Bioresource Technology 106 (2012) 110–116

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Continuous two stage acetone–butanol–ethanol fermentation with integrated solvent removal using *Clostridium acetobutylicum* B 5313

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ARTICLE INFO

Article history:
Received 25 October 2011
Received in revised form 30 November 2011
Accepted 1 December 2011
Available online 8 December 2011

Keywords:
Butanol
Two stage chemostat
Oleyl alcohol
Sugar cane bagasse
Clostridium acetobutylicum

ABSTRACT

The objective of this study was to optimize continuous acetone–butanol–ethanol (ABE) fermentation using a two stage chemostat system integrated with liquid–liquid extraction of solvents produced in the first stage. This minimized end product inhibition by butanol and subsequently enhanced glucose utilization and solvent production in continuous cultures of *Clostridium acetobutylicum* B 5313. During continuous two-stage ABE fermentation, sugarcane bagasse was used as the cell holding material for the both stages and liquid–liquid extraction was performed using an oleyl alcohol and decanol mixture. An overall solvent production of 25.32 g/L (acetone 5.93 g/L, butanol 16.90 g/L and ethanol 2.48 g/L) was observed as compared to 15.98 g/L in the single stage chemostat with highest solvent productivity and solvent yield of 2.5 g/L h and of 0.35 g/g, respectively. Maximum glucose utilization (83.21%) at a dilution rate of 0.05 1/h was observed as compared to 54.38% in the single stage chemostat.

1. Introduction

Depletion of petroleum fuel-reserves and various environmental issues like greenhouse effects, global warming, and climate change are the current issues to be resolved worldwide. This decline of reserve, the rising price and the concerns on the environmental impact of petroleum-based fuel have initiated interest in renewable biofuels (Ramos et al., 2009; Koh and Ghazoul, 2008). The acetone–butanol–ethanol (ABE) fermentation process continues to receive attention as a source of fuel and chemical feedstock based on renewable resources. The traditional batch fermentation process, however, suffers from various problems which impede its commercial development. End-product inhibition, low product concentration, large volumes of fermentation broth, requirement of large bioreactors, and high costs associated with generating the steam required to distill the fermentation products from broth has largely contributed to the decline in fermentative ABE production.

Low reactor productivity and severe product inhibition which accompanies the process, limits the ABE concentration in the broth to 20 g/L. In Clostridial ABE fermentation, butanol inhibition limits product titer which in turn contributes to the high cost of product recovery (Ezeji et al., 2005). These problems can be solved either by genetic manipulation of the Clostridium bacterium or by advanced bioprocess developments of ABE production and recovery. Process development for ABE fermentation with marked improvements in reactor productivity have been reported *viz.* continuous culture and immobilized cells culture techniques (Qureshi and Maddox, 1995). However, product recovery from dilute solutions and disposal of large volumes of effluent are the drawbacks with these systems. As was attempted in the present study, recycling the reactor effluent so as to achieve complete sugar utilization with continuous removal of the inhibitory solvents may lead to higher product concentrations. In particular, *in situ* solvent extractive fermentation has been proposed as one of the approaches to minimize butanol inhibition and increase product titer in fermentation broth (Ezeji et al., 2005; Ishizaki et al., 1999; Grobben et al., 1993).

In order to improve the extraction process, several solvents have been identified and tested on the basis of their selectivity for butanol and biocompatibility with the fermentative organism (Shukla et al., 1988; Job et al., 1989). Among these solvents, oleyl alcohol (Qureshi and Maddox, 1995; Davison and Thompson, 1993), decanol (Evans and Wang, 1988), dibutyl phthalate (Qureshi and Maddox, 1995) and polypropylene glycol (Barton and Daugulis, 1992) have been extensively used at the laboratory scale for *in situ* solvent extraction. Methylated crude palm oil (Ishizaki et al., 1999) and methylated sunflower oil (Grobben et al., 1993) as a butanol extractant have also been successfully studied to maintain a low butanol level in fermentation broth.

The solvent productivity of continuous cultivation can be increased to values between 0.5 and 1.0 g/L h in a culture of freely suspended cells (Maddox, 1989). Modest increase in productivity can be achieved by increasing the cell concentration in the medium. According to several methods described in the literature, cell

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doi:10.1016/j.biortech.2011.12.005

immobilization is found to be the most advantageous method for continuous ABE fermentation. The use of carrier material and formation of cell aggregates are useful to increase the biomass concentration and therefore the productivity of microbial process (Beeftink et al., 1988). Our previous study of continuous production of ABE with SO_2–ethanol–water spent liquor from spruce chips has successfully employed immobilized culture in a column to enhance the solvent productivity (Survase et al., 2011a).

Attempts to improve the productivity of ABE fermentation using two-stage continuous bioreactor (Mutschlechner et al., 2000; Bahl et al., 1982) and *in situ* recovery in continuous bioreactor (Qureshi and Maddox, 1995, 2005; Gapes et al., 1996) have been reported. These approaches have given low productivities of the order of 0.25–1.5 g/L h. We hypothesized that an inclusion of *in situ* extraction of ABE between the two stages of continuous extraction would overcome the toxicity of the solvent system to the organism and also increase the productivity by better utilization of the substrate. Besides, it would enable the reactor to be used continuously for a longer time. This approach has not been used so far for ABE fermentation. Accordingly, the present work aimed to achieve and maintain the conditions which allow for degeneration-free, long term stability, and continuous two stage cultivation of *Clostridium acetobutylicum* B 5313 with a high overall solvent productivity. Sugarcane bagasse as a carrier material was used in both stages. A liquid–liquid extraction module was integrated in between the two stages to counter the product inhibition, and allow high productivities with maximum sugar utilization.

2. Methods

2.1. Organism, maintenance and inoculum preparation

C. acetobutylicum B 5313 (DSM 792, ATCC 824) was obtained from the Russian National Collection of Industrial Microorganisms at the Institute of Genetics and Selection of Industrial Microorganisms (Moscow, Russia). Initially, sporulated cells were activated by heat shock at 80 °C for 10 min. The activated spore culture (2.5 mL) was inoculated in 100 mL sterile reinforced clostridia medium (RCM) in 125 mL air tight, anaerobic glass bottles and grown for 20 h at 37 °C. RCM medium contained (g/L) meat extract, 10; peptone, 5.0; yeast extract, 3.0, glucose, 30; starch, 1.0; sodium chloride, 5.0; sodium acetate, 3.0; L-cysteine, 0.50 and pH was adjusted to 6.8 ± 0.2.

2.2. Production medium

The production medium reported by Monot et al. (1982) was used in the present study, which contained (g/L): glucose, 60; ammonium acetate, 2.2; K_2HPO_4, 0.5; KH_2PO_4, 0.5; *p*-aminobenzoic acid, 0.1; thiamine hydrochloride, 0.1; biotin, 0.01; $FeSO_4$, 0.01; $MnSO_4{\cdot}H_2O$, 0.01; $MgSO_4$, 0.2; NaCl, 0.01. The medium pH was adjusted to 6.5 with HCl. The medium was then autoclaved at 10^5 Pa (121 °C) for 20 min and cooled.

2.3. Continuous fermentation with immobilized cells

In batch fermentation, reactor productivity is in the order of 0.35–0.40 g/L h (Qureshi and Maddox, 1995). Such low reactor productivity requires large reactor volumes which results in high capital and operational costs. In order to reduce the cost of production, high reactor productivity is desirable, which can be achieved using immobilized cell reactors or membrane cell recycle reactors. Since immobilized cell reactors are cheaper and are simple to operate, this technique was employed to achieve high reactor productivity. Sugarcane bagasse was used as a support material for cells.

2.3.1. Single-stage chemostat cultivation

Single-stage chemostat cultivations were carried out in 1 L jacketed glass bioreactor with a 500 mL fermentation volume. The bioreactor contained sugarcane bagasse (size, 5–10 mm), packed in a nylon mesh basket which served as a carrier material for bacterial cells. The carrier material to liquid ratio was 1:4 (previously optimized, data not shown). The bioreactor was inoculated with 10% inoculum of highly motile cells of *C. acetobutylicum* B 5313. The fermentation was allowed to proceed in batch mode for 24 h, after which fermentation feed medium was continuously pumped into the bioreactor at different dilution rates, wherever mentioned. The cells were adsorbed onto the sugarcane bagasse during batch growth, before starting continuous fermentation. The working volume of the fermenter was kept constant by removing excess medium from the bioreactor at a rate equal to the feed rate. Fresh medium was continuously introduced into the bioreactor using a peristaltic pump (Watson–Marlow Ltd., Falmouth, England). Dilution rates were varied from 0.05 to 0.70 1/h with reactor temperature of 37 °C. During the course of fermentation, samples were collected at regular intervals and analyzed for biomass, acetone, butanol, ethanol, residual sugar and acids. Unless otherwise stated, all continuous fermentations were carried out in duplicates and results reported are the average of two fermentations. Samples for biomass and product analysis were taken after five reactor volumes for two consecutive days at steady state condition. The steady state was confirmed by stable biomass and product values at a specific dilution rate.

2.3.2. Two-stage chemostat integrated with liquid–liquid extraction

The two stage chemostat set up was adopted from our previous study (Survase et al., 2011b) with some modifications. The experimental setup with a two stage bioreactor (different volume in both) was carried out with sugarcane bagasse as the cell holding material and integrated with a liquid–liquid extraction module for *in situ* solvent removal. The bioreactors (stage 1 and stage 2) contained sugarcane bagasse (size 5–10 mm), packed in a nylon mesh basket which served as a carrier material (ratio with liquid phase was 1:4) for bacterial cells. The assembly consisted of four glass jacketed bioreactors with a total volume of 1 L. Bioreactors were arranged sequentially and designated as stage one (R1), extractor (E), settling vessel (S) and stage two (R2) (Fig. 1). Culture volumes (including medium and carrier material) in the R1 and R2 stages were maintained as 350 mL and 700 mL, respectively. Fresh medium was continuously introduced into R1 with the help of a peristaltic pump. The dilution rate of R1 was varied between 0.05 and 1.0 1/h which resulted in a dilution rate of 0.025–0.5 1/h in R2.

The bioreactors were inoculated with 10% v/v highly motile cells of *C. acetobutylicum* B 5313. Fermentation was allowed to proceed in the batch mode for 24 h, after which fermentation feed medium was continuously pumped into R1 at different dilution rates as mentioned. Steady state was confirmed by stable biomass and product values at specific dilution rates. Samples for biomass and product analysis were taken after five working volume changes at steady state conditions and for two consecutive days. All the vessels were maintained at 37 °C and medium from both bioreactors was agitated at 200 rpm using a magnetic stirrer.

An extraction vessel (E) contained the extractant (oleyl alcohol:decanol, 4:1) to remove the solvents generated during continuous cultivation from R1. Culture broth and extractant were mixed by continuous agitation (400 rpm) with the help of a magnetic stirrer and the volume of extractor was maintained at 500 mL by maintaining the flow rate to separator and back flow of extractant from separator to extractor (Fig. 1). The mixture was allowed to separate into an organic phase and an aqueous phase in the separator (S) and latter was pumped to the R2 where further fermentation was carried out using immobilized cells. The organic phase in the separator