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Simeon Gavrailov
Viara Ivanova

Authors' address:
Department of Microbiology,
University of Food Technologies,
26, Maritsa Blvd.
4002, Plovdiv, Bulgaria.

Correspondence:
Viara Ivanova
Department of Microbiology,
University of Food Technologies,
26, Maritsa Blvd.
4002, Plovdiv, Bulgaria.
Tel.: +359 32 6036221
e-mail: vn.ivanova@abv.bg

Isolation and characteristics of a thermophilic *Bacillus* strain, producer of inulinase

ABSTRACT

A strain of *Bacillus* sp. SG7, isolated from thermal water samples from the region of Velingrad, Bulgaria, showed good capacity to produce extracellular inulinase. The enzyme was synthesized in presence of monosaccharides and pure inulin, as carbon sources. With regard to the nitrogen source, the best results were obtained with yeast extract and other sources of proteic nitrogen, comparatively to the mineral nitrogen. Optimal growth temperature was 55-60°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 optimum pH of the crude enzyme for inulin hydrolysis was found between 6.0 and 7.0 and the optimun temperature at 60°C. The enzyme showed capacity to hydrolyse sucrose, raffinose and inulin from which it liberated only fructose units showing, therefore, an exo-action mechanism. Acting on inulins from several sources, the enzyme showed hydrolysis of the polissaccharide from chicory (*Cichorium intibus*), the inulins from dahlia (*Dahlia pinnata*) and Jerusalem artichoke (*Helianthus tuberosus*) roots.

Key words: inulinase, *Bacillus*, thermophile, hydrolysis, inulin

Introduction

Inulin is a natural storage polymer found widely in plants. 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. Fructose residues are linked together by β-(2,1) or β-(2,6) glycoside bonds (Gupta & Kaur, 1997). In general, plant inulins contain between 20 and several thousand fructose units. Smaller compounds are called fructooligosaccharides, the simplest being 1-kestose, which has 2 fructose units and 1 glucose unit. Different types of inulin differ in their degree of polymerization and have different functional properties. The degree of polymerization depends on the plant source, climate and growing conditions, storage time after harvest, etc. (Chi et al., 2009). Inulin is produced naturally in over 45 000 plants worldwide (Hendry, 1993; Chi et al., 2011). Most of the inulin-containing plants are dicotyledonous, belonging to the *Asteraceae* and *Campanulaceae* families, but a small amount is also found in some monocotyledonous plants from the *Poaceae*, *Liliaceae* and *Amaryllidaceae* families (Frank & Leenheer, 2002). It appears as a reserve carbohydrate in garlic, asparagus root, Jerusalem artichoke, dahlia tubers or chicory roots as well as in plants such as dandelion, goldenrod and burdock (Pandey et al., 1999; Singh & Gill, 2006; Kango, 2008). The content of inulin (in percents), based on a live weight from these sources varies from 10% to 70% (Stolzenburg, 2005). In nature, it is the second most abundant storage carbohydrate after starch. Some inulin containing plants commonly used in human nutrition are leek, onion, garlic, asparagus, Jerusalem artichoke, dahlia, chicory, yacon, etc. (Cazetta et al., 2005). The degree of polymerization (DP) is one of the important properties of inulin, because it influences the functionalities of the fructans (Kierstan, 1978; Zittan, 1981). The DP of plant inulin is rather low (DP < 200) and varies according to the plant species, weather conditions, and physiological age of the plant.

Inulin is hydrolyzed by group of enzymes called inulinases - enzymes that degrade the β-(2,1) linkages of β-fructans, like inulin. Inulinases are classified into endo- and exo-inulinases, depending on their mode of action (Ertan et al., 2003, Kango & Jain, 2011). They act using two mechanisms: exo-inulinases (EC 3.2.1.80) sequentially split-

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off the terminal β-(2,1) fructofuranosidic bonds, while endo-inulinases hydrolyze (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) the internal linkages in inulin and release inulooligosaccharides. The exo-inulinase removes the terminal fructose residues from the non-reducing end of inulin, whereas the endo-inulinase acts on the internal linkages of the inulin molecule but lacks invertase activity. Inulinases have different catalytic properties (molecular weight, optimum pH, optimum temperature, stability), depending especially upon their provenience. Generally, the inulinase activity (I) is accompanied by invertase activity (S) and the enzymatic complex is characterized by S/I (or I/S) ratio. When S/I ratio is lower than 50, the enzyme complex has a preponderate inulinase activity, while for invertase activity the S/I ratio is from 1600 to 2800 (Belamri et al., 1994). Natural inulinase substrates include inulin, sucrose and levan (Singh & Gill, 2006). It is a versatile enzyme and is subject to catabolite repression (Vandamme & Derycke, 1983).

The most common of inulinase producers are of the genera *Aspergillus* and *Kluyveromyces* together with those of the genera *Pseudomonas*, *Xanthomonas*, *Penicillium*, *Chrysosporium* and *Bacillus* (Singh & Gill, 2006) (Table 1).

The production levels of inulinases in bacteria are not comparable to those of yeast and fungi. However, due to the ability of many bacteria to survive at high temperatures, attempts have been made to isolate bacterial strains which can produce high quantities of thermally stable inulinase.om *P. cyclopium* was the most labile (Singh & Gill, 2006).

Table 1. *Microbial sources of inulinases*.

Microorganism	Productive microorganism	Reference
Fungi	*Penicillium* sp.	Nakamura & Nakatsu, 1977
	A. ficuum	Uhm et al., 1999, Mutanda et al., 2009
	Rhizopus sp. & *A. versicolor*.	Kochhar et al., 1997
	A. candidus	Kochhar et al., 1999
	Penicillium janczewskii	Pessoni et al.,1999
	Alternaria alternata, *Aspergillus niger* and *Trichoderma harzianum*	Ertan & Ekinci, 2002
	Aspergillus fumigatus	Gouda, 2002; Gill et al., 2004
	Rhizoctonia solani	Ertan et al., 2003
	Alternaria alternata	Sanal et al., 2005
	A. niveus	Souza-Motta et al., 2005
	Penicillium purpurogenum	Nandagobal & Kumari, 2006
	A. niger	Derycke & Vandamme, 1984; Skowronek & Fidurek, 2006; Goosen et al., 2008
	A. tamarii Ar.I.N9	Saber & El-Naggar, 2009
Yeasts	*Saccharomyces fragilis*	Snyder & Phaff, 1962
	Pichia guilliermondii	Gong et al., 1976
	Kluyveromyces fragilis	Gupta et al., 1994
	Kluyveromyces marxianus	Silva-Santisteban & Filho, 2005
Bacteria	*Flavobacterium multivorum*	Allais et al., 1986
	Bacillus subtilis	Vullo et al., 1991
	Geobacillus (Bacillus) stearothermophilus	Belmari et al., 1994
	Xanthomonas campestris	Ayyachamy et al., 2007
	Streptomyces spp.	Sharma & Gill, 2007
	Bacillus cereus	Meenakshi et al., 2013

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In this paper, we describe the isolation and characterization of thermophilic bacterial strain with inulinase activity. The strain synthesize 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. SG7 was isolated from thermal water samples from the region of Velingrad (Kostandovo) with temperature 58°C and pH 7.5. 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 because the methods for isolation of *Bacillus* strains are based on the resistance of their spores towards elevated temperatures (Sneath, 1986). After chilling, 5 ml from these suspensions were mixed again with 5 ml isolation medium and cultivated 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 Petri dishes were incubated at 37°C and 50°C for 3 days. 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. SG7 achieved the highest enzyme activity, and it was selected for further studies and stored at 4°C.

Fermentation medium

The mediun 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 volume) were charged with 50 ml of medium, inoculated (2%) 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 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) 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 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 addition of 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

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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 (Bradford, 1976), using bovine serum albumin as standard. The culture growth was determined by the absorbance at 650 nm 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 (typicaly 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 OD650 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 and glucose in concentration 2.5 mg/ml (Sigma, USA) were used as standards.

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, by 2 h and the residual activity was estimated. The optimum temperature was obtained 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, pH 7.0, was maintained for 30 min in the same temperature range with or without addition 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) and inulin (Jerusalem artichoke, Sigma-Aldrich, USA), 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 (*Helianthus tuberosus*) (Sigma-Aldrich, USA) 5.0% inulin solutions in 0.05 M phosphate buffer, pH 7.0.

Preparation of onion and garlic extract

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

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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 2), 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.

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 2). 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 SG7 isolate

The selected *Bacillus* isolate was Gram-positive, motile, aerobic rod-shaped bacterium (0.6-0.8 x 1.7-4.0 μm) forming ellipsoidal terminal spores swelling the sporangia (Table 3). It grew at high temperatures - from 45 to 65°C. Optimal growth temperature was 55-60°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 8.5 with optimum at pH 8.0-8.5. The following tests were positive: acid from glucose, arabinose, 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; 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 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 3, Table 4).

Table 2. *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

Legend: * 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.

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Table 3. *Morphological and physiological characteristics of the Bacillus sp. SG7 strain.*

Strain characteristics	***Bacillus* sp. SG7**
Source	Velingrad (Kostandovo)
Gram stain	Gram-positive
Cell morphology	Rod-shaped bacterium 0.6-0.8 x 1.7-4.0 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	45-65°C
Temperature optimum	55-60°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	-

According to these results, the *Bacillus* isolate SG7 showed no differences in comparison to *Geobacillus* (*Bacillus) stearothermophilus* strains described by Gordon et al. (1973), Sneath, 1986, Bergey's Manual of Determinative Bacteriology (Holt, 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. SG7.

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Enzyme production by batch cultivation

The kinetics of growth and enzyme production of strain *Bacillus* sp. SG7 were studied in batch cultivation in 300 ml Erlenmeyer flasks with 50 ml medium (initial pH 8.0) in water-bath shaker, for 36 h, at 200 rpm. 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 2 h 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). Also a biphasic type of growth was observed - maximal OD at the 4 hour of cultivation followed by decrease and a second peak about the 16 h. This fact would be explained by an assumption that the produced enzyme is exo-inulinase and splits-off terminal fructose units from the nonreducing end of the inulin molecule. Fructose is a rapidly metabolized carbon source and exerted a repressive effect on enzyme synthesis (general phenomenon for the synthesis of extracellular bacterial enzymes). By that reason the inulinase activity curves and the invertase activity curves showed a certain delay in the hours of fructose accumulation in the medium (data for increased reducing sugars not shown). Utilization of fructose (an increase of optical density) abolished the catabolite repression and led to an increase of the enzyme level (Figure 1).

Maximal inulinase activity of 2.3 U/ml and invertase activity of 24.5 U/ml were reached in the cultural liquid after 16 hours of fermentation. 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.SG7 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.

Table 4. *Assimilation of sugars by the strain SG7.*

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	+	-

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

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Temperature and pH optima for growth and enzyme production

The effect of temperature and pH on strain SG7 was investigated. The strain grew at temperatures from 45 to 65°C (Figure 2). As noted above, 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 from these data, pH 8.0 was selected for further experiments (Figure 3).

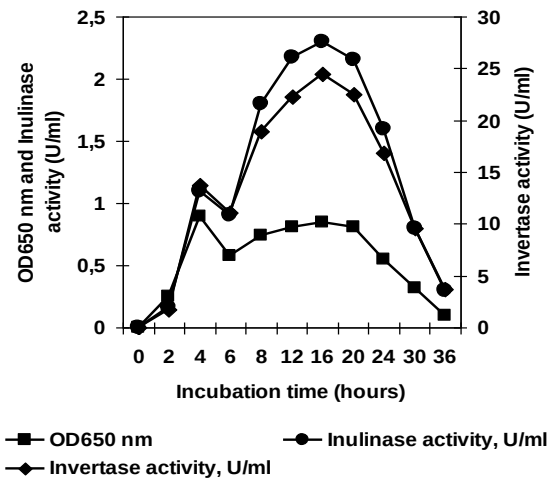

Figure 1. *Growth profile and inulinase production by Bacillus sp. SG7.*

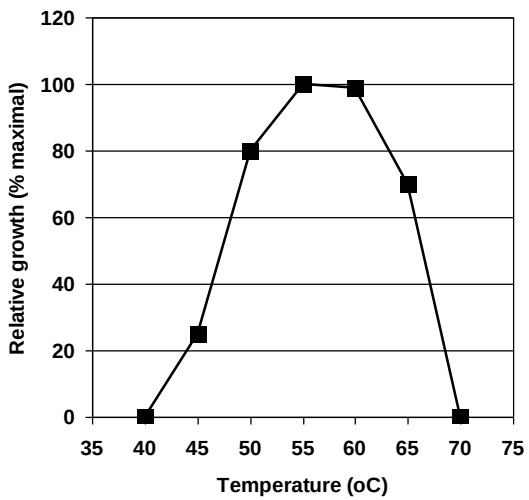

Figure 2. *Temperature optimum for growth of Bacillus sp. SG7.*

Thermophilic strain *Bacillus* sp. SG7 possesses some advantages for a large-scale production: i) extracellular inulinase synthesis at high temperature (60°C); ii) a short production time (24 h); iii) a high inulinase yield (2.33 U/ml, similar or higher to that from other bacteria and yeasts).

Effect of carbon and nitrogen sources

Inulinase production by the *Bacillus* sp. SG7 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 5). Growth on inulin, garlic and onion extract, Jerusalem artichoke tubers and stems extract was almost identical (optical density in the range of 0.75 to 0.89).

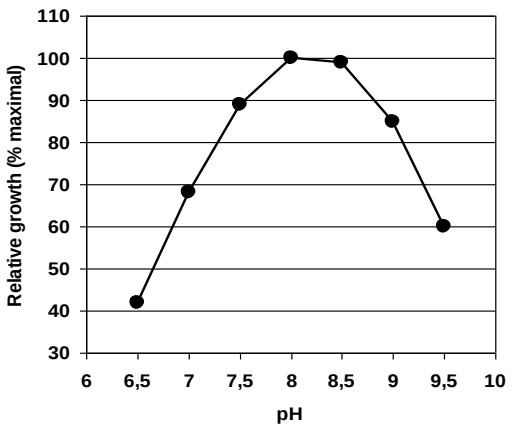

Figure 3. *pH optimum for growth of strain Bacillus sp. SG7.*

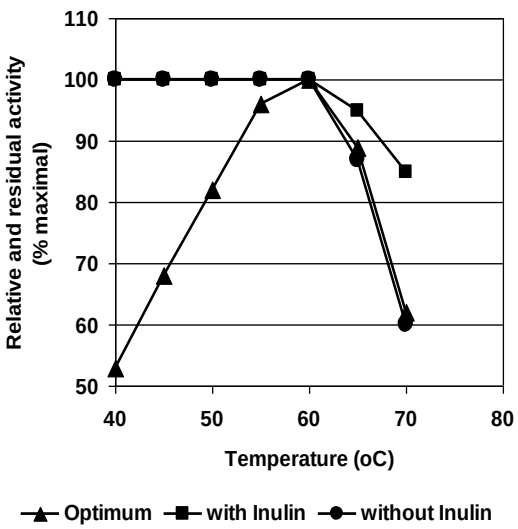

Figure 4. *Effect of temperature on the activity and stability of the inulinase from Bacillus sp. SG7.*

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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 6). 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-source (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 9.7-fold concentration and 1.7-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.

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 60% of the measured maximal activity (Figure 4).

Table 5. *Effect of the carbon source on the inulinase production by Bacillus sp. SG7 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	0.045 ± 0.03	0.516 ± 0.02	11.46	0.55 ± 0.03
Fructose	0.073 ± 0.05	0.800 ± 0.02	10.95	0.50 ± 0.03
Saccharose	0.075 ± 0.03	0.825 ± 0.03	11.00	0.55 ± 0.02
Inulin	**2.330 ± 0.05**	**24.550 ± 0.06**	**10.54**	**0.89 ± 0.03**
Sugar cane molasse	0.087 ± 0.03	0.933 ± 0.03	10.72	0.45 ± 0.03
Soluble starch	0.053 ± 0.03	0.575 ± 0.04	10.85	0.33 ± 0.02
Garlic extract	1.920 ± 0.05	20.650 ±0.05	10.75	0.79 ± 0.03
Onion extract	1.870 ± 0.05	20.400 ± 0.04	10.90	0.78 ± 0.03
Tubers flour	0.960 ± 0.03	11.560 ± 0.07	12.04	1.10^8 CFU/ml
Stems flour	0.850 ± 0.02	10.000 ± 0.07	11.76	5.10^7 CFU/ml
Tubers extract	2.160 ± 0.05	22.900 ± 0.06	10.60	0.80±0.02
Stems extract	1.900 ± 0.05	20.240 ± 0.04	10.65	0.75±0.02

Legend: * 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 6. *Effect of the nitrogen source on the inulinase production by Bacillus sp. SG7 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	2.15 ± 0.04	22.79 ± 0.07	10.60	0.75 ±0.03
Beef extract	2.28 ± 0.05	24.00 ± 0.03	10.53	0.77 ± 0.03
Yeast extract	**2.30 ± 0.05**	**24.50 ± 0.06**	**10.65**	**0.80 ± 0.03**
Soy flour	1.76 ± 0.03	18.83 ± 0.03	10.70	0.65 ± 0.03
$NaNO_3$	0.55 ± 0.03	7.25 ± 0.03	13.18	0.43 ± 0.03
KNO_3	0.47 ± 0.03	6.33 ± 0.03	13.47	0.38 ± 0.03
$(NH_4)_2SO_4$	0.74 ± 0.03	9.85 ± 0.03	13.31	0.57 ± 0.03
$(NH_4)H_2PO_4$	0.96 ± 0.03	12.38 ± 0.03	12.90	0.66 ± 0.03

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

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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 87% residual inulinase activity of the enzyme. At higher temperature (70°C) inulinase activity dropped sharply.

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 95%. A heat treatment at 70°C for 30 min led to approximately 85% residual enzyme activity.

Enzyme thermostability was similar towards enzyme 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. SG7-enzyme was significantly higher (Vandamme & Derycke, 1983).

pH optimum and stability

The enzyme activity on substrates inulin and sucrose was studied at pH values from 3.5 to 9.0 and temperature 60°C. The enzyme was the most active at pH 6.0-7.0. When the enzyme reaction was performed at pH 8.0, the relative inulinase activity was 85% from the established maximal activity. At pH 9.0 the relative activity was 55% of the maximal.

The preliminary incubation of the enzyme for 2 hours at pH values from 3.5 to 9.0 showed that the residual inulinase activity was retained 92-100% at pH range 5.5-8.0 (Figure 5).

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 melezitose (Table 7). Enzyme activity on substrates raffinose and melezitose was determined using the calibration curve with standard fructose solutions.

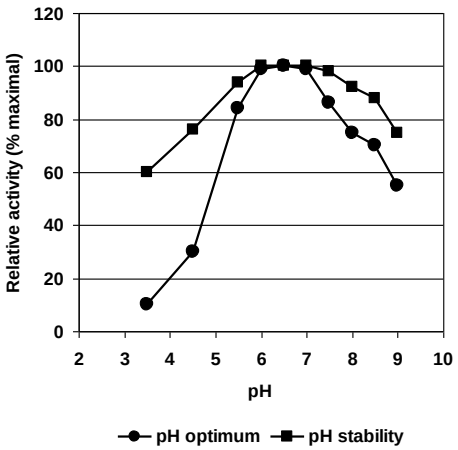

Figure 5. *Effect of pH on the activity and stability of the inulinase from Bacillus sp. SG7.*

Table 7. *Substrate specificity of the crude enzyme concentrate.*

Substrate	Enzyme activity (U/ml)*
Inulin	21.25
Sucrose	223.85
Raffinose	53.12
Melezitose	0

Legend: * Per ml of crude enzyme concentrate.

The enzyme splitted inulin, sucrose, raffinose, whereas melezitose was not hydrolyzed. 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 the Figure 6. The hydrolysis products from inulin were analyzed by thin layer chromatography. Only fructose was the first produced product. Fructooligosaccharides were not detected after an initial 20 min of the process. The results testified to a typical mode of action of an exo-inulinase splitting fructose residues from fructose side of inulin molecule.

Higher degree of hydrolysis of inulin from Jerusalem artichoke was achieved as compared to the hydrolysis of inulin from dahlia tubers and 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

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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 10.54.

In conclusion, enzyme produced by *Bacillus* sp. SG7 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 (10.54). This exo-inulinase could be applied at high temperatures (60-65°C) and pH ranges 5.5-8.0 in contrast to the industrial preparations from yeasts and from *Aspergillus* fungi.

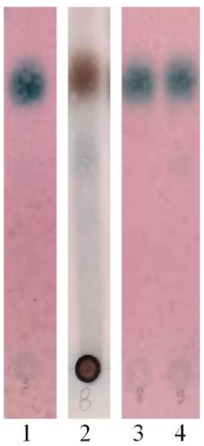

Figure 6. *TLC of reaction products from hydrolysis of inulin.*
Legend: *1, 3 – Standards 2.5 mg/ml (Sigma, USA) – 1, 3 – fructose; 2 – products from 4% chicory inulin; 4 – products from 1% inulin from Jerusalem artichoke; 50 °C, 20 minutes, pH 7.0, enzyme activity 0.90 U/ml.*

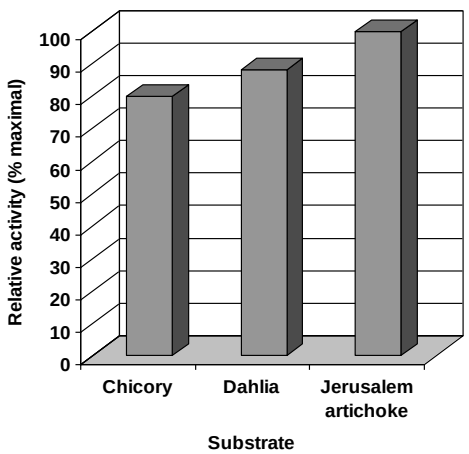

Figure 7. *Action of the inulinase on several substrates.*

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