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Novel in situ product removal technique for simultaneous production of propionic acid and vitamin B12 by expanded bed adsorption bioreactor

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ABSTRACT

A new type of in situ product removal (ISPR) technique of expanded bed adsorption (EBA) bioreactor was studied to simultaneously produce extracellular propionic acid and intracellular vitamin B12 by *Propionibacterium freudenreichii* CICC 10019. Resin screening experiments showed that the ZGA330 resin have the best biocompatibility and highest adsorption for propionic acid. Through the EBA bioreactor, propionic acid could be recovered efficiently by semi-continuous recirculation of the unfiltered broth, which eliminated the feedback inhibition of propionic acid. Fed-batch fermentation was carried out using the EBA system, resulting in a propionic acid concentration of 52.5 g L^{-1} and vitamin B12 concentration of 43.04 mg L^{-1} at 160 h, which correspond to product yields of 0.66 g g^{-1} and 0.54 mg g^{-1}, respectively. The present study suggests that the EBA bioreactor can be utilized for the simple and economical production of propionic acid and vitamin B12 in a single fermentation process.

1. Introduction

As the essential vitamin in the metabolic process of human tissues, vitamin B12 has various physiological functions and has been widely used in pharmaceutical and food industries. In the process of producing food-grade vitamin B12 by anaerobic fermentation of *Propionibacterium freudenreichii* (*P. freudenreichii*), propionic acid, an extracellular product contained in fermentation broth, might accumulate to a high concentration of 20–30 g L^{-1}, which would causes feedback inhibition to the growth of microbial cells (Gu et al., 1998; Suwannakham and Yang, 2005). On the other hand, this weak bioorganic acid, together with its salts, could be applied as human food antifungal agent, food additive in animal feed and a substrate for conversion to methane (Lueck, 1980; Huitson, 1968; Draughon et al., 1982; Guo et al., 2011). Accordingly, on-line removing propionic acid from the fermentation process of vitamin B12 could not only change it from harmfulness to usefulness but also eliminate the inhibition, then increase the productivity of vitamin B12.

In situ product removal (ISPR) is a useful technique to overcome product inhibition. Its capability to obtain a high productivity has been proved for many fermentation processes. For example, ISPR have been reported for producing organic acids (Moldes et al., 2001; Einicke et al., 1995; Cao et al., 1996; Roddick and Britz, 1997). The use of various ISPR techniques to produce propionic acid has also been reported (Feng et al., 2011; Gupta and Srivastava, 2001). However, few works have been done on the applicability of two products system such as propionic acid/vitamin B12. Moreover, most previous research required the use of additional membrane separation device to remove microbial cells to prevent from the column clogging. This would result in extra operation unit and increase the cost for the process system.

Expanded bed adsorption (EBA), a recently developed technology in separation field, has some potential advantages when used in ISPR. For EBA, the adsorption happens when the column in an expended state. This allows particles in the fermentation broth to directly pass the chromatographic column and avoids the congestion that characterizes fixed-bed absorption. Currently, this technology is applied in the direct separation of protein from broth or tissue homogenate (Mattiasson, 1999), extraction of ephedrine hydrochloride from raw materials (Chen et al., 2004), and extraction of human epidermal growth factor from recombinant *Escherichia coli* fermentation broth (Wang et al., 2005). So, coupling EBA to a fermentation reactor directly can omit the membrane unit between the fermenter and the chromatographic column. However, there is no report on the application of EBA technology to ISPR until now.

In this paper, a novel ISPR equipment that integrated EBA and fermentation reactor was designed and applied to simultaneously produce propionic acid and vitamin B12 in a single fermentation process. Nine alkalescence anion exchange resins were used first

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to determine their propionic acid adsorption capacity. In the EBA based ISPR, microbial cells would contact directly with the chromatographic resin, which might be harmful to the cell's growth. So, the biocompatibility of best resin was also investigated. Based on the decided condition, fed-batch fermentation was carried out and a high concentration of propionic acid and vitamin B12 was obtained. The present results suggested that the EBA bioreactor could be utilized for the simple and economical production of propionic acid and vitamin B12 in a single fermentation process.

2. Methods

2.1. Microorganism and media

P. freudenreichii CICC 10019 was obtained from the Chinese Industrial-microorganism Conservation Center. The stock culture was incubated in deep agar slants, stored at 4 °C and transferred to new agar monthly.

The preculture media contained the following materials (per liter of deionized water): glucose, 35 g; corn steep liquor (CSL), 21 g; ammonium sulfate 5 g; potassium dihydrogen phosphate, 4 g; and cobalt chloride, 0.005 g. pH before autoclaving was within 6.8–7.0 (adjusted by 12% ammonia solution). The stock sugar solution was autoclaved separately prior to mixing with the rest of the medium.

The fermentation media contained the following materials (per liter of deionized water): glucose, 60 g; corn steep liquor (CSL), 40 g; potassium dihydrogen phosphate, 4.6 g; and cobalt chloride, 0.0127 g. pH before autoclaving was within 6.8–7.0. For medium preparation, glucose was autoclaved separately.

2.2. Analytical methods

The eluents from the resins were analyzed without sample preparation for the quantification of propionic acid in the aqueous phase. On the other hand, the supernatants from 1 mL culture broth were used after centrifugation at 10,000×*g* for 10 min. The samples were analyzed by HPLC using Beckman C18 column (5 μm, 4.6 μm × 25 cm) with a flow rate of 1.0 mL min^{-1} and a wavelength of 215 nm at 25 °C. The mobile phase consisted of 0.02 M potassium dihydrogen phosphate buffer solution/acetonitrile (pH 2.8, adjusted with phosphoric acid solution) with 4% acetonitrile isocratic elution. Commercially available propionic acid was used as external standard.

2.3. Measurement of cell concentration

To measure growth kinetics, cell concentrations were analyzed photometrically at 600 nm. Before measuring cell concentrations, 1 mL broth was centrifuged at 10,000×*g* for 10 min and cell pellets were resuspended in 1 mL phosphate buffer after removing the supernatant. The error produced by the removal of the very small fraction of glucose was negligible. OD600 nm values were converted to dry cell weight (DCW) values using the following empiric equation based on the measurement of 11 different cell samples: DCW (g L^{-1}) = OD600 nm × 0.30. Standard deviation = 0.06. DCW was determined by using 5 mL liquid culture as described by Mirata et al. (2008).

2.4. Screening of ion-exchange resin for propionic acid adsorption and elution

Nine alkalescence anion exchange resins were tested. Characteristics of these nine resins included skeleton type, function group, particle size, and moisture capacity (Table 1). To determine the capacity of the resins under fermentation conditions, adsorption experiments were firstly performed in an aliquot of propionic acid solution. An amount of resin corresponding to a 10-fold equivalent was added to a 500 mL shaking flask filled with 50 mL propionic acid solution. The assays were performed under the conditions of 30 °C and 120 rpm for 4 h. Propionic acid adsorption was determined by analyzing the concentrations in the supernatant before and after the experiments via HPLC. Furthermore, for the comparison of the adsorption and elution performance of the best resin that was screened from shaking flask experiment, an amount of resin was added to a 500 mL glass column and, 1500 mL fermentation broth (5.8 mg mL^{-1} glucose, 2.1 mg L^{-1} amino nitrogen, 5.1 g dcw L^{-1} and 20 mg mL^{-1} propionic acid) was pumped through it. Sampling and analysis of glucose, amino nitrogen, cell biomass and propionic acid after adsorption were conducted on a regular basis (50 mL) throughout the process.

2.5. Biocompatibility investigation with anion exchange-based in situ product removal

Biocompatibility between *P. freudenreichii* CICC 10019 and ZGA330 resin was determined based on the metabolic activity and propionic acid production of batch cultivations of *P. freudenreichii*. The batch culture of *P. freudenreichii* containing different resin concentrations between 0 g L^{-1} and 80 g L^{-1} was carried out in 50 mL shaking flasks filled with 20 mL fermentation medium, and was performed for 5 days at 30 °C and 220 rpm. Sampling and analysis were conducted with a volume of 0.2 mL and a frequency of 12 h.

2.6. EBA-based bioreactor construction and propionic acid/vitamin B12 production in the EBA-based bioreactor

The EBA-based bioreactor was mainly an external recovery loop composed primarily of a 7 L stirred-tank fermenter (Bioflo 110, New Brunswick Scientific, USA) (Fig. 1, Detail 1) and three paralleled EBA columns (25 mm o.d., length 500 mm, total volume 200 mL; purchased from Jinhua Co., Shanghai, China) (Fig. 1, Detail

Table 1
The partial properties of resins, including skeleton type, function group, particle size, and moisture capacity.

Resin type	Skeleton type	Function group	Particle size (mm)	Moisture capacity (g g^{-1})
ZGA454	Acrylic acid	$-N(CH_3)_2$	0.315–1.250	0.663
ZGA456	Acrylic acid	$-N(CH_3)_2$	0.315–1.250	0.468
D301	Styrene-DVB	$-N(CH_3)_2$	0.315–1.250	0.529
ZGA330	Epoxy	$-C_2H_4NH_2$–C_2H_4NHR $C_2H_4NR_2$-$C_2H_4NR_3$	0.355–1.50	0.641
312	Acrylic acid	$-N(CH_3)_2$	0.315–1.250	0.614
ZGD730	Acrylic acid	$-N+(R_3)$	0.45–1.250	0.694
D301R	Styrene-DVB	$-N(CH_3)_2$	0.315–1.250	0.556
D301T	Styrene-DVB	$-N(CH_3)_2$	0.3–1.2	0.654
D380	Styrene-DVB	$-NH_2$	0.315–1.250	0.56