RESEARCH ARTICLE

Biosynthesis of inulinases by *Bacillus* bacteria

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

ABSTRACT

Biosynthesis of extracellular inulinases by bacteria *Bacillus licheniformis* 44MB82, *Bacillus licheniformis* 44MB82-A, *Bacillus licheniformis* 44MB82-G, *Bacillus amyloliquefaciens* CCM 3502, *Bacillus circulans* ATCC 28782 was studied. Different carbohydrates were tested for determining of their effect as carbon sources on inulinase synthesis. Various mono-, di- and polysaccharides were tested; flours from topinambour tubers and stems were used as source of inulin. The effect of nitrogen sources – mineral or organic – on the cells growth and enzyme activities was studied. The enzyme biosynthesis was activated by the presence of carbohydrates. *Bacillus licheniformis* 44MB82, *Bacillus licheniformis* 44MB82-A, *Bacillus amyloliquefaciens* CCM 3502 displayed maximum activities on starch media, *Bacillus licheniformis* 44MB82-G and *Bacillus circulans* ATCC 28782 – on glucose and starch media. With regard to the nitrogen source, the best results were obtained with yeast extract, casein and other sources of proteic nitrogen, comparatively to the mineral nitrogen. Optimal parameters for growth were pH 7.0-7.2, temperature 30-37°C, growth duration 72 hours. For *Bacillus circulans* ATCC 28782 they were pH 9.8, 40°C, growth duration 48 hours.

Key words: inulinases, *Bacillus*, carbon sources, nitrogen sources

Introduction

Inulin is a linear β-2,1-linked polyfructose chain, terminated by a glucose residue through a sucrose-type linkage at the reducing end (Gupta & Kaur, 1997). The polymer is produced naturally in over 45 000 plants worldwide (Hendry, 1993; Sharma et al., 2006) (Table 1). It is found as a carbohydrate reserve in the roots and tubers in plants such as Jerusalem artichoke, chicory, dahlia and also in burdock, goldenrod and dandelion (Singh & Gill, 2006). Inulins are different in their degree of polymerization, having also different functional properties. The degree of polymerization depends upon plant source, climate and growing conditions, storage time after harvest etc. (Neagu-Bonciu & Bahrim, 2011). Inulin is a recognised source for the production of either ultra-high fructose syrups, alternatively to the multi-enzyme hydrolysis of starch or the less favoured inverted sugar production with invertase, or for the production of oligofructose syrups (Zittan, 1981; Vandamme & Derycke, 1983; Rocha et al., 2006). The polymer is degraded by inulinase, which cleaves glycoside bonds to form largely (95 %) D-fructose by a single-step process (Singh & Gill, 2006).

Inulinases are fructofuranosyl hydrolases that act on the β-2,1 linkage of inulin, resulting in the formation of fructose, glucose and inulooligosaccharides depending on the pattern of action of inulinase. These enzymes can be divided into exo-inulinases and endo-inulinases. 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 (Neagu-Bonciu & Bahrim, 2011; Pouyez et al., 2012).

Microbial inulinases are important industrial enzymes, which are usually inducible and extracellular (Jain et al., 2012). They are produced by a wide array of microorganisms, comprehending bacteria, fungi and yeast (Catana et al., 2007). Although inulinases from various organisms have been reported (Zherebtsov et al. 2002; Sharma et al. 2006; Singh et al. 2007; Yuan & Bai 2008), only a few of these organisms have been able to produce sufficient inulinases which fulfill the desired characteristics of high temperature optimum and thermal stability for the successful application

RESEARCH ARTICLE

of inulinases in the various industries. Inulin-hydrolyzing activity has been reported from various microbial strains, but yeasts together with *Aspergillus* spp. have proved to be the most versatile source of inulinases (Singh & Gill, 2006; Kango & Jain, 2011). Among the yeasts that can produce inulinases, *Kluyveromyces* spp., *Pichia* spp. and *Candida* spp. have high potential for producing high yields of inulinase activity (Neagu-Bonciu & Bahrim, 2011). However, the yeast inulinases show only modest thermostability, due to which their potential for commercial scale applications appears to be limited (Singh & Gill, 2006). As far as fungi are concerned, inulinase activity has been obtained using different mould strains, *Aspergillus* spp. being the favourite species for inulinase production. *Chrysosporium pannorum*, isolated from soil, was found also as a very active inulinase producer (Neagu-Bonciu & Bahrim, 2011). Of the different inulinases reported from other *Penicillium* spp., the inulinase from *P. aculeatum* was more thermoresistant, whereas the inulinase from *P. cyclopium* was the most labile (Singh & Gill, 2006).

Data on inulinases biosynthesis using bacterial strains are scarce and mainly concern endo-inulinases (Jain et al., 2012). 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 (Singh & Gill, 2006). *Streptomyces* spp. was found as a good producer of inulinases. Bacteria of genus *Bacillus* are also active producers of extracellular inulinase – 42.36 U/ml inulinase activities were obtained on sucrose as substrate (Zherebtsov et al., 2002). *Pseudomonas* spp. (Kim et al., 1997) and *Arthrobacter* spp. (Kang et al., 1998) were also tested for the ability to produce inulinases.

Inulinase is a versatile enzyme used in many fields, it is subject to catabolite repression (Kim et al., 2008; Pouyez et al., 2012) and is used for the production of ultra-high fructose syrup from inulin, bioethanol production, inulo-oligosaccharides, single-cell oil and single-cell protein, some chemicals like citric acid, butanediol, alcohols, acetone, butanol pullulan, gluconic acid, sorbitol and lactic acid (Pandey et al., 1999; Chi et al., 2009; Liu et al., 2010; Chi et al., 2011; Erdal et al., 2011).

In the present study, the production of extracellular inulinases by five *Bacillus* strains in liquid cultures is reported. Specific features of biosynthesis by these bacterial strains are studied.

Materials and Methods

Bacterial strains

Bacillus licheniformis 44MB82, *Bacillus licheniformis* 44MB82-A, *Bacillus licheniformis* 44MB82-G, *Bacillus amyloliquefaciens* CCM 3502, *Bacillus circulans* ATCC 28782 (supplied by the National Bank of Microorganisms and Cell Cultures, Sofia, Bulgaria) were used in this investigation. Pure cultures were grown on beef-extract agar for 4 days at 30-37°C.

Inoculum preparation

Bacillus licheniformis 44MB82, *Bacillus licheniformis* 44MB82-A and *Bacillus amyloliquefaciens* CCM 3502 strains were cultivated 24 h at 38°C in nutrient medium containing (g/l): soluble starch (Sigma-Aldrich, USA), 2.0; peptone (Oxoid, Basingstoke, UK), 5.0; yeast extract (Oxoid), 5.0; $MgSO_4$, 0.2 and K_2HPO_4, 1.0; pH before sterilization 6.5.

Table 1. *Distribution of fructans in the angiosperms (adapted from Hendry, 1993).*

	Families	Estimated number of species
Dicotyledons	*Asteraceae*	25000
	Campanulaceae	2250
	Dipsacaceae	60
	Polemoniaceae	2340
	Ericaceae	150
Monocotyledons	*Poaceae* (also called *Gramineae* or true grasses)	8000
	Liliaceae	7200
Total number		45000

RESEARCH ARTICLE

Bacillus licheniformis 44MB82-G strain was grown in a nutrient broth supplemented with (in g/l): glucose, 60.0; beef extract (Lab-Lemco powder, Oxoid, UK), 15.0; peptone (Oxoid), 15.0; K_2HPO_4, 10.4; cornsteep liquor, 6.6; $CaCl_2$, 1.1; pH 6.5. *Bacillus circulans* ATCC 28782 was cultivated at 40°C in nutrient medium containing (g/l): soluble starch (Sigma-Aldrich, USA), 2.0; peptone (Oxoid, Basingstoke, UK), 5.0; yeast extract (Oxoid), 5.0; $MgSO_4$, 0.2 and K_2HPO_4, 1.0. Sterile sodium carbonate was used to adjust the medium to pH 9.8-10.0 after autoclaving. These media are optimized for amylase and CGTase synthesis by the studied strains.

Fermentation medium

The mediun used for inulinase 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 from 6.5 to 10.0 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 30°C-40°C in water-bath (Julabo SW22) shaker, for 96 h, at 200 rpm.

Effect of nitrogen sources

Effect of different nitrogen sources including peptone, beef extract, yeast extract, casein, soy flour (organic N-sources) and $NaNO_3$, KNO_3, $(NH_4)_2SO_4$ and $(NH_4)H_2PO_4$ (inorganic N-sources) was studied by incorporating 0.4% (w/v) of each N- source in the fermentation medium on the place of the peptone and yeast extract. 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, saccharose, 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 7.5) and 100 µl enzyme solution. After incubation at 40°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 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 7.5) 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.

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.

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

RESEARCH ARTICLE

resulting flours were used in the next experiments without fractionation. The stems 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

The effects of carbon nutrition on the inulin synthesis by bacteria were studied using the following substrates: glucose, fructose, saccharose, inulin, sugar cane molasse, soluble starch, garlic and onion extracts, flours from Jerusalem artichoke tubers and stems, extracts from these flours. The specific medium for each strain was used as a control. Inulinase and invertase activities and optical density at 650 nm were measured.

Optimal parameters for growth and inulinase production from strain *Bacillus licheniformis* 44MB82 were pH 7.0 and temperature 35°C; for *Bacillus amyloliquefaciens* CCM 3502 strain they were pH 7.2 and 30°C; for *Bacillus licheniformis* 44MB82-A and *Bacillus licheniformis* 44MB82-G strains - pH 7.0 and optimal temperature of production 37°C. Maximal inulinase activity with these strains was reached after 72 hours of cultivation on the different carbon sources. For *Bacillus circulans* ATCC 28782 the optimal growth and enzyme production parameters were pH 9.8, 40°C, growth duration 48 hours.

Various microorganisms such as fungi, yeasts, bacteria, and actinomycetes, can synthesize inulinase on inulin, saccharose, fructose, lactose, xylose, raffinose, and maltose as carbon sources (Vullo et al., 1991; Gern et al., 2001; Kango & Jain, 2011). The biosynthesis of enzymes from microorganisms is dependent on their genetic characteristics but the regulation of the synthesis by changes in the nutrient medium composition is of great importance.

The inulinase activity of the studied strains on the specific media was good. Very low or absent activity was not observed, the enzyme was synthesized in low quantities without the presence of inulin or inulosaccharides. Although the enzyme has been synthesized in presence of inulin and garlic and onion extracts, Jerusalem artichoke flours and extracts, saccharose and sugar cane molasse, the productivity was higher when the microorganisms were inoculated in media formulated with soluble starch, as carbon source. Growth was better and the biomass was higher on these starch media. Only the catabolite derepressed strain *B. licheniformis* 44MB82-G and the *B. circulans* ATCC 28782 strain showed similar growth on starch and on glucose media; *Bacillus licheniformis* 44MB82, *Bacillus licheniformis* 44MB82-A, *Bacillus amyloliquefaciens* CCM 3502 displayed maximum activities on starch media (Tables 2-6). The effect of different carbohydrates decreased in the following order: for *B. licheniformis* 44MB82: Starch > saccharose > inulin > onion extract > garlic extract > tubers extract > glucose; for *B. licheniformis* 44MB82-A: Starch > garlic extract > onion extract and inulin; for *B. licheniformis* 44MB82-G: Starch and glucose > inulin > saccharose > garlic and onion extract; for *B. amyloliquefaciens* CCM 3502: Starch > inulin and saccharose; for *B. circulans* ATCC 28782: Glucose and starch > inulin, saccharose, garlic and onion extract.

Table 2. *Effect of the carbon source on the inulinase production by Bacillus licheniformis 44MB82 strain at optimal pH 7.0 and optimal temperature of production 35°C.*

Carbon source	Inulinase** U/ml ± SD	Invertase** U/ml ± SD	S/I ratio	Optical density* OD650nm
Glucose	0.030±0.02	0.035±0.02	1.17	1.72±0.02
Fructose	0.033±0.02	0.044±0.02	1.33	1.58±0.02
Saccharose	0.025±0.02	0.038±0.02	1.52	1.62±0.03
Inulin	0.105±0.02	0.144±0.03	1.38	1.88±0.04
Sugar cane molasse	0.042±0.02	0.057±0.02	1.36	0.97±0.04
Soluble starch	**0.145±0.03**	**0.209±0.03**	**1.44**	**5.32±0.02**
Garlic extract	0.099±0.02	0.134±0.02	1.35	1.88±0.04
Onion extract	0.102±0.03	0.142±0.02	1.39	1.81±0.03
Tubers flour	0.078±0.02	0.082±0.02	1.05	-
Stems flour	0.065±0.02	0.066±0.02	1.01	-
Tubers extract	0.096±0.02	0.128±0.03	1.33	1.90±0.02
Stems extract	0.088±0.02	0.119±0.03	1.36	1.83±0.04

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

RESEARCH ARTICLE

Table 3. *Effect of the carbon source on the inulinase production by Bacillus licheniformis 44MB82-A strain at optimal pH 7.0 and optimal temperature of production 37°C.*

Carbon source	Inulinase** U/ml ± SD	Invertase** U/ml ± SD	S/I ratio	Optical density* OD650nm
Glucose	0.034 ± 0.02	0.046 ± 0.02	1.34	1.89± 0.03
Fructose	0.028 ± 0.02	0.039 ± 0.02	1.38	1.86 ± 0.04
Saccharose	0.027 ± 0.02	0.032 ± 0.02	1.18	1.79 ± 0.03
Inulin	0.098 ± 0.02	0.113 ± 0.04	1.16	1.85 ± 0.04
Sugar cane molasse	0.056 ± 0.02	0.069 ± 0.02	1.23	0.75 ± 0.03
Soluble starch	**0.157 ± 0.03**	**0.185 ± 0.03**	**1.18**	**5.23 ± 0.04**
Garlic extract	0.110 ± 0.03	0.128 ± 0.03	1.16	1.55 ± 0.03
Onion extract	0.098 ± 0.03	0.130 ± 0.03	1.27	1.41 ± 0.03
Tubers flour	0.045 ± 0.02	0.056 ± 0.02	1.23	-
Stems flour	0.030 ± 0.02	0.038 ± 0.02	1.27	-
Tubers extract	0.077 ± 0.02	0.096 ± 0.02	1.25	1.84±0.03
Stems extract	0.072 ± 0.02	0.089 ± 0.02	1.24	1.81±0.03

Legend: * OD_{corr}, 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 0.05, n = 5; I -Inulinase; S – Invertase.

Correlation between the inulinase and the invertase activity was observed – significant fluctuations of the S/I ratio on inulin or inulin-containing substrates and saccharose were not measured suggesting that the strains synthesized a single enzymes responsible for hydrolysis of both inulin and saccharose.

The effect of nitrogen sources – mineral or organic – on the cells growth and enzyme activities was studied using fermentation media supplemented with starch as carbon source. Complex nitrogen sources were better than inorganic nitrogen sources. The microorganisms were more efficient in the inulinase production when inoculated in media containing proteic nitrogen in relation to those formulated with mineral nitrogen as it can be verified in Tables 7 and 8. Yeast extract was found to be the best nitrogen source to be used for inulinase production followed by beef extract and the casein. 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).

On ammonium sulphate, sodium nitrate, potassium nitrate as nitrogen sources, the averages inulinase and invertase activities were considerably inferior to all the media formulated with proteic nitrogen. The S/I ratio for every single *Bacillus* strain was not affected by the organic nitrogen sources in comparison to the results, obtained with the inorganic nitrogen sources (Table 8).

In conclusion, various carbon sources were evaluated for production of inulinases by the bacterial strains *Bacillus licheniformis* 44MB82, *Bacillus licheniformis* 44MB82-A, *Bacillus licheniformis* 44MB82-G, *Bacillus amyloliquefaciens* CCM 3502, *Bacillus circulans* ATCC 28782, producers of other extracellular enzymes - amylases, proteinases and cyclodextrin glucanotransferase. The strains showed good growth on a simple media, containing different carbohydrates – mono, di- and polysaccharides. Varied inulinase levels were noticed on different C- sources - inulin, soluble starch, garlic and onion extracts, flours from Jerusalem artichoke tubers and stems, extracts from these flours. Although inulinases are usually inducible enzymes, they were produced without the presence of inulin or inulosaccharides – the studied *Bacillus* strains synthesized similar inulinase activity on starch than on inulin or inulin-containing substrates. Among various nitrogen sources tested, yeast extract was found to be the best source followed by beef extract, casein and peptone.

RESEARCH ARTICLE

Table 4. *Effect of the carbon source on the inulinase production by Bacillus amyloliquefaciens CCM 3502 strain at optimal pH 7.2 and optimal temperature of production 30°C.*

Carbon source	Inulinase** U/ml ± SD	Invertase** U/ml ± SD	S/I ratio	Optical density* OD650nm
Glucose	0.017 ± 0.02	0.022 ± 0.02	1.27	0.85 ± 0.03
Fructose	0.015 ± 0.02	0.019 ± 0.02	1.28	0.83 ± 0.04
Saccharose	0.039 ± 0.02	0.045 ± 0.02	1.18	1.88 ± 0.03
Inulin	0.090 ± 0.02	0.111 ± 0.03	1.23	1.96 ± 0.04
Sugar cane molasse	0.055 ± 0.02	0.062 ± 0.02	1.13	0.66 ± 0.03
Soluble starch	**0.123 ± 0.03**	**0.141 ± 0.03**	**1.14**	**3.98 ± 0.03**
Garlic extract	0.082 ± 0.02	0.106 ± 0.03	1.29	1.85 ± 0.04
Onion extract	0.080 ± 0.02	0.103 ± 0.03	1.29	1.85 ± 0.04
Tubers flour	0.045 ± 0.02	0.060 ± 0.02	1.33	-
Stems flour	0.035 ± 0.02	0.051 ± 0.02	1.45	-
Tubers extract	0.083 ± 0.02	0.097 ± 0.02	1.16	1.88 ± 0.04
Stems extract	0.077 ± 0.02	0.094 ± 0.02	1.21	1.80 ± 0.04

Table 5. *Effect of the carbon source on the inulinase production by Bacillus licheniformis 44MB82-G strain at optimal pH 7.0 and optimal temperature of production 37°C.*

Carbon source	Inulinase** U/ml ± SD	Invertase** U/ml ± SD	S/I ratio	Optical density* OD650nm
Glucose	**0.207 ± 0.05**	**0.195 ± 0.04**	**0.94**	**4.98 ± 0.04**
Fructose	0.040 ±0.02	0.045 ± 0.02	1.12	1.22 ± 0.04
Saccharose	0.046 ± 0.02	0.048 ± 0.02	1.07	2.12 ± 0.04
Inulin	**0.194 ± 0.04**	**0.058 ± 0.02**	**0.30**	2.98 ± 0.03
Sugar cane molasse	0.023 ± 0.02	0.026 ± 0.02	1.04	0.90 ± 0.03
Soluble starch	**0.204 ± 0.05**	**0.198 ± 0.05**	**0.97**	**5.20 ± 0.04**
Garlic extract	0.135 ± 0.03	0.078 ± 0.02	0.58	1.10 ± 0.04
Onion extract	0.140 ± 0.03	0.083 ± 0.02	0.59	1.05 ± 0.04
Tubers flour	0.047 ± 0.02	0.015 ± 0.02	0.32	-
Stems flour	0.035 ± 0.02	0.012 ± 0.02	0.34	-
Tubers extract	0.128 ± 0.03	0.040 ± 0.02	0.31	0.90 ± 0.03
Stems extract	0.120 ± 0.03	0.047 ± 0.02	0.39	0.78 ± 0.04

Table 6. *Effect of the carbon source on the inulinase production by Bacillus circulans ATCC 28782 strain at optimal pH 9.8 and optimal temperature of production 37°C.*

Carbon source	Inulinase** U/ml ± SD	Invertase** U/ml ± SD	S/I ratio	Optical density* OD650nm
Glucose	0.095 ± 0.02	0.097 ± 0.02	1.02	**0.85 ± 0.04**
Fructose	0.027 ± 0.02	0.030 ± 0.02	1.11	0.50 ± 0.04
Saccharose	0.013 ± 0.02	0.014 ± 0.02	1.08	0.55 ± 0.02
Inulin	**0.112 ± 0.03**	**0.011 ± 0.02**	**0.10**	0.43 ± 0.03
Sugar cane molasse	0.027 ± 0.02	0.033 ± 0.02	0.35	0.45 ± 0.04
Soluble starch	**0.153 ± 0.03**	**0.155 ± 0.04**	**1.01**	**0.83 ± 0.04**
Garlic extract	0.122 ± 0.02	0.105 ± 0.03	0.86	0.39 ± 0.03
Onion extract	0.117 ± 0.02	0.093 ± 0.03	0.79	0.38 ± 0.04
Tubers flour	0.076 ± 0.02	0.060 ± 0.02	0.79	-
Stems flour	0.070 ± 0.02	0.065 ± 0.02	0.93	-
Tubers extract	0.124 ± 0.02	0.098 ± 0.03	0.79	0.30 ± 0.03
Stems extract	0.086 ± 0.02	0.066 ± 0.02	0.77	0.25 ± 0.03

Legend: * OD_{corr}, 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 0.05, n = 5; I – Inulinase; S – Invertase

RESEARCH ARTICLE

Table 7. *Effect of the organic nitrogen sources on the inulinase production by Bacillus strains at optimal pH and temperatures.*

Organic nitrogen source/Bacterial strain	Inulinase U/ml ± SD	Invertase U/ml ± SD	S/I ratio	Optical density* OD650nm
Peptone				
1 - 44MB82	0.135 ± 0.03	0.211 ± 0.05	1.56	4.95 ± 0.06
2 - 44MB82-A	0.140 ± 0.03	0.179 ± 0.04	1.28	4.98 ± 0.05
3 - 44MB82-G	0.176 ± 0.03	0.164 ± 0.03	0.93	4.75 ± 0.07
4 - CCM 3502	0.115 ± 0.02	0.132 ± 0.02	1.15	3.89 ± 0.03
5 - ATCC 28782	0.146 ± 0.03	0.149 ± 0.03	1.06	0.75 ± 0.07
Beef extract				
1 - 44MB82	0.130 ± 0.03	0.210 ± 0.03	1.62	5.20 ± 0.04
2 - 44MB82-A	0.138 ± 0.03	0.180 ± 0.03	1.30	5.15 ± 0.04
3 - 44MB82-G	0.180 ± 0.04	0.165 ± 0.03	0.92	5.10 ± 0.07
4 - CCM 3502	0.112 ± 0.02	0.131 ± 0.03	1.17	3.88 ± 0.02
5 - ATCC 28782	0.140 ± 0.03	0.155 ± 0.03	1.11	0.81 ± 0.07
Yeast extract				
1 - 44MB82	0.140 ± 0.03	0.224 ± 0.05	1.60	5.32 ± 0.06
2 - 44MB82-A	0.153 ± 0.03	0.183 ± 0.04	1.20	5.20 ± 0.05
3 - 44MB82-G	0.195 ± 0.04	0.180 ± 0.04	0.92	5.18 ± 0.06
4 - CCM 3502	0.115 ± 0.02	0.135 ± 0.02	1.18	3.90 ± 0.04
5 - ATCC 28782	0.150 ± 0.03	0.164 ± 0.02	1.09	0.83 ± 0.07
Casein				
1 - 44MB82	0.140 ± 0.03	0.219 ± 0.05	1.56	4.25 ± 0.05
2 - 44MB82-A	0.150 ± 0.03	0.182 ± 0.05	1.21	4.20 ± 0.04
3 - 44MB82-G	0.180 ± 0.04	0.165 ± 0.04	0.92	4.80 ± 0.05
4 - CCM 3502	0.115 ± 0.03	0.137 ± 0.03	1.17	2.90 ± 0.04
5 - ATCC 28782	0.150 ± 0.04	0.155±0.03	1.03	0.63 ± 0.06
Soy flour				
1 - 44MB82	0.120 ± 0.03	0.201 ± 0.04	1.68	3.20 ± 0.05
2 - 44MB82-A	0.133 ± 0.02	0.180 ± 0.03	1.35	3.08 ± 0.06
3 - 44MB82-G	0.162 ± 0.03	0.139 ± 0.02	0.86	4.64 ± 0.06
4 - CCM 3502	0.100 ± 0.02	0.129 ± 0.02	1.29	1.83 ± 0.04
5 - ATCC 28782	0.129 ± 0.02	0.127 ± 0.02	0.98	0.48 ± 0.05

Legend: 1 - *B. licheniformis* 44MB82; 2 - *B. licheniformis* 44MB82-A; *B. licheniformis* 44MB82-G; *B. amyloliquefaciens* CCM 3502; *B. circulans* ATCC 28782. Statistical significance ($p < 0.05$), Data are mean values ± SD from 0.02 to 0.07, n = 5; * OD_{corr}; I – Inulinase; S – Invertase.

Table 8. *Effect of the inorganic nitrogen sources on the inulinase production by Bacillus strains at optimal pH and temperatures.*

Inorganic nitrogen source/Bacterial strain	Inulinase U/ml ± SD	Invertase U/ml ± SD	S/I ratio	Optical density* OD650nm
$NaNO_3$				
1 - 44MB82	0.054 ± 0.02	0.121 ± 0.02	2.24	0.58 ± 0.02
2 - 44MB82-A	0.070 ± 0.02	0.109 ± 0.03	1.56	0.62 ± 0.02
3 - 44MB82-G	0.063 ± 0.02	0.140 ± 0.02	2.22	0.88± 0.03
4 - CCM 3502	0.040 ± 0.02	0.085 ± 0.03	2.12	0.43 ± 0.02
5 - ATCC 28782	0.025 ± 0.02	0.101 ± 0.02	4.04	0.35 ± 0.03
KNO_3				
1 - 44MB82	0.045 ± 0.02	0.105 ± 0.03	2.33	0.53 ± 0.02
2 - 44MB82-A	0.060 ± 0.02	0.098 ± 0.03	1.63	0.58 ± 0.02
3 - 44MB82-G	0.048 ± 0.02	0.122 ± 0.02	2.54	0.90 ± 0.04
4 - CCM 3502	0.030 ± 0.02	0.076 ± 0.03	2.53	0.48 ± 0.02
5 - ATCC 28782	0.030 ± 0.02	0.095 ± 0.03	3.17	0.49 ± 0.03
$(NH_4)_2SO_4$				
1 - 44MB82	0.050 ± 0.02	0.107 ± 0.02	2.14	0.60 ± 0.02
2 - 44MB82-A	0.073 ± 0.02	0.101 ± 0.02	1.38	1.60 ± 0.02
3 - 44MB82-G	0.068 ± 0.02	0.135 ± 0.03	1.98	0.95 ± 0.03

RESEARCH ARTICLE

4 - CCM 3502	0.060 ± 0.02	0.081 ± 0.02	1.35	0.53 ± 0.02
5 - ATCC 28782	0.084 ± 0.02	0.099 ± 0.02	1.18	0.55 ± 0.03
$(NH_4)H_2PO_4$				
1 - 44MB82	0.084 ± 0.02	0.109 ± 0.02	1.30	0.93 ± 0.04
2 - 44MB82-A	0.090 ± 0.02	0.134 ± 0.03	1.49	1.85 ± 0.03
3 - 44MB82-G	0.095 ± 0.02	0.166 ± 0.03	1.75	1.15 ± 0.05
4 - CCM 3502	0.080 ± 0.03	0.103 ± 0.02	1.29	0.75 ± 0.02
5 - ATCC 28782	0.105 ± 0.02	0.173 ± 0.03	1.65	0.65 ± 0.06

Legend: 1 - *B. licheniformis* 44MB82; 2 - *B. licheniformis* 44MB82-A; *B. licheniformis* 44MB82-G; *B. amyloliquefaciens* CCM 3502; *B. circulans* ATCC 28782. Statistical significance ($p < 0.05$), Data are mean values ± SD from 0.02 to 0.06, n = 5; * OD_{corr}; I – Inulinase; S – Invertase.

References

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

Catana R, Eloy M, Rocha JR, Ferreira BS, Cabral JMS, Fernandes P. 2007. Stability evaluation of an immobilized enzyme system for inulin hydrolysis. Food Chem., 101(1): 260-266.

Chi Z, Zhang T, Liu G, Xue L. 2009. Inulinase-expressing microorganisms and applications of inulinases. Appl. Microbiol. Biotechnol., 82(2): 211-220.

Chi Z-M, Zhang T, Cao TS, Liu XY, Cui W, Zhao CH. 2011. Biotechnological potential of inulin for bioprocesses. Bioresour. Technol., 102(6): 4295-4303.

Erdal S, Canli O, Algur OF. 2011. Inulinase production by *Geotrichum candidum* using Jerusalem artichoke as sole carbon source. Romanian Biotechnol. Letters, 16 (4): 6378-6383.

Gern RM, Furlan SA, Ninow JL, Jonas R. 2001. Screening for microorganisms that produce only endo-inulinase. Appl. Microbiol. Biotechnol., 55(5): 632-635.

Gupta AK, Kaur N. 1997. Fructan storing plants - a potential source of high fructose syrups. J. Sci. Ind. Res., 56(8): 447-452.

Hendry G. 1993. Evolutionary origins and natural functions of fructans: a climatological, biogeographic and mechanistic appraisal. New Phytol., 123(1): 3-14.

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

Kang S, Chang YJ, Oh SJ, Kim SI. 1998. Purification and properties of an endo-inulinase from an *Arthrobacter* sp., Biotechnol. Letters, 20(10): 983-986.

Kango N. 2008. Production of inulinase using tap roots of dandelion (*Taraxacum officinale*) by *Aspergillus niger*. J. Food Eng., 85(3): 473-478.

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

Kim DH, Choi YJ, Song SK, Yun JW. 1997. Production of inulo-oligosaccharides using endo-inulinase from a *Pseudomonas* sp. Biotechnol. Letters, 19(4): 369-372.

Kim KY, Nascimento AS, Golubev AM, Polikarpov I, Kim CS, Kang SI, Kim SI. 2008. Catalytic mechanism of inulinase from *Arthrobacter* sp. S37. Biochem. Biophys. Res. Commun., 371(4): 600-605.

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

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31(3): 426-428.

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

Pandey A, Soccol CR, Selvakumar P, Soccol VT, Krieger N, Fontana JD. 1999. Recent developments in microbial inulinases, its production, properties, and industrial applications. Appl. Biochem. Biotechnol., 81(1): 35-52.

Pouyez J, Mayard A, Vandamme A-M, Roussel G, Perpète EA, Wouters J, Housen I, Michaux C. 2012. First crystal structure of an endo-inulinase, INU2, from *Aspergillus ficuum*: Discovery of an extra-pocket in the catalytic domain responsible for its endo-activity. Biochimie, 94(11): 2423-2430.

Rocha JR, Catana R, Ferreira BS, Cabral JMS, Fernandes P. 2006. Design and characterisation of an enzyme system for inulin hydrolysis. Food Chem., 95(1): 77-82.

Sharma AD, Kainth S, Gill PK. 2006. Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces* sp. J. Food Eng., 77(3): 486-491.

Singh P, Gill PK. 2006. Production of inulinases: recent advances. Food Technol. Biotech., 44 (2): 151-162.

Singh RS, Sooch BS, Puri M. 2007. Optimization of medium and process parameters for the production of inulinase from a newly isolated *Kluyveromyces marxianus* YS-1. Bioresour. Technol., 98(13): 2518-2525.

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

Vullo DL, Coto CE, Sineriz F. 1991. Characteristics of an inulinase produced by *Bacillus subtilis* 430A, a strain isolated from the rhizosphere of *Vernonia herbacea* (Vell Rusby). Appl. Environ. Microbiol., 57(8): 2392-2394.

Yuan W, Bai, F. 2008. Optimization of medium and process parameters for the production of inulinase from *Kluyveromyces marxianus* Y1. J. Biotechnol., 136S: S317-318.

Zherebtsov NA. Shelamova SA, Abramova IN. 2002. Biosynthesis of inulinases by *Bacillus* bacteria. Appl. Biochem. Microbiol., 38(6): 544-548.

Zittan L. 1981. Enzymatic hydrolysis of inulin - an alternative way to fructose production. Starch, 33(11): 373-377.