ISOLATION AND IDENTIFICATION OF CLOSTRIDIUM SPP. FROM NATURAL SAMPLES THAT PERFORMS EFFECTIVE CONVERSION OF GLYCEROL TO 1,3-PROPANEDIOL

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Abstract. Indigenous bacteria in the natural environment can produce a wide range of metabolites more efficiently. The aim of this work was to isolate from the natural environment non-pathogenic Clostridium strains that are able to convert glycerol to 1,3-propanediol and other metabolites of potential uses in industry. The effective methods of selection and maintenance of anaerobic cultures in the laboratory conditions were also investigated. Samples were pre-cultured on modified PY medium consisted 50 g/l of glycerol. Isolated colonies growth on TSC medium were screened on the basis of morphological characters typical for Clostridium sp. Isolated bacterial strains were allowed to growth in selective media such as RCM and modified PY. The metabolites of bacteria were investigated by the HPLC technique. The bacteria strains were identified by 16S rRNA technique. The highest percentage of isolates of the genus Clostridium were obtained from excrements of animals, compost, and silages. Nearly 60% were able to convert glycerol to 1,3-propanediol. The highest capacity for utilization of glycerol to 1,3-propanediol was observed in case of the species of Clostridium bifermentans and Clostridium sordelli. The most of examined microflora were also able to short-chain organic acids and ethanol synthesis.

Key words: Clostridium spp., isolation, 1,3-propanediol, microflora of natural environment, short-chain organic acids

INTRODUCTION

The natural environment offers microorganisms which may be used in many branches of industry. Isolation of such microflora and investigation the possibility of its industrial application is an important step to replace the chemical synthesis by the biotechnological
processes. The chemical syntheses generate a lot of by-products which pollute natural environment. Moreover, it is lack of specificity. These problems can be overcome by using the biotechnological methods [Kaeberlein et al. 2002].

Strains isolated from the natural environment (bottom sediments, composts, soils, and excrements of domestic and wild animals) posses metabolites production capability. Many microbial strains, capable of industry useful metabolites production, are available commercially, among other from the ATTC collection. However, indigenous bacteria in the soil can product a wide range of metabolites more efficiently [Lopez et al. 2004]. An example of the end-products for different microorganisms during glycerol degradation are presented in the Figure 1.

Fig. 1. The end-products from the conversion of glycerol by microbiological way [da Silva et al. 2009]
An important group of environmental microorganisms is *Clostridium* spp. The clostridia are a heterogeneous group of bacteria which share a small number of common features: they are anaerobic, Gram-positive, endospore-forming rods, without the capacity for dissimulator sulfate reduction. The species have wide biotechnological potential, among other in the conversion of renewable biomass to commodity chemicals, and in produce potent toxins which are causative agents of disease. Clostridia are able to metabolize an extremely wide range of organic molecules, among others sugars and other carbohydrates, organic acids, alcohols, aromatic compound, amino acids, amines, purines, and pyrimidine. They can be use to solvent (butane, acetone, and ethanol) and acids (succinic, acetic, butyric, and lactic) produce [Mitchell 2001]. The *Clostridium* sp. are also able to 1,3-propanediol (1,3-PD) biosynthesis. 1,3-PD, a typical product of glycerol fermentation. It is one of the most interesting raw materials for chemical industries due to its wide use in different fields, e.g. it is a valuable chemical intermediate applied in organic synthesis. It is also used as a monomer for the production of biodegradable polymers (polyesters, polyether, polyurethanes, etc.), cosmetics, lubricants, medicines, and as an intermediate for the synthesis of heterocyclic compounds [Menzel et al. 1997, Biebl et
al. 1999, Katrlik et al. 2007]. Recently, 1,3-PD is also used as a monomer to synthesize a new type of a polyester – polytrimethylene terephthalate [Biebl et al. 1999, Zeng and Biebl 2002, Liu et al. 2007, Zhang et al. 2007].

The aim of this work was to isolate from the natural environment non-pathogenic Clostridium strains that are able to convert glycerol to 1,3-PD and other metabolites of potential uses in industry. The effective methods of selection and maintenance of anaerobic cultures in the laboratory conditions were also investigated.

MATERIALS AND METHODS

Collection of samples

Samples from excrements of animals and composts, composts and silages, samples from biogas works, soils, active sludge, rivers’ sludge and wastes from food industry were collected from the Wielkopolska District during January – December 2010. Samples were collected in sterile plastic jars and stored in refrigerator until experimentation.

Isolation of microorganisms and maintenance of cultures

Samples were pre-cultured on modified PY medium according to Biebl and Spöer [2002]. The modified PY medium consisted of (g/l): BactoPeptone 10; yeast extract 10; glycerol 50; CaCl₂, MgSO₄ x 7H₂O 0.96; K₂HPO₄ 2; NaHCO₃ 20; NaCl 4. The pre-cultivation step was conducted under anaerobic condition using Anaerobic Jar with Gas Generating Kit (Oxoid, UK) at 30°C for 7 days. After incubation period, samples were pretreated at 80°C for 12 min., diluted with sterile solution of sodium chloride and then spread onto TSC agar plates (Biocorp, Poland). The plates were incubated for 24 h under anaerobic condition using Anaerobic Jar with Gas Generating Kit (Oxoid, UK) at the temperature of 30°C. Isolated colonies were screened on the basis of morphological characters (black colonies or black with a 2-4mm opaque white zone surrounding the colonies as a result of lecithinase activity). To make pure culture and maintain culture conditions for the bacteria, screened colonies were transferred on both TSC agar plates (Biocorp, Poland) and RCM broth (Biocorp, Poland).

Screening of bacterial isolates for producing 1,3-PD

Isolated bacterial strains were allowed to grow in modified PY medium for 7 days at 30°C. After incubation period the broths were centrifuged at 3000 rpm for 10 min. The cell free supernatants was collected and used for estimation of 1,3-PD production via high liquid performance chromatography (HPLC) technique. In the experiments Hewlett Packard system consisted of autosampler, pump and refractive index detector was carried out. Analysis were performed isocratically at flow rate 0.6ml/min. at 65°C, on column Aminex HPX-87H 300x7.8 (BIO-RAD). 0.5mN H₂SO₄ as a mobile phase was used. Standards were applied to identify peaks in chromatograms, and peak areas were used to determine the samples concentration. It was conducted by computer integration (ChemStation, Agilent) operated in the mode of external standards.

Sequencing and phylogenetic analysis

Total DNA from bacteria was extracted using Genomie Mini AX Bacteria Kit (A&A Biotechnology, Gdańsk, Poland) after initial incubation in 50 mg/mL lysozyme (Sigma)
for 1 h at 37°C. Sequences encoding small subunit of rRNA were amplified in PCR re-
action using primers SDBact0008aS20 and SUuniv1492bA21 [Suau et al.1999]. PCR
products were purified using Clean-up Kit (A&A Biotechnology, Gdańsk, Poland) and
sequenced at Genomed, Warszawa, Poland with primers used for PCR and additionally for
inner sequence with GTGCCAGCMGCCGCTAA primer. Obtained sequences were
arranged into contigs and identified in BLAST service of the GenBank database [Altschul
et al. 1990].

RESULTS AND DISCUSSION

Despite a wide range available selective media for isolating specific groups of bacte-
ria from food and clinical samples, there are relatively few media available for isolation
anaerobic microflora colonizing natural samples. George et al. [1979], Karasawa et al.
[1995], Barbirato et al. [1998], and Biebl and Spöer [2002] made an effort to developing
media for Clostridium sp. However to date no medium has been extensively examined and
accepted for routine isolation and maintenance of environmental isolates in laboratory. In
this study, different plating media and different enrichment selective broths were tested
according to their ability for isolation and enumeration of anaerobic bacteria of the genus
of Clostridium from natural samples (our unpublished data). However, the procedure that
gave the highest yield of isolates of Clostridium spp. from each tested samples consisted of
selective enrichment PY and RCM broths followed by plating out onto TSC agar.

In the work all tested natural samples yielded Clostridium spp. isolates (Tab. 1). The
highest percentage of isolates of the genus of Clostridium came from excrements of animals
(726 isolates) and composts and silages (774 isolates). In addition in our study all bacterial
strains were tested for their ability to grow with glycerol in the medium and to converse
this substrate to 1,3-PD. Of 2278 isolates tested nearly 60% fermented glycerol to 1,3-PD.
Excrements of animals as well as compost and silages contained the highest number of
isolates able to produce 1,3-PD (Tab. 1). Amplification and sequence analysis showed that
all 1,3-PD-positive strains have a molecular profile consistent with Clostridium spp. (ap-
proximately 98% confidence). Isolates of the highest capacity for utilization of glycerol
to 1,3-PD belonged to the species of Clostridium bifermentans and Clostridium sordelli.
Interestingly, the glycerol fermentation by these species has not been investigated.

In the study we also aimed to characterize the products of glycerol fermentation by
examined Clostridium bifermentans and Clostridium sordelli. The work of Papanikolo-
lau et al. [2004], González-Pajuelo et al. [2006], and Rehman et al. [2008] concluded
that Clostridium acetobutylicum, Clostridium butyricum, and Clostridium pasteurianum
obtained from the culture collection are able to convert glycerol to 1,3-PD and other
metabolites, such as ethanol, acetic and butyric acids on the high level. In the literature
there are any information concerns 1,3-PD production by microflora existed in natural
environment. It is high probability that wild strains can produce above mentioned meta-
bolites in highest level.
### Table 1. Samples processed and obtained isolates

<table>
<thead>
<tr>
<th>Materials</th>
<th>No of isolates of <em>Clostridium</em> spp</th>
<th>No of 1,3-PD-positive isolates of <em>Clostridium</em> spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>excrements of animals</td>
<td>726</td>
<td>480</td>
</tr>
<tr>
<td>odchody zwierzęce</td>
<td>774</td>
<td>473</td>
</tr>
<tr>
<td>composts and silages</td>
<td>256</td>
<td>122</td>
</tr>
<tr>
<td>samples from biogas works</td>
<td>243</td>
<td>141</td>
</tr>
<tr>
<td>soils</td>
<td>225</td>
<td>97</td>
</tr>
<tr>
<td>active sludge</td>
<td>31</td>
<td>9</td>
</tr>
<tr>
<td>rivers' sludge</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>wastes from food industry</td>
<td>2278</td>
<td>1344</td>
</tr>
</tbody>
</table>

### Total

Całkowicie

Table 2 performs the amounts of products formed per gram of glycerol fermented by selected microflora. 1,3-PD was the main product, but acetic acid, lactic acid, formic acid and ethanol were also formed. Isolates of *Clostridium bifermentans* obtained from silages showed 1,3-PD production at the level of approximately 9.8 g/l. These two isolates possess also ability to produce the lactic acid. The production of the lactic acid metabolite exceeded the value of 8.0 g/l. In most cases the production of 1,3-PD by isolates of *Clostridium bifermentans* obtained from soil crossed the value of 11.0 g/l, whereas the lactic acid was synthesized at lesser amount (Tab. 2). During glycerol fermentation these strains produced also acetic and formic acids. Similar trend was observed for *Clostridium sordelli*. The products of glycerol fermentation composed mainly of 1,3-PD (7.31 g/l) and lactic acid (5.21 g/l). In this experiment the amount of acetic acid, formic acid and ethanol crossed the level of 1.0g/l (Tab. 2). As it was mentioned, some bacteria strains, but only a few of all, are able to 1,3-PD biosynthesis. The conversion of glycerol to 1,3-PD is necessary for them because it provides energy for cell growth [Biebl et al. 1999]. Production of organic acids by microorganisms plays a key role in the process of controlling of appearance of other genus of bacteria in particular environment [Ishii et al. 2000]. Siragusa and Dickson [1992] stated that a small amount of short-chain organic acids reduced growth of coexisting non sporogenes microflora.
Isolation and identification...

Table 2. Profile of metabolites of glycerol fermentation by selected *Clostridium* spp.
Tabela 2. Profil metaboliczny fermentacji glicerolu przez wybrane szczepy *Clostridium*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source of isolation</th>
<th>1,3-PD</th>
<th>Lactic acid</th>
<th>Formic acid</th>
<th>Acetic acid</th>
<th>Ethanol [g/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bifermentans</em></td>
<td>silage obornik</td>
<td>8.71</td>
<td>4.58</td>
<td>1.13</td>
<td>1.48</td>
<td>2.43</td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td>silage obornik</td>
<td>10.18</td>
<td>8.67</td>
<td>2.34</td>
<td>3.73</td>
<td>0.49</td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td>silage obornik</td>
<td>9.78</td>
<td>8.19</td>
<td>1.94</td>
<td>3.81</td>
<td>nd</td>
</tr>
<tr>
<td><em>sordelli</em></td>
<td>soil gleba</td>
<td>16.98</td>
<td>3.23</td>
<td>0.28</td>
<td>1.46</td>
<td>0.46</td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td>soil gleba</td>
<td>14.89</td>
<td>6.66</td>
<td>1.36</td>
<td>3.04</td>
<td>0.44</td>
</tr>
<tr>
<td><em>bifermentans</em></td>
<td>soil gleba</td>
<td>12.88</td>
<td>1.29</td>
<td>1.77</td>
<td>2.17</td>
<td>nd</td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td>soil gleba</td>
<td>11.94</td>
<td>1.34</td>
<td>2.33</td>
<td>5.23</td>
<td>1.93</td>
</tr>
<tr>
<td><em>bifermentans</em></td>
<td>soil gleba</td>
<td>11.54</td>
<td>0.96</td>
<td>nd</td>
<td>1.67</td>
<td>nd</td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td>soil gleba</td>
<td>11.34</td>
<td>7.17</td>
<td>1.76</td>
<td>2.65</td>
<td>1.73</td>
</tr>
<tr>
<td><em>sordelli</em></td>
<td>silage obornik</td>
<td>7.31</td>
<td>5.21</td>
<td>1.08</td>
<td>1.62</td>
<td>1.33</td>
</tr>
</tbody>
</table>

nd – not detected
nd – nie wykryto

**CONCLUSION**

Our data support the notion that the natural environment might be colonized by the strains which are industrially useful. Some of them are non-pathogenic and has an ability to converse glycerol to 1,3-PD and other metabolites, such as short-chain organic acids and alcohols. The best 1,3-PD producers were identified by the rRNA technique as *Clostridium bifermentans* and *Clostridium sordelli*. The improving of the effective methods of isolation and maintenance of bacterial cultures in the laboratory might increase the production of industrially useful metabolites. In further investigations we plan to optimize the composition of the culture medium and other cultivations condition to increase 1,3-PD production by selective bacteria.

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REFERENCES


IZOLACJA I IDENTYFIKACJA NOWYCH GATUNKÓW CLOSTRIDIUM SP. ZE ŚRODOWISKA NATURALNEGO ZDOLNYCH DO EFEKTYWNEJ KONWERSJI GLICEROLU DO 1,3-PROPANODIOLU I INNYCH METABOLITÓW

Streszczenie. Szereg procesów metabolicznych jest efektywniej przeprowadzanych przez mikroflorę kolonizującą środowisko naturalne. Celem pracy była selekcja niepatogennych kultur bakterii z rodzaju Clostridium ze środowiska naturalnego zdolnych do konwersji glicerylu do 1,3-propanodiolu i innych metabolitów o znaczeniu przemysłowym. Badania dotyczyły także opracowania efektywnych procedur izolacji oraz hodowli mikroorganizmów beztlenowych w warunkach laboratoryjnych. Wstępna adaptację mikroflory obecnej w próbach środowiskowych przeprowadzono na podłożu PY zawierającym glicerol w stężeniu 50 g/l. Hodowle prowadzono w warunkach beztlenowych w anaerostatach. Wyraźnie oddzielone kolonie o morfologii typowej dla Clostridium sp. na podłożu TSC posiewano na podłożu wybiorczo-namnażającym RCM oraz PY. Zawartość metabolitów w płynie pohodowlanym oceniano za pomocą techniki HPLC. Identyfikacji gatunkowej dokonano metodą amplifikacji sekwencji kodującej 16S rRNA. Najwyższy odsetek kultur bakteryjnych Clostridium sp. pozyskano z odchodów zwierzęcych, kompostów i obornika. Blisko 60% uzyskanych izolatów wykazywało zdolność syntezy 1,3-propanodiolu z glicerolu. Najwyższa zdolność do utylizacji glicerylu do 1,3-propanodiolu charakteryzowała się szczepy Clostridium bifermentans oraz Clostridium sordelli. Większość przebadanych drobnoustrojów wykazywała także zdolność do syntezę krótkołańcuchowych kwasów organicznych i etanolu.

Słowa kluczowe: Clostridium sp., izolacja, 1,3-propanodiol, mikroflora środowiska naturalnego, krótkołańcuchowe kwasy organiczne...

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