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Short communication

Butyric acid production with *Clostridium tyrobutyricum* immobilised to PVA gel

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ABSTRACT

Clostridium tyrobutyricum was entrapped into lens-shaped polyvinyl alcohol particles (LentiKats®). Immobilised cells were propagated in series of repeated batches and reached average productivity of 1.74 g/L h and yield of 0.44 g/g during 31 repetitions with glucose as an initial substrate, which was 5 times higher productivity compared with a free cell system under the same conditions. Immobilised cells were successfully applied for more than 71 repeated batches and demonstrated remarkable process stability. Application of immobilised cells to a continuous system resulted in 5.8 times higher productivity compared with free cells, with 6.8 g/L of butyric acid and residual glucose of 5.8 g/L.

1. Introduction

Butyric acid as a short-chain fatty acid with many applications in the chemical, food and pharmaceutical industries is currently produced predominantly via the petroleum-based oxosynthesis of butyraldehyde from propylene [1–4]. However, with the decreased supply of world crude oil, the increased demand of bio-products such as butyric acid by is observed [5].

Under anaerobic conditions, butyric acid is a common metabolite produced by bacteria strains of *Clostridium* genus such as: *Clostridium tyrobutyricum*, *C. butyricum*, *C. populeti* and *C. thermobutyricum* [1,6,7]. Especially strain *C. tyrobutyricum*, known as a gram-positive, rod-shaped, spore-forming, and obligate anaerobic bacterium is one of the most promising microorganisms for industrial production of butyric acid from various carbohydrates [4,8].

However, conventional microbial fermentation production of butyric acid is not competitive with the petroleum routes, because of its relatively low yield of butyric acid and the low reactor productivity caused by the simultaneous production of acetic acid as a by-product [1,2,4]. Methods how to deal with these disadvantages can be established on in situ product removal [9] or on whole cell immobilisation [10,13]. One of the methods used for whole cell immobilisation is entrapment into lens-shaped polyvinyl alcohol (PVA) hydrogel particles (LentiKats®). Compared with other gel systems this immobilisation offers simple gel preparation, it is available for large-scale production, and the particles are easy to separate from the reaction mixture (diameter: 3–4 mm). Particles have also low diffusion limitation (thickness: 200–400 μm) and demonstrate excellent mechanical stability [11,12]. However, particles are made under strict aerobic conditions, which may be a problem for anaerobic microorganism immobilisation.

The aim of this study was to apply a method developed for immobilisation of anaerobic bacteria by method LentiKats® [13] for immobilisation of *C. tyrobutyricum* and verify the butyric acid productivity, yield and stability in both repeated batch and continuous fermentations.

2. Materials and methods

2.1. Microorganism and media

C. tyrobutyricum DSM 2637 was used in all experiments. Stock culture was kept on Reinforced Clostridial Agar (MERCK, Germany) at 34 °C in an anaerobic chamber (Bactron I, Shel Lab, USA). Inoculation medium contained per L: 20 g glucose; 6 g tryptone (Fluka, USA); 2 g yeast extract (Gistex®, DSM, Germany); 2.5 g KH_2PO_4; 2.5 g K_2HPO_4; 0.01 g $FeSO_4 \cdot 7H_2O$; 3 g ammonium acetate; and 0.5 g cysteine hydrochloride. pH was adjusted to 6.2. Media in reagent bottles sealed by Suba-Seal® (Sigma-Aldrich Co., USA) was

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sparged with N_2 for 15 min and then autoclaved (120 °C, 20 min). $MgSO_4 \cdot 7H_2O$ (0.3 g in 10 mL of distilled water) was autoclaved separately and added to the production medium before the inoculation.

The production medium was the same as the inoculation one, but 2 g of tryptone, 0.5 g of KH_2PO_4 and 0.5 g of K_2HPO_4 were used. The medium was autoclaved in a fermentor vessel and before inoculation $MgSO_4 \cdot 7H_2O$ was added and sparged with N_2 (15 min). Fed media for continuous culture had the same composition but two different glucose concentrations (20 and 60 g/L) were used.

2.2. Biomass preparation

Durham bottles (100 mL) (Fisherbrand®, Fisher Scientific, USA) were filled with 50 mL of inoculation medium and magnetic blender. A single colony from a Petri dish was transferred to the medium and cultivated at 34 °C in an anaerobic chamber with magnetic stirring (220 rpm). After 10 h of cultivation ($OD_{600\,nm}$ = 2.0 – 5.0), the culture (5% v/v) was used as inoculum for 1 L of production medium (free cell fermentation) or 900 mL of production medium and 100 g of immobilised cells in a 1.3-L fermentor (BioFlow 115, Eppendorf, Germany). For immobilisation the biomass was prepared in 700 mL of inoculation medium (in 1 L Durham bottles), inoculated with 2% v/v inoculum. Biomass was centrifuged (2750 g, 30 min, 4 °C) after 16 h of growth ($OD_{600\,nm}$ = 8.9) and suspended in 70 mL of anaerobic sterile distilled water in the anaerobic chamber.

2.3. Immobilisation

Immobilisation of *C. tyrobutyricum* was performed in an immobilisation pilot plant in company LentiKat's a.s. (Czech Republic, www.lentikats.eu). At first, 1.4 L of PVA hydrogel in liquid form was prepared by melting of defined amount of PVA and polyethylene glycol in distilled water during water bath at a temperature 85–90 °C, following the manufacturer's protocol [24]. Mixture was than cooled down to 40 °C and homogenized with concentrated biomass (2.8 g dry cell weight) regarding to modified manufacturer's protocol for immobilisation of anaerobic bacteria [13]. Lens-shaped particles with entrapped cells were prepared by passing the gel mixture through thin nozzles on a hard surface followed by subsequent drying in an airflow cabinet at temperature 40 °C until were dried down to 30% initial mass. After that, particles are swollen in stabilising solution (0.1 M Na_2SO_4) for 1 h. Empty particles for verification of repeated batch fermentations were prepared as above without biomass addition.

2.4. Fermentation experiments

1.3-L *BioFlo*® 115 fermentors (Eppendorf, Germany), filled with 1 L of the production medium, were inoculated with 50 mL of a 5% (v/v) inoculum. Batch fermentations were carried out at pH 4.8, 5.0, 5.5, 6.0 (with automatic addition of 5 M KOH) and different glucose concentrations (20 and 70 g/L) at 37 °C, and 150 rpm stirring. Before inoculation production the medium was sparged in a fermentor by N_2 for 15 min (0.1 vvm). Sparging was stopped when the microorganism reached the log phase. Continuous fermentation with free cells of *C. tyrobutyricum* started the batch fermentation (glucose 20 g/L, pH 5.0). When glucose dropped to 4 g/L, continuous fermentation was initiated. Stock production media were not sparged by N_2.

Repeated batch fermentations with immobilised cells were performed as described above, but 900 mL of production medium was inoculated with 100 g of immobilised *C. tyrobutyricum* (load of immobilised microorganism 10% w/v) and the stirring was set to 370 rpm (glucose 20 g/L, pH 5.0, 37 °C). Fermentor with immobilised cells and production medium was also inoculated with 50 mL of inoculum 5% (v/v). When residual concentrations of glucose decreased (concentration higher than 1 g/L), the whole volume of production medium was drained through a sieve (to avoid washing out the particles). The fermentor was subsequently fed with a fresh production medium, and the whole process was repeated for consecutive batch fermentations.

After 40 repeated batches as described above, the production medium was reduced to the final volume of 500 mL in the fermentor, to increase concentration of the immobilised microorganism (load 20% w/v) and used for 31 consecutive batch fermentations similarly to load 10% w/v. After all repeated batch fermentations, the continuous fermentation with immobilised cells (load 20% w/v) was set to 500 mL with the production medium and the fermentor was fed with production media (glucose 20 g/L) at a dilution rate of 0.24 h^{-1}. Control repeated batch fermentations with empty PVA particles were realised as described above with 10% w/v particles load and inoculation 5% (v/v) in the 1st batch fermentation. Ten consecutive batch fermentations were realised.

2.5. Analytics and calculations

The biomass concentration (OD) was measured at 600 nm with a spectrophotometer (BioSpectometer®, Eppendorf, Germany). Glucose and acids were analysed by HPLC on a 250 × 8-mm column Polymer IEX H^+ form (Watrex, Czech Republic) at 32 °C, mobile phase 9 mM H_2SO_4 (0.7 mL/min), with a RI 2000 (refractive index) detector (Schambeck SFD, Germany). The volumetric productivity (g/L h) was calculated as the concentration of butyric acid produced (g/L) divided by the time of fermentation until the microorganism was active. Yield of butyric acid was calculated as gram of butyric acid produced per gram of sugar utilised, expressed as gram per gram.

3. Results and discussion

C. tyrobutyricum is a stable butyric acid producer, which was successfully applied to various processes: e.g., glucose and xylose utilisation by mutant strains [4], hydrogen production [14], extractive fermentation [9] and even *n*-butanol production by metabolically engineered strains [15]. The improvement of this fermentation process by immobilisation was already demonstrated [2,14,16–18]. However, the immobilisation to PVA hydrogel, which is available on an industrial scale, was quite challenging. Immobilisation to LentiKats® is an aerobic process which may result in excessive inhibition of immobilised anaerobic microorganism *C. tyrobutyricum*.

3.1. Free cell fermentations

The optimum pH for butyric acid production is a key factor for the yield and productivity of *C. tyrobutyricum*. Most of the effective productivities were reported in the range pH = 5 to 6.3 [19]. The most suitable pH for the fermentation process was therefore investigated in this range for 20 g/L of glucose, as a main substrate. Typical free cell fermentation process (Table 1) at pH = 5 was completed within 27.7 h, resulting in production of 9.8 g/L of butyric acid, which is a 94% yield of the theoretical amount according to Huang et al. (maximum theoretical yield 0.489 g/g, g of butyric acid per g of glucose) [1] and the process reached volumetric productivity of 0.35 g/L h (Table 1). Varying the pH of the process did not improve productivity and the product yield was even significantly reduced. The highest concentration of butyric acid (26.6 g/L) was reached in a batch system with initial glucose concentration of 75.2 g/L. However, the lower product yield (0.43 g/g) and high residual glucose did not make this fermentation an attractive option.