

Production and Properties of a Bacterial Thermostable Exo-Inulinase

Kristina Uzunova^{a,§}, Anna Vassileva^a, Margarita Kambourova^a, Viara Ivanova^a, Dimitrina Spasova^b, Rositza Mandeva^a, Anna Derekova^a, Alexandra Tonkova^{a,*}

^a Department of Extremophilic Bacteria

^b Department of Morphology of Microorganisms and Electron Microscopy, Institute of Microbiology, Bulgarian Academy of Sciences, 26 Acad. G. Bonchev str., 1113 Sofia, Bulgaria. Fax: (+359 2) 700 109; E-mail: tonkova@microbio.bas.bg

* Author for correspondence and reprint requests

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Enzyme production of newly isolated thermophilic inulin-degrading *Bacillus* sp. 11 strain was studied by batch cultivation in a fermentor. The achieved inulinase and invertase activities after a short growth time (4.25 h) were similar or higher compared to those reported for other mesophilic aerobic or anaerobic thermophilic bacterial producers and yeasts. The investigated enzyme belonged to the exo-type inulinases and splitted-off inulin, sucrose and raffinose. It could be used at temperatures above 65 °C and pH range 5.5–7.5. The obtained crude enzyme preparation possessed high thermostability. The residual inulinase and invertase activities were 92–98% after pretreatment at 65 °C for 60 min in the presence of substrate inulin.

Introduction

Inulin-degrading enzymes (exo-inulinase, EC 3.2.1.80; endo-inulinase, EC 3.2.1.7 and invertase, EC 3.2.1.26) hydrolyze the plant polymer inulin practically into pure fructose syrups. Inulin is of great interest from biotechnological point of view as cheap plant substrate for microbial production of the so-called Ultra High Fructose Glucose Syrups. Recently, these syrups were applied widely and successfully in medicine, food and agriculture industries as a substitute of sucrose, for ethanol and single cell protein production.

The enzyme hydrolysis of inulin is an alternative and economically efficient way for production of high fructose syrups (with fructose content 95%) and competes the used difficult conventional process of starch hydrolysis needing at least four enzymatic step (including α -amylase, pululanase, amyloglucosidase and glucose isomerase action) for production of fructose-glucose syrups with fructose content up to 60% (Nigam and Singh, 1995; Vandamme and Derycke, 1983).

Among microorganisms producing inulin-degrading enzymes the thermophilic members of ge-

nus *Bacillus* deserve a special attention. The aerobic thermophilic bacilli (Allais *et al.*, 1987a and b; Belamri *et al.*, 1994) in comparison with fungi (Ferreira *et al.*, 1991; Ongen-Baysal *et al.*, 1994), yeasts (Bajpai and Margaritis, 1985; Guiraud *et al.*, 1982), mesophilic or anaerobic bacterial producers (Drent and Gottschal, 1991; Drent *et al.*, 1991; Kim *et al.*, 1997; Muramatsu *et al.*, 1992) possess certain advantages: i) realization of extracellular enzyme synthesis; ii) growth at high temperatures 60–70 °C with minimal risk of contamination; iii) high thermostability of the produced enzymes, an important feature necessary for the industrial polysaccharide hydrolysis where the preferable temperatures are above 60 °C.

In the present article the production of an extracellular inulin-degrading enzyme by *Bacillus* sp. 11 and some properties of the enzyme were studied.

Materials and Methods

Bacterial strain and growth conditions

Strain *Bacillus* sp. 11 was isolated from a thermal Bulgarian spring (with temperature 102 °C, pH 8.0–8.8, near Sapareva banya village). The characterization of the strain was performed using the methods of Gordon *et al.* (1973) and Bergey's Manual of Systematic Bacteriology (1986). The

§ Present address: Institut für Genetik, Zülpicherstr. 47, D-50674, Köln, Germany.



specimen for scanning electron microscopy (SEM) were prepared as described by Tonkova *et al.* (1994).

The medium used for maintenance of the strain and enzyme production had the following composition ($\text{g} \cdot \text{l}^{-1}$): peptone (Oxoid, Basingstoke, UK) – 2; yeast extract (Oxoid) – 2; K_2HPO_4 – 0.4; MgSO_4 – 0.08 and inulin (from *Dahlia* tubers, Fluka, Buchs, Switzerland) – 2. 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 8.5 after autoclaving.

The inoculum culture was grown in 100 ml Erlenmeyer flasks with 20 ml medium (initial pH 8.5). After overnight incubation at 60 °C on Gyrotory G-76 water bath shaker (240 rpm, New Brunswick Sci. Co. Inc., Edison, NJ, USA) culture (A650 nm – 0.6–0.7) was transferred into fermentor (Bioflo C-32, New Brunswick, working volume 350 ml) under the following conditions: temperature 60 °C, aeration rate 1.0 (vvm) and three different agitation rates – 300, 400 and 500 rpm.

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.

Inulinase assay

Inulinase activity was measured by determination of the reducing sugars released from substrate inulin by DNS-method (Bernfeld, 1955). The reaction mixture contained 50 μl substrate inulin ($10 \text{ g} \cdot \text{l}^{-1}$, phosphate buffer pH 7.0) and 50 μl enzyme solution. After incubation at 60 °C for 20 min the reaction was stopped by addition of 100 μl DNS-reagent. Reducing sugars were determined by calibration curve obtained using a standard solution of fructose. 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 activity

Invertase activity was determined under the conditions described above, with the difference that sucrose (Reachim, Moscow, Russia, 10 $\text{g} \cdot \text{l}^{-1}$, phosphate buffer pH 7.0) was used as a substrate. The 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 sucrose 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 a standard. The culture growth was determined by the absorbance at 650 nm.

Thin-layer chromatography (TLC)

TLC was performed on Silica gel 60 precoated plates (Merck, Darmstadt, Germany, 25 × 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.

Results

Morphological and physiologo-biochemical characteristics of strain *Bacillus* sp.11

The selected *Bacillus* isolate was Gram-positive, aerobic rod-shaped bacterium ($0.6\text{--}0.7 \times 2.7\text{--}8.0 \mu\text{m}$) forming ellipsoidal terminal spores swelling the sporangia (established by SEM). It grew at high temperatures – from 45 to 70 °C. Optimal growth temperature was 60–65 °C. No growth was established at 40 °C and 73 °C. It also showed rapid, good growth in wide pH range – from 5.7 to 10.0 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 6.8; decomposition of casein and tyrosine; formation of dihydroxyacetone; growth at pH 5.7 and 6.8; growth in 2 and 5% NaCl; production of acid in litmus milk. Negative reactions

were: Voges-Proskauer; catalase; anaerobic growth; growth in 0.02% azide broth; gas from glucose, arabinose, xylose, mannitol; utilization of citrate; deamination of phenylalanine; reduction of nitrate to nitrite; formation of indole; growth in 7 and 10% NaCl; resistance to lysozyme.

According to these results, the *Bacillus* isolate 11 showed five differences in comparison to *Bacillus stearothermophilus* strains described by Gordon *et al.* (1973) and Bergeys Manual of Systematic Bacteriology (1986). A specific 16S rDNA analysis of the strain 11 is required for taxonomical identification. In the present paper the object of studies was denoted as *Bacillus sp.* 11.

Enzyme production by batch cultivation in fermentor

The kinetics of growth and enzyme production of strain *Bacillus sp.* 11 were studied in a fermentor at three different agitation rates (Fig. 1, the curves at 300 and 500 rpm not shown).

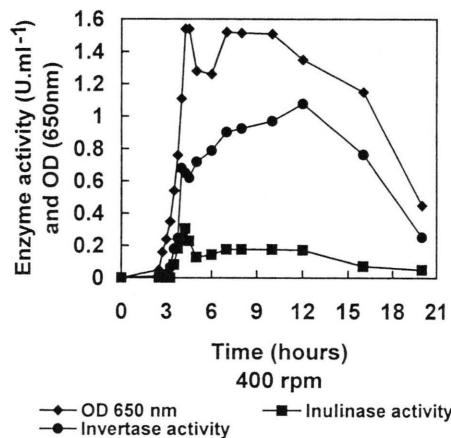


Fig. 1. Kinetics of enzyme production in fermentor by *Bacillus sp.* 11 (400 rpm).

The data obtained showed that the specific growth rate was enhanced from 1.27 to 2.28 h⁻¹ with an increase of the agitation rate from 300 to 500 rpm. Accordingly, the double time was decreased from 32.7 to 18.2 min.

It is established biphasic growth of the culture. 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 (further TLC studies confirmed this assumption). 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 drop in the hours of fructose accumulation in the medium (data for increased reducing sugars not shown). Rapidly utilization of fructose (an increase of optical density) abolished the catabolite repression and led to an increase of the enzyme level (Fig. 1).

At optimal agitation rate (400 rpm) the specific growth rate was 1.72 h⁻¹ and the double time 24.2 min. Maximal inulinase yield of 0.305 U·ml⁻¹ was achieved after 4–4.25 h at optical density 1.54.

Enzyme concentrate

Bacterial cells from the cultural liquid were harvested by centrifugation and the clear supernatant was concentrated by ultrafiltration. An 8.9-fold concentration and 1.3-fold purification of the cultural supernatant was achieved with a negligible loss of inulinase activity. More than 93% from the initial enzyme activity of the supernatant was detected in the concentrate. The industrial application of inulinases for obtaining of fructose syrups does not require a complete enzyme purification. By that reason, the further studies on the enzyme properties were performed using a crude enzyme preparation.

Temperature optimum and stability

The effect of temperature on inulinase and invertase activities 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 65 °C. When enzyme reaction was performed at 70 °C the relative inulinase and invertase activities were 87% and 94% of the measured maximal activity, respectively.

Thermostability of the enzyme concentrate was checked after a preliminary treatment at 50, 60, 65 and 70 °C for 15, 30 and 60 min in the absence or in the presence of a substrate inulin (Table I).

Enzyme activity on both substrates (inulin and sucrose) was retained 100% after a heat treatment of the concentrate at 50 °C for 15–60 min in the absence of substrate inulin. The increase of temperature up to 60 °C for 60 min led to 73% resid-

Table I. Thermostability of the crude enzyme concentrate.

Pretreatment		Without substrate inulin		With substrate inulin	
		Residual activities (%)		Inulinase	Invertase
		Inulinase	Invertase		
50 °C	15 min	100	100	100	100
50 °C	60 min	100	100	100	100
60 °C	15 min	78	91	100	100
60 °C	60 min	73	86	100	100
65 °C	15 min	34	13	97	100
65 °C	60 min	17	1	92	98
70 °C	15 min	7	1	85	67
70 °C	60 min	2	0	56	43

ual inulinase activity and 86% residual invertase activity of the enzyme. At higher temperatures (65–70 °C) both activities dropped sharply.

The performance of the preliminary heat treatment in the presence of substrate inulin (10 g·l⁻¹) showed a significant increase of the enzyme thermostability. In this case at 65 °C for 60 min the residual inulinase activity was 92% and the residual invertase activity 98%. A heat treatment at 70 °C for 60 min led to approximately 50% residual enzyme activity.

pH-optimum and stability

The enzyme activity on substrates inulin and sucrose was studied at pH values from 6.0 to 9.0 and temperature 60 °C. The enzyme was the most active at pH 6.5–7.0. When the enzyme reaction was performed at pH 8.0, the relative inulinase and invertase activities were 58% and 49% from the established maximal activity, respectively.

The preliminary incubation of the enzyme for 10 min at pH values from 5.5 to 9.0 showed that the residual activities on both substrates were retained 95–100% at pH range 5.5–7.5.

Reaction products from inulin hydrolysis by the crude enzyme preparation

The hydrolysis products from inulin were analyzed by thin layer chromatography allowing the separation of inulin, fructose, glucose and fructooligosaccharides. Only fructose was the first produced product. Fructooligosaccharides were not detected after an initial 1–10 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. Glucose and fruc-

tooligosaccharides were not detected also in the next 20–60 min at the denoted experimental conditions: enzyme – 0.83 U·ml⁻¹; inulin – 10 g·l⁻¹; reaction time – 60 min.

Hydrolysis of high sucrose syrups

The crude enzyme obtained from *Bacillus* sp. 11 showed a high invertase activity and was used for a hydrolysis of a high sucrose syrups (Fig. 2).

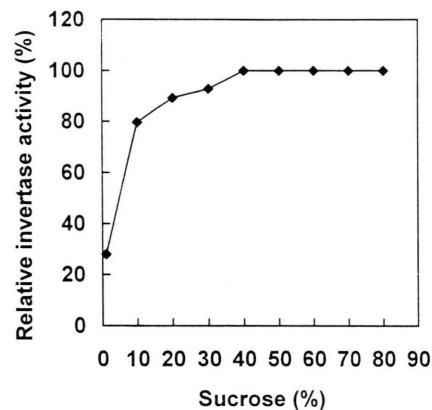


Fig. 2. Effect of a high sucrose concentration on the invertase activity of the preparation. Enzyme reaction was carried out at the standard conditions (60 °C, pH 7, 20 min). The relative activity was determined as percentage towards maximal activity.

The results demonstrated the possibility for a considerable enzyme action in the presence of 40–80% sucrose concentration. The increase of sucrose concentration from 1 to 40% enhanced the invertase activity. A maximal activity was reached in the presence of 400 g·l⁻¹ sucrose in the reaction mixture. Further increase of the substrate concen-

tration to $800 \text{ g} \cdot \text{l}^{-1}$ retained the invertase activity at the same level.

Kinetics of inulin and sucrose hydrolysis

Crude exo-inulinase from *Bacillus sp.* 11 was compared with a commercial inulinase preparation from *Aspergillus niger* (kat.No 57620, Fluka, Buchs, Switzerland). Both enzymes in equal concentrations ($0.83 \text{ U} \cdot \text{ml}^{-1}$ for enzyme from *Bacillus sp.* 11 and $0.85 \text{ U} \cdot \text{ml}^{-1}$ for enzyme from *Aspergillus niger*) were used for a hydrolysis of $10 \text{ g} \cdot \text{l}^{-1}$ inulin. The process was carried out at the optimal action conditions for each enzyme, respectively at 60°C and pH 7 for *Bacillus* sp.-enzyme and at 37°C and pH 4.1 for *Aspergillus niger*-enzyme (Fig. 3).

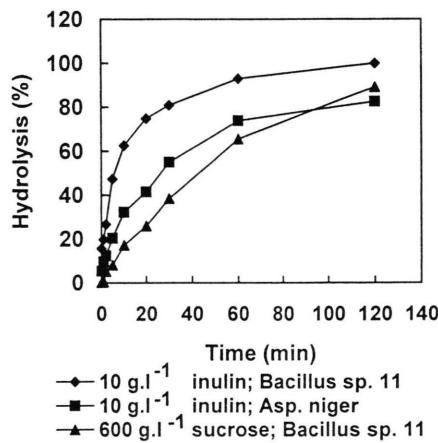


Fig. 3. Inulin and sucrose hydrolysis by the obtained exo-inulinase and its comparison with a commercial fungus inulinase from *Aspergillus niger* (using substrate inulin).

The data showed that after 1 h action of both enzymes, $9.3 \text{ g} \cdot \text{l}^{-1}$ reducing sugars (measured as fructose) were established using the bacterial exo-inulinase and $7.4 \text{ g} \cdot \text{l}^{-1}$ reducing sugars using the fungus preparation. A complete hydrolysis (100%) of inulin ($10 \text{ g} \cdot \text{l}^{-1}$) was attained for 2 h in the presence of *Bacillus* sp. 11-enzyme. At the same time the hydrolysis yield with the firm inulinase was 82.5%. Another advantage of the obtained bacterial preparation was the possibility for its application at high temperatures (60 – 65°C), preferable for the industrial processes of inulin hydrolysis.

The time course of hydrolysis of high concentrated sucrose solution ($600 \text{ g} \cdot \text{l}^{-1}$) using exo-inulinase from *Bacillus* sp. 11 ($0.83 \text{ U} \cdot \text{ml}^{-1}$) was also presented in Fig. 3. The yield of reducing sugars was $393 \text{ g} \cdot \text{l}^{-1}$ after 60 min reaction time. In the next hour (2 h) the percentage of hydrolysis was increased to 89%.

Substrate spectrum

For determination of the substrate specificity of the obtained exo-inulinase, the enzyme reaction was carried out using 4 substrates ($10 \text{ g} \cdot \text{l}^{-1}$): inulin, sucrose, raffinose and melizitose (Table II). Enzyme activity on substrates raffinose and melizitose was determined using the calibration curve with standard fructose solutions.

The enzyme splitted inulin, sucrose, raffinose, whereas melizitose was not hydrolyzed.

It is generally accepted that the ratio of the activity on inulin versus sucrose (I/S ratio) characterizes inulin-degrading enzymes: for inulinases (exo- or endo-) the I/S ratio is higher than 10^{-2} while for invertases it is lower than 10^{-4} (Ettalibi and Baratti, 1987). In our case this ratio I/S was 0.105.

According to Belamri *et al.* (1994) inulin-degrading enzymes are characterized by S/I ratio. For inulinases this ratio is lower than 50 while for invertases S/I values vary from 1600 to 2800. In our case this ratio S/I was 9.5.

Enzyme produced by *Bacillus* sp. 11 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 ratio I/S (0.105) or S/I ratio (9.5).

Table II. Substrate specificity of the enzyme.

Substrate	Enzyme activity ($\text{U} \cdot \text{ml}^{-1}$) [*]
Inulin	2.0
Sucrose	19.0
Raffinose	6.2
Melizitose	0

* In the presence of substrates inulin, raffinose and melizitose one unit of enzyme activity was defined as the amount of enzyme that liberates one μmol of fructose per minute under the assay conditions (pH 7.0, 60°C , 20 min reaction time). In the presence of substrate sucrose one unit of enzyme activity was defined as described in Materials and Methods.

Discussion

The kinetics of inulinase production by thermostophilic *Bacillus* sp. 11 was studied in a fermentor to explore the possibility for industrial production. The inulinase yield was $0.305 \text{ U} \cdot \text{ml}^{-1}$ (Fig. 1). Similar enzyme levels in cultural liquid have been reported for other mesophilic and thermophilic bacterial producers, for example, $0.200 \text{ U} \cdot \text{ml}^{-1}$ by *Bacillus* sp. LCB41 (Allais *et al.*, 1987a and b); $0.400 \text{ U} \cdot \text{ml}^{-1}$ by *Arthrobacter* sp. EM278 (Elyachioui *et al.*, 1992); $0.330\text{--}0.460 \text{ U} \cdot \text{ml}^{-1}$ by *Flavobacterium multivorum* (Allais *et al.*, 1986). Usually these inulinase yields were obtained after a longer growth time of 11 to 18 h, compared with 4.25 h in our case. Furthermore, inulinase from *Flavobacterium multivorum* (Allais *et al.*, 1986) was found to be endocellular, so its use in the industry is limited by its isolation which requires preliminary cell treatment. In comparison with yeast inulinases, the enzyme level in cultural liquid of *Bacillus* sp. 11 was at the same range, for example, $0.390 \text{ U} \cdot \text{ml}^{-1}$ was reported for *Kluyveromyces fragilis* (Negoro, 1978) and $0.222 \text{ U} \cdot \text{ml}^{-1}$ for *Candida kefyr* (Negoro and Kito, 1973), but these activities were obtained after a long cultivation time of 48 to 96 hours.

Thermophilic strain *Bacillus* sp. 11 possesses some advantages for a large-scale production: i) extracellular inulinase synthesis at high temperature (60°C); ii) a short production time (4.25 h); iii) high specific growth rate (1.72 h^{-1}); iv) a high inulinase yield ($0.305 \text{ U} \cdot \text{ml}^{-1}$, similar or higher to that from other bacteria and yeasts).

The data concerning pH- and temperature optimum of *Bacillus* sp. 11-enzyme were similar to those reported for numbers bacterial inulinases (Allais *et al.*, 1987a; Belamri *et al.*, 1994; Drent and Gottschal, 1991; Drent *et al.*, 1991; Elyachioui *et al.*, 1992).

The results in regard to enzyme thermostability were similar towards enzyme from *B. stearother-*

mophilus (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); inulinase from *Clostridium thermosuccinogenes* possessed 38% residual activity after pretreatment at 63°C for 30 min (Drent *et al.*, 1991). In comparison with inulinases from yeasts and fungi, thermostability of *Bacillus* sp. 11-enzyme was significantly higher (Vandamme and Derycke, 1983).

Enzyme from *Bacillus* sp. 11 belongs to exo-type inulinases yielding fructose as a sole reaction product. Our data were in agreement with those obtained by Azhari *et al.* (1989). These authors have been worked with inulinase NovoZym 230, solution of crude material from *Aspergillus* fungi. After a separation of exo- and endo-inulinases from crude preparation, TLC of the products from inulin hydrolysis by that exo-inulinase has showed that only fructose is produced at the initial stages of the process. Liberation of a small amount of glucose has been detectable after 1, 3 and 24 h reaction time.

Exo-inulinase from *Bacillus* sp. 11 competes yeast invertases and it can be successfully applied for hydrolysis of a high sucrose syrups (60–80%) at relative invertase activities 100% (Fig. 2). At the same time, sucrose concentration of 50% led to 97% relative activities for *Bacillus* sp. LCB41-enzyme and 62% for the *Kluyveromyces fragilis*-inulinase (Allais *et al.*, 1987a).

In conclusion, the exo-inulinase from thermostophilic *Bacillus* sp. 11 could be applied at high temperatures (above 65°C) and pH range 5.5–7.5 in contrast to the industrial preparations used up to now. The lack of a bacterial inulinase preparation on the world market determines the importance of the presented studies.

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