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Strategies for Reducing Solvent Toxicity in Extractive Ethanol Fermentation

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ABSTRACT

Extractive fermentation is a widely preferred technique in which the products of fermentation are removed from the fermentation medium by a proper solvent, in order to avoid the inhibitory effects of the products. In this work, decanol, which has a high distribution coefficient with respect to the biocompatible solvents, was used in extractive ethanol fermentation. In order to reduce decanol toxicity, *Saccharomyces cerevisiae* cells were immobilized in calcium alginate gel. Further, sunflower oil and Al_2O_3 were added to the immobilization media. Experiments were performed in 250-mL Erlenmeyer flasks that were placed in the constant-temperature bath of a constant stirring-rate shaker. Ethanol concentrations were measured to observe the effect of various parameters on ethanol production. Immobilization media included 10, 20, and 30% sunflower oil, or 5, 10, and 20% Al_2O_3, or Al_2O_3 and sunflower oil together. The ratio of the volume of aqueous phase to that of decanol phase ranged from 2:1 to 6:1. It was observed that protection depends on the oil, Al_2O_3, and decanol amounts. Utilization of sunflower oil (30%) and Al_2O_3 (5%) together yielded best results.

Index Entries: Extractive ethanol fermentation; toxicity; decanol; *Saccharomyces cerevisiae;* sunflower oil; Al_2O_3.

Nomenclature: C_{so}, initial glucose concentration in the fermentation media (g/L); C_s, residual glucose concentration in the fermentation media (g/L); $C_{p,a.}$, final ethanol concentration in the aqueous phase (g/L); $C_{p,sol.}$, final ethanol concentration in the solvent phase (g/L);

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$C_{p,\,con}$, final ethanol concentration in the aqueous phase without decanol (g/L); $C_{p,\,tot}$, total ethanol concentration in the aqueous phase and solvent phase; K_D, distribution coefficient of ethanol between solvent and aqueous phases (g ethanol/L solvent)/(g ethanol/L aqueous); R, $V_a/V_{sol.}$; V_a, volume of aqueous phase (mL); $V_{sol.}$, volume of decanol phase (mL); $Y_{P/S}$, yield (g ethanol/g glucose).

INTRODUCTION

In conventional ethanol fermentation, inhibitory effect of the product decreases the rate of ethanol production and cell concentration. Removing ethanol as soon as it is produced will reduce these limitations. Vacuum fermentation *(1,2)*, extractive fermentation *(3–8)*, supercritical CO_2 extraction *(9)*, and adsorption *(10)* are various methods for removing the produced ethanol from fermentation broth. It has been demonstrated that product costs are decreased in extractive fermentation *(11,12)*. In the literature, *in situ* liquid extraction of ethanol has been studied extensively *(13,15)*, and it has been found that the choice of a suitable extracting solvent is very important *(15,16)*. The solvent must be biocompatible and must have a high capacity for ethanol, but biocompatible solvents generally have low distribution coefficients.

Arenson et al. *(17)* showed that a mixture of cresol and chloroform has a high mass distribution coefficient to ethanol (over 2.0), but this mixture is known to be highly toxic to all living cells. Minier and Goma *(5)* showed that the distribution coefficient of ethanol increases with the decrease in carbon (C) number of aliphatic alcohols. Alcohols with 2–12 C numbers show strong inhibitory effects for ethanol production by *Saccharomyces cerevisiae*. The only insoluble alcohols that do not inhibit ethanol production, substrate consumption, and cell growth have C numbers greater than 12. Bruce and Daugulis *(16)* mixed a biocompatible, but poor, solvent with a toxic solvent that has a better extracting capability, to obtain a mixture with improved solvent characteristics that is still biocompatible. The increase in distribution coefficient was about 12%.

Bruce and Daugulis *(18)*, in a later work, classified the toxic action of solvents into two major classes, physical and dissolved toxicity. They defined the critical solvent concentration in the aqueous phase that is required to cause total loss of activity, and the corresponding critical solvent concentration in the membrane. Matsumara and Mark1 *(19)* tried to eliminate the toxic effect of sec-octanol on *Saccharomyces uvarum* using Porapack Q and some surface-active reagents in the immobilization media. Honda et al. *(20)* used castor oil to prevent the toxic effects of *o*-isopropylphenol and *o*-tert-butylphenol (OTBP), which have high distribution coefficients for ethanol. The oil eliminated toxicity in the presence

of a very low amount (1% v/v) of OTBP for *S. cerevisiae*. Tanaka *(12)* was able to reduced the toxic effect (0.1% v/v) of 2-octanol using vegetable oils. In all these studies the amount of the toxic solvent in the fermentation media was low.

In this study, decanol, which has a higher distribution coefficient than commonly used nontoxic solvents, was used as a solvent. In order to reduce decanols toxicity, *S. cerevisiae* cells were immobilized in calcium alginate gel. Further, to protect the cells from the water-soluble decanol, sunflower oil, and/or Al_2O_3 were added to the immobilization media.

MATERIALS AND METHODS

Microorganisms and Media

S. cerevisiae Y-567 was obtained from the National Center for Agricultural Utilization Research in Peoria, IL. The growth medium was the one given by Bazua and Wilke *(22)*. Ethanol production media used in experiments contained 150 g/L glucose (Merck, Germany), 0.05 *M* $CaCl_2$ (Merck) and salts given in Bazua and Wilke *(22)*.

Experimental Apparatus and Procedures

The experiments were performed in 250-mL Erlenmeyer flasks. The mixing rate and media temperature were kept constant with the aid of a constant-temperature shaker set to 200 rev min^{-1}. Twenty hours after inoculation, the growth medium was centrifuged at 6720 g for 10 min. Four grams of this concentrated wet cell mass was added to 10 mL 3% sodium alginate solution obtained from *Macrocystis pyrifera* (Sigma, St. Louis, MO), which also contained definite amounts of sunflower oil (Komili, Türkiye) and/or Al_2O_3 (Merck). This mixture was fed to 0.2 *M* $CaCl_2$ solution through a hypodermic needle (0.65 × 30 mm) with the aid of a peristaltic pump. The diameter of the beads obtained varied between 3 and 3.5 mm; 2.5 g of the beads thus obtained were added to the Erlenmeyer flasks containing decanol (Merck) and 50 mL fermentation media. Temperature was kept constant at 30°C. Ethanol and glucose analysis in the aqueous phase were made 44 h after the beginning. Solvent-phase ethanol concentrations were calculated using the K_D value of ethanol between the solvent and aqueous phases (0.48 [g ethanol/L solvent]/[g ethanol/L aqueous]). The results presented were the averages of two experiments.

Analytical Methods

Ethanol in the aqueous phase was analyzed by gas chromatography (Varian-3400, Ireland) equipped with Chromosorb 101 (80–100 mesh) column

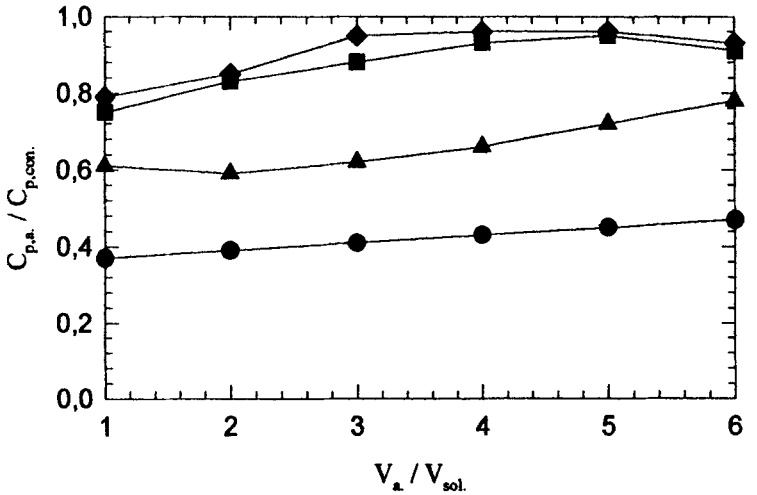

Fig. 1. Effect of immobilization and sunflower oil on the ethanol concentrations in the aqueous phase. ● 0% sunflower oil; ▲ 10% sunflower oil; ■ 20% sunflower oil; ◆ 30% sunflower oil.

and utilizing *n*-propanol as the internal standard. Glucose was analyzed with Glucometer-Reflolux S (Boehringer-Mannheim, Mannheim, Germany).

RESULTS AND DISCUSSION

Effect of Sunflower Oil on Decanol Toxicity

The extractive fermentation was performed by using *S. cerevisiae* cells immobilized in 3% calcium alginate gel, which included 10, 20, and 30% sunflower oil, with the V_a (mL)/$V_{sol.}$ (mL) ratio ranging from 2:1 to 6:1. The ethanol concentrations in the phases were measured, and the results were compared with the control runs performed without decanol. For $V_a/V_{sol.}$ = 2:1, adding 30% oil only yielded 85% ethanol production in the aqueous phase, compared to the control run (Fig. 1). This value is 40% for immobilization alone. The reasons for the low ethanol concentrations in the aqueous phase can either be toxicity of the decanol or extraction. It was observed that $C_{p.a.}/C_{p,con.}$ values increase with increasing protection for the same $V_a/V_{sol.}$ ratio. This shows that toxicity of decanol is more effective for the low aqueous-phase ethanol concentrations. As can be seen from Table 1, immobilization alone cannot remove the toxic effect. Even with V_a (mL)/$V_{sol.}$ (mL) = 6:1, total ethanol production is only 51% of the control run. When 10% sunflower oil is added to the immobilization media, toxic effect is reduced, and total ethanol production increased to 84% of control run. Addition of 20–30% sunflower oil increased production close to 100%.

Table 1
Effect of Immobilization and Sunflower Oil on Total Ethanol Production

	$C_{p,tot}/C_{p,con.}$			
V_a/V_{sol}	Immobilization	10% Sunflower oil	20% Sunflower oil	30% Sunflower oil
Control	1.00	1.00	1.00	1.00
1:1	0.54	0.90	1.11	1.17
2:1	0.49	0.73	1.03	1.05
3:1	0.47	0.71	1.02	1.10
4:1	0.47	0.74	1.04	1.07
5:1	0.49	0.78	1.04	1.05
6:1	0.51	0.84	0.98	0.99

The oil in the gel absorbs the amount of decanol that has diffused into the immobilized beads, and thus prevents the inhibitory effect on the cells. It is clear that the protection depends on the oil and solvent amounts.

Effect of Al_2O_3 on Decanol Toxicity

Ethanol production via extractive fermentation, using *S. cerevisiae* immobilized in 3% calcium alginate gel that included 5, 10, and 20% Al_2O_3, was studied at different aqueous-phase–decanol-phase ratios. The ratios of aqueous-phase ethanol concentration to the control run are shown in Fig. 2. For $V_a/V_{sol.} = 5:1$, using different Al_2O_3 percentages, total ethanol production increased from 84 to 100%, compared to the control run for $V_a/V_{sol.} = 5:1$ (Table 2).

Although less effective at low $V_a/V_{sol.}$ ratios, it is apparent that Al_2O_3 exhibits a protective behavior. It can be claimed that Al_2O_3 absorbs the decanol that is dissolved in the aqueous phase, thus reducing its toxicity. In addition, because the beads containing Al_2O_3 are heavier, they move away from the interface between the two phases, and thus do not get into contact with decanol. Honda *(20)* also used Al_2O_3 to prevent the beads from floating. It must be stated that, when the Al_2O_3 amount increases, various problems appear in the immobilization stage, like plugging of the needles.

Effect of Sunflower Oil Plus Al_2O_3 on Decanol Toxicity

Ethanol concentrations in the aqueous and solvent phases were determined in the experiments performed with *S. cerevisiae* immobilized in a

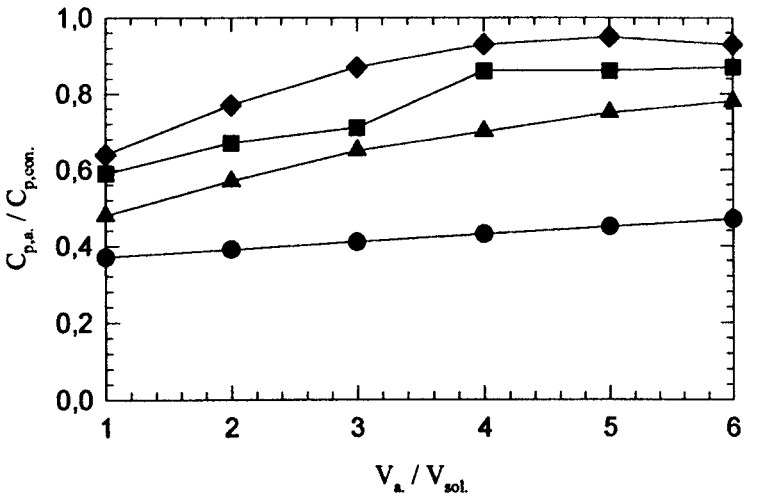

Fig. 2. Effect of immobilization and Al_2O_3 on the ethanol concentrations in the aqueous phase. ●, 0% sunflower oil; ▲, 5% Al_2O_3, ■, 10% Al_2O_3; ◆, 20% Al_2O_3.

Table 2
Effect of Immobilization and Al_2O_3 on Total Ethanol Production

	$C_{p,\,tot.}/C_{p,\,con.}$			
$V_a/V_{sol.}$	Immobilization	10% Al_2O_3	20% Al_2O_3	30% Al_2O_3
Control	1.00	1.00	1.00	1.00
1:1	0.54	0.71	0.87	0.95
2:1	0.49	0.71	0.83	0.95
3:1	0.47	0.75	0.82	1.01
4:1	0.47	0.78	0.96	1.04
5:1	0.49	0.82	0.94	1.04
6:1	0.51	0.84	0.94	1.00

media containing sunflower oil and Al_2O_3 together. Figure 3 shows the results for 10% sunflower oil, 20% Al_2O_3 and 10% sunflower oil plus 20% Al_2O_3 together. When $V_a/V_{sol.}$ values were higher than 4:1, the ethanol concentrations in the aqueous phases were above the control run for 20% Al_2O_3 plus 10% sunflower oil. This shows that the toxicity of decanol is completely prevented. Figure 4 shows the results for 5% Al_2O_3, 30% sunflower oil, and 30% sunflower oil and 5% Al_2O_3 together. Toxicity of decanol was completely prevented when 30% sunflower oil and 5% Al_2O_3 were used together. Tables 3 and 4 show the ratios of the total ethanol amounts in the aqueous and solvent phases to the control run. It was observed that the increase in the total ethanol amount was about 27% for

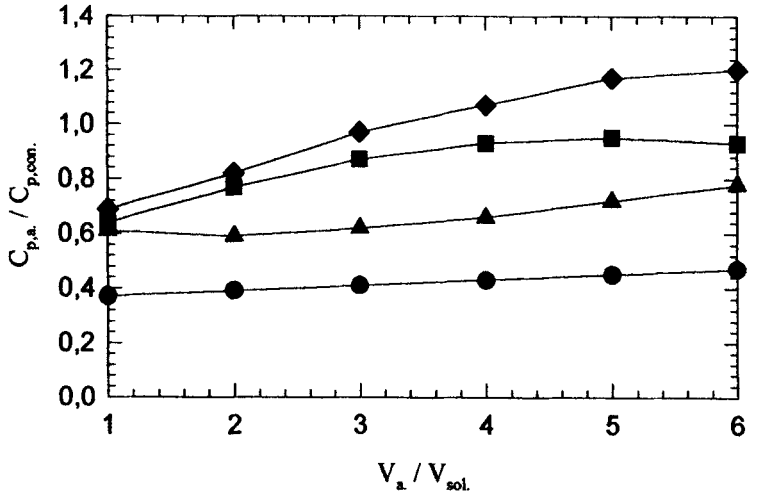

Fig. 3. Effect of immobilization, 10% sunflower oil, and 20% Al_2O_3 on the ethanol concentrations in the aqueous phase. ●, 0% sunflower oil; ▲, 10% sunflower oil; ■, 20% Al_2O_3; ◆, 10% sunflower oil and 20% Al_2O_3.

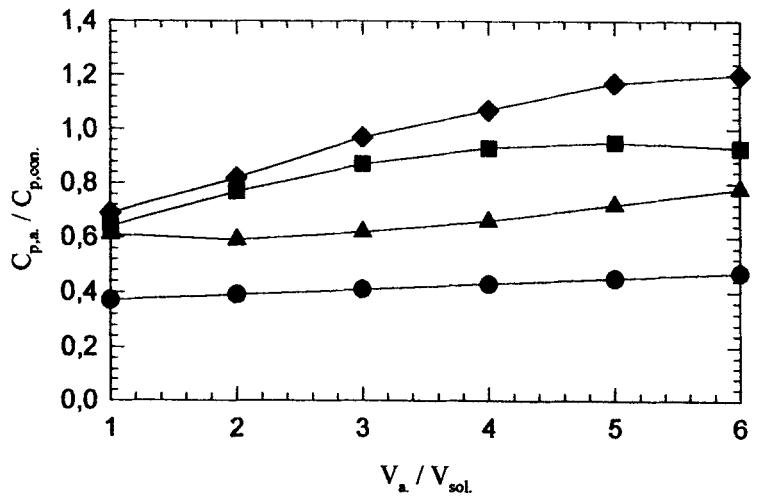

Fig. 4. Effect of immobilization, 30% sunflower oil and 5% Al_2O_3 on the ethanol concentrations in the aqueous phase. ●, 0% sunflower oil; ▲, 5% Al_2O_3; ■, 30% sunflower oil; ◆, 30% sunflower oil and 5% Al_2O_3.

30% sunflower oil plus 5% Al_2O_3. Considering the practical problems caused by high Al_2O_3 amounts, 30% oil and 5% Al_2O_3 can be recommended.

In addition, residual glucose concentrations are also shown in Tables 3 and 4. Yield values, $Y_{P/S}$, were calculated using glucose data (Fig. 5). $Y_{P/S}$ values did not change much for different $V_a / V_{sol.}$ values for 10% sunflower

Table 3
Effect of Immobilization with 10% Sunflower Oil Plus 20% Al_2O_3 on Total Ethanol Concentrations

V_a/V_{sol}	$C_{p,a}$ (g/L)	(1/R) $C_{p,sol.}$ (g/L)	$C_{p,tot.}$ (g/L)	$C_{p,tot.}/C_{p,con.}$	C_S (g/L)
Control	54	–	54.00	1.00	42.84
1:1	37	17.76	54.76	1.01	49.72
2:1	44	7.04	51.04	0.95	47.93
3:1	52	8.32	60.32	1.12	39.12
4:1	58	6.96	64.96	1.20	27.98
5:1	63	6.05	69.05	1.28	17.19
6:1	65	5.20	70.20	1.30	15.23

Table 4
Effect of Immobilization with 30% Sunflower Oil Plus 5% Al_2O_3 on Total Ethanol Concentrations

V_a/V_{sol}	$C_{p,a}$ (g/L)	(1/R) $C_{p,sol.}$ (g/L)	$C_{p,tot.}$ (g/L)	$C_{p,tot.}/C_{p,con}$	C_S (g/L)
Control	52	–	52.00	1.00	47.13
1:1	45	21.60	66.60	1.28	27.57
2:1	50	12.00	62.00	1.19	32.53
3:1	58	9.28	67.28	1.29	24.28
4:1	59	7.08	66.08	1.27	25.62
5:1	60	5.76	65.76	1.26	23.59
6:1	61	4.88	65.88	1.27	20.63

oil plus 20% Al_2O_3 and for 30% sunflower oil plus 20% Al_2O_3. This shows that protection and solvent addition have no effect on yield.

CONCLUSIONS

It was found that, in extractive ethanol fermentation, decanol can be used as a solvent, if the *S. cerevisiae* cells are immobilized in calcium alginate gel together sunflower oil and Al_2O_3. Distribution coefficient of ethanol between decanol and aqueous phases is 0.48 (g/L)/(g/L) *(23)*. This value is higher than the K_D value of ethanol in Adol 85 NF (0.266 [g/L]/[g/L]) and the mixture of Adol 85 NF and 4-heptanon (0.298 [g/L]/[g/L]) *(16)*. When 30% sunflower oil and 5% Al_2O_3 were added, toxic effect of the decanol was prevented for $V_a/V_{sol.} \geq 3:1$. Adding sunflower oil and Al_2O_3 to the matrix could prevent the diffusion of solvent

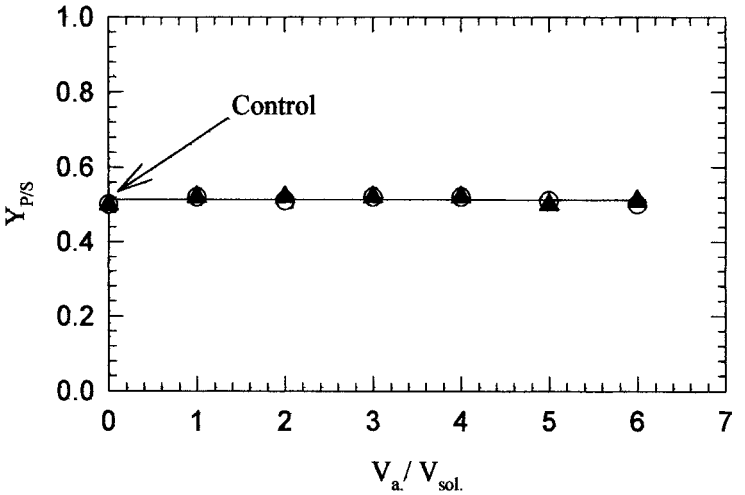

Fig. 5. Effect of protection and $V_a/V_{sol.}$ on the yield ○, 10% sunflower oil plus 20% Al_2O_3, ▲, 30% sunflower oil plus 5% Al_2O_3 on the yield.

molecules to the cell membrane, and thus could reduce solvent concentration in the membrane below the critical value, which in turn eliminates the toxic effect of the water-soluble solvent. This immobilization method seems to be ideal for application in the extractive fermentation. Using sunflower oil and Al_2O_3 is more economic than Porapack Q which was used by Matsumura and Markl *(19)*. Daugulis et al. *(12)* concluded that when K_D increases from 0.25 to 0.4, the cost of production decreases only 4%. Further studies must be performed to investigate the reusability of immobilized beads and the regeneration of decanol-saturated beads with oil. In addition, similar methods can be applied for the production of fine chemicals.

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