

Original Research Article

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Production of Amylase from *Bacillus thuringiensis* J2 Using Apple Pomace as Substrate in Solid State Fermentation

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ABSTRACT

Keywords

Solid state fermentation, Amylase, Hot water spring, *Bacillus thuringiensis* and Yeast extract.

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Amylase producing thermophilic bacterial strain was isolated from Jeori hot water spring, Rampur Bushahr of Shimla district of Himachal Pradesh, India and identified as *Bacillus thuringiensis* J2 using 16S rRNA gene sequencing and deposited in NCBI gene bank vide accession number [KY990713]. Medium components and process parameters were optimized with respect to substrate concentration, carbon sources, nitrogen sources, incubation period, pH and temperature for an enhanced amylase production. Highest amylase activity of 61.35 IU was obtained in apple pomace as a low cost substrate at a temperature of 45°C, pH 9 and incubation period of 72 h with starch and yeast extract as best carbon and nitrogen sources.

Introduction

Due to the increasing demand for enzymes in various industries, there is enormous interest in research on enzymes suitable for commercial applications and their cost effective production techniques. The microbial production of amylase is more effective than the other sources as the technique is easy, cost effective and consistent which can be modified to obtain enzymes of desired characteristics (Panneerselvam and Elavarasi, 2015). The amylases dominate about 25 per cent of enzyme trade in commercial applications especially for hydrolysis of starch in various

industries. Amylases are classified into three categories based on their mode of action viz., α -amylases, β -amylases and glucoamylases. Among these amylases, α -amylase ranks first in terms of commercial exploitation as α -amylases are active over a broad pH (5.0-9.0) and temperature (35-105°C) (Tiwari *et al.*, 2014). α -amylase (EC 3.2.1.1) is a well-known calcium containing enzyme which catalyzes hydrolyses of starch and related carbohydrates by randomly cleaving internal α -D-(1,4) glycosidic linkages, yielding glucose, maltose and other oligosaccharides (Senthilkumar *et al.*, 2012).

The thermostability and compatibility of enzymes at high temperature is one of the unique features from an industrial point of view (Kovacic *et al.*, 2016). Therefore, isolation of thermophiles has received considerable importance due to their capability in producing thermostable enzyme that are not usually denatured by high temperature and are active at elevated temperature. Advances in the use of microbial amylases in industry have been possible with the isolation of thermophilic microorganisms from ecological niches of earth and subsequent extraction of useful enzymes from them (Kohilu *et al.*, 2001; Haki and Rakshit, 2003). One of the important natural habitats of the thermophilic bacteria is hot water spring which is produced by the emergence of geothermal-heated ground water from the earth crust.

Solid state fermentation (SSF) has been traditionally used for the preparation of food and recently it is gaining importance in the production of microbial enzymes. SSF refers to the growth of microbes on solid substrate without the presence of free flowing water. The solid state fermentation with low cost substrate have numerous advantages like superior productivity, simple technique, low capital investment, low energy requirement, less waste water output and better product recovery (Pandey *et al.*, 1999). Therefore, the present investigation was undertaken for isolation, cheap production, characterization and evaluation of amylase enzyme from hot water spring.

Materials and Methods

Sample collection and isolation of amylase producing bacteria

Water samples were collected in sterilized screw capped tubes from Jeori hot water spring, Rampur Bushahr of Shimla district of

Himachal Pradesh, India and brought to the Microbiology Laboratory, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan for further isolation and characterization work in aseptic conditions.

The water samples were analyzed for physico-chemical characteristics and stored in a refrigerator at 4°C for further processing. Isolation of amylase producing bacteria was done by the standard procedure of Subba Rao (1999).

Amylase assay

Qualitative assay

Starch hydrolysis test was performed by Shaw *et al.*, (1995). The starch agar plates were spot inoculated with the isolated strains and incubated at 45°C for 72 h.

The growth thus obtained was flooded with 2 ml of iodine solution. Bacterial colonies producing clear zones were selected and purified using streak plate technique on the starch medium and refrigerated at 4°C for further studies.

Quantitative assay

Amylase activity was determined by measuring the amount of hydrolyzed starch using the method of Xiao *et al.*, (2006). 0.5 ml of enzyme solution was incubated with 0.2 per cent starch at 37°C for 15 min. 3 ml of DNSA reagent was added to it and the mixture was heated on boiling water bath for 15 min. After cooling down to room temperature, absorbance of reaction mixture was read at 540 nm. The standard curve was prepared by using soluble starch. One International Unit (IU) of amylase activity is defined as the disappearance of an average of 1µmol of iodine binding starch material per minute in the assay reaction.

Morphological and biochemical characterization of *Bacillus thuringiensis* J2

Morphological and biochemical identification of *Bacillus thuringiensis* J2 were performed as the method of Sherman and Cappuccino (2005).

16S rRNA sequencing of *Bacillus thuringiensis* J2

Genomic DNA of *Bacillus thuringiensis* J2 isolate was extracted by conventional method (Sambrook *et al.*, 1989). The purified fragments were sequenced from commercial sequencing facility (Xleris lab). The comparison of 16S rDNA gene sequence was performed via NCBI databases by employing BLAST algorithm. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 6.

Amylase production in solid state fermentation

20 ml of the basal salt medium was dispense into 250 ml flask containing 10 g of different substrates i.e. apple pomace, wood dust and wheat bran and autoclaved at 15 psi pressure for 20 min. The medium was supplemented with 0.5 per cent of yeast extract.

The inoculum was prepared by making a suspension of 24 h old growth of culture on starch nutrient medium slants containing 0.3 per cent starch. The flasks were inoculated with 2 ml of bacterial suspension (O.D 1.0 at 660 nm) and incubated in orbital incubator. At the desired interval the flask were taken out and the content were extracted with 45 ml sterilized buffer. The flask content was

centrifuged at 5300 rpm for 30 min at 4°C. The culture supernatant was used as crude amylase preparation.

Effect of incubation period, pH and temperature on amylase activity

The bacterial isolates using best substrate were grown on medium at different incubation period (24 to 72 h), temperature (25°C to 80°C) and pH (3.0 to 9.0) for maximum amylase production. The optimum incubation period, temperature and pH suitable for the growth were selected on the basis of turbidity caused by the bacterial growth.

Effect of carbon and nitrogen sources on amylase activity

Various carbon sources viz., sucrose, maltose, glucose and starch were used for exploring their effect on amylase production at a concentration of 1 per cent. Similarly, various nitrogen sources viz., yeast extract, urea, casein, NaNO₃ and NH₄Cl at a concentration of 1 per cent were tested for their effects on amylase production.

Statistical analysis

The data recorded for various parameters under laboratory conditions were statistically analyzed as described by Gomez and Gomez (1984).

Results and Discussion

Variation in pH and temperature of water collected from hot spring

The pH of collected water ranged between 4.5 and 5.4 and temperature ranged from a minimum of 45.0°C to a maximum of 75.0°C. The variation in pH and temperature of water collected from different hot spring may be

attributed to the type of microorganism present in them. Our results are in confirmation with Fooladi and Sajjadian (2010) who reported temperature ranged between 46°C to 82°C and the pH ranged from 6.5 to 7.0 of three Iranian hot springs namely, Larijan, Mahallat and Meshkinshahr.

Similarly, Pathak and Rekadwad (2013) also concluded that the physical properties of the water are responsible for the presence of type of organism and characteristics of the organism. They concluded that the water of Mukhya Kund hot spring has exhibited temperature of 61.7°C with pH of 7.0 and that of Surya Kund showed temperature of 48.0°C with pH 7.3.

Isolation and screening of amylase producing bacteria

In total, five isolates (J2, J3, J4, J12 and J32) showed clear zones of starch hydrolysis with varying diameters from the pooled samples of water as depicted in table 1. The zone size of bacterial colonies ranged between 4.70 to 10.30 mm with enzyme index of 12.80 to 30.60. *Bacillus thuringiensis* J2 exhibited maximum zone (10.30 mm) with enzyme index of 30.60 and was selected for further studies.

Fooladi and Sajjadian (2010) screened thermophilic amylase producing bacterial strains from three Iranian hot springs namely, Larijan (67°C, pH 6.5), Mahallat (46°C, pH 7) and Meshkinshahr (82°C, pH 6). They found that Meshkinshahr hot spring was rich in amylase producing bacteria.

Pathak and Rekadwad (2013) isolated twelve amylase producing strains from the hot spring of Mukhya Kund (61.7°C, pH, 7.0) and Surya Kund (48.0°C, pH 7.3) in Unkeshwar district of Maharashtra.

Morphological and biochemical characterization of *Bacillus thuringiensis* J2

On the basis of morphological and biochemical characteristics, the J2 isolate was identified as *Bacillus* sp. as per the criteria of Bergey's Manual of Systematic Bacteriology (Table 2). Similar morphological and biochemical characteristics of *Bacillus* have been reported by Kirti *et al.*, (2016).

16S rRNA sequencing of *Bacillus thuringiensis* J2

The sequence analysis of 16S rRNA of strain *Bacillus thuringiensis* J2 (KY990713) (Fig. 1) showed maximum identity of 99.00 per cent to *Bacillus thuringiensis* (NR112780).

Substrate for amylase production

Apple pomace was found to be best substrate for amylase production in comparison to wood dust and wheat bran. *Bacillus thuringiensis* J2 exhibited highest amylase activity (52.58 IU) with specific activity of 2.18 IU/mg [Fig. 2 (A)]. The peak growth of microorganism coincides with the maximum amylase production. The highest amylase activity in apple pomace may be due to high non-reducing and total sugars which induces the enzyme activity. Use of apple pomaces as substrate for amylase production has been reported by Gama *et al.*, (2015).

Effect of incubation period, pH and temperature on amylase activity

Bacillus thuringiensis J2 exhibited highest amylase activity of 46.96 IU with specific activity of 2.02 IU/mg at 72 h of incubation [Fig. 2 (B)]. At 24 h, a low enzymatic activity was noticed which increased up to 72 h and the activity followed a declining trend thereafter.

Table.1 Characteristics of amylase producing bacterial isolates from hot water spring

Isolates	Zone size (mm)	Enzyme Index
J2	10.30	30.60
J3	5.30	13.90
J4	9.00	12.80
J12	6.38	18.70
J32	4.70	19.40
S.E. _m	0.43	0.07
CD	1.26	0.20

Table.2 Morphological and biochemical characteristics of the Selected amylase producing bacterial isolates

Characteristics	J2
Morphological	
Form	Circular
Elevation	Raised
Margin	Entire
Opacity	Translucent
Color	Purple
Gram's reaction	+
Shape	Rod
Endospore formation	-
Biochemical	
Oxidase test	+
Catalase test	+
Indole test	-
Methyl red test	+
Voges Proskauer test	-
H ₂ S test	+
Simmon citrate test	-
Glucose test	+
Sucrose test	+
Lactose test	+
Urease test	-
H ₂ S test	+

(+) indicates positivity of test; (-) indicates negativity of test

Fig.1 Effect of (A) Substrate, (B) Incubation period (h), (C) pH, (D) Temperature (°C), (E) Carbon sources and (F) Nitrogen sources on amylase production from *Bacillus thuringiensis* J2

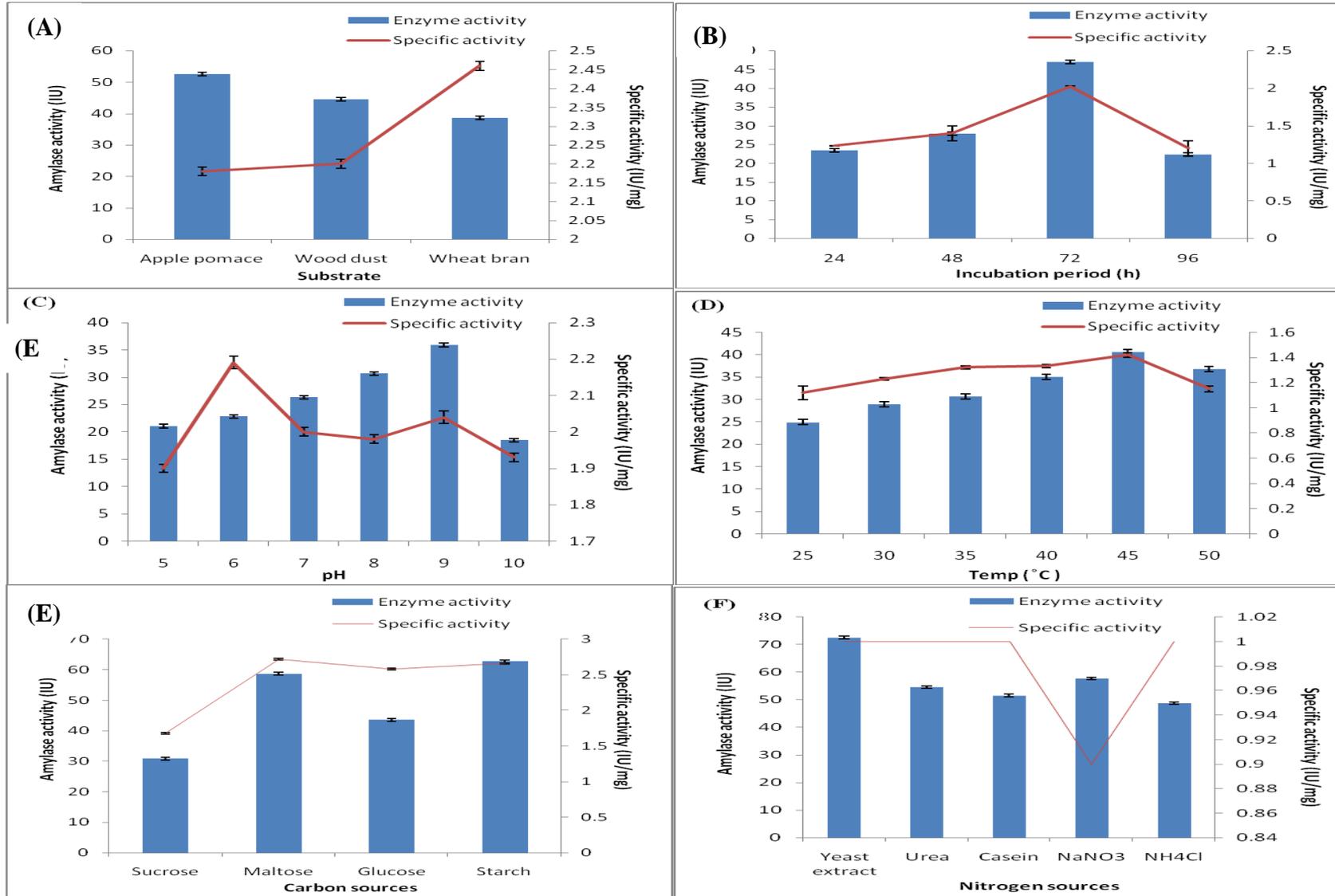
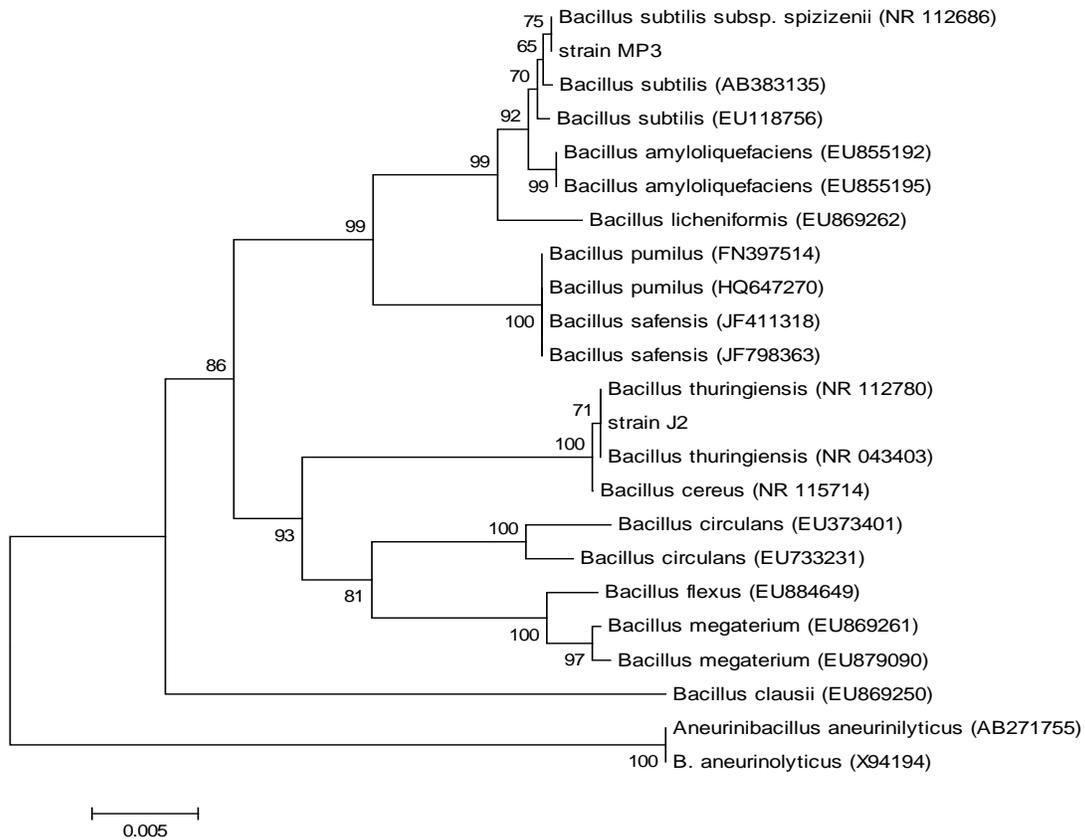


Fig.2 Neighbor joining tree based on 16S rDNA sequences showing the phylogenetic Relationship of *Bacillus thuringiensis* J2 with the analyzed sequences



Maximum production of amylase at 72 h of incubation has also been reported by Vidyalakshmi *et al.*, (2009) and Chauhan *et al.*, (2011). Possible reason for amylase activation after 24 h might be due to release of high levels of intracellular proteases and/or secondary metabolites in the culture medium at the end of exponential phase. After 72 h, there was a decrease in the amylolytic activity, which may be due to the depletion of nutrients, thereby causing a stressed microbial physiology resulting in an inactivation of enzyme (Flores *et al.*, 1997). Another reason could be the catabolite repression i.e. the increase in production of reducing sugars, which after a certain period of growth could exhibit inhibitory effect on enzyme production, since α -amylase is an inducible enzyme (Premila, 2013).

The production of enzyme activity initially increased with increase in pH of medium up to 9.0 and thereafter the activity decreased [Fig. 2 (C)]. *Bacillus thuringiensis* J2 showed highest amylase activity of 35.93 IU at pH 9.0 with specific activity of 2.04 IU/mg.

At lower pH of 5.0, a respective amylase activity of 21.03 IU and 1.90 IU/mg specific activity was recorded. The pH of the medium influences the growth of microorganisms and plays an important role in terms of inducing enzyme production and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannam, 2006). Our results are in agreement with Zaferanloo *et al.*, (2014) who have reported maximum activity of amylase at pH 9.0 by *Preussia minima*.

Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and usually varies from one organism to another (Banerjee and Bhattacharyya, 1992; Kumar and Takagi, 1999). *Bacillus thuringiensis* J2 exhibited amylase activity of 40.72 IU with 1.42 IU/mg of specific activity at an optimum temperature of 45°C [Fig. 2 (D)]. However, with the further increase in temperature, the amylase activity decreased significantly. Maximum enzyme activity at optimum temperature may be due to an increased metabolic activity of the cells resulting in an increased extracellular enzyme production in culture supernatant. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins, thus lowering the enzyme activity. Similar optimum incubation temperature of 45°C for an enhanced amylase production has been reported by Matthias (2013) and Wang (2016).

Effect of carbon and nitrogen sources on amylase activity

Bacillus thuringiensis J2 exhibited highest amylase activity of 62.73 IU [Fig. 2 (E)] and specific activity of 2.66 IU/mg with supplementation of starch among all other carbon sources. The maximum amylase activity with starch was comparable with apple pomace which may be due to the presence of polysaccharides, non-reducing sugars and maltose in the apple pomace. Starch has also been found to increase enzyme production by *Bacillus sonorensis* GV2 and *Bacillus* sp. as reported by Vyas and Sharma (2015) and Khusro *et al.*, (2017), respectively.

Nitrogen is the most important compounds for the growth and metabolism of microorganisms. The nature of these

compounds and the concentration used may stimulate or slow down the production of enzymes (Sharma and Singh, 2012). *Bacillus thuringiensis* J2 exhibited the highest amylase activity of 72.58 IU with specific activity of 1.00 IU/mg [Fig. 2 (F)]. Yeast extract as the best nitrogen source can be attributed to higher content of minerals, vitamins, and coenzymes as reported by several workers (Ashwini *et al.*, 2011; Demirkan *et al.*, 2011; Vijayabhasker *et al.*, 2012). It has also been observed that inorganic sources of nitrogen give better amylase production than the organic sources (Sankaralingam, 2012).

In conclusion, Solid state fermentation carried out with apple pomace served as a cost effective substrate enabling the growth of *Bacillus thuringiensis* J2 from hot water spring. The optimum reaction temperature, pH and incubation period for enzyme activity were 45°C, 9.0 and 72 h, respectively. It could be claimed that significant thermostability of the *Bacillus thuringiensis* J2 makes a good candidate for amylase production for use in the industrial applications which are normally carried out at very high temperatures.

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