Research Paper

Biotechnological potential of *Clostridium butyricum* bacteria

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Abstract

In response to demand from industry for microorganisms with auspicious biotechnological potential, a worldwide interest has developed in bacteria and fungi isolation. Microorganisms of interesting metabolic properties include non-pathogenic bacteria of the genus *Clostridium*, particularly *C. acetobutylicum*, *C. butyricum* and *C. pasteurianum*. A well-known property of *C. butyricum* is their ability to produce butyric acid, as well as effectively convert glycerol to 1,3-propanediol (38.2 g/L). A conversion rate of 0.66 mol 1,3-propanediol/mol of glycerol has been obtained. Results of the studies described in the present paper broaden our knowledge of characteristic features of *C. butyricum* specific isolates in terms of their phylogenetic affiliation, fermentation capacity and antibacterial properties.

Key words: 1,3-propanediol, *Clostridium* spp., *C. butyricum*, glycerol, metabolic activity.

Introduction

Microbiology and industrial biotechnology extensively utilize the immense potential of microorganisms. An important role is ascribed to bacteria of the genus *Clostridium* as they are microorganisms with numerous applications in several branches of industry (Zhang *et al.*, 2009; Wang *et al.*, 2011; Wilknes *et al.*, 2011; Kaur *et al.*, 2012; Metsoviti *et al.*, 2012). The genus *Clostridium* is one of the largest in the Kingdom of Procaryota. These are anaerobic heterogenic bacteria, typically Gram-positive. Their distinctive feature is a cylindrical shape. Cells of bacteria of the genus *Clostridium* are ciliated, mobile and capable of forming endospores (Bahl and Dürre, 2001). For most species, the optimum growth temperature falls within the 30-40 °C range, while the optimum pH is contained in the 6.5-7.5 range. Guanine and cytosine contents in DNA amount to 24-54% mol. Commonly found in nature, the main habitats of *Clostridium* genus bacteria are soil, river sludge, active sludge, animal excrements, etc. (Schlegel, 1993; Bahl and Dürre, 2001).

These bacteria are characterized by intensive fermentation metabolism. They may use numerous organic compounds as sources of carbon and nitrogen. Products of their metabolism include CO_2, H_2, as well as organic compounds (butyric, lactic, acetic and succinic acids) and solvents (butanol, acetone, isopropanol) (Ezji *et al.*, 2007; Ren *et al.*, 2007; Skonieczny and Yargeau, 2009; Song *et al.*, 2011; Wang *et al.*, 2011). Bacteria of the genus *Clostridium*, *e.g.*, *C. butyricum*, *C. pasteurianum*, *C. diols*, *C. butylicum* and *C. perfingens* also participate in biotransformation processes (Colin *et al.*, 2000; Hao *et al.*, 2008). The above mentioned bacterial strains are capable of effectively converting glycerol to 1,3-propanediol (Kubiak *et al.*, 2012). Another species of great value for industry is the *C. thermocellum* strain, which is an anaerobic thermophile capable of converting waste cellulose into ethanol (Colin *et al.*, 2000; Demain *et al.*, 2005; Ezeji *et al.*, 2007; Ren *et al.*, 2007; Leja *et al.*, 2011; Song *et al.*, 2011; Kubiak *et al.*, 2012). By fermenting pectins, pectinolytic species of the genus *Clostridium* (*e.g. C. pectinovorum*) loosen plants' tissue structure and facilitate a rapid separation of cellulose fibers, which appears particularly attractive in terms of biomass conversion(Perry, 2008). By fermenting sugars (glucose, sucrose, lactose), *C. acetobutylicum* and *C. butyricum* are capable of producing hydrogen (Oh *et al.*, 2009; Beckers *et al.*, 2010). An increasing number of reports on the probiotic potential of *C. butyricum* have been published (Takahashi *et al.*, 2000, 2004; Zhang *et al.*, 2002; Seki *et*

al., 2003; Araki *et al.*, 2004; Shimbo *et al.*, 2005). An interesting example is the *C. butyricum* MIYAIRI 588 strain, which exhibits probiotic properties and is recommended as an additive to animal feeds (Shimbo *et al.*, 2005). Bacteria of the *C. butyricum* and *C. pectinovorum* species have also found applications in ecology, as they play a considerable role in soil mineralization processes and conversion of organic matter.

Besides a sizeable representation of the genus *Clostridium* characterized by significant positive potential for industrial application, there also exist several species exhibiting adverse effects on foodstuffs, as well as on human and animal health. Mesophilic proteolytic species such as *C. putrefaciens* frequently cause food spoilage. *C. botulinum* and *C. tetani* species are among the most dangerous bacteria, and their pathogenic character manifests itself in their capacity to produce very strong exotoxins. On the other hand, the botulin toxin produced by *C. botulinum* is used in medicine (to alleviate neurological symptoms) and in cosmetology, as an agent effectively reducing mimic wrinkles (Schlegel, 1993; Ting and Freiman, 2004).

The primary application of *C. butyricum* bacteria is the production of butyric acid. The aim of the present study was to indicate other potential areas of industrial application.

Materials and Methods

Microorganisms

The examined microorganisms (original environmental isolates) came from the Department of Biotechnology and Food Microbiology (Poznan University of Life Sciences, Poland).

Microbiological media

The Reincorfed Clostridial Medium RCM (BIOCORP, Poland) was used as the proliferation medium for the bacteria of the genus *Clostridium*.

The production medium (*Rich Medium*) was prepared according to Himmi *et al.* (1999) and supplemented with crude glycerol (Wratislawia, Poland) at a concentration of 70 g/L. Glycerol feedstock had the following composition (in w/w): 86% glycerol; 8-10% water; 5-6% NaCl; free fatty acids, and < 0.01% methanol.

Bacteria identification

Complete DNA of the analyzed microorganisms was isolated using a Genomic Mini AX Bacteria Kit (A&A Biotechnology, Gdansk, Poland), following incubation with lysozyme (Sigma-Aldrich, Poland) at a concentration of 50 mg/mL for 1 h at 37 °C. For amplification of the small rRNA, subunit primers SDBact0008aS20 and SUniv1492bA21 were used (Suau *et al.*, 1999). PCR products were purified with the use of Clean-up Kit (A&A Biotechnology, Poland), and next sequenced (Genomed,

Poland). Obtained sequences of 16S rRNA of the analyzed strains were compared with the sequences available at the GenBank using the BLAST program.

Phylogenetic analysis

A phylogenetic analysis of newly discovered strains makes it possible to determine the species affiliation, as well as assess affinity of the new strains to those already described in literature.

16S rRNA sequences (obtained at the identification stage) of DSP1, DSP5, DO14 and DO16 *C. butyricum* strains were compared with the *Clostridium* strains available at the GenBank. Clustal program implemented in BioEdit (ver. 7.0.9) was used to fit the DNA sequences (http://www.mbio.ncsu.edu/bioedit/ bioedit.html). The phylogenetic tree was constructed in MEGA 4.0 program (Tamura *et al.*, 2007). The neighbor joining method and the p-distance model were used.

Bioreactor culture

The first stage of culture consisted in proliferation of bacteria in the Hungate test tube in anaerobe culture chamber (Whitley MG500 by Scientific). After the adopted incubation time (24 h), the preinoculum was transferred by means of a sterile syringe to a bottle (Duran®) integrated with a 30 L bioreactor (Sartorius Stedim Ltd., Germany). The bottle was placed in a water bath (32 °C) and incubated for 24 h to proliferate bacterial biomass. After incubation, the contents of the bottle was pumped to a bioreactor with a working capacity of 10 L (total capacity of 30 L) with the use of a peristaltic pump. Incubation was repeated at 32 °C for 24 h. Then 10 L proliferated bacterial biomass was used to inoculate 90 L production medium. Prior to inoculation the medium was sterilized for 30 min. at a temperature of 121 °C (internal sterilization) in a bioreactor (New Brunswick Scientific, USA). Fermentation was run for 34 h at 36 °C and constant agitation rate of 60 rpm. The pH value of 7.0 was maintained by the addition of 20% NaOH.

Biochemical properties

Metabolic potential of the analyzed microorganisms was assessed using biochemical ANAEROtest 23® (Pliva-Lachema Diagnostica, Czech Republic). The ANAEROtest 23® kit is typically used for routine identification of anaerobic bacteria found in clinical material and foodstuffs. The kit makes it possible to determine 23 characteristic features of a given microorganism. For identification of a strain, assays were placed in wells on microtitration plates, at three rows with eight wells. Biochemical traits were determined based on chemical reactions occurring in the specifically composed growth media (found in plate wells). Macroscopic readings of test results included observations of growth, observations of changes in medium color occurring as a result of microbial metabolism, activation of color reaction by means of introducing a reagent reacting with the

metabolite produced by a given microorganism or gas release.

Comparison of isolated C. butyricum strains with indicator microorganisms

In order to identify antagonism of the tested *C. butyricum* strains in relation to indicator microorganisms, analyses were conducted, including preparation of culture media of *C. butyricum* DSP1 and *C. butyricum* DO14 strains, separation of culture media into fractions (the supernatant and precipitate), preparation of indicator microorganisms, as well as analyses of activity of the obtained culture medium and the supernatant by the well method.

Preparation of culture liquid media

Antibacterial activity was determined using 24 h cultures (on RCM) of *C. butyricum* DSP1 and *C. butyricum* DO14. Cultures were run under strictly anaerobic conditions at a temperature of 32 °C.

Separation of the culture liquid media into fractions

In order to obtain supernatant (S) cultures, the analyzed strains were centrifuged (5000 *g*; 10 min.). Analyses of the supernatant aimed to determine the activity of bacterial exocellular metabolites.

Preparation of indicator microorganisms

Indicator microorganisms (*Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonella enteritidis, Staphylococcus aureus, Bacillus subtilis, Escherichia coli*) were transferred to test tubes containing 10 mL broth medium with an addition of 2% glucose (to proliferate biomass). Cultures were run at 37 °C for 24 h. Next, in order to obtain a distinct confluent layer, the liquefied agar medium was inoculated with 10% (v/v) 24 h indicator culture and poured onto Petri dishes.

Liquid culture medium and supernatant fraction antibacterial activity analyses

After solidification of the broth medium inoculated with indicator microorganisms, wells were made using a cork borer. Each well was supplemented with 150 μL liquid culture medium and 150 μL supernatant fraction of the analyzed strain. Next, diameters of growth inhibition or reduction of indicator bacteria were measured. Growth inhibition of the indicator microorganism, which manifested itself in complete lightening around the place where the liquid culture/supernatant was transferred, indicated bactericidal activity of the tested strain. Bacteriostatic properties were determined by measuring the diameter of the growth inhibition zone (growth limitation of indicator strain). An increase in the density of confluent layer around the well indicated a stimulatory action of the tested culture/supernatant in relation to the applied indicator. Growth inhibition of the indicator microorganism, manifested in a complete lightening around the place where the supernatant fraction was transferred, indicated antibacterial activity of exocellular metabolites. Bacteriostatic properties or stimulatory action of the supernatant were determined in the same way as with the liquid culture medium.

Analytical methods

1,3-PD, glycerol and organic acids were assayed by high performance liquid chromatography (HPLC) on an Agilent Technologies 1200 series chromatograph (the set included automatic sample feeder G1329B, and double G1312B pump with a G1362A refractometric detector). Assays were performed on a Rezex ROA column using 0.005 N H_2SO_4 as the mobile phase, at a flow rate of 0.6 mL/min at 40 °C. Samples were transferred on a column at 10 μL. Quantitative and qualitative identifications were performed by the external standard method based on peak areas (measurements and computer integration using ChemStation for LC 3D systems, Agilent).

Results and Discussion

Phylogenetic analysis

One of the first stages in the examination of the new environmental isolates was to determine their species affiliation based on biochemical tests or 16S rRNA sequences. The capacity of the isolated strains of the *C. butyricum* species to synthesize 1,3-PD was presented in subchapter "Synthesis of 1,3-PD from crude glycerol in batch culture", and in a study by Szymanowska-Powalowska *et al.* (2013). In order to present interdependencies between strains of *C. butyricum* DSP1, *C. butyricum* DSP5 and *C. butyricum* DO16, as well as other strains of the genus *Clostridium*, a phylogenetic tree recreated on the basis of the nucleotide sequence of the 16S rRNA coding gene was constructed. In case of *C. butyricum* DO14 strain, the sequencing quality did not make it possible to include this strain in the graphic illustration of interstrain dependencies.

In most cases, strains selected from the data base for the purpose of phylogenetic analyses are capable of using glycerol as a source of carbon (Biebl and Spröer, 2002). Some of the compared microorganisms exhibit capacity to synthesize 1,3-PD (*C. butyricum* VIP 3266, *C. butyricum* DSM 2478, *C. butyricum* DSM 523, *C. diolis* DSM 5430, *C. diolis* DSM 5431, *C. diolis* E5) (Biebl and Spröer, 2002). The phylogenetic tree presented in Figure 1 shows the relationship between the new *C. butyricum* isolates and the strains described in literature as auspicious to 1,3-PD producers.

Biochemical properties of the genus *Clostridium* isolates

A major stage in the characteristics of the new strains, particularly those revealing industrial applicability potential, was an examination of their biochemical properties (such as the capacity to assimilate, ferment or decompose
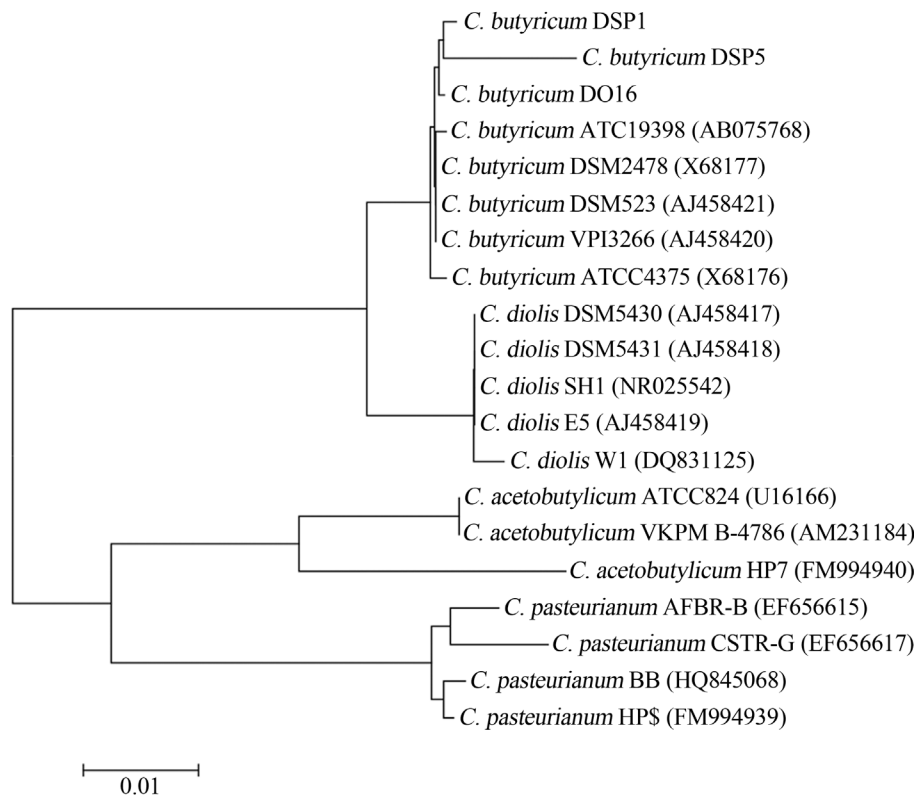
Figure 1 - A phylogenetic tree constructed on the basis of the 16S rRNA nucleotide sequence showing evolutionary relationships between *C. butyricum* DSP1, DSP5 and DO16, and other strains from the genus *Clostridium*.

chemical compounds). Assimilation consists in conversion of a nutrient to compounds used as building blocks for the organism and to energy in the form of ATP. Fermentation was defined as anaerobic enzymatic conversion of organic compounds, particularly carbohydrates, to simpler products, mainly alcohols, and to energy in the form of ATP. Different types of microorganisms were characterized by specific enzymatic systems active in the processes of assimilation, fermentation, or decomposition. Identification of microorganisms' capacity to assimilate, ferment and decompose different substrates determined their potential application in different branches of industry.

   Table 1 presents data recorded as a result of biochemical tests conducted on the novel bacterial strains of *C. butyricum* species (DSP1, DSP5, DO14 and DO16). A total of 23 biochemical traits were investigated. All isolates exhibited capacity to ferment mono- (six and five carbon) and disaccharides, which was also reported for other stains, particularly with reference to the metabolism of glucose into butyric acid (Quilaguy-Ayure *et al.*, 2010). The capacity of *C. butyricum* bacteria to produce butyric acid using simple sugars is the basic trait used in the chemical industry. Strains analyzed in this study did not exhibit capacity to metabolize hexahydroxyl alcohols, such as sorbitol or mannitol, while being capable of using glycerol as a source of carbon, as evidenced in subchapter "Synthesis of 1,3-PD from crude glycerol in batch culture," and in the study by

Szymanowska-Powalowska *et al.* (2013). Conversion of glycerol to 1,3-PD (the primary product of glycerol metabolism) is an essential characteristics of significant industrial potential. 1,3-PD is used as a monomer in the production of polyesters, lubricants and organic solvents. Moreover, growing demand for polyesters and polypropylenes (PTT) has made it necessary to increase production of 1,3-PD used in the synthesis of polyurethanes. Biotechnological conversion of glycerol to 1,3-PD using *C. butyricum* bacteria can become an attractive alternative to the chemical synthesis, which is not only costly, but also brings harmful environmental consequences (Metsoviti *et al.*, 2012; Kaur *et al.*, 2012; Kubiak *et al.*, 2012; Venkataramanan *et al.*, 2013). Biochemical tests have proved that the analyzed isolates are capable of fermenting certain trisaccharides. Rafinose may serve as an example. DSP1, DSP5, DO14 and DO16 strains of *C. butyricum* exhibited β-glucosidase activity, which is associated with the ability to ferment cellobiose and can be applied in the process of plant biomass conversion. The strains did not show ability to metabolize N-acetyl-βglucosamine, a polysaccharide constituting a source of carbon and nitrogen for bacteria of the genera *Flavobacterium*, *Bacillus*, and *Pseudomonas,* as well as fungi of the genera *Mucor* or *Aspergillus*. Similar results of biochemical tests of new *C. butyricum* isolates were also obtained by other authors (Petidemange *et al.*,

Table 1 - Biochemical properties of new *Clostridium* isolates in comparison to reference strains

Biochemical properties	New *C. butyricum* isolates				Reference *C. butyricum* strains		
	DSP1	DSP5	DO14	DO16	IP 3044*	E5**	DSM 5431***
Indole	-	-	-	-	-	-	NA
Glucose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	NA
Fructose	+	+	+	+	+	+	NA
Galactose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	NA
Trehalose	+	+	+	+	NA	NA	+
Ramnose	-	-	+	-	+	+	NA
Mannose	+	+	+	-	+	+	NA
Rafinose	+	+	+	+	NA	NA	-
Cellobiose	+	+	+	+	W	W	+
Xylose	+	+	+	+	+	+	+
Arabinose	-	-	+	+	+	+	W
Melecitose	-	-	+	-	NA	NA	W
Salicin	+	+	+	+	NA	+	+
Sorbitol	-	-	+	-	NA	NA	W
Glycerol	+	+	+	+	+	+	+
Mannitol	-	-	+	-	+	+	+
Esculine	+	+	+	+	+	W	W
N-acetyl-β glucosamiase	-	-	-	-	NA	NA	NA
b-Glucosidase	+	+	-	-	NA	NA	NA
Urease	-	-	-	-	NA	NA	NA
Nitrates	-	-	-	-	NA	NA	NA

(+) - positive result; (-) - negative result; (NA) - not analyzed; (W) - weak reaction, weak acidification.
*IP 3044 - *Clostridum butyricum* from Institut Pasteur Collection (Paris, France) (Petidemange *et al.*, 1995).
**E5 - *Clostridum butyricum* (Petidemange *et al.*, 1995).
DSM 5431 - *Clostridium butyricum* (Biebl and Spröer, 2002).

1995). One of the best isolates of the investigated strains (in terms of 1,3-PD synthesis) showed a comparable biochemical characteristics to *C. butyricum* E5 strain. Polymers, such as starch, xylene and pectins found in plant biomass, may constitute important sources of substrates for wild bacterial strains of the genus *Clostridium*. These bacteria were capable of degrading enzymes or enzymatic complexes, which makes biomass available for microorganisms (*C. thermocellum*, *C. cellulovorans*, *C. celluloticum*)(Biebl and Spröer, 2002). Similar effects were achieved when compounds like sucrose and xylose were used as sources of carbon; this was confirmed for isolates described both in the present study and in other reports (Taguchi *et al.*, 1995; Biebl and Spröer, 2002; Yung-Chung *et al.*, 2010). Besides cellulose and lignin, plant biomass contains hemicellulose.

Xylose, in turn, is a monosaccharide contained in hemicellulose, and accouns for 30% of its composition. Reports confirm the existence of numerous representatives bacteria of the genus *Clostridium* capable of synthesizing xylanases (Bahl and Dürre, 2001; Wang *et al.*, 2011; Yung-Chung *et al.*, 2010). Metabolic capacity associated with decomposition and fermentation of biomass is particularly important in the production of 2nd generation biofuels (ethanol from cellulose) (Maki *et al.*, 2009; Williams *et al.*, 2013). Sucrose and xylose are used as sources of carbon in the microbiological synthesis of hydrogen by the CGS2 *C. butyricum* strain (Yung-Chung *et al.*, 2010). The use of hydrogen carries great potential for such applications, as production of nitrogen fertilizers, or crude oil processing. It can also be used in fat industry, metallurgy, and, as fuel, in the power

industry (Urbaniec *et al.*, 2012). Wang *et al.* (2011) investigated the effect of carbohydrate substrates on the growth of *C. butyricum* TK2 strain. Glucose, sucrose and lactose were selected as carbon sources. An increase in the number of microorganisms was observed in all the examined cases: 5×10^8 cfu/mL (glucose), 2.5×10^8 cfu/mL (lactose) and 2.4×10^8 cfu/mL (sucrose). Identification of bacteria's metabolic capacity, particularly this associated with decomposition of complex compounds, may be a sign of enzymatic activity. Decomposition of starch polysaccharides, which indicates the presence of the amylolytic enzyme profile by a given microorganism, may serve here as an example. The ability to synthesize amylases by *Clostridium* spp. bacteria was also reported by other authors (Annous and Blaschek, 1990). Important biochemical indexes also included the ability to decompose tryptophane to indol, and to reduce nitrates (reduction of nitrates (V) to nitrates (III) or nitrogen). These are main characteristic features to determine bacteria's affiliation to the family *Enterobacteriaceae*. For the new isolates of the genus *Clostridium*, which constitute the subject of the present study, the result for these indexes was negative. Within the scope of the present study, no capacity was found in these strains to decompose urea (to ammonia and carbon dioxide), whereas this trait was characteristic of the bacteria from the genera *Klebsiella* or *Proteus*, and had even found certain applications in the area of environment protection (ammonification) (Bahl and Dürre, 2001).

Synthesis of 1,3-PD from crude glycerol in batch culture

Glycerol obtained in the course of biofuel production is a crude product that is very difficult to utilize. However, it needs to be stressed that this crude product with a very limited utilization potential contains several contaminants which adversely affect microorganisms. Contaminations found in crude glycerol determine its potential for use in industry. According to sources, a 6% concentration of methanol inhibits microbial growth by as much as 90%. Ions of heavy metals retained in glycerol after the process of biodiesel production may have a negative effect on the viability of microbial cells. Moreover, high concentrations of salts contained in crude glycerol have an inhibitory effect on the growth of *Clostridium* species' cells. On the other hand, in some investigations with yeast high concentration of salt stimulated production of erythritol by selected strain of *Yarrowia lipolytica* (Rywinska *et al.*, 2013) or had no effect on biosynthesis of citric acid by *Y. lipolytica* on media containing crude glycerol (Rymowicz *et al.*, 2010). Unsaturated free fatty acids also influence microbial metabolism and viability. Another problem, particularly in commercial production conditions, is the variability of the raw material (especially its quality), which may have adverse effect on process yields (Bahl and Dürre, 2001; Kaur *et al.*, 2012; Kubiak *et al.*, 2012). A potential method of the crude product utilization may be its biotechnological conversion to

1,3-PD. The biological process of glycerol conversion to diols involves microorganisms, *e.g.* bacteria of the species *C. butyricum* (Kaur *et al.*, 2012; Kubiak *et al.*, 2012). Figure 2 and Table 2 show analytical results obtained during the process of 1,3-PD synthesis from crude glycerol in a batch culture. Batch fermentation was run in a bioreactor of 100 L operating capacity. The initial glycerol concentration equaled 70 g/L. Conducted analyses made it possible to obtain a 1,3-PD concentration of 38.2 g/L. At the assumed glycerol concentration of 70 g/L and its complete conversion, the efficiency of 1,3-PD synthesis was 0.66 mol/mol glycerol.

Literature of the subject presents a growing amount of data on the synthesis of 1,3-PD using crude glycerol, as it is only application of this type of substrate that is auspicious in terms of potential industrial use. In a batch culture, Metsoviti *et al.* (2012) obtained approx. 32 g/L 1,3-PD from 55 g/L crude glycerol and a very similar profile of waste metabolites in terms of their quantities and quality. In a fed-batch culture of AKR102a *C. butyricum* (at a temperature of 32 °C), Wilkens *et al.* (2011) obtained 1,3-PD concentrations of 93.7 and 76.2 g/L from for pure and crude glycerol, respectively. In their study, Chatzifragkou *et al.* (2011) also described the effect of crude glycerol on the parameters of 1,3-PD synthesis for the *C. butyricum* VPI 1718 strain. The efficiency of 1,3-PD synthesis for pure and crude glycerol was identical, and amounted to 0.67 mol/mol glycerol.

Literature of the subject, as well as the results presented in the current study, demonstrated the potential applicability of *C. butyricum* bacteria in the conversion of crude glycerol to synthesize 1,3-PD. Substitution of anhydrous glycerol with crude product would considerably lower the process cost, as well as facilitate development of an integrated process in which crude product from one process (glycerol obtained in the production of biodiesel) would become the primary raw material (the only source of carbon and energy) for another process, this of microbiological conversion of crude glycerol to 1,3-propanediol.

Antibacterial activity

Bacteria of the genus *Clostridium* are frequently associated with dangerous pathogens which pose a threat to health or even life of humans and animals. Novel strains of microorganisms exhibiting antibacterial properties constitute an interesting prospect, particularly for the feed industry. The Fodders Act of 22 July 2006 imposed a ban on the use of antibiotic growth stimulators in the feed industry. An alternative solution is the use of probiotics (besides acidifiers, enzymes, and plant extracts). According to WHO definition, probiotics are live microorganisms, which, when administered in adequate quantities, have a positive effect on the host organism. One of probiotics' characteristic features is their operation against harmful microflora. It manifests itself, for example, in the inhibition of pathogen
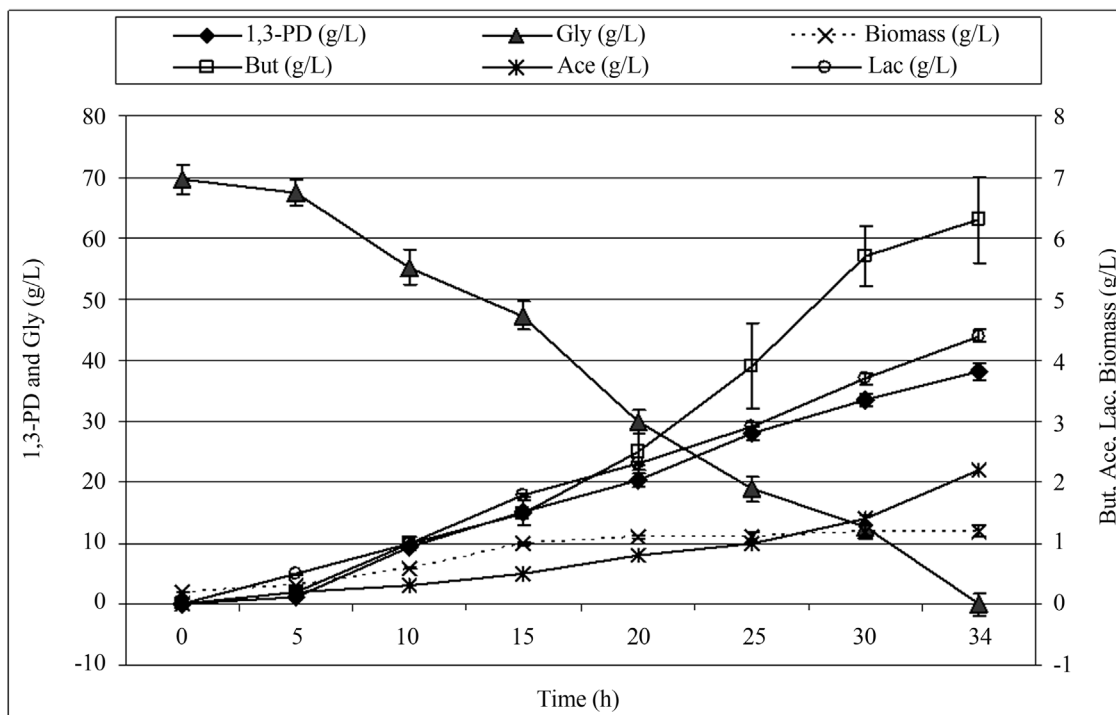
Figure 2 - Kinetics of 1,3-PD synthesis from crude glycerol by *C. butyricum* DSP1. Fermentation conditions: initial glycerol concentration: 70 ± 1.0 g/L, temperature 36 °C; pH 7.0; culture vessel: bioreactor of 100 L. The experiment was conducted in two replications.

Table 2 - Basic parameters of the fermentation process.

Microorganisms	Max.$_{1,3\text{-PD}}$ (g/L)	Utilization of glycerol (%)	Max.$_{biomass}$ (g/L)	Yield (mol/mol)	Productivity (g/Lh)
C. butyricum DSP1	38.2 ± 1.5	100	1.2 ± 0.2	0.66	1.12

Max.$_{1,3\text{-PD}}$ - maximum concentration of 1,3-propanediol; Max.$_{biomass}$ - maximum biomass concentration.

growth. This is the result of the microorganisms' capacity to secrete organic acid (mainly lactic acid), hydrogen peroxide, and bacteriocins (*i.e.* small proteins of specific bactericidal qualities). The best known microorganisms which are attributed characteristic probiotic properties are the lactic fermentation bacteria. However, the greatest amount of information available on *C. butyricum* bacteria concentrates on their pathogenic qualities (Tanaka *et al.*, 2000; Takahashi *et al.*, 2004; Shimbo *et al.*, 2005; Zhang *et al.*, 2009).

A further stage in the study of characteristic qualities revealed by the new strains of *C. butyricum* was to investigate their operation against the most frequent human and animal pathogens. Table 3 shows the results of the study on adverse operation of *C. butyricum* bacteria (DSP1, DSP5, DO14, DO16) upon pathogenic microorganisms of *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Bacillus subtilis,* and *Escherichia coli* species.

The highest level of bactericidal and bacteriostatic properties against Pseudomonas aeruginosa, Salmonella enteritidis and *Escherichia coli* was found with the *C.*

butyricum DSP1 strain. No such activity, in turn, was observed with any of the analyzed isolates against *Listeria monocytogenes* and *Staphylococcus aureus* strains. Numerous reports concentrate on the inhibitory effect of *C. butyricum* on the pathogenic bacteria *Helicobacter pylori* (Tanaka *et al.*, 2000; Takahashi *et al.*, 2004; Shimbo *et al.*, 2005). Takahashi *et al.* (2004) described similar properties of *C. butyricum* MIYAIRI 588 against *Escherichia coli* O157:H7. In their opinion, what determines the antibacterial potential of the *Clostridium* genus bacteria is their capacity to synthesize organic acids. The inhibitory effect of lactic acid and butyric acid of the tested *C. butyricum* MIYAIRI 588 strain was observed at neutral and low pH, which is significant information with view to its applicability potential (*e.g.* tolerance to gastrointestinal juices). Antibacterial properties of lactic acid were also discussed by other authors (Ammor *et al.*, 2006). It needs to be stressed that *C. butyricum* bacteria also exhibit antagonistic interactions with *Candida albicans*, *C. difficile*, *Klebsiella* spp., *Salmonella* spp., *Vibrio* spp. (Chen *et al.*, 1987; Fujita and Takashi, 1987; Kuroiwa *et al.*, 1990; Takahashi *et al.*, 2004).

Table 3 - Antibacterial activity of *C. butyricum* DSP1 and *C. butyricum* DO14 against selected pathogens.

Indicator bacteria	Antibacterial activity (mm)			
	Well method			
	C. butyricum DO14		*C. butyricum* DSP1	
	P	S	P	S
Pseudomonas aeruginosa	12(bg)	10(bd)	10(bd)	10(bd)
Listeria monocytogenes	NF	NF	NF	NF
Salmonella enteritidis	NF	NF	12(bd)	12(bd)
Staphylococcus aureus	NF	NF	NF	NF
Bacillus subtilis	NF	NF	16(S)	16(S)
Escherichia coli	12(bg)	10(bd)	12(bg)	12(bg)

P - culture liquid, S - supernatant, bd - bacteriostatic activity (reduction density); bg - bacteriostatic activity (complete growth inhibition); S - growth stimulation; NF - not found.

EU Commission's implementing regulation no. 373/2011 admitted C. butyricum FERM-BP 2789 preparation for use as a feed additive for birds and pigs. Use of the bacterial strain specified in the regulation is intended to increase weight gains and improve feed conversion rates in the above mentioned species. Imase *et al.* (2008) also described the effect of *C. butyricum* CMB 588 bacteria on the pathogenic strain *Helicobacter pylori.* Additionally, these authors observed an increase in the level of beneficial intestinal microflora (*e.g.* bacteria of the genus *Bifidobacterium*) during administration of an antibiotic therapy containing bacteria of the genus *Clostridium*. Other authors analyzed the probiotic effect of *C. butyricum* MIYAIRI 588 on a dangerous pathogen of the same species, the *C. difficile*(Woo *et al.*, 2011).

Conclusions

In conclusion, the non-pathogenic clostridia reveal a large potential for industrial application. The study was designed to present new interesting potential applications of *C. butyricum*. The biochemical profile of the novel *C. butyricum* isolates indicates their capacity to ferment a wide range of sugars (simple and complex). The *C. butyricum* DSP1 strain was capable of synthesizing 1,3-PD from crude glycerol in the batch culture with an efficiency of 0.66 mol/mol glycerol. In addition, the *C. butyricum* DSP1 and *C. butyricum* DO14 isolates exhibit antibacterial qualities against *Pseudomonas aeruginosa* and *Escherichia coli.*

Acknowledgments

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