J Ind Microbiol Biotechnol (2004) 31: 442–446
DOI 10.1007/s10295-004-0168-z

ORIGINAL PAPER

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Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 using a synthetic medium and raw glycerol

Received: 3 November 2003 / Accepted: 12 August 2004 / Published online: 16 September 2004

Abstract Growth inhibition of *Clostridium butyricum* VPI 3266 by raw glycerol, obtained from the biodiesel production process, was evaluated. *C. butyricum* presents the same tolerance to raw and to commercial glycerol, when both are of similar grade, i.e. above 87% (w/v). A 39% increase of growth inhibition was observed in the presence of 100 g l^{-1} of a lower grade raw glycerol (65% w/v). Furthermore, 1,3-propanediol production from two raw glycerol types (65% w/v and 92% w/v), without any prior purification, was observed in batch and continuous cultures, on a synthetic medium. No significant differences were found in *C. butyricum* fermentation patterns on raw and commercial glycerol as the sole carbon source. In every case, 1,3-propanediol yield was around 0.60 mol/mol glycerol consumed.

Keywords *Clostridium butyricum* · Raw glycerol · 1,3-Propanediol

Introduction

1,3-Propanediol, an emerging bulk chemical, is a monomer for producing plastics with special properties, such as biodegradability, and is the base of the new polyester PTT [3, 16, 21]. 1,3-Propanediol is currently derived from acrolein, a petroleum derivative and harmful reagent, making the monomer production process relatively expensive and dangerous [3]. As a biological alternative to the chemical process, it has been shown that some bacteria are able to produce 1,3-propanediol from glycerol [2, 8–10, 17, 19], and *Clostridium butyricum* is probably the best producer described. One approach towards develop an economically viable process for the microbial production of 1,3-propanediol is to use low price renewable feedstock as substrates. In recent years, there has been an increasing interest in biodiesel as a less polluting, alternative fuel [4, 7, 11]. Biodiesel is produced by the transesterification of plant seed oils and yields glycerol as the main by-product (about 10% by weight). Since 50% of the entire cost of the microbial production of 1,3-propanediol is due to the price of raw materials [6], raw glycerol from biodiesel production processes may be an interesting renewable carbon source for *C. butyricum* if glycerol costs were significantly lower than current bulk prices. In this work, growth inhibition of *C. butyricum* VPI 3266 by different grades and types of glycerol were evaluated. Two raw glycerol types (65% w/v and 92% w/v), by-products of different biodiesel production processes, were used without any prior purification in order to evaluate their potential toxic effect on growth and 1,3-propanediol production by *C. butyricum* VPI 3266 in batch and continuous cultures with a synthetic medium.

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Materials and methods

Organism

Clostridium butyricum VPI 3266 (Virginia Polytechnic Institute Culture Collection, Blackburn, Va.) was maintained in the synthetic medium described below, with 0.2 M MOPS (3-[*N*]-morpholino]propanesulphonic acid), in spore form, at −20°C. This strain is available in other culture collections as *C. butyricum* NCIMB 7423 (National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland, UK) and *C. butyricum* CECT 361 (Colección Española de Cultivos Tipo; Universitat de Valencia, Valencia, Spain).

Culture media

Experiments to study growth inhibition were performed in reinforced clostridial medium (RCM, Oxoid). The synthetic medium used contained (per litre of deionised water): glycerol 30–60 g; KH_2PO_4 0.5 g; K_2HPO_4 0.5 g; $MgSO_4{\cdot}7H_2O$ 0.2 g; $CoCl_2{\cdot}6H_2O$ 0.01 g; $FeSO_4{\cdot}7H_2O$ 0.01 g; biotin, 0.04 mg; *p*-aminobenzoic acid 8 mg; acetic acid, 2 g. The pH of the medium was adjusted to 6.5 with 6 *N* NH_4OH. For batch cultures without pH regulation, MOPS was added to the synthetic medium (final concentration, 0.2 M). The feed medium for continuous cultures was the synthetic medium described above, without acetic acid, and with 0.028 g l^{-1} $FeSO_4{\cdot}7H_2O$ (instead of 0.01 g l^{-1}), 1.5 g l^{-1} NH_4Cl and 1 ml H_2SO_4 17.4 M; the pH of the medium was not adjusted in this case. Commercial glycerol, a purified 87% (w/v) grade glycerin, or raw glycerol, a by-product of biodiesel production processes without any prior purification, were used as carbon source. Commercial glycerol was purchased from Riedel-de Haën (Seelze, Germany). Raw glycerol, from the transesterification process for biodiesel production using rapeseed oil was kindly supplied by Novance (Compiègne, France) and contained the following components (as provided by the supplier): glycerol, 65% (w/v); sodium salts, less than 5% (w/v); non-glycerol organic matter, less than 1% (w/v); metals, less than 1,000 ppm; heavy metals, less than 5 ppm. A second type of raw glycerol from a biodiesel production process, with a higher grade (92% w/v), was kindly provided by Diester Industrie (Grand Couronne, France). Commercial and raw glycerol grades were confirmed by HPLC analysis.

Batch fermentations without pH regulation

After preparation, the culture medium was boiled, distributed in 100 ml flasks and sparged with O_2-free nitrogen for 15 min. The flasks were then sealed with butyl rubber stoppers and autoclaved (121°C, 20 min). The media were inoculated (10% v/v) with a cell suspension in exponential growth phase. Cultures were grown at 35°C.

Continuous cultures

Continuous cultures were performed in a 2-l bioreactor (Biostat MD; Braun, Melsungen, Germany) with a working volume of 1,250 ml. To create anaerobic conditions, the sterilised synthetic medium in the vessel was flushed with sterile O_2-free nitrogen until room temperature was attained. After sterilisation, the feed medium was also sparged with sterile O_2-free nitrogen, until it reached room temperature. A growing culture on the synthetic medium with MOPS, 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. During the experiments, the feed medium was maintained under N_2 at 30 mbar (3,000 Pa), to avoid O_2 entry. All tubing was made of butyl rubber and the bioreactor gas outlet was protected with a pyrogallol arrangement [20]. The culture was stirred at 200 rpm, temperature was set to 35°C and pH was maintained constant by automatic addition of 6 *N* NH_4OH.

Measurement of growth inhibition

Growth inhibition was measured in cultures performed in tubes containing 9 ml RCM, with different glycerol concentrations (0, 20, 40, 65, 100 g l^{-1}), inoculated with 1 ml cells in early exponential growth phase and incubated at 35°C. For this study, commercial glycerol (87% w/v) and the two types of raw glycerol (65% w/v and 92% w/v) were tested. Experiments were performed in duplicate. Growth was monitored every 2 h by optical density (OD) measurement at 620 nm. The maximum specific growth rate (μ) of *C. butyricum* VPI 3266, on the different types and concentrations of glycerol, was determined from the slope of the least square regression lines of the logarithm of OD vs time data.

Inhibition (%) in each case was determined from the following equation:

$$\%\ \text{Inhibition} = (1 - \mu_i/\mu_0) \times 100$$

where, μ_i is the maximum specific growth rate in experiment i and μ_0 is the maximum specific growth rate of the control experiment (no glycerol added to the medium).

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, Calif.). Separation was performed on a Bio-Rad Aminex HPX-87H column (300 mm ×7.8 mm; Bio-Rad, Richmond, Calif.) 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 [20].

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

Results

Growth inhibition of *C. butyricum* VPI 3266 by different types of glycerol

The effect of two types of raw glycerol (65% w/v and 92% w/v) and of commercial glycerol (87% w/v) on the growth of *C. butyricum* VPI 3266 is presented in Fig. 1. No inhibitory effect was observed with 20 g l^{-1} of any kind of glycerol. The percentage of growth inhibition increased linearly with commercial and raw glycerol concentrations between 20 and 100 g l^{-1} . Up to 100 g l^{-1}, the percentages of growth inhibition by commercial and 92% (w/v) raw glycerol were very similar (0, 21, 41 and 62% of growth inhibition for glycerol concentrations of 20, 40, 60 and 100 g l^{-1}, respectively). The inhibition effect was more evident when 65% (w/v) raw glycerol was used; a growth inhibition of 86% was observed when the medium contained 100 g l^{-1} of this type of glycerol.

Batch cultures of *C. butyricum* VPI 3266 on a synthetic medium with raw glycerol

The ability of *C. butyricum* VPI 3266 to produce 1,3-propanediol from commercial and raw glycerol types was shown in batch cultures without pH regulation. Samples for product analysis were taken 24 h after inoculation, when the pH of the medium was between 5.0 and 5.2. The results in Table 1 show that 1,3-propanediol was the major measured fermentation end-product. Organic acids, such as acetic and lactic acid, were also produced; however, butyric acid, an organic acid usually synthesised by *C. butyricum*, could not be quantified from these experiments (as it could not be separated from MOPS by HPLC analysis).

Continuous cultures of *C. butyricum* VPI 3266 on a synthetic medium with raw glycerol

The possibility of producing 1,3-propanediol from raw glycerol by *C. butyricum* was also shown in continuous cultures. Commercial glycerol and the two types of raw glycerol (65% w/v and 92% w/v) were used as the sole carbon source at two different concentrations (30 and 60 g l^{-1}). The results are shown in Table 2.

1,3-Propanediol was the major fermentation end-product. Acetic and butyric acid were also synthesised. Lactic acid production was not observed in continuous cultures, in contrast to results obtained in batch fermentations. Glycerol consumption was around 100%, whether the glycerol concentration in the feed tank was 30 g l^{-1} or 60 g l^{-1} . In every case, 1,3-propanediol yield was around 0.60 mol 1,3-propanediol/mol glycerol consumed. The final 1,3-propanediol titre increased with the initial glycerol concentration, 30 g l^{-1} being obtained from 60 g l^{-1} of glycerol as carbon substrate. The fermentation patterns of *C. butyricum* grown on raw or commercial glycerol were similar.

Discussion

In this work, the inhibitory effect of raw glycerol, from biodiesel production processes, on the growth of *C. butyricum* VPI 3266 was shown. Petitdemange et al. [15]

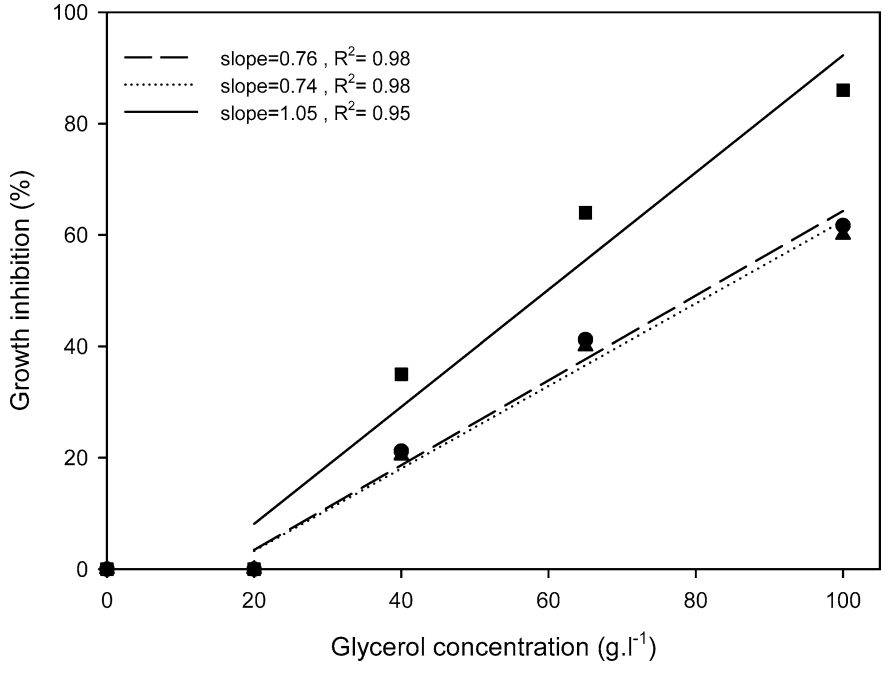

Fig. 1 Growth inhibition of *Clostridium butyricum* VPI 3266 by different types of glycerol. *Filled circles* Commercial glycerol (87% w/v), dashed regression line; *filled triangles* raw glycerol (92% w/v), dotted regression line, *filled squares* raw glycerol (65% w/v), solid regression line

Table 1 Batch cultures of *Clostridium butyricum* VPI 3266 on commercial and raw glycerol (synthetic medium without pH regulation, 35°C)

Type of glycerol (grade w/v)	Initial glycerol concentration (g l^{-1})	Glycerol consumed (%)	Products (mol/mol glycerol consumed)		
			1,3-Propanediol	Lactic acid	Acetic acid
Commercial (87%)	39.6	41	0.58	0.026	0.072
Raw (92%)	44.1	47	0.51	0.023	0.073
Raw (65%)	33.5	54	0.56	0.011	0.087

Table 2 Continuous cultures of *C. butyricum* VPI 3266 on different types of glycerol. Synthetic medium, dilution rate (D) = 0.1 h^{-1}, pH 6.5, 35°C. $Y_{1,3\text{-Pdiol}}$ 1,3-propanediol yield (mol/mol glycerol consumed), $Q_{1,3\text{-Pdiol}}$ 1,3-propanediol volumetric productivity, $q_{1,3\text{-Pdiol}}$ 1,3-propanediol specific formation rate

	Type of glycerol					
	Commercial glycerol (87% w/v)		Raw glycerol (92% w/v)		Raw glycerol (65% w/v)	
Feed glycerol (g l^{-1})	30.2	58.0	35.0	60.6	30.8	62.1
Residual glycerol (g l^{-1})	0.48	0.18	0.55	0.19	0.30	0.20
Biomass (g l^{-1})	1.15	2.18	1.33	2.71	1.09	2.46
Product concentration (g l^{-1})						
1,3-Propanediol	14.8	29.7	16.4	30.0	15.8	31.5
Acetate	0.47	2.50	0.65	2.42	0.62	2.38
Butyrate	3.88	5.31	4.50	5.61	4.09	5.61
$Y_{1,3\text{-Pdiol}}$ (mol/mol)	0.60	0.62	0.57	0.60	0.62	0.61
$Q_{1,3\text{-Pdiol}}$ (g l^{-1} h^{-1})	1.54	2.98	1.67	3.02	1.59	3.15
$q_{1,3\text{-Pdiol}}$ (g g dry weight^{-1} h^{-1})	1.32	1.36	1.25	1.11	1.46	1.28
Carbon recovery (%)[a]	97	97	96	96	96	96

[a]For calculation of carbon recovery, carbon dioxide concentration was estimated from end-product concentrations based on Papoutsakis [14]

showed the effect of commercial glycerol on the growth of *C. butyricum* DSM 5431. At 100 g l^{-1} commercial glycerol, growth was inhibited by 59%. This value is close to those obtained here with commercial and 92% raw glycerol. Although these authors also showed that some strains from bacterial culture collections were unable to grow on raw glycerol, in our experiments *C. butyricum* VPI 3266 was able to grow on two different types of raw glycerol. *C. butyricum* VPI 3266 seems to have the same tolerance to raw and to commercial glycerol when both have a similar grade (above 87%). An appreciably higher growth inhibition of *C. butyricum* VPI 3266 was observed when 65% raw glycerol was used. Due to its production process, raw glycerol contains other substances such as sodium salts and heavy metals in concentrations that might interfere with cell division.

The effect of raw glycerol on batch and continuous cultures was minimal, and it did not interfere with 1,3-propanediol production. In every case, the consumption of raw glycerol yielded about 0.60 mol 1,3-propanediol/mol glycerol consumed. These results are similar to those obtained by Saint-Amans et al. [18] with 30 g l^{-1} commercial glycerol, where 14 g l^{-1} 1,3-propanediol, 0.4 g l^{-1} acetate and 5.1 g l^{-1} butyrate were obtained in continuous fermentation, leading to a yield of 0.65 mol 1,3-propanediol/mol glycerol consumed. Petitdemange et al. [15] also showed that four of ten isolated strains of *C. butyricum* were able to grow on raw glycerol. One of these strains, *C. butyricum* E5, produced 58 g/l 1,3-propanediol, in fed-batch culture, from 109 g l^{-1} raw glycerol in a medium containing 1 g l^{-1} yeast extract. Another newly isolated strain of *C. butyricum* was also shown to consume raw glycerol (65% grade) in a medium containing 1 g l^{-1} yeast extract [13]; this strain, *C. butyricum* F2b, yielded around 0.66 mol 1,3-propanediol/mol glycerol consumed in a continuous culture fed with 90 g l^{-1} glycerol.

Recently, the use of raw glycerol has also been reported for lipid production by *Yarrowia lipolytica* [12] and for butanol production by *Clostridium acetobutylicum* ATCC 824 [1].

In the present work, the collection strain *C. butyricum* VPI 3266 was able to produce 1,3-propanediol from raw glycerol on a synthetic medium. It was shown that when raw glycerol fermentations were compared to a commercial glycerol fermentation, no significant differences were observed concerning final product concentration and 1,3-propanediol yield and volumetric productivity. These facts show that raw glycerol is an interesting substrate for 1,3-propanediol biological production. The economical viability of this process is dependent on raw

glycerol price and availability. As a carbon feedstock for fermentations, glycerol could be competitive if its price is not higher than US$ 0.3/kg [5].

Acknowledgements This work was financially supported by the European FAIR project (Contract No. FAIR-CT96-1912). M. González-Pajuelo was supported by PRAXIS XXI with a PhD grant (BD/16036/98).

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