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Original Research

The Application Potential of Newly Isolated Bacteria Strains to 1,3-PD Production

Agnieszka Drożdżyńska, Katarzyna Leja*, Ewa Dzwonkowska, Tomasz Jamruszka, Natalia Dąbrowska, Monika Drobna, Agnieszka Kujawa, Jan Wolski, Katarzyna Czaczyk

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

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Abstract

Because of an increasing interest in "green" processes for the production of chemicals, researchers are constantly looking for new strains. The natural environment offers a rich isolation source. Strains from natural probes have strong metabolic properties because they must adapt to variable conditions, and they are also able to produce a wide range of metabolites efficiently.

This work is a very early report on some capacities of a few bacteria from *Hafnia* and *Citrobacter* genera, isolated from environmental probes to 1,3-PD and organic acids (fumaric, succinic, and acetic) production from glycerol. In this report, the predisposition to effective synthesis of 1,3-PD by investigated strains, based on feedback tests and resistance of bacteria to raw material, have been described.

Keywords: *Citrobacter freundii*, environmental strains, *Hafnia alvei*, resistance of isolates, 1,3-PD

Introduction

Natural environment probes are constantly screened in order to isolate new bacteria strains [1, 2]. Strains isolated from diverse sources are metabolically stronger than the ones collected by us because they must adapt to variable conditions [3, 4]. Thus, indigenous bacteria in the natural environment can produce a wide range of metabolites more efficiently [1, 5, 6].

Since the biodiesel industry is growing rapidly, simultaneously the amount of crude glycerol obtained is also increasing [7]. One solution to that problem is to utilized crude glycerol and convert it into 1,3-PD as well as organic acids via a microbial method [6, 8-10]. Many researchers have worked on screening the natural environment in search of new strains that can effectively convert crude glycerol to metabolites. However, most of the available literature is all about the same bacteria species: *Clostridium butyricum* [11], *Clostridium pasteurianum* [12, 13], *Clostridium diolis*, *Clostridium acetobutylicum*, *Clostridium butylicum*, *Clostridium perfingens*, [14, 15], *Klebsiella pneumonia* [16], *Klebsiella oxytoca* [17], *Klebsiella aerogenes* [16], *Lactobacillus reuterii*, *Lactobacillus buchnerii*, *Lactobacillus collinoides* [18], *Enterobacter agglomerans* [19], *Citrobacter freundii* [20-22], *Pelobacter carbinolicus*, *Rautella planticola*, and *Bacillus welchii* [23]. Thus, the screening of the natural environment for the new strains able to convert glycerol into industrial metabolites is a very important

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

issue. Moreover, during such research it has occurred that some yeasts also are able to convert glycerol into valuable products. As a result, Mirończuk et al. [24] published the first report describing erythritol production from industrial glycerol by the *Yarrowia lipolytica* strain. Because of the high biotechnological potential of *Y. lipolytica*, this microorganism has been successfully subjected to ultraviolet mutagenesis with the aim of enhancing erythritol production and minimizing by-product formation [25].

In our previous work research on the isolation of new bacteria strains from a few species which are not known as a 1,3-PD producers was carried out. As a result, 2,278 strains from *Enterobacter* genera able to 1,3-PD (1,3-propanediol) synthetize from glycerol were obtained [5]. Among them were strains belonging to *C. freundii* and *H. alvei* species [6, 26].

The present work is a very early report on capacities of some bacteria from *Hafnia* and *Citrobacter* genera, isolated from environmental probes, to metabolite production from glycerol. Additionally, it presents diverse features of the isolated bacteria.

Material and Methods

Microorganisms

Five selected bacteria strains were found to be able to assist in industrially important metabolite production. Bacteria were isolated from environmental probes collected in the Wielkopolska District, Poland, between January 2010 and July 2011. The complete isolation procedure, media used, and identification methods are described in Drożdżyńska et al. [26]. The taxonomic affiliation and isolation source of those strains are presented in Table 1.

Bacteria were cultivated in modified Barbirato medium [19]: $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$*7 H_2O 0.4 g/L, $CaCl_{2*}$2 H_2O 0.1 g/L, $CoCl_2$ *6 H_2O 4 mg/L, yeast pepton 2 g/L, bactopepton 2.5 g/L, meat pepton 1.5 g/L, K_2HPO_4 48 g/L, KH_2PO_4 12 g/L, and glycerol 50 g/L.

Inoculum Preparation

All organisms were subculture from stock vials onto bacterial agar slants and passaged at least twice to ensure viability. Incubation conditions throughout were 30ºC in the air. The inoculum was prepared by picking colonies from 24-h-old cultures and suspending the material in 10 ml of sterile 0.85% saline. The resulting suspension was vortexed for 15 s, and the cell density was adjusted by the Wickerham card method. Turbidity was approximately the same as the turbidity standard (0.5 McFarland), which was used for comparison.

Characterization of Strains

Resistance to Glycerol

The investigated strains were cultivated in modified Barbirato medium [19] with glycerol in the level of 20 up to 200 g/L. The control probe was cultivated without glycerol. The resistance to the increasing level of carbon source was tested as was 1,3-PD formation ability. Cultivations were carried out through 168 hours at 30^0C without mixing and gas purge. Metabolic production ability was tested by HPLC technique. Additionally, the efficiency of product formation was calculated as product [g/L] per substrate [g/L]. In this step, pharmaceutical glycerol (PG) (99%) was used.

Technique Glycerol Utilization Ability

The strains were tested to crude glycerol utilization. Glycerol was obtained from four diverse refineries assigned as R1, R2, R3, and R4. The contamination level of raw material was tested using atomic absorption spectrometry (ASA) and described as a chlorides concentration [g/L] in glycerol. This research step was carried out in the Chemistry Department, Poznan University of Life Sciences. A modified Barbirato medium [19] was used. It was supplemented with 50 g/L of glycerol.

Crude glycerol utilization ability was observed as a 1,3-PD production by the investigated strains, which was tested by HPLC technique. Glycerol (PG) was use as a control probe pharmaceutic. Cultivations were carried out over 168 hours at 30^0C.

Feedback Tests

Resistance to main products of glycerol utilization by investigated strains – such as 1,3-PD, ethanol, methanol, lactic (LA) and acetic (AA) acids – was investigated. Cultivations were carried out in a modified Barbirato

Table 1. Taxonomic affiliation and isolation source of investigated strains.

No.	Isolation source	Bacteria morphology	Taxonomic affiliation	Strain symbol
1	Food product	Bacilli G(-)	*Hafnia alvei*	AD27
2	Food product	Bacilli G(-)	*Citrobacter freundii*	AD65
3	Aquarium water	Bacilli G(-)	*Citrobacter freundii*	AD728
4	Pond	Bacilli G(-)	*Citrobacter freundii*	AD970
5	Food product	Bacilli G(-)	*Citrobacter freundii*	AD119

Table 2. Resistance of bacteria to increasing glycerol levels in cultivation medium.

Strain	Glycerol [g/L]	0	20	40	60	80	100	120	140	160	180	200
C. freundii AD119	Δ 1,3-PD [g/L]	0	10.54	19.38	21.71	23.06	22.72	21.86	18.24	19.74	18.51	16.89
	efficiency of 1,3-PD formation[P/S]	0	0.53	0.53	0.46	0.38	0.41	0.30	0.33	0.34	0.25	0.26
C. freundii AD970	Δ 1,3-PD [g/L]	0	11.25	19.49	19.69	21.32	19.99	20.42	19.89	19.26	18.7	17.56
	efficiency of 1,3-PD formation[P/S]	0	0.56	0.53	0.47	0.43	0.29	0.37	0.37	0.33	0.33	0.29
C. freundii AD728	Δ 1,3-PD [g/L]	0	11.74	19.24	21.52	22.47	21.94	20.08	20.87	19.13	19.93	20.91
	efficiency of 1,3-PD formation[P/S]	0	0.69	0.52	0.48	0.44	0.30	0.40	0.38	0.34	0.36	0.34
C. freundii AD65	Δ 1,3-PD [g/L]	0	7.42	13.97	16.91	17.95	16.12	15.93	15.89	16.23	13.65	9.77
	efficiency of 1,3-PD formation[P/S]	0	0.37	0.35	0.36	0.34	0.23	0.28	0.29	0.30	0.26	0.22
C. freundii AD27	Δ 1,3-PD [g/L]	0	8.73	5.14	5.23	17.65	15.37	10.29	4.96	4.95	4.99	0.00
	efficiency of 1,3-PD formation[P/S]	0	0.44	0.17	0.08	0.36	0.37	0.18	0.29	0.16	0.21	0.00

medium [19] with 50 g/L of glycerol as a carbon source. The medium was additionally supplemented with aforementioned chemicals in a concentration determined by the ability of bacteria to produce different metabolites (0 to 200 g/L of 1,3-PD, 1 to 27 g/L acetic acid, 0 to 250 g/L of methanol, and 0 to 80 g/L of ethanol and lactic acid). The control probe was cultivated on modified Barbirato medium [19] with 50g/L of glycerol, without additions. Cultivations were carried out over 168 hours at 30ºC.

Alternative Carbon Source Utilization Ability Tests

These tests used the Biolog System phenotypic matrix PM1. Experiments were carried out according to the manufacturer's instructions.

Antibiotics Sensitivity Test

To characterize antibiotic sensitivities of the isolates they were tested against batteries of both traditional and non-traditional antibiotics using a rotary test method. Bacteria strains were spread onto plate dishes and the modified Barbirato medium [19] was used. Then, the rotaries with different antibiotics were put onto plate dishes. After 24 hours, in the case of strains that were sensitive to the antibiotic, a bright zone (ø>4mm) around the rotary was observed. All the tests were done in duplicate.

Metabolic Pathways of Investigated Strains

Metabolite production abilities by *C. freundii* AD119, *C. freundii* AD970, *C. freundii* AD727, *C. freundii* AD65, and *H. alvei* AD27 were determined using high liquid performance chromatography (HLPC). Modified Barbirato [19] medium with 50 g/L of glycerol was used. Cultivations were carried out over 168 hours at 30ºC, without mixing and gas purge.

Determination of Metabolite Procedures

Determinations of organic acids (acetic, lactic, butyric, and succinic), 1,3-PD, and glycerol were carried out on VWR-HITACHI LaChrom Elite system consisting of an autosampler (model L-2200), pump (model L-2130), refractive index detector (model L-2490), and UV detector (*L-2400*) connected in series. Analyses were performed isocratic ally at a flow rate of 0.6 ml/min at 40°C, on column Remex ROA-organic acid H+, 300x7.8mm (Phenomena). 0,005 N sulfuric acid was used as a mobile phase. Standards were used to identify peaks in chromatograms, and peak area was used to determine sample concentration. This test was done by computer integration (Escrow Elite, Version 3.3.2 SP2) operated in the mode of external standard.

Results

In our research, four selected *C. fundi* and 1 *H. alvei* strains were tested for their ability to metabolite from glycerol production. For this purpose, first we investigated resistance to raw material (glycerol), main metabolic products (1, 3-PD, ethanol, and organic acids), and methanol (a main contaminant of crude glycerol). High concentrations of glycerol and 1,3-PD cause high osmotic

Table 3. The ability of investigated bacteria to facilitate crude glycerol utilization (PG is pharmaceutical glycerol).

Strain	Glycerol source	Contamination level [g/L]	Glycerol [%]	1,3-PD [g/L]
C. freundii AD119	PG	0,01±0.00	99.99	22.72
	R1	1.24±0.32	98.76	10.80
	R2	30.72±0.34	69.28	15.60
	R3	38.61±0.98	61.39	13.50
	R4	37.98±0.41	62.02	13.60
C. freundii AD970	PG	0.01±0.00	99.99	19.99
	R1	1.24±0.32	98.76	18.19
	R2	30.72±0.34	69.28	15.71
	R3	38.61±0.98	61.39	19.01
	R4	37.98±0.41	62.02	15.01
C. freundii AD728	PG	0.01±0.00	99.99	21.94
	R1	1.24±0.32	98.76	14.21
	R2	30.72±0.34	69.28	18.85
	R3	38.61±0.98	61.39	21.06
	R4	37.98±0.41	62.02	18.35
C. freundii AD65	PG	0.01±0.00	99.00	16.12
	R1	1.24±0.32	98.76	15.20
	R2	30.72±0.34	69.28	13.89
	R3	38.61±0.98	61.39	13.83
	R4	37.98±0.41	62.02	9.96
H. alvei AD27	PG	0.01±0.00	99.99	15.37
	R1	1.24±0.32	98.76	4.33
	R2	30.72±0.34	69.28	6.62
	R3	38.61±0.98	61.39	8.47
	R4	37.98±0.41	62.02	5.53

pressure in cultivation environment, thus the ability of strains to survive and maintain metabolic activity in such conditions is a key issue testifying to the potential of strains to produce metabolites on an industrial scale. Also, the resistance of strains to acidification caused by organic acids synthetized from glycerol is very important in the scale-up of 1,3-PD production. The strains should also have an opportunity for the occurrence of alcohols during fermentation, especially to methanol, which is a major contaminant in crude glycerol. In the case of glycerol, the range of 0 up to 200 g/L of this addition in medium was used. The ability of 1,3-PD production from glycerol is presented in Table 2. The tested strains demonstrated different resistance to an increased glycerol level, but all of them were able to 1,3-PD synthetize even from 200 g/L of glycerol (with the exception of the *H. alvei* AD27, for which the upper limit in capacity of 1,3-PD production was 160 g/L). The 1,3-PD production level was highest in the case of the addition of 80 g/L of glycerol for all *C. fundi* strains as well as for *H. alvei* AD27. Moreover, in the case of *Citrobacter* strains, the tendency to increase metabolic production was noted, with an increasing level of added glycerol in the range of 20 to 80 g/L. Further supplementation of medium with glycerol caused a decrease in 1,3-PD synthesis. Surprisingly, *H. alvei* AD27 formed more 1,3-PD from 20 g/L of glycerol than from 40 and 60 g/L. Based on these observations, it can be concluded that bacterial cells are able to survive and maintain the ability of glycerol to 1,3-PD convert in high concentration of this raw material. However, taking into account the efficiency of product formation depending on the addition of glycerol, it must be stated that a high concentration of this raw material is a limiting factor. The highest efficiency of 1,3-PD is observed when glycerol is added in the lowest concentration of 20 g/L.

As biodiesel producers for many years have to struggle with the problem of management of huge amounts of waste glycerol, the optimal solution would be to use it as

Table 4. Resistance to main fermentation product (1,3-PD) presented as a % of utilized glycerol.

Strain/ 1,3-PD [g/L] in medium	0	20	40	60	80	100	120	140	160	180	200
C. freundii AD 119	89.70	90.34	86.08	84.50	80.82	76.60	69.12	67.90	64.04	48.28	34.56
C. freundii AD970	82.72	81.74	76.34	73.76	69.65	65.36	62.24	60.70	57.15	56.89	55.21
C. freundii AD728	91.67	90.05	87.53	83.35	63.26	57.15	57.03	54.72	53.34	51.72	50.83
C. freundii AD65	89.12	88.82	87.01	85.70	63.27	59.05	52.75	51.27	50.64	48.96	47.39
H. alvei AD27	79.77	79.74	78.91	76.06	74.67	23.26	23.11	22.93	21.67	20.95	18.84

a raw material in industrially useful metabolic production. Therefore, all strains investigated in this work were cultivated in medium with the addition of crude glycerol (50 g/L) and their ability to 1,3-PD from this raw material was tested. Crude glycerol from four different sources was used in this experiment. The level of contamination in all of these sources was tested. The results are presented in Table 3. The ability of diol production from crude glycerol is compared with the level of metabolites formed from pure glycerol.

The contamination level in used glycerol has no impact on the 1,3-PD production ability by investigated strains. The purity of R1 glycerol was comparable with the PG one used in control probes. However, this raw material did not give satisfactory results because in the case of only two strains (*C. freundii* AD65) from all five, it decreases 1,3-PD production level only about 5.71% (*C. freundii* AD65) and 7.65% (*C. freundii* AD970). In the case of other strains, glycerol limited production of about 53% (*C. freundii* AD119), 35.24% (*C. freundii* AD728), and 71.83% (*H. alvei* AD27). Two *Citrobacter* strains (AD728 and AD970) were able to effectively synthetize 1,3-PD from R3 glycerol. The levels of obtained metabolite, in comparison with PG, were only 4.01% and 4.90% lower, respectively. R2 glycerol caused a decrease in 1,3-PD synthesis by more than 10% in all strains (31.44 for AD119, 21.42% for AD970, 14.19 for AD728, 13.84% for AD65, and 57% for AD27). These results were comparable in the case of LCD glycerol. Generally, *Citrobacter* strains seem to be more resistant to contamination from glycerol than the *Hafnia* strain. Additionally, the investigated bacteria were able to survive and 1,3-PD synthesis from all types of crude glycerol. Because of such conclusions, further research based on this preliminary study about crude glycerol to industrial metabolic conversion was carried out.

The metabolic activity of bacteria strains are often limited due to an accumulation of products formed during raw material conversion. Thus, resistance of the investigated strains to the main products of the glycerol pathway were tested. The results of the resistance to 1,3-PD were described as glycerol utilization ability (Table 4), and to the rest of metabolites as an 1,3-PD production ability in case of an increasing concentration of such products in bacteria medium (see Table 5). The only significant conclusion from the 1,3-PD resistance test is that all the investigated bacteria strains are able to survive in the presence of a high level of 1,3-PD (up to 200 g/L). It cannot be calculated how much 1,3-PD in the post-fermented broth is a new synthetics because bacteria did not utilize all 1,3-PD from medium and it is detected together with that added 1,3-PD.

These strains were also able to survive and produce 1,3-PD in the presence of ethanol and methanol in the range of 10 up to 80 g/L in the case of ethanol and up to 150 g/L in the case of methanol. The efficiency of diol synthesis decreased linearly with increasing amounts of alcohols. The addition of the highest level of ethanol (80 g/L) into fermentation medium decreased the 1,3-PD amount only to 22.77% and 23.21% in *H. alvei* AD27 and *C. freundii* AD970, respectively. In the remaining strains this limitation was on a higher level (*C. freundii* AD119 38.25%, *C. freundii* AD728 58.21%, and *C. freundii* AD65 65.25%). The smallest decline of 1,3-PD production in the case of the addition of the highest amount of methanol (150 g/L) was observed for *H. alvei* AD27 (35.27%) and *C. freundii* AD970 (35.87%). In the remaining strains this limitation was on a higher level (*C. freundii* AD119 69.55%, *C. freundii* AD728 46.59%, and *C. freundii* AD65 52.73%). Generally, the highest resistance for a high concentration of alcohols in a fermentation environment occurred with *H. alvei* AD27 and *C. freundii* AD970. Moreover, resistance of strains to lactic and acetic acid was investigated. Lactic acid was added into medium in the amount of 10 to 80 g/L and acetic acid in the amount of 2.5 to 25 g/L. In all cultivations bacteria cells were able to survive and a small amount of 1,3-PD formed. The higher resistance to lactic acid was observed in *H. alvei* Ad27; synthesis of 1,3-PD decreased by only 8.72% (in the remaining strains it was more than 30%). An addition of 25 g/L of acetic acid resulted in limited 1,3-PD production to 28.43% in *H. alvei* AD27, and more than a 31% in the case of other strains. Generally, the above-described results showed that the main products of glycerol conversion in *H. alvei* and *C. freundii* do not demonstrate the feedback process and their presence in the fermentation medium does not inhibit 1,3-PD synthesis.

This work also tested the abilities of other carbon source fermentation by all the investigated strains (see Table 6). This information allows elaboration on the co-substrates for fermentation for individual strains. Despite the fact that the four strains belong to the same species

Table 5. Feedback tests (A - ethanol, B - methanol, C - lactic acid, D - acetic acid) presented as an ability to 1,3-PD synthetize [g/L].

A.

Strain/ ethanol [g/L] in medium	0	10	20	30	40	50	60	70	80
C. freundii AD 119	22.72	20.83	19.14	17.93	16.70	15.76	14.70	14.04	14.03
C. freundii AD970	19.99	18.64	18.32	17.31	16.68	15.94	15.87	15.59	15.37
C. freundii AD728	21.94	20.15	19.15	16.76	15.22	13.40	8.71	11.45	9.19
C. freundii AD65	16.12	16.03	15.87	15.10	14.45	10.86	9.23	6.80	5.44
H. alvei AD27	15.37	15.07	15.01	15.10	14.12	13.24	12.23	13.08	12.04

B.

Strain/ methanol [g/L] in medium	0	10	20	40	60	80	100	150
C. freundii AD 119	22.72	19.71	17.13	17.11	15.98	14.79	14.07	6.92
C. freundii AD970	19.99	18.54	15.12	15.08	14.95	14.48	13.89	12.82
C. freundii AD728	21.94	14.86	14.72	13.87	13.55	12.22	12.14	11.72
C. freundii AD65	16.12	15.65	14.45	12.25	11.80	11.07	10.01	7.62
H. alvei AD27	15.37	14.95	14.53	12.53	12.23	11.29	10.57	9.95

C.

Strain/ LA [g/L] in medium	0	10	20	30	40	50	60	70	80
C. freundii AD 119	22.72	17.17	15.70	15.48	15.33	15.30	15.27	14.89	14.22
C. freundii AD970	19.99	16.79	15.29	13.29	12.65	12.65	11.11	10.56	10.46
C. freundii AD728	21.94	18.16	14.35	14.18	14.06	14.02	13.86	11.92	11.62
C. freundii AD65	16.12	14.1	12.79	12.34	11.77	11.75	11.74	11.65	11.09
H. alvei AD27	15.37	15.30	15.22	15.03	14.97	14.32	14.29	14.15	14.03

D.

Strain/ AA [g/L] in medium	0	2.5	3.5	6	9	10	15	17	20	25
C. freundii AD 119	22.72	20.43	19.75	17.02	15.49	11.62	9.30	7.78	7.14	7.05
C. freundii AD970	19.99	16.88	15.23	12.62	9.05	7.98	7.62	7.57	7.16	6.96
C. freundii AD728	21.94	16.11	14.84	10.67	9.35	19.23	9.15	9.03	9.02	8.86
C. freundii AD65	16.12	15.23	13.73	10.45	6.67	6.24	3.76	3.25	3.21	3.02
H. alvei AD27	15.37	14.84	14.81	14.67	13.67	11.98	11.16	11.14	11.04	11.00

LA – lactic acid, AA – acetic acid

(*Citrobacter* spp), they show different saccharides and acid utilization ability. Also, the test of antibiotic sensitivity (see Table 7) is evidence of diversity of the strains under investigation. Resistance to antibiotics is an important property for strains applied in industry. Strains with multiple antibiotic resistance should not enter the natural environment because it could lead to the formation of mechanisms of resistance in cells of bacteria naturally occurring in the environment.

Since *C. freundii* and *H. alvei* strains revealed the ability to utilize a large amount of glycerol, the ability to maintain main metabolic pathways under stressed conditions (large amounts of alcohols and acids in fermentation broth), the main hypothetical glycerol pathways in these strains were investigated. We found that all the investigated strains were capable of 1,3-PD, ethanol, lactic, acetic, succinic, and fumaric acid production (Fig. 1). Despite the fact that the metabolic profiles of the investigated bacteria were similar, the quantitative ratios between individual products were different in all four strains. The percentage of individual metabolites in all synthetized products is also presented in Fig. 1. The main metabolite in all the

Table 6. The ability to use other carbon sources by the investigated strains.

Source of carbon	*C. freundii* AD970	*C. freundi*i AD65	*C.freundii* AD728	*C. freundii* AD119	*H. alvei AD27*
L-arabinose; N-acetylo-D-glukozoamine; D-galactose; D-asparagine acid; D-trehalose; D-mannose; glycerol; D-glucoronic acid; D-gluconic acid; glycerol phosphate; D-xylose; L-lacic acid; D-mannitol; glucose-6-phosphate; D,L-malic acid; D-rybose; D-fructose; D-glucose; maltose; tymidine; L-asparagine; uridine; glucose-1-phosphate; fructose-6-phosphate; β-metyloglucoside; maltoriose; deoxyadenosine; adenosine; fumaric acid; inosine; L-seryne; monomethyl succinian; L-malic acid; D-galacturonic acid; pyruvate acid; L-galacturonic acid lactone; succinic acid; D-sorbitol; D-asparagine acid; methyle-D-galactoside; D-lactose; glycyl asparagine acid; citric acid; bromosuccinic acid; 1,2,3-propan tricarboxylic acid; L-treonine; methyl pyruvate; D-malic acid	+	+	+	+	+
L-proline; D-alanine; L-alanyl-glycyne; glycyl-L-proline	+	+	+	-	-
D-seryne; L-fucose	+	-	+	+	+
L-ramnose	+	+	-	+	+
N-acetylo-D-mannosamine	+	+	+	+	+
D-glucaric acid; muconic acid; glycyl glutamic acid	+	+	+	-	+
saccharose	-	+	+	+	-
propionic acid	+	+	+	-	-
D-celobiose	+	-	+	-	-
D-melibiose	+	+	+	+	-
glucosamine acid	-	+	-	+	-
α-keto-butyric acid; L-glutamic acid; α –hydroxy-butyric acid	+	+	-	-	+
dulcitol	+	-	+	-	-
inositol	-	-	+	-	-
acetic acid	+	-	-	-	-
formic acid	-	+	-	-	-
L-lyxose	-	-	-	-	-
L-glutamine acid; Tween 20; 1,2-propanediol; Tween 40; α –keto-glutaric acid; lactulose; tartaric acid; Tween 80; α – hydroxyglutaric acid lactone; ribitol; treonine; glicole acid; glioxalic acid; acetylacetic acid; tyramine; glucuronic acid amid; phenyloethylamine; ethanoloamine. D-galacturonic acid lactone; 4-hydroxyphenyloacetic acid; 3-hydroxyphenyloacetic acid; D-psicose	-	-	-	-	+

strains was 1,3-PD, which included more than 70% of all fermentation products. The bacteria also formed a lot of lactic acid (despite *H. alvei* AD27, which synthetized only trace amounts of this acid) and concerns ca. 40% of all the metabolites. Acetic acid was synthetized in all the strains on the level of ca. 4.5% (*C. freundii* AD65) to 9% (*C. freundii* AD119, and *C. freundii* AD970) of total metabolites. Small amounts of succinic acid were formed (2-3%) in the case of all strains. The level of synthetized ethanol was between 2% (*C. freundii* AD119 and *C. freundii* AD728) up to 15% (*C. freundii* AD65 and *H. alvei* AD27).

Discussion

The natural environment is screening new bacteria strains that can be used in many branches of industry for metabolite production. Bacteria isolated from natural probes have a unique character and adapt to many undesirable conditions for the collection strains [27]. In the Department of Biotechnology and Food Microbiology at Poznan University, new strains able to produce 1,3-PD from glycerol were isolated from probes collected from natural sources. During this research many strains from species known as natural 1,3-PD producers have been

Table 7. Antibiotic sensitivity tests.

Antibiotic/strain	*C. freundii* AD119	*C. freundii* AD970	*C. freundii* AD728	*C. freundii* AD65	*H. alvei* AD27
Amoxycillin 10 ug	+	-	+	-	+
Ampicillin 10 ug	+	+	-	-	-
Azithromycin 15 ug	+	+	+	+	+
Bacitracin 10 U	-	-	-	-	-
Chloramphenicol 30 ug	+	+	+	+	+
Cefixime 5 ug	+	+	+	+	+
Ciprofloxacin 5 ug	+	+	+	+	+
Gentamicin 30 ug	+	+	+	+	+
Cefotaxime 30 ug	+	+	+	+	+
Doxycycline 30 ug	+	+	+	+	+
Erythromycin 15 ug	-	+	+	-	+
Enrofloxacin 5 ug	+	+	+	+	+
Fusidic acid 10 ug	-	-	-	-	-
Imipenen 10 ug	+	+	+	+	+
Kanamycin 30 ug	+	+	+	+	+
Cephalothin 30 ug	+	+	+	-	-
Meropenem 10 ug	+	+	+	+	+
Lincomycin 15 ug	-	-	-	-	-
Neomycin 30 ug	+	+	+	+	+
Norfloxacin 10 ug	+	+	+	+	+
Nystatin 100 U	-	-	-	-	-
Novobiocin 30 ug	-	-	-	-	+
Penicillin G 1 U	-	-	-	+	-
Penicillin G 10 U	+	-	+	-	+
Polymyxin B 300 U	+	+	+	+	+
Rifampicin 5 ug	+	+	+	-	+
Streptomycin 10 ug	+	+	+	+	+
Tetracycline 30 ug	+	+	+	+	+
Ticarcillin/Clavulanic acid 7.5:1 85 ug	+	+	+	+	+
Vancomycin 30 ug	-	-	+	-	-

+ resistant to antibiotic - sensitive to antibiotic

acquired [5, 26] but also new 1,3-PD producers developed, such as *Clostridium bifermentans* and *Clostridium sordelli* [5], and *H. alvei* [26]. In that work, predispositions to 1,3-PD and other industrial metabolites of one *H. alvei* strain and four *C. freundii* strains were investigated. In the first step of the experiment, resistance of the investigated strains to increasing concentration of raw materials, glycerol, and main metabolic products (1,3-PD, ethanol, acetic, lactic, fumaric, and succinic acids) were tested. It occurred that all these strains have an ability to survive in such conditions and they maintain the 1,3-PD metabolic pathways. *H. alvei* and *C. freundii* strains were isolated from food products, aquarium water, and ponds so that they were in a natural way exposed to variable and not optimal conditions. We demonstrated that these strains adapt to these prevailing conditions and they became more resistant [28-32]. Thus, these strains may be applied in large-scale metabolic production.

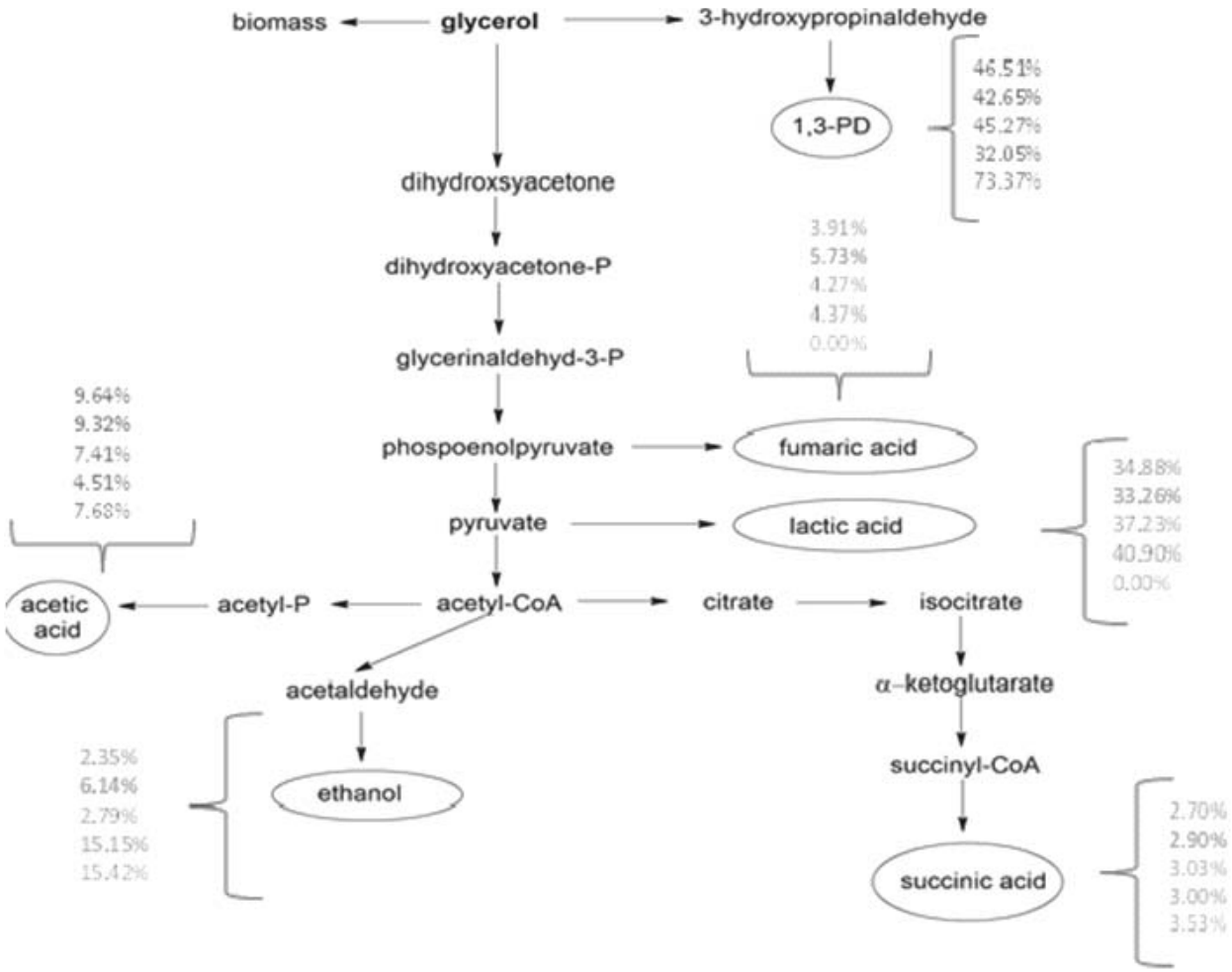

Fig. 1. Hypothetical glycerol pathway in the investigated strains (the percentages of individual metabolites in all synthetized products are presented: red AD119, blue AD 970, green AD728, orange AD65, grey AD27 strains).

It has been estimated that up to 40,000 tons of crude glycerol (the main byproduct in the biodiesel industry) will be produced each year by 2020 [7]. Thus, in the next step of the present work the ability of the investigated strains to four types of crude glycerol fermentation to 1,3-PD and other metabolites were tested. The used types of glycerol were of varying quality. However, the quantity of formed 1,3-PD was not dependent on the level of impurities in it. This is probably because of the biodiversity of strains and their resistance to some impurities, such as chlorides. Additionally, some of these impurities (e.g., potassium, sodium, and magnesium chlorides) may be used as enzyme cofactors [33]. In another work researchers reported that the efficiency of 1,3-PD production was the same for both types of glycerol: pure and crude [34]. A few reports have demonstrated that efficiency of 1,3-PD production is higher from crude glycerol than from pure [35-37]. Accordingly, it may be true that the quality of impurities of glycerol has less impact on 1,3-PD synthesis than quantity [33].

The investigated strains are responsible for high oncentrations of all products formed during glycerol fermentation. This is an important feature, as it facilitates the use of bacteria on an industrial scale. In the available literature there is only limited information about a feedback phenomenon in other 1,3-PD producers. Mostly morphological, physiological, and biochemical characteristics have been researched so far [38]. However, Abbad-Andaloussi [39] described the cell growth inhibitions in high concentrations of glycerol and 1,3-PD in *Clostridium butyricum* DSM5431. Scientists try to improve the resistance of the bacteria to raw material and main fermentation products by a mutagenesis technique. In the case of the investigated isolates in the present work, it was redundant as strains had natural resistance to glycerol, 1,3-PD, ethanol, and organic acids.

Because the strains used in this work were isolated from natural probes, their biochemical properties have not been studied in more detail. The Biolog Identification System (Biolog, Inc., Hayward, Calif.) was used to test the ability of microorganisms to oxidize a panel of 95 different carbon sources. It occurred that strains (even the ones that belong to the same genera) indicated biodiversity. The majority of carbon sources were utilized by *C. freundii* AD970, *C. freundii* AD65, and *C. freundii* AD728. *C. freundii* AD119 and *H. alvei* AD27 showed low enzymatic activity associated with disposal of individual chemical compounds. These differences

between strains are probably the result of their adaptation to different environments of their natural existence [40]. Moreover, resistance to 30 different antibiotics was tested. All strains were resistant to the majority of them (ca. 20), and only a small diversity between strains was observed. The reason for this significant resistance is an easiness with which bacteria adapted to diverse environments. This results from the fact that bacteria are haploid organisms. Thus even a single point mutation gives a phenotypic effect [41].

Conclusion

Strains isolated from natural probes have a wide range of properties and that are interesting for several industrial applications. The incoherence in properties of *C. freundii* and *H. alvei* in different research results is a symptom of a great variety of isolated strains. It may also be connected with different sources of their isolation. Cells haploidy makes them genetically flexible, which leads to genetic changes due to the lack of sensitivity to stressor factors. In summary, the hypothetical glycerol pathway in all the investigated strains was prepared (see Fig. 1). Additionally, the percentage of individual metabolites in all the synthetized products was indicated.

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References

1. LOPEZ M.J., NICHOLS N.N., DIEN B.S., MORENO J., BOTHAST R.J. Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. Appl. Microbiol. Biotechnol. **64**, 125, **2011**.
2. MOHSENI M. Isolation and Optimization of Ethanol Producing Bacteria from Natural Environments of Mazandaran Province in Iran. J. Gen. Res. **1** (1), 35, **2015**.
3. MELO A.D.B., FIGUEIREDO A.A., BITTENCOURT S.G.L.F., A.C.L. BATISTA, HORÁCIO R.M. Antimicrobial effect against different bacterial strains and bacterial adaptation to essential oils used as feed additives. Canadian J. Vet. Res. **79** (4), 285, **2015**.
4. SNAUWAERT I., STRAGIER P., VUYST L.D., VANDAMME P. Comparative genome analysis of *Pediococcus damnosus* LMG 28219, a strain well-adapted to the beer environment. BMC Genomics **16**, 267, **2015**.
5. LEJA K., MYSZKA K., KUBIAK P., WOJCIECHOWSKA J., OLEJNIK-SCHMIDT A.K., CZACZYK K., GRAJEK W. Isolation and identification of *Clostridium* spp. from natural samples that performs effective conversion of glycerol to 1,3-propanediol. Acta Sci. Pol., Biotechnologia **10** (2), 25, **2011**.
6. LEJA K., MYSZKA K., SCHMIDT-OLEJNIK A.K., JUZWA W., CZACZYK K. Selection and characterization of *Clostridium bifermentans* strains from natural environment capable of producing 1,3-propanediol under microaerophilic conditions. **8** (11), 1187, **2014**.
7. BRUNO H.A., DE ANDRADE B.A., KLEBER Z.A.C., DA SILVA-FILHO C., LACERDA V. Recent advances in using niobium compounds as catalysts in organic chemistry. Curr. Org. Synth. **12** (5), 570, **2015**.
8. TALARICO T.L., AXELSSON L.T., NOVOTNY J., FIUZAT M., DOBROGOSZ W.J. Utilization of glycerol as a hydrogen acceptor by *Lactobacillus reuteri*: purification of 1,3-propanediol : NAD+ oxidoreductase1990. Appl. Environ. Microbiol. **56** (4), 943-948, **1990**.
9. BOENIGK R., BOWIEN S., GOTTSCHALK G. Fermentation of glycerol to 13-propanediol in continuous cultures of *Citrobacter freundii*. Appl. Microbiol. Biotechnol. **38**, 453, **1993**.
10. WOJTUSIK M., RODRÍGUEZ A., RIPOLL V., SANTOS V.E., GARCÍA J.L., GARCÍA-OCHOA F. 1,3-Propanediol production by *Klebsiella oxytoca* NRRL-B199 from glycerol. Medium composition and operational conditions. Biotechnol. Rep. **6**, 100, **2015**.
11. COLIN T., BORIES A., MOULIN G. Inhibition of *Clostridium butyricum* by 1,3-propanediol and diols during glycerol fermentation. Appl. Microb. Biotechnol. **54** (2), 201, **2000**.
12. BIEBL H., MARTEN S., HIPPE H., DECKWER W.D. Glycerol conversion to 1,3- propanediol by newly isolated clostridia. Appl. Microbiol. Biotechnol. **36**, 592, **1992**.
13. DABROCK B., BAHL H., GOTTSCHALK G. Parameters affecting solvent production by *Clostridium pasteurianum*. Appl. Environ. Microbiol. **58**, 1233, **1992**.
14. YOUNGLESON J.S., JONES W.A., JONES D.T., WOODS D.R. Molecular analysis and nucleotide sequence of the adh1 gene encoding an NADPH-dependent butanol dehydrogenase gene in the gram-positive anaerobe *Clostridium acetobutylicum*. Gene **78**, 355, **1998**.
15. HAO J., WEI W. ‚JIESHENG T., JILUN L., DEHUA L. Decrease of 3-hydroxypriopionaldehyde accumulation in 1,3-propanediol production by over-expressing dhaT gebe in Klebsiella pneumonia TUAC01I. Ind. Microbiol. Biotechnol., **35**, 735, **2008**.
16. BIEBL H., MENZEL K., ZENG A.P., DECKWER W.D. Microbial production of 1,3-propanediol. Appl. Microbiol. Biotechnol. **52**, 289, **1998**.
17. HOMANN T., TAG C., BIEBL H., DECKWER W.D., SCHINK B. Fermentation of glycerol to 13-propanediol by *Klebsiella* and *Citrobacter* strains. Appl. Microbiol. Biotechnol. **33**, 121, **1990**.
18. DA SILVA G.P., MACK M., CONTIERO J. Glycerol: A promising and abundant carbon source for industrial microbiology. Biot. Adv. **27**, 30, **2009**.
19. BARBIRATO F., HIMMI E. H., CONTE T., BORIES A. 1,3-propanediol production by fermentation: An interesting way to valorize glycerin from the ester and ethanol industries. Ind. Crops Products. **7**, 281, **1998**.
20. BOENIGK R., BOWIEN S., GOTTSCHALK G. Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii*. Appl. Microbiol. Biotechnol. **38** (4), 453, **1993**.
21. DANIEL R., BOENIGK R., GOTTSCHALK G. Purification of 1,3-propanediol dehydrogenase from *Citrobacter freundii* and cloning sequencing and overexpression of the corresponding gene in *Escherichia coli*. J. Bacteriol. **3**, 2151, **1995**.

22. MALINOWSKI J. Evaluation of liquid extraction potentials for downstream separation of 1,3-propanediol. Biot. Tech. **13**, 127, **1999**.
23. SAXENA R.K., PINKI A., SAURABH S., JASMINE I. Microbial production of 13-propanediol: Recent developments and emerging opportunities. Biot. Adv. **27**, 805-913, **2009**.
24. MIROŃCZUK A.M., RAKICKA M., BIEGALSKA A., RYMOWICZ W., DOBROWOLSKI A. A two-stage fermentation process of erythritol production by yeast *Y. lipolytica* from molasses and glycerol. Biores. Technol. **198**, 445, **2015**.
25. MIROŃCZUK A.M., DOBROWOLSKI A., RAKICKA M., RYWIŃSKA A., RYMOWICZ W. Newly isolated mutant of *Yarrowia lipolytica* MK1 as a proper host for efficient erythritol biosynthesis from glycerol. Proc. Biochem. **50** (1), 61, **2015**.
26. DROŻDŻYŃSKA A., PAWLICKA J., KUBIAK P., KOŚMIDER A., PRANKE D., OLEJNIK-SCHMIDT A., CZACZYK K. Conversion of glycerol to 1,3-propanediol by *Citrobacter freundii* and *Hafnia alvei* – newly isolated strains from the *Enterobacteriaceae*. New Biotechnol. **31** (5), 402, **2014**.
27. TORSVIK V., DAAE F.L., SANDAA R.A., ØVREÅS L. Novel techniques for analysing microbial diversity in natural and perturbed environments. J. Biotechnol. **64** (1), 53, 1998.
28. CASES, DE LORENZO V., OUZOUNIS CH.A. Transcription regulation and environmental adaptation in bacteria. Trends in Microbiol. **11** (6), 248, **2003**.
29. GIAOURIS E., CHORIANOPOULOS N., NYCHAS G.J. Acquired acid adaptation of *Listeria monocytogenes* during its planktonic growth enhances subsequent survival of its sessile population to disinfection with natural organic compounds. **64**, 896, **2014**.
30. MORADIGARAVAND D., ENGELSTÄDTER J. The impact of natural transformation on adaptation in spatially structured bacterial populations. BMC Evolution. Biol. **14**, 141, **2014**.
31. KOPF M., KLÄHN S., SCHOLZ I., HESS W.R., VOß B. Variations in the non-coding transcriptome as a driver of inter-strain divergence and physiological adaptation in bacteria. Scientific Reports. DOI: 10.1038/srep09560
32. KRAEMER S.A., KASSEN R. Patterns of local adaptation in space and time among soil bacteria. The American Naturalist.**185** (3), 317, **2015**.
33. SAMUL D., LEJA K., CZACZYK K. Impurities of crude glycerol and their effect on metabolite production. Ann. Microbiol. **64**, 891, **2014**.
34. CHATZIFRAGKOU A., DIETZ D., KOMAITIS M., ZENG A.P., PAPANIKOLAOU S. Effect of biodiesel-derived waste glycerol impurities on biomass and 1,3-propanediol production of *Clostridium butyricum* VPI 1718. Biotechnol. Bioeng. **107**, 76, **2010**.
35. RYWIŃSKA A., RYMOWICZ W., ŻLAROWSKA B. Biosynthesis of citric acid from glycerol by acetate mutants of *Yarrowia lipolytica* in fed-batch fermentation. Food Technol. Biotechnol. **47**, 1, **2009**.
36. JUN S.A., MOON C., KANG C.H., KONG S.W., SANG B.I., UM Y. Microbial fed-batch production of 1,3-propanediol using raw glycerol with suspended and immobilized *Klebsiella pneumoniae*. Appl. Biochem. Biotechnol. **161**, 491, **2010**.
37. JITRWUNG R., YARGEAU V. Optimization of media composition for the production of biohydrogen from waste glycerol. Int. J. Hydrogen Energy **36**, 9602, **2011**.
38. ZHANG G.L., MA B.B., XU X.L., LI CH., WANG L. Fast conversion of glycerol to 1,3-propanediol by a new strain of *Klebsiella pneumoniae*. Biochem. Eng.J. **37** (3), 256, **2007**.
39. ABBAD-ANDALOUSI S., MANGINOT-DURR C., AMINE J., PETITDEMANGE E., PETITDEMANGE H. Isolation and characterization of *Clostridium butyricum* DSM 5431 mutants with increased resistance to 1,3-propanediol and altered production of acids. Appl. Environm. Microbiol. **61** (12), 4413, **1995**.
40. DAHM H. Metabolic activity of bacteria isolated from soil, rhizosphere and mycorrhizosphere of pine (*Pinus silvestris* L.). Acta Microbiol. Polonica **33** (2), 157, **1984**.
41. HAYES W. The genetics of bacteria and their viruses. Studies in basic genetics and molecular biology. In: The genetics of bacteria and their viruses. Studies in basic genetics and molecular biology. pp 734, pp.014, **1964**.