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ISOLATION OF SUCCINIC ACID-PRODUCING *ESCHERICHIA COLI* FROM ANIMAL FAECES

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ABSTRACT. Succinic acid is currently used in the agricultural, food and pharmaceutical industries, is one of the key basic chemicals used in the preparation of biodegradable polymers. The first step in the fermentative production of succinic acid is the screening of bacterial strains. Therefore we were isolated a number of 65 *E. coli* strains from different animal faeces. Out of these strains, we identified a number of six strains by sequencing, and we examined their ability to produce succinic acid with chromatography. Based on the resulting chromatograms, 38 strains of the all *E. coli* strains produced succinic acid.

Keywords: *Escherichia coli, succinic acid, isolation, identification, ARDRA, chromatography*

INTRODUCTION

Succinic acid is a dicarboxylic acid containing four carbon atoms with chemical formula $C_4H_6O_4$ and it was first prepared from amber by Georgius Agricola in 1546 [1]. Succinic acid has a wide variety of applications and can be used as a starting material for many chemical products, such as biodegradable polymers and solvents [2], adipic acid, 1,4-butanediol and γ-butyrolactone [1]. Currently, succinic acid is produced on industrial scale by catalytic hydrogenation of petrochemically derived maleic anhydride [3]. However, in recent times, has received much attention the bio-based production of succinic acid by bacterial fermentation from renewable resources, because the petroleum is a finite resource and the price of oil is increasing [1, 4]. The US Department of Energy listed succinic acid in the top 12 chemical building blocks, which can be used for the production of various high value-added derivatives from renewable resources by microorganisms [5].

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Sccinic acid is an intermediate metabolite, and many bacteria are capable to produce it under different environmental conditions [6]. Three well-known natural succinic acid-overproducing strains have been studied: *Mannheimia succiniproducens* [7, 8], *Anaerobiospirillum succiniproducens* [9] and *Actinobacillus succinogenes* [10, 11]. There are some strains that produce no or only very small amounts of succinic acid, but the genetically modified version of these strains already produce, such as *Escherichia coli* [12-15], *Corynobacterium glutamicum* [16], *Saccharomyces cerevisiae* [17, 18].

Escherichia coli is one of the most widely used microorganism in industry and biotechnology [3]. It is a gram-negative, facultative anaerobe bacterium, which has a few advantages over other microorganisms as follows: culters can be grown faster in minimal media, wide range of substrate utilization, hence it is a strong candidate to be used in different biotechnologycal processes [6, 19]. The availability of genetic engineering tools for this organism and the well understood methods makes to be widely used in metabolic engineering [20].

The main aim of our study was to isolate *Escherichia coli* strains from different animal faeces, which are able to produce succinic acid. In the present study we discuss the isolation of *E. coli*, biochemical properties of isolated strains and identification of the strains with Amplified Ribosomal DNA Restriction Analysis (ARDRA), as well as which strains produce succinic acid.

RESULTS AND DISCUSSION

As we know *Escherichia coli* is commonly found in the diggestive tracts of the normal intestinal flora of different species of animals including humans too, so the isolation was carried out from animal faeces. The faeces were collected from farms from different animals and birds (dog, cow, horse, sheep, pig, hen and pigeon).

All the *E. coli* isolates, which can ferment lactose appaer as colonies with a green metallic sheen or blue-black to brown color on the Eosin Methylene Blue (EMB) agar and as yellow colonies on the Tergitol-7 agar. Bacteria that can utilize acetic acid turn the medium blue on the Acetate Differential Agar (ADA) [21]. Out of total 23 samples, 121 isolates were obtained: 75 isolates from EMB agar, 28 isolates from ADA and 18 isolates from Tergitol-7 agar.

Out of these isolates, 92 isolates gave positive tests for indol production, 100 isolates gave positive results for methyl red test, 112 isolates gave negative results for Voges-Proskauer test and 115 isolates gave negative tests for citrate utilization. Those strains which showing results + + - - during the reactions IMViC were confirmed as *Escherichia coli*. Based on the biochemical tests, 78 isolates showed similarities with biochemical properties of *E. coli*.

These 78 isolates were analyzed by ARDRA, thus, the 16S rDNA caracterization was realized for all strains. From the digested products obtained with the two restriction endonucleases, *AluI* and *HaeIII*, 11 isolates are shown in Figure 1. and Figure 2.

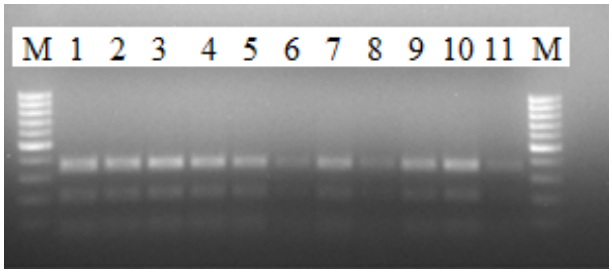

Figure 1. Restriction patterns of the 16S rDNA region of strains after digestion with *AluI* (M: 1 kb molecular weight marker, Fermentas, 1: M3/2 10^{-2}, 2: M10/2 10^{-1}, 3: EMB4/3, 4: Ter8/1 10^{-3}, 5: M4/7 10^{-4}, 6: ADA M7 10^{-2}, 7: M8/2 10^{-2}, 8: M3/4 10^{-1}, 9: M3/1, 10: Ter 4 10^{-3}, 11: Ter3/2)

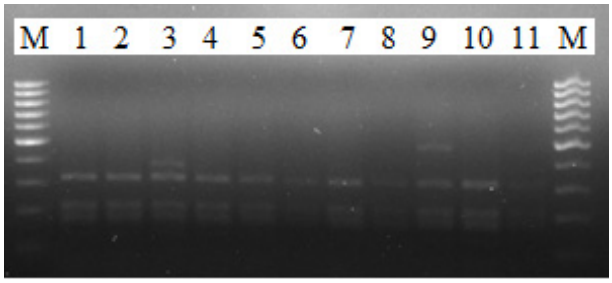

Figure 2. Restriction patterns of the 16S rDNA region of strains after digestion with *HaeIII* (M: 1 kb molecular weight marker, Fermentas, 1: M3/2 10^{-2}, 2: M10/2 10^{-1}, 3: EMB4/3, 4: Ter8/1 10^{-3}, 5: M4/7 10^{-4}, 6: ADA M7 10^{-2}, 7: M8/2 10^{-2}, 8: M3/4 10^{-1}, 9: M3/1, 10: Ter 4 10^{-3}, 11: Ter3/2)

On the basis of cluster analysis (Figure 3.), isolated bacterial strains resulted in five different ARDRA groups. The first group includes 65 isolates, including *E. coli* strain ATCC 13706, which was used as a reference strain. The second group includes seven isolates and the remaining three groups each including two strains.

From each group one strain was sequenced, except for the first group, from which six were sequenced. The obtained sequences were compared with sequences found in the NCBI database by the Blast search program. The result of the sequencing are shown in the Figure 3., where instead of the codes of isolates, the name of the strains are listed.

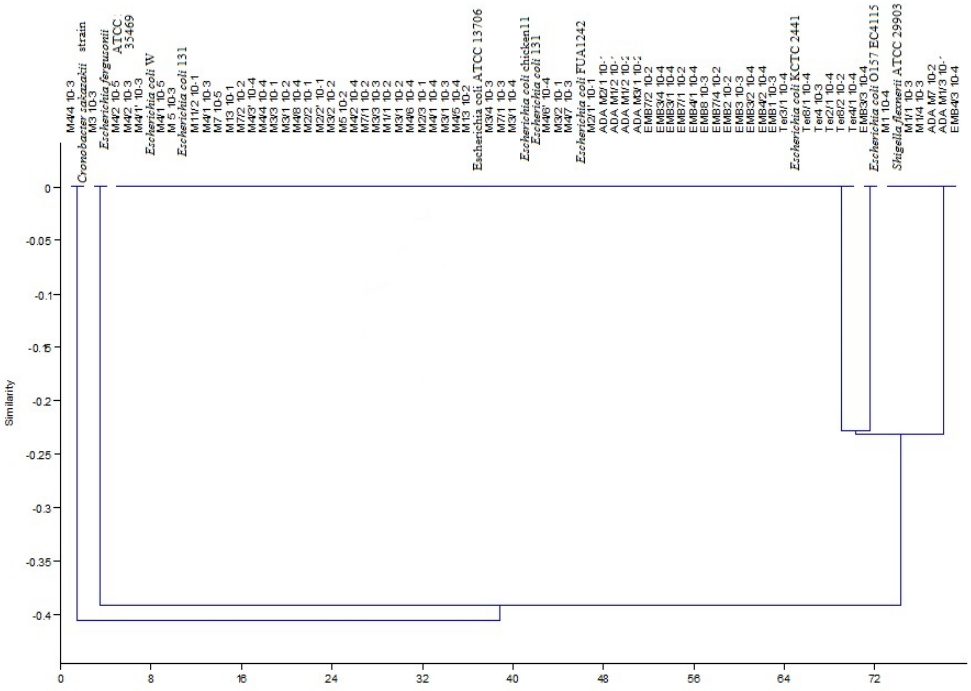

Figure 3. Genetic variability of the isolated bacterial strains

The isolated *E. coli* strains were screened based on their abilities to produce succinic acid using glycerol as carbon source under microaerobic conditions as described in Experimental Section. Identification of succinic acid was performed in a gas chromatography–mass spectrometry (GC/MS) experiment. In the fermentation mixtures the succinic acid was identified as trimethylsilylated succinate at 10'71" retention time. A number of 38 *E. coli* isolates from the 65 studied, were able to produce succinic acid. The results of the GC/MS analysis in the case of two strains are presented in Figure 4. The Figure 4A shows that the Ter8/1 10^{-3} strain was able to produce succinic acid in minimal medium, but in case of M8/2 10^{-2} strain the level of succinic acid is extremely low (B).

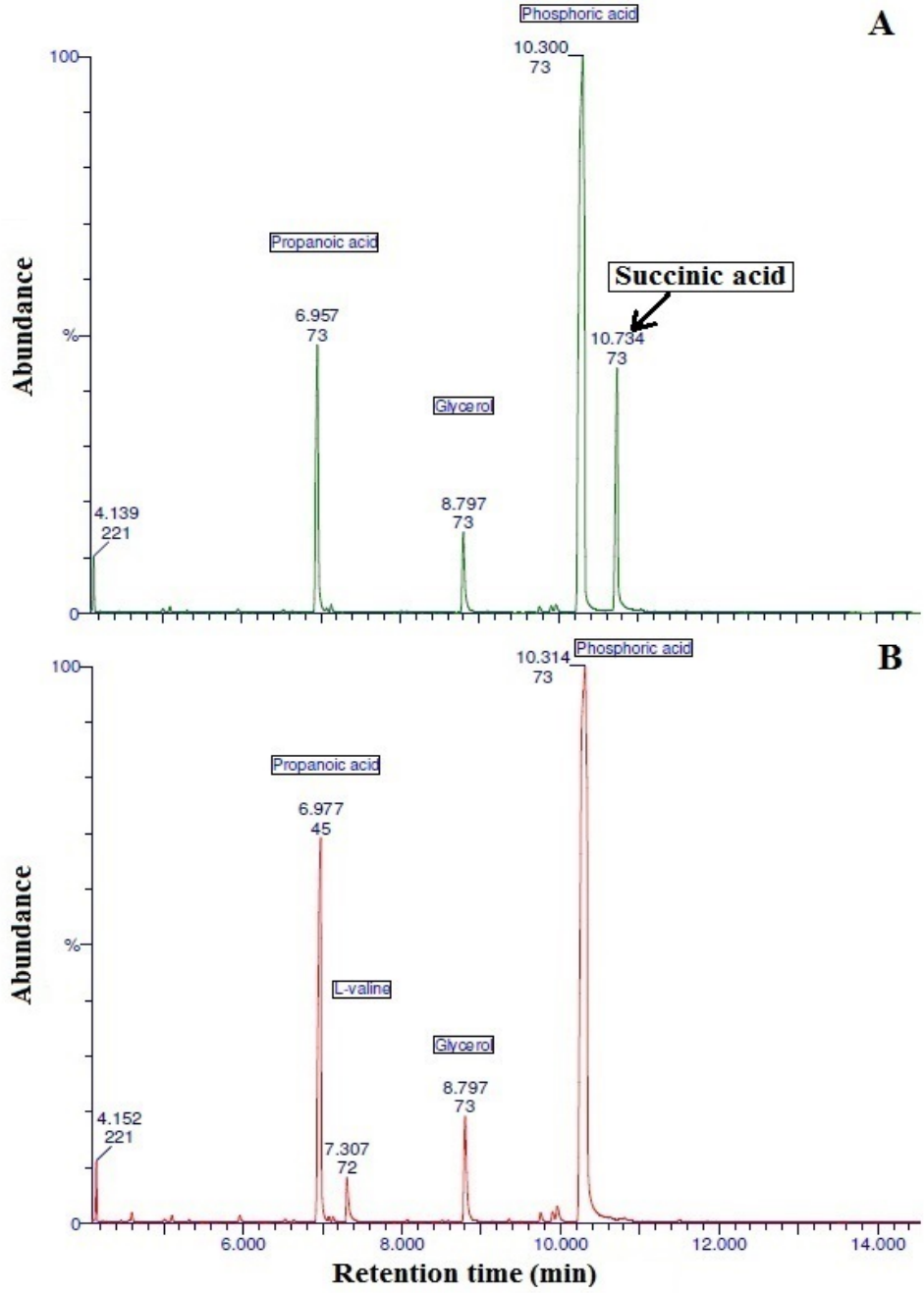

Figure 4. GC chromatograms of metabolits in case of *E. coli* KCTC 2441 strain (Ter8/1 10^{-3}) (A) and *E. coli* W strain (M8/2 10^{-2}) (B)

CONCLUSIONS

In the industrial production of succinic acid the first important step is the isolation and characterization of possible producing strains. In this study we managed to isolate and characterize different *E. coli* strains from different faeces possible candidates for succinic acid production. Out of total 23 samples, 121 isolates were obtained, from which 78 isolates were confirmed as *Escherichia coli* on the basis of their biochemical properties. All of the isolates were analysed for the differences in the ribosomal DNA with *AluI* and *HaeIII* restriction endonucleases, and five ARDRA groups were obtained. We identified at least one isolate from each group, and six *E. coli* strains were identified by sequencing. After the identification, all of the 65 *E. coli* strains were analyzed according to the ability to produce succinic acid, using GC-MS. Based on the resulting chromatograms, we can conclude that 38 of the 65 *E. coli* strains are able to produce succinic acid.

In the future quantitative analysis will be performed for succinic acid from one of the screened strain. This strain will be genetically modified in order to improve succinic acid production level and create an industrially important strain.

EXPERIMENTAL SECTION

Collection and culture of the samples

Samples were collected aseptically and analyzed within 24 hours. From the center of each samples were weighed out 1 g and homogenized with 9 mL physiological solution. From this stock solution 5-fold serial dilutions was prepared and a volume of 1 mL of each solutions was spreaded onto three selective agar: EMB, ADA and Tergitol-7 agar [21] and incubated at 37°C for 24 hours. The organisms showing characteristic colony morphology of *E. coli* were taken onto nutrient agar and repeatedly subcultured onto nutrient agar until the pure cultures with homogenous colonies were obtained. The pure cultures were taken onto nutrient agar slant and used for biochemical assays.

Biochemical tests

Each pure culture was subjected to four biochemical tests, namely IMViC: indole production, methyl red test, Voges-Proskauer test and citrate utilization.

Glucose-peptone broth (glucose 1 g/L, peptone from casein 20 g/L, peptone from meat 5 g/L, KNO_3 1 g/L, $Na_2S_2O_3 \cdot 5H_2O$ 0.2 g/L, pH=7.3±0.1)

was used for indol test. The strains were cultured in 5 mL of this broth at 37°C for 48 hours. After 48 hours 0.3 mL Kovac's reagent was gently added to the medium and formation of a red colored ring on the top of the medium indicated positive result.

MR-VP broth (glucose 5 g/L, peptone from casein 5 g/L, KH_2PO_4 5 g/L, pH=7.5±0.1) was used for methyl red test and Voges-Proskauer test. The strains were cultured in 5 mL MR-VP broth at 37°C. After 48 hours 3-4 drops of methyl red solution was added to the culture to determine the color formation. Formation of red color indicated positive result and yellow color indicated negative result. For the strains which were cultured for the Voges-Proskauer test 5 mL of 10% potassium hydroxide was added and incubated at 37°C for 24 hours. After 24 hours the pink-red color of broth indicated positive result.

Citrate agar (NaCl 5 g/L, $MgSO_4$ 0.2 g/L, $(NH_4)H_2PO_4$ 1 g/L, K_2HPO_4 1 g/L, sodium citrate 2.67 g/L, agar 18 g/L, 4 ml of 1% bromthymol blue, pH=6.8±0.1) was used for the citrate utilization test. A single colony was transferred onto the surface of the agar with a steril needle and incubated at 37°C. After 48 hours, if the color of agar is intese blue, then the test result is positive and if no color change, then the result is negative.

Strains showing results + + - - during the reactions IMViC were confirmed as *E. coli*.

Identification of the isolated bacterial strains

DNA isolation from 3 mL overnight pure cultures was performed using the Wizard Genomic DNA Isolation Kit (Promega) according to the manufacturer's instructions. The 16S rDNA gene was amplified by PCR using 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5' TACGGYTACCTTGTTACGACTT3') universal primers. The volume of the reaction mixture was 50 µL and contained 0.1 mM of each deoxynucleoside triphosphate, 1 U of Taq DNA polymerase, 2 mM $MgCl_2$, 0.4 µM of each primer and about 20 ng of genomic DNA template in 1X Taq Buffer with $(NH_4)_2SO_4$ (Fermentas). The amplification reaction was performed in Corbett Palm-Cycler thermocycler (Cornett CG1-96) using the following protocol: an initial denaturation at 94°C for 3 min, followed by 32 cycles of amplification (94°C for 30 sec; 46°C for 30 sec; 72°C for 1 min), a final extension at 72°C for 7 min and a subsequent incubation at 4°C.

The amplified products were checked on a 1% agarose gel stained with ethidium bromide and visualized under UV light by using GelDoc System from BioRad.

Isolates were grouped by ARDRA with two different restriction endonucleases: *HaeIII* and *AluI*. Fragments were separated on 2% agarose gel and ARDRA group representatives were identified by sequencing.

The nucleotide sequence determination was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and capillary electrophoresis of the samples was performed by Biomi Ltd (Gödöllő, Hungary). The obtained sequences were compared to the GenBank databases by the Blast search program. Sequence similarity over 99% was accepted as species level identification.

GC/MS analysis

The isolated *E. coli* strains were grown in 20 mL minimal medium supplemented with 0.2% (v/v) glycerol under microaerobic condition at 37°C. The cells were incubated in serum bottles (50 mL) with shaking (250 rpm). After 24 h, the cells were harvested by centrifugation (10 minutes at 14000 rpm) and the supernatants were filtered with 0.22 µm Millipore® nitrocellulose membranes. The samples were subjected to trimethylsilyl (TMS) derivatization, and this derivatized sample was used for GC/MS (6890N/5975 Agilent). Compounds were identified by comparing the obtained mass spectra with commercially available NIST 98 spectra library.

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