M. González-Pajuelo · J. C. Andrade · I. Vasconcelos

Production of 1,3-Propanediol by *Clostridium butyricum* VPI 3266 in continuous cultures with high yield and productivity

Abstract The effects of dilution rate and substrate feed concentration on continuous glycerol fermentation by *Clostridium butyricum* VPI 3266, a natural 1,3-propanediol producer, were evaluated in this work. A high and constant 1,3-propanediol yield (around 0.65 mol/mol), close to the theoretical value, was obtained irrespective of substrate feed concentration or dilution rate. Improvement of 1,3-propanediol volumetric productivity was achieved by increasing the dilution rate, at a fixed feed substrate concentration of 30, 60 or 70 g l^{-1}. Higher 1,3-propanediol final concentrations and volumetric productivities were also obtained when glycerol feed concentration was increased from 30 to 60 g l^{-1}, at $D = 0.05$–0.3 h^{-1}, and from 60–70 g l^{-1}, at $D = 0.05$ and 0.1 h^{-1} · 30 g l^{-1} of 1,3-propanediol and the highest reported value of productivity, 10.3 g l^{-1} h^{-1}, was achieved at $D = 0.30$ h^{-1} and 60 g l^{-1} of feed glycerol. A switch to an acetate/butyrate ratio higher than one was observed for 60 g l^{-1} of feed glycerol and a dilution rate higher than 0.10 h^{-1}; moreover, at $D = 0.30$ h^{-1} 3-hydroxypropionaldehyde accumulation was observed for the first time in the fermentation broth of *C. butyricum*.

Keywords *Clostridium butyricum* · 1,3-propanediol · Continuous cultures · 3-hydroxypropionaldehyde

Introduction

1,3-propanediol is a versatile degradable intermediate compound for the synthesis of heterocycles and a monomer for the production of polymers, such as polyesters and polyurethanes. The classic route to produce this monomer is the chemical process from acrolein, a very harmful reagent. As an alternative, it has been shown that some bacteria are able to produce 1,3-propanediol from glycerol [2, 7–9, 15, 16]. The recent development of PTT, a new polyester based on 1,3-propanediol and terephtalic acid, has increased the number of studies on microbial conversion of glycerol to 1,3-propanediol [3]. Yield, final product concentration and volumetric productivity are the main issues for the feasibility of an industrial production process. A high final product concentration in the effluent turns the down-stream separation process less expensive and, at the same time, contributes to achieve a high volumetric productivity [3]. *Clostridium butyricum* VPI 3266 was described as probably the best natural 1,3-propanediol producer since, unlike other microorganisms, production of 1,3-propanediol by this strain is not a B_{12}-vitamin dependent process, which is clearly an economical advantage for an industrial application [13]. Furthermore, production of 1,3-propanediol by this strain using a synthetic medium and low-price raw glycerol has been recently reported [4]. Glycerol is metabolised by *C. butyricum* following two pathways. One pathway leads to glycerol oxidation to dihydroxyacetone (DHA) by a NAD^+ -dependent glycerol dehydrogenase, followed by DHA phosphorilation to DHA-phosphate (DHAP) by a DHA kinase; DHAP enters the glycolytic pathway. In the other pathway glycerol is dehydrated to 3-hydroxipropionaldehyde (3-HPA) via a glycerol dehydratase; 3-HPA is then reduced to 1,3-propanediol by a 1,3-propanediol dehydrogenase with NADH consumption, leading to a theoretical yield of 0.70 mol/mol in conditions of no butyrate and no H_2 formation [2, 3, 18].

Since an industrial utilisation of *C. butyricum* VPI 3266 may be suggested, continuous glycerol fermentations of this strain have been carried out in the present work in order to increase 1,3-propanediol concentration and productivity by manipulating dilution rate and glycerol feed concentration.

M. González-Pajuelo · J. C. Andrade · I. Vasconcelos (✉)
Escola Superior de Biotecnologia,
Universidade Católica Portuguesa,
Rua Dr. António Bernardino de Almeida,
4200 Porto, Portugal
E-mail: ivasc@esb.ucp.pt
Tel.: +351-225-580049
Fax: +351-225-090351

Materials and methods

Organism

Clostridium butyricum VPI 3266 (Virginia Polytechnic Institute Culture Collection, Blacksburg, Va.) was maintained in the synthetic medium described below, in spore form, at −20°C. This strain is available from other culture collections as *C. butyricum* NCIMB 7423 (National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland, United Kingdom) and *C. butyricum* CECT 361 (Colección Española de Cultivos Tipo; Universitat de Valencia, Valencia, Spain).

Culture media

The synthetic medium used in the experiments contained per litre of deionized water: glycerol, 30–80 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $CoCl_2.6H_2O$, 0.01 g; $FeSO_4.7H_2O$, 0.01 g; biotin, 0.04 mg; *p*-aminobenzoic acid, 8 mg; acetic acid, 2 g. The medium pH was adjusted to 6.5 with 6N NH_4OH. The feed medium for continuous cultures was the synthetic medium described above, without acetic acid, and with 0.028 g l^{-1} of $FeSO_4.7H_2O$ (instead of 0.01 g l^{-1}), 1.5 g l^{-1} of NH_4Cl and 1 ml of $H_2SO_4$17.4 M; medium pH was not adjusted in this case.

Continuous cultures conditions

Continuous cultures were performed in a 2-l bioreactor (Biostat MD; Braun, Melsungen, Germany), with a working volume of 1,250 ml, and in a 400 ml glass bioreactor, with a working volume of 300 ml. The culture was stirred at 200 rpm, temperature was set to 35°C and pH was maintained constant by automatic addition of 6N NH_4OH. To create anaerobic conditions, the sterilised medium in the vessel was flushed with sterile O_2-free nitrogen until room temperature was attained. A growing culture taken at the early exponential growth phase was used as inoculum (10% v/v). The culture was first grown batchwise and continuous feeding was started once the exponential growth phase was reached. After sterilisation, the feed medium was sparged with sterile O_2-free nitrogen, until room temperature was reached. During the experiments, the feed medium was maintained under nitrogen at 30 mbar, to avoid O_2 entry. All tubing was made of butyl rubber and the bioreactor gas outlet was protected with a pyrogallol arrangement [17].

Analytical procedures

Cell concentration was measured turbidometrically, at 620 nm, and correlated with cell dry weight determined directly. Glycerol, 1,3-propanediol, ethanol and acetic, butyric and lactic acid concentrations were determined by HPLC (System Gold; Beckman, Fullerton, CA, USA). Separation was performed on a Biorad Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Richmond, CA, USA) and detection was achieved by refractive index. Operating conditions were as follows: mobile phase, sulphuric acid 0.5 mM; flow rate, 0.5 ml/min; temperature, 30°C. A qualitative HPLC analysis of 3-hydroxypropionaldehyde in fermentation broth was also performed in the conditions described. As 3-HPA is not commercially available, it was chemically synthesised following the method described by Hall and Stern [5].

1,3-propanediol volumetric productivity ($Q_{1,3\text{-propanediol}}$) and specific 1,3-propanediol formation or glycerol consumption rate ($q_{1,3\text{-propanediol or glycerol}}$) in chemostat cultures were calculated as $Q_{1,3\text{-propanediol}} = C_{1,3\text{-propanediol}} \times D$ and $q_{1,3\text{-propanediol or glycerol}} = C_{1,3\text{-propanediol or glycerol}} \times D/X$ respectively, where $C_{1,3\text{-propanediol}}$ is the 1,3-propanediol mass concentration, C_{glycerol} is the consumed glycerol mass concentration, D is the dilution rate of the chemostat and X is the cell mass concentration.

Results

Effect of dilution rate on continuous 1,3-propanediol production

Dilution rate was increased from 0.05 to 0.50 h^{-1} in chemostat cultures of *C butyricum* VPI 3266, with a constant glycerol feed of 30 g l^{-1}. 1,3-propanediol was the major fermentation end-product, attaining 15 g l^{-1} (Fig. 1a), and acetate and butyrate were the main by-products. No ethanol or lactate, other possible fermentation products, were synthesised. Residual glycerol exceeded 1 g l^{-1} only when dilution rate increased above 0.3 h^{-1} (Fig. 1b). At higher dilution rate of 0.5 h^{-1}, residual glycerol had reached 12.5 g l^{-1} and severe decreases in biomass (0.2 g l^{-1}) and 1,3-propanediol (8.1 g l^{-1}) were observed (data not shown). Up to a dilution rate of 0.40 h^{-1}, 1,3-propanediol concentration was constant, leading to an increase in the volumetric productivity, with a maximum value of 5.5 g l^{-1} h^{-1} (Fig. 2). For every D value, conversion of glycerol into 1,3-propanediol was between 0.60 and 0.68 mol 1,3-propanediol/mol glycerol consumed (Fig. 1a), indicating that the carbon flux through the 1,3-propanediol pathway is under a strong regulation.

Effect of glycerol feed concentration on continuous 1,3-propanediol production

The effect of glycerol feed concentration on the fermentation pattern of *C. butyricum* VPI 3266 in chemostat was also studied. Feed glycerol concentration varied from 30 to 70 g l^{-1} at different dilution rates (0.05–

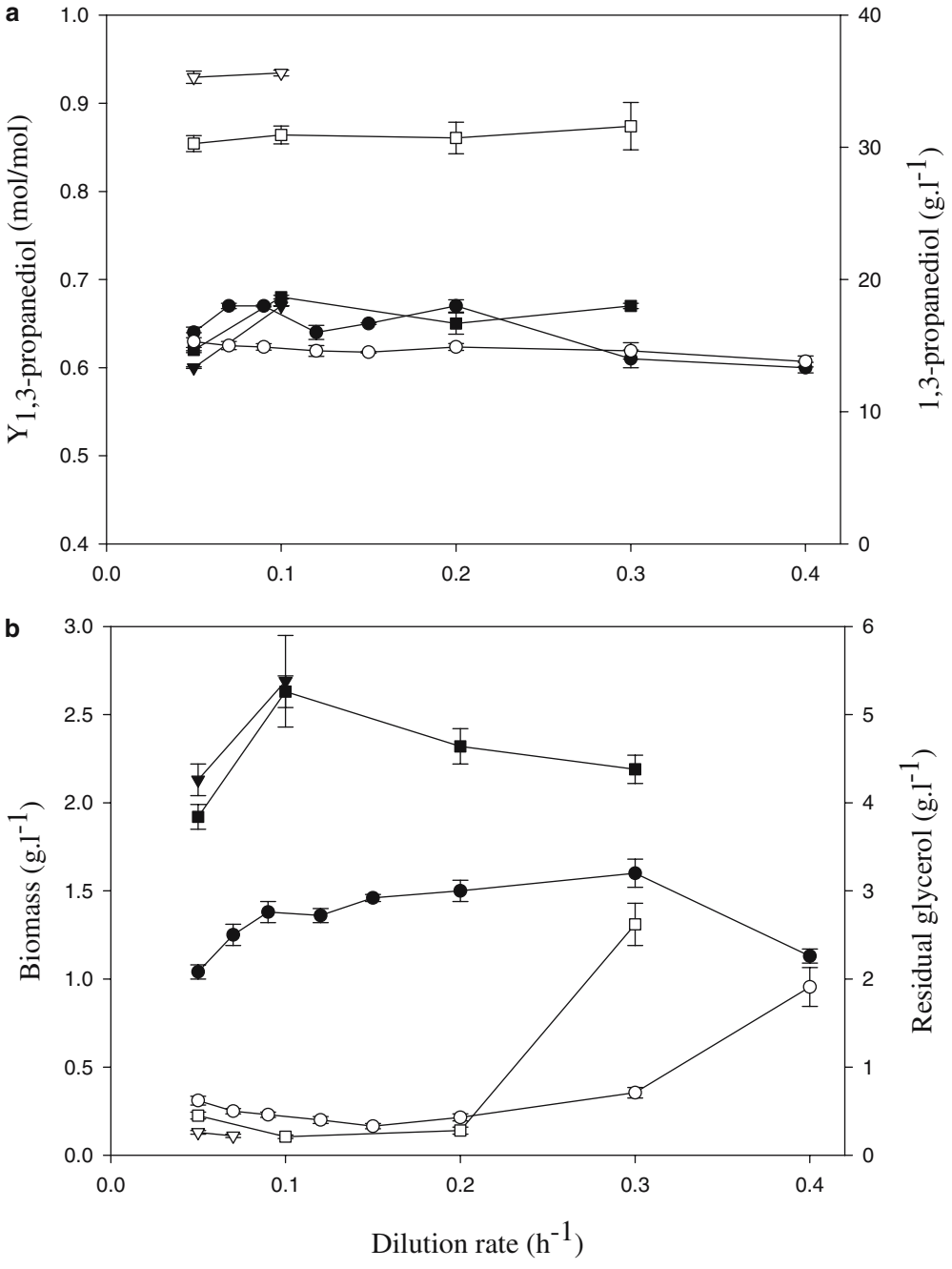

Fig. 1 Influence of glycerol feed concentration and dilution rate on a- 1,3-propanediol molar yield (*solid symbols*) and 1,3-propanediol concentration (*open symbols*) and on b- biomass concentration (*solid symbols*) and residual glycerol (*open symbols*) in continuous cultures of *C. butyricum* VPI 3266 (pH 6.5, 35°C). *circle*, *filled circle* 30 g l^{-1} of feed glycerol; *square*, *filled square* 60 g l^{-1} of feed glycerol; *inverted triangle*, *closed inverted triangle* 70 g l^{-1} of feed glycerol. *Vertical bars* represent standard deviation values

0.30 h^{-1}). However, when 70 g l^{-1} of glycerol were fed at a dilution rate of 0.20 h^{-1} washout of the reactor occurred.

For every dilution rate, an increase in glycerol feed concentration led to an increase in 1,3-propanediol concentration, which achieved 35 g l^{-1} for 70 g l^{-1} of feed glycerol, and 30 g l^{-1} for 60 g l^{-1} of feed glycerol (Fig. 1a). 1,3-propanediol yield was not affected by increasing glycerol feed concentration and was always around 0.65 mol 1,3-propanediol/mol glycerol consumed. Therefore, an improvement of volumetric productivity was observed with increasing dilution rate or glycerol feed concentration (Fig. 2). The highest volumetric productivity value, 10.3 g l^{-1} h^{-1}, was obtained at a dilution rate of 0.30 h^{-1} with 60 g l^{-1} of feed glycerol. Biomass concentration was also affected by glycerol feed concentration, since up to a two fold increase was achieved when substrate concentration was changed from 30 to 60 and 70 g l^{-1} (Fig. 1b). For every glycerol feed concentration, a decrease of biomass concentration was associated with an increase of residual glycerol in fermentation broth.

For a 30 g l^{-1} glycerol feed, acetate and butyrate concentrations were constant, although the dilution rate increased; furthermore, butyrate concentration was always higher than acetate concentration (Fig. 3). How-

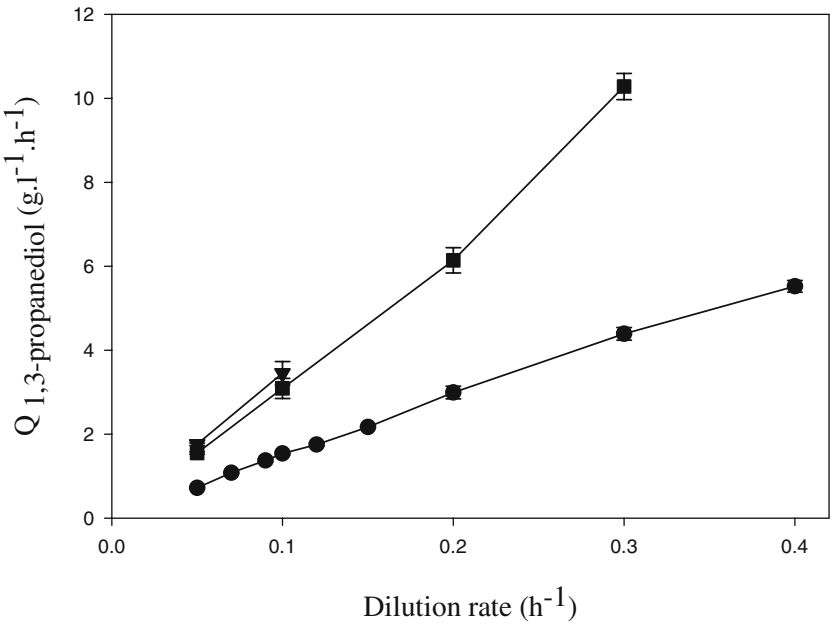

Fig. 2 Influence of glycerol feed concentration and dilution rate on 1,3-propanediol volumetric productivity in continuous cultures of *C. butyricum* VPI 3266 (pH 6.5, 35°C). *Filled circle* 30 g l^{-1} of feed glycerol, *filled square* 60 g l^{-1} of feed glycerol, *inverted triangle* 70 g l^{-1} of feed glycerol. *Vertical bars* represent standard deviation values

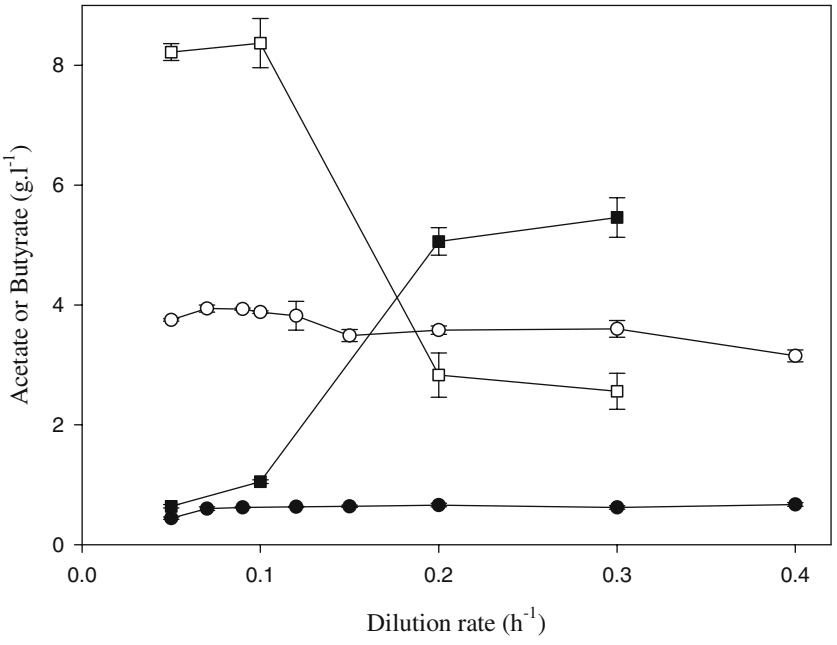

Fig. 3 Influence of glycerol feed concentration and dilution rate on acetate (*solid symbols*) and butyrate (*open symbols*) production in continuous cultures of *C. butyricum* VPI 3266 (pH 6.5, 35°C). *circle, filled circle* 30 g l^{-1} of feed glycerol; *square, filled square* 60 g l^{-1} of feed glycerol. *Vertical bars* represent standard deviation values

ever, a switch in the acetate/butyrate ratio occurred for a glycerol feed of 60 g l^{-1}, at dilution rates of 0.20 and 0.30 h^{-1}, indicating a change in the metabolic carbon flux. At $D = 0.30$ h^{-1}, 3-HPA was detected for the first time in the fermentation broth of *C. butyricum*, although quantification was not possible, as the pure compound was not available.

Discussion

Clostridium butyricum VPI 3266 has been described as the best candidate for 1,3-propanediol production, since it was demonstrated that this strain carries out a B_{12}-vitamin independent process [13]. It has also been shown that this strain is able to produce 30 g l^{-1} of 1,3-propanediol, with a molar yield of 0.61, in continuous cultures using a synthetic medium and raw glycerol [4]. However, there are no reports in literature establishing high substrate consumption values for high growth rate continuous cultures, as pointed out by Biebl et al. [3]. In chemostat cultures with a feed glycerol concentration of 10 g l^{-1}, *C. butyricum* DSM 5431 could grow up to a maximum growth rate of 0.26 h^{-1}, with exhaustive glycerol consumption [1]; biomass decreased markedly at 0.30 h^{-1}, corresponding to glycerol accumulation.

Klebsiella pneumoniae has also been considered a good 1,3-propanediol producer. Menzel et al. [9] showed that this microorganism was able to produce 35.2–48.5 g l^{-1} of 1,3-propanediol in continuous fermentation and volumetric productivities of 4.9–8.8 g l^{-1} h^{-1} were obtained at dilution rates between 0.10 and 0.25 h^{-1}; however, in these experiments more than 45 g l^{-1} of residual glycerol were observed in the fermentation broth. Moreover, *K. pneumoniae* is an opportunist pathogen microorganism and special care must be taken when used in fermentation. In another attempt to increase the volumetric productivity, *C. butyricum* DSM 5431 was cultivated in a cell recycle bioreactor with 55 g l^{-1} of feed glycerol [12]. In this case, a 1,3-propanediol concentration of 26.5 g l^{-1} was maintained up to a dilution rate of 0.50 h^{-1}, which corresponds to a high volumetric productivity (13.3 g l^{-1} h^{-1}). Nevertheless, this process could only be run for short time periods as the microfiltration membrane was prone to clogging. Other bacteria genus have been used for 1,3-propanediol production. Pflugmacher and Gottschalk [11] developed an immobilised cell reactor for 1,3-propanediol production by *Citrobacter freundii*. However, 10 g l^{-1} of residual glycerol were observed and only 18.7 g l^{-1} of 1,3-propanediol were produced with 60 g l^{-1} of feed glycerol and $D = 0.30$ h^{-1}. These results lead to a relatively low 1,3-propanediol yield (0.46 mol/mol) and a poor productivity (5.6 g l^{-1} h^{-1}). Hartlep et al. [6] reported a two step-process for 1,3-propanediol production, wherein glucose was first converted to glycerol by a recombinant *Escherichia coli* strain; in a second stage, glycerol was converted to 1,3-propanediol by *K. pneumoniae*. A fed-batch cultivation under limiting glucose supply resulted in a production of 14 g l^{-1} of 1,3-propanediol in the second stage, leading to a productivity of 2 g l^{-1} h^{-1}. The 1,3-propanediol productivity observed in the present work, 10.3 g l^{-1} h^{-1}, is the highest value reported for chemostat cultures.

The switch in acetate/butyrate ratio observed in this work was reported before for *C. butyricum* DSM 5431 growing in glycerol [1]: whereas at D values from 0.05 to 0.26 h^{-1} cells produced more butyrate than acetate, at a D value of 0.30 h^{-1} a switch was observed and the cells produced more acetate than butyrate. *C. tyrobutyricum* grown on glucose also showed a decrease of the selectivity for butyrate with an increase in glucose concentration [10]; this decrease was observed at both $D = 0.10$ h^{-1} and $D = 0.20$ h^{-1}, but was faster at $D = 0.20$ h^{-1}. Up to now, accumulation of 3-HPA, described as a toxic compound to the cells [1], was never reported for *C. butyricum*. In this work, 3-HPA was detected in fermentation broth from chemostat cultures with 60 g l^{-1} of feed glycerol at a dilution rate of 0.30 h^{-1}, when the acetate/butyrate ratio was higher than one. Formation of butyrate is redox-neutral, but acetate synthesis generates NADH excess, which could be used for the production of 1,3-propanediol from 3-HPA. The 1,3-propanediol yield observed in this case, 0.68 mol/mol, is very close to the theoretical maximum yield (0.70 mol/mol) calculated by Zeng [18] in conditions of no butyrate and no H_2 formation. The strain VPI 3266 is known to produce no molecular H_2 when grown on glycerol as the sole carbon and energy source [14]. Therefore, the inversion of the acetate/butyrate rate may be a mechanism to avoid accumulation 3-HPA by increasing NADH availability.

In this work it was shown that *C. butyricum* VPI 3266 is able to produce up to 30 g l^{-1} of 1,3-propanediol in continuous cultures from 60 g l^{-1} of feed glycerol, at high dilution rate, leading to a volumetric productivity of 10.3 g l^{-1} h^{-1}. This value is the highest ever reported for a chemostat culture of *C. butyricum*. A constant propanediol yield, close to the theoretical value, was also obtained in this work, irrespective of substrate concentration or dilution rate. Since an economic production of 1,3-propanediol from glycerol requires high final concentration and productivity and considering that 1,3-propanediol production by *C. butyricum* VPI 3266 is a B_{12}-vitamin independent process, this strain seems to be the best natural candidate for a 1,3-propanediol industrial process.

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