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Newly characterized butyrate producing *Clostridium* sp. strain 4a1, isolated from chickpea beans (*Cicer arietinum L.)*

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Butyric acid is a valuable chemical with wide application in various industries. The interest in its biotechnological production is revived in view to the application in the production of alternative fuels from biomass feedstocks. Microorganisms of genus *Clostridium* are well known as producers of butyric acid. *Clostridium* species was found to dominate the system in the spontaneous chickpea fermentation. In the present study a *Clostridium* sp. strain 4a1 from chickpea beans fermentation was selected as a good producer of butyric acid. Some process parameters as initial pH of the medium (7.5), temperature (37 ºC), medium composition and glucose concentration (20 g/l) were determined. The strain was identified as *Clostridium beijerinskii* (97% similarity) on the basis of classical and modern polyphasic taxonomy methods.

Keywords: Butyric acid, Chickpea beans, Fermentation, *Clostridium*, Polyphasic taxonomy.

INTRODUCTION

Butyric acid ($C_4H_8O_2$, IUPAC name butanoic acid) is a chemical with wide application in several industries like food, pharmaceutical, chemical industry, etc. Among the main applications of butyric acid is the production of plastics for textile fibers, of butyrate esters as food and perfume additives, and of biodegradable biopolymers. Because of its ability to neutralize the activity of food carcinogens [1], to lower cholesterol levels [2] and to inhibit tumor cells [3] butyric acid is subject to increased research interest. Derivatives of butyric acid are used for the production of anti-thyroid drugs and vasopressors [4], as well as of antioxidants. In the recent years, butyric acid is used in the production of biobutanol, not only as an intermediate in acetone-butanol-ethanol (ABE) fermentation, but as a co-fermenting substrate, leading to significant enhancement of butanol yield [5, 6].

Butyric acid can be produced *via* chemical or biotechnological routes. Bacteria involved in the production of butyric acid are divided into two groups – producing acid as a final product and producing mostly butanol. The ABE fermentation is one of the oldest industrial fermentations and in the first half of the twentieth century it has become one of the largest biotechnological processes [7]. Later on, the fermentative production of butyric acid could not compete to the low price of acid produced *via* petrochemical route and nowadays butyric acid is mainly produced *via* oxidation of butyraldehyde derived from propylene by an oxo process. The annual production of butyric acid is estimated to be around 50,000 t [8].

In the last 20 years, the interest towards the fermentative production of butyric acid has revived. This is due to the increasing demand for butyric acid from microbial fermentation, on one hand because of strong demand for bio-based products and decreasing resources of crude oil and environmental issues on the other hand. The development of *in-situ* separation processes, helping to overcome low final product concentration and product inhibition problems, as well as the possibility of using agricultural and industrial byproducts and wastes as substrates also play an important role.

According to Zhang *et al*. [7] there are more than 10 butyrate-producing bacteria species from at least seven genera with butyric acid-producing capacity being investigated for potential industrial application. The microorganisms belong to the genera *Clostridium*, *Butyrvibrio*, *Butyribacterium*, *Eubacterium*, *Fusobacterium*, *Megasphera*, and *Sarcina* and all of them are anaerobic [9]. In view of industrial application, the strains of *Clostridium* (*Cl. butyricum*, *Cl. tyrobutiricum* and *Cl. thermobutiricum*) have been largely studied for the production of butyric acid. However, because acetic acid is also produced as a byproduct, genetic engineering and process development have been

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attempted to increase butyric acid yield and selectivity [10].

Chickpea (*Cicer arietinum L.*) is one of the most important legumes in the world. It is known from ancient times and now is grown in many countries – in the Balkans, Mediterranean, Middle East, Indian subcontinent, and Americas. There are two distinct types of cultivated chickpea, Desi, and Kabuli – they differ by size and color [11]. Chickpea is utilized either in whole or paste form as a main or side dish after cooking or as a snack food after roasting. In some Mediterranean countries, fermented chickpea is being used as a leavening agent to make traditional bread and rusks [12]. By the addition of fermented chickpea beans in the wheat flour, besides the enhancement of the nutritional quality, the product's shelf life is also expanded [13]. In the most of the Mediterranean countries, fermented chickpea is used as a leavening agent to make baked products [14]. Bread prepared with chickpea yeast ("simmits") is a very popular product in Bulgaria, some of the Balkans countries and the Near East [15].

The data for the microbial community in fermented chickpea are very limited in the scientific literature. There are only a few published research results. According to Tangüler [16] in studies done in Turkey, lactic acid bacteria such as *Lb. plantarum, Lb. pentosus, Lb. bifermantans, Str.thermophilus, Lc. ssp. lactis, Lb. brevis, Lb. plantarum, Lb. pentosus*, coccobacillus *Weissella confusa* and yeasts such as *S. cerevisiae* were identified. Katsaboxakis and K. Mallidis [17] studied the microflora of soak water during natural fermentation of coarsely ground chickpea seed at different temperatures. A gas-producing *Clostridium* species was found to dominate this fermentation system, particularly at higher temperatures. Gram-negative bacteria and yeasts were not found. *Bacillus* species, as well as *Lactobacillus, Corynebacterium, Micrococcus* and *Pediococcus spp.* were isolated. Kyyaly *et al*. [18] investigated the major bacteria genera in the soaked fermented chickpea and isolated the following species: *Clostridium sartagoforme, Bacillus thuringiensis* and *Enterococcus faecium*. Only *Clostridium s*p. produced gas from sugar and hydrolysed gluten during dough fermentation. In a study of changes in numbers and kinds of bacteria during a chickpea submerged fermentation used as a leavening agent for bread production Hatzikamari *et al*. [12, 19] isolated only representatives of *bacilli* (*B. cereus, B. thur*ingiensis and *B. licheniformis*) and *clostridia* (*Cl. perfringens* and *Cl. beijerinckii*). *B. cereus* and *C. perfringens* predominantly growing during fermentation do not seem to form any toxins. Antonova-Nikolova *et al*. [15] studied the dynamics of the development of chickpeas fermentation microflora. They found only representatives of the genera *Bacillus* and *Clostridium* and suggested additional studies to prove the possible participation of lactic acid bacteria. On the basis of phenotypic characteristics and numerical taxonomy clostridial strains were related in 3 clusters of *Cl. aceticum, Cl. acidurici* and *Cl. polysaccharolyticum* and bacilli in 8 groups of *B. lentus, B. pumilus, B. subtilis, B. coagulans, B. sphaericus, B. alvei, B. polymyxa* and *B. cereus* [20].

Fermentation of chickpea beans (*Cicer arietinum L.),* as it was shown in previous investigations [15, 20, 21], was caused by members of the genus *Clostridium.* Therefore, it could be used as a source for isolation of desirable clostridial strains. With this aim 14 new strains were isolated from four samples of chickpea crops harvested from different geographical regions of North and South Bulgaria during 1996 and 1997. Initial characterization of the metabolic activity of these 14 pure cultures isolated from chickpea beans fermentation showed the strain 4a1 as a promising isolate [22].

In the present study, the *Clostridium* sp. strain 4a1 from chickpea beans was characterized as a good butyric producing strain and was identified according to the modern polyphasic taxonomy.

MATERIALS AND METHODS

Chickpea beans fermentation

Coarsely ground chickpea beans were put in a bottle with a narrow long neck and washed twice with boiled and slightly salted water. They were poured on to the bottle neck and kept at about 40° C until thick foam was rising and started to overflow, and the fermentation liquid clarified [23]. The duration of fermentation varied between 8 and 18 h.

Microorganisms, media and culture conditions

A protocol for *Clostridium* sp. isolation from different samples with fermented chickpea beans was designed including steps of initial enrichment followed by cultivation and pure cultures isolation [23]. The isolation of pure cultures and their maintenance were carried out on nutrient agar with 3% (w/v) glucose. A chickpea infusion medium was used as enrichment medium and for determination of gas and butyric acid formation. It was prepared as 1 part of coarsely ground chickpeas was poured with 5 parts of tap water. After autoclaving (1 atm/30 min) the mixture was

filtered through cheesecloth and the filtrate was centrifuged at 4000 rpm for 30 min. The separated supernatant was adjusted to pH 7.5 with 1N NaOH and sterilized again at 0.8 atm for 20 min. All isolates were sub-cultured on blood agar (BulBio, Bulgaria) and the hemolysis was monitored.

Disinfectants examinations were performed using PY (peptone – yeast extract) broth, PYG (peptone – yeast extract – glucose) broth, PYG broth with 1% fructose or 20% bile, yolk agar, nutrient agar with 6.5 % NaCl, gelatin, starch agar by procedures described in Bergey's manual [24] using Winogradsky's N-free selective medium for anaerobic nitrogen fixation.

The isolates were lyophilized in protective medium (10% skimmed milk, 1.5% gelatin and 10% sucrose) and stored at 4ºC until tests [25].

The morphological, physiological and biochemical characterizations of the isolated strains were carried out under anaerobic conditions in an anaerobic jar (Anaerocult, Merck) at 40 °C according to [24].

Phenotypic characterization

The presumptive colonies of *Clostridium spp*. were described macroscopically on blood agar and characterized microscopically by using Gram's staining. Starch inclusions and spore-formation were confirmed by staining [26]. Motility of the strains was examined by the classical hanging drop method. Bacterial growth at different temperatures was determined after 3 days at 40 °C and 55 °C, after 5 days at 22 °C, and after 21 days at 4 °C in nutrient agar with 1% glucose. The growth in nutrient agar with 6.5% sodium chloride was recorded on the 5th day. Nitrogen fixation was observed after 24-48 h, as a positive reaction was gas formation.

Biochemical characterization

Several biochemical tests such as carbohydrate fermentation test, methyl red and Voges–Proskauer (MR-VP) tests, indole reaction, starch, gelatin and esculin hydrolysis, enzyme activity (catalase, lecithinase, lipase, urease, reductase), bile susceptibility, H_2S and butyric acid production, hemolysis were performed according to the procedures described in [26].

Numerical taxonomy

Using the K-mean method, a program was set up for clustering of isolated clostridial strains on the basis of similarity to the type of clostridial cultures.

Molecular identification

A butyrate producing strain was identified to the species level, according to the modern polyphasic taxonomy. Isolation of genomic DNA from a pure exponential culture of the selected *Clostridium* strain was performed according to the method of Delley *et al*. [27] in modification. The obtained DNA sample was analyzed by 1% v/v agarose gel electrophoresis and the concentration and purity were checked. The DNA, stored at -20°C, was used as a target in the PCR assay and following sequencing analysis. Amplification of 16S rDNA gene was carried out with the following pairs of primers: forward primer fD1 (5`-AGA GTT TGA TCC TGG CTC AG-3`) and reverse primer rD1 (5`-AAG GAG GTG ATC CAG CC-3`) Amplification reactions were performed with 2×PCR TaqMixture kit (HiMedia, India), primers rD1 and fD1 (0.6 µM each) and 1 ng of DNA/µl. They were conducted on the above-mentioned apparatus under the following amplification conditions: initial denaturation at 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min, 32 cycles of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56.5 °C for 75 s, elongation at 72 °C for 75 s, and a final synthesis at 72 °C for 5 min. The amplified products of approximately 1550 bp were purified and sequenced using a 3730×l DNA analyzer (Thermo Fisher Scientific, USA) by Macrogen Inc. (Belgium). The obtained sequences were analyzed with *Chromas* 2.3 programme (*Technelysium Pty* Ltd., *Australia*) and species identification of the strain was performed by BLASTN analysis.

Butyric acid production

In view to investigate the influence of different parameters on butyric acid production nutrient medium CM with composition (g/l): yeast extract - 5; K_2HPO_4 - 1; KH_2PO_4 - 1; $CaCl_2.2H_2O$ – 0.01; $(NH_4)_2SO_4$ – 0.1; $FeSO_4.7H_20$ – 0.005; $MgSO_4.7H_20$ – 0.1 was used. The medium was sterilized for 20 min at 121ºC. Glucose (20 g/l) was used as a substrate and the fermentations were carried out without pH control (initial pH was 7.5).

Analytical methods

The concentrations of glucose and butyric acid were determined by HPLC. Samples were analyzed on a chromatographic system consisting of a pump Smartline S-100, Knauer, RI detector - Perkin - Elmer LC- 25RI, column Aminex HPX- 87H, Biorad, 300×7.8 mm and specialized software EuroChom, Knauer. 0.01 N H_2SO_4 was used as mobile phase at a flow rate of 0.6 ml/min.

RESULTS AND DISCUSSION

Clostridia and its role in the fermentation of various non-milk substrates are relatively poorly studied. At the same time, the interest in the so-called "functional foods" with beneficial properties increases. The use of butyrate-producing microorganisms for food production is also among the weakly studied issues. Therefore, a key part of the study of spontaneous chickpea fermentation was the characterization of the species involved.

Butyric acid production by newly isolated bacteria from chickpea beans

In the present work a newly isolated strain from spontaneous fermented chickpea beans was characterized as a butyrate producer and was identified. This strain is a part of a group of 21 newly isolated bacteria from laboratory made spontaneous fermentations of chickpea beans in water. A pre-selection between isolates was made on the base of classical microbiological characteristics. Thus, 14 strains were characterized as belonging to the genus *Clostridium* and were estimated as butyrate producers. The strain 4a1 showed the highest butyric acid production. In addition, optimization of some process parameters was carried out.

Optimization of some process parameters - pH of the medium and temperature

The influence of pH on the growth and butyric acid production was investigated in the interval 4.5 -10.0. As it can be seen on Fig.1 the maximum production of butyric acid (~6 g/l) was observed at initial pH=7.5.

The temperature influence was studied at four levels – 4, 22, 37 and 55 ^{o}C. The best growth and maximal production was observed at 37 ^{o}C (Fig. 2).

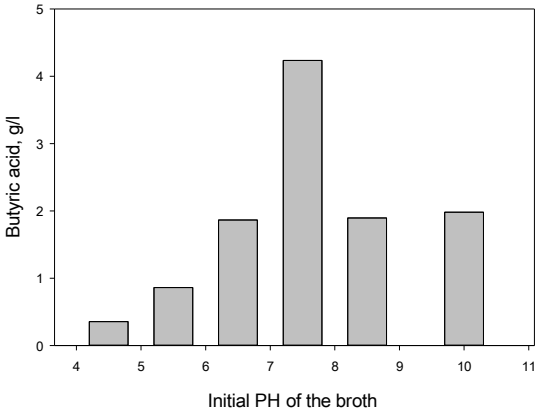


Fig. 1. Influence of the initial pH on the butyric acid production by 4a1 strain.

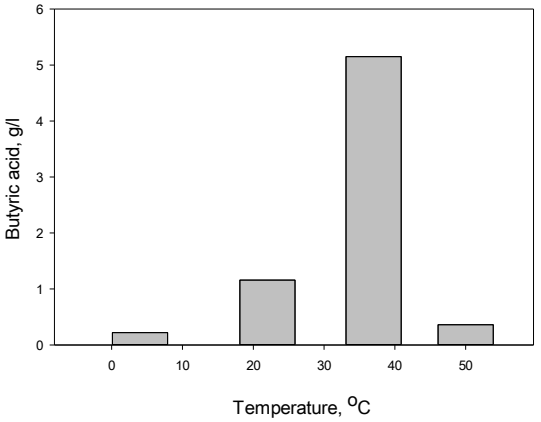


Fig. 2. Influence of temperature on butyric acid production by 4a1 strain.

Effect of medium composition

Seven medium compositions with different amounts of various nitrogen sources and microelements were tested (Table 1). The composition CM7 was found as optimal for maximal butyric acid production.

Identification of pre-selected butyric acid producing strain 4a1

All strains with industrial potential and especially for food-associated application have to be identified to the species level, according to the

Table 1. Media composition for butyric acid production by 4a1 strain

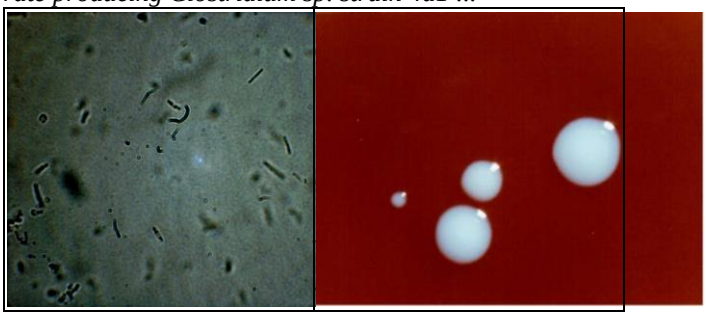
	Glucose	Peptone	Yeast Extract	Meat Extract	KH_2PO_4	K_2HPO_4	$MgSO_4 \times 7H_2O$	$CaCl_2 \times 2H_2O$	$FeSO_4 \times 7H_2O$	NaCl
CM1	5	10	3	10	-	-	-	-	-	5
CM2	5	-	1	-	0.5	0.5	0.21	0.001	-	-
CM3	5	2	5	-	1	1	0.1	0.01	0.005	-

	Glucose	Peptone	Yeast Extract	Triptone	KH_2PO_4	K_2HPO_4	$MgSO_4 \times 7H2O$	$CaCl_2 \times 2H_2O$	$FeSO_4 \times 7H_2O$	$MnCl_2 \times 4H_2O$	$CoCl_2 \times 6H_2O$	$Na_2MoCl_4 \times 2H_2O$
CM4	5	2	5	2	1	1	0.025	0.015	0.01	0.002	0.025	0.025
CM5	10	2	6	2	1.2	5.5	0.025	0.015	0.01	0.002	0.025	0.025
CM6	15	5	6.5	2	1.2	3.5	0.025	0.015	0.01	0.002	0.025	0.025
CM7	20	2	6	2	2	5.5	0.025	0.015	0.01	0.002	0.025	0.025

modern polyphasic taxonomy. Initial taxonomic characterization was carried out according to Bergey's Manual of systematic bacteriology [24]. The strain Aa1 was initially identified as *Clostridium sp.* on the base of morphological characteristics, as Gram-positive staining, catalase-negative and spore-forming, motile, rod-shaped bacteria, with polymorphism of cells (Fig. 3a). Subterminal spores are oval and swell vegetative cells. The strain 4a1 forms round, large, white, glossy non-hemolytic colonies, convex, with regular edge and uniform consistency after anaerobic growth for 72 h on blood agar (BulBio, Bulgaria) (Fig.3b).

Several biochemical tests, usually applied for Gram-positive bacteria from the genus *Clostridium* were carried out and the results are summarized in Table 2.

The investigated strain does not grow under aerobic conditions. The most abundant growth is observed at 37 °C, showing that the strain belongs to mesophilic clostridia.



A **B**

Fig. 3. Cell-morphology (A) and colony appearance on Blood agar (B) of the *Clostridium* strain 4a1

*Light microscopy Boecko microscope, magnitude 1000×

The strain 4a1 is susceptible to high concentration of bile but can grow weakly at 6.5% NaCl. It ferments glucose with acid production but does not hydrolyze gelatin and casein. In the opposite, it can hydrolyze polysaccharides as esculin and starch. The strain 4a1 can fix molecular nitrogen when is grown in Winogradski's N_2-free liquid medium. It does not produce indole, acetoin and H_2S, as well as the enzymes lecithinase, lipase and urease. A weak carbolytic activity had to be pointed when the fermentation of 19 carbohydrates by isolated pure culture was examined.

Table 2. Physiological and biochemical characterization of the investigated strain 4a1

Characteristics		*Results*	*Characteristics*		*Results*
Aerobic growth		-	Acid from glucose		+
Hemolysis		-	Gelatin hydrolysis		-
Catalase		-	Nitrogen fixation		+
Growth at:	4 °C	+	Production of	Indole	-
	22 °C	+		H_2S	-
	37 °C	+++		Lecithinase	-
	55 °C	+		Lipase	-
	6.5% NaCl	+/-		Urease	-
	20% bile	-			
Nitrate reduction		-	Voges-Proskauer reaction		-
Methyl red test		-	Neutral red reduction		-
Hydrolysis of Esculin		+	Hydrolysis of Starch		+
Acid production from	Arabinose	-	Acid production from	Mannitol	-
	Galactose	-		Manose	-
	Sucrose	+/-		Starch	-
	Inositol	-		Rhamnose	+
	Inulin	-		Raffinose	-
	Xylose	-		Ribose	+
	Lactose	+/-		Sorbitol	-
	Melibiose	-		Fructose	-
	Maltose	-		Cellobiose	-
Milk coagulation		+/-	Casein hydrolysis		-
Milk reaction-methylene blue reduction					+

Legend:
(+) – weak growth; positive biochemical activity
(+/-) – doubtful growth; doubtful biochemical activity
(+++) – abundant growth
(-) – absence of growth; negative biochemical activity

The strain ferments glucose, rhamnose and ribose and weakly or no utilizes sucrose and lactose. On the basis of two characteristics (acid formation from glucose and gelatin hydrolysis), the clostridia are grouped into four groups: 1) non-carbolytic and non-proteolytic; 2) carbolytic and non-proteolytic; 3) non-carbolytic and proteolytic, and 4) carbolytic and proteolytic [24]. Using the methods of numerical taxonomy, the strain 4a1 was related to the group of carbolytic and non-proteolytic clostridia with type strain *Cl. Saccharolyticum* (at similarity of 50%), together with *Cl. butyricum*, *Cl. tyrobutiricum*, *Cl. beijerinckii* (Fig. 4). As it was mentioned above, these clostridial species are good producers of butyric acid. Some differences were established when the phenotypic characteristics of the investigated strain 4a1 and the type strains were compared. The differences between strain 4a1 and *Cl. butyricum* and *Cl. beijerinckii* were mainly found in the utilization of sugars, whereas differences between strain 4a1 and *Cl. saccharolyticum* and *Cl. tyrobutiricum* were in nitrate reduction, indole production, nitrogen-fixation and absence of motility. Ttherefore, it was not possible to make a definitive identification on the basis of phenotypic features alone.

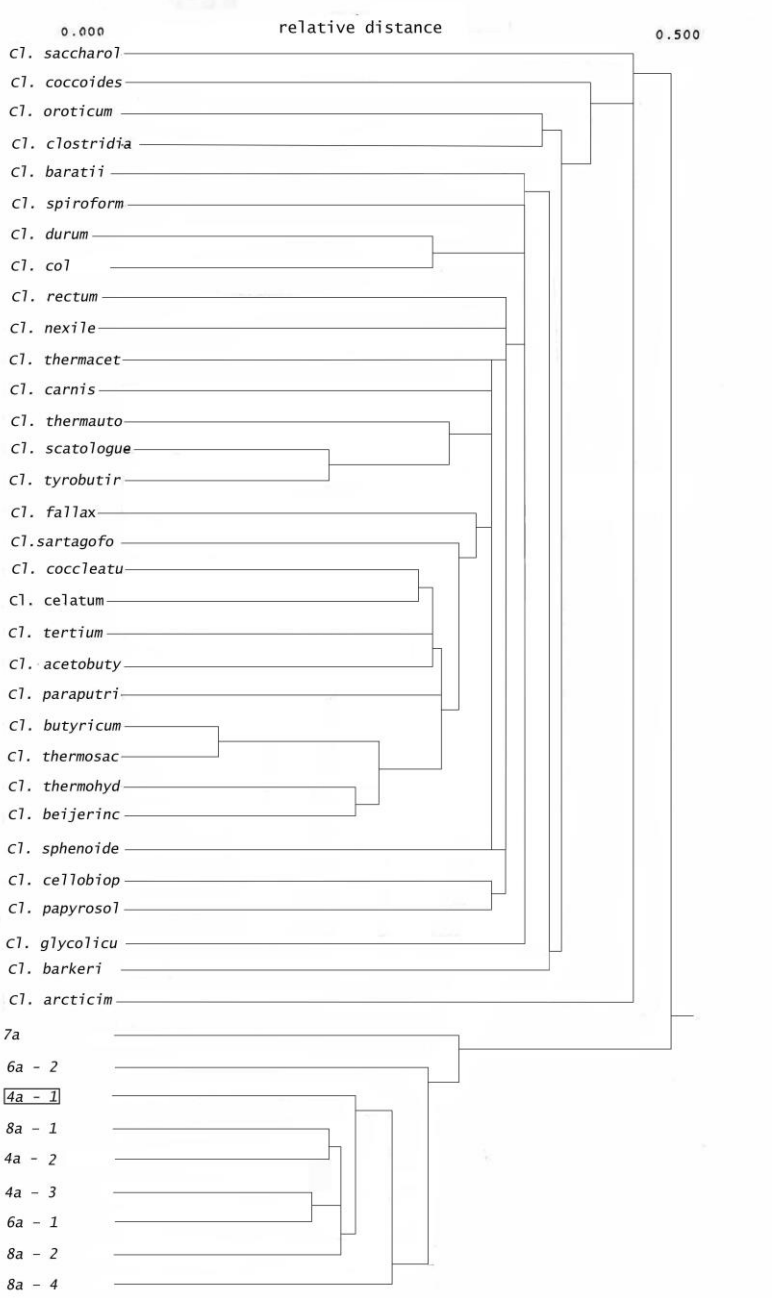


Fig. 4. Dendrogram for location of the *Clostridium* strain 4a1 depending on the relative distance in the group of clostridia, producing acid of glucose (0) and non-hydrolyzing gelatin (1).

The *Clostridium* has long been recognized as a phenotypically heterogeneous genus. Therefore, molecular – based methods have to be applied for species identification. One of the main stages in the study of newly isolated strains is to determine their species by a sequence analysis of parts of the ribosomal operon. This approach was successfully applied for the genus *Clostridium* [28-30].

In order to identify to the species level selected *strain* 4a1, additionally, molecular – genetic approach was applied. A total DNA from exponential *Clostridium* culture was extracted by the modified method of Delley *et al*. [27] and subjected to PCR amplification with universal primers for 16S rRNA gene of ribosomal operon (*fD1* и *rD1*), according to Weisburg *et al.*, [31]. The amplified PCR product (~ 1550 bp) was sequenced in Macrogen (The Netherlands) using ABIPRISM®310 DNA Genetic Analyzer, (PE Applied Biosystems). The golden 16S rDNA sequence analysis was applied. Obtained sequences were edited by Chromas. The comparative analysis of the obtained partial 16S rDNA sequence for unidentified strain 4a1, with the extensive GenBank database was used to assign the isolate to the species. The strain was identified as *Cl. beijerinckii* with 97% similarity. The sequence was deposited to the NCBI - GenBank - JN244676 and a phylogenetic tree was constructed (Fig. 5).

Clostridium beijerinckii species are ubiquitous in nature and routinely isolated from soil samples. It was reported as a species during a chickpea submerged fermentation by M. Hatzikamari *et al*. [19].

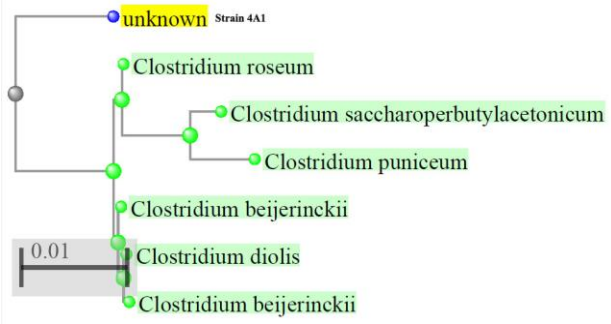


Fig. 5. A phylogenetic tree showing the interrelationships within a cluster formed by the newly identified *Clostridium beijerinskii* strain 4a1 and other *Clostridium* species. Tree was constructed on the base of 16S rDNA sequences, using BLAST pairwise alignments (NCBI), by Newburgh Joining tree method at max. sequences distances 0.75. (Tree Viewer 1.17.0 (October 24, 2017).

CONCLUSION

A newly isolated strain 4a1 from chickpea beans fermentation was identified as *Clostridium beijerinskii* according to modern polyphasic taxonomy, combining classical phenotypic and molecular-genetic methods. It is capable to produce butyric acid from glucose in a relatively broad temperature interval (from 4ºC to 55ºC). The optimum initial pH (7.5), temperature (37 ºC) and optimized fermentation medium for butyric production with good yield were determined. However, further optimization of the process parameters is necessary and is still in progress.

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НОВООХАРАКТЕРИЗИРАН ЩАМ *CLOSTRIDIUM* SP. 4A1, ИЗОЛИРАН ОТ ЗЪРНА НАХУТ (*CICER ARIETINUM L.),* ПРОИЗВЕЖДАЩ БУТИРАТ

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(Резюме)

Бутировата киселина е ценно съединение с широко приложение в различни клонове на индустрията. Интересът към нейното биохимично производство се възражда с оглед на приложението й за получаване на алтернативни горива от биомаса. Микроорганизмите от вида *Clostridium* са добре известни като производители на бутирова киселина. Установено е, че те доминират при спонтанната ферментация на зърна от нахут. В настоящото изследване щамът *Clostridium* sp. 4a1 от ферментацията на зърна от нахут е избран като производител на бутирова киселина. Определени са някои параметри на процеса като първоначално pH на средата (7.5), температура (37 ºC), състав на средата и концентрация на глюкозата (20 g/l). С помощта на класически и съвременни полифазни таксономични методи щамът е идентифициран като *Clostridium beijerinskii* с 97% подобие.