J Ind Microbiol Biotechnol (2009) 36:313–317
DOI 10.1007/s10295-008-0501-z

ORIGINAL PAPER

In situ recovery of 2,3-butanediol from fermentation by liquid–liquid extraction

Masumeh Anvari · Gholam Khayati

Received: 19 July 2008 / Accepted: 4 November 2008 / Published online: 27 November 2008

Abstract End-product conversion, low product concentration and large volumes of fermentation broth, the requirements for large bioreactors, in addition to the high cost involved in generating the steam required to distil fermentation products from the broth largely contributed to the decline in fermentative products. These considerations have motivated the study of organic extractants as a means to remove the product during fermentation and minimize downstream recovery. The aim of this study is to assess the practical applicability of liquid–liquid extraction in 2,3-butanediol fermentations. Eighteen organic solvents were screened to determine their biocompatibility, and bioavailability for their effects on *Klebsiella pneumoniae* growth. Candidate solvents at first were screened in shake flasks for toxicity to *K. pneumoniae*. Cell density and substrate consumption were used as measures of cell toxicity. The possibility of employing oleyl alcohol as an extraction solvent to enhance end product in 2,3-butanediol fermentation was evaluated. Fermentation was carried out at an initial glucose concentration of 80 g/l. Oleyl alcohol did not inhibit the growth of the fermentative organism. 2,3-Butanediol production increased from 17.9 g/l (in conventional fermentation) to 23.01 g/l (in extractive fermentation). Applying oleyl alcohol as the extraction solvent, about 68% of the total 2,3-butanediol produced was extracted.

Keywords 2,3-Butanediol · Oleyl alcohol · Liquid–liquid extraction · Solvent selection

M. Anvari (✉)
Department of Biology,
Islamic Azad University,
Rasht branch, Rasht, Iran
e-mail: anvari@iaurasht.ac.ir

G. Khayati
Department of Chemical Engineering,
Engineering Faculty,
Guilan University, Rasht, Iran
e-mail: khayatiir@yahoo.com

Introduction

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 downstream separation costs [1].

In situ product extraction during fermentation has been shown to be one technique that enhances product recovery by reducing end-product effect. Solvents for conventional extractions are selected with respect to a low tendency to form emulsions, sufficient density difference to the culture broth, and high partition equilibria for the products to be extracted [2].

A major problem for in situ extraction is the toxicity of commonly used organic solvents. The biocompatibility of a particular solvent depends on the organism employed, necessitating solvent screening [3]. It is accepted that low polarity high molecular weight solvents exhibit less microbial toxicity. Furthermore, solvent biocompatibility (with low or no toxic effect) was found to be related to the logarithm of the partition coefficient of the solvent (log *P*) in a

standard octane/water system (1:1 v/v). It was reported that solvents with log P >4 were found to be mildly toxic [4].

Conventional fermentative production of 2,3-butanediol by *Klebsiella pneumoniae* has the disadvantage of product conversion of other metabolites by the organism. Alternatives to overcome this problem have met with limited success. Extractive fermentation has been shown to solve this problem [5, 6]. An effort has been made in this study to use for the extractive fermentation of 2,3-butanediol using oleyl alcohol as extractant.

The aim of this work was to evaluate the feasibility of application of a number of selected solvents for the in situ extractive fermentation of *K. pneumoniae* in batch to overcome the accumulation effect of 2,3-butanediol. In this study, the kinetics of the process has been compared to those of conventional batch fermentation.

Materials and methods

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 appropriately select a solvent for in situ extraction of 2,3-butanediol with *K. pneumoniae*, it was necessary critical log P was determined using 11 solvents with a range of log P values from 0.79 to 5.45. The 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 the organism's growth in the aqueous phase is not adversely affected by the presence of the solvent. The critical log P of the bacterium was determined using method of Janikowski et al. [7]. After determination 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. The bioavailability of these solvents to the bacterium was tested as described by Janikowski et al., and those solvents that could be used as a carbon source by the bacterium were excluded from consideration for use in the extractive fermentation [7].

Solubility of 2,3-butanediol in solvents

The maximum solubility of 2,3-butanediol + solvent system was determined at 34 ± 0.5°C as described in our previous paper [8].

Organism and growth conditions

Microorganism

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

Fermentation

Submerged fermentation experiments were carried out in 1-l ferment or with shaking 180 rpm at 34°C. It was operated at working volume of 400 ml that included 10% inoculums. The extractant was then aseptically added to 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 the addition of an extractant. 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°C injector temperature, 300°C detector temperature, and 175°C column temperature, and using *n*-butanol as the internal standard. Acetoin was not quantified 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 (DCW) obtained gravimetrically.

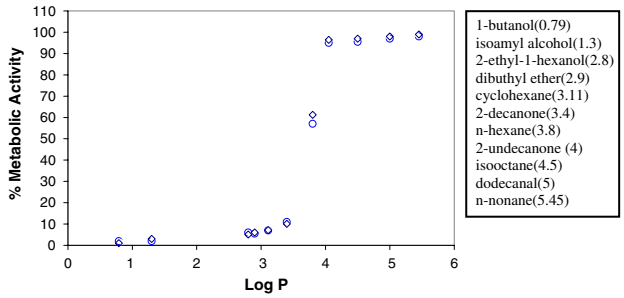

Fig. 1 Biocompatibility of solvents with *K. pneumoniae* based on percentage metabolic activity, (*diamond*) change in cell dry weight, (*empty circle*) glucose consumed

Glucose in the aqueous supernatants was measured using glucose oxidase kit.

Results and discussion

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 [9]. 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 Fig. 1. This method was previously described in the case biodegradation of polycyclic aromatic hydrocarbons [10] and 6-pentyl-α-pyrone production [4]. 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 other gram-negative bacteria [11, 12]. It has shown significant correlation is between the microbial activity and log *P* [13].

Bioavailability of solvents

Properties of solvents tested as to their bioavailability shown in Table 1. Cell growth, using the solvent as a carbon source, of less than 15% of the maximum growth compared to negative control medium without any carbon source and positive control medium with corn oil as carbon source 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 [7]. The results are shown in Fig. 2. The 1-decanol and lauraldehyde showed growth greater than 15% and, therefore, were excluded for use with *K. pneumoniae* in extractive fermentation. Collins and Daugulis [14] 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 2. 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, 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

Table 1 Properties of various biocompatible solvents

No.	Name	Chemical formula	Density (g/cm)	Boiling point	Solubility (g/l)	Log *P*	Bioavailability
1	*n*-Decane	CH3(CH2)8CH3	0.73	174	Ins.	5.98	No
2	1-Decanol	CH3(CH2)9OH	0.83	220–235	0.037	4	Yes
3	Dodecane	CH3(CH2)10CH3	0.75	216.3	Ins.	6.6	No
4	*n*-Hexadecane	CH3(CH2)14CH3	0.77	287	Ins.	8.8	No
5	Lauraldehyde	C12H24O	0.83	238	Ins.	4.8	Yes
6	*n*-Octane	CH3(CH2)6CH3	0.7	125–126	Ins.	4.58	No
7	Oleyl alcohol	C18H36O	0.85	330–360	Ins.	7.5	No

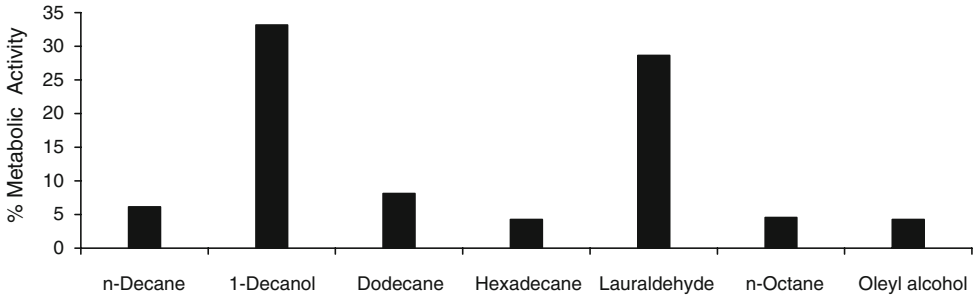

Fig. 2 Metabolism of *Klebsiella* in the presence of various solvents, with log *P* values greater than 3.8, relative to growth in the presence of corn oil

Table 2 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

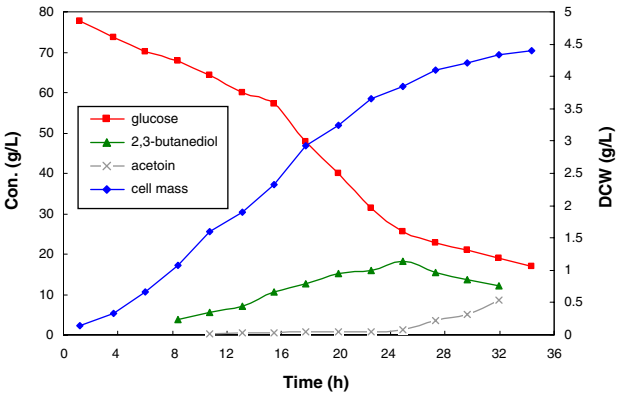

Fig. 3 Time course of 2,3-butanediol production and glucose utilization by *K. pneumoniae*

that oleyl alcohol has also been selected as extracting solvent for mesophilic acetone–butanol fermentation by *Clostridium acetobutylicum* and also Zigova et al. determined that oleyl alcohol has been shown to be a good extractant for use in extractive fermentation [1, 15].

Fermentation

In this study, batch fermentation was carried out at an initial glucose concentration of 80 g/l by both extractive and conventional fermentations. Attempts to ferment more than 90 g/l 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. Figure 3 displays the results of typical batch fermentation. In this figure, the concentrations of 2,3-butanediol and acetoin products are presented (acetic acid, also produced, was never greater than 0.5 g/l). The maximum specific growth rate was 0.2/h, and this was the same as that obtained during extractive fermentation in the presence of oleyl alcohol. The cell yield was approximately 0.10 g/g glucose, while the 2,3-butanediol yield was 0.30 g/g, nearly indicated to that obtained by Qin et al. [16]. The 2,3-butanediol productivity was 0.5 g/l h.

After 25 h, the 2,3-butanediol had been completely accumulated, and so the organisms then converted it to form acetoin so 2,3-butanediol production in batch culture is limited by conversion to acetoin. 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 [17]. 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 [5, 6, 18].

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 h of fermentation, oleyl alcohol was 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 under conventional fermentation to 23.01 g/l.

Oleyl alcohol was therefore added for extraction of 2,3-butanediol from the broth since it could extract approximately 68% of the 2,3-butanediol, thereby eliminating its end-product accumulation effect. Figure 4 shows the relative distribution of 2,3-butanediol during extractive fermentation. The relatively high 2,3-butanediol concentration in oleyl alcohol phase was attributed to its high 2,3-butanediol partition coefficient. Oleyl alcohol did not extract glucose or microorganisms appreciably. 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 h in the control to 0.66 g/l h in oleyl alcohol. On the addition of oleyl alcohol, the aqueous concentration of 2,3-butanediol

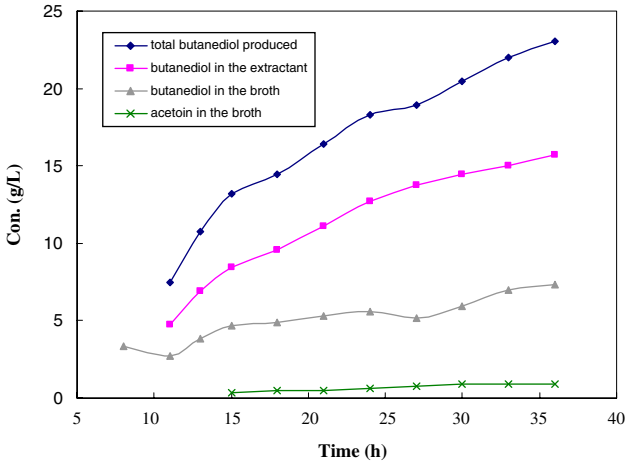

Fig. 4 Effect of solvent extraction on 2,3-butanediol production

was reduced from 17.9 to 7.32 g/l, but the total concentration increased only from 17.9 to 23.01 g/l (Fig. 4).

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

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 [2, 19–21]. Our finding confirmed that increased productivity and no longer acetoin production.

References

1. Shi Z, Zhang C, Chen J, Mao Z (2005) Performance evaluation of acetone–butanol continuous flash extractive fermentation process. Bioprocess Biosyst Eng 27:175–183. doi:10.1007/s00449-004-0396-7
2. Cheng HC, Wang FS (2007) Trade-off optimal design of a biocompatible solvent for an extractive fermentation process. Chem Eng Sci 62:4316–4324. doi:10.1016/j.ces.2007.05.010
3. Martik J, Rosenberg M, Schlosser S, Kristofikovi C (1995) Toxicity of organic solvents used in situ microbial fermentation. Biotechnol Tech 9(4):247–252. doi:10.1007/BF00151570
4. Serrano-Carreón L, Balderas-Ruíz K, Galindo E, Rito-Palomares M (2002) Production and biotransformation of 6-pentyl-α-pyrone by *Trichoderma harzianumin* two-phase culture systems. Appl Microbiol Biotechnol 58:170–174. doi:10.1007/s00253-001-0874-7
5. Eiteman MA, Gainer JL (1989) In situ extraction versus the use of an external column in fermentation. Appl Microbiol Biotechnol 30:614–618. doi:10.1007/BF00255368
6. Ghosh S, Swaminathan T (2003) Optimization of process variables for the extractive fermentation of 2,3-butanediol by *Klebsiella oxytoca* in aqueous two-phase system using response surface methodology. Chem Biochem Eng Q 17(4):319–325
7. Janikowski TB, Velicogna D, Punt M, Daugulis AJ (2002) Use of a two-phase partitioning bioreactor for degrading polycyclic aromatic hydrocarbons by a *Sphingomonas* sp. Appl Microbiol Biotechnol 59:368–376. doi:10.1007/s00253-002-1011-y
8. Ghanadzadeh Gilani H, Khayati G, Haghi AH (2006) Liquid–liquid equilibria of (water + 2, 3-butanediol + 2-ethyl-1-hexanol) at several temperatures. Fluid Phase Equil 247:199–204. doi:10.1016/j.fluid.2006.06.025
9. Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F (2007) Solvent-tolerant bacteria for biotransformation in two-phase fermentation systems. Appl Microbiol Biotechnol 74:961–973. doi:10.1007/s00253-006-0833-4
10. MacLeod CT, Daugulis AJ (2003) Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailable solvent. Appl Microbiol Biotechnol 62:291–296. doi:10.1007/s00253-003-1297-4
11. Pudge IB, Daugulis AJ, Dubois C (2003) The use of *Enterobacter cloacae* ATCC 43560 in the development of a two-phase partitioning bioreactor for the destruction of hexahydro-1,3,5-trinitro-1,3,5-*s*-triazine (RDX). J Biotechnol 100:65–75. doi:10.1016/S0168-1656(02)00229-8
12. Aono R, Tsukagoshi N, Miyamoto T (2001) Evaluation of the growth inhibition strength of hydrocarbon solvents against *Escherichia coli* and *Pseudomonas putida* grown in a two-liquid phase culture system consisting of a medium and organic solvent. Extremophiles 5:11–15. doi:10.1007/s007920000167
13. Lotter J, Botes AL, Van Dyk MS, Breytenbach J (2004) Correlation between the physicochemical properties of organic solvents and their biocompatibility toward epoxide hydrolase activity in whole-cells of a yeast, *Rhodotorula* sp. Biotechnol Lett 26:1191–1195. doi:10.1023/B:BILE.0000036595.09808.c2
14. Collins LD, Daugulis AJ (1999) Benzene/toluene/*p*-xylene degradation. Part I. Solvent selection and toluene degradation in a two-phase partitioning bioreactor. Appl Microbiol Biotechnol 52:354–359. doi:10.1007/s002530051531
15. Zigova′ J, Sturdı'k E, Vanda'k D, Schlosser S (1999) Butyric acid production by *Clostridium butyricum* with integrated extraction and pertraction. Process Biochem 34:835–843. doi:10.1016/S0032-9592(99)00007-2
16. Qin J, Xiao Z, Ma C, Xie N, Liu P, Xu P (2006) Production of 2,3-Butanediol by *Klebsiella oxytoca* using glucose and ammonium phosphate. Chin J Chem Eng 14(1):132–136. doi:10.1016/S1004-9541(06)60050-5
17. Syu MJ (2001) Biological production of 2,3-butanediol. Appl Microbiol Biotechnol 55:10–18. doi:10.1007/s002530000486
18. Yu EKC, Saddler JN (1983) Fed-batch approach to production of 2, 3-butanediol by *Klebsiella pneumoniae* grown on high substrate concentrations. Appl Environ Microbiol 46(3):630–635
19. Ishizaki A, Michiwaki S, Crabbe F, Kobayashi G, Sonomoto K, Yoshino S (1999) Extractive acetone–butanol–ethanol fermentation using methylated crude palm oil as extractant in batch culture of *Clostridium saccharoperbutylacetonicum* Nl-4 (ATCC 13564). J Biosci Bioeng 87(3):352–356. doi:10.1016/S1389-1723(99)80044-9
20. Wang Y, Achenie LEK (2002) Computer aided solvent design for extractive fermentation. Fluid Phase Equil 201:1–18. doi:10.1016/S0378-3812(02)00073-0
21. Porto TS, Monyeiro TIR, Moreira KA, Lima-Filho JL, Silva MPC, Porto ALF (2005) Liquid–liquid extraction of an extracellular alkaline protease from fermentation broth using aqueous two-phase and reversed micelles systems. World J Microbiol Biotechnol 21:655–659. doi:10.1007/s11274-004-3570-9