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Isolation of Novel Alkaliphilic *Bacillus* Strains for Cyclodextrin Glucanotransferase Production

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Abstract New alkaliphilic *Bacillus* producers of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) were isolated from 17 Bulgarian alkaline and normal habitats (springs and soils) by three steps of a selection. None of the isolates obtained, producing CGTase, appeared to be thermophilic in character. One hundred and thirty-seven strains were estimated for CGTase activity by batch cultivation in a liquid alkaline medium. Twenty-seven of them had a detectable CGTase activity in their culture supernatants under the enzyme assay conditions, despite of the significant growth of all isolates. The phenotypic properties of three selected strains (20RF, 8SB and 24WE) were determined. They were aerobic endospore-forming *Bacillus* strains: two of them were obligated alkaliphiles (20RF and 8SB) and one, alkalitolerant (24WE). Both obligated alkaliphiles were further characterised by 16S rRNA analysis. According to the full 16S rRNA gene sequences obtained and deposited to the NCBI GenBank database, both isolated obligated alkaliphiles 20RF and 8SB were clustered into the group of alkaliphilic *Bacillus* species. The exhibited CGTase production by them (230–250 U ml^{-1} for 20RF and 130–160 U ml^{-1} for 8SB) defined these new isolates as promising producers of the enzyme, especially *Bacillus* sp. 8SB synthesising thermostable alkaline β-CGTase. Both new enzymes from 20RF and 8SB *Bacillus* strains formed only two types of cyclodextrins, beta and gamma, which could be of interest for their easy separation and industrial production.

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Introduction

Alkaline-adapted microorganisms can be classified into two main groups: alkaliphiles (also called alkalophiles) and alkalitolerants. The term "alkaliphile" is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near-neutral pH value. Microorganisms, capable of growing at pH values more than 9 or 10, but with optimum growth rates at around neutrality or less, are referred as alkalitolerant [1, 2]. Besides the known habitats of alkaliphiles (groundwaters bearing high Ca^{2+} concentration and soda lakes and deserts bearing high concentration of Na_2CO_3), many alkaliphiles have been isolated from neutral environments, sometimes even from acidic soil samples [1, 3]. Recently, studies on the isolation of alkaliphilic *Bacillus* strains, on their phylogenetic and phenotypic characteristics, on their cell-surface features depending on high alkaline culture pH and on the molecular basis of alkaliphily have been increased [1, 4–9]. This big interest is in connection with a great impact that the alkaliphilic bacilli have made by their valuable and commercially important enzymes [1, 3, 10]. The enzymes from alkaliphilic bacilli, such as proteases, amylases, cellulases and pullulanases, are widely employed in laundry detergents, paper pulp bleaching, treatment of agricultural and food wastes. Another important application is the industrial production of cyclodextrins (CD) by alkaline cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19). This enzyme reduced the production cost and paved the way for cyclodextrin use in large quantities in foodstuffs, chemicals and pharmaceuticals [1].

The present paper reports the isolation of new alkaliphilic *Bacillus* strains from Bulgarian alkaline and normal habitats (springs and soils) and their evaluation for using of β-CGTase production.

Materials and Methods

Isolation of CGTase Active Alkaliphilic Bacillus Strains from Bulgarian Habitats

Plate Screening The alkaline medium used for isolation of alkaliphilic *Bacillus* strains comprised (g l^{-1}): soluble starch (Poland), 2; peptone (Oxoid, Basingstoke, UK), 5; yeast extract (Oxoid), 5; $MgSO_4$, 0.2 and K_2HPO_4, 1. Sterile sodium carbonate was used to adjust the medium to pH 10 after autoclaving. Five milliliters from samples (water or approximately 1 g of soil, suspended in 5 ml sterile water) were mixed with 5 ml alkaline medium and incubated at 40 and 60 °C for 48 h for enrichment. After that suspensions were heated at 80 °C for 10 min because the methods for isolation of *Bacillus* strains are based on the resistance of their spores towards elevated temperatures [11, 12]. After chilling, 5 ml from these suspensions were mixed again with 5 ml alkaline medium and cultivated 48 h at 40 and 60 °C. Thirty microliters of the samples was plated out on alkaline agar medium containing 1.5% (*w*/*v*) agar (Oxoid), 0.02% (*w*/*v*) phenolphthalein and 0.01% methyl orange and incubated at 40 and 60 °C, respectively [13]. Cultures producing β-CGTase were surrounded by clear yellow color zones on the plate. Alkalitolerant *Bacillus circulans* ATCC 21783, producer of β-CGTase (supplied by the National Bank of Microorganisms and Cell Cultures, Sofia, Bulgaria) was used as a reference strain. The active cultures were transferred several times on

the same agar medium but without phenolphthalein and methyl orange, and then individual colonies were isolated.

Screening for CGTase Active Strains by Batch Cultures The isolated strains were evaluated by batch cultivation process. They were inoculated into 100 ml Erlenmeyer flasks, containing 20 ml of the alkaline liquid medium (initial; pH 10). After overnight incubation at 40°C in a water bath shaker (New Brunswick, USA, 220 rpm), the inoculum suspensions (2%, *v*/*v*) were transferred into 20 ml alkaline medium and cultivated 48 h in a shaker at 40 °C.

Screening for CGTase Active Lines by a Continuous Culture Continuous cultivation system was applied for enrichment and isolation of high productive CGTase lines from the most active strain 20RF. Continuous culture experiments were carried out in a laboratory bioreactor Bioflo (New Brunswick, USA) with a 700-ml fermentor vessel and 350-ml working volume under the following growth conditions: inoculum concentration 2% (*v*/*v*), an initial pH of the alkaline nutrient medium 10, temperature 40 °C, air flow rate 1 *v*/*v*/min and agitation rate 400 rpm. A batch operation was performed during the initial 9 h (a late exponential growth phase; OD_{650nm} 1.00–1.03), and then continuous operation was started. Four different dilution rates (D, h^{-1}), corresponding to 11.1–3.7 h residence time, were switched over. It was considered that the system is in a steady state after at least five replacement volumes (residence times), and then at each dilution rate, triplicate samples at an interval of 1 h were taken to measure the CGTase activity, enzyme productivity, protein content, cell growth and to isolate individual colonies (lines) on agar alkaline medium.

Morphological and Physiological Characteristics of the Chosen Isolates

Characterisation of the isolates was performed as described by Sneath [12]. The media for the phenotypic tests were adjusted to pH 10 with sterile Na_2CO_3 (after autoclaving).

Phylogenetic Analysis of the Isolated Obligated Alkaliphiles

DNA Isolation and PCR Amplification of 16S rRNA Genes The pure chromosomal DNA was isolated from 5 ml 16-h old cultures, using GFX Genomic blood DNA purification kit (Amersham Biosciences).

Polymerase chain reaction (PCR) was performed with primer pair, universal for eubacterial 16S rRNA genes. The forward primer fD1: 5′-AGAGTTTGATCCTG GCTCAG-3′ and the reverse primer rD1: 5′-AAGGAGGTGATCCAGCC-3′ [14] were purchased from MWG-Biotech, Germany. The PCR mixtures, in a total volume 50 μl, were prepared with PuReTaq™ Ready to Go™ PCR beads (Amersham Biosciences), according to the manufacturer's instructions. The final concentrations of primers were 1 pmol μl^{-1}. The template DNA was 1–2 ng μl^{-1}. The PCR amplification was done in a Progene termocycler (Techne, UK) under the following temperature profile: 95 °C for 5 min followed by 35 cycles of heat denaturation at 94 °C for 1 min, primers annealing at 55 °C for 1 min, 72 °C for 2 min and final elongation of the amplified DNA at 72°C for 5 min. The 1.5 kb PCR products were visualised in 1% agarose gel in Tris–borate buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) with ethidium bromide staining [15].

DNA Sequencing The 16S DNA PCR products were purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences). The nucleotide sequences of the

PCR products were determined by MWG-Biotech Company (Germany) using automated sequencer (Applied Biosystems 3700) and the dye-deoxy termination procedure. Primer pair, used for sequencing, was the above-described fD1/rD1. The sequence analysis was performed by Chromas and CAP3 Sequence Assembly Program. Sequence comparison was done by the basic local alignment search tool (BLAST) program [16]. All 16S rRNA gene sequences of the alkaliphilic bacterial strains were taken from the GenBank of NCBI.

Phylogenetic Tree and Accession Numbers The phylogenetic tree was constructed with the neighbor-joining method after multiple sequence alignments, using ClustalW [17] and BioEdit programs.

The 16S rRNA ribosomal gene sequences obtained in this study are deposited to the NCBI GenBank database under accession numbers EF589779 (for *Bacillus* sp. 20RF) and EF589780 (for *Bacillus* sp. 8SB).

Preliminary Evaluation of the Isolated Obligated Alkaliphiles for Cyclodextrin Production

Crude enzymes were obtained as follows: active culture liquids were centrifuged at 4,000×*g* for 20 min, and the supernatant solutions were concentrated and partially purified by ultrafiltration using Millipore Minitan^{TM} Ultrafiltration System (Bedford, Massachusetts, USA; membrane PM 30 kDa).

The pH profile of the crude enzymes was studied over the pH range of 5.0 to 10.0, using the following buffers: 0.1 M Na–acetate–CH_3COOH (pH 5.0–6.0), 1/15M potassium–sodium phosphate (pH 6.0–8.0) and 0.1 M glycine–NaOH–NaCl (pH 8.5–10.0).

Thermal stability of the crude enzymes was measured as a residual activity after a heat pretreatment at 60 °C for 1 h and at 65 °C for 30 min in the presence of 1 mM $CaCl_2$ and 1% (*w*/*v*) substrate. The enzyme activity of an untreated sample corresponded to 100%.

Starch hydrolysates were prepared and analysed for CD production by using 40 g l^{-1} starch (Fluka) and crude CGTases (12 U/g substrate for *Bacillus* sp. 20RF and 16 U/g substrate for *Bacillus* sp. 8SB). The enzyme reaction was carried out at 60 °C, and at defined time intervals, it was stopped by placing the samples in a boiling water bath for 5 min. Starch hydrolysates were chilled, centrifuged at 6,000×*g* for 15 min and analysed for CDs by HPLC.

Analytical Methods

CGTase cyclising activity was assayed by the method of Kaneko et al. [18] based on the reduction in the colour intensity of phenolphthalein after complexation with β-cyclodextrin. One unit of CGTase activity was defined as the amount of enzyme that formed 1 μg of β-CD min^{-1} under standard conditions (substrate starch soluble acc. to Zulkowsky, Fluka; phosphate buffer pH 6.0; 60 °C; 20 min reaction time).

Total protein content was determined by the method of Bradford [19] using bovine serum albumin as a standard.

Cell growth was measured by absorbance at 650 nm.

CD production was analysed by HPLC system Perkin Elmer LC-25 with YMC-Pack-ODS-AQ column (150×4.6 mm) at a room temperature. Samples were applied to the column after a membrane filtration (0.22 μm, Millipore). The mobile phase was methanol/water (7:93, *v*/*v*) with a flow rate 1.2 ml min^{-1}. Injection volume was 20 μl.

Results and Discussion

Isolation of CGTase Active *Bacillus* Strains

Enrichment and Plate Screening Water and soil samples from 17 Bulgarian regions were studied after treatment at 80 °C for 10 min as it was described above (Table 1). CGTase active thermophilic *Bacillus* isolates (incubated at 60 °C) were not found, despite of the good cell growth and the high alkaline pH of the culture medium in 23 from of the 29

Table 1 Isolation of alkaliphilic CGTase producers from the genus *Bacillus* from Bulgarian regions.

Source	Cultivation at 40 °C		Cultivation at 60 °C	
	pH after enrichment	CGTase active *Bacillus* isolates	pH after enrichment	CGTase active *Bacillus* isolates
Sapareva bania				
Water	9.5	No	9.5	No
Water+slime	7.0	No	8.8	No
Water+soil	9.7	+	8.5	No
Belchin bania				
Water	9.3	No	9.3	No
Water+slime	6.0	+	9.0	No
Water+soil	5.5	+	5.5	No
Blagoevgrad				
Water	9.3	No	9.8	No
Water+slime	9.3	No	8.0	No
Water+soil	6.0	No	7.0	No
Sofia, Central bania				
Water	9.0	+	9.5	No
Water+slime	7.0	No	9.2	No
Sofia region				
Potato+soil 1	8.0	No	8.0	No
Potato+soil 2	8.0	No	8.0	No
Kaloianovo, rice field				
Rice root+soil	10.0	+	10.0	No
Rice blade	9.0	+	9.5	No
Welingrad				
Maize root	10.0	No	9.0	No
Aitos–water	7.0	No	7.0	No
Chepino—1 water	6.0	No	8.0	No
Chepino–2 water	6.0	No	7.8	No
Kostandovo–water 1	7.0	No	7.5	No
Kostandovo–water 2	7.5	No	7.5	No
Momina salsa–water	6.0	No	9.0	No
Hisaria–water	6.0	No	9.2	No
Staro jelezare–water	10.0	No	10.0	No
Rakitovo–water	10.0	No	10.0	No
Chepino–water+soil	8.9	No	9.2	No
Welingrad				
Water 1	10.0	No	9.5	No
Water+soil 2	9.7	+	9.8	No
Water+slime 3	9.9	No	10.0	No

samples studied. Further selection by growth in alkaline agar medium containing phenolphthalein and methyl orange was based on the formation of yellow halo zones around the isolates as a result from the formed β-cyclodextrin-phenolphthalein inclusion complex. Beta-cyclodextrin can also form inclusion complex with methyl orange, but the interaction between the molecules is weak due to the larger size of the β-cyclodexrtin cavity and the extent of methyl orange decolorisation is very low [20]. Seven clear yellow halo zones around the samples grown at 40 °C on alkaline agar medium with indicators were observed. Three of these samples (with final pH 9.7–10.0) were chosen for further isolation of the individual colonies: Sapareva Bania (water+soil), Kaloianovo (rice field, rice root+soil) and Welingrad (water+soil).

Evaluation and Selection by a Batch Culture One hundred and thirty-seven isolated alkaliphilic strains grown in a liquid alkaline medium for 48 h at 40 °C were estimated for CGTase activity. However, only 27 isolates had detectable CGTase activity in their culture supernatants under the assay conditions (Table 2). Despite of the significant growth of all isolates, the overall CGTase levels in liquid medium were quite low and did not seem to correlate with the activity detected on agar plates. Thirteen strains isolated from Kaloianovo rice field (RF) exhibited CGTase activity in range 90–140 U ml^{-1} after 24 h batch cultivation and more of them maintained or enhanced this enzyme level after 48 h. In most cases, the high CGTase production correlated with high-specific CGTase activity. The isolates from Sapareva bania (SB) and Welingrad (WE) had low CGTase activities except the strains 8SB and 24 WE (in range, 129–134 U ml^{-1} and 25–35 U ml^{-1}, respectively).

Selection of CGTase Active Lines from the Isolate 20RF by a Continuous Culture The results for isolation of high productive lines from the most active isolate 20RF by using of continuous culture are presented in Table 3. CGTase activities in range 200–284 U ml^{-1} were found at a residence time 5.9–6.7 h (corresponding to 0.17 and 0.15 h^{-1} dilution rate, respectively). The enzyme productivity, representing the efficiency of the system, was maximal at a dilution rate 0.15 h^{-1} (42.6 U ml^{-1} h^{-1}; calculated by U ml^{-1}/residence time). The used system for a continuous CGTase production by isolate 20RF provided a significantly higher CGTase yield and specific CGTase activity in comparison to 24- and 48-h batch cultures of the same isolate: namely, 2.1-fold higher CGTase yield and 1.6-fold higher specific CGTase activity compared to 24-h batch; and 1.6-fold higher enzyme activity and 1.2-fold higher specific CGTase activity compared to 48-h batch (Table 3 compared to data from Table 2).

The culture liquids collected at a dilution rate 0.15 h^{-1} at the steady state conditions were used for the isolation of high productive lines of the isolate 20RF (from individual colonies).

After a new batch cultivation of these isolated lines in flasks, the culture supernatants obtained were with CGTase activities 230–250 U ml^{-1}.

Morphological and Physiological Characteristics of Three Selected Strains

Isolates from three different Bulgarian regions were chosen for further studies: 20RF (from Kaloianovo, rice field), 8SB (from Separeva Bania, water+soil) and 24 WE (from Welingrad, water+soil). Their phenotypic characteristics summarised in Table 4 indicated that they possessed the key properties for the genus *Bacillus*. The characteristics, identical for the three strains were Gram-positive, sporulating, rod-shaped microorganisms with ellipsoidal spores and positive for catalase test, starch hydrolysis, reduction of nitrate to nitrite and formation of indole; they were negative for gas production from carbohydrates,

Table 2 Selection of CGTase active *Bacillus* isolates by batch cultures.

Bacillus isolates, No.	After 24 h batch cultivation			After 48 h batch cultivation		
	OD_{650nm}[a]	CGTase activity (U ml^{-1})[b]	Specific CGTase activity (U mg^{-1} protein)	$OD_{650\ nm}$	CGTase activity (U ml^{-1})	Specific CGTase activity (U mg^{-1} protein)
1SB	1.02	44.8	203.6	0.80	12.5	83.3
2SB	1.28	9.1	82.7	0.78	0	0
3SB	1.08	10.6	81.4	0.87	0	0
4SB	0.90	5.25	47.7	0.72	0	0
5SB	1.04	20.8	148.6	0.85	0	0
6SB	1.02	14.8	98.7	0.84	0	0
7SB	0.54	117.6	470.4	0.41	165.1	589.6
8SB	1.28	129.2	680.0	0.82	134.4	840.0
9RF	1.21	109.9	578.4	0.84	147.2	865.9
10RF	0.68	124.7	319.7	0.55	110.7	299.2
11RF	1.08	44.7	203.2	1.07	23.2	154.7
12RF	1.01	133.2	444.0	1.28	158.1	465.0
13RF	1.06	145.8	502.7	1.31	157.1	506.8
14RF	1.09	136.8	427.5	0.88	92.3	288.4
15RF	0.80	129.0	379.4	0.74	161.8	449.4
16RF	0.81	130.2	372.0	0.73	143.2	461.9
17RF	1.10	28.0	200.0	1.18	49.0	376.9
18RF	0.85	100.3	401.2	0.90	153.0	900.0
19RF	0.83	93.3	405.6	0.85	141.8	834.1
20RF	0.90	134.5	896.7	0.90	174.4	1162.7
21RF	0.83	111.2	654.1	0.85	155.0	911.8
22RF	0.82	96.1	565.3	0.87	151.0	888.2
23RF	0.80	97.7	542.8	0.84	122.7	681.7
24WE	1.18	34.6	266.1	1.17	25.3	194.2
25WE	1.24	45.5	216.7	1.21	10.8	51.4
26WE	1.31	38.7	203.7	1.29	12.5	59.5
27WE	0.99	9.8	51.6	1.20	0	0

[a] Data are mean values±SD from 0.05 to 0.07, $n=3$

[b] Data are mean values±SD from 0.14 to 0.23, $n=3$

casein hydrolysis, formation of dihydroxyacetone, deamination of phenylalanine, and degradation of tyrosine.

Concerning the other studied phenotypic properties, the isolates 20RF and 8SB grew in nutrient broth at pH between 9.0–11.0 with an optimum at pH 9.8–10.3, indicating that they

Table 3 Selection of CGTase active lines of the isolate 20RF by continuous culture at different dilution rates.

Dilution rate (h^{-1})	0.09	0.15	0.17	0.27
pH	10.0	10.0	10.0	10.0
OD_{650nm}	1.60	0.94	0.99	0.88
CGTase activity (U ml^{-1})[a]	134.8	284.1	200.3	88.7
Specific CGTase activity (U mg^{-1} protein)	1685.0	1420.5	1430.7	739.2
Enzyme productivity (U ml^{-1} h^{-1})	12.14	42.59	34.06	23.97

[a] Data are mean values±SD 0.07, $n=3$

Table 4 Phenotypic properties of 20RF, 8SB and 24WE isolates (each property for each strain was studied in three replicates).

Property	20RF	8SB	24WE
Form	Rod	Rod	Rod
Spores			
Round	−	−	−
Ellipsoidal	+	+	+
Central or paracentral	+	+	−
Subterminal or terminal	−	−	+
Swelling the sporangium	−	−	+
Gram reaction	+	+	+
Catalase	+	+	+
Anaerobic growth	+ (weak, after 72 h)	+	+
Voges–Proskauer test	−	−	+ (weak)
pH in V-P broth	9.5	9.5	6.0
Temperature of growth:			
Maximum	45 °C	45 °C	50 °C
Optimum	38–40 °C	35–38 °C	40 °C
Minimum	15 °C	15 °C	15 °C
Growth in lysozyme (0.001%)	−	−	+
Growth in azide (0.02%)	+	+	−
Growth in			
Media, pH 5.7	−	−	−
pH 6.8	−	−	+
pH 7.0	−	−	+
pH 8.0	−	−	+
pH 9.0	+ (weak)	+	+
pH 10.0	+	+	+
pH 11.0	+	+	+
NaCl (2%)	+	+	+
NaCl (5%)	−	+	−
NaCl (7%)	−	+	−
NaCl (10%)	−	+	−
Acid from			
Glucose	+	+	+
Arabinose	+ (weakly)	+ (weakly)	+ (weakly)
Xylose	+	+	+
Mannitol	+ (weakly)	+ (weakly)	+
Gas from carbohydrates	−	−	−
Hydrolysis of			
Starch	+	+	+
Casein	−	−	−
Utilization of citrate	+ (weakly)	+	−
Reduction of nitrate to nitrite	+	+	+
Formation of indole	+ (weakly)	+	+
Formation of dihydroxyacetone	−	−	−
Deamination of phenylalanine	−	−	−
Degradation of tyrosine	−	−	−
Litmus milk	Alkaline reaction of litmus, without clotting	Alkaline reaction of litmus, without clotting	Neutral reaction of litmus, without clotting
Colony shape, colour	Circular, whitish	Circular, white	Circular, white

are obligated alkaliphiles. The strain 24 WE was alkalitolerant as seen by its ability to grow in pH interval from 6.8 to 11.0 with an optimum at pH 10.0. Spores of strains 20RF and 8SB were located centrally to paracentrally, not swelling the young sporangium. These two isolates were negative for Voges–Proskauer test (pH 9.5 in V-P broth) and growth in lysozyme. They were positive for growth in azide, utilisation of glucose, arabinose, xylose, mannitol and citrate and showed alkaline reaction of litmus milk.

The facultative alkaliphilic strain 24WE differed from the obligated alkaliphiles (20RF and 8SB): Spores were located subterminally or terminally with swelling the sporangium; it was positive for Voges–Proskauer test (pH 6.0 in V-P-broth) and growth in lysozyme and negative for utilisation of citrate and growth in azide. Litmus milk showed neutral reaction.

Further phylogenetic analyses were carried out with both obligated alkaliphilic strains 20RF and 8SB exhibiting some differences in their phenotypic properties: Isolate 20RF showed a weak and slow anaerobic growth compared to isolate 8SB; isolate 20RF grew at a higher alkaline pH, between 10.0 and 11.0, whereas isolate 8SB showed a good growth in a more wide range of pH, from 9.0 to 11.0; isolate 20RF grew in nutrient medium with only 2% (*w*/*v*) NaCl, unlike the isolate 8SB, growing in the presence of 2, 5, 7 and 10% (*w*/*v*) NaCl; the colonies of both isolated 20RF and 8SB differed, whitish and white, respectively; and only isolate 20RF showed some weak flocculation in a liquid medium.

Phylogenetic Analysis

The full 16S rRNA gene sequences obtained for the isolated obligated alkaliphiles 20RF and 8SB were aligned to the database for the alkaliphilic bacterial strains available in NCBI-GenBank. Both strains were grouped with *Bacillus* sp.169 (accession number AB043841), *Bacillus* sp. 135 (accession number AB043840) and *Bacillus* sp. C-59-2 (accession number AB043848) with a sequence identity of 99% (Fig. 1). A lower identity of studied strains was found with *Bacillus pseudalcaliphilus* DSM 8725, 98% [21], *Bacillus alcalophilus* DM 485^{T}, 97% [21], *Bacillus* sp. JAMB-750, 97% (accession number AB128830), *Bacillus* sp. A-5A, 97% (accession number AY 914066) and *Bacillus* sp. A-5B, 97% (accession number AY914067).

Evaluation of the CGTases from *Bacillus* sp. 20RF and *Bacillus* sp. 8SB for a Future Exploration in Terms of CD Production

The exhibited CGTase production by the new isolated alkaliphilic *Bacillus* stains 20RF and 8SB was significantly higher, compared to that reported for other alkaliphilic *Bacillus* produces [10, 22–24]. For example, a culture supernatant from alkaliphilic *Bacillus* sp. TS1-1 contains CGTase with 84 U ml^{-1} activity after an optimisation procedure [23] compared with 250 and 140 U ml^{-1} established in the culture supernatants of *Bacillus* sp. 20RF and *Bacillus* sp. 8SB, respectively, by using the same method for CGTase assay. The comparison of the data obtained for the CGTase level and the specific CGTase activity with other reported for alkaliphilic *Bacillus* isolates is getting more complicated because of the use of maltodextrin as a substrate for the enzyme reaction instead of starch [10, 22].

In connection with the future use of the isolated obligated alkaliphiles *Bacillus* sp. 20RF and *Bacillus* sp. 8SB for CD production, crude CGTases with specific activities 1,597 and 1,420 U mg^{-1}, respectively, were obtained by ultrafiltration.

For the industrial application of CGTase, the enzyme thermal stability and pH profile are important properties because the first step in the starch liquefaction is performed at a high

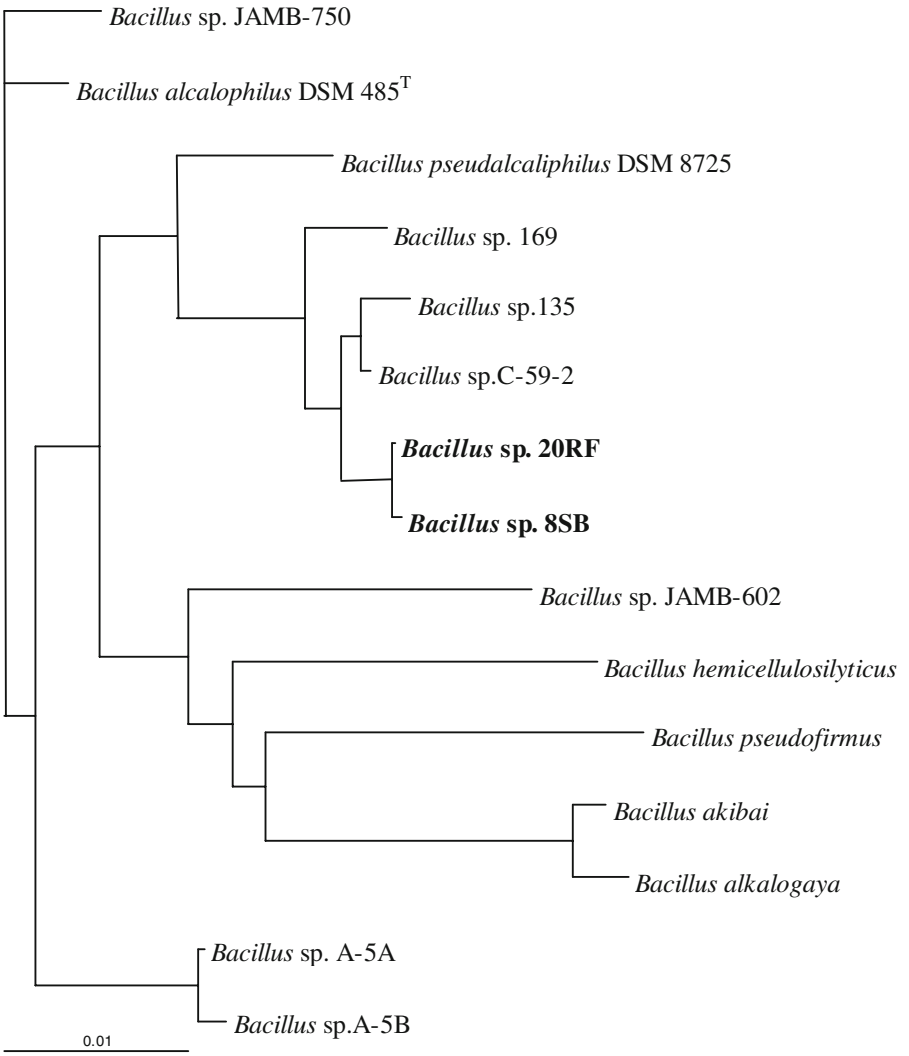

Fig. 1 Phylogenetic tree of the isolated obligated alkaliphiles *Bacillus* sp. 20RF and *Bacillus* sp. 8SB based on the full 16S rRNA gene sequences using the neighbor-joining method (BioEdit-program) after multiple sequence alignment by ClustalW [17]

temperature under the appropriate pH. Both crude CGTases obtained exhibited two pH optima, in the acidic range at pH 6.0 and in the alkaline range at pH 9.0 (Fig. 2). Maximum enzyme activity was established at pH 6.0 for both CGTases, whereas the performance of the reaction at pH 9.0 led to 64.2% relative activity for *Bacillus* sp.20RF CGTase and 93.3% relative activity for *Bacillus* sp. 8SB CGTase.

Both studied CGTases showed some differences in their temperature stability, measured as a residual activity after a heat pretreatment at defined temperatures and time intervals. As the enzyme is protected against heat denaturation by Ca^{2+} and substrate, the experiments were carried out in the presence of these protectors. The data obtained were compared to those for other CGTases from alkaliphilic *Bacillus* strains (Table 5). It is obvious that the new CGTases

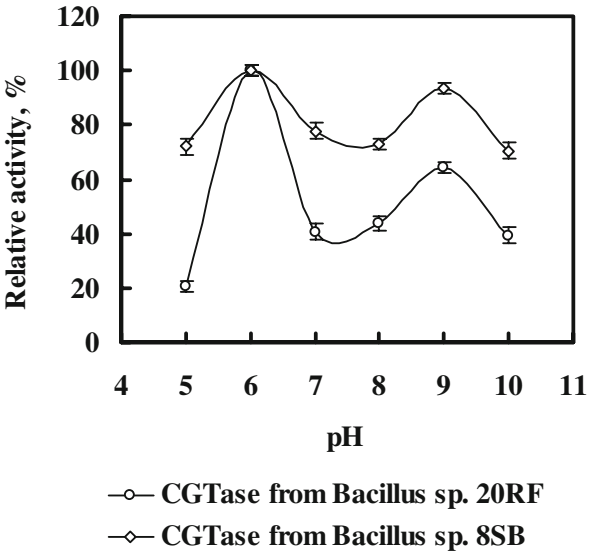

Fig. 2 pH-profile of crude CGTases from *Bacillus* sp. 20RF and *Bacillus* sp. 8SB. *Bars* represent the standard deviation from triplicate determinations

exhibit a promising thermal stability, without detailed studies including higher temperatures, the presence of different concentrations of Ca^{2+} or other reagents and different concentrations of starch or maltodextrin at a defined time for heat pretreatment. The established residual enzyme activities for crude CGTase of *Bacillus* sp. 20RF and crude CGTase of *Bacillus* sp. 8SB after pretreatment at 60°C for 1 h in the presence of 1 mM Ca^{2+} and 1% substrate were 73.3% and 100%, respectively, whereas CGTase from *Bacillus firmus* NCBI 5119 is thermolabile and rapidly loses its activity above 30 °C [24]; CGTase from *Bacillus agaradhaerens* LS-3C after 1 h pretreatment in the presence of 5% maltodextrin or 5 mM Ca^{2+} is stable at a temperature up to 45–50 °C [22]; CGTase from *Bacillus ohbensis* is stable

Table 5 Comparison of pH optimum and thermal stability of CGTases from alkaliphilic *Bacillus* strains.

Producer	pH optimum	Thermostability	References
Bacillus firmus NCBI 5119	5.5–8.5	Up to 30 °C	[24]
Bacillus agaradhaerens LS-3C	9.0	45 °C/1h (+5mM $CaCl_2$)–87% residual activity	[22]
Bacillus sp. G1	6.0	Up to 60 °C	[26]
Bacillus firmus	7.5 and 8.5	Up to 55 °C	[13]
Bacillus ohbensis C-1400	5.0 and 10.0	Up to 45 °C/30 min	[25]
Bacillus sp. AL-6	7.5 and 10.5	40 °C/30 min, 100% residual activity	[27]
Bacillus sp. 20RF	6.0 and 9.0	60 °C/1 h (+1 mM Ca^{2+}+1% starch)–73% residual activity; 65 °C/30 min (+1mM Ca^{2+}+1% starch)–42% residual activity	This work
Bacillus sp. 8SB	6.0 and 9.0	60 °C/1h (+1mM Ca^{2+} + 1% starch)–100% residual activity; 65 °C/30 min (+1mM Ca^{2+}+1% starch)–64.9% residual activity	This work

below 45 °C [25]. The established high cyclising activity at pH 9.0 by crude CGTase of *Bacillus* sp.8SB defined this isolate as a promising producer of a thermostable alkaline CGTase (Table 5 and Fig. 2).

Up to 17–22% conversion to cyclodextrins by crude CGTases from both obligated alkaliphiles 8SB and 20RF was obtained from 40 g l^{-1} starch (Fluka) without using any additives and without an optimisation of the bioconversion conditions, such as a kind, nature and concentration of the substrate, enzyme concentration, temperature, pH and reaction time (Table 6). Both new enzymes formed only two types of CDs, beta and gamma, a feature which could be of interest for their easy separation and industrial production. The crude 20RF CGTase produced β- and γ-CD in the ratio (%) 81.5:18.5 and the crude 8SB CGTase in the ratio (%) 82.7:17.3 after 24 h reaction time at the conditions denoted in Table 6. Moreover, the quantities of the formed γ-CDs were relatively high and comparable to CGTase from *Thermoanaerobacter* sp. (from Novo Nordisk) forming α-, β- and γ-CDs in ratio (%) 30:50:20, or to CGTase from *Bacillus circulans* 251 forming α-, β- and γ-CDs in ratio (%) 15:65:20 at almost identical reaction conditions [28]. In most processes for CD production, selective complexing agents (as trichloroethylene, toluene, bromobenzene, ethanol, etc.) are used to drive the reaction towards the formation of one particular type of CD, but specific applications in food and pharmaceuticals require CDs completely free of these toxic complexing agents. Complete removal of these chemicals is often difficult and expensive. Therefore, the new studied CGTases forming relatively high yield of γ-CD without the use of any agents and without optimisation of the starch conversion process are commercially desirable.

Conclusion

New alkaliphilic *Bacillus* strains, producing CGTase, were isolated from Bulgarian habitats by three steps of a selection.

Both obligated alkaliphiles were clustered into the group of alkaliphilic *Bacillus* species according to the full 16S rRNA gene sequences obtained and deposited to the NCBI GenBank database.

The new isolated obligated alkaliphilic *Bacillus* strains 20RF (accession number EF589779) and 8SB (accession number EF589780) are candidates for further exploration of their CGTases by the use of free and magnetically immobilised cells for the enzyme synthesis and purification.

Table 6 CD production by crude CGTases from *Bacillus* sp. 20RF and *Bacillus* sp. 8SB (40 g l^{-1} starch, 60 °C, pH 6.0, without additives).

Time, h	*Bacillus* sp. 20RF CGTase (12 U per gram substrate)				*Bacillus* sp. 8SB CGTase (16 U per gram substrate)			
	A-CD (mg ml^{-1})	β-CD (mg ml^{-1})	γ-CD (mg ml^{-1})	Conversion into CDs,%	α-CD (mg ml^{-1})	β-CD (mg ml^{-1})	γ-CD	Conversion into CDs, %
2	0	3.86	0.55	11.0	0	4.81	0.94	14.4
3	0	4.84	0.78	14.1	0	4.90	1.02	14.8
4	0	5.11	1.13	15.6	0	4.91	1.08	15.0
24	0	7.23	1.65	22.2	0	5.74	1.19	17.3

The crude CGTases obtained from the above-mentioned isolates possess two pH optima, high temperature stability and converted starch into β- and γ-CDs, which could be of interest for industrial CD production.

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