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The methane fermentation medium as an attractive source of bacteria from genus *Clostridium* capable of converting glycerol into 1,3-propylene glycol

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Abstract

Microorganisms that efficiently conduct several metabolic processes can be found among the microflora that colonizes the natural environment. The aim of this study was to select non-pathogenic cultures of bacteria from the genus *Clostridium* which capable of converting glycerol into 1,3-propylene glycol from the glycerol-supplemented methane fermentation medium. Moreover, analyses was performed in which best isolates were tested in terms of 1,3-propylene glycol production using both pure and waste glycerol. Trials in a microreactor scale were performed for the best strains identified. Thes trials were aimed at the determination of the effect of pH and temperature conditions on the effectiveness and duration of 1,3-PD synthesis. Metabolite contents in the culture fluid were assessed using HPLC. Species were identified by amplification of 16S rRNA coding sequence. A total of 67 isolates were identified, of which a vast majority was capable of synthesizing 1,3-PD. The best results were obtained for strain *C butyricum* DS30. Maximum concentration of 1,3-PD for this strain exceeded 38.56 g/l. At a temperature of 37°C and constant pH of 7.0, the efficiency of this strain fell within the range of 0.66-0.67 mol/mol glycerol in the case of laboratory and bioreactor scales using pure and waste glycerol.

Key words: *Clostridium* spp., isolation, 1,3-propylene glycol, glycerol, microflora of natural environment

Introduction

1,3-propylene glycol (1,3-PD) is one of the products of glycerol metabolism in many bacteria. Biotechnologically, it can be obtained in fermentations performed by *Clostridium* sp. (Saxena et al., 2009; Papanikolaou et al., 2008; Barbirato et al., 1998). Production of this metabolite is considered a possible way to utilize waste glycerol, a biodiesel byproduct, and obtain a value-added chemical (Zeng and Sabra, 2012). 1,3-PD finds many applications in the chemical industry as a monomer for polymerization, or an ingredient of solvents or lubricants (Barbirato et al., 1998).

1,3-propylene glycol–producing clostridia has been studied extensively for more than two decades (Biebl, 1991). To date several strains of this genus have become well-established in the field of research on biotechnological 1,3-PD biosynthesis. It seems that their metabolic capabilities are well-known and that their upper limits of 1,3-PD production efficiency have been reached. Most of these 1,3-propylene glycol producers belong to *C. butyricum* or *C. Diolis*. These species are considered best fit for the process, as the yield with which their members convert glycerol to 1,3-PD is better than that of other *Clostridium* spp. To a greater extent, the available reports cover *C. diolis* DSM 5430 (formerly *C. butyricum*) (Kretschmann et al., 1989), *C. butyricum* VPI 3266 (Saint-Amas et al., 1994), *C. butyricum* VPI 1718 (Chatzifragkou et al., 2011) and *C. butyricum* CNCM 1211 (Himmi, 1999).

Researchers aim at improving the overall outcome of the process by both genetic engineering and isolation of new strains (Abbad-Andaloussi et al., 1995; González-Pajuelo et al., 2006; Otte et al., 2009). The main objective of these efforts is to maximize the final 1,3-PD concentration obtained. For this purpose, strains showing high tolerance to elevated levels of toxic metabolites and

other compounds present in the fermentation broths are desired. Recently reports have been released that describe the process of 1,3-PD production using novel strains (Hirschmann et al., 2005, Gungormusler et al., 2010, Ringel et al., 2012, Leja et al., 2011). The study by Ringel et al. (2012) is an example of how the results of bacterial screening can be improved when the source of isolation and the screening strategy are carefully selected. A strain isolated from a habitat rich in fats performed exceptionally well (Wilkens et al., 2012).

In this paper we present the results of isolation of 1,3-PD that produces anaerobes from the environment of the anaerobic digestion of cattle manure. In order to enhance the probability of isolating a promising 1,3-PD producer, this environment was modified by the addition of crude glycerol at different concentrations.

Materials and methods

Media, microorganisms and culture conditions

The cattle manure used for the isolation experiments was obtained from the experimental farm of the Poznan University of Life Sciences (Przybroda, Poland). Three types of glycerol were used for media supplementations, i.e. pure glycerol (POCH, Poland), crude glycerin (M1) from BIOPALIWA S.A. (Malbork, Poland) containing 83.5% pure glycerol, and crude glycerin (S2) from BIOPAL (Borek Wielkopolski, Poland) containing 18.06% pure glycerol and 13.3% methanol.

All the microorganisms used for the experiments were isolated from an anaerobic digestion system presented below. The material obtained from the anaerobically digested manure was incubated on TSC-base agar plates (Biocorp, Poland) for 24h, after which the selected colonies were transferred into tubes containing RCM broth (Biocorp, Poland).

The medium for the fermentation tests was a modified Rich Medium (Himmi et al.,1999), composed of the following: 3.4 g K_2HPO_4, 1.3 g KH_2PO_4, 2.0 g $(NH_4)_2S0_4$, 0.2 g $MgSO_4$ x $7H_2O$, 2.0 g $CaCO_3$, 0.02 g $CaCl_2$ x $2H_2O$, 5 mg $FeCl_2$ x $7H_2O$, 2.0 g yeast extract and 2 ml trace element solution SL7 (Papanikolaou et al., 2000) per liter of demineralized water. In all the fermentation experiments, the medium contained 70 g/l glycerol, either pure or crude. When fermentation experiments were conducted in tubes, the medium was supplemented with 1% bromocresol purple solution at 2 ml/l, which allowed pH regulation with 20% NaOH. The pH was regulated according to color changes of this indicator.

In the fermentation experiments, with the use of bioreactors, the broth was continuously sparged with sterile nitrogen and stirred at 60 rpm. The pH was regulated at 7.0 with 20% NaOH. Vessels of 5000 ml total working volume were filled up to 2000 ml. The volume of 200 ml cultures cultivated on RCM was used as inoculum.

Except for the bioreactor fermentations, all the work was performed in an anaerobic workstation at 30°C.

Anaerobic digestion

The anaerobic digestion of cattle manure was performed using a system presented in Figure 1.

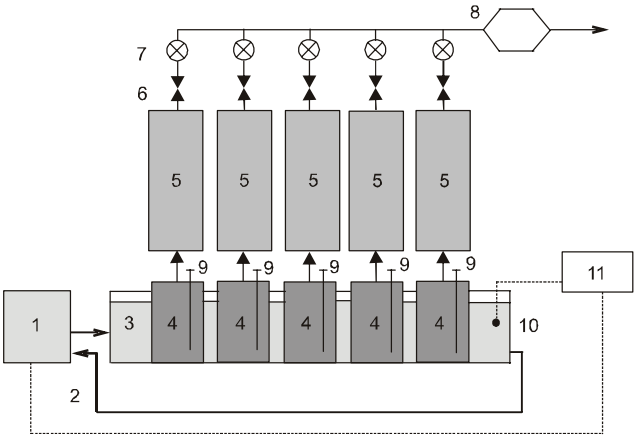

Fig. 1. The anaerobic digestion system: 1 – thermostated water heater, 2 – isolated water transfer tubes, 3 – heating jacket, 4 – biofermentors, 5 – biogas container with volumetric scale, 6 – cut-off valves, 7 – gas flow meters, 8 – gas analyzer, 9 – sample collection tubes, 10 – thermometer, 11 – control unit (Adamski et al., 2009)

The digestion process was carried out batch-wise with microorganisms natively present in cattle manure at 39°C until gas evolution stopped. No pH correction was used. The concentration of the manure was 70 g/l dry matter. The concentrations of the glycerin fraction (M1) of 0, 50, 100 and 150 g/l were used.

Analytical techniques

Chromatographic methods

Samples of fermentation broth were collected, centrifuged at 10.000 × *g* for 10 min and the cell-free supernatants were filtered through syringe filters prior to the HPLC analysis. A Hewlett Packard 1050 system equipped with a refractive index detector was used. Analyses were performed isocratically at a flow rate of

0.6 ml/min on an Aminex HPX-87H 300 × 7.8 column (BIO-RAD, CA, USA) thermostated at 65 °C. The mobile phase comprised 0.5 mN H_2SO_4. External standards were applied for identification and quantification.

Bacteria identification

Total DNA from bacteria was extracted using a Genomic Mini AX Bacteria Kit (A&A Biotechnology, Gdańsk, Poland) after initial incubation in 50 mg/ml lysozyme (Sigma) for 1 h at 37 °C. Sequences encoding small subunits of rRNA were amplified in PCR using SDBact0008aS20 and SUniv1492bA21 primers (Suau et al., 1999). PCR products were purified using a Clean-up Kit (A&A Biotechnology, Gdańsk, Poland) and sequenced at Genomed (Warszawa, Poland) with primers used for PCR and additionally for the inner sequence with the GTGCCAGCMGCCGCCCTAA primer. Obtained sequences were arranged into contigs and identified with the BLAST program of the GenBank database (Altschul et al., 1990).

Results

Methane fermentation of cattle manure supplemented with glycerol

The process of methane fermentation was not the main concern of this study and served mostly as an isolation source for new strains applicable to fermentative 1,3-propylene glycol production. However, its progress was monitored and differences were observed as crude glycerol was being introduced to cattle manure at increasing concentrations. The results of measurements taken throughout this procedure are provided in Table 1.

It is known that for efficient methanogenesis, the pH of the fermentation broth should be maintained at 6.8 to 7.5 (Ángel et al., 2009). This is often achieved by adding fresh manure. In the present experiment, the initial pH of the fermented material was 8.1. However, a rapid decrease in pH to acidic values was observed in the processes carried out with added glycerol. This pH drop was probably one of the factors that shifted the quantitative composition of the formed biogas towards carbon dioxide at the expense of methane. Even though an increased gas evolution was recorded, the total methane volume was reduced at higher glycerol additions in the fermented manure.

These observations may be attributed to the differences in the quantitative and qualitative composition of the consortium used for biogas production (Table 1). The introduction of glycerol may have triggered growth of bacteria capable of its utilization and, among them, 1,3-propylene glycol producers.

It needs to be emphasized that an increasing amount of information has recently been added in literature on combining methane fermentation with the process of 1,3-PD synthesis (Friedman and Zeng, 2008; Bizukojc et al., 2010). The application of microbial consortia aims at the removal of inhibitors (acetic and lactic acids, ethanol) from the fermentation medium. Moreover, processes based on the combination of fermentation bacteria, methane-producing bacteria and archaebacteria may prove to be an effective method of purifying the fermentation broth from metabolites other than 1,3-PD (after fermentation) and biogas production. Apart from the combination of 1,3-PD synthesis with methane production, a consortium of microorganisms capable of synthesizing hydrogen and 1,3-PD on the substrate with waste glycerol is an interesting option. Selembo et al. (2009), in their study, reached an efficiency of 0.31 mol/mol glycerol for hydrogen and 0.59-0.69 mol/mol glycerol for 1,3-PD.

Isolation and characterization of 1,3-propylene glycol producing anaerobes

Samples of the fermented manure were collected and used to isolate anaerobic bacteria. This resulted in the isolation of 67 strains. All the strains were tested for 1,3-propylene glycol production from glycerol, using a production medium containing 70 g/l pure glycerol. The isolation results are shown in Table 2.

From the pool of the isolated strains, the best 1,3-propylene glycol producers were selected for identification and further studies. Examples of the results from the isolation and strain selection procedure are given in Table 3.

The highest yield and final concentration of 1,3-PD was obtained in fermentations performed by strains isolated from the manure fermented with 10% (w/v) glycerol addition. The best strains, i.e. DS28, DS30 and DS52, were identified phylogenetically as *C. butyricum*. Strain DS30 fermented 70 g/l pure glycerol to 38.56 g/l 1,3-PD with the molar yield of 0.67. A similar glycerol fermentation yield, 0.69 mole of 1,3-PD per mole of glycerol, was obtained in batch fermentation with *C. butyricum* CNCM 1211 (Himmi et al., 1999). The exceptionally high molar yield, with which *C. butyricum* converts

Table 1. Results of biogas fermentation at different crude glycerol concentration

Crude glycerol concentration [% (w/v)]	Total fermentation time [days]	Final pH	Total biogas [dm^3]	Methane in biogas [dm^3]	CO_2 in biogas [dm^3]
0	44	8.0	12.8	7.1	2.5
5	43	6.1	16.4	5.5	> 7.2
10	21	6.0	27.9	2.2	> 13.2
15	13	5.7	30.1	1.64	> 23.76

Table 2. Results of isolation of anaerobic 1,3-PD producing strains from fermented cattle manure

Strain	Glycerol addition in the cattle manure [% (w/v)]	Final 1,3-PD concentration [g/l]	Glycerol utilization [%]	1,3-PD yield [$mol_{13\text{-}PD}/mol_{glycerol}$]
DS1	0	1.76	11	0.28
DS3	0	2.09	14	0.25
DS4	0	2.56	10	0.44
DS5	0	3.61	19	0.33
DS13	5	12.98	52	0.43
DS17	5	17.77	75	0.41
DS19	5	24.99	95	0.46
DS24	5	22.98	92	0.41
DS28	10	37.87	100	0.65
DS30	10	38.56	100	0.67
DS44	10	25.43	82	0.53
DS52	10	36.99	98	0.66
DS62	15	34.21	100	0.59
DS66	15	31.75	100	0.54
DS71	15	29.89	100	0.52
DS72	15	29.07	100	0.52

Table 3. Selected results of the isolation procedure

Glycerol addition in the cattle manure [% (w/v)]	Total number of isolated strains	Number of strains capable of 1,3-PD production
0	10	4
5	14	14
10	31	30
15	12	10
Totality	67	58

glycerol to 1,3-PD, seems to be the characteristic of this species.

Utilizing different *Clostridium* species, other authors did not obtain comparatively satisfying results. Taconi et al. (2009) reported achievement of a maximum 1,3-PD yield from glycerol of 0.5 mol/mol and a final 1,3-PD concentration of 7 g/l in a batch culture. Gungormusler et al. (2010) obtained 0.36 moles of 1,3-PD from

Table 4. Fermentation products of pure and crude glycerol by best isolates of bacteria from the species *C. butyricum*

Strain	1,3-PD [g/l]	Glycerol [g/l]	Butyric acid [g/l]	Acetic acid [g/l]
Pure glycerol				
C. butyricum DS28	37.34	0.00	3.84	1.43
C. butyricum DS30	38.56	0.00	2.12	0.51
C. butyricum DS52	36.99	0.91	2.55	1.98
Crude glycerol M1				
C. butyricum DS18	34.65	0.00	3.91	1.45
C. butyricum DS19	32.19	0.00	3.87	1.09
C. butyricum DS22	29.89	0.00	2.54	1.32
Crude glycerol S1				
C. butyricum DS18	0.54	15.54	0.07	0.05
C. butyricum DS19	1.01	15.76	0.09	0.01
C. butyricum DS22	1.06	16.43	0.04	0.1

Table 5. Products of glycerol fermentation by *C. butyricum* DS30 at pH 6.0-8.0

pH	Strain	Max concentration of 1,3-PD [g/l]	Max yield [$M_{1,3\text{-PD}}/M_{glicerol}$]	Butyrate/acetate [g/g]
6.0	*C. butyricum* DS30	12.02	0.37	0.24
6.5		35.14	0.61	0.48
7.0		38.05	0.67	0.44
7.5		24.85	0.53	0.33
8.0		0.03	0.00	0.00

1 mole of glycerol using the newly isolated *C. saccharobutylicum* NRRL B-643.

Analyses conducted at this stage showed that the methane fermentation medium supplemented with glycerol is a good source of isolates with a potential of commercial-scale importance.

Fermentation of pure and crude glycerol by selected isolates from the species C. butyricum

When industrial 1,3-PD production is discussed, crude glycerol, a by-product of biodiesel production, is considered the substrate of choice. This raw material, however, contains various substances that are inhibitory to the growth and metabolism of microorganisms in quantities depending on its purity. These substances include sodium salts, heavy metal ions, soaps, methanol and free fatty acids. For example, sodium ions inhibit the growth of *C. butyricum* at a concentration of 12 g/l, lead ions at 25 mg/l (Homann et al., 1990; Francis and Dodge, 1987) and nickel, contained in the glycerin fraction, also have inhibitory effects (Keeling and Cater, 1998).

For this reason, selected strains were tested in order to verify their ability to grow and synthesize 1,3-PD on the media containing crude glycerol. Two glycerin fractions from biodiesel production plants were utilized (M1 and S1). Fermentations carried out on pure glycerol were the controls in this experiment and the results are listed in Table 4. The highest concentration of 1,3-PD (38.56 g/l) was obtained in the fermentation with isolate DS30 using pure glycerol. In all the fermentations using pure glycerol, the final 1,3-PD titer was higher than in those using crude glycerol. However, considering the fact that the total pure glycerol concentration in these runs was lower than in those using pure glycerol, the results obtained with crude glycerol M1 were comparable. Additionally, the molar 1,3-PD yield from fermented glycerol did not vary significantly between fermentations perfor-

med on pure glycerol and M1. Crude glycerol S1 did not provide conditions suitable for any of the tested strains.

Available literature sources present an increasing number of reports on the synthesis of 1,3-PD from crude glycerol.

C. butyricum strain F2b was described as capable of producing 48 g/l of 1,3-PD (efficiency of 0.66 mol/mol) using crude glycerol (Papanikolaou and Aggelis, 2003). *C. butyricum* CNCM 1211 produced 63.4 g/l 1,3-PD from crude glycerol with an efficiency of 0.69 mol/mol (Colin et al., 2000). It needs to be stressed that high concentrations of 1,3-PD reported by other authors frequently result from the type of fermentation runs, i.e. fed-batch or continuous. Results showing that isolation of new strains can still improve the outcome of glycerol fermentation to 1,3-PD were recorded by Ringel et al. (2012). These authors isolated non-pathogenic *C. butyricum* strains AKR102a and AKR91b resistant to high concentrations of 1,3-PD, and at the same time capable of an effective conversion of waste glycerol (approx. 100 g/l 1,3-PD). However, is noteworthy that the fraction applied by the authors did not contain any considerable amounts of contaminations. Also several other authors investigated the effect of waste glycerol on the efficiency of 1,3-PD synthesis using the raw material containing maximum of 1% methanol (Rehman et al., 2008; Papanikolaou et al., 2000). In this study much lower concentrations of 1,3-PD were obtained for the S1 fraction, in which glycerol concentration was 18.01%. Moreover, the raw material contained approx. 14% methanol and 45% sodium soaps of fatty acids, which obviously had an adverse effect on the very low efficiency of the fermentation process.

C. butyricum strain DS30, which on pure glycerol and the M1 fraction provided the efficiency of 1,3-PD synthesis at a level comparable to the theoretical one, S1 fraction usage was not capable of producing 1,3-PD at more than 1 g. Literature sources contain many reports on the inhibitory effect of waste glycerol components on the growth and metabolism of microorganisms (González-Pajuelo et al., 2004; Rehman et al., 2008; Chatzifragkou et al., 2011; Ringel et al., 2012). In the investigated case, methanol, a dominant component of the glycerin fraction was the main inhibitor of fermentation. The solution to this problem is to separate methanol by distillation. However, it is a very costly solution. Possibly the application of a mixed culture capable of metabolizing glycerol and methanol would be a cost-effective solution. Strains capable of utilizing methanol have been discussed by many authors (Braun and Stolp, 1985; Mechichi et al., 1999). *Clostridium xylanovorans* isolated from the methanol fermentation medium may serve as an example in this respect (Mechichi et al., 1999).

The effect of temperature on productivity of C. butyricum strain DS30

In order to improve the efficiency of technological processes, particularly those based on the utilization of microorganisms, it is a key issue to determine optimal parameters of their culture. Temperature is one of these parameters. There are several published reports that describe the dependency between the efficiency of 1,3-PD synthesis with the application of *C. butyricum* and the temperature of the culture medium (Colin et al., 2000; Himmi et al., 1999; Barbirato et al., 1998).

Thus, in the next stage, the effect of temperature on the rate of 1,3-PD synthesis was investigated for the most promising isolate, i.e. *C. butyricum* DS30. Analyses were conducted in bioreactors, with the application of the optimal production broth, at a concentration of pure glycerol amounting to 70 g/l in six temperature variants (30, 32, 34, 36, 38, 40°C). Within the temperature range of 32-38°C the final concentration of 1,3-PD was similar, amounting to 34-37 g/l. The maximum efficiency was 0.67 mol/mol glycerol. However, an increase in temperature to 38°C resulted in an increase in 1,3-PD productivity from 0.47 to 1.44 g/l/h, and thus it reduced fermentation time to 30 h (Fig. 2).

Using the fed-batch culture at a temperature of 35°C for *C. butyricum* DSM5431, Reimann and Biebl (1996), obtained an efficiency of 1,3-PD synthesis at 0.62 mol/mol

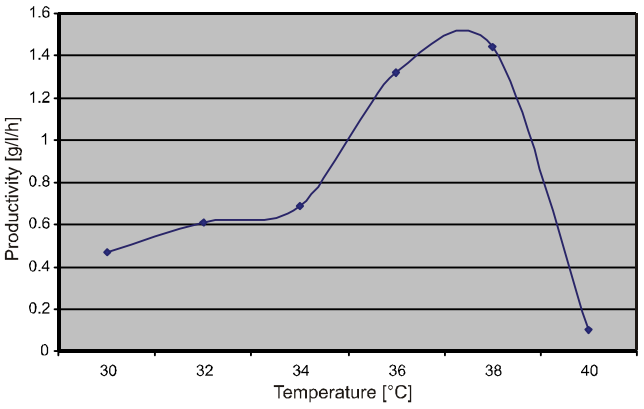

Fig. 2. Relationship between the temperature and the 1,3-PD productivity

and productivity of 2.4 g/l/h during the period of 20 h, while Himmi et al. (1999), applying the RM medium, at 37°C and the *C. butyricum* strain CNCM 1211 obtained a maximum productivity of 1.85 g/l/h and efficiency of 0.66 mol/mol. In turn, Barbirato et al. (1998), when investigating a *C. butyricum* strain capable of synthesizing 1,3-propylene glycol, after 9-hour fermentation, at 37°C obtained the efficiency of 0.62 mol/mol glycerol. The temperature of 40°C prevented the growth of bacteria and synthesis of 1,3-PD (0.04 g/l). Such a high temperature inhibits cell growth by inactivation of enzymes participating in glycolysis and the Krebs cycle, which, as a consequence, reduces or inhibits carbon metabolism. Moreover, an elevated temperature leads to the denaturation of enzymatic proteins (Hochachka, 1986).

The source of isolate origin provides a good estimation of the experimentally confirmed fact on the optimal temperature (36-38°C) of fermentation. Firstly, liquid manure came from breeding animals whose body temperature fell within the above-specified range. Secondly, in the secondary environment of isolates (methane fermentation), the temperature even exceeded the specified value, and therefore microorganisms that inhabited that medium seemed to have been adapted to the elevated temperature of the culture medium.

The effect of pH on efficiency of 1,3-propylene glycol synthesis by C. butyricum DS30

The pH value of the medium is an important parameter that influences the production of 1,3-propylene glycol in the fermentation process. This mechanism is related to the activity of enzymes and metabolic pathways. Optimal pH for microbial growth is highly varied and depends on the genus or even species affiliation. It needs to be stressed that by modifying the pH value of the medium the intensity of synthesis for specific metabolites can be influenced (Colin et al., 2001; Barbirato et al., 1998; Sattayasamitsathit et al., 2012). Thus the pH effect was analyzed in this study not only for the efficiency of 1,3-propanediol synthesis, but also for the production of organic acids for the best isolate, i.e. *C. butyricum* DS30. The fermentation process was run in a bioreactor with an infinitely variable pH adjustment (range of 6.0-8.0), while the concentration of (pure) glycerol in the production broth was 70 g/l. Results presented in graph 1 show that an increase in medium pH results in an increased production of 1,3-PD, but only to a certain value. The boundary value of pH for *C. butyricum* strain is 7.5. At pH of 8.0, the level of 1,3-PD did not exceed the concentration of 0.1 g/l. Within the range from 6.5 to 7.5, a complete utilization of glycerol occurred and the highest concentration of 1,3-PD was reached, amounting to 36.61 and 38.05 g/l. Similar conclusions were also reached by other authors. Biebl (1991) investigated the effect of pH on the efficiency and time of fermentation. Similar as in this study, the best results were found for pH of 6.5 and 7.0 (19.1 and 20.9 g/l of 1,3-PD from 50 g/l glycerol). A lower efficiency at pH 6.5, amounting to 0.54 g/g glycerol (with no infinitely variable adjustment of pH) and 0.58 g/g glycerol (with pH adjustment to 6.8) was obtained by Metsoviti et al. (2012). Barbirato et al. (1998) reported an almost complete utilization of glycerol at pH 6.6 and the maximum (although lower than that recorded in this study) concentration of 1,3-propylene glycol amounting to 247.6 mM (22.83 g/l). These authors investigated the pH range of 4.9-6.6. In another study, Biebl et al. (1992), described a pH range very similar to that found in this paper, i.e. pH 5.5-8, and determined the productivity of 1,3-PD as well as concentrations of butyric and acetic acids. The optimal pH value, ensuring the maintenance of the highest productivity, was 7.0. Similar to this study, at a pH above 7.5, the level of 1,3-PD dropped dramatically. The range of pH similar to that investigated within this study was described by Colin et al. (2001). At pH 7.0, these authors obtained the highest efficiency of 0.68 mol/mol glycerol.

A significant element in the microbial synthesis of 1,3-PD is associated with the profile of short-chain organic acids, particularly butyric and acetic acids. It is worth mentioning that the qualitative and quantitative characteristics of the end by-products depend on the species and fermentation conditions (Chatzifragkou, et al., 2011; Wilkens et al., 2012; Biebl and Spröer, 2002; Biebl, 2001). The pH value is an important parameter. A certain dependency was observed with an increase in pH. The higher the pH value of the fermentation medium, the higher the concentration of acetic acid and the higher the final concentration of 1,3-PD. Colin et al. (2001) published the results confirming the effect of acetate and butyrate on the efficiency of 1,3-PD synthesis in fed cultures of *C. butyricum* with an addition of either of these acids. When butyrate was added, its production in the cell was reduced and the efficiency of 1,3-PD synthesis increased. Similar studies were conducted by Heyen-

drix et al. (1991) on *C butyricum* and *C. pasteurianum.* In *C. butyricum* supplementation with acetate resulted in an increase in the synthesis of butyrate and a reduction of 1,3-PD production. Regulatory mechanisms need to be identified for the purpose of controlled management of glycerol metabolism. According to Zeng (1996), obtaining maximum efficiency of 1,3-PD production per 1 mol utilized glycerin is possible when the by-product, i.e. butyric acid, is formed only in the oxidation pathway and hydrogen is not be released. Moreover, the same author pointed towards the inhibitory effect of undissociated acids on microorganisms. This aspect seems to be particularly significant in fed-batch and continuous cultures (Zeng et al., 1994).

Conclusions

The methane fermentation medium may undoubtedly constitute a good source of microorganisms with a potentially commercial-scale applicability. Unfortunately, in this study, supplementation of cattle slurry (considered a very good substrate for methane fermentation) with glycerol had no advantageous effect on the course of fermentation or the production of biogas. This seems, however, an effect of rapid acidification of fermentation environment towards pH values far from the optimal range for methanogens. This problem is often coped with by neutralization resulting from the addition of fresh feedstock in semi-continuous or continuous process setups.

The approach used in this study, while not having beneficial impact on methane production, made it possible to obtain attractive isolates capable of efficient synthesis of 1,3-propylene glycol from glycerol. There still remains the issue of adaptation mechanisms in microorganisms. The environment of methane fermentation supplemented with glycerol proved to be advantageous for bacteria of the *Clostridium* genus. A more extensive analysis (at the level of transcriptome and proteome) could provide information concerning the influence of glycerol concentration on cellular regulatory and functional elements that are responsible for increased osmotic tolerance and the resulting ability to synthesize 1,3-propylene glycerol at high concentrations.

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