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Continuous production of *n*-butanol by *Clostridium pasteurianum* DSM 525 using suspended and surface-immobilized cells

Alessandro Gallazzi[a,b,c], Barbora Branska[a], Flavia Marinelli[b,c], Petra Patakova[a,*]

[a] Department of Biotechnology, University of Chemistry and Technology, Prague, Czech Republic
[b] Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy
[c] The Protein FactoryResearch Center, Politecnico of Milano, ICRM CNR Milano and University of Insubria, Varese, Italy

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ABSTRACT

For *n*-butanol production by *Clostridium pasteurianum* DSM 525, a modified reinforced *Clostridium* medium was used, where glucose was alternated with glycerol and two kinds of continuous fermentation were tested using suspended and surface immobilized cells on corn stover pieces. A steady state, with butanol productivity of 4.2 g/L h, was reached during the packed-bed continuous fermentation at a dilution rate of 0.44 h^{-1}. The average *n*-butanol concentration, yield and the ratio of *n*-butanol/liquid by-products were 10.4 g/L, 33 % and 2.5, respectively. Unexpectedly, during continuous fermentation with suspended cells, at a dilution rate of 0.01 h^{-1}, steady-state was not achieved and regular oscillations occurred in all measured variables, i.e. concentrations of glycerol, products and the number of cells stained with the fluorescent dyes carboxy fluorescein diacetate and propidium iodide. A possible explanation for oscillatory/steady-state behavior of suspended/surface-attached cells, respectively, may be specific butanol toxicity (toxicity per cell), which was higher/lower in respective cases, and which might be caused by lower/higher cell numbers respectively in both systems.

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1. Introduction

Fluctuating crude oil prices, unstable political situations in countries exporting the oil and a growing consciousness of environmental problems, such as global warming, attributed to the use of fossil fuels, has brought significant attention to the production of biofuels from biomass. In Europe, 20-20-20 targets, which aim, *inter alia,* to cover 20 % of energy consumption from renewable sources by the year 2020, resulted in increased production of biodiesel and bioethanol. Mostly, biodiesel is produced from triglycerides present in various plant oils, animal fats or waste oils, by transesterification with methanol using acidic, alkaline or enzymatic catalysts (Demirbaş, 2003); this results in the formation of glycerol as an abundant by-product and, in general, lowers the refined glycerol world market price. However the actual glycerol price also depends on the price of oil plants (for recent fluctuations of refined glycerol price and the impact of biodiesel production on glycerol market, see Ciriminna et al., 2014). Nevertheless, glycerol has become an attractive fermentable substrate due to its availability, low cost and high degree of reduction. Use of glycerol can be also considered a model example of functioning biorefinery concept idea where biodiesel waste is consumed in the following production of other valuable compound, butanol.

Biobutanol, a promising biofuel candidate, can be produced by acetone–butanol–ethanol (ABE) fermentation by selected species of the genus *Clostridium*. Butanol can be blended both with gasoline and diesel and due to several advantages over ethanol, such as higher energy content, lower water miscibility and lower corrosivity, has attracted considerable attention in recent years (Dürre, 2008). The strain *Clostridium pasteurianum* DSM 525 differs from the most well-known *Clostridium acetobutylicum* ATCC 824 strain in its fermentation pattern and can ferment glycerol to *n*-butanol and 1,3-propanediol as main products together with ethanol, butyric and lactic acids, hydrogen and carbon dioxide as by-products (Dabrock et al., 1992; Biebl, 2011). This or closely related strains deposited in other culture collections i.e., ATCC 6013 and MTCC 116 have already been used for glycerol fermentation (Dabrock et al., 1992; Biebl, 2011; Moon et al., 2011; Jensen et al., 2012; Khanna et al., 2013; Gallardo et al., 2014) but not using packed-bed continuous fermentation with corn stover pieces as immobilization support material. This work is focused on a comparison of continuous *n*-butanol production from glycerol by *C. pasteurianum* DSM 525 using suspended and surface (on corn stover) immobi-

* Corresponding author at: Department of Biotechnology, University of Chemistry and Technology, Technicka 5, CZ 16628 Prague 6, Czech Republic.
E-mail address: petra.patakova@vscht.cz (P. Patakova).

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lized cells. In addition, double fluorescent staining with propidium iodide (PI) and carboxy fluorescein diacetate (CFDA), followed by flow cytometry (FC), were used to monitor the physiological state of cells during fermentation. To the best of our knowledge, neither a comparison of different types of continuous fermentation nor flow cytometric data have been used previously for this *Clostridium* strain.

2. Materials and methods

2.1. Microorganism and culture conditions

The strain *C. pasteurianum* DSM 525 was stored frozen at −70 °C and an inoculum was grown in the modified reinforced clostridium medium (RCM) (containing in g/L: tryptone (Merck, Germany) 10, meat extract (Merck, Germany) 10, yeast extract (Merck, Germany) 3, NaCl 5, sodium acetate 3, glucose 15), at 37 °C in an anaerobic chamber (Sony Technology Centre, Glamorgam, UK) for 18–24 h in Erlenmeyer flasks.

2.2. Batch and continuous production with suspended culture

Multifors (Infors HT, Switzerland) bioreactors were used for batch and continuous fermentations with suspended cells. Both batch and steady-state working volumes in the bioreactor were kept at 400 mL and cultivation parameters were maintained at 200 rpm and 37 °C. Bioreactors with suspended cultures were run in duplicate. The carbon source, originally glucose in the modified RCM, was replaced with pure glycerol (Penta, Czech Republic) and its initial concentration in the medium was 40 or 45 g/L. Prior to inoculation, the bioreactors were bubbled with CO_2 for 10 min to establish an anaerobic atmosphere and the pH was adjusted to 6.6–7.0. During fermentation, pH was not regulated. After inoculation (ratio 1:10), the cultivation was operated in batch mode for 18 h followed by continuous fermentation wherein regular samplings were carried out. Dilution rate during the continuous period was kept constant, at 0.01 h^{-1}, and the glycerol concentration in the feed medium was 60 g/L.

2.3. Continuous production with the culture immobilized on corn stover

Immobilized cultivations were carried out in a glass, in-house made, jacketed, packed-bed bioreactor (250 mL total volume and 200 mL max. working volume) heated by water to 37 °C. A schematic of the bioreactor system is shown in Fig. 1. The bioreactor was filled with cut corn stover pieces of 1 cm^3 approximate size. A laboratory pH meter (WTW, Germany) was used for online pH measurements in a through-flow cell at the bioreactor outlet. The feed rate was calculated from weight loss, as measured by a laboratory balance (KERN, Germany). The bioreactor was filled with immobilization material and 200 mL of the RCM culture medium with glycerol as a carbon source prior to sterilization, and was then sterilized in an autoclave at 121 °C for 20 min. After cooling, the bioreactor was placed in a sterile inoculation box and inoculated with 20 mL of the seed culture. After inoculation, the cell culture was grown batch-wise for 18 h, with an initial glycerol concentration of 54 g/L, and with recirculation of the medium allowing colonization of the bacteria on the corn stover.

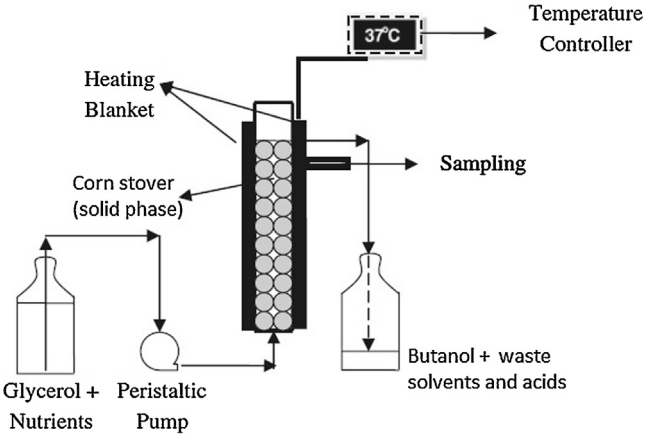

Fig. 1. Schema of the packed-bed bioreactor system.

The steady-state working volume was kept constant at 180 mL and the medium feed was set to reach the required dilution rate (D). Dilution rates adopted were: 0.13 h^{-1} during the first 140 h and after that, 0.44 h^{-1} until the end of the fermentation. The concentrations of glycerol in the feeding solutions were: 45 g/L for $D = 0.13\ h^{-1}$ and 35 g/L for $D = 0.44\ h^{-1}$.

2.4. Analyses

Growth was followed as an optical density (OD) measured at 600 nm using a spectrophotometer (Varian, Spain).

Concentrations of carbon source, glycerol, and products (*n*-butanol, 1,3 propanediol, lactic acid, butyric acid and ethanol) were determined by HPLC analysis (Agilent Series 1200HPLC; Agilent, Spain) using a Polymer IEX H^+ column (Watrex, Czech Republic) heated to 60 °C with a mobile phase of 5 mM H_2SO_4 (flow rate 0.5 mL/min) and equipped with refractive index detection.

2.5. Flow cytometric analysis and fluorescent staining

Cell fluorescence was monitored by microscopic observation as described previously (Linhova et al., 2010) and by flow cytometric analysis. FC analyses were conducted after fluorescent staining with two probes, CFDA and PI; both purchased from Sigma Aldrich. The samples were filtered through a 30 μm filter, centrifuged (2000 × *g*, 3 min), washed twice in sterile 0.8% (w/v) NaCl and diluted in the same solution to an OD of 0.20 ± 0.01. The fluorescent probes, PI and CFDA, were added to the cell suspension to a final concentration of 10.0 μg/mL. The cultures were incubated in the dark, at room temperature, for 10 min and subsequently analyzed. Stained suspensions were analyzed with an Accuri C6 cytometer (BD Accuri Cytometer Inc., USA) equipped with an argon laser (488 nm). FSC and SSC signals were used as a trigger, and green (FL1; 515–565 nm) and red (FL3; >605 nm) fluorescence were used to measure the percentage of PI or CFDA stained cells. The sample flow rate was set to 10 μL/min for all experiments.

2.6. Electron microscopy

SEM microphotographs of the material (corn stover) with attached cells were performed using SEM Hitachi S-4700 (Hitachi, Japan) device. Prior the microscopy, the samples were mounted using double stick carbon tape, dried in thermostat at 50 °C, and coated by Au/Pd (approx. 15 nm).

3. Theory/calculation

From an industrial point of view, the preferred method for producing *n*-butanol is continuous production. However, common drawbacks of continuous production with solventogenic clostridia are culture instability, strain degeneration and alternating growth and sporulation phases that cause a decrease or loss of solvent production. This behavior has been studied in detail for *C. acetobutylicum* ATCC 824 (Clarke et al., 1988). However in the case of *C.*

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