Extractive Capacity of Oleyl Alcohol on 2, 3-Butanediol Production in Fermentation Process with Use of *Klebsiella pneumoniae* PTCC 1290

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ABSTRACT: *Recovery of metabolites from fermentation broth by solvent extraction can be used to optimize fermentation processes. End-product reutilization, low product concentration and large volumes of fermentation broth and the requirements for large bioreactors, in addition to the high cost largely contributed to the decline in fermentative 2,3-butanediol production. Extraction can successfully be used for in-situ alcohol recovery in 2,3-butanediol fermentations to increase the substrate conversion. In the present work organic extraction of 2,3-butanediol produced by Klebsiella pneumoniae fermentation was studied to determine solvent effect on 2,3-butanediol production. The aim of this project was liquid-liquid extractive fermentation systems evaluation as an alternative to overcome the end product effect and to increase of 2,3-butanediol production by K.pneumoniae because Conventional fermentative production of 2,3-butanediol by K. pneumoniae has the disadvantage of product reutilization by the organism. Alternatives to overcome this problem have met with limited success. Extractive fermentation has been shown to solve this problem. An effort has been made in this study to use for the extractive fermentation of 2,3-butanediol using oleyl alcohol as extract-ant. Eighteen organic solvents were examined to determine their biocompatibility for in situ extraction of fermentation products from cultures of the K. pneumoniae. From 18 tested solvents, 13 of which were non-toxic to K.pneumoniae. The highest 2,3-butanediol production (23.01 g l^{-1}) was achieved when oleyl alcohol was used. In situ removal of end products from K.pneumoniae resulted in increased productivity. In conclusion 2,3-butanediol productivity increased from 0.5 g $l^{-1}h^{-1}$ to 0.66 g $l^{-1}h^{-1}$ in extractive fermentation using oleyl alcohol as the extraction solvent.*

KEY WORDS: *2,3-butanediol, Oleyl alcohol, Liquid-liquid extraction, Solvent selection.*

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INTRODUCTION

Extractive fermentation, or in situ removal of products as they are formed, represents an alternative and scalable technology to improve productivity. A reasonable approach to increase productivity and effectively recover the products may be the utilization of extractive fermentation system, which could remove the metabolites from the broth by in situ liquid-liquid extraction [1].

With the extractive fermentation system, the product concentration in the extract-ant phase is higher as compared to the fermentation broth and this helps to reduce down-stream separation costs. Extractive fermentation is advantageous, because the product recovery is not associated with a water flow through the bioreactor and solid-liquid separation of cells is not necessary if the organic solvent comes in direct contact with the culture broth [1].

In fermentation the suitability of a chemical as a solvent, however, is mainly determined by its toxicity with respect to the micro-organism. A number of articles has dealt with this toxicity of chemicals at saturation in water [2-4]. The most important characteristic required for extractant is to dissolve the solute preferentially, with a high partition coefficient [5]. Other properties of a chemical such as the density, viscosity and surface tension will become important for extractor selection [6].

Conventional fermentative production of 2,3-butanediol by *K. pneumoniae* has the disadvantage of product reutilization by the organism. In situ solvent extraction of 2,3-butanediol during cultivation of *K. pneumoniae* appears to be a potentially viable alternative to overcome product reutilization. The in-situ recovery of 2,3-butanediol from fermentation has gained considerable attention in recent years [7, 8]. It is considered that in-situ recovery is one of the major developments that may attribute to the commercial revival of the 2,3-butanediol fermentation. In-situ recovery will reduce the effect of product accumulation by 2,3-butanediol and will enable the conversion of a concentrated feed and lead to a high productivity, hence lowering the production costs. Another aim in research on in-situ recovery is development a separation method which consumes less energy than the conventional distillation [6].

The purpose of this study was to survey the effects of oleyl alcohol on 2,3-butanediol formation by *K. pneumoniae*.

EXPERIMENTAL

Chemicals

Organic solvents from different chemical groups and of varying molecular mass and polarity were chosen on the basis of general availability, and were obtained from Aldrich and Merck.

Solvent selection

Biocompatibility experiments

In order to appropriate1y select a solvent for in situ extraction of 2,3-butanediol with *K. pneumoniae*, it was critical log $P_{octanol}$ (log P) was determined using 11 solvents with a range of log P values from 0.79 to 5.45. The log P of a solvent is defined as the logarithm of its partition coefficient when placed into a two phase system consisting of octanol and water. Lower the solvent log P, the more hydrophilic and therefore the more likely that the toxic effects of a solvent will affect a bacterium growing in the aqueous phase. The critical log P for an organism is defined as the log P of a solvent at which organism's growth in the aqueous phase is not adversely affected by the presence of the solvent. The experiment was carried out in 125 mL Erlenmeyer flasks that contained 50 mL of maintenance medium and 5-mL of stock culture. To each flask, 10 mL of solvent was added with the exception of the control that had no solvent addition. The flasks were placed on a shaker incubator at 34 °C and 160 rpm and incubated for 48 h. After 24 and 48 h, 3 mL aqueous samples were removed from the flasks. Cell growth was assessed by measurement of optical density at OD_{620}, and this value was compared with a standard curve to determine cell dry weight in grams per liter. The samples were then centrifuged to separate phases, and analysis of glucose concentration was conducted using glucose oxidase kit and glucose concentration determined by comparison to a standard curve. Net changes in cell dry weight (CDW) and glucose concentration were used to calculate percent metabolic activity of the cultures relative to the control, and the critical log P value of the organisms was assessed [9]. After determining of the critical log P value, solvents were selected using the following criteria: a log P value

greater than the previously determined critical log P value; a high boiling point; a high partition coefficient; and low water solubility. To determine the potential bioavailability of the selected solvents (i.e. their potential consumption as carbon and energy sources), to 125 mL Erlenmeyer flasks, containing 50 mL maintenance medium, 5 mL of inoculum's was added. The maintenance medium did not contain any glucose, but did contain 10 mL solvent. Solvent was not added to one flask, which was used as a negative control. In addition, 10 mL corn oil was added to one flask, to serve as a positive control. Flasks were placed on the shaker incubator as above for 72 h. The cell density in each flask was measured every 24 h. The net change in cell density was then compared to the change in cell density seen in the negative and positive controls. A change in cell density less than or equal to that seen in the negative control was seen as evidence that the solvent was not consumed by the cells, whereas a change in cell density greater than that seen in the control was taken as evidence that the solvent was consumed by the cells. A change in cell density greater than the positive control indicated that the solvent could be readily used as a substrate by the cell culture and those solvents that could be used as a carbon source by the bacterium were excluded from consideration for use in the extractive fermentation [9].

Solubility of 2,3-butanediol in solvents

The maximum solubility of 2, 3-butanediol+solvent system was determined at 34±0.5 ºC as described in our previous paper [10].

Organism and growth conditions

Microorganism

Bacterial strain used in this study was *Klebsiella pneumoniae* PTCC 1290, obtained from the Iranian Research Organization for Science and Technology (IROST). The strain was maintained on nutrient agar slants at 4 ºC and sub-cultured monthly. The pre-culture medium was nutrient broth containing 2.0 g L^{-1} yeast extract, 5.0 g L^{-1} peptone, 5.0 g L^{-1} NaCl, and 1.0 g L^{-1} beef extract, sterilized at 121 ºC for 15 min. Cells in exponential growth were used as inoculums.

Fermentation

Submerged fermentation experiments were carried out in cotton plugged 500 mL Erlenmeyer flasks containing 100 mL of production medium on a shaker incubator with shaking 180 rpm at 34 °C. The extract-ant was then aseptically added the surface of the broth after 10 h of cultivation. A volume ratio (solvent volume/medium volume) of 0.20 was used. A control experiment was carried out without any extract-ant. Periodically, samples were withdrawn and the concentrations of cell, residual glucose, 2,3-butanediol and acetoin were determined.

All experiments were repeated at least three times in order to acquire high accuracy. This procedure gave consistent and reproducible results.

Analytical methods

The product concentrations in each liquid phase were measured by gas chromatography (Carlo Erba, Milan, Italy) using a Chromos-orb 101 column (Supelco, Bellefonte, PA) operated with N_2 as the carrier gas, at 250 ºC injector temperature, 300 ºC detector temperature, and 175 ºC column temperature, and using n-butanol as the internal standard. Acetoin were not quantitated in the organic phase because it did not distribute significantly into this phase. Aqueous samples were centrifuged to remove the cells.

Dry cell mass concentration was estimated by measuring the optical density of the sample at 600 nm in a spectrophotometer and by its correlation with the dry cell weight, obtained gravimetrically.

Glucose in the aqueous supernatants was measured using glucose oxidase

RESULTS AND DISCUSSION

Fermentation

In this study, batch fermentation was carried out at an initial glucose concentration of 80 g l^{-1} by both extractive and conventional fermentations. Attempts to ferment more than 90 g L^{-1} of glucose in batch extractive fermentation, however, were unsuccessful due to catabolite repression of the cells by the high concentrations of glucose in the medium.

Before operating an extractive fermentation, several batch fermentations were conducted without the presence of any solvent. Fig. 1 displays the results of typical batch fermentation. In this Fig. the concentrations of 2,3-butanediol and acetoin products are presented (acetic acid, also produced, was never greater than 0.5 g L^{-1}).

The maximum specific growth rate was 0.2 h^{-1} and this was the same as that obtained during extractive

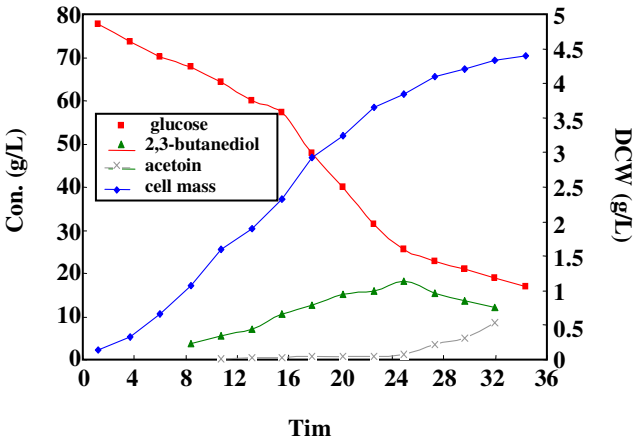

Fig. 1: Time course of 2,3-butanediol production and glucose utilization by K.pneumoniae.

fermentation in the presence of oleyl alcohol. The cell yield was approximately 0.10 g g^{-1} glucose, while the 2,3-butanediol yield was 0.30 g g^{-1}, nearly indicated to that obtained by *Qin et al.* [11]. The 2,3-butanediol productivity was 0.5 g $L^{-1}h^{-1}$.

After 25 h the 2,3-butanediol had been completely accumulated, and the organisms then converted it to form acetoin so 2,3-butanediol production in batch culture is limited by reutilization of microbial product. Acetoin is an intermediate metabolite immediately prior to the formation of 2,3-butanediol during fermentation. The metabolic conversion of acetoin to 2,3-butanediol is reversible [12]. However, on longer incubation the level of butanediol subsequently declined. This appeared to be due to its reoxidation to form acetoin (or acetyl methyl carbonyl), which progressively increased during prolonged fermentation [7, 8, 13].

The conditions for the in situ extractive fermentation remained the same as for the preceding control experiments, except that now about 20 % of the total volume was oleyl alcohol. After 10 hours of fermentation, oleyl alcohol were added to batch culture. The results showed that, in the presence of oleyl alcohol, 2,3-butanediol increased by 28.5 % from 17.9 g L^{-1} under conventional fermentation to 23.01 g L^{-1}. An increase in 2,3-butanediol production was observed on the addition of oleyl alcohol compared with the control. The maximal rate of 2,3-butanediol production also increased from 0.5 g $L^{-1}h^{-1}$ in the control to 0.66 g $L^{-1}h^{-1}$ in oleyl alcohol. On the addition of oleyl alcohol, the aqueous concentration of 2,3-butanediol was reduced from 17.9 to 16.8 g L^{-1}, but the total concentration increased only from 17.9 to 23.01 g L^{-1}.

Oleyl alcohol was therefore added for extraction of 2,3-butanediol from the broth since it could extract approximately 27 % of the 2,3-butanediol, thereby eliminating its end-product accumulation effect. Oleyl alcohol did not extract glucose, acetic acid nor microorganisms appreciably.

Compared to regular batch fermentation, in batch extractive fermentation using oleyl alcohol, 2,3-butanediol productivity and glucose consumption were increased 32 % and 28 % respectively.

Our results have clearly indicated that oleyl alcohol can be used as an alternative 2,3-butanediol extractant in fermentation. In general, it was concluded from the experiments that liquid-liquid extraction can successfully be used for in-situ recovery, which is in agreement with literature [3, 4, 14, 15].

Biocompatibility of solvents and the critical log P

Solvent selection is a key step in the development of two-phase system. The ability of a microorganism to tolerate a solvent can be predicted from the critical log P of the microbes [16]. It was therefore necessary to determine the critical log P for the organism to be used in the two-phase system, as all solvents to be considered for use in the two-phase system. They would have to possess log P values greater than this critical value.

The metabolic activity of the microorganisms, in the presence of each of 11 solvents with log P values between 0.79 and 5.45, is shown in table 1. Extractive fermentation was previously described in the case biodegradation of polycyclic aromatic hydrocarbons [17] and 6-pentyl-α-pyrone production [18]. The cells did not consume any glucose and show an increase in cell density in the presence of solvents with log P values below approximately 3.8. Solvents with log P values above 3.8 did not appear to have any negative effect on the metabolic activity of the microbes. n-Hexane, a solvent with a log P value of 3.8, had a moderately negative effect on the activity of the cells, but did not show complete inhibition. It was therefore concluded that the critical log P for this organism was 3.8, and the initial solvent screening procedures was done for solvents with log P values greater than this critical value. The critical log P of *K. pneumoniae* is similar to the values found for

Table 1: Biocompatibility of solvents with K.pneumoniae based on percentage metabolic activity.

Solvents	Log P	Percentage metabolic activity*	
		cell dry weight	glucose consumed
1-Butanol	0.79	0.98	2.25
Isoamyl alcohol	1.3	3.09	1.97
2-Ethyl-1-hexanol	2.8	4.92	6.06
Dibuthyl ether	2.9	5.91	5.21
Cyclohexane	3.11	7.04	6.91
2-Decanone	3.4	10.14	11.00
n-Hexane	3.8	61.26	56.97
2-Undecanone	4	96.47	95.05
Isooctane	4.5	96.920	95.47
Dodecanal	5	98.02	97.03
n-Nonane	54.45	98.87	98.15

** Net changes in cell dry weight and glucose concentration were used to calculate percent metabolic activity of the cultures relative to the control*

Table 2: Properties of various biocompatible solvents.

No	Name	Chemical formula	Density (g cm^{-3})	Boiling point (ºC)	Solubility(g l^{-1})	LogP	Bioavailability
1	n-Decane	$CH_3(CH_2)_8CH_3$	0.73	174	Insoluble	5.98	-
2	1-Decanol	$CH_3(CH_2)_9OH$	0.83	220-235	0.037	4	+
3	Dodecane	$CH_3(CH_2)_{10}CH_3$	0.75	216.3	Insoluble	66	-
4	n-Hexadecane	$CH_3(CH_2)_{14}CH_3$	0.77	287	Insoluble	8.8	-
5	Lauraldehyde	$C_{12}H_{24}O$	0.83	238	Insoluble	4.8	+
6	n-Octane	$CH_3(CH_2)_6CH_3$	0.7	125-126	Insoluble	4.58	-
7	Oleyl alcohol	$C_{18}H_{36}O$	0.85	330-360	Insoluble	7.5	-

other gram-negative bacteria [19-20]. As shown in table 1, *Lotter et al.* [21] have been reported that a significant correlation is between the microbial activity and log P.

Bioavailability of solvents

Properties of solvents tested as to their bioavailability shown in table 2. Cell growth, using the solvent as a carbon source, of less than 15 % of the maximum growth was assumed to be acceptable and these solvents were considered for further use in the extractive fermentation. Solvents that yielded cell growth of 15 % or more of the maximum growth was excluded [9]. The 1-decanol and lauraldehyde showed growth greater than 15 % and, therefore, were excluded for use with *K.pneumoniae* in extractive fermentation (Fig. 2). Collins and Daugulis [22] also reported that 1-decanol is bioavailable as organic phase in a two-phase partitioning bioreactor for benzene degradation. The solvents that showed less than 15 % growth included n-octane, n-decane, dodecane, oleyl alcohol and hexadecane were considered to be non-bioavailable to the bacterium and further considered for use in the extractive fermentation.

Solubility of 2,3-butanediol in solvents

Following final solvent selection, the solubility of 2, 3-butanediol in solvents was measured. The results are summarized in table 3. As shown in the Table, in case of *K. pneumoniae,* oleyl alcohol appears to be the best suitable solvent for the in situ extraction of 2,3-butanediol. It has good relative solubility for 2,3-butanediol, is biocompatible but not bioavailable, it shows good phase stability (i.e. no emulsion-forming tendencies), and it has a log P value significantly above the critical log P value. It should be noted that oleyl alcohol has also been selected as extracting solvent for mesophilic acetone-butanol fermentation by

Table 3: Evaluation of solvents based on 2,3-butanediol solubility in different solvents.

Solvents	2,3-butanediol solubility (mg 2,3-BD per ml solvent)
n-Decane	50
Dodecane	72
n-Hexadecane	230
n-Octane	42
Oleyl alcohol	Perfect soluble

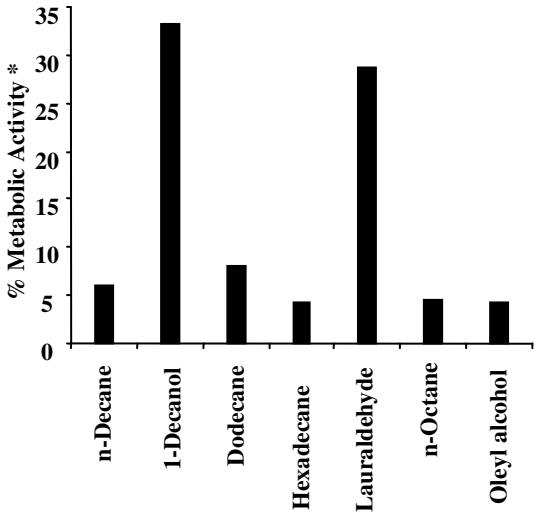

Fig. 2: Metabolism of Kelebsiella in the presence of various solvents, with log P values greater than 3.8, relative to growth in the presence of corn oil.

Clostridium acetobutylicum [5]. *Zigova et al.* [23] has been detected that oleyl alcohol has been shown to be a good extractant for use in extractive fermentation.

CONCLUSIONS

Recovery of metabolites from fermentation broths by solvent extraction can be used to optimize fermentation processes. With the extractive fermentation system, the product concentration in the extractant phase is higher as compared to the fermentation broth and this helps to reduce down-stream separation costs. Eighteen organic solvents were screened to determine their biocompatibility and bioavailability for their effects on *Klebsiella pneumoniae* growth. The possibility of employing oleyl alcohol as an extraction solvent to enhance end product in 2,3-butanediol fermentation was evaluated. Oleyl alcohol did not inhibit the growth of the fermentative organism. 2,3-butanediol production increased from 17.9 g L^{-1} (in conventional fermentation) to 23.01 g L^{-1} (in extractive fermentation). Applying oleyl alcohol as the extraction solvent, about 27 % of the total 2,3-butanediol produced was extracted. Our results have clearly indicated that oleyl alcohol can be used as an alternative 2,3-butanediol extractant in fermentation.

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