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Hypothetical glycerol pathways of newly isolated strains capable of 1,3-propanediol production

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Study presented here demonstrates the ability of three newly isolated strains, obtained from environmental probes (manure, bottom sediment, and food waste) and identified as *Clostridium bifermentans*, *Clostridium butyricum*, and *Hafnia alvei,* to synthesize 1,3-propanediol (1,3-PD), organic acids (such as lactic, acetic, fumaric, succinic, and butyric acids), and ethanol from glycerol. The production of 1,3-PD as well as the glycerol pathways in *C. bifermentans* and *H. alvei* cells have not been investigated and described yet by others. Moreover, there is no data in the available literature on the products of glycerol utilization by *H. alvei* and there is only some incoherent data (mainly from the first half of the twentieth century) about the ability of *C. bifermentans* to carry out glycerol degradation. Additionally, this study presents complete hypothetical glycerol pathways and the basic fermentation kinetic parameters (such as yield and productivity) for both strains as well as for the newly isolated *C. butyricum* strain.

Key words: *Clostridium bifermentans*, *Clostridium butyricum*, *Hafnia alvei*, 1,3-propanediol

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INTRODUCTION

Bacteria from *Clostridium* species commonly occur in such environmental probes as water, soil, sewage (Nachman *et al*., 1989), sediment and river sludge (Wang *et al*., 2003), and animal excrements (Bergey *et al*., 1984; Leja *et al*., 2011). *H. alvei* also exists in the natural environment; i.e. it can be isolated from soil, water, plants, food, intestines of animals and humans (Brenner *et al*., 2005).

Different types of microorganisms are characterized by specific enzymatic systems, active in the processes of assimilation, fermentation or decomposition. Identification of the capacity of microorganisms to assimilate, ferment and decompose different substrates is crucial for determination of their potential application in specific branches of industry. Bacteria from the genus *Clostridium* spp. are characterized by an intense fermentation metabolism. The main products of fermentation carried out by *Clostridium* species are organic acids (acetic, butyric, fumaric, propionic and benzoic) (Bergey *et al*., 1984; Wu & Yang, 2003), solvents (butanol, acetone, and ethanol) (Jones & Woods, 1986), and gases (carbon dioxide, hydrogen, and ammonium) (Khanal *et al*., 2004; Levin *et al*., 2006). However, there is no information in the literature as to which metabolites are produced by *Hafnia alvei*. On the other hand, some information about the metabolic activity of *C. bifermentans* can be found in the available literature. For example, Brooks and Epps (Brooks & Epps, 1958) stated that *C. bifermentans* are able to carry out glycerol, glucose, fructose, maltose, sorbitol, and mannose fermentation. Other scientists, namely Dezfulian and coworkers (Dezfulian *et al*., 1994)), investigated strains isolated from soil and sewage probes that are able to perform maltose and glucose fermentation. Significantly, these strains were not able to utilize such saccharides as arabinose, cellobiose, lactose, melibiose, raffinose, trehalose, xylose, and glycerol. The presented data about metabolic properties of *C. bifermentans* strains are not complaisant and, moreover no papers about metabolic pathways in these bacteria have been published lately. It is well-known that bacteria from the genus *C. butyricum* are able to ferment glucose, maltose, fructose, galactose, lactose, sucrose, ramnose, mannose, xylose, arabinose, glycerol, mannitol (Petitdemange *et al*., 1995). *C. butyricum* is known as a classical acid producer and it typically ferments glucose to butyrate, acetate, carbon dioxide and molecular hydrogen (Jungermann *et al*., 1973). When *C. butyricum* is growing on glycerol, this substrate is oxidized to dihydroxyacetone (DHA) and subsequently phosphorylated to yield DHA-phosphate, or it is dehydrated to 3-hydroxypropionaldehyde (3-HPA); the latter compound is then reduced to 1,3-PD (Biebl *et al*., 1998; Dabrock *et al*., 1992). Despite the fact that *H. alvei* is described as a strain capable of glycerol fermentation, there is no literature data about its ability for 1,3-PD production. Thus, we decided to investigate and present hypothetical pathways of glycerol in the environmental isolates of *C. bifermentans*, *C. butyricum*, and *H. alvei*.

MATERIALS AND METHODS

Microorganisms. *C. bifermentans* KM 371 was isolated from a manure sample (obtained in the Wielkopolska Region, Poland). This strain was identified and precisely characterized in a previous work by Myszka and coworkers (Myszka *et al*., 2012). *C. butyricum* DO14 was isolated from a bottom sediment (in the Kujawsko-Pomorskie Region, Poland). This strain was identified in a previous

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Abbreviations: 1,3-PD, 1,3-propanodiol; LA, lactic acid; FA, fumaric acid; AA, acetic acid; SA, succinic acid; BA, butyric acid; Et, ethanol

work by Orczyk and Szymanowska-Powałowska (Orczyk & Szymanowska-Powałowska, 2012).

H. alvei AD 27 was isolated from food waste.

Stocks of investigated strains are kept at the culture collection of the Department of Biotechnology and Food Microbiology at Poznań University of Life Sciences.

Cultivation medium. *C. bifermentans* bacteria were cultivated in the modified PY medium (Biebl & Spröer, 2002) consisting of (g/L): BactoPeptone 10; yeast extract 10; $CaCl_2$, $MgSO_4 \times 7H_2O$ 0.96; K_2HPO_4 2; $NaHCO_3$ 20; NaCl 4. The medium was supplemented with glycerol (50 g/L).

C. butyricum was cultivated in the modified Rich Medium (Himmi *et al*., 1999) consisting of (g/L): $K_2HPO_4 \times 3H_2O$ 3.4; KH_2PO_4 1.3; $(NH_4)_2SO_4$ 2.0; $MgSO_4 \times 7H_2O$; 0.2 $CaCl_2$ 0.02; $FeSO_4 \times 7H_2O$ 0.05; yeast extract 2.0 and 2 ml trace element solution SL_7 (Papanikolaou *et al*., 2000). The medium was supplemented with glycerol (50 g/L).

H. alvei was cultivated in the production medium consisting of (g/L): K_2HPO_4 2.4; KH_2PO_4 0.6; $(NH_4)_2SO_4$ 2; $MgSO_4 \times 7H_2O$ 0.4; $CaCl_2 \times 2H_2O$ 0.1; $CoCl_2 \times 2H_2O$ 0.004; yeast extract 2; bactopeptone 2.5; meat extract 1.5. The medium was supplemented with glycerol (50 g/L).

Batch fermentation procedures. A preculture of *C. bifermentans* strain was carried out in a 500 ml flask containing 300 ml PY medium with glycerol at 37°C for 24 h. It was inoculated into a 5L bioreactor (Sartorius Stedim, Germany) with 3L PY medium. According to Myszka and coworkers (Myszka *et al*., 2012), a blanket of a high-purity grade gas mixture of 5% O_2 and 95% CO_2 was maintained during cultivation. Gas flow rate was at up to 1.0 L/min, the stirrer speed varied between 200 and 500 rpm. The fermentation was run under microaerophilic conditions, at 30°C for 7 days.

A preculture of *C. butyricum* strain was carried out in a Hungate test tube in a culture chamber for anaerobes (Whitley MG500 by Scientific). After the adopted incubation time (24 h, 32°C), the preinoculum was transferred, using a sterile syringe, to a bottle (Duran®) integrated with a 5 L bioreactor (Sartorius Stedim, Germany). The bottle was placed in a water bath (37°C) and incubated for 24 h to proliferate bacterial biomass. After incubation, the contents of the bottle were pumped to a bioreactor with a working capacity of 2 L with the use of a peristaltic pump. Fermentation was run for 72 h at 37°C, pH 7.0 was maintained by addition of 20% NaOH. A blanket of a high-purity grade nitrogen gas was maintained during cultivation to ensure anaerobic conditions. Gas flow rate was at up to 1.0 L/min, the stirrer speed was constant at 70 rpm.

A preculture of *H. alvei* strain was carried out in a 250 ml flask containing 100 ml of production medium with glycerol at 30°C for 24 h. It was inoculated into 5L bioreactor (Sartorius Stedim, Germany) with 1L of production medium. The fermentation was carried out under controlled conditions: pH 7.0, temperature 30°C , and agitation rate 80 rpm for 40 h. No air or nitrogen sparging was used before and during the fermentation process.

Next the fermentations probes were taken out and analyzed by HPLC technique.

Analytical procedures. The ability of metabolite production by *C. bifermentans* KM371, *C. butyricum* DO14, and *H. alvei* AD27 was determined with a high liquid performance chromatography (HPLC) technique. The Hewlett Packard system consisting of an auto sampler and a pump, and a refractive index detector was used. The analysis was performed isocratically at a flow rate 0.6 mL/min. at 65°C, on a Aminex HPX-87H300×7.8 column (Bio-Rad,USA). 0.5 mNH_2SO_4 was also used as a mobile phase. Standards were applied to identify peaks in chromatograms, and peak areas were measured to determine the samples' concentration (ChemStation, Agilent, USA).

RESULTS

In the earlier research carried out in the Department of Biotechnology and Food Microbiology (Poznan University of Life Sciences) several new strains capable of 1,3-PD production, from the species of *C. bifermentans*, *C. butyricum*, and *H. alvei* among others, were obtained (Myszka *et al*., 2012; Orczyk & Szymanowska-Powałowska, 2012). Because *C. bifermentans* and *H. alvei* have never been characterized as a 1,3-PD producers, we decided to investigate their glycerol metabolic pathway and compare it with *C. butyricum* pathway, a typical 1,3-PD producer.

As a result, it was found out that all three investigated strains were capable of 1,3-PD, lactic acid, acetic acid, and succinic acid, acid production. Additionally, *C. butyricum* DO14 and *H. alvei* AD27 synthetized ethanol, *C. bifermentans* and *C. butyricum* synthetized fumaric acid, and only *C. butyricum* produced butyric acid. The hypothetic pathways of glycerol in the above mentioned strains are presented in Fig. 1.

Despite the fact that the metabolite profiles of investigated bacteria were similar, the quantitative ratios between individual products were different in all strains. The highest level of 1,3-PD was synthetized by *C. butyricum* strain. *C. bifermentans* produced the highest amount of organic acids: lactic, fumaric, acetic, and succinic. Butyric acid was produced only by one strain — *C. butyricum* (Fig. 2).

Additionally, the percentage of individual metabolites in all synthetized products, the metabolite yields and productivity are presented in Table 1. In case of all three investigated strains, 1,3-PD was the main metabolite: in *C. butyricum* DO14 it constituted 60.11% of all produced metabolites, in *H. alvei* AD27 — 55.39%, and 32.69% in *C. bifermentans* KM371. The highest P_a for 1,3-PD was obtained for *C. butyricum* — the maximum level of this metabolite was obtained after 72 h of cultivation. Significantly, this strain was able to synthetize about 2.5 times more 1,3-PD than *C. bifermentans* and ca. 2 times more than *H. alvei*, which was cultivated for the shortest time. Quantitatively, the second metabolite in *C. bifermentans* was lactic acid (27.37% of all metabolites), in *C. butyricum* — butyric acid (14.55%), and in *H. alvei* — acetic acid (19.87%).

Also, the level of glycerol utilization and its kinetics were different in all investigated strains (Fig. 3). The end of the culture was regarded as the moment when the level of metabolites has no longer increasing. Thus, this time is different for all three strains. The most effective utilization of glycerol was observed for *C. butyricum* strain. During 72 hours of cultivation total glycerol was consumed. Despite the fact that the utilization of glycerol (and metabolite production) in *C. bifermentans* required 168 hours of cultivation, the level of its utilization was high — 94.32%. *H. alvei* consumed less glycerol (56.48%), however the maximum amount of metabolites was obtained 3.5 times faster, already after 48 hours of cultivation; additionally, raw material was utilized by this strain more intensively than by *C. bifermentans*.

These preliminary studies of metabolism of *C. bifermentans* and *H. alvei* demonstrate that these strains are capable of 1,3-PD production from glycerol. Because

Figure 1. Hypothetic glycerol pathways in investigated strains: (A) *C. bifermentans* KM371, (B) *C. butyricum* DO14, (C) *H. alvei* AD27

this feature has as yet not been investigated by other scientists, and there in no literature data about this ability, some part of our work was done intuitively. The yield of the main metabolite — 1,3-PD and its productivity are lower in comparison with *C. butyricum* strain. Thus,

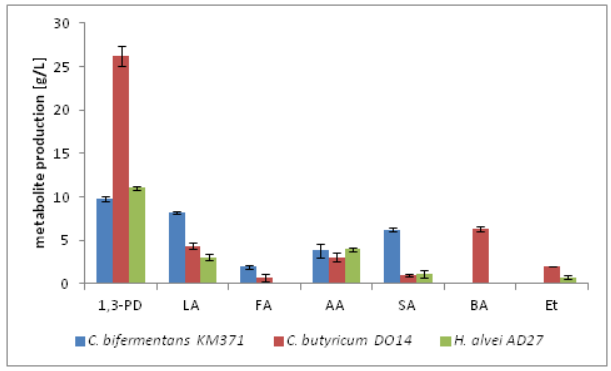

Figure 2. The level of metabolite production by *C. bifermentans* KM371, *C. butyricum* DO14, *H. alvei* AD27
1,3-PD — propanediol; LA — lactic acid; FA — fumaric acid; AA — acetic acid; SA — succinic acid; BA — butyric acid; Et — ethanol

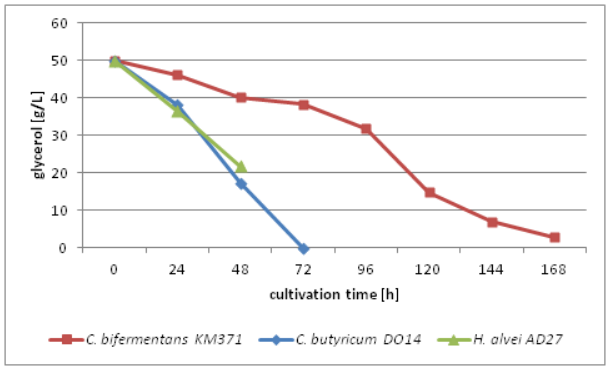

Figure 3. The kinetics of glycerol utilization in *C. bifermentans* KM371, *C. butyricum* DO14, and *H. alvei* AD27

further experiments on the optimization of cultivation medium and fermentation parameters for *C. bifermentans* and *H. alvei* should be done.

DISCUSSION

The natural environment is a rich source of industrially useful microorganisms. Isolating such bacteria and developing methods of application in a number of industry branches is an important step in substituting chemical synthesis by microbiological processes. Chemical synthesis produces significant quantities of by-products, contaminating the natural environment; moreover, it has a low specificity. On the other hand, biotechnological methods circumvent these problems (Kaeberlein *et al.*, 2002; Nicol *et al.*, 2012).

In our earlier experiments we obtained strains capable of 1,3-PD production from glycerol. Some of these strains have not been described as yet by other scientist as 1,3-PD producers, such as *C. bifermentans* and *H. alvei*. *C. butyricum* strains that are known to be able to synthesize 1,3-PD, were also obtained. The metabolism of *C. bifermentans* and *H. alvei* has not been investigated in detail yet, thus we decided to compare it with *C. butyricum*.

In the literature there is some incoherent information about the metabolism of *C. bifermentans*. For example, Dezfulian and coworkers (Dezfulian *et al.*, 1994) isolat-

Table 1 Basic kinetic parameters of fermentation in investigated strains

Strain	Product	%	$Y_{p/s}$	P_a
C. bifermentans	1,3-PD	32.69	0.20	0.06
	LA	27.37	0.16	0.05
	FA	6.48	0.04	0.01
	AA	12.73	0.08	0.02
	SA	20.72	0.12	0.04
	BA	nd	nd	nd
	Et	nd	nd	nd
C. butyricum	1,3-PD	60.11	0.52	0.36
	LA	9.97	0.09	0.06
	FA	1.67	0.01	0.01
	AA	6.99	0.06	0.04
	SA	2.20	0.02	0.01
	BA	14.55	0.13	0.09
	Et	4.51	0.04	0.03
H. alvei	1,3-PD	55.39	0.22	0.23
	LA	15.40	0.06	0.06
	FA	nd	nd	nd
	AA	19.87	0.08	0.08
	SA	5.52	0.02	0.02
	BA	nd	nd	nd
	Et	3.81	0.02	0.02

1,3-PD — propanediol; LA — lactic acid; FA — fumaric acid; AA — acetic acid; SA — succinic acid; BA — butyric acid; Et — ethanol; % — the percentage of individual metabolites in all synthetized products; $Y_{p/s}$ — production yield (product/substrate $g \times g^{-1}$); P_a — productivity ($g \times dm^{-3} \times h^{-1}$)

ed 20 strains of *C. bifermentans* from samples from desert tortoise *Gopherus agassizii* in California. It was found that none of them were capable of glycerol utilization. However, earlier studies carried out by McCoy and McClung (Mccoy & Mcclung, 1936) and Brooks and Epps (Brooks & Epps, 1958) indicated that *C. bifermentans* are able to carry out glycerol fermentation. In the work presented here this ability was also proven. Because there is no literature data about the glycerol pathway in *C. bifermentans* strains, we decided to investigate it. As a result, it was found that by-products from glycerol utilization such as organic acids (such as lactic, acetic, succinic, and formic acid) are synthetized. Additionally, the level of lactic acid synthesis is high. This ability has not been presented yet by other scientists. In the literature there is more information about amino acid utilization by *C. bifermentans* and volatile acid synthesis (Mead, 1971; Elsden *et al.*, 1976; Elsden & Hilton, 1978) and utilization of glucose into ethanol and acetic acid (Turton *et al.*, 1982).

The ability of *H. alvei* for glycerol utilization has already been described (Homann *et al.*, 1990; Rodriguez *et al.*, 1998; McBee & Schauer, 2006; Hao *et al.*, 2008; Rossi *et al.*, 2012). However, the capability of 1,3-PD production by *H. alvei* has been not investigated yet by any other scientists. Significantly, the metabolic pathway of glycerol in this strain has not been presented. During the course of this work it was found that a by-products from glycerol to 1,3-PD utilization include organic acids such as lactic, acetic, and succinic and ethanol.

C. butyricum is well-known as a 1,3-PD producer from glycerol (Biebl *et al.*, 1998; Hao *et al.*, 2008; Biebl *et al.*, 1992; Kubiak *et al.*, 2012; Wilkens *et al.*, 2012; Samul *et al.*, 2013; Ringel *et al.*, 2012). According to the literature data, *C. butyricum* utilizes glycerol to 1,3-PD and by-products: ethanol and lactic, butyric and acetic acids (Zeng & Biebl, 2002). A new strain from this species was obtained and its glycerol metabolic pathway has been presented in this work. It is also able to produce ethanol and organic acids: lactic, acetic, fumaric, succinic and butyric acid, unlike *C. bifermentans* and *H. alvei*. It occurred that the metabolic pathway of the newly isolated *C. butyricum* DO14 strain was slightly different than in the strain described by Zeng and Biebl (Zeng & Biebl, 2002) – it produces also fumaric and succinic acids.

These results indicate that a natural environment is a good source of bacterial strains with a huge industrial potential. Moreover, these strains show significant biodiversity. The number of strain isolation studies is still on the increase. Scientists are screening the natural environment for new strains capable of glycerol conversion to usable metabolites, including 1,3-PD (Ringel *et al.*, 2012; Jungermann *et al.*, 1973; Hiremath *et al.*, 2011; Sattayasamitsathit *et al.*, 2011; Gelder *et al.*, 2012; Hong *et al.*, 2013). As a result, new strains were obtained, among them: *Trichococcus flocculiformis* (Gelder *et al.*, 2012), *Klebsiella pneumoniae* and *Pantoea agglomerans* (Rossi *et al.*, 2012), *C. butyricum* (Ringel *et al.*, 2012). However, no research focused on the isolation of bacteria from environmental samples of unknown capacity for the synthesis of 1,3-PD has been conducted yet. In the present work, two strains (*C. bifermentans* and *H. alvei*) with so far unknown capacity for the synthesis of 1,3-PD are described.

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