Polish J. of Environ. Stud. Vol. 19, No. 6 (2010), 1249-1253

Original Research

Propionic Acid Production by *Propionibacterium freudenreichii* ssp. *shermanii* Using Crude Glycerol and Whey Lactose Industrial Wastes

Alicja Kośmider*, Agnieszka Drożdżyńska, Kalina Blaszka, Katarzyna Leja, Katarzyna Czaczyk

Department of Biotechnology and Food Microbiology, Faculty of Food Science and Nutrition, Poznań University of Life Sciences, Wojska Polskiego 48, 60-627 Poznań, Poland

Received: 9 December 2009
Accepted: 1 June 2010

Abstract

Our study shows the possibilities of industrial waste (glycerol and whey lactose) utilization in a microbiological way. In these investigations, propionic acid production by *Propionibacterium freudenreichii* ssp. *shermanii* was studied using pure glycerol, crude glycerol, and whey lactose as a carbon sources in culture media. Bacterial growth was carried out in batch fermentation at 20 g/L and 40 g/L initial concentration of carbon sources. Results of conducted experiments show that greater production of propionic acid was achieved with 4% carbon source content in culture media. The highest propionic acid accumulation was 22.57 g/L using whey lactose only in culture media, 24.47 g/L and 24.80 g/L using whey lactose with pure glycerol and whey lactose with crude glycerol as substrates, respectively. It was shown that simultaneous fermentation of whey lactose and pure or crude glycerol did not reduce propionic acid yield compared to whey alone as a substrate in culture media. Moreover, a replacement of 50% of whey lactose by crude or pure glycerol caused lower acetic acid accumulation. Reduction of this by-product should simplify extraction of propionic acid from fermentation broth in the distillation process. These results indicate that industrial wastes could be excellent substrates for propionic acid production.

Keywords: propionic acid, glycerol, whey lactose, batch fermentation

Introduction

Propionic acid is widely used as an antifungal in food [1], feed [2] and as an intermediate in the synthesis of herbicides, cellulose acetate – propionate plastics, solvents and pharmaceuticals [3, 4]. Chemical synthesis from petroleum feedstock is currently the main source of propionic acid. Fermentation processes utilizing low-value industrial by-products as carbon sources may become an attractive alternative [5].

Biodiesel production in the European Union was over 7.7 million tons in 2008, 35.7% more than in 2007 [6]. It is estimated that in 2010 biodiesel demand may increase to 10 million tons per year [7]. Currently, methyl or ethyl esters of fatty acids produced by transestrification from vegetable oil are the most widely used biodiesel fuels [8]. For every 3 moles of ethyl esters 1 mol of crude glycerol is produced [9]. If biodiesel production increases as predicted, the supply of crude glycerol will pose a serious problem, its utilization will become an urgent topic. Biodiesel producers have limited funds to refine this by-product and, since it contains methanol, it is considered a hazardous waste.

*e-mail: kosmider@up.poznan.pl

Obtaining tolerant microorganisms resistant to inhibitory components, such as salts and organic solvents present in crude glycerol, is an important challenge in low-grade crude glycerol utilization through biotechnological means [10].

Whey is a major co-product of the dairy industry with its volume increasing at about the same rate as milk production volume (>2% annually) [11]. Exhibiting a biochemical oxygen demand (BOD5) of 30,000-50,000 ppm and chemical oxygen demand (COD) of 60,000-80,000 ppm, it poses a great threat to the environment [12, 13]. Although several possibilities of whey utilization have been considered over the last 50 years, half of world whey production is not treated [11, 14].

Many substrates have been used for propionic fermentation, including glucose, xylose, maltose, sucrose, lactic acid, and whey lactose. Despite the investigations on glycerol metabolic pathways in propionic acid bacteria – carried out by de Vries et al. [15] – pure or crude glycerol have rarely been considered carbon sources in the discussed fermentation [4]. There are no reports on simultaneous fermentation of glycerol and whey lactose to propionic acid.

The aim of this study was to evaluate the advantages of using glycerol in mixtures with whey as attractive carbon sources for propionic fermentation and demonstrate an interesting possibility for industrial waste utilization.

Table 1. Variants of carbon sources in culture media.

Number of culture medium/ Type of substrate	Carbon source content* (g/L)	Carbon source (g/L)	
		Glycerol	Lactose
1	20	20	-
2		20	-
3		-	20
4		10	10
5		10	10
6	40	40	-
7		40	-
8		-	40
9		20	20
10		20	20

Substrates:
1, 6 – pure glycerol; 2, 7 – crude glycerol (from biodiesel production); 3, 8 – powdered whey; 4, 9 – powdered whey and pure glycerol; 5, 10 – powdered whey and crude glycerol.
*Carbon source content correspond to lactose and glycerol concentrations in appropriate substrates.

Experimental Procedures

Substrates

Powdered whey was obtained from P.P.H.U. LAKTOPOL, Suwałki, Poland. The whey contained 60% of lactose in dry matter, which determined the carbon source in culture media. Crude glycerol (partially refined, desalinated and devoided of methanol) was obtained as a by-product from biodiesel production (SG BODDINGS GmbH, Germany) and contained 86% of pure glycerol in dry matter, which determined the carbon source in culture media. Pure glycerol came from Chempur, Poland.

Microorganisms

Propionibacterium freudenreichii ssp. *shermanii* 1 came from our own collection in the Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences (Poznań, Poland). The strain was maintained on a liquid inoculum medium supplemented with 20% glycerol. Stock cultures for inoculation were stored in a refrigerator at -20ºC. For experiments, inoculum of propionic acid bacteria cultivated on a fresh inoculum medium was used.

Inoculum Medium

Inoculum cultures were prepared in 250 ml Erlenmeyer flasks containing, per liter: 20 g glucose (POCH, Poland), 5 g casamino acid (Difco, USA), 10 g casitone (Difco, USA), 1.76 g K_3PO_4 (POCH, Poland), 2.29 g $NaH_2PO_4 \cdot 2H_2O$ (POCH, Poland), 0.3 mg biotin (Merck, Germany), 4 mg calcium pantothenate (Koch-Light, England), 5 mg $FeSO_4 \cdot 7 H_2O$ (POCH, Poland), 2 mg $CoSO_4 \cdot 6H_2O$ (POCH, Poland), 10 mg $MnCl_2 \cdot 4 H_2O$ (POCH, Poland), 2 mg $ZnCl_2$ (POCH, Poland), and 0.2 g $MgCl_2 \cdot 6 H_2O$ (POCH, Poland). Medium was modified according to the method of Pędziwilk [16].

Culture Media

Ten growth media differing in the type of carbon source and its proportional content (2% and 4%) were used. The composition of basal medium for propionic acid production was the same as inoculum medium (without glucose). Carbon source variants applied in culture media are given in Table 1. All media were adjusted to 6.8 pH using 25% ammonia solution in water (Chempur, Poland) and sterilized at 100ºC for 25 min. in a Koch apparatus. In culture media where whey was used as a substrate, significant precipitation occurred during heating.

Fermentation

Propionic acid fermentation was carried out in 250 ml Erlenmeyer flasks containing 200 ml proper fermentation broth. The experimental culture was inoculated with 10% (v/v) inoculum of propionic acid bacteria grown for 72 h. The culture was incubated statically under relatively anaerobic conditions (carbon dioxide gassing) at 30ºC and pH was adjusted daily to 6.8 pH using 25% ammonia solution. Culture samples were obtained at 24, 48, 72, 120, 168, and 240 hours of process duration. All fermentation experiments were run in duplicate.

Table 2. The organic acids biosynthesis profile of *Propionibacterium freudenreichii* ssp. *shermanii* 1 using various carbon sources at a point of total substrate utilization or at the end of experiments.

Number of culture medium/ Type of substrates	Carbon source content	Time of total waste of substrate	Propionic acid	Acetic acid	Succinic acid	Dry weight	Yield of propionic acid
	(g/L)	(h)	(g/L)	(g/L)	(g/L)	(g/L)	(g/ g of substrate)
1	20	120	13.75^{ab}	0.23^{a}	3.47^{de}	10.73^{e}	0.68^{e}
2		120	11.16^{a}	0.99^{b}	3.47^{de}	10.62^{d}	0.56^{bcde}
3		120	10.02^{a}	2.67^{c}	1.96^{abc}	9.85^{c}	0.50^{bc}
4		120	10.11^{a}	1.33^{b}	1.30^{a}	9.59^{a}	0.50^{bc}
5		240	13.22^{ab}	2.65^{c}	2.49^{bc}	9.64^{a}	0.66^{de}
6	40	240*	17.21^{b}	0.75^{ab}	3.77^{e}	9.64^{a}	0.45^{b}
7		240*	9.37^{a}	0.82^{ab}	2.67^{bcd}	9.67^{ab}	0.25^{a}
8		120	22.57^{c}	5.95^{e}	1.90^{ab}	9.59^{a}	0.56^{bcde}
9		120	24.37^{c}	2.80^{c}	2.84^{cd}	9.96^{bc}	0.63^{cde}
10		240	24.80^{c}	3.62^{d}	5.61^{f}	9.60	0.60^{cde}

*incomplete substrate utilization.
Substrate:
1, 6 – pure glycerol; 2, 7 – crude glycerol (from biodiesel production); 3, 8 – powdered whey; 4, 9 – powdered whey and pure glycerol; 5, 10 – powdered whey and crude glycerol.
a-f – significantly different at α=0.05 (the experiments were done in two replicates).

Analytical Methods

After filtration (0.45 μm porosity membranes) and appropriate dilution of samples, substrates and fermentation end-products (propionic, acetic and succinic acid) were quantified by HPLC. The apparatus, a MERCK-HITACHI system comprised autosampler (model L-7250), pump (model L-7100), and refractive index detector (model L-7490). Analyses were performed isocratically at a flow rate of 0.6 ml/min at 60ºC, on Aminex HPX-87H, 300x7, 8 mm (BIO-RAD) column. 1 mM sulfuric acid as a mobile phase was used. External standards were used to identify peaks in chromatograms and peak area was measured to determine sample concentrations. Computer integration was used (Chromatography Data Station Software, MERCK-HITACHI).

Propionic acid bacteria concentration was determined by dry cell weight analysis. 20 ml samples of the fermentation broth were centrifuged (4,000 g) at 20ºC for 10 min. The received biomass was suspended in 5 ml of distilled water and dried to constant weight at 105ºC. In all cases where precipitates from whey were present in samples, the amount of biomass was quantified by measuring a difference between dry weight of cell paste (cells plus precipitates) and dry weight of appropriate culture medium before inoculation (after centrifugation and suspension of the precipitates in distilled water).

Statistical calculations were performed with STATISTICA 6.0. PL StatSoft, Inc. (2003) using Tukey's honest significance test at α=0.05.

Results

In this study the potential of pure glycerol, crude glycerol (a waste from biodiesel industry), and whey lactose (a dairy industry waste) use as a carbon sources in the biosynthesis of propionic acid by *P. freudenreichii* ssp. *shermanii* 1 was estimated.

Comparison of organic acid biosynthesis profiles using various carbon sources and its proportional content at a point of total substrates utilization is shown in Table 2.

Propionic Acid Fermentation Using Culture Media with 2% Carbon Source Content

Cell growth reached the stationary phase at 24 h of fermentation, irrespective of carbon source and its proportional content in culture media. At that time the maximal dry weight of 10.73 g/L was observed. The substrate was completely utilized at 120 h in most experimental variants. Only in the fermentation of crude glycerol with whey lactose did utilization of substrates last longer (240 h). Propionic acid production was the highest in culture media containing pure glycerol or crude glycerol with whey lactose as substrates (13.57 g/L and 13.22 g/L, respectively). Also, the highest propionic acid yield was achieved in the mentioned variants (0.68 g/g and 0.66 g/g, respectively). It was found that the addition of whey lactose to the culture media caused an increase of acetic acid production and decreased succinic acid production at the same time.

Of all fermentation variants with 2% carbon source, the process utilizing pure glycerol as the sole carbon source was the most effective. In that case the highest productivity of 0.11 g/L/h was achieved and a trace amount (0.23 g/L) of acetic acid was determined. The course of that fermentation is given in Fig. 1.

Propionic Acid Fermentation Using Culture Media with 4% Carbon Source Content

In most of the experiments, the increase of substrate concentration from 20 g/L to 40 g/L caused an extension of process duration (240 h or longer). Only the fermentations utilizing whey lactose and whey lactose with pure glycerol ended at 120 h, reaching maximal propionic acid concentration of 22.57 g/L and 24.37 g/L, respectively. In the mentioned variants propionic acid yield (0.56 g/g and 0.63 g/g, respectively) and productivity (0.18 g/L/h and 0.20 g/L/h, respectively) were the highest observed in the experiment. Similar values of propionic acid production (24.80 g/L) and propionic acid yield (0.60 g/g) were obtained in the fermentation of whey lactose with crude glycerol, but productivity was two-fold lower (0.05 g/L/h) in that variant compared to variants discussed above.

Results indicate that the mixture of whey lactose with pure glycerol was the most effective carbon source with 0.20 g/L/h productivity and significantly low acetic acid amount (2.30 g/L). The course of that fermentation is given in Fig. 2.

In all experiments, when whey lactose with pure or crude glycerol was used, irrespective of its proportional content in culture media, utilization of whey lactose was faster than glycerol.

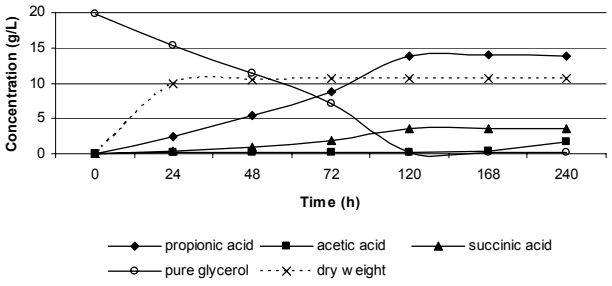

Fig. 1. The course of propionic acid fermentation processes with 2% carbon source content: pure glycerol.

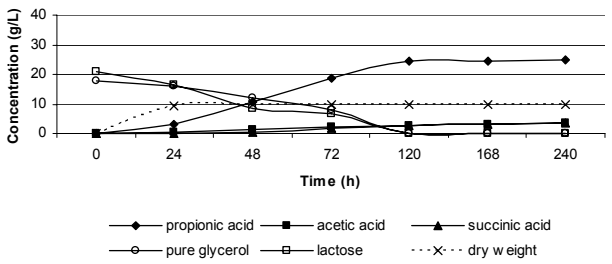

Fig. 2. The course of propionic acid fermentation processes with 4% carbon source content: pure glycerol and whey lactose.

Discussion

Results of conducted experiments indicate that culture media containing 40 g/L whey lactose and whey lactose mixed with crude or pure glycerol grant the highest propionic acid production by *P. freudenreichii* ssp. *shermanii* 1, 22.57g/L (120 h), 24.47 g/L (120 h), and 24.80 g/L (240 h), respectively. Barbirato et al. [3] report obtaining similar results with *P. acidipropionic*i grown on medium containing glycerol, 26.5 g/L after 140 h of fermentation. Goswami et al. [17] report propionic acid concentration of 20.75 g/L in 85 h batch fermentation utilizing lactose (47.7 g/L) as substrate. Shorter process duration is probably a result of different pH control approach. In hereby presented experiments pH was controlled to 6.8 in daily intervals, pH control was automatic in examples given above. It was revealed that propionibacteria growth is greatly inhibited in pH below 5.0; pH value of 6.5-7.0 is reported as optimal for propionic acid production [4, 18].

Utilizing glycerol as sole carbon source resulted in high propionic acid accumulation (17.21 g/L), it was lower, however, in comparison to accumulations achieved with other 4% carbon source media. Glycerol is generally metabolized by propionic acid bacteria at a lower rate than lactose. At the end of fermentation (240 h) incomplete utilization of glycerol was observed. A comparable observation was recorded by Coral et al. [19] in processes with glycerol and lactose as culture media carbon sources. At 130 h of batch fermentation lactose introduced with medium was totally utilized, while half of glycerol initial amount remained. A faster rate of lactate metabolism comes from the fact that it does not need to be degraded via a glycolytic pathway [19].

An increase of crude glycerol concentration from 20 g/L to 40 g/L in culture medium did not contribute to higher propionic acid production (11.16 g/L versus 9.37 g/L). This is probably a result of introducing higher concentrations of inhibitors and contaminants present in this waste product.

Moreover, acetic acid formation was decreased by 53% when whey lactose with pure glycerol were used, and 40% when whey lactose with crude glycerol were used, compared to fermentation with whey lactose alone. In all experiments during which glycerol was utilized as substrate, trace amounts of acetic acid were observed. A decrease in acetic acid production caused by glycerol addition was previously reported [3, 4], the fact that glycerol is highly reduced and the necessity to balance the intracellular redox potential being likely causes. As glycerol and propionic acid are of the same redox state, there is no need for conversion to a different metabolite [4]. Simplification of distillation procedure due to decreased acetic acid amount is another result of introducing glycerol as a carbon source in propionic acid fermentation [3, 4].

Conclusions

Batch fermentations utilizing pure glycerol, crude glycerol, and whey lactose as carbon sources at 20 g/L and 40 g/L concentrations were conducted. In the majority of

experiments, average final propionic acid concentrations of 10 g/L and 20 g/L, respectively, were achieved. Based on experimental data obtained, simultaneous fermentation of whey lactose in mixtures with pure or crude glycerol proved to be the most effective, yielding, respectively, 0.68 g/g and 0.60 g/g. Additionally, propionic acid/acetic acid ratio was 8.7:1 and 8.65:1 in above-mentioned variants, respectively, versus 3.8:1 when whey lactose only was used (with no difference in propionic acid yield). Fermentation duration with whey lactose and crude glycerol was twice the duration of the process with whey lactose and pure glycerol, which contributed to a twofold decrease in productivity.

A few studies on glycerol and whey lactose utilization to propionic acid by microbiological means have been reported [3, 4, 17, 20]. To the best of our knowledge, this is the first report concerning propionic acid fermentation with simultaneous usage of glycerol and whey lactose as carbon sources, and since glycerol became an industrial waste derived from biodiesel production, it is worthwhile to further investigate biotechnological production of value-added-products from waste mixtures.

Developing and testing feeding strategies, culture conditions and reactor volume to enhance microbial production of propionic acid will be topics of our future investigations.

References

1. RAZAVI-ROHANI S.M., GRIFFITHS M.W. Antifungal effects of sorbic acid and propionic acid at different pH and NaCl conditions. J. Food Safety. **19**, (2), 109, **1999**.
2. VALE M.M., MENTEN J.F.M., MORAIS S.C.D., BRAINER M.M.A. Mixture of formic and propionic acid as additives in broiler feeds. Sci. Agr. **61**, (4), 371, **2004**.
3. BARBIRATO F., CHEDAILLE D., BORIES A. Propionic acid fermentation of glycerol: comparison with conventional substrates. Appl. Microbiol. Biotechnol. **47**, 441, **1997**.
4. HIMMI E.H., BORIES A., BOUSSAID A., HASSANI L. Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermanii*. Appl. Microbiol. Biotechnol. **53**, 435, **2000**.
5. PLAYNE M.J. Propionic and butyric acids. In: Moo-Young M. (Ed) Comprehensive biotechnology, Vol **3**. Pergamon, New York, pp. 731-755, **1985**.
6. GACA J. Crude glycerol from biodiesel production - waste or valuable material? Czysta Energia. **11**, 34, **2006** [In Polish].
7. http://www.ebb-eu.org/stats.php
8. SENDZIKIENE E., MAKAREVICIENE V., JANULIS P. Oxidation stability of biodiesel fuel produced from fatty wastes. Polish J. of Environ. Stud. **16**, (1), 17, **2007**.
9. KARINEN R.S., KRAUSE A.O.I. New biocomponents from glycerol. Appl. Catal. A. Gen. **306**, 128, **2006**.
10. CHOI, WON J. Glycerol-based biorefinery for fuels and chemicals. Recent Patents on Biotechnology. **2**, (3), 173, **2008**.
11. SMITHERS G.W. Whey and whey proteins- From 'guter-to-gold'. Int. Dairy J. **18**, 695, **2008**.
12. BULLERMAN L.B., BERRY E.C. Use of cheese whey for vitamin B_{12} production. Appl. Microbiol. **14**, 353, **1966**.
13. SISO M.I.G. The biotechnological utilization of cheese whey. A review. Biores. Technol. **57**, 1, **1996**.
14. SIENKIEWICZ T., RIEDEL C.L. Whey and whey utilization: Possibilities for utilization in agriculture and foodstuffs production. Gelsenkirchen-Buer, Berlin, Germany Verlag, Th. Mann, **1990**.
15. DE VRIES W., VAN WYCK-KAPTEYN W.M., STOUTHAMER A.H. Generation of ATP during cytochrome-linked anaerobic electron transport in propionic acid bacteria. J. Gen. Microbiol. **76**, 31, **1973**.
16. PĘDZIWILK F. A simple plating method for the isolation and enumeration of *Propionibacteria*. Acta Alim. Pol. **25**, 127, **1975**.
17. GOSWAMI V., SRIVASTAVA A.K. Fed-batch propionic acid production by *Propionibacterium acidipropionici*. Biochem. Eng. J. **4**, 121, **2000**.
18. HSU S.T., YANG S.T. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*: effect of pH. Biotechnol. Bioeng. **38**, 571, **1991**.
19. CORAL J., KARP S.G., DE SOUZA VANDENBERGHE L.P., PARADA J.L., PANDEY A., SOCCOL C.R. Batch fermentation model of propionic acid production by *Propionibacterium acidipropionici* in different carbon sources. App. Biochem. Biotechnol. **151**, 333, **2008**.
20. CHAMPAGNE C.P., BAILLARGEON-COTE C., GOULET J. Whey fermentation by immobilized cells of *Propionibacterium shermanii.* J. Appl. Bacteriol. **66**, 175, **1989**.