl^{-1})	Y_{ps} (gg^{-1})	E_y(%)	P(gl^{-1})	Y_{ps}(gg^{-1})	E_y(%)
1	28.06	0.14	27.4	50.41	0.25	49.3
2	57.14	0.38	74.5	86.32	0.43	84.4
3	75.84	0.37	74.2	87.25	0.43	85.3
4	80.21	0.40	80.2	87.18	0.43	85.3
5	80.43	0.39	76.9	87.20	0.43	85.3
6	77.50	0.38	75.8	87.24	0.43	85.3

P: ethanol concentration; $Y_{ps:}$ ethanol yield, Ey (%) ;fermentation efficiency

The amount of ethanol present in 150 g molasses L^{-1} after repeated batch fermentation for six cycles after 18, 24 and 36 hr using 200 beads are shown in Table 4. These results also indicate that the amount of ethanol was produced in the first cycle was less compared to subsequent cycles. The maximum amount of ethanol produced in the first cycle after 36 hr of incubation using 150 g molasses L^{-1} was 40 g ethanol L^{-1}. The maximum amount of ethanol produced after sixth cycle was 71 g ethanol $L^{-1.}$ More ethanol was produced with 400 beads than 200 beads. About 85% of the ethanol produced in 16 hr of fermentation by increasing the bead number to 400.

The amount of ethanol present in 200 g molasses L^{-1} after repeated batch fermentation for six cycles after 18, 24 and 36 hr using 400 beads are shown in Table 5. These results also indicate that the amount of ethanol was produced in the first cycle was less compared to subsequent cycles. The maximum amount of ethanol produced 87 g after 36 h. about 85% of the ethanol was produced in 18 h of fermentation by increasing the bead number to 400.

In the later experiments the bead number was increased from 400 to 600 (1.0 g of yeast cells to 1.5 g). Samples were collected after 9, 18, 24 and 36 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 400 beads). Thus the maximum amount of ethanol produced from 150 g molasses using 600 beads was 35, 60, 68 and 71 g after 9, 18, 24 and 36 h. About 80% of ethanol was produced within 18 h using 600 beads. In this experiment the bead number was increased from 400 to 600. Samples were collected after 9,18, 24 and 36 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 400 beads). About 75% of ethanol was produced within 18 h using 600 beads (data was not showed).Similarly, immobilization technique was also adapted to industrial strain of *S. pombe*and NCIM 3570. 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. This table presents the results of the linear regression for the ethanol concentration model in which the concentration of ethanol is taken as the dependant variable. The R^2 denotes multiple determinations of the dependant variable. These results are taken for three different strains i.e. NCIM3640, *S.pombe*, and NCIM3570. NCIM 3640 strain is the test strain taken by the researcher, *S.pombe* strain taken from Industrial usage and NCIM 3570 Strain taken as a reference strain. With regard to the NCIM 3640 strain for all the models the multiple determinations denoted by R^2 is ranging from 91-74%. This can be explained as the independent variables taken were explaining that much percentage of change in the dependant variable. With regard to coefficients associated with the number of beads, they show a positive and highly significant relationship (both at 1 and 5% level) with the ethanol concentration in all the models. The variation in ethanol concentration caused by a one percent change in the number of beads is in the range of 95-34% percent respectively.

Table: 6. Linear regression table for ethanol production using 200, 400, 600 beadswith different molasses concentrations using the strains of NCIM 3640,*S.pombe* and NCIM 3570

Model No	N	R^2	Adjusted R2	No of Beads		No of Cycles		Time		Sugar concentration	
				β	ţ	β	ţ	β	ţ	β	ţ
NCIM 3640											
1	12	.859	.752	.708	3.810*	.531	2.236**	.783	4.164*	.063	1.28***
2	12	.918	.857	.580	1.835**	.614	1.456**	.741	5.176*	.207	1.16
3	12	.779	.696	.733	3.175*	.731	3.407*	.538	3.241*	.52	2.242**
4	12	.849	.792	.343	1.819**	.780	4.734*	.213	1.950**	.187	1.837**
5	12	.746	.607	.950	1.765**	.511	1.359***	.113	1.356***	.003	.008
S. pombe											
1	12	.813	.672	.637	2.510**	.637	2.327**	.699	1.370**	.125	.422
2	12	.937	.891	.590	1.956**	.701	1.804**	.677	1.817**	.010	1.826**
3	12	.876	.830	.580	1.755**	.740	1.805**	.545	1.86**	.032	2.401**
4	12	.847	.790	.209	1.736**	.744	2.500**	.170	1.834**	.248	1.944**
5	12	.909	.875	.647	1.476***	.407	2.190*	.837	1.839**	.080	.628
NCIM3570											
1	12	.954	.919	.544	1.662**	.731	1.793**	.690	1.428**	.068	1.501**
2	12	.919	.858	.845	1.802**	.647	1.843**	.606	1.255**	.100	1.130
3	12	.916	.884	.973	1.835**	.807	1.881**	.484	1.719**	.028	2.208**
4	12	.955	.937	.820	1.761**	.828	1.168**	.319	1.227**	.157	1.843**
5	12	.867	.817	.781	2.287**	.351	2.274**	.846	1.561**	.062	.401

* Significant at 1% level, ** Significant at 5% level, *** Significant at 10% level

With regard to the coefficient associated with number of cycles they show a positive relationship with the ethanol concentration at 1 and 5% level of probability. With regard to coefficient associated with time expect sixth model they show a positive and highly significant relationship with the ethanol concentration. With regard to the coefficient associated with sugar concentration except in the third and four models, they show in significant relationship with the level of ethanol concentration. As a whole it can be interpreted that the independent variables taken were showing positive and statistically significant relationship. That is the independent variables were significantly explaining the change in the dependant variable.

With regard to the *S.pombe* strain (Predominantly used for industrial preparation of ethanol) the multiple determinations denoted by R^2 is ranging from 93.7-81.3%. With regard to the coefficient associated with number of beads they show a positive and highly significant relationship with the level of ethanol concentration. With regard to the coefficient associated with number of cycles they show positive and significant relationship with the level of ethanol concentration. With regard to the coefficient associated with time they show positive and significant relationship with level of ethanol concentration. With regard to the coefficient associated with sugar concentration they expect in one and fifth models they show positive and significant relationship with the level of ethanol concentration. As a whole it can be concluded that the variables taken were positive and significant in determining the level of ethanol concentrations. The level of significance is in the range of five to ten percent level of probability.

With regard to the NCIM 3570 strain, used as reference i.e. as table values to estimate the veracity of the work done. The multiple determinations denoted by $R^2$is ranging from 95-86%. With regard to the coefficient associated with the number of beads, number of cycles, time taken and sugar concentration, they show positive and significant relationship with the level of ethanol concentration. The significance is confined to 5 and 10 per cent level of probability. To sum up, the result of the strains taken into consideration are positive and shows significant relationship between the dependant and independent variables but the results of the strain taken by the researcher i.e. NCIM 3640 shows positive and highly significant relationship among the dependent and independent variables.Nigam *et al.,* 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 *Kluyveromycesmarxianus*IMB3 immobilized in mineral kissiris [19]. But NCIM3640 strain gave maxima of 71 and 87g ethanol L^{-1}. Though ethanol production by calcium alginate immobilization is frequently used, the high ethanol yield produced by *S. cerevisiae*NCIM3640 and viability of the system for more than 400 h makes the process novel and economical for ethanol production. These results indicate that *S. cerevisiae*NCIM3640 is suitable for producing high ethanol by alginate immobilization with less expensive technology. Our results were supported earlier study (2).

CONCLUSION

Over all the immobilization technique was considered as a useful process. Surviving the yeast cells about 12 cycles of repeated batch fermentations in the three strains under

study.Repeated batch fermentation in the free cells were appeared up to four cycles and a gradual decrease was observed from second cycle onwards. Hence, the immobilization of the yeast cells were understand as an effective means for improving the repeated batch fermentation. Finally, the results presented in this paper show the good potential in using the NCIM 3640 culture in industrial alcohol production and may create new areas of interest in biotechnological and industrial fermentation applications.

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