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Characterisation of a newly isolated thermophilic *Bacillus* strain and of the produced exo-inulinase

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ABSTRACT

A strain of *Bacillus* sp. SG10, isolated from thermal water samples from the region of Velingrad, Bulgaria, showed good capacity to produce extracellular inulinase. The enzyme was synthesized in the presence of monosaccharides and inulin, as carbon sources. The best results were obtained with yeast extract and other sources of organic nitrogen, comparatively to the mineral nitrogen. Optimal growth temperature was 60°C. No growth was established at 40°C and 70°C. It also showed rapid growth in wide pH range from 6.5 to 9.5 with optimum at pH 8.0-8.5. The optimum pH of the crude enzyme for inulin hydrolysis was found at pH 7.0 and the optimum temperature at 60°C. The enzyme showed capacity to hydrolyse sucrose, raffinose and inulin from which it liberated only fructose units, therefore showing an exo-action mechanism. Acting on inulins from several sources, the enzyme showed hydrolysis of the polissaccharide from chicory, dahlia and Jerusalem artichoke tubers.

Keywords: inulinase, *Bacillus*, thermophile, hydrolysis, inulin

Introduction

Inulin is present as a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia etc. Such inulin sources have recently received a great deal of attention as they represent a renewable, low-cost and abundant raw material for the production of ultra-high fructose syrup (Zittan, 1981). They can be used for bioethanol production as well as a versatile source of inulo-oligosaccharides, single-cell oils and proteins and some chemicals like citric acid, butanediol, alcohols and lactic acid (Pandey et al., 1999; Chi et al., 2009a; Liu et al., 2010; Chi et al., 2011; Kango and Jain, 2011).

Inulin belongs to a class of carbohydrates known as fructans. Inulins are polymers composed mainly of fructose units and typically have a terminal glucose molecule. It consists of linear chains of β-2,1-linked D-fructofuranose residues attached to a terminal sucrose molecule (Vandamme and Derycke, 1983).

Inulin can be hydrolyzed by two different types of inulinases: 1/exoinulinase (β-D-fructan fructohydrolase, EC 3.2.1.80) that liberates fructose from the non-reducing end of the β-2→1-glycosidic linkage by plants (exohydrolases, 1-FEH I and 1-FEH II) and microorganisms, including filamentous fungi (*Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus ficuum* and *Aspergillus niger* 12) (Derycke and Vandamme, 1984), yeast (*Candida kefyr*, *Kluyveromyces fragilis* and *Debaromyces cantarellii*) and bacteria (Vandamme and Derycke, 1983; Chi et al., 2009b; Kango & Jain, 2011); and 2/endoinulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) that produces oligosaccharides (Shuichi & Norio, 1992; Skowronek and Fiedurek, 2004). Microbial inulinases are exo-acting in their nature, with the exception of *Aspergillus niger* 12, *A. ficuum*, *Penicillium purpurogenum* and *Chrysosporium pannorum*, which secrete both endo- and exoinulinases that act in synergy for efficient fructose recovery. The action of endoinulinase on inulin results in the production of inulotriose, inulotetraose and inulopentaose. The enzyme lacks invertase (enzyme catalyzing the hydrolysis of sucrose into glucose and fructose) activity as observed in most exoinulinases (Nakamura et al.,1995).

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The increasing interest in fructooligosaccharide production using enzyme hydrolysis is due to factors such as enzyme substrate specificity, controllable hydrolysis reactions that avoid the production of undesirable by-products, in comparison to chemical processing methods. Moreover, enzymes can be immobilized on solid supports for continuous processing and are biodegradable (MacCabe et al., 2002).

In this paper, we describe the isolation and characterization of thermophilic bacterial strain with inulinase activity. The strain synthesizes thermostable enzyme which is of potential industrial use for inulin or sucrose hydrolysis. The paper reports the characteristic of the crude inulinase from the newly isolated *Bacillus* strain.

Materials and Methods

Bacterial strain isolation

The strain of *Bacillus* sp. SG10 was isolated from thermal water samples with temperature 58°C and pH 7.5 from the region of Velingrad (Kostandovo). Five milliliters from samples were mixed with 5 ml isolation medium and incubated at 37°C and 50°C for 48 h for enrichment. After that suspensions were heated at 80°C for 10 min for isolation of *Bacillus* strains (Sneath, 1986). After chilling, 5 ml from these suspensions were mixed again with 5 ml isolation medium and cultivated for 48 h at 37°C and 50°C. Then the samples were serially diluted prior to plating 30 µl on meat agar (1.5% (w/v), Oxoid) containing inulin for isolation of single colonies. Plates were incubated at 37°C and 50°C for 3 days. Pure colonies were obtained by repetitive dilution streaking on peptone-yeast extract agar with additional inulin (0.2%, w/v) as carbon source which helped in the selection of colonies having inulinase enzyme activity. The active cultures were transferred several times on the same agar medium, and then individual colonies were isolated. Ten strains of *Bacillus* were thus isolated. The strains were screened for exo-inulinase production. The strain designated as *Bacillus* spp. SG10 was selected for further studies and stored at 4°C.

Fermentation medium

The medium used for strain isolation, maintenance and enzyme production had the following composition (g/l): peptone (Oxoid, Basingstoke, UK) – 2.0; yeast extract (Oxoid) – 2.0; K_2HPO_4 – 0.4; $MgSO_4$ – 0.08 and inulin (from *Dahlia* tubers, Fluka, Buchs, Switzerland) – 2.0. Inulin was sterilized separately for 20 min at 110°C and added to the medium before inoculation. Sterile sodium carbonate was used to adjust the medium to pH 8.0-8.5 after autoclaving. Erlenmeyer flasks (300 ml) were charged with 50 ml of medium, inoculated (2% v/v) with a culture previously incubated for 18 h, and incubated at 37°C-70°C in water-bath (Julabo SW22) shaker for 36 h at 200 rpm.

Physiological and biochemical tests

The characterization of the strain was performed using the specific tests for Gram staining, motility, spore formation, hydrolysis of starch, casein, gelatin, indol formation, assimilation of arabinose, maltose, sucrose, mannitol, nitrate reduction, etc. These tests were performed using the methods of Gordon et al. (1973), Bergey's Manual of Systematic Bacteriology (Holt et al., 1986; Vos et al., 2009), and Bergey's Manual of Determinative Bacteriology (Holt, 1994).

Effect of nitrogen sources

Effect of different nitrogen sources including peptone, beef extract, yeast extract, casein, soy flour (organic N-sources) and $NaNO_3$, KNO_3, $(NH_4)_2SO_4$ and $(NH_4)H_2PO_4$ (inorganic N-sources) was studied by incorporating 0.4% (w/v) of each N- source in the fermentation medium. From each experimental design, 5 flasks were inoculated and the results submitted to variance analysis for verification of statistical significance (Tukey's range test).

Effect of carbon sources

For the experiments on the effect of the carbon sources, the media were formulated with 2.0% of glucose, fructose, sucrose, inulin, sugar cane molasse, soluble starch, garlic and onion extracts, flours from topinambour (Jerusalem artichoke, *Helianthus tuberosus*) tubers and stems, extracts from these flours, and 0.2% of peptone and yeast extract, as nitrogen sources. From each experimental design, 5 flasks were inoculated and the results, submitted to variance analysis for verification of statistical significance (Tukey's range test).

Inulinase assay

The culture medium was centrifuged at 4000 rpm for 15 min and the supernatant was used as the inulinase source. Inulinase activity was measured by determination of the reducing sugars released from substrate inulin by DNS-method (Miller, 1959). The reaction mixture contained 100 µl

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substrate inulin (from *Dahlia* tubers, Fluka, Buchs, Switzerland; 20 g/l, phosphate buffer pH 7.0) and 100 µl enzyme solution. After incubation at 60°C for 20 minutes the reaction was stopped by 200 µl DNS-reagent. Reducing sugars were determined by calibration curve obtained using a standard solution of fructose (Scharlab S.L., Spain). One unit of inulinase activity was defined as the amount of enzyme that liberates one µmol of fructose per minute under the assay conditions.

Invertase assay

Invertase activity was determined under the conditions described above with the difference that saccharose (sucrose) (Scharlab S.L., Spain; 20 g/l in phosphate buffer, pH 7.0) was used as a substrate. A calibration curve was obtained using an equimolar standard solution of glucose and fructose. One unit of invertase activity was defined as the amount of enzyme that hydrolyzes 1 µmol of saccharose per minute under the assay conditions.

Determination of protein, cell growth

Total protein content in enzyme solution was measured by the Bradford method (1976), using bovine serum albumin as standard.

The culture growth was determined by the absorbance at 650nm and by the CFU/ml. A graph correlating CFU/ml to OD was used. Serial dilution of samples with measured optical density was prepared. The diluted samples were plated (typically from 10^{-4} to 10^{-7} dilution) on agar plates in triplicate and after incubation the colonies were counted, the CFU/ml was calculated and the graph correlating CFU/ml to $OD_{650\ nm}$ was prepared.

Thin-layer chromatography (TLC)

TLC was performed on Silica gel 60 pre-coated plates (Merck, Darmstadt, Germany, 25 x 25 cm). A mixture of n-propyl alcohol/ethyl acetate/water (7:1:2, v/v/v) was used as a developing solvent. Sugars were detected by spraying of the air-dried plates with staining reagent containing ethanol/acetic acid/sulfuric acid/anis aldehyde (9:0.1:0.5:0.5, v/v/v/v). Carbohydrates were revealed after heating for 10 min at 120°C and were visualized as dark green spots. Fructose, glucose, raffinose, melezitose, inulin, saccharose in concentration 2.5 mg/ml (Sigma, USA) were used as standards. Garlic and onion extracts and extract from flour of Jerusalem artichoke tubers were also tested.

Crude enzyme preparation

The active cultural liquid was centrifuged at 4000 rpm for 20 min to remove the bacterial cells. The clear supernatant was concentrated and partially purified by ultrafiltration using a Millipore PM 30 membranes (Millipore, Bedford, MA, USA). The concentrated solution obtained was used as a crude enzyme preparation.

Enzyme characterization

The effect of pH on crude inulinase activity was investigated by measuring the enzyme activity at 50°C over the pH range 3.5 to 6.5 (0.1 M acetate buffer), 6.8 to 7.7 (0.1 M phosphate buffer) and 8 to 9.0 (0.1 M Tris-HCl buffer). For the pH stability determination, aliquots of 0.5 ml of enzyme plus 0.5 ml of the same buffer in mentioned pH range were maintained at 4°C for 2 h and the residual activity was estimated. The optimum temperature was determined by measuring the enzymatic activity in 0.1 M phosphate buffer, pH 7.0 in the temperature range from 40°C to 70°C. For the thermal stability determination of the inulinase, a reaction medium composed of 0.5 ml of enzyme solution and 0.5 ml of 0.1 M phosphate buffer with a pH value of 7.0, was maintained for 30 min in the same temperature range with or without the presence of inulin (10.0 g/l) as stabilizer and the residual activity was measured as described in enzyme assay.

Inulinase action pattern

Aliquots of 0.5 ml of the partially purified enzyme were incubated, separately in 2.0 ml of raffinose solution (Sigma-Aldrich, USA), sucrose (Scharlab S.L., Spain), melezitose (Sigma-Aldrich, USA), inulin (Jerusalem artichoke, Sigma-Aldrich, USA), garlic and onion extracts and extract from flour of Jerusalem artichoke tubers, in a final concentration of 1.0% in 0.1 M phosphate buffer, pH 7.0, at 50°C. The profile of the enzymatic reaction was followed by thin layer chromatography (TLC). The liberation of glucose and fructose from sucrose and fructose from raffinose and inulin was also determined, respectively by the DNS-method. The values obtained in the raffinose reaction were divided by 2, because to each molecule of liberated fructose corresponds 1 reducing unit of melibiose.

Hydrolysis of inulin from different sources

For the hydrolysis studies of inulin from different origins, the reaction system was composed by 0.5 ml (14 U/ml) of partially purified enzyme, 5 ml of dahlia (*Dahlia pinnata*), chicory (*Cichorium intibus*) or Jerusalem artichoke

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(*Helianthus tuberosus*) (Sigma-Aldrich, USA) 5.0% inulin solutions in 0.05 M phosphate buffer, pH 7.0.

Preparation of onion and garlic extract

2 kg of the bulbs or cloves were peeled and chopped, then heated up to 90°C with 2 litters of distilled water. The slurry obtained was allowed to cool down and to stand for sedimentation. Afterwards, it was filtered through muslin cloth and the filtrate was used in media formulation.

Preparation of Jerusalem artichoke flours from tubers and stems

Jerusalem artichoke (*Helianthus tuberosus* L.) tubers and stalks were collected from several farms. The tubers were washed, peeled and cut, then were dried at 80°C to constant weight. The dried tubers were grounded mechanically and the resulting flours were used in the next experiments without fractionation. The stalks were processed similarly. The inulin present in the stalks and tubers and the soluble carbohydrates were extracted with a steam jet (twice for a 10 min).

Results and Discussion

Isolation of bacterial strains

Ten bacterial strains growing on a medium containing inulin as the sole carbon and energy source were isolated by enrichment culture as described in Materials and Methods. Six strains were able to grow at both 50°C and 37°C, showing a typical thermotolerant pattern (Table 1); one was able to grow only at 37°C. The three remaining strains were determined to be thermophilic since they could grow at 50°C, but not at 37°C.

Table 1. *Isolation of thermophilic inulin-degrading bacterial strains and activity on inulin and sucrose.*

Strain	Growth at		Bacterial activity (U/ml) on:		Sucrose/Inulin ratio
	37°C	50°C	Inulin*	Saccharose**	
Bacillus spp. SG1	+	+	0.90	25.30	28.11
Bacillus spp. SG2	+	+	1.24	10.82	8.73
Bacillus spp. SG3	+	+	1.62	27.85	17.19
Bacillus spp. SG4	+	+	0.73	33.93	46.48
Bacillus spp. SG5	+	+	1.12	43.74	39.05
Bacillus spp. SG6	+	+	1.14	17.46	15.32
Bacillus spp. SG7	-	+	2.33	24.55	10.54
Bacillus spp. SG8	+	-	1.64	32.54	19.84
Bacillus spp. SG9	-	+	1.42	18.36	12.93
Bacillus spp. SG10	-	+	1.95	19.04	9.76

* Statistical significance ($p<0.01$), Data are mean values ± SD from 0.01 to 0.03, n=5;
** Statistical significance ($p<0.05$), Data are mean values ± SD from 0.02 to 0.06, n=5;

Activity on inulin and saccharose

Strains SG1 to SG10 were cultivated on the inulin medium, the culture medium was centrifuged at 4000 rpm for 15 min and the supernatant was used for assaying the enzyme activity toward inulin and sucrose (Table 1). All of the strains displayed activity on inulin and on sucrose, in the ranges of 0.73 to 2.33 U/ml for inulin as substrate. The saccharose-hydrolyzing activity - from 10.82 to 43.74 U/ml, was higher than that on inulin. The sucrose-inulin ratios were from 8.73 to 46.48. According to Belamri et al. (1994) inulin-degrading enzymes are characterized by the S/I ratio. For inulinases this ratio is lower than 50 while for invertases S/I values vary from 1600 to 2800, therefore all enzymes produced by the isolated *Bacillus* sp. strains were inulinases.

Morphological and physiological characterization of the SG10 isolate

The selected *Bacillus* isolate was Gram-positive, motile, aerobic rod-shaped bacterium (0.5-0.7 x 1.4-2.5 μm) forming ellipsoidal terminal spores swelling the sporangia (Table 2). It grew at high temperatures - from 50 to 60°C. Optimal growth temperature was 60°C. No growth was established at 40°C and 70°C. It also showed rapid, good growth in wide pH range - from 6.5 to 9.5 with optimum at pH 8.0-8.5. The following tests were positive: acid from glucose, xylose, mannitol; hydrolysis of starch; pH in Voges-Proskauer broth 5.7; decomposition of casein and tyrosine; growth at 6.8; growth in 2 and 5% NaCl; production of acid in litmus milk;

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reduction of nitrate to nitrite; decomposition of gelatin, catalase and oxidase tests. Negative reactions were: growth at pH 5.7; formation of dihydroxyacetone, acetyl methyl carbinol is not produced - Voges-Proskauer test is negative; anaerobic growth; growth in 0.02% azide broth; gas from glucose, arabinose, xylose, mannitol; utilization of citrate; deamination of phenylalanin; formation of indole; decomposition of casein; growth in 7 and 10% NaCl; resistance to lysozyme; methyl red test, hydrogen sulfide formation Tables 2 and 3). According to these results, the *Bacillus* isolate SG10 showed no differences in comparison to *Geobacillus* (*Bacillus) stearothermophilus* strains described by Gordon et al. (1973), Sneath, 1986, Bergey's Manual of Determinative Bacteriology (1994), Zeigler, 2001 and Slepecky & Hemphill, 2006. Because of the requirement of specific 16S rDNA analysis of the strain for taxonomical identification, in the present paper the object of studies was denoted as *Bacillus* sp. SG10. The isolate differs from the *Bacillus* sp. SG7 strain (Gavrailov and Ivanova, 2014) by its cell morphology, temperature interval of growth and temperature optimum.

Enzyme production by batch cultivation

Figure 1 shows the growth and production curves of the strain. The biosynthesis of the inulinase follows with a certain delay to the log phase of the bacterial growth, starting at the second hour of incubation and the largest enzyme liberation was between the 8 and 20 h of fermentation. Such behavior is common for this enzyme - in other microorganisms such as yeasts and molds it reaches the maximum activity in the medium after 72 h of fermentation (Allais et al., 1986; Neagu (Bonciu) & Bahrim, 2011). Maximal inulinase activity of 1.95 U/ml and invertase activity of 19.04 U/ml were reached in the cultural liquid after 14 hours of fermentation. These activities are similar to those obtained with strain Bacillus sp. SG7 (Gavrailov and Ivanova, 2014). Lower enzyme levels in cultural liquid have been reported for other mesophilic and thermophilic bacterial producers, for example, 0.200 U/ml by *Bacillus* sp. LCB41 (Allais et al., 1987a, b); 0.400 U/ml by *Arthrobacter* sp. EM278 (Elyachioui et al., 1992); 0.330-0.460 U/ml by *Flavobacterium multivorum* (Allais et al., 1986). In comparison with yeast inulinases, the enzyme level in cultural liquid of *Bacillus* sp.SG10 was at the same range as the reported for *Kluyveromyces fragilis* (Gupta et al., 1994) and for *Kluyveromyces marxianus* (Silva-Santisteban & Filho, 2005), but these activities were obtained after a long cultivation time of 48 to 96 hours.

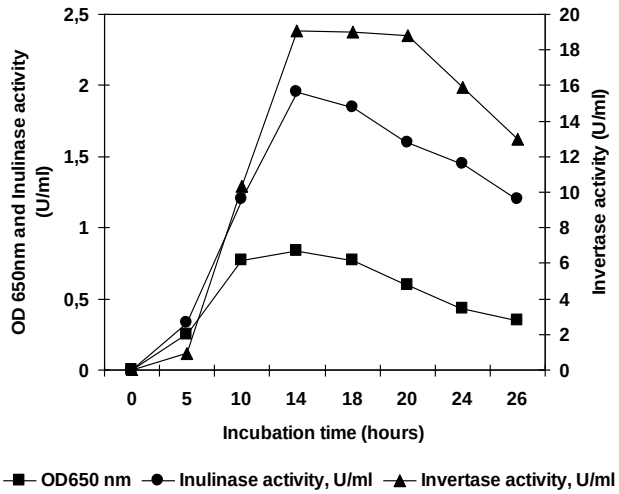

Figure 1. *Growth profile and inulinase production by Bacillus sp. SG10.*

Temperature and pH optima for growth and enzyme production

The effect of temperature and pH on strain SG10 was investigated. The strain grew at temperatures from 45 to 65°C (Figure 2), no growth was observed at 40°C and 70°C. A temperature of 60°C was selected for further experiments. At pH values lower than 6.5, no growth was observed. At pH 8.0-8.5 the activity was almost constant and pH 8.0 was selected for further experiments (Figure 3).

Thermophilic strain *Bacillus* sp. SG10 possesses some advantages for a large-scale production: i) extracellular inulinase synthesis at high temperature (60°C); ii) a short production time (14-20 h); iii) a high inulinase yield (1.95 U/ml, similar or higher to that from other bacteria and yeasts).

Effect of carbon and nitrogen sources

Inulinase production by the *Bacillus* sp. SG10 strain was influenced greatly by different carbon sources (including inulin, fructose, glucose, sucrose, garlic and onion extract, Jerusalem artichoke tuber flour extract) in the medium (Table 4). Growth on inulin, garlic and onion extracts, Jerusalem artichoke tubers and stems extracts was almost identical (optical density in the range of 0.76 to 0.84).

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Table 2. *Morphological and physiological characteristics of the Bacillus sp. SG10 strain.*

Strain characteristics	***Bacillus* sp. SG10**
Source	Velingrad (Kostandovo)
Gram stain	Gram-positive
Cell morphology	Rod-shaped bacterium 0.5-0.7 x 1.4-2.5 μ
Spore formation	+
Type of spores	Ellipsoidal terminal spores swelling the sporangia
pH interval of growth	6.5-9.5
pH optimum of growth	8.0-8.5
Temperature interval of growth	50-60°C
Temperature optimum	60°C
Motility test	+
Growth at pH 5.7	-
Anaerobic growth	-
Utilization of citrate	-
Hydrolysis of starch	+
Decomposition of casein	-
Decomposition of tyrosine	-
Deamination of phenylalanine	-
pH in Voges-Proskauer broth (acid)	5.7
Voges-Proskauer	-
Formation of dihydroxyacetone	-
Growth in NaCl solution	
2%	+
5%	+
7%	-
10%	-
Acid in litmus milk	+
Growth in 0.02% azide broth	-
Reduction of nitrate to nitrite	+
Formation of indole	-
Catalase test	+
Oxidase test	+
Methyl red test	-
Gelatin test	+
H_2S test	-
Resistance to lysozyme	-

Yeast extract was found to be the best nitrogen source to be used in combination with inulin from dahlia for inulinase production followed by beef extract and peptone (Table 5). Complex nitrogen sources were better than inorganic nitrogen sources. Kango (2008) also found yeast extract to be the best N-source in media containing dandelion roots, while meat extract and corn steep liquor have also been reported to be better N-sources (Jain et al., 2012).

Enzyme concentrate

Bacterial cells from the cultural liquid were harvested by centrifugation and the clear supernatant was concentrated by ultrafiltration. An 8.8-fold concentration and 1.55-fold purification of the cultural supernatant was achieved with a negligible loss of inulinase activity. More than 94% from the initial enzyme activity of the supernatant was detected in the concentrate. The further studies on the enzyme properties were performed using this crude enzyme preparation.

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Table 3. *Assimilation of sugars by the strain SG10.*

Carbon source	Assimilation	Acid formation/Gas formation
Glucose	+	+/-
Fructose	+	-/-
Arabinose	+	-/-
Xylose	+	+/-
Mannitol	+	+/-
Lactose	+	-/-
Maltose	+	-/-
Saccharose	+	-/-
Soluble starch	+	+/-
Sugar cane molasse	s	-
Inulin (dahlia)	+	-/-
Garlic extract	+	-
Onion extract	+	-
Tubers flour	s	-
Stems flour	s	-
Tubers extract	+	-
Stems extract	+	-

+: positive; -: negative; s: slow.

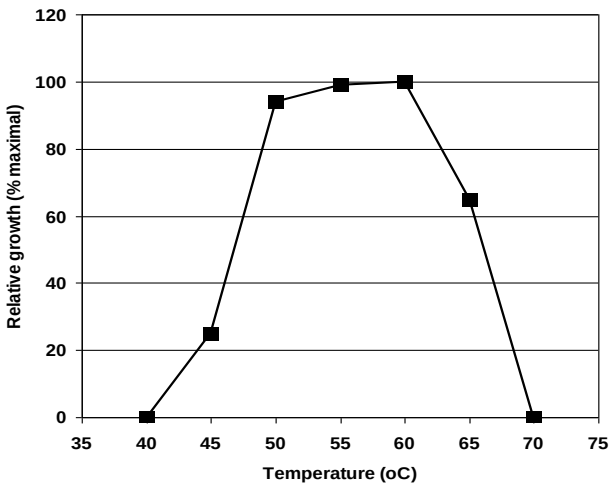

Figure 2. *Temperature optimum for growth of Bacillus sp. SG10.*

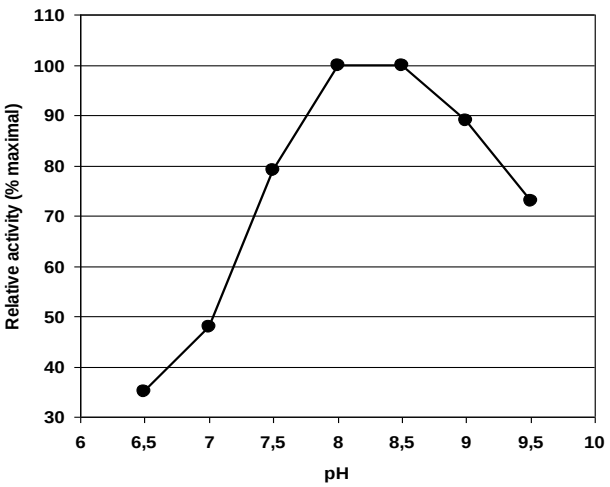

Figure 3. *pH optimum for growth of strain Bacillus sp. SG10.*

Temperature optimum and stability

The effect of temperature on inulinase activity of the crude preparation was studied from 40 to 70°C at pH 7.0. Optimal activity on both substrates (inulin and sucrose) was established at 60°C. When enzyme reaction was performed at 70°C the relative inulinase activity was 51% of the measured maximal activity (Figure 4).

Thermostability of the enzyme concentrate was measured after a preliminary treatment at 40, 50, 60 and 70°C for 30 min in the absence or in the presence of a substrate inulin (Figure 4). Enzyme activity on inulin as substrate was retained 100% after a heat treatment of the concentrate at 40-60°C for 30 min in the absence of substrate inulin. The increase of temperature up to 65°C for 30 min led to 82% residual inulinase activity of the enzyme.

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Table 4. *Effect of the carbon source on the inulinase production by Bacillus sp. SG10 strain at optimal pH 8.0 and optimal temperature of production 60°C.*

Carbon source	Inulinase** U/ml ± SD	Invertase** U/ml ± SD	S/I ratio	Optical density* OD650nm
Glucose	0.040±0.03	0.455±0.02	11.37	0.50±0.03
Fructose	0.065±0.05	0.700±0.02	10.77	0.45±0.03
Saccharose	0.075±0.03	0.725±0.03	9.67	0.55±0.02
Inulin	**1.950±0.05**	**19.045±0.06**	**9.76**	**0.84±0.03**
Sugar cane molasse	0.077±0.03	0.833±0.03	10.82	0.40±0.03
Soluble starch	0.053±0.03	0.515±0.04	9.72	0.33±0.02
Garlic extract	1.915±0.05	19.250±0.05	10.05	0.80±0.03
Onion extract	1.900±0.05	19.400±0.04	10.21	0.80±0.03
Tubers flour	1.160±0.03	11.590±0.07	9.99	1.10^8 CFU/ml
Stems flour	0.850±0.02	9.000±0.07	10.59	2.10^7 CFU/ml
Tubers extract	1.760±0.05	18.900±0.06	10.73	0.76±0.02
Stems extract	1.350±0.05	14.240±0.04	10.55	0.78±0.02

* Statistical significance ($p<0.01$), Data are mean values ± SD from 0.01 to 0.04, n=5;
** Statistical significance ($p<0.05$), Data are mean values ± SD from 0.02 to 0.07, n=5; I – Inulinase; S – Invertase.

Table 5. *Effect of the nitrogen source on the inulinase production by Bacillus sp. SG10 strain at optimal pH 8.0 and optimal temperature of production 60°C.*

Nitrogen source	Inulinase U/ml ± SD	Invertase U/ml ± SD	S/I ratio	Optical density OD650nm
Peptone	1.75 ±0.04	18.29±0.07	10.45	0.75±0.03
Beef extract	1.88±0.05	18.90±0.03	10.05	0.80±0.03
Yeast extract	**1.95±0.05**	**19.04±0.06**	**9.76**	**0.84±0.03**
Soy flour	1.66±0.03	16.33±0.03	9.84	0.70±0.03
$NaNO_3$	0.55±0.03	5.25±0.03	9.55	0.41±0.03
KNO_3	0.47±0.03	4.23±0.03	9.00	0.36±0.03
$(NH_4)_2SO_4$	0.74±0.03	7.85±0.03	10.61	0.47±0.03
$(NH_4)H_2PO_4$	0.96±0.03	10.38±0.03	10.81	0.56±0.03

Statistical significance ($p<0.05$), Data are mean values ± SD from 0.02 to 0.07, n=5; I – Inulinase; S – Invertase.

At higher temperature (70°C) inulinase activity dropped sharply to 55% of the initial.

The preliminary heat treatment in the presence of substrate inulin (10 mg/ml) showed a significant increase of the enzyme thermostability. In this case at 65°C for 30 min the residual inulinase activity was 94%. A heat treatment at 70°C for 30 min led to approximately 88% residual enzyme activity.

Enzyme thermostability was similar to that of inulinase from B. *stearothermophilus* (Belamri et al., 1994) or higher compared with a number of bacterial inulinases. For example, exo-inulinase from *Bacillus* sp. LCB41 retained 70% from its initial activity at 60°C and treatment time 10 min (Allais et al., 1987a), exo-inulinase from *Arthrobacter* sp. showed 50% residual activity at 55°C and incubation time 30 min (Elyachioui et al., 1992). In comparison with inulinases from yeasts and fungi, thermostability of *Bacillus* sp. SG10-enzyme was significantly higher (Vandamme & Derycke, 1983).

Action of the inulinase on several substrates and substrate spectrum

For determination of the substrate specificity of the obtained exo-inulinase, the enzyme reaction was carried out using 4 substrates (10 mg/ml): inulin, sucrose, raffinose and

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melezitose (Table 6). Enzyme activity on substrates raffinose and melezitose was determined using the calibration curve with standard fructose solutions.

The enzyme split inulin, sucrose, raffinose, whereas melezitose was not hydrolyzed.

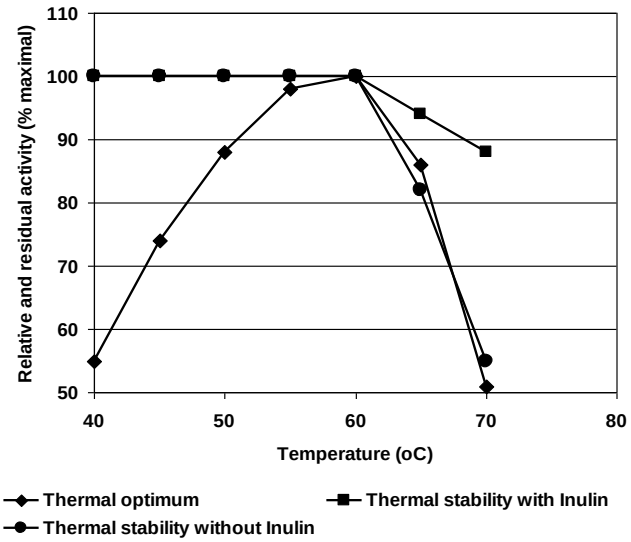

Figure 4. *Effect of temperature on the activity and stability of the inulinase from Bacillus sp. SG10.*

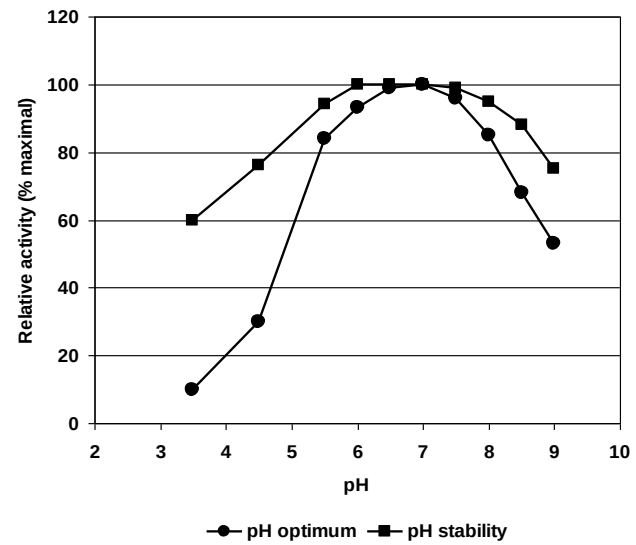

Figure 5. *Effect of pH on the activity and stability of the inulinase from Bacillus sp. SG10.*

Table 6. *Substrate specificity of the crude enzyme concentrate.*

Substrate	Enzyme activity (U/ml)*
Inulin	16.05
Sucrose	156.55
Raffinose	73.65
Melezitose	0

The obtaining of fructose as the only hydrolysis product from inulin and its liberation from the sucrose and raffinose suggest an exo-action mechanism of the enzyme. This action pattern was confirmed also by the chromatogram on Figure 6. The hydrolysis products were analyzed by thin layer chromatography. The only hydrolysis product was fructose. Fructo-oligosaccharides were not detected after an initial 20 min of the process from all substrates except raffinose and melezitose. Results showed that the enzyme could partially hydrolyze raffinose (Figure 6). Fructose from hydrolysis of melezitose was not detected.

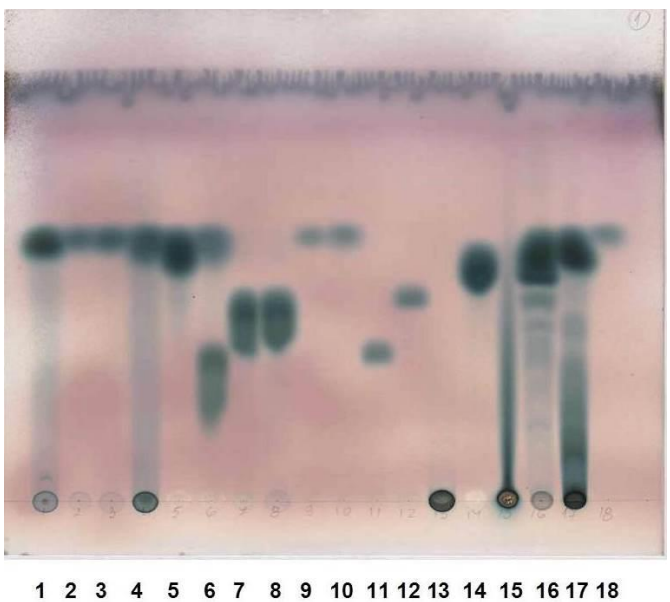

Figure 6. *TLC of reaction products from: 1, garlic extract; 2, onion extract; 3, extract from topinambour flour; 4, inulin; 5, saccharose; 6, raffinose; 7, 8, melezitose. Standards 2.5 mg/ml (Sigma, USA) – 9, 18 glucose; 10, fructose; 11, raffinose; 12, melezitose; 13, inulin from Jerusalem artichoke; 14, saccharose; 15, crude garlic extract; 16, crude onion extract; 17, crude extract from topinambour flour; 50°C, 20 minutes, pH 7.0, enzyme activity 0.90 U/ml*

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The obtained results testified to a typical mode of action of an exo-inulinase splitting fructose residues from fructose side of inulin molecule from different origin.

Higher degree of hydrolysis of inulin from dahlia tubers and Jerusalem artichoke was achieved as compared to the hydrolysis of inulin from chicory (Figure 7).

It is generally accepted that the ratio of the activity on inulin versus sucrose (I/S ratio) and the activity on sucrose versus inulin (S/I ratio) characterizes inulin-degrading enzymes. According to Belamri et al. (1994) the S/I ratio for inulinases is lower than 50 while for invertases S/I values vary from 1600 to 2800. In our case this ratio S/I was 9.76.

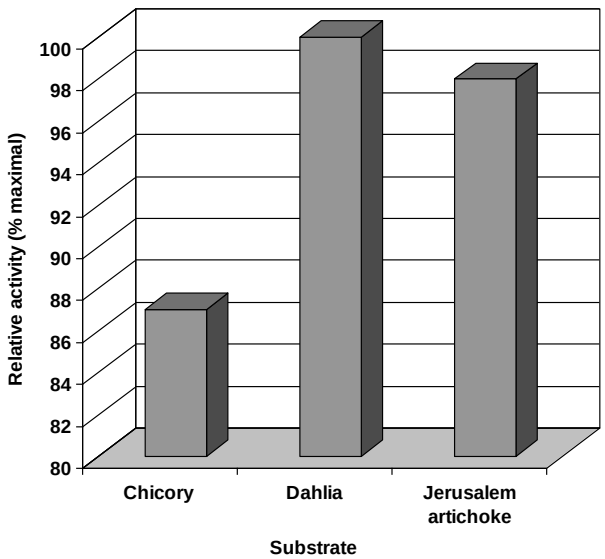

Figure 7. *Action of the inulinase on some substrates.*

In conclusion, enzyme produced by *Bacillus* sp. SG10 is exo-inulinase according to the presented data about: i) reaction products of inulin hydrolysis (fructose); ii) substrate spectrum (inulin, sucrose, raffinose); iii) and the S/I ratio (9.76). This exo-inulinase could be applied at high temperatures (60°C) and pH ranges 6.0-8.0 in contrast to the industrial preparations from yeasts and from *Aspergillus* fungi.

References

Allais J-J, Kammoun S, Blank P, Grirrad C, Baratti JC. 1986. Isolation and characterization of bacterial strains with inulinase activity. Appl. Environ. Microbiol., 52: 1086-1090.

Allais J-J, Hoyos-Lopez G, Baratti J. 1987a. Characterization and properties of an inulinase from thermophilic bacteria. Carbohydrate Polymers, 7: 277-290.

Allais J-J, Hoyos-Lopez G, Kammoun S, Baratti J. 1987b. Isolation and characterization of thermophilic bacterial strains with inulinase activity. Appl. Environ. Microbiol., 53: 942-945.

Belamri M, Sassi AH, Savart M, Tantaoui-Elaraki A, Cottin P. 1994. Purification and properties of an extra cellular inulinase-like β-fructosidase from *Bacillus stearothermophilus*. Lett. Appl. Microbiol., 19(6): 410-413.

Bergey's Manual of Systematic Bacteriology, vol. 2. 1986. (Editor-in-chief Holt JG.), (Editors: Sneath PHA, Mair NS, Sharpe HS.) Baltimore, Williams& Wilkins, 2, ISBN: 0-683-07893-3

Bergey's Manual of Determinative Bacteriology. 1994. Ninth edition, (eds. Holt, J.G. et al.). Williams & Wilkins, Baltimore, Maryland, ISBN 978-0-683-00603-2.

Bergey's Manual of Systematic Bacteriology, 2nd ed. 2009. (eds. Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K-H, Whitman WB.), New York: Springer, ISBN: 978-0-387-68489-5.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principale of protein-dye binding. Anal. Biochem., 27: 248-254.

Chi Z, Zhang T, Liu G, Li J, Wang X. 2009a. Production, characterization and gene cloning of the extracellular enzymes from the marine-derived yeasts and their potential applications. Biotechnology Advances, 27(3):236-255.

Chi Z, Zhang T, Liu G, Xue L. 2009. Inulinase-expressing microorganisms and applications of inulinases. Appl. Microbiol. Biotechnol., 82: 211–220.

Chi ZM, Zhang T, Cao TS, Liu XY, Cui W, Zhao CH. 2011. Biotechnological potential of inulin for bioprocesses. Biores. Technol., 102: 4295-4303.

Deryck DG, Vandamme EJ. 1984. Production and properties of *Aspergillus niger* inulinase. J. Chem. Technol. Biotechnol., 34: 45-51.

Elyachioui M, Hornez JP, Tailliez R. 1992. General properties of extracellular bacterial inulinase. J. Appl. Bacteriol. 73: 514-519.

Gavrailov S, Ivanova V. 2014. Isolation and characteristics of a thermophilic *Bacillus* strain, producer of inulinase. J. BioSci. Biotechnol., SE/ONLINE: 83-94.

Gordon RE, Haynes WC, Pang CH-N. 1973. The Genus *Bacillus*. Washington DC: US Department of Agriculture, Handbook No 427.

Gupta AK, Singh DP, Kaur N, Singh R. 1994. Production, purification and immobilisation of inulinase from *Kluyveromyces fragilis*. J. Chem. Technol. Biotechnol., 59(4): 377-385.

Jain SC, Jain PC, Kango N. 2012. Production of inulinase from *Kluyveromyces marxianus* using dahlia tuber extract. Braz. J. Microbiol., 43(1): 62–69.

Kango N. 2008. Production of inulinase using tap roots of dandelion (*Taraxacum officinale*) by *Aspergillus niger*. J. Food Eng., 85: 473-478.

RESEARCH ARTICLE

Kango N, Jain SC. 2011. Production and properties of microbial inulinases: recent advances. Food Biotechnol., 25(3): 165-212.

Liu XY, Chi Z, Liu GL, Wang F, Madzak C, Chi Z.M. 2010. Inulin hydrolysis and citric acid production from inulin using the surface-engineered *Yarrowia lipolytica* displaying inulinase. Metabolic Engineering, 12(5): 469-476.

MacCabe AP, Orejas M, Tamayo EN, Villaneuva A, Ramon D. 2002. Improving extracellular production of food-use enzymes from *Aspergillus nidulans*. J. Biotechnol., 96: 43-54.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 3: 1426-428.

Nakamura T, Ogata Y, Shitara A, Nakamura A, Ohta K. 1995. Continuous production of fructose syrups from inulin by immobilized inulinase from *Aspergillus niger* mutant 817. J. Ferment. Bioeng., 80: 164–169.

Neagu (Bonciu) C, Bahrim G. 2011. Inulinases - a versatile tool for biotechnology. Innovative Romanian Food Biotechnol., 9: 1-11.

Pandey A, Soccol CR, Selvakumar P, Soccol VT, Krieger N, Fontana JD. 1999. Recent developments in microbial inulinases, its production, properties, and industrial applications. Appl. Biochem. Biotechnol., 81: 35-52.

Shuichi O, Norio S. 1992. Purification and subsite affinities of exo-inulinase from *Penicillium trzebinskii.* Biosci., Biotechnol. Biochem., 56(9): 1443-1447.

Silva-Santisteban BOY, Filho FM. 2005. Agitation, aeration and shear stress as key factors in inulinase production by *Kluyveromyces marxianus*. Enzyme Microb. Technol., 36: 717-724.

Skowronek M, Fiedurek J. 2006. Purification and properties of extracellular endoinulinase from *Aspergillus niger* 20 OSM. Food Technol. Biotechnol., 44(1): 53-58.

Slepecky RA, Hemphill HE. 2006. The Genus *Bacillus*—Nonmedical. Prokaryotes, 4: 530–562.

Sneath PHA. 1986. In: Sneath PHA, Mair NS, Sharpe HS. (Eds.) Bergey's Mannual of Systematic Bacteriology, pp. 1104–1141. Baltimore MD, USA: Williams & Wilkins, ISBN: 0-683-07893-3.

Vandamme EJ, Derycke DG. 1983. Microbial inulinases: fermentation process, properties and applications. Adv. Appl. Microbiol., 29: 139-176.

Zeigler DR. 2001. *Bacillus* Genetic Stock Center, Catalog of Strains, Seventh Edition,Volume 3: The Genus *Geobacillus*, Ohio State University, USA.

Zittan L. 1981. Enzymatic-hydrolysis of inulin an alternative way to fructose production. Staerke, 33: 373–377.