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PURIFICATION OF AN EXO-INULINASE FROM *BACILLUS* SP. SG7

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ABSTRACT

An exo-inulinase from strain *Bacillus* sp. SG7 was isolated and purified. A two-phase system PEG/Dextran, size-exclusion chromatography and ion-exchange chromatography were used in the purification process. The enzyme was purified to homogeneity with specific inulinase activity 18.47 U/mg protein and specific invertase activity 196.5 U/mg protein, purification fold of 10.44 and 27.4% yield. The molecular mass of the purified enzyme was estimated to be 56 000 Da. Strong inhibitors of enzyme activity are Pb, Hg, Zn and Cu ions with inhibition levels rising up to 55% for Cu and 95% for Pb. SDS totally inhibited the purified inulinase. The kinetic constants Km and Vmax for inulin as substrate were determined to be 1.0 mg/mL and 6.25 mg/mL.h, respectively. The pH optimum is at pH 7.0 and the enzyme is stable between pH 6.0 and pH 7.5, while retaining 100% of its initial activity between pH 6.5 and pH 7.0. The temperature optimum for the purified inulinase from strain *Bacillus* sp. SG7 was at 60°C. In the presence of inulin the purified inulinase sustains its activity at 100% for 55 minutes at 65°C. After the 70th minute the residual activity is 63% of the initial. The enzyme showed capacity to hydrolyse sucrose, raffinose and inulin from which it liberated only fructose units showing, therefore, an exo-action mechanism. The inulins from chicory (*Cichorium intibus*), from dahlia (*Dahlia pinnata*) and Jerusalem artichoke (*Helianthus tuberosus*) roots were hydrolysed by the purified enzyme.

Keywords: inulinase, *Bacillus*, thermophile, purification, characterization

INTRODUCTION

Inulin belongs to a class of carbohydrates known as fructans – polymers composed mainly of fructose units, and typically with a terminal glucose molecule. Fructose residues are linked together by β – (2,1) or β – (2,6) glycosidic bonds **(Gupta and Kaur, 1997; Rocha *et al*., 2006)**. Inulin type fructans are linear compounds in which fructose residues are mostly linked by β-(2,1) linkages **(Rocha *et al*., 2006)**. Inulin is naturally synthesized by over 45 000 plants. It acts as a storage carbohydrate in garlic, asparagus roots, topinambour, dahlia, chicory rhizomes and roots, as well as in dandelion, burdock and the European goldenrod. Inulin quantities in fresh mass from these sources range from 10% to 20% **(Rocha *et al*., 2006; Kango and Jain, 2011)**.
Fructans are typical prebiotics and have a proven stimulating effect on bifidobacteria in animals and humans. These sugars have a low caloric value and act as dietary fiber because the digestive enzymes in the upper part of the gastrointestinal tract are unable to hydrolyze β-fructosyl bonds. Fructans are selectively metabolized in the large intestine by bifidobacteria and lactobacilli, which suppress the growth of pathogenic species such as *Escherichia coli*, *Clostridium* sp. and *Salmonella* sp. Bifidobacteria and lactobacilli produce short-chained fatty acids and lactic acid from inulin, which in turn leads to a higher Calcium and Magnesium assimilation, vitamin B synthesis, lower levels of serum cholesterol, and large intestine cancer prevention. It is believed that fructooligosaccharides can inhibit tumor growth **(Gao *et al*., 2009)**.
Inulin can be hydrolyzed at pH 1.0-2.0 and temperature of 80-100°C, but low pH levels cause fructose degradation and can also lead to the formation of difructose anhydrides. Chemical hydrolysis of inulin with organic or mineral acids or through heterogeneous catalysis with solid acid catalysts, such as acid cation exchange resins or oxidized activated carbon, is disadvantageous because it results in unwanted side products and coloring compounds. This then requires special treatment, thus rendering the process inefficient. These disadvantages can be overcome if a specialized enzyme method is applied. Pure fructose syrup from inulin can be derived through treatment with microbial inulinases.
Inulinases are fructofuranosyl hydrolases produced by a wide range of microorganisms **(Singh and Gill, 2006)**. These enzymes also possess an invertase activity (hydrolysis of saccharose). During the past few decades a wide variety of microorganisms such as bacteria (*Clostridium* spp., *Xanthomonas* spp., *Bifidobacterium* spp., *Geobacillus* spp., *Bacillus* spp., *Thermotoga* spp., *Pseudomonas* spp.), yeast (*Kluyveromyces* spp.) and ascomycetous fungi (*Penicillium* spp., *Fusarium* spp., *Aspergillus* spp.) has been used for inulinase production **(Pandey *et al*., 1999; Kango and Jain, 2011; Neagu (Bonciu) and Bahrim, 2011)**. Amongst the most commonly used genera are *Aspergillus*, *Kluyveromyces* **(Pandey *et al*., 1999)** along with *Pseudomonas*, *Xanthomonas*, *Penicillium*, *Chrysosporium* (**Kango and Jain, 2011; Neagu (Bonciu) and Bahrim, 2011**) and *Bacillus* **(Zherebtsov *et al*., 2002; Kim *et al*., 2004)**.
Bacterial inulinases are essential to the industry as they are inductive, extracellular **(Jain *et al*., 2012)** and thermostable **(Ettalibi and Baratti, 2001)**. Due to bacteria's ability to withstand extremely high temperatures, there have been attempts to isolate such strains which can produce high quantities of thermostable inulinases. Successful isolation of number of *Arthrobacter ureafaciens* mutants that produce a thermostable inulinase was reported. According to **Allais *et al*., (1986)**, the inulinases are secreted into the growth medium.
It has been proven that β-fructofuranosidase, purified from *Arthrobacter*, has an exo-enzyme type of action at optimal pH value of 6.0 and optimal temperature of 50°C **(Elyachioui *et al*., 1992)**. **Takahashi *et al*. (1985)** purified to homogeneity an exo-inulinase with molecular mass of 83 kDa from *Streptococcus salivarius*.
Bacteria from genus *Bacillus* are also active producers of extracellular inulinases **(Uzunova *et al*., 2002)**. *Bacillus subtilis* 430A, isolated from the rhizospehere of *Vernonia herbacea*, produces an extracellular inulinase **(Vullo *et al*., 1991)**. **Meenakshi *et al*., (2013)** have isolated an extracellular inulinase producer that has been identified as *Bacillus cereus* MU-31, which has the ability to produce large quantities of the enzyme - 3783 U/mL after 36 hours of cultivation. *Achromobacter* sp. has been reported to have high enzymatic activity of 333 U/L after 22 h of cultivation **(Jenny *et al*., 2012)**. Another species of the *Bacillus* genus – *Bacillus safensis*, can produce an endoinulinase with maximum inulinase activity of 12.56 U/mL **(Singh *et al*., 2013)**.
Allais *et al*. (1987a) isolated a thermophillic strain from genus *Bacillus* that produces an inulin inductive inulinase. The partially purified inulinase from *Bacillus subtilis* shows higher specificity towards inulin (Km 8 mM) compared to saccharose (Km 56 mM). The thermophillic soil isolate *Bacillus stearothermophilus* KP1289, which develops at temperatures ranging from 41 to 69°C, produces an inulin inductive extracellular inulinase with molecular mass of

54 kDa and pI of 5.0 **(Kato *et al*., 1999)**. **Zherebtsov *et al.* (2002)** studied the synthesis of extracellular inulinase from *Bacillus polymyxa* 29, *Bacillus polymyxa* 722, and *Bacillus subtilis* 68. Maximum enzyme production was observed after 72 hours of incubation at temperatures between 33 and 35°C and pH 7.0. **Gao *et al*., (2009)** used a medium containing only inulin as a carbon source and isolated a new thermophillic inulinase producing strain with optimal development temperature of 60°C. Following a 16S rDNA analysis the strain was identified as *Bacillus smithii*. Using only inulin as a carbon source, **Allais *et al*., (1987b)** have successfully isolated 5 thermotolerant and 4 thermophillic species from genus *Bacillus*, whilst **Drent *et al*., (1991)** isolated thermophills from genus *Clostridium*. Based on their ability to secrete a large amount of succinate during fermentation, the latter have been suggested to be named *Clostridium thermosuccinogenes*.

The gene coding one of the most thermostable bacterial inulinases, which can sustain 85% of its original activity for up to 5 hours at 80°C and pH 7.0, has been cloned from *Thermotoga maritima* **(Liebl *et al*., 1998)**. *Thermotoga maritima* is a strictly anaerobic heterotroph with optimal growth at 90°C.

Industrial fructose and fructooligosaccharide production happens at approximately 60°C **(Vandamme and Derycke, 1983; Vijayaraghavan *et al*., 2009; Chi *et al*., 2011)**. For the most part, inulinase activity drops after a few hours at said temperature and addition of enzyme increases the cost of the end product. Therefore the isolation of thermostable inulinases is essential, as it would solve this problem. Inulinases from various microbial sources have been isolated and characterized. Unfortunately, only a small part of which have a temperature optimum of 60°C and a considerable thermal stability **(Ettalibi and Baratti, 1987; Ettalibi and Baratti, 2001; Kwon *et al*., 2003; Sharma and Gill, 2007; Neagu (Bonciu) and Bahrim, 2011; Shen *et al*., 2015)**. It is for these reasons that the matter of thermostable inulinases, produced by thermophillic microorganisms remains relevant.

MATERIAL AND METHODS

Bacterial strain isolation

The strain of *Bacillus* sp. SG7 was isolated from thermal water samples from the region of Velingrad (Kostandovo, Bulgaria) with temperature 58°C and pH 7.5. Five milliliters from the 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 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 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* sp. SG7 achieved the highest enzyme activity, and it was selected for further studies and stored at 4°C **(Gavrailov and Ivanova, 2014)**.

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 volume) were charged with 50 mL of the 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.

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 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 saccharose (Scharlab S.L., Spain; 20 g/L in phosphate buffer, pH 7.0) as 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.

Enzyme purification

A two-phase separation method was used for the purification of inulinase from strain *Bacillus* sp. SG7 **(Ivanova *et al*., 1993)**. 100 mL of the supernatant were mixed with a 50% PEG 6000 solution and a 30% dextran 500 solution respectively, thus obtaining solutions with 15% and 6% final concentration. The dextran fraction was used in a size-exclusion chromatography column (2.6 x 75 cm, LKB system) with Sephadex G-100 (Pharmacia Fine Chemicals), balanced with 0.025M Tris-HCl buffer with pH 7.3. Samples of 4 mL each were collected at 15 mL/h flow rate. The samples were analysed for protein by the Bradford method. The fractions with inulinase and invertase activities were collected and concentrated with ultrafiltration system Amicon (Amicon Corp., USA, pore size 10 000 Da and pressure 0.5 MPa) and were afterwards subjected to ion-exchange chromatography through a column containing CM Sepharose CL-6B (Pharmacia Fine Chemicals, 1.6 x 40 cm), balanced with 0.05M sodium-acetate buffer with pH 5.0. Fractions of 1 mL each were collected at flow rate 40 mL/h. A 0→2.0 M NaCl gradient in sodium acetate buffer, pH 5.0, was applied. The inulinase and invertase fractions were collected, subjected to dialysis in 0.025M Tris-HCl buffer with pH 7.3 at 4°C and concentrated.

Enzyme homogeneity and molecular mass determination

SDS-PAGE electrophoresis was used. For the molecular mass determination of the separated protein fractions, molecular standards from Bio-Rad (Bio-Rad, Bio-Rad Laboratories, USA) with 7 proteins with respective masses of 200, 97, 69, 45, 30, 21, 14 kDa were applied. Polyacrylamide gel electrophoresis at denaturizing conditions was conducted according to the Laemmli method **(Laemmli, 1970)**, using a Mini-PROTEANII (Bio-Rad Laboratories, USA). A 14% separating gel and 6% concentrating gel with 1.5 mm width were used. The electrophoretical separation was performed at constant voltage of 120V, at room temperature for 100 minutes. After electrophoresis the gels were either silver stained **(Blum *et al*., 1987)** or stained with a Coomassie Brilliant Blue R-250 solution.

Enzyme characterization

pH and temperature optima and stability

The effect of pH on the purified inulinase was investigated by measuring the enzyme activity at 60°C over the pH range 3.5 to 6.5 (0.1 M acetate buffer), 6.8-7.7 (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 (1.0 U/mL) plus 0.5 mL of the same buffer in the mentioned pH range were maintained at 20°C for 1 h and the residual activity was estimated. The activity of a control assay with no pH treatment is regarded as the maximum activity of 100%.

The optimum temperature was determined by measuring the enzyme 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 (1.0 U/mL) and 0.5 mL of 0.1 M phosphate buffer with a pH value of 7.0, was maintained from 5 to 60 min at 65°C in the presence of 1% inulin or without inulin and the residual activity was measured as described in the enzyme assay.

Determination of kinetic constants

Km and Vmax for an inulin substrate were determined at the optimal temperature for enzyme action. The concentration of the inulin substrate from dahlia (*Dahlia* tubers, Fluka, Buchs, Switzerland) was between 2.5 and 10.0 g/L; the enzyme concentration was 0.9 µg/mL. The constants were calculated using the Lineweaver-Burk plot **(Dixon and Webb, 1979)**.

Effect of metal ions and reagents

The effects of the following metal ions and reagents on the inulinase activity of the purified enzyme were studied: Mn^{2+} (as $MnSO_4.H_2O$), Fe^{2+} ($FeCl_2$), Fe^{3+}

($FeCl_3$), Pb^{2+} ($Pb(CH_3COO)_2.3H_2O$), Co^{2+} ($Co(NO_3)_2$), Mg^{2+} ($MgCl_2.7H_2O$), Hg^{2+} ($HgCl_2$), K^+ (KCl), Ag^+ ($AgNO_3$), Ca^{2+} ($CaCl_2$), Na^+ (NaCl), Na_2EDTA, SDS and 2-Mercaptoethanol (all in concentration of 1.0 mM). The enzyme was incubated in the presence of the particular salt at 25°C for 60 minutes. The residual activity was determined afterwards. The activity of a control assay with no added ions was regarded as the maximum activity of 100%.

Inulinase action pattern

Aliquots of 0.5 mL of the purified enzyme (1.0 U/mL) were incubated, separately in 2.0 mL of raffinose (Sigma-Aldrich, USA), sucrose (Scharlab S.L., Spain), melezitose (Sigma-Aldrich, USA) and inulin (Jerusalem artichoke, Sigma-Aldrich, USA) solutions, in a final concentration of 1.0% in 0.1 M phosphate buffer, pH 7.0, at 60°C. The liberation of fructose from raffinose and inulin was determined by the DNS-method. The values obtained for the hydrolysis of raffinose reaction were divided by 2, because to each molecule of liberated fructose, corresponds 1 reducing unit of melibiose. The profile of the enzyme reaction was visualized by thin layer chromatography (TLC).

Hydrolysis of inulin from different sources

For the hydrolysis studies of inulin from different origins, the reaction system was composed by 0.5 mL (4.0 U/mL) of 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.

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.

RESULTS AND DISCUSSION

Purification

Bacterial cells were separated by centrifuging at 4000 rpm for 20 min and the clear supernatant was used in the next purification steps. The results from the two-phase separation in system of PEG 6000 and dextran 500 used for the purification of an inulinase from strain *Bacillus* sp. SG7 are shown on Table 1. The major part of the inulinase was retained in the dextran fraction. Only 8% of the inulinase were retained in the PEG fraction, with 90% remaining in the dextran fraction. The purification fold at this stage was 7.22, the specific activity increased from 1.77 U/mg protein to 12.78 U/mg protein. The separation from other proteins through size-exclusion chromatography was not completely effective as a few lines were observed after an SDS-electrophoresis (Figure 1A). Because of this the active fractions were concentrated and subjected to ion-exchange chromatography. Thus the inulinase was purified 10.44-fold with 24.7% yield and 18.47 U/mg specific inulinase activity. The homogeneity of the purified enzyme was tested with SDS-PAGE (Figure 1B). The inulinase-active fractions were combined and concentrated to protein content 1.7 mg/mL.

These results are comparable to those of **Martirosyan (2004)**, who purified over 50 fold an intracellular inulinase from *Bacillus subtilis* using ion-exchange and adsorption chromatography and size-exclusion chromatography. A specific enzyme activity of 42.7 U/mg was reached. **Ma *et al.* (2016)** purified a thermostable inulinase from *Kluyveromyces cicerisporus* through ion-exchange chromatography with 64% yield and purification fold 4.13. The purified enzyme had a specific activity of 88.7 U/mg.

Specific invertase activity of the enzyme from strain *Bacillus* sp. SG7 increased from 18.85 U/mg protein (supernatant) to 136.1 U/mg protein in the dextran fraction, to 153.8 U/mg protein after separation with size-exclusion chromatography on Sephadex G-100 and to 196.5 U/mg protein after ion-exchange chromatography. According to **Belamri *et al.* (1994)** the inulin degrading enzymes are characterized by the S/I ratio, which is lower than 50 for inulinases and between 1600 and 2800 for invertases. It is evident from the results that the saccharose/inulin (S/I) ratio for the studied protein was 10.64 (after ion-exchange chromatography), thus proving that the enzyme from strain *Bacillus* sp. SG7 is inulinase.

Table 1 Purification of the inulinase from strain *Bacillus* sp. SG7

Purification	Volume (mL)	Total protein (mg)	Total inulinase activity (U)	Specific inulinase activity (U/mg)	Yield (%)	Purification fold
Supernatant	200	260.0	460.0	1.77	100	(1.0)
PEG-fraction	276	210.2	40.0	-	-	-
Dextran fraction	56	32.4	414.0	12.78	90.0	7.22
Sephadex G-100	374	18.35	264.4	14.41	57.6	8.15
CM Sepharose CL-6B	106	6.16	113.8	18.47	24.7	10.44

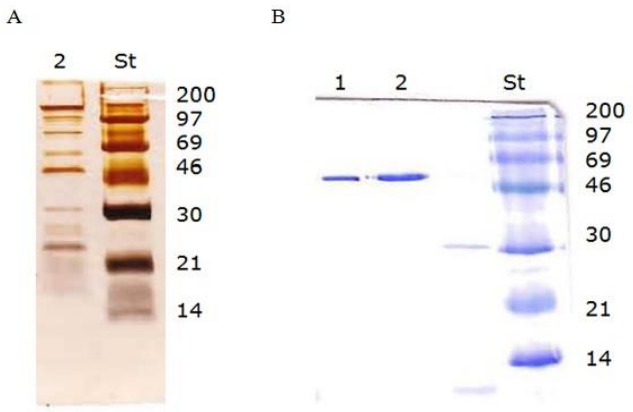

Figure 1 A: SDS-PAGE after size-exclusion chromatography, silver staining, 2 – inulinase from *Bacillus* sp. SG7, St – molecular standards Bio-Rad. B: SDS-PAGE after ion-exchange chromatography, staining with Coomassie Brilliant Blue R-250. 1, 2 – inulinase from strain *Bacillus* sp. SG7 (line 2 has a higher protein concentration); St- molecular standards Bio-Rad.

Molecular mass determination

The molecular mass of the enzyme has been determined based on the results from the two SDS-PAGE electrophoresis (Figure 1) and was calculated to be 56 kDa through the Rf values of individual protein markers and the logarithm of their molecular mass.

The molecular mass of the inulinase from strain *Bacillus* sp. SG7 is similar to the molecular mass of other purified bacterial, yeast and fungi inulinases. It has been established that most microbial inulinases have a molecular mass over 50 kDa **(Chi *et al.*, 2009)** and under 60 kDa. For example, the molecular masses of 9 inulinases are between 49.8 and 59.1 kDa **(Liebl *et al.*, 1998; Kwon *et al.*, 2000; Tsujimoto *et al.*, 2003; Kim *et al.*, 2004; Nagem *et al.*, 2004; Kobayashi *et al.*, 2012; Gao *et al.*, 2014; Liu *et al.*, 2014)**. **Kwon *et al.* (2003)** found the molecular mass of exo-inulinase from *Bacillus polymyxa* to be 55 522 Da.

Kang *et al.* (1998) characterized endoinulinases from *Arthrobacter* sp. and determined their molecular mass to be 75 kDa. **Takahashi *et al.* (1985)** purified to homogeneity an exo-inulinase with mass of 83 kDa from *Streptococcus salivarius.* **Shen *et al.* (2015)** determined the molecular weight of an exo-inulinase from *Arthrobacter* sp. HJ7 to be 95.1 kDa. **Sharma and Gill (2007)**, purified an extracellular exo-inulinase from *Streptomyces* sp. with molecular mass of 45 kDa. The thermophillic soil species *B. stearothermophilus* KP1289, which grows at temperatures ranging from 41 to 69°C, produces an inulin inductive extracellular inulinase with molecular mass and pI of 54 kDa and 5.0, respectively **(Kato *et al.*, 1999)**.

The molecular masses of inulinases produced by bacteria are similar to those produced by yeast. **Sheng *et al.* (2008)** purified and characterized an inulinase from *Cryptococcus aureus* and calculated its molecular mass to be 60 kDa. The inulinases obtained from *Pichia guilliermondii* have a molecular mass of 50 kDa according to **Gong *et al.* (2008)** and 54 kDa according to **Chi *et al.* (2009)**. Inulinases with a molecular mass of 250 kDa are produced by *Kluyveromyces fragilis* **(Pandey *et al.*, 1999)**.

The molecular mass of fungi produced inulinases varies between 50 kDa and 300 kDa: *Aspergillus ochraceus* – 79 kDa **(Guimaraes *et al.*, 2007)**, *Penicillium* spp. – 68 kDa **(Chi *et al.*, 2009)**, *Fusarium oxysporum* – 300 kDa **(Pandey *et al.*, 1999; Neagu (Bonciu) and Bahrim, 2011)**.

pH-optimum and pH-stability

The pH-optimum and the pH-stability range of the purified inulinase are displayed in Figures 2 and 3. Optimum pH is at pH 7.0 and the enzyme is stable

between pH 6.0 and pH 7.5 as it sustains 100% of its activity in pH 6.5-7.0, which slightly differentiates it from the partially purified enzyme with its pH-stability ranging from pH 5.5 to pH 8.0 **(Gavrailov and Ivanova, 2014)**.

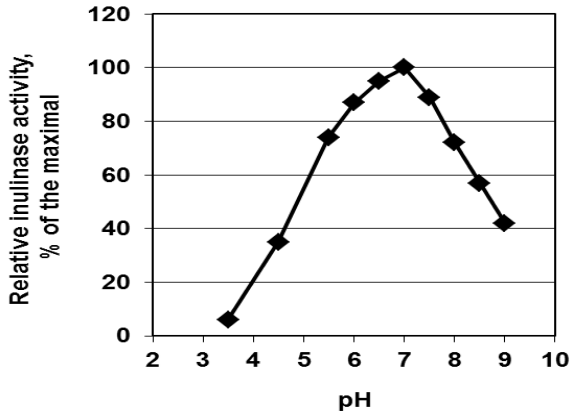

Figure 2 pH-Optimum of purified inulinase from strain *Bacillus* sp. SG7.

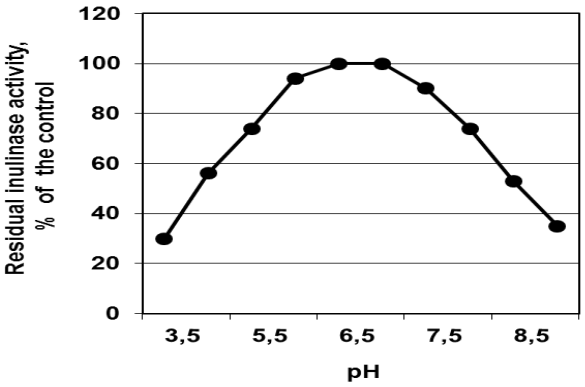

Figure 3 pH-Stability of purified inulinase from strain *Bacillus* sp. SG7.

Temperature optimum and temperature stability

Temperature optimum of the purified inulinase from strain *Bacillus* sp. SG7 has been determined to be at 60°C (Figure 4), same as the one of the partially purified inulinase **(Gavrailov and Ivanova, 2014)**. At 70°C the enzyme shows 30% of its maximal activity as it is slightly more sensitive to this temperature, compared to its partially purified counterpart.

In the presence of inulin, the purified inulinase retained 100% of its activity for 55 min at 65°C. After 70 min the remaining activity is 65% of the initial one (Figure 5). At temperature of 65°C its half-life is 72 min without inulin and 77 min in the presence of inulin.

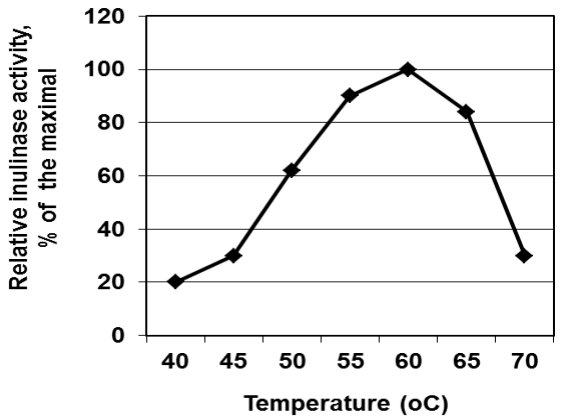

Figure 4 Temperature optimum of purified inulinase from strain *Bacillus* sp. SG7.

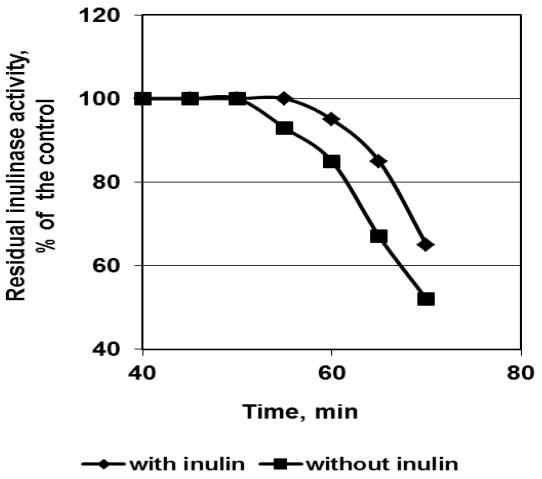

Figure 5 Thermal stability of purified inulinase from strain *Bacillus* sp. SG7 at 65°C.

Sharma and Gill (2007) purified an extracellular exo-inulinase from *Streptomyces* sp. with temperature optimum 70°C and pH optimum at pH 6.0. **Ma *et al.* (2016)** successfully purified a thermostable inulinase from *Kluyveromyces cicerisporus*, the temperature and pH optimum for which are at 55°C and pH 4.5, respectively. This inulinase can sustain 90% of its activity during one hour incubation at 50°C. **Shen *et al.* (2015)** found that the exo-inulinase from *Arthrobacter* sp. HJ7 has a temperature optimum range of 40-45°C and a pH optimum ranging between pH 5.0 and 5.5. The enzyme is stable at pH 6.0-10.0 and can sustain up to 70% of its activity when incubated for 1h. At temperatures of 50°C and 60°C its half-life is 30 min and 10 min, respectively.

The thermostability of purified inulinase from strain *Bacillus* sp. SG7 is similar to that of the enzyme from *Bacillus stearothermophilus* **(Belamri *et al.*, 1994)** or higher than that of several bacterial inulinases. For example, the exo-inulinase from *Bacillus* sp. LCB41 retains 70% of its initial activity after 10 min at 60°C **(Allais *et al.*, 1987a)**, the exo-inulinase from *Arthrobacter* sp. shows 50% residual activity at 55°C after 30 min of incubation **(Elyachioui *et al.*, 1992)**. In comparison, the thermal stability of the enzyme from *Bacillus* sp. SG7 is significantly higher than the thermostabilities of inulinases from yeasts and fungi **(Vandamme and Derycke, 1983)**.

Effects of metal ions and reagents

The effects of metals ions and EDTA on enzymatic activity in 1mM concentration were studied and the results are displayed in Table 2.

Table 2 Effects of metal ions and reagents

Metal ions and reagents Concentration 1.0 mM	**Residual activity (%)**
Standard probe containing no ions or reagents	100
Al^{3+}	85
Mn^{2+}	77
Fe^{2+}	50
Fe^{3+}	71
Pb^{2+}	5
Co^{2+}	75
Mg^{2+}	99
Hg^{2+}	26
Ca^{2+}	100
Cu^{2+}	45
Zn^{2+}	35
Na^{+}	97
K^{+}	95
Ag^{+}	78
Na_2EDTA	65
SDS	0
2-mercaptoethanol	99

No activation effect was observed. Magnesium, Calcium, Sodium, Potassium and 2-mercaptoethanol ions do not affect enzyme activity. Al^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ag^{+} inhibit 15-30% of enzyme activity. Fe^{2+} and EDTA have a bigger impact on the enzyme and inhibit 35-50% of its activity. Other strong inhibitors are Lead ions (95% inhibition), Zinc, Copper (55% inhibition) and Mercury (74% inhibition). SDS completely inhibits the purified inulinase. Lead ions affect sulfhydryl groups and their significant inhibitory properties could be linked to the fact that sulfhydryl groups are strongly connected to enzymatic activity, as previously discovered by **Ettalibi and Baratti (1987)** and **Kochhar *et al.* (1997)**.

Kwon *et al.* (2003) proved that the exo-inulinase from *Bacillus polymyxa* is completely inactivated when treated with 0.001M Ag^{+} and Hg^{+} and concluded that the active site contains thiol groups. Cu $^{2+}$, Zn^{2+} and Ni^{2+} also inhibit said inulinase.
Shen *et al.* (2015) determined that the exo-inulinase produced by *Arthrobacter* sp. HJ7 is not affected by 1.0 and 10.0 mM Zn^{2+} and Pb^{2+} ions. Meanwhile, **Ma *et al.* (2016)** found that the exo-inulinase from *Kluyveromyces cicerisporus* is considerably inhibited by Cu^{2+}, Co^{2+}, Fe^{3+} and Ag^{+}.
Many exo-inulinases can be inhibited by 1.0 and 5.0 mM of Zn^{2+} and Pb^{2+} **(Kwon *et al.*, 2000; Kwon *et al.*, 2003; Gill *et al.*, 2006; Wang *et al.*, 2011)** with the exception of the exo-inulinases produced by *Bacillus subtilis* ssp. 168 **(Wanker *et al.*, 1995)**, *Penicillium* sp. TN-88 **(Moriyama *et al.*, 2002)**, InuAMN8 **(Zhou *et al.*, 2015)**, rInuAHJ7 **(Shen *et al.*, 2015)**.

Determination of kinetic constants

Km and Vmax for an inulin substrate were determined at the optimal temperature for enzyme action. The constants were calculated using the Lineweaver-Burk plot **(Dixon and Webb, 1979)** and the initial velocities of the enzyme reaction with different substrate concentrations. Said constants were calculated to be 1.0 mg/mL (Km) and 6.25 mg/mL.h (Vmax). The concentration of the inulin substrate from dahlia (Dahlia tubers, Fluka, Buchs, Switzerland) was between 2.5 and 10.0 g/L; the enzyme concentration was 0.9 µg/mL.
In comparison, **Trivedi *et al.* (2015)** determined Km of an exo-inulinase from *Aspergillus tubingensis* CR16 to be 7.4 mg/mL and Vmax to be 52.0 µmol/mL.min. **Sharma and Gill (2007)** purified an extracellular exo-inulinase from *Streptomyces* sp. with inulin Km of 1.63 mM. Km and Vmax of a purified endoinulinase from *Xanthomonas oryzae* No.5 **(Cho and Yun, 2002)** are 16.7 mg/mL and 12.1 mg/mL.h, respectively.

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 (concentration10 mg/mL): inulin, sucrose, raffinose and melezitose (Table 3). Enzyme activity on substrates raffinose and melezitose was determined using the calibration curve with standard fructose solutions. The enzyme degrades inulin, sucrose, raffinose, whereas melezitose was not hydrolyzed.

Table 3 Substrate specificity

Substrate	Enzyme activity (U/mL)*
Inulin	31.15
Sucrose	331.44
Raffinose	48.36
Melezitose	0

* Per mL of purified enzyme

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 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).

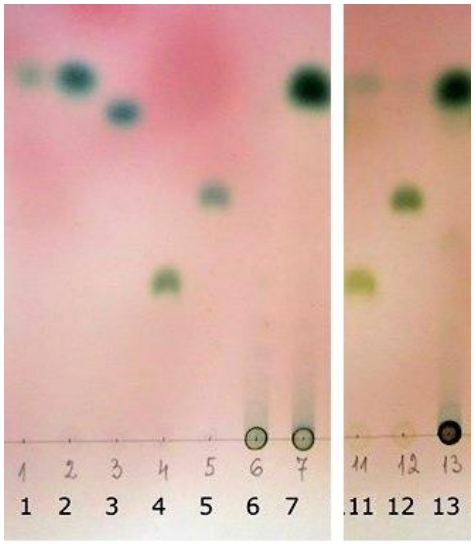

Figure 6 TLC of reaction products from hydrolysis of inulin, sucrose, raffinose and melezitose. Standards 2.5 mg/mL (Sigma, USA): 1–glucose, 2 – fructose; 3 – sucrose; 4 – raffinose, 5 – melezitose, 6 – inulin. Hydrolysates: 7, 13 – inulin, 11 – raffinose, 12 – melezitose; 60°C, 20 minutes, pH 7.0, enzyme activity 1.0 U/mL.

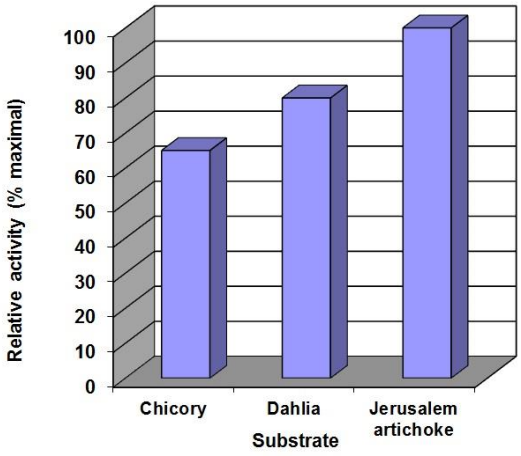

Figure 7 Action of the inulinase on several substrates, enzyme activity 4.0 U/mL.

CONCLUSION

The application of inulinases to obtain fructose syrups could resolve two main issues in the industry: inulin has a limited solubility at room temperature and microbial contamination is highly probable at this temperature. To avoid contamination the industrial process happens at 60°C. Most known inulinases lose their activity after a few hours at said temperature and the addition of more enzyme increases the cost of the final product.
Amongst the yeasts, producing inulinases are *Kluyveromyces* spp., *Pichia* spp. and *Candida* spp., which could potentially synthesize large quantities of the enzyme **(Neagu (Bonciu) and Bahrim, 2011)**. These inulinases have a pH optimum of 4.5-5.5 and a temperature optimum of 40-55°C. The molecular weight of the enzymes is approximately 60 kDa. Although the enzymes produced by *Kluyveromyces* strains are stable at 50°C, incubation at 55°C and 60°C leads to rapid loss of activity, which explains their limited industrial application **(Singh and Gill, 2006)**.
Various fungi have also been reported to synthesize inulinases, which have a pH optimum between 5.5 and 6.5 and a temperature optimum of 35-45°C. Thermophillic fungi could produce inulinases with a higher temperature optimum. The molecular weight of these enzymes ranges between 50 kDa and 300 kDa.
The exo-inulinase from strain *Bacillus* sp. SG7 was purified to homogeneity with specific inulinase activity of 18.47 U/mg protein and specific invertase activity of 196.5 U/mg protein. The purification fold was 10.44 and the yield 27.4%.
The molecular mass of the purified enzyme from strain *Bacillus* sp. SG7 was determined based on the SDS-PAGE electrophoresis results and was calculated to be 56 000 Da. Al^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ag^{+} inhibit the enzyme activity at the studied ion concentrations. The inhibition ranges between 15% and 30%. Fe^{2+} and EDTA affect the enzyme more strongly and inhibit its activity, beginning at 35% up to 50%. Potent inhibitors are also Pb^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, with inhibition

reaching 55% for Cu^{2+} and 95% for Pb^{2+}. SDS completely inhibits the purified inulinase. Km and Vmax for an inulin substrate are 1.0 mg/mL (Km) and 6.25 mg/mL.h (Vmax). The pH-optimum is at pH 7.0 and the enzyme is stable at pH interval from pH 6.0 to 7.5, whilst retaining 100% of its initial activity at pH interval 6.5-7.0, which distinguishes it from the partially purified enzyme and its pH-stability range from pH 5.5 to 8.0 **(Gavrailov and Ivanova, 2014)**. The temperature optimum of the purified inulinase from strain *Bacillus* sp. SG7 is 60°C, which is the same as the temperature optimum of the enzyme in the concentrate. In the presence of inulin, the purified inulinase retains 100% of its activity for 55 minutes at 65°C. After the 70th minute the activity decreases to 65%.

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 (10.64). This exo-inulinase could be applied at high temperatures (60-65°C) and pH ranges 5.5-7.5 in contrast to the industrial preparations from yeasts and from *Aspergillus* fungi.

The production of inulinases from thermophiles is characterised by: brief fermentation, due to the rapid growth of thermophiles; low viscosity of the growth medium at high temperatures, which makes aeration and mass exchange easier; reduced risk of microbial contamination; higher substrate solubility; thermostability of the enzyme molecule; suitability for use in the food industry, determined by the thermophiles and their products' nonpathogenic nature.

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REFERENCES

Allais, J.J., Kammoun, S., Blanc, Ph., Girard, Ch., Baratti, J.C. (1986). Isolation and characterization of bacterial strains with inulinase activity. *Applied and Environmental Microbiol*ogy, 52(5), 1086-1090.

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

Allais, J.J., Hoyos-Lopez, G., Kammoun, S., Baratti, J.C. (1987b). Isolation and characterization of thermophilic bacterial strains with inulinase activity. *Applied and Environmental Microbiology*, 53, 942-945.

Belamri, M., Sassi, A.H., Savart, M., Tantaoui-Elaraki, A., Cottin, P. (1994). Purification and properties of an extra cellular inulinase-like β-fructosidase from *Bacillus stearothermophilus*. Letters in Applied Microbiology, 19(6), 410-413. https://doi.org/10.1111/j.1472-765X.1994.tb00969.x

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principale of protein-dye binding. *Analytical Biochemistry*, 27, 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Blum, H., Beier, H., Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, 8, 93-99. https://doi.org/10.1002/elps.1150080203

Chi, Z., Zhang, T., Liu, G., Xue, L. (2009). Inulinase-expressing microorganisms and applications of inulinases. *Applied Microbiology and Biotechnol*ogy, 82, 211–220. https://doi.org/10.1007/s00253-008-1827-1

Chi, Z.M., Zhang, T., Cao, T.S., Liu, X.Y., Cui, W., Zhao, C.H. (2011). Biotechnological potential of inulin for bioprocesses. *Bioresource Technology*, 102, 4295-4303. http://doi.org/10.1016/j.biortech.2010.12.086

Dixon, M., Webb, E. (1979). In: Dixon, M., Webb, E. (Eds.) *Enzymes*, 3rd editin, Longman, USA.

Drent, W.J., Lahpor, G.A., Weigant, W.M., Gottschal, J.C. (1991). Fermentation of inulin by *Clostridium thermosuccinogenes* sp. nov., a thermophilic anaerobic bacterium isolated from various habitats. *Applied and Environmental Microbiology*, 57(2), 455-462.

Elyachioui, M., Hornez, J.P., Tailliez, R. (1992). General properties of extracellular bacterial inulinase. *Journal of Applied Microbiology*, 73(6), 514–519. https://doi.org/10.1111/j.1365-2672.1992.tb05014.x

Ettalibi, M., Baratti, J.C. (1987). Purification, properties and comparison of invertase, exoinulinases and endoinulinases of *Aspergillus ficuum*. *Applied Microbiology and Biotechnology*, 26, 13–20. https://doi.org/10.1007/BF00282143

Ettalibi, M., Baratti, J.C. (2001). Sucrose hydrolysis by thermostabe immobilized inulinases form *Aspergillus ficuum*. *Enzyme and Microbial Technol*ogy, 28, 596 – 601. https://doi.org/10.1016/S0141-0229(00)00342-2

Gao, W., Bao, Y., Liu, Y., Zhang, X., Wang, J., An, L. (2009). Characterization of thermostable endoinulinase from a new strain *Bacillus smithii* T7. *Applied Biochemistry and Biotechnology*, 157, 498–506. https://doi.org/10.1007/s12010-008-8313-1

Gao, J., Xu, Y.Y., Yang, H.M., Xu, H., Xue, F., Li, S., Feng, X.H. (2014). Gene cloning, expression, and characterization of an exo-inulinase from *Paenibacillus polymyxa* ZJ-9. *Applied Biochemistry and Biotechnology*, 173, 1419-1430. https://doi.org/10.1007/s12010-014-0950-y

Gavrailov, S., Ivanova, V. (2014). Isolation and characteristics of a thermophilic *Bacillus* strain, producer of inulinase. *Journal of BioScience and Biotechnology*, SE/ONLINE, 83-94.

Gill, P.K., Manhas, R.K., Singh, P. (2006). Purification and properties of a heat-stable exoinulinase isoform from *Aspergillus fumigatus*. *Bioresource Technology*, 97, 894-902. https://doi.org/10.1016/j.biortech.2005.04.034

Gong, F., Zhang, T., Chi, Z., Sheng, J., Li. J., Wang, X. (2008). Purification and characterization of extracellular inulinase from a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the purified inulinase. *Biotechnology and Bioprocess Engineering*, 13, 533-539. https://doi.org/10.1007/s12257-007-0177-7

Guimaraes, L.H.S., Terenzi, H.F., Polizeli, M.L., Jorge, J.A. (2007). Production and characterization of a thermostable extracellular β-Dfructofuranosidase produced by *Aspergillus ochraceus* with agroindustrial residues as carbon sources. *Enzyme and Microbial Technology*., 42, 52-57. https://doi.org/10.1016/j.enzmictec.2007.07.021

Gupta, A.K., Kaur, N. (1997). Fructan storing plants - a potential source of high fructose syrups. *Journal of Scientific and Industrial Research*, 56(8), 447-452.

Ivanova, V.N., Dobreva, E.P., Emanuilova, E.I. (1993). Purification and properties of thermostable alpha-amylase from *B. licheniformis* 44MB82-A. *Journal of Biotechnology*, 28, 277-289. http://dx.doi.org/10.1016/0168-1656(93)90176-N

Jain, S.C., Jain, P.C., Kango, N. (2012). Production of inulinase from *Kluyveromyces marxianus* using dahlia tuber extract. *Brazilian Journal of Microbiology*, 62-69, ISSN 1517-8382. http://dx.doi.org/10.1590/S1517-83822012000100007

Jenny, S.A., Kavitha, C., Vidyadharan, G., Priya, R., Dhandapani, R. (2012). Isolation of inulinase producing bacteria from sugarcane soil. International Journal of Applied Biology and Pharmaceutical Technology, 3(4), 320-326.

Kango, N., Jain, S.C. (2011). Production and properties of microbial inulinases: recent advances. *Food Biotechnology*, 25, 165-212. http://dx.doi.org/10.1080/08905436.2011.590763

Kato, K., Araki, T., Kitamura, T., Morita, N., Moori, M., Suzuki, Y. (1999). Purification and properties of a thermostable inulinase (β-D-fructan fructohydrolase) from *Bacillus stearothermophilus* KP1289. *Starch*, 51, 253–258. https://doi.org/10.1002/(SICI)1521-379X(199907)51:7<253::AID-STAR253>3.0.CO;2-7

Kim, K.Y., Koo, B.S., Jo, D., Kim, S.I. (2004). Cloning, expression, and purification of exoinulinase from *Bacillus* sp. snu-7. *Journal of Microbiology and Biotechnology*, 14, 344-349.

Kobayashi, T., Uchimura, K., Deguchi, S., Horikoshi, K. (2012). Cloning and sequencing of inulinase and β–fructofuranosidase genes of a deep-sea microbulbifirespecies and properties of recombinant enzymes. *Applied and Environmental Microbiology*, 78, 2493-2495. PMID:22286980; http://dx.doi.org/10.1128/aem.07442-11

Kochhar, A., Kaur, N., Gupta, A.K. (1997). Inulinase from *Aspergillus versicolor*. A potent enzyme for producing fructose from inulin, *Journal of Scientific and Industrial Research*, 56, 721–726.

Kwon, J.M., Kim, H.Y., Choi, Y.J. (2000). Cloning and characterization of *Pseudomonas mucidolens* exoinulinase. *Journal of Microbiology and Biotechnology*, 10, 238-243.

Kwon, H.J., Jeon, S.J., You, D.J., Kim, K.H., Jeong, Y.K., Kim, Y.H., Kim, Y.M., Kim, B.W. 2003. Cloning and characterization of an exoinulinase from *Bacillus polymyxa*. *Biotechnology Letters*, 25, 155–159. https://doi.org/10.1023/A:1021987923630

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685. http://dx.doi.org/10.1038/227680a0

Liebl, W., Brem, D., Gotschlich, A. (1998). Analysis of the gene for β-fructosidase (invertase, inulinase) of the hyperthermophilic bacterium *Thermotoga maritima* and characterization of the enzyme expressed in *Escherichia coli*. *Applied Microbiology and Biotechnology*, 50, 55–64. https://doi.org/10.1007/s002530051256

Liu, G.L., Fu, G.Y., Chi, Z., Chi, Z.M. (2014). Enhanced expression of the codon-optimized exo-inulinase gene from the yeast *Meyerozyma guillermondii* in *Saccharomyces* sp.W0 and bioethanol production from inulin. *Applied Microbiology and Biotechnology*, 98, 9129-9138. https://doi.org/10.1007/s00253-014-6079-7

Ma, J-Y., Cao, H-L., Tan, H-D., Nu, X-J., Liu, W-J., Du, Y-G., Yin, H. (2016). Cloning, expression, characterization and mutagenesis of a thermostable exoinulinase from *Kluyveromyces cicerisporus*. *Applied Biochemistry and Biotechnology*, 178, 144-158. https://doi.org/10.1007/s12010-015-1864-z

Martirosyan, G.V. (2004). Inulinase of *Bacillus subtilis*: extraction, purification and characterization. *Electronic Journal of Natural Sciences*, 2(1), 38-42.

Meenakshi, S., Umayaparvathi, S., Manivasagan, P., Arumungam, M., Balasubramanian, T. (2013). Purification and characterization of inulinase from marine bacterium, *Bacillus cereus* MU – 31. *Indian Journal of Geo-Marine Sciences*, 42(4), 510 – 515.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 3, 1426-428. https://doi.org/10.1021/ac60147a030

Moriyama, S., Akimoto, H., Suetsugu, N., Kawasaki, S., Nakamura, T., Ohta. K. (2002). Purification and properties of an extracellular exoinulinase from *Penicillium* sp. strain TN-88 and sequence analysis of the encoding gene. *Bioscience, Biotechnology and Biochemistry*, 66, 1887-1896. https://doi.org/10.1271/bbb.66.1887

Nagem, R.A.P., Rojas, A.L., Golubev, A.M., Korneeva, O.S., Eneyskaya, E.V., Kulminskaya, K.N., Neustroev, K.N., Polikarpov, I. (2004). Crystal structure of exo-inulinase from *Aspergillus awamori*: the enzyme fold and structural determinants of substrate recognition, *Journal of Molecular Biology*, 344, 471-480. https://doi.org/10.1016/j.jmb.2004.09.024

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

Pandey, A., Soccol, C.R., Selvakumar, P., Soccol, V.T., Krieger, N., Fontana, J.D. (1999). Recent developments in microbial inulinases, its production, properties, and industrial applications. *Applied Biochemistry and Biotechnology*, 81, 35-52. https://doi.org/10.1385/ABAB:81:1:35

Rocha, J.R., Catana, R., Ferreira, B.S., Cabral, J.M.S., Fernandes, P. (2006). Design and characterisation of an enzyme system for inulin hydrolysis. Food Chemistry, 95: 77–82.

Sharma, A.D., Gill, P.K. (2007). Purification and characterization of heat-stable exo-inulinase from *Streptomyces* sp. *Journal of Food Engineering*, 79, 1172-1178. https://doi.org/10.1016/j.jfoodeng.2006.04.008

Shen, J., Zhang, R., Li, J., Tang, X., Li, R., Wang, M., Huang, Z., Zhou, J. (2015). Characterization of an exo-inulinase from Arthrobacter: A novel NaCl-tolerant exo-inulinase with high molecular mass. *Bioengineered*, 6(2), 99–105. https://doi.org/10.1080/21655979.2015.1019686

Sheng, J., Chi, Z., Gong, F., Li, J. (2008). Purification and characterization of extracellular inulinase from a marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the purified inulinase. *Applied Biochemistry and Biotechnology*, 144, 111-121. https://doi.org/10.1007/s12010-007-8025-y

Singh, P., Gill, P.K. (2006). Production of Inulinases. *Food Technology and Biotechnology*, 44 (2), 151–162. https://doi.org/10.1.1.918.708&rep=rep1&type=pdf

Singh, R.S., Bhermi, H. (2008). Production of extracellular exoinulinase from *Kluyveromyces marxianus* YS-1 using root tubers of *Asparagus officinalis*. *Bioresource Technology*, 99(15), 7418-7423.

Singh, R.S., Singh, R.P., Yadav, M. (2013). Molecular and biochemical characterization of a new endoinulinase producing bacterial strain of *Bacillus safensis* AS-08. *Biologia*, 68(6), 1028-1033. https://doi.org/10.2478/s11756-013-0259-2

Sneath, P.H.A. (1986). In: Sneath, P.H.A., Mair, N.S., Sharpe, H.S. (Eds.) *Bergey's Mannual of Systematic Bacteriology*, Baltimore MD, USA: Williams & Wilkins, ISBN: 0-683-07893-3, pp. 1104–1141.

Takahashi, N., Mizuno, F., Takamori, K. (1985). Purification and preliminary characterization of exo-beta-D-fructosidase in *Streptococcus salivarius* KTA-19. *Infection and Immunity*, 47, 271– 276.

Trivedi, S., Divecha, J., Shah, T., Shah, A. (2015). Rapid and efficient bioconversion of chicory inulin to fructose by immobilized thermostable inulinase from *Aspergillus tubingensis* CR16. *Bioresources and Bioprocessing*, 2, 32-42. https://doi.org/10.1186/s40643-015-0060-x

Tsujimoto, Y., Watanabe, A., Nakano, K., Watanabe, K., Matsui, H., Tsuji, K., Tsukihara, T., Suzuki, Y. (2003). Gene cloning, expression, and crystallization of a thermostable exo-inulinase from *Geobacillus stearothermophilus* KP1289. *Applied Microbiology and Biotechnology*, 62, 180-185. https://doi.org/10.1007/s00253-003-1261-3

Uzunova, K., Vassileva, A., Ivanova, V., Spasova, D., Tonkova, A. (2002). Thermostable exo-inulinase production by semicontinuous cultivation of membrane-immobilized *Bacillus* sp. 11 cells. *Process Biochemistry*, 37(8), 863-868. https://doi.org/10.1016/S0032-9592(01)00283-7

Vandamme, E.J., Derycke, D.G. (1983). Microbial inulinases: fermentation process, properties, and applications. *Advances in Applied Microbiology*, 29, 139-176. https://doi.org/10.1016/S0065-2164(08)70356-3

Vijayaraghavan, K., Yamini, D., Ambika, V., Sowdamini, N.S. (2009). Trends in inulinase production-a review. *Critical Reviews in Biotechnology*, 29, 67-77. https://doi.org/10.1080/07388550802685389

Vullo, D.L., Coto, C.E., Sineriz, F. (1991). Characteristics of an Inulinase Produced by *Bacillus subtilis* 430A, a Strain Isolated from the Rhizosphere of *Vernonia herbacea* (Vell Rusby). *Applied and Environmental Microbiology*, 57(8), 2392-2394.

Wang, L., Huang, Y., Long, X., Meng, X., Liu, Z. (2011). Cloning of exoinulinase gene from *Penicillium janthinellum* strain B01 and its high level expression in *Pichia pastoris*. *Applied Microbiology*, 111, 1371-1380. https://doi.org/10.1111/j.1365-2672.2011.05145.x

Wanker, E., Huber, A., Schwab, H. (1995). Purification and characterization of the *Bacillus subtilis* levanase produced in *Escherichia coli*. *Applied and Environmental Microbiology*, 61(5), 1953-1958.

Zherebtsov, N.A., Shelamova, S.A., Abramova, I.N. (2002). Biosynthesis of inulinases by *Bacillus* bacteria. *Applied Biochemistry and Microbiology*, 38(6), 634-638. https://doi.org/10.1023/A:1020722510374

Zhou, J.P., Lu, Q., Peng, M.Z., Zhang, R., Mo, M.H., Tang, X.H., Li, J.J., Xu, B., Ding, J.M., Huang, Z.X. (2015). Cold-active and NaCl-tolerant exo-inulinase from a cold-adapted *Arthrobacter* sp. MN8 and its potential for use in the production of fructose at low temperatures. *Journal of Biosciences and Bioengineering*, 119, 267-274. https://doi.org/10.1016/j.jbiosc.2014.08.003