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Molecular and biochemical characterization of a new endoinulinase producing bacterial strain of *Bacillus safensis* AS-08*

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Abstract: Microbial inulinases are an important class of industrial enzymes, which are used for the production of fructooligosaccharides and high-fructose syrup. Endoinulinase producing bacterial strains were isolated from soil samples taken from the vicinity of *Asparagus* sp. root tubers. All the bacterial strains were screened for inulinase activity. The primary screening was carried out based on hydrolytic zone on agar plates containing inulin-based medium and Lugol's iodine solution. Thus 30 inulinase producing bacterial strains were isolated. Out of 30 strains, 5 bacterial strains were found endoinulolytic, whereas 25 were exoinulolytic on the basis of action pattern of the enzyme. In tertiary screening, the bacterial isolate AS-08 was found to be most efficient for inulinase activity. Morphological, biochemical and physiological characteristics of the bacterial isolate AS-08 confirmed it as *Bacillus* sp. Furthermore, species-specific identification by 16S rDNA sequencing and phylogenetic analysis revealed the isolate as *Bacillus safensis. Bacillus pumilus* SH-B30 was found to be the nearest homolog. The strain showed maximum inulinase activity (12.56 U/mL) after 20 h of incubation at 37 °C.

Key words: *Bacillus safensis*; 16S rDNA sequence; endoinulinase; biochemical characterization; thin layer chromatography; phylogenetic analysis.

Abbreviations: TLC, thin layer chromatography.

Introduction

Inulin is a well known fructan particularly abundant in some plants belonging to families Asteraceae, Campanulaceae, Poaceae, Liliaceae and Amaryllidaceae (Kaur & Gupta 2002; Singh & Singh 2010). It is made of linear chains of D-fructofuranose molecules linked by β-2,1-glycosidic bonds and has a D-glucose moiety at the reducing end. Inulin and its partially hydrolyzed products (fructooligosaccharides) have gained significant importance in food and pharmaceutical industries. Fructooligosaccharides are popular functional food components due to their beneficial health properties, such as bifidogenic nature, low calorie diet and rich source of dietary fibre (Sangeetha et al. 2005; Meyer & Stasse-Wolthuis 2009; Sabater-Molina et al. 2009; Singh & Singh 2010).

The development in industrial enzymology has enabled the large scale production of fructooligosaccharides by enzymatic synthesis either from sucrose by glycosyl transfer reaction using fructosyl transferase or from inulin by hydrolysis using endoinulinases. Inulinases are important microbial enzymes, which hydrolyze inulin to produce fructose (Ricca et al. 2007) and fructooligosaccharides (Singh & Singh 2010; Kango & Jain 2011). Depending upon their mode of action, inulinases are exoinulinase (EC 3.2.1.80), which cleaves β-2,1 linkage from the non-reducing end thus releasing fructose, and endoinulinase (EC 3.2.1.7) acting randomly on the internal linkages to form fructooligosaccharides (Ohta et al. 2004; Kango & Jain 2011). A number of fungi, yeasts and bacterial sources have been reported for endoinulinase production (Ohta et al. 2004; Singh & Singh 2010; Kango & Jain 2011). Amongst bacteria, *Xanthomonas oryzae* (Cho et al. 2001; Cho & Yun 2002), *Xanthomonas campestris* (Naidoo et al. 2009), *Pseudomonas* sp. (Park & Yun 2001), *Arthrobacter* sp. (Kang et al. 1998) and *Bacillus smithii* (Gao et al. 2009) are reported as potent sources of endoinulinases. A number of bacterial strains have been described for the production of fructooligosaccharides from inulin at laboratory scale (Singh & Singh 2010; Kango & Jain 2011). However, bacterial sources producing high yield of endoinulinases are still required for scale-up of an industrial process.

In the present study, endoinulinase producing bacterial strains have been isolated and screened for enzyme activity. The bacterial strain of *Bacillus safensis*

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AS-08 showing maximum inulinase activity has been characterized from both molecular and biochemical points of view. In the best of our knowledge, this is the first report on the endoinulinase activity from *Bacillus safensis.*

Material and methods

Collection of soil samples
Root tubers of *Asparagus* sp. are well known for their high inulin content (Gupta & Kaur 1997; Singh & Singh 2010). Therefore, for the isolation of endoinulinase producing bacteria, soil samples were taken from the fields of *Asparagus* sp. planted area of Botanical Garden, Punjabi University, Patiala, India.

Isolation of inulinase producing bacterial strains
Soil sample (2%, w/v) was added in the sterilized enrichment medium (100 mL in Erlenmeyer's flask) containing (w/v) inulin 2%, yeast extract 0.5%, $(NH_4)_2SO_4$ 0.5% and adjusted to pH 7.0 (Gao et al. 2009), under aseptic conditions. The inoculated flasks were incubated at 37 °C for 24 h with constant shaking (150 rpm) on a rotary incubator shaker (Innova 4337, New Brunswick, USA). Thereafter, spreading of serially diluted cultured broth was carried out on agar plates of the same medium. Agar plates were incubated at 37 °C for 24 h in a BOD incubator (NSW-152, Narang Scientific Works, India). Healthy bacterial colonies were selected for screening of inulinase activity. All bacterial cultures were maintained periodically on nutrient agar slants and stored at 4 °C, until further use.

Screening of inulinase producing bacterial strains
All the bacterial strains were screened based on hydrolytic zone on inulin-based medium and action pattern of enzyme on inulin. Only endoinulolytic bacterial cultures were screened further for enzyme activity.

Primary screening. Primary screening was performed on the basis of Lugol's iodine screening assay (Li et al. 2011). The bacterial isolates were grown at 37 °C for 24 h on agar plates of enrichment medium used for isolation of bacterial strains. The plates were then flooded with Lugol's iodine solution containing potassium iodide (1.5%, w/v) and iodine (1.0%, w/v) for 2 min. Thereafter, the plates were washed three times with sterilized distilled water and further kept open for 30 min. The formation of clear hydrolytic zone was considered positive for inulinase activity and respective bacterial cultures were selected for secondary screening.

Secondary screening. All the bacterial isolates that exhibited considerable hydrolytic zone were subjected to secondary screening. In this step, the action pattern of inulinase was studied by thin layer chromatography (TLC) (Reiffová & Nemcová 2006). Crude enzyme (1.0 mL) was mixed with an equal volume of substrate solution (2% inulin in 0.1 M sodium acetate buffer, pH 5.5) and incubated at 50 °C for 20 h. The enzyme was deactivated by incubating the reaction mixture in boiling water bath for 10 min. The reaction mixture was spotted on pre-coated TLC plates (SD Fine Chemicals Ltd., India) and air dried. In addition to reaction mixture, pure inulin and glucose, fructose and sucrose were also run on the plates as standard for comparative analysis. The spotted TLC plates were kept in the developing solvent containing butanol:ethanol:water (50:30:20, v/v). The plates were air dried and sprayed with aniline diphenylamine reagent (diphenylamine 1%, aniline 1%, phosphoric acid 10%), and thereafter incubated in hot air oven at 120 °C for 15 min. The developed spots of different samples were visually analyzed.

Tertiary screening. Only endoinulolytic bacterial strains were selected for tertiary screening. Tertiary screening was performed on the basis of inulinase activity. The endoinulolytic bacterial isolates were cultured in nutrient broth at 37 °C under shaking (150 rpm) for 12 h. The harvested broth was used as inoculum (5%, v/v) for enzyme production in medium containing (w/v) inulin 2%, yeast extract 2%, $(NH_4)H_2PO_4$ 0.2%, $(NH_4)_2HPO_4$ 0.5%, $MnCl_2.4H_2O$ 0.05%, KCl 0.05%, $MgSO_4.7H_2O$ 0.05%, $FeSO_4.7H_2O$ 0.001%, and adjusted to pH 7.0 (Cho & Yun 2002). Inulinase activity of all the selected bacterial isolates was assayed as described by Mutanda et al. (2008) with slight modifications. Briefly, crude enzyme (100 μL) was mixed with substrate solution (2.0% inulin in 0.1 M sodium acetate buffer, pH 5.5). The reaction mixture was incubated at 50 °C for 15 min. The enzyme was inactivated by incubating the reaction mixture at 100 °C for 10 min. The resultant deactivated hydrolysate was analyzed by dinitrosalicylic acid method (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme that produces one micromole of fructose per min under standard assay conditions.

Morphological, biochemical and physiological characterization of bacterial isolate AS-08
The morphological characteristics, Gram's reaction, spore formation and motility of the bacterial isolate AS-08 were examined by microscopic observations. The isolate was characterized for various biochemical characteristics including esculin hydrolysis, Voges-Proskauer test, citrate utilization, casein hydrolysis, starch hydrolysis, nitrate reduction, arginine dihydrolase, oxidase, DNase and growth in the presence of Tween 40, 60 and 80. The isolate was also investigated for fermentation of different carbohydrates including cellobiose, trehalose, fructose, mannitol, dulcitol, sucrose, dextrose, raffinose, galactose, mannose, inositol and melibiose. Physiological aspects of bacterial isolate AS-08 including growth at a temperature range of 4–55 °C, varying pH 5.0-11.0 and effect of different concentrations of NaCl (0.5–10%, w/v) on the growth were also investigated.

DNA isolation and PCR amplification of 16S rDNA gene sequence
The bacterial isolate was cultured in nutrient broth overnight. Thereafter, cells were harvested and washed twice with sterilized distilled water to remove any traces of nutrient broth. Genomic DNA was isolated using GeneiUltrapure^{TM} bacterial genomic DNA purification kit (Bangalore Genei, India). Isolated DNA preparation was checked by agarose gel electrophoresis (0.8%) for concentration and shearing, if any.

Genomic DNA was used as template for 16S rDNA gene amplification using consensus primers (Bangalore Genei, India). The reaction mixture (50 μL) consisted of 10X Taq buffer A (Bangalore Genei, India) to a final concentration of 1X, dNTP mix (2.5 mM each), forward and reverse oligonucleotide (each 100 ng), Taq DNA polymerase (3 U) and rest part of nuclease free water. The reaction was carried out in a thermal cycler (Master Cycler Personal, Eppendorf AG, Germany). The program consisted of initial denaturation at 94 °C for 5 min subsequently followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s. The final extension was carried out at 72 °C for 10 min. Amplification of

target sequence was confirmed by agarose gel electrophoresis (1.0%) with StepUp^{TM} 500 bp DNA ladder (Bangalore Genei, India).

16s rDNA gene sequencing and phylogenetic analysis

The purified PCR product was sequenced bidirectionally with forward and reverse primers using 16 capillary automated DNA sequencer ABI3100 (Applied Biosystems, USA) at Bangalore Genei, India. The nucleotide sequence obtained was searched for homology in the NCBI nucleotide database using BLAST tool (Altschul et al. 1990). The 16S rRNA gene sequence was aligned against the reference nucleotide sequences retrieved from GenBank (Benson et al. 2013) using Clustal W program (Thompson et al. 1994). The sequence was analyzed to find the nearest homolog of the microbe. The distances were computed on basis of Kimura-2 parameter (Kimura 1980) and a phylogenetic tree was constructed using neighbour-joining method (Saitou & Nei 1987) with the help of MEGA 3.1 software (Kumar et al. 2004) with the bootstrap value of 1,000 replicates.

Inulinase activity of B. safensis AS-08 as a function of time

Inulinase activity of *B. safensis* AS-08 was studied as a function of time at shake-flask level. The seed culture was prepared in nutrient broth at 37℃ under shaking (150 rpm) for 12 h. The seed (5%, v/v) was inoculated in the pre-sterilized production media containing (w/v) inulin 2%, yeast extract 2%, $(NH_4)H_2PO_4$ 0.2%, $(NH_4)_2HPO_4$ 0.5%, $MnCl_2.4H_2O$ 0.05%, KCl 0.05%, $MgSO_4.7H_2O$ 0.05%, $FeSO_4.7H_2O$ 0.001% and adjusted to pH 7.0 (Cho & Yun 2002). The inoculated flasks were incubated under shaking (150 rpm) at 37℃ for 28 h. The samples were withdrawn at regular time intervals to determine inulinase activity and biomass. Inulinase activity was determined as described above under the section Tertiary screening.

Determination of growth

Growth was measured in terms of optical density. Optical density of four times diluted fermentation broth was estimated at 600 nm in a UV-Visible Spectrophotometer (Pharmaspec 1700, Shimadzu, Japan) against the cell free broth as blank.

Nucleotide sequence accession number

The 16S rDNA gene sequence of the strain has been submitted to the GenBank database (Benson et al. 2013) with the accession number JX849661.

Results and discussion

Isolation of inulinase producing bacterial strains

Total 50 bacterial strains were isolated on the basis of their growth (appearance and size of colony) on agar plates containing inulin as sole carbon source. The rapid growth and healthy colonies of these isolates on inulin-based media indicated positive inulinase activity. This has also been reported as the most common technique used for the isolation of inulinase producing microorganisms (Allais et al. 1986).

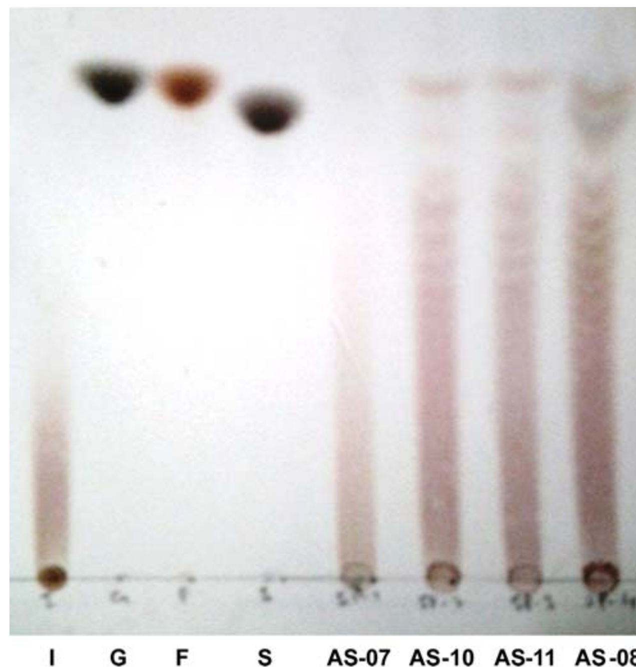

Fig. 1. Thin layer chromatogram of hydrolyzed products formed by hydrolysis of inulin by crude enzyme from *Bacillus safensis* AS-08. Lane I, inulin; G, glucose; F, fructose; S, sucrose. Lanes AS-07, AS-10, AS-11 and AS-08 show reaction products of inulin by crude endoinulinase from bacterial isolates AS-07, AS-10, AS-11 and AS-08, respectively.

Screening of inulinase producing bacterial strains

Primary screening. Amongst the 50 isolates screened for hydrolytic zone on inulin-based medium, bacterial isolate AS-08 and 29 other isolates showed clear hydrolytic zone on agar plates. Plate screening assay is commonly used for detection of extracellular hydrolytic enzymes produced by microorganisms. The existence of inulin-hydrolyzing enzyme on agar plates can be detected by a clear zone surrounding the bacterial colony after incubation. The simple and rapid plate assay used was found very effective and efficient for primary screening of inulin hydrolyzing enzymes. The simple plate assay containing pure inulin allows screening of a large number of inulin-hydrolyzing colonies from environmental samples in a very short time, while dye-labelled substrates require more time for substrate dye binding and are also expensive.

Secondary screening. Out of 30 isolates, only 5 bacterial isolates were found endoinulolytic in nature. Rest of them exhibited exoinulolytic activity. Generally, the individual constituents of the fructooligosaccharides are separated according to their molecular mass. TLC analysis (Fig. 1) of hydrolyzed inulin showed the presence of fructooligosaccharides with varied degree of polymerization. The upper most spots (extreme from the origin) on TLC plate belonged to fructose and sucrose, which are the basic units of fructooligosaccharides and were in a minute quantity. The presence of fructooligosaccharides on the TLC plates confirmed the endoinulolytic nature of the selected bacterial isolates. The endoinulolytic nature of various bacterial strains like *Strep-*

Table 1. Biochemical characteristics of *Bacillus safensis* AS-08.

Test	Result	Carbohydrate	Result
Catalase production	+	Cellobiose	+
Oxidase production	+	Trehalose	+
Voges-Proskauer	−	Fructose	+
Casein hydrolysis	+	Mannitol	−
Citrate utilization	−	Dulcitol	−
Nitrate reduction	−	Sucrose	+
Arginine dihydrolase	−	Dextrose	+
Gelatin hydrolysis	+	Raffinose	−
Starch hydrolysis	−	Galactose	−
Esculin hydrolysis	+	Mannose	+
DNase	−	Inositol	−
		Melibiose	−

tomyces sp. (Laowklom et al. 2012), *Bacillus smithi* T7 (Gao et al. 2009), *Nocardiopsis lucentensis* (Li et al. 2011), *Xanthomonas oryzae* (Cho & Yun 2002) and *Marinimicrobium koreense* (Li et al. 2011) have also been reported.

Tertiary screening. Among the endoinulolytic bacterial cultures, the bacterial isolate AS-08 showed maximal inulinase activity (12.20 U/mL) followed by AS-10 (10.01 U/mL). Other 3 isolates AS-07, AS-09 and AS-11 displayed 9.42, 9.16 and 9.33 U/mL, respectively, of inulinase activity. The inulinase activity of bacterial isolate AS-08 was compared with other endoinulolytic bacterial sources reported in literature. Bacterial isolate AS-08 showed higher inulinase activity than *Pseudomonas* sp. (Park & Yun 2001), *Streptomyces rochei* (Yokata et al. 1995) and *Xanthomonas campestris* (Naidoo et al. 2009). Based on the enzyme activity, the bacterial isolate AS-08 was selected for further biochemical and molecular characterization.

Morphological, biochemical and physiological characterization of bacterial isolate AS-08

The bacterial isolate AS-08 was found Gram positive, motile and sporulating. Colonies were opaque, circular with entire margin and raised elevations. The surface texture was smooth and slimy. It was found to be positive for catalase, oxidase, casein hydrolysis, gelatin hydrolysis and esculin hydrolysis, but showed absence of DNase activity (Table 1). The isolate was found to grow in the presence of Tween 20, while its growth was inhibited when Tween 40, 60 and 80 were supplemented in the medium. Satomi et al. (2006) have also reported inhibition of growth of *Bacillus safensis* by Tween 80. The isolate was found to produce acid from cellobiose, trehalose, fructose, sucrose, dextrose and mannose, while it did not react with mannitol, dulcitol, raffinose, galactose, inositol and melibiose. The morphological, biochemical and physiological characteristics of *Bacillus* sp. are well established. Similar biochemical characteristics of *Bacillus safensis* have been reported earlier (Satomi et al. 2006; Raja & Omine 2012; Sitdhipol et al. 2012) which corroborates our results. The bacterial isolate AS-08 showed growth at pH range of 6.0–9.0 and temperature range of 25–42 ℃. The strain was found to be salt resistant as it grew well in the presence of high concentration of NaCl (0.5–12.0%). Satomi et al. (2006) have also reported growth of *Bacillus safensis* in presence of high concentrations of NaCl (10%, w/v).

On the basis of morphological, biochemical and physiological characteristics, the bacterial isolate AS-08 was determined to be *Bacillus* sp. The characteristics, however, failed to identify the isolate AS-08 up to species level. Therefore, the bacterial isolate AS-08 was further subjected to 16S rDNA sequencing for species specific identification.

Molecular characterization of bacterial isolate AS-08

16S rDNA gene was successfully amplified with consensus primers. Agarose gel electrophoresis revealed amplification of 1,500 bp fragment. Nucleotide sequencing confirmed the size of purified PCR product. The nucleotide sequence was searched for homology against the nucleotide data available on the NCBI GenBank using the BLAST tool. Alignment of nucleotide sequence with retrieved data showed 99% identity with *Bacillus safensis* (GenBank accession No. HQ625386). On the basis of alignment results and homology, the strain *Bacillus* sp. AS-08 was identified as *Bacillus safensis* and named accordingly as *Bacillus safensis* AS-08.

Bacillus safensis has been reported as an important bacterial strain for industrial and scientific studies but there is no report on inulinase activity from this bacterium. *Bacillus safensis* has been reported as a potent bacterial source of alkaline phosphatase (Sitdhipol et al. 2012). Raja & Omine (2012) have reported arsenic, boron and salt resistant strain of *Bacillus safensis*, which can be useful in remediation of salt and metal polluted soils. *Bacillus safensis* has also been shown to incresase the phytoremediation efficiency of nickel polluted soil (Motesharezadeh & Savaghebi-Firoozabadi 2011). Khaneja et al. (2010) reported *Bacillus safensis* as a source of carotenoids. Our studies reveal the industrial importance of *Bacillus safensis* as a source of endoinulinase, which could be used for the production of fructooligosaccharides from inulin.

Phylogenetic analysis

Phyologenetic analysis was based on nucleotide sequence homology. Unrooted phylogenetic tree (Fig. 2) was constructed using the neighbour-joining method. The distances were computed on the basis of Kimura-2 parameters. Based on the nucleotide homology and phyologenetic analysis, *Bacillus pumilus* SH-B30 (FJ549019) was found to be the nearest homolog. Other close homologs included *Bacillus pumilus* HPS1 (JQ308558), bacterium Te68R (AY587832), *Bacillus* sp. 05-4004 (HQ699617) and *Bacillus* sp. HY11 (HM579 802).

Inulinase activity of B. safensis AS-08 as a function of time

Maximal inulinase activity (12.56 U/mL) was observed after 20 h (Fig. 3) of growth at 37 ℃. Increase in enzyme activity as well as biomass was observed up to

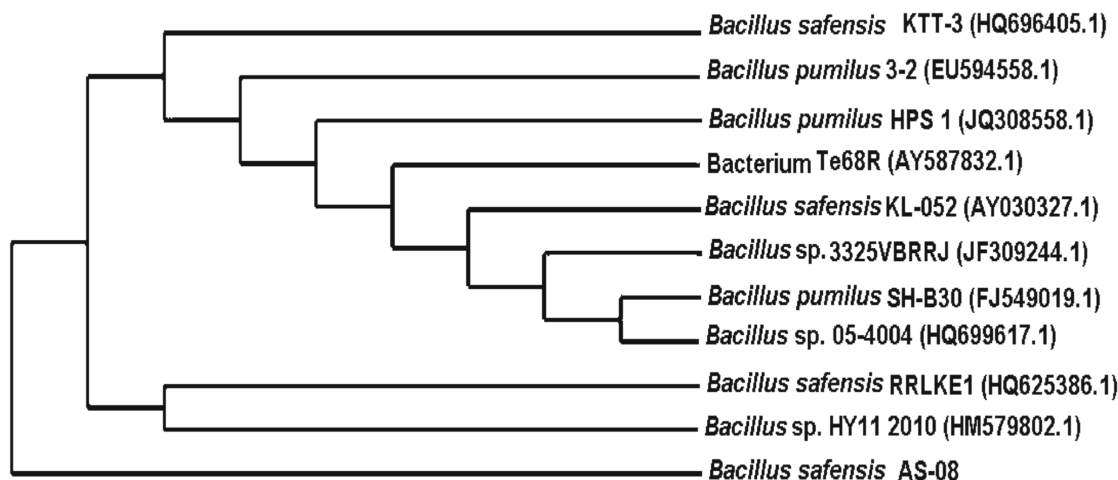

Fig. 2. Phylogenetic tree based on neighbour-joining method showing relationship among the 16S rDNA sequence of the *Bacillus safensis* and other close homologs. The distances were computed on the basis of Kimura-2 parameters. Bootstrap values were based on 1,000 replicates.

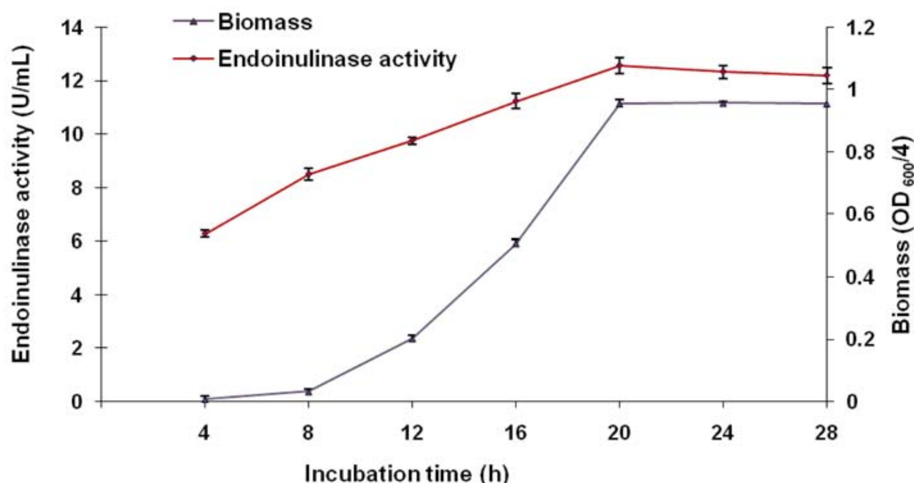

Fig. 3. Endoinulinase activity of *Bacillus safensis* AS-08 as a function of time.

20 h, which shows growth-associated production of inulinase. Growth-associated production of inulinase has also been reported earlier for *Bacillus macerans* and *Xanthomonas oryzae* (Park et al. 2001; Cho & Yun 2002). A little decrease in enzyme activity after 20 h of growth may be due to the secretion of some proteases in the stationary phase of growth, which slightly inhibit the enzyme activity.

Conclusions

Out of 50 isolated bacterial strains, only 5 strains were endoinulolytic in nature. Among these bacterial isolates, the strain AS-08 was found to be the most efficient endoinulinase producer. Morphological, biochemical and physiological characterization identified the isolate AS-08 as a *Bacillus* sp. Further characterization by its 16S rDNA gene sequence identified the isolate up to a species level and the isolate was identified as *Bacillus safensis*. *Bacillus pumilus* was found to be its nearest homolog. The strain exhibited maximum inulinase activity after 20 h of growth. Earlier reports revealed industrial importance of *Bacillus safensis* in area of enzymology, microbial remediation and phytoremediation. This report introduces the new property of *Bacillus safensis* as an endoinulinase producer. This study will help to execute further research focused on the endoinulinase production, purification and characterization of the enzyme. In addition, the purified endoinulinase can be used for the preparation of fructooligosaccharides from inulin.

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