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Amylolytic activity and its parametric optimization of an endophytic bacterium *Bacillus subtilis* with an ethno-medicinal origin*

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Abstract: Amylolytic enzymes are a starch hydrolyzing important group of industrial enzymes with a multi-dimensional utility. In the present study, endophytic bacteria were isolated from *Potentilla fulgens* Wall. and screened for their amylolytic activity. Out of total 13 endophytic bacteria isolated, 3 of them showed positive results for amylolytic activity, i.e. production of halo zone against blue background in starch-containing media. The most promising isolate was identified as *Bacillus subtilis* subsp. *inaquosorum* PR-1. After 72 h of incubation, PR-1 exhibited maximum biomass (0.017 mg) and the overall specific growth rate was recorded as 0.0126 h^{-1}. Amylolytic enzyme activity of PR-1 was found to be optimal at 10 mg/mL starch concentration in pH 7 at 50 °C temperature after 30 min of incubation. This indicates that the production of amylolytic enzyme by the representative isolate was growth-dependent as well as preferable for industrial application. The enzyme remained stable in the presence of surfactants, whereas it was inhibited by ethylenediaminetetraacetic acid. Metal ions Ca^{2+}, Na^{+} and Fe^{2+} were found to increase the activity, while Ba^{2+}, Mg^{2+} and Mn^{2+} inhibited the activity. Activity staining for amylolytic enzyme showing one major band in the starch native polyacrylamide gel electrophoresis confirmed its active amylolytic activity with absence of isozymes with a molecular weight of 97.4 kDa. The amylolytic activity of PR-1 was also enumerated against the raw starch of *Dioscorea alata* L. where the highest amylolytic activity (50.46 U/mL) was achieved in 10 mg/mL of raw starch after 2 h of incubation.

Key words: *Potentilla fulgens*; *Bacillus subtilis* subsp. *inaquosorum*; amylolytic enzyme; PAGE; endophytic bacteria; *Dioscorea alata*.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenyl methylsulfonyl fluoride; SDS, sodium dodecyl sulphate.

Introduction

Endophytic bacteria are those, which are present in a plant tissue either intercellular or intracellular without any substantial harm to the host accompanied by some metabolic activities (Zinniel et al. 2002). Endophytic bacteria have been isolated from a range of plant species including various economical crop as well as medicinal plants (Castro et al. 2014). They colonize inside plants, get nutrition and protection from the host plants and, in return, they produce a variety of secondary metabolites and enzymes, which have the potential to hydrolyze several plant-derived macromolecules (Zaferanloo et al. 2013). This interaction also provides the plants to increase their ability to utilize nutrients from soil (Deeb et al. 2013). Of the myriad ecosystems on earth, generally a region with utmost biodiversity seems to have more variety of endophytic bacteria (Strobel et al. 2004), which are relatively unstudied and offer a potential sources of novel natural products with biotechnological potential (Castro et al. 2014).

In general, microorganisms are important sources of enzyme production due to their high production capability with low cost and propensity to genetic manipulations (Dalvi & Anthappan 2007). The enzymes produced by the endophytic microorganisms occupy relatively an unexplored site with elevated demand in industries due to their potentialities and industrial applications (Zaferanloo et al. 2014). Amylolytic enzymes are an important group of industrial enzymes, which can hydrolyze the glycosidic linkages in starch leading to the formation of soluble maltose and glucose (Rasiah & Rehm 2009). They have different application in many fields, such as clinical, medicinal and analytical chemistries (Mishra & Behera 2008). Out of the different source of derivation of an amylolytic enzyme, micro-

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bial sources meet the industrial demand (Pandey et al. 2000) with unique properties with respect to temperature, pH, substrate concentration, utilization of various agents; which persuade the screening of endophytic microbes for the production of these enzymes (Najafi et al. 2005; Liu & Xu 2008).

In starch-processing industries, there is an urgent need to explore amylolytic enzymes having competent ability to hydrolyze raw starch under high concentration (Goyal et al. 2005). Concerning amylolytic enzymes from endophytic microorganisms, endophytic fungi were reported as their efficient producers (Sunitha et al. 2012; Zaferanloo et al. 2014). Different raw starch degrading amylase producing endophytic fungi, such as *Gibberella pulicaris*, *Acremonium* sp., *Synnematous* sp. and *Nodilusporium* sp., were reported by Marlida et al. (2000). But only a few reports are available on amylase production from endophytic bacteria as well as its raw starch utilizing ability (Pimentel et al. 2011). This entreaty has driven the exploitation of endophytic bacteria as enzyme sources for promising industrial applications in agriculture, medicine and food industry.

Meghalaya, which is a part of the Indo-Burma biodiversity hotspot, is the house of several herbal plants, which have an immense scope for ethno-botanical studies (Hynniewta & Kumar 2008). *Potentilla fulgens* Wall., belonging to the family Rosaceae, is a medicinal plant, which is consumed by the indigenous peoples of Meghalaya to get rid of various ailments (Roy et al. 2010). But amylolytic potential of endophytic bacteria harboured in this plant has not been explored so far. Understanding the importance of amylolytic enzyme activity of endophytic bacteria, the present investigation dealt with isolation of endophytic bacteria from *Potentilla fulgens* Wall., their amylolytic activity and raw starch degradation efficiency, which have an immense industrial momentousness.

Material and methods

Sample collection and isolation of endophytic bacteria

Twenty healthy *Potentilla fulgens* Wall. plants were collected from natural forest covering the East-Khasi Hills and Jaintia Hills districts of Meghalaya, India. Taxonomic identity of the plant was confirmed with the help of Herbarium Curator of the parent University. The plants were placed in sterile polyethylene bags, which were brought to the laboratory in an ice box and processed within 24 h after collection. Root, stem and leaf of the plants were separated, washed thoroughly under running tap water to remove adhering soil particles and the microbes. Root, stem and leaf of the plants were surface sterilized according to Fisher et al. (1992) with some minor modifications. The plant parts were sterilized by sequential immersion in 10 mL of 70% (v/v) alcohol for 5 min and 10 mL of sodium hypochlorite solution (0.9%, w/v, available chlorine) for 20 min followed by washed in sterile distilled water for 3–4 times to remove surface sterilization agents.

To validate the effectiveness of surface sterilization, the surface sterilized samples were washed in sterile distilled water (thrice), then soaked in 5 mL sterile water and this obtained sterilized sample (plant part) – water mixtures were stirred for 1 min. An aliquot of 0.5 mL water obtained above (after removing the plant parts) was then inoculated on nutrient agar medium and the plates were examined for bacterial growth after incubation at 30 °C for 48 h. Samples, that were not detected as contaminated by cultivable microorganisms, were considered as successfully surface disinfected and used for isolation of endophytic bacteria (Schulz et al. 1993).

Each surface-sterilized samples were aseptically sectioned horizontally into 0.5 cm (diameter) for root and stem and 1.0 cm × 2.0 cm rectangular pieces for leaf and plated on nutrient agar plates (2 sections per plate) supplemented with 50 µg/mL cycloheximide to inhibit the growth of fungus followed by incubation at 30 °C for 7 days. These plates were observed regularly for the emergence of bacterial colonies. All morphologically different isolated bacterial colonies were repeatedly sub-cultured for purification and purified bacterial cultures were maintained at 15% (v/v) glycerol suspensions at –80 °C for long-term preservation.

Microscopic elucidation of abundant endophytic bacteria colonizing the tissue of P. fulgens

Surface-sterilized thin, free-hand-cut tissue sections prepared employing a fine razor blade and inoculated on nutrient agar medium for 24 h followed by staining with Trypan Blue were examined after mounting on acetone-washed autoclaved microscope slides under phase contrast microscope (Thomas & Reddy 2013).

Screening of endophytic bacteria for amylolytic enzyme production

Preliminary screening of the obtained isolates for amylolytic enzyme production was performed by streaking the isolates on starch agar plates supplemented with (%, w/v): 0.05 g peptone, 0.01 g KCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.01 g $(NH_4)_2SO_4$, 0.01 g NaH_2PO_4, and 1 g starch, in triplicate, followed by incubation at 30 °C for 24 h. Production of the enzyme was detected by flooding the plates with Gram's iodine reagent (0.01 M I_2/KI solution) (Srivastava & Baruah 1986).

Enzyme assay and protein concentration

The strain marked as PR-1 showing the largest halo zone in starch agar plate was selected and incubated in starch-beef extract broth (1.0 g starch, 1.0 g beef extract, 0.2 g yeast extract, 0.01% $MgSO_4$, pH 7) (Kar & Ray 2008) at 30 °C with continuous agitation in orbital shaker at 120 rpm. After every 24 h of incubation the culture was centrifuged at 12,000 rpm for 5 min at 4 °C and cell-free supernatant was used for amylase assay. The reaction mixture containing 1 mL of 1% soluble starch, 0.1 mL of 0.1 M phosphate buffer (pH 7) with 1 mL crude enzyme was incubated at 30 °C for 30 min for enzyme-substrate reaction, which influence the release of reducing sugar. To stop the enzyme-substrate reaction, 0.25 mL of 0.1 M HCl was used and the amount of reducing sugar produced was calculated by dinitrosalicylic acid method (Miller 1959) at 540 nm against blank. All the experiments were conducted in triplicate and mean of the three was represented as the units of enzyme produced per mL of substrate. One unit of amylase activity was defined as the amount of enzyme that liberates 1 µmol of glucose per min from starch under standard assay condition (Mishra & Behera 2008). The percentage of relative values of amylase activity in respect to control was determined using the following formula: relative activity (%) = (obtained amount of sugar concentration / added substrate concentration) × 100; where 1 mL enzyme filtrate with 1 mL of

1% soluble starch, 0.1 mL of 0.1 M phosphate buffer (pH 7) with incubation at 30 °C for 30 min for enzyme-substrate reaction served as control.

The total soluble protein concentration was also determined with bovine serum albumin as a standard (Lowry et al. 1951). The specific activity of the enzyme was calculated by dividing the enzyme activity to protein content to determine the purity and quality of the enzyme (Ram et al. 1986).

Effect of temperature, pH and incubation time on amylolytic enzyme activity

The effect of temperature on amylolytic enzyme was determined by incubating the reaction mixture (1 mL of 1% soluble starch, 1 mL crude enzyme and 0.1 mL of 0.1 M phosphate buffer, pH 7) at various temperature ranges from 10 °C to 90 °C in 20 °C increments for 30 min and the amount of reducing sugar was calculated at each temperature. The optimum pH of amylolytic activity was determined by incubating the reaction mixture at different pH ranging from 4 to 10 at 30 °C for 30 min of incubation. Since every buffer has different pH range, hence pH 4–5 was maintained with 0.1 M acetate buffer, pH 6–8 with 0.1 M potassium phosphate buffer and pH 9–10 with 0.1 M glycine–NaOH buffer. The effect of incubation time on enzyme production was monitored by incubating the reaction mixture under assay condition (1 mL of 1% soluble starch, 1 mL crude enzyme and 0.1 mL of 0.1 M phosphate buffer, pH 7) for the range of 10 min to 110 min.

Substrate specificity

The K_m and V_{max} values as important factors determining the specificity of the enzyme to the substrate were determined by varying the substrate (soluble starch) concentration from 1 to 20 mg/mL using a double reciprocal Lineweaver-Burk plot.

Effect of different chemical reagents, metal ions and organic solvents on amylolytic enzyme activity

Influence of various denaturing and metal chelating agents such as sodium dodecyl sulphate (SDS), Triton X-100, Tween-80, β-mercaptoethanol, hydrogen peroxide, ethylenediaminetetraacetic acid (EDTA) and phenyl methylsulfonyl fluoride (PMSF) (1 mM and 5 mM concentration each) on amylolytic enzyme activity was determined by pre-incubation with enzyme at 30 °C for 1 h followed by standard amylase assay. Similarly various heavy metal ions were added as chloride salts at 1 mM and 5 mM concentrations to carry out for the amylase assay. The effect of various organic solvents on the enzyme activity was carried out by the addition of 10% and 20% of polar solvents, such as acetone, ethanol, methanol, butanol and isoamyl alcohol by incubating for 1 h at 30 °C followed by standard assay.

Polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE profiling of amylolytic enzyme

Amylolytic enzyme activity pattern of the strain PR-1 was detected by starch-native-PAGE (7%). After running the gel, it was soaked in a 50 mM phosphate buffer for 30 min, and after that immediately transferred to 1% (w/v) soluble starch solution for 30 min. Then, the gel was stained with an acidic iodine solution (0.2% I_2 and 2% KI in 0.2 M HCl) at 37 °C till the development of a clear and transparent zone in the brown background indicating the location of amylolytic enzyme activity (Whitehead & Cotta 1995). For resolving the molecular weight of the amylolytic enzyme band, starch-SDS-PAGE (7%) was carried out with medium range molecular markers (Merck Millipore, India). After running the samples, the slice containing markers was cut and stained with 0.1% coomassie brilliant blue and rest of the gel was gently shaken in 2.5% Triton X-100 at 4 °C for 1 h to remove SDS and reinstate the enzymatic activity (Martinez et al. 2000) followed by staining with acidic iodine solution. By comparing both the gel pieces, molecular weight of the amylolytic enzyme band was calculated depending on relative mobility of the protein with the molecular weight markers (Whitehead & Cotta 1995).

Growth kinetics

The growth of PR-1 was measured in terms of cell biomass. For biomass study, PR-1 was grown in 100 mL of starch-beef extract broth at 30 °C in orbital shaker at 120 rpm followed by collection of 1 mL sample during each interval of 24 h and was centrifuged at 8,000 rpm for 10 min at 4 °C. The supernatant was decanted from the tubes, air dried for overnight and weighed to determine the biomass. The specific growth rate (μ) of the bacteria was calculated using the formula: $\mu = [3.322 \times \log (Mt_2/Mt_1)]/dt$, where Mt_2 and Mt_1 is biomass at t_2 and t_1 time point, respectively (Singh & Singh 2011).

Degradation efficiency of amylolytic enzyme of PR-1 on raw starch

For the extraction of starch from the tubers of *Dioscorea alata* L., the freshly harvested tubers (100.0 g) were peeled and homogenized with 1 M NaCl (900.0 mL) solution using a commercial blender. The mixture was filtered through triple layered cheesecloth and the granules of starch were allowed to settle and water was discarded followed by centrifugation at 3,000 $\times$ *g* for 10 min. Starch was allowed to air dry overnight at room temperature (Riley et al. 2006). For amylase assay, the reaction mixture contained 1 mL of 5–30 mg/mL raw starch of *Dioscorea alata* L., 0.1 mL of 0.1 M phosphate buffer (pH 7) with 1 mL crude enzyme. The mixture was incubated at 30 °C for 2 h and 8 h, respectively, for enzyme-substrate reaction and the release of reducing sugar was calculated by using dinitrosalicylic acid method (Miller 1959) at 540 nm against blank.

Morphological and biochemical characterization of PR-1

Morphological criteria including Gram-staining, acid-fast staining and spore staining were recorded using binocular optical microscope (Dialux 20, Leitz, Germany). Biochemical characterization of PR-1 was also performed following the standard protocol (Holt et al. 1994). PR-1 was mounted on stubs, splutter-coated with gold, and viewed on the scanning electron microscope (JEOL Ltd., Japan JSM-6360) at an accelerating voltage of 20 kV for viewing the morphology of PR-1. The photomicrographs were recorded on Kodak film (New York, USA).

DNA isolation, 16S rRNA gene amplification and sequencing

PR-1 was grown in Luria-Bertani broth at 30 °C for 24 h in an orbital shaking incubator. Total genomic DNA was isolated by using standard procedure (Sambrook et al. 1989). 16S rRNA gene was amplified with universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') on GeneAmp PCR system 9700 (Applied Biosystems, USA) (Song et al. 2011). The PCR reaction mixture consisted of 5 µL 10X buffer (with Mg^{2+}), 8 µL dNTP mixture (1.25 mM each), 0.5 µL of each primer, 1 µL of template DNA and

1.0 µL of *Taq* polymerase (Merck Millipore, India) in a final volume of 50 µL. PCR amplification parameters were as follows: 94 °C for 3 min of initial melting; 30 cycles of 94 °C, 45 s, 55.5 °C, 30 s, and 72 °C, 90 s, and a final extension at 72 °C for 7 min (Bhattacharjee et al. 2012). PCR products were visualized on 1% agarose gels and the products were purified with QIAquick Gel extaction kit (Qiagen, Germany) by following the manufacturer's instructions and sequenced with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA).

16S rRNA gene sequences of the isolated strain and its closely relative species were retrieved from EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) and aligned using ClustalX2 programme (Larkin et al. 2007). The tree topologies were evaluated by bootstrap analyses based on 1,000 replications with MEGA 6.0 (Tamura et al. 2013) and consensus phylogenetic tree was inferred using neighbour-joining and maximum likelihood methods considering *Escherichia coli* as outgroup with Kimura's 2 parameter model. Mr. Bayes v3.1.2 (Ronquist & Huelsenbeck 2003) was used for Bayesian phylogenetic reconstructions adopting the general time reversible model with γ distributed rates and invariant sites (GTR + I + G) of nucleotide substitution and run until the mean standard deviation of split frequencies was below 0.01. A consensus tree was constructed following a visually determined burn-in of 25% (Bhattacharjee et al. 2012).

Statistical analysis

All experiments in the present study were performed in triplicates ($n = 3$) and the results were expressed in mean of the three (average value ± SD). The test data were analyzed with the Student's t test (paired) and paired Z test for the significance of differences between the two groups. Statistical analyses for comparison of variances in experimental data were performed using Levene's test and Bartlett's homogeneity of variances test. All the tests were conducted at the significance level of 0.05. The Microsoft Office Excel 2003 (Microsoft Corp., USA), XLStat v7.5.2 (Addinsoft, USA) and OriginPro 8 (OriginLab Corporation, USA) were used for all statistical analysis.

Results

Isolation and identification of endophytic bacteria

To evaluate the population of endophytic bacteria in *Potentilla fulgen* Wall. (Fig. S1), a total of thirteen endophytic bacteria were isolated from surface-sterilized root, stem and leaf of the plant (Fig. S2). The highest number of endophytic bacteria was isolated from root ($n = 7$) followed by leaf ($n = 4$) and stem ($n = 2$). The presence of endophytic bacteria in the host cells under phase contrast microscope was also characterized by their active motility and wriggling movement. Live bacterial cells exclude trypan blue and remain clear and translucent in the microscopic field. In contrast, the dye penetrates membranes of dead cells and stains them a dark blue colour (Fig. S3).

On screening of all the isolates for amylolytic enzyme activity it was observed that out of thirteen isolates only three of them were positive for amylolytic enzyme activity having halo zone against blue background. The best isolate PR-1 showing the largest halo zone was identified and characterized (Table 1). Based on microscopic observation, the PR-1 strain was revealed as rod-shaped, Gram-positive with distinct biochemical characteristics (Fig. 1, Table 2). It was identified up to species level using the 16S rRNA based on phylogenetic analysis. A band of ∼1,500 bp was obtained from PCR-amplified product of total genomic DNA of PR-1. PR-1 belongs to the genus *Bacillus* was supported by the tested treeing algorithms showing a high bootstrap value in the neighbour-joining analysis (Fig. 2) and also higher degree of taxon separation in Bayesian phylogeny (Fig. S4). The isolate PR-1 was identified as *Bacillus subtilis* subsp. *inaquosorum* PR-1 and its sequence was submitted to NCBI GenBank (Benson et al. 2013) under the accession number JX566818.

Table 1. Zone of amylolytic enzyme activity of endophytic bacteria on starch agar plate.[a]

Isolate	Zone Y	Colony X	Amylolytic activity
PS-2	2.8	0.3	2.5
PR-1	10.0	4.0	6.0
PR-3	2.4	0.2	2.2

[a] Zone Y – diameter of the zone (Y, in mm); colony X – diameter of the colony (X, in mm); amylolytic activity – zone of amylolytic enzyme activity (Y–X, in mm).

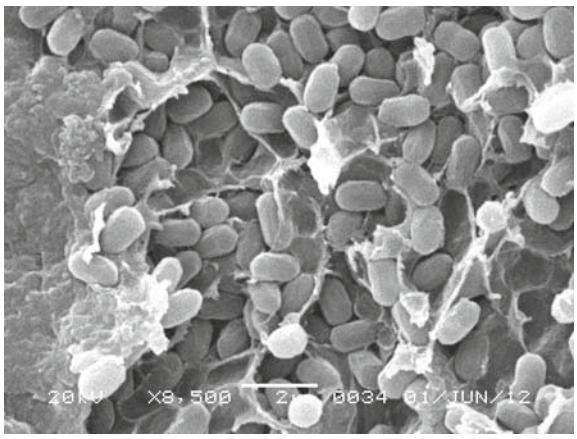

Fig. 1. Scanning electron micrograph showing morphology of *Bacillus subtilis* subsp. *inaquosorum* PR-1.

Growth kinetics

The total biomass of *B. subtilis* subsp. *inaquosorum* PR-1 was found to be 0.0013 mg after 24 h of growth followed by highest biomass after 72 h of growth (0.017 mg) and then declined progressively (Fig. 3). The overall specific growth rate of *B. subtilis* subsp. *inaquosorum* PR-1 was recorded as 0.0126 per h using the formula concerning specific growth rate (μ).

Quantification of amylolytic enzyme activity

Amylolytic enzyme production of *B. subtilis* subsp. *inaquosorum* PR-1 gradually initiated at 24 h of growth. Maximum amylolytic activity was observed at 72 h of growth (50.46 U/mL) and it remains stable after

Table 2. Comparison of morphological and biochemical characteristics of the selected endophytic isolate (PR-1) with the closest strain (KCTC 13429).[a]

Characteristics	PR-1	KCTC 13429
Shape	Rod	Rod
Motility test	+	+
Sporulation	+, centrally present	+, centrally or paracentrally present
Pigmentation	Brownish black or none[b]	None
Gram's stain	+	+
Indole	–	–
Citrate test	+	+
Methyl red	+	+
Voges-Proskauer	+	+
Citrate utilization	+	+
Urease test	–	–
Oxidase test	+	+
Catalase test	+	+
Phosphatase test	+	+
Nitrate production test	+	+
Growth at		
40 °C	+	+
60 °C	–	–
3% NaCl	+	+
5% NaCl	+	+
9% NaCl	+	+
Degradation		
Starch	+	+
Tween-80	–	ND
Urea	–	ND
Aesculin	+	+
Casein	+	+
Elastin	–	ND
Acid from		
Glycogen	+	+
Glucose	+	+
Melibiose	+	+
Mannitol	+	+
Sorbitol	+	+
Antibiotics resistance		
Kanamycin	–	ND
Penillin G	–	ND
Ampicillin	–	ND

[a] PR-1 and KCTC 13429 mean *Bacillus subtilis* subsp. *inaquosorum* PR-1 and *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429, respectively; "+" and "–" indicate positive and negative, respectively; ND – not determined.
[b] Brownish black on glucose-containing media, none on tyrosine-containing media.

96 h. By comparing the amylolytic activity of *B. subtilis* subsp. *inaquosorum* PR-1 with its growth kinetics, it was observed that growth and amylolytic enzyme production patterns were closely associated (Fig. 3). The protein content of PR-1 after 72 h of incubation was 1.73 mg/mL with a specific activity of amylolytic enzyme 29.16 U/mg, which in turn represents the purity of the enzyme.

Effect of temperature, pH and incubation time

In experimental data, significant differences were noted between the different factors (temperature, pH and incubation time) with enzyme activity at significance level of 0.05. Amylolytic enzyme activity of *B. subtilis* subsp. *inaquosorum* PR-1 was tested over the range of 10–90 °C in 20 °C increments. The activity remains stable from 30 °C to 50 °C with the maximum activity being recorded at 50 °C (58.03 U/mL) after 30 min of incubation; thereafter it declined subsequently ($p < 0.05$) (Fig. 4a). The effect of different pH on activity of amylolytic enzyme was detected over a wide range of pH levels ranging from 4 to 10 and amylolytic enzyme activity showed an increasing trend in the pH range (pH 4–7) reaching maximum at pH 7; after that it decreased gradually ($p < 0.0001$) (Fig. 4b). By incubating the amylolytic enzyme at 30 °C in pH 7 for the range of 30–110 min concluded that the enzyme exhibited maximum activity in the range 30–70 min of incubation ($p < 0.05$) (Fig. 4c). Results from the analysis of comparison of variances using Levene ($F = 5.231$, $p < 0.001$) and Bartlett ($\chi^2 = 26.941$, $p < 0.0001$) tests infers the significant inequality of variances in enzyme factors.

Enzyme kinetics

By observing the effects of various substrate concentrations (1–20 mg/mL) on amylolytic enzyme activity, it was found that enzyme worked best in between 5–10 mg/mL of soluble starch concentration with optimum at 10 mg/mL; further increase in starch concentration did not influence the amylase production (Fig. 5). The K_m and V_{max} values were calculated as

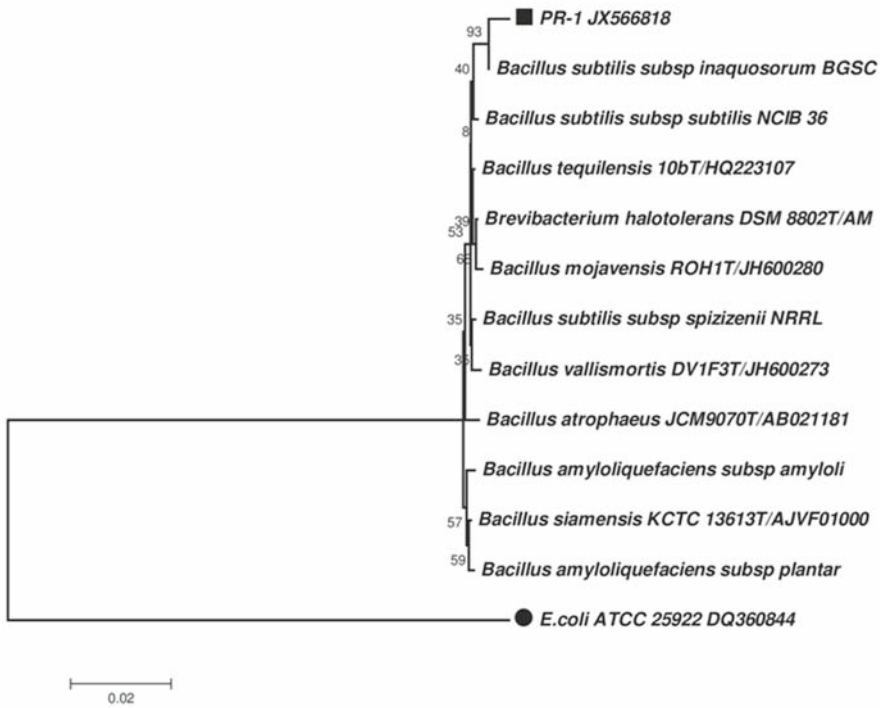

Fig. 2. Neighbour-joining method analysis of 16S rRNA gene sequences of selected strains and its closest phylogenetic neighbours considering *Escherichia coli* as an outgroup. Bootstrap values, expressed as percentage of 1,000 replications, are indicated at nodes. Bar means 2% sequence divergence. Filled square box and filled circle represent the studied isolate and the outgroup, respectively.

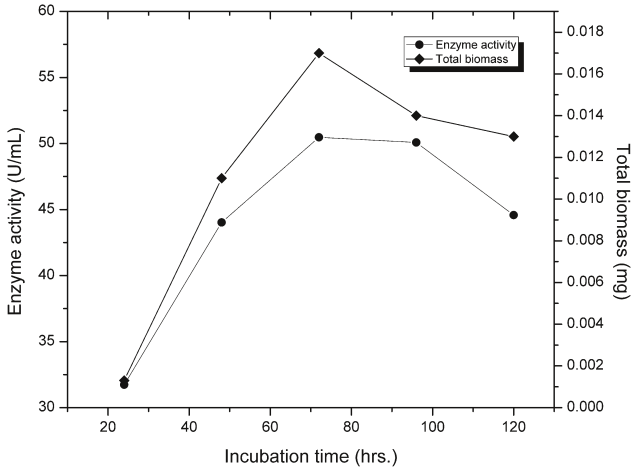

Fig. 3. Amylolytic enzyme activity pattern of the isolate and its relationship with cell biomass at respective hours of incubation.

2.7 mg/mL and 40.74 U/mL using a double reciprocal Lineweaver-Burk plot.

Effect of different chemical reagents, metal ions and organic solvents on amylolytic enzyme activity
Various agents documented that the amylolytic enzyme activity remained almost stable with SDS, Tween-80 and Triton X-100, but it was inhibited by EDTA, PMSF, H_2O_2 and β-mercaptoethanol up to 30–40% in comparison with control (Table 3). Note that the control was the reaction mixture containing 1 mL of 1% soluble starch, 0.1 mL of 0.1 M phosphate buffer (pH 7) with 1 mL crude enzyme, which was incubated at 30 ℃ for 30 min for enzyme-substrate reaction. The action of metal ions on amylolytic enzyme activity varied. One mM Ca^{2+}, Na^{+}, Fe^{2+} increase the activity by 64%, 12% and 36%, respectively, however 5 mM Ca^{2+}, Na^{+} and Fe^{2+} increase the activity by 58%, 5% and 27%, respectively, in respect to control. The remaining metal ions, such as Ba^{2+}, Mg^{2+} and Mn^{2+} inhibited the amylolytic enzyme activity by 14–48%. The impact of organic solvents on amylolytic enzyme activity was also studied and showed an inhibition of the activity up to 35–38% (Table 3).

PAGE and SDS-PAGE profiling of amylolytic enzyme
Presence of a single band in activity staining of starch-native-PAGE confirmed the secretion of active amylolytic enzyme production. The molecular weight of the band was estimated 97.4 kDa in starch-SDS-PAGE (Fig. 6).

Raw starch degradation efficiency of amylolytic enzyme
The potential of raw starch degrading activity of amylolytic enzyme from *B. subtilis* subsp. *inaquosorum* PR-1 was increased from 5 to 10 mg/mL of raw starch where maximum was obtained with 10 mg/mL after 2 h of incubation (50.46 U/mL). This subsequently declined with increasing concentration of raw starch. Similar tendency of degradation efficiency was also observed with 8 h of incubation where maximum activity was observed with 10 mg/mL raw starch (49.94 U/mL). The results demonstrated that the maximum hydrolysis occurs during 2 h of incubation at 10 mg/mL concentration of raw starch from *Dioscorea alata* L. (Fig. 7).

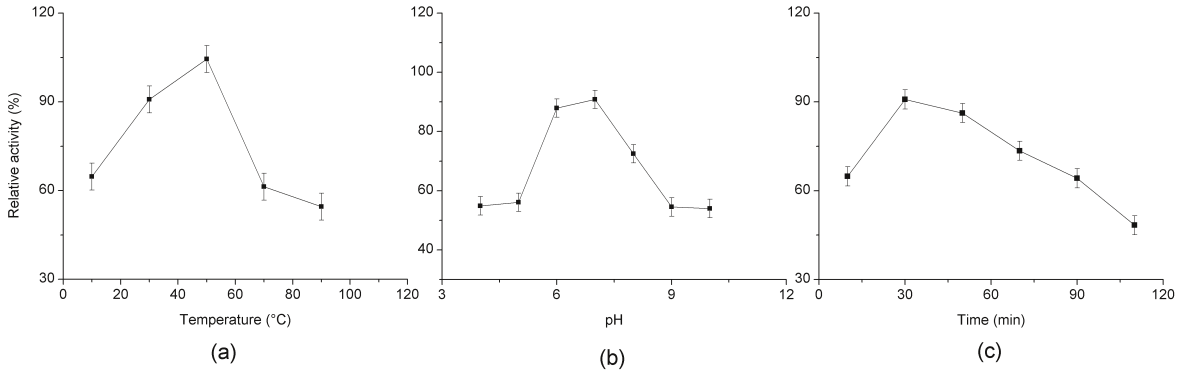

Fig. 4. Amylolytic enzyme activity pattern of the isolate after 72 h of incubation at different incubation temperature (a), pH (b) and incubation time (c).

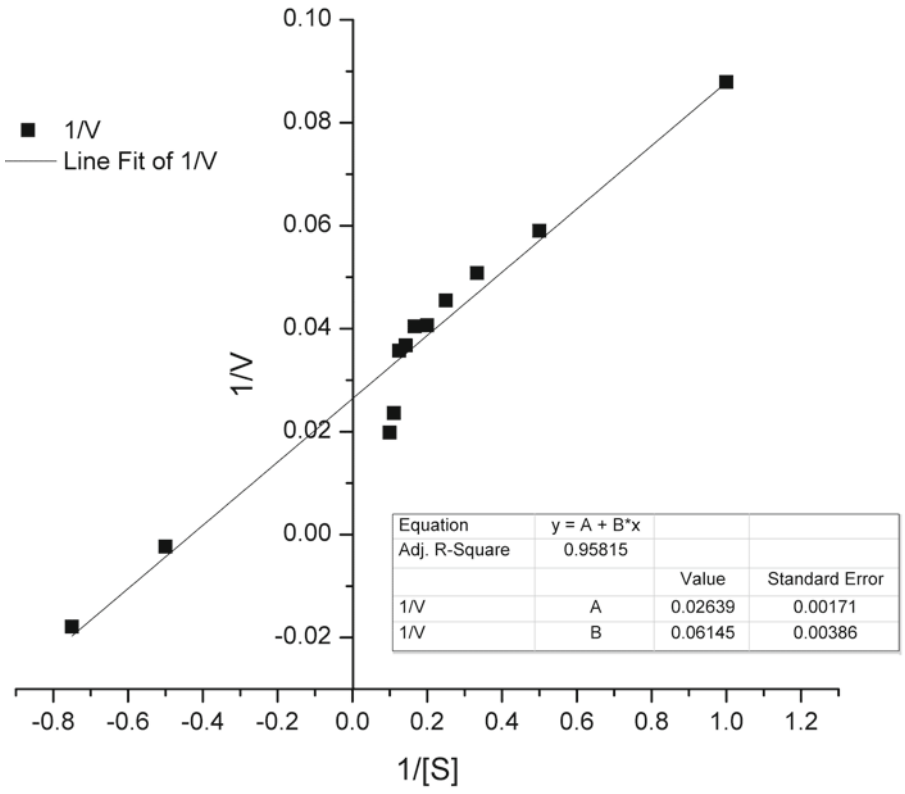

Fig. 5. Effect of substrate concentration on enzyme activity showing with double reciprocal Lineweaver-Burk plot.

Discussion

Endophytic bacterial communities interact with plants and obtain nutrients without any harm to the host. Due to their capability of producing secondary metabolites, endophytic bacteria mainly belonging to the genera *Pseudomonas*, *Burkholderia* and *Bacillus* are important for biotechnological studies (Lodewyckx et al. 2002). Previously, endophytic bacteria were isolated from different medicinal plants including *Gynura procumbens*, *Azadirachta indica*, *Curcuma longa*, *Eucalyptus globules*, *Musa paradiasica. Zingiber officinale*, *Strobilanthes crispa*, *Withania somnifera*, *Tridax procumbens*, *Aloe vera* and *Lantana camara* (Bhore et al. 2010; Ingle 2011; Jalgaonwala & Mahajan 2011; Bhore and Tiong 2012; Janardhan & Vijayan 2012; Preveena & Bhore 2013; Jasim et al. 2014; Prakash et al. 2014). The enzymes from microbial sources are considered more important in agriculture, industry and human health as they are more stable than the enzymes derived from plants and animals sources (Jalgaonwala & Mahajan 2011). Among bacterial community, *Bacillus subtilis*, *Bacillus amyloliquifaciens*, *Bacillus cladolyticus*, *Pseudoalteromonas* sp. were known as an efficient degrader of starch by producing amylolytic enzymes (Thippeswamy et al. 2006; Tao et al. 2008).

The present research work was undertaken to explore amylolytic enzyme from a bacterium of endophytic origin, *B. subtilis* subsp. *inaquosorum* PR-1 from *Potentilla fulgen*, and to optimize its culture conditions with raw starch digesting ability. This may lead to using the enzyme as an additive in starch-processing indus-

Table 3. Influence of metal ions, surfactants, oxidizing agents, reducing agents and organic solvents on amylolytic enzyme activity.

Agents	Relative activity (%)
Control	90.83
1 mM Na^+	103.02
5 mM Na^+	96.36
1 mM Ba^{2+}	55.80
5 mM Ba^{2+}	41.00
1 mM Ca^{2+}	154.62
5 mM Ca^{2+}	148.56
1 mM Mg^{2+}	60.70
5 mM Mg^{2+}	45.85
1 mM Mn^{2+}	65.77
5 mM Mn^{2+}	41.97
1 mM Fe^{2+}	127.62
5 mM Fe^{2+}	117.80
SDS	86.99
Triton X-100	88.15
Tween-80	82.69
β-Mercaptoethanol	57.24
H_2O_2	51.25
1 mM EDTA	53.52
5 mM EDTA	51.76
1 mM PMSF	53.16
5 mM PMSF	52.98
10% Acetone	53.06
20% Acetone	51.93
10% Butanol	53.79
20% Butanol	53.24
10% Ethanol	53.74
20% Ethanol	54.03
10% Isoamyl alcohol	53.03
20% Isoamyl alcohol	55.65
10% Methanol	53.38
20% Methanol	54.97

try. The occurrence of the highest number of endophytic bacteria was observed in the case of root of *Potentilla fulgen*, which can be supported by the previous study (Jin et al. 2014).

Generally, an amylase production is affected by cellular growth of bacteria (Riaz et al. 2008). Most of bacteria produce maximum amylase during their logarithmic or the exponential phase of growth with a restriction in the stationary phase (Davis et al. 1980). The results recorded in the present study showed conformity with the amylase production by *Nocardiopsis* sp. of yam bean (Stamford et al. 2001). On the other hand *Bacillus coagulans* was reported to produce a maximum thermostable amylase after 48 h up to 72 h of incubation (Babu & Satyanarayana 1993) and *Bacillus* sp. isolated from industrial waste was reported to require 80 h for optimum amylase production (Thippeswamy et al. 2006). The relation between growth of bacteria and its amylase activity suggests that the cellular development is associated with amylase production (Cordeiro et al. 2002). Again, the amylolytic enzyme activity of *B. subtilis* subsp. *inaquosorum* PR-1 was found to be superior than those of *Nocardiopsis* sp. – an endophytic actinomycete isolated from yam bean (Stamford et al. 2001), and *Streptosporangium* sp. – an endophyte of maize leaves (Stamford et al. 2002).

Changing of temperature, pH, substrate concentration are some of important factors for the performance

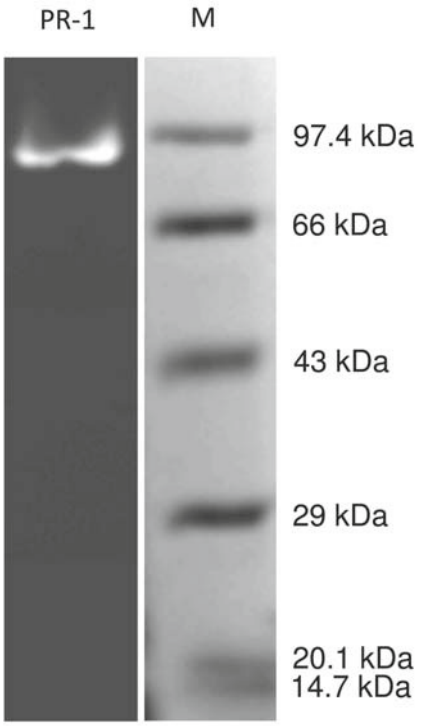

Fig. 6. Electrophoretic profiling of amylolytic enzyme of the isolate in 7% starch-SDS-PAGE. Left lane: amylase; right lane: protein molecular weight markers.

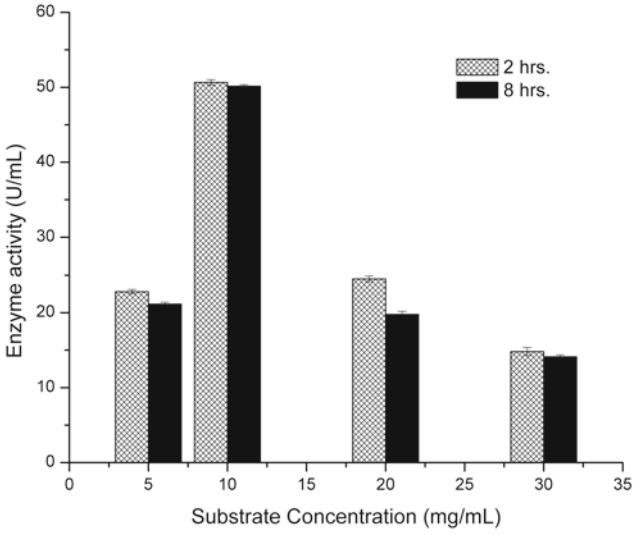

Fig. 7. Amylolytic enzyme activity pattern of the isolate after 2 h and 8 h of incubation at different concentration of raw starch of *Dioscorea alata* L.

of amylase activity (Nielsen et al. 2001). Optimum amylase activity at 50 ℃ or above is a characteristic feature of moderately thermostable microorganisms of industrial importance (Kar & Ray 2008). Amylolytic enzyme activity described in the present research work reveals that the optimum amylolytic enzyme production was at 50 ℃, which makes it suitable for industrial applications. The result is analogous with an alkalophilic *Bacillus* sp. strain GM8901 (Kim et al. 1995). Different strains of *Bacillus* sp. were also reported to require 60 ℃ and 70 ℃ for optimum α-amylase activity (Goyal

et al. 2005; Thippeswamy et al. 2006).

Each microorganism requires a specific pH range for its growth and activity (Sivaramakrishnan et al. 2007). Generally, *Bacillus* sp. showed maximum amylase activity between the pH range 5.0–8.0 (Sajedi et al. 2005). But some of the amylases exhibited their optimum activity at lower pH values (Sajedi et al. 2005), which makes them attractive from the industrial point of view. Amylases active at alkaline pH are also significant for industrial purposes (Annamalai et al. 2011). During the assay period, the amylolytic enzyme activity of *B. subtilis* subsp. *inaquosorum* PR-1 showed a high range of pH stability from acidic to neutral pH. A similar result was also reported from an amylase produced by *Bacillus* sp. (Cordeiro et al. 2002).

B. subtilis subsp. *inaquosorum* PR-1 exhibited maximum amylolytic enzyme production at 1% starch concentration, which is similar with the observations by Kar & Ray (1995). In contrast, maximum enzyme activity was also observed at 2% of starch concentration (Mishra & Behera 2008). Generally, increase in concentration of starch (above 1%) did not increase the enzyme activity, since enzyme production was comparatively lower with higher concentration of starch as the time required to reach the maximum enzyme level was longer (Baks et al. 2006). The K_m value differs in various species of *Bacillus* (Bano et al. 2011). Lower the K_m value indicates higher specificity of the enzyme towards substrate. The K_m value of *B. subtilis* subsp. *inaquosorum* PR-1 was lower than that of alkaliphilic *Bacillus* sp. isolate ANT-6 (Burhan et al. 2003) and almost similar to that of *B. subtilis* KIBGE HAS (Bano et al. 2011).

Organic solvents tolerating enzymes are mainly important for industries dealing with bioremediation (Shafiei et al. 2011). But amylolytic enzyme activity described in this study was found to be inhibited in presence of the organic solvents.

Kumar et al. (2010) reported that the enzymes independent of surfactants are generally useful for industrial purpose. However, Vijayabaskar et al. (2012) reported that SDS and Tween-80 stimulate the amylase activity. According to Bano et al. (2009) Triton X-100 increases the activity, but its decrease by SDS and Tween-80 indicate that surfactants have different effect on the same type of enzyme. Among the various surfactants tested in the present study, amylolytic enzyme production was found to be little effected by SDS, Tween-80 and Triton X-100, which is similar to the results of Kumar et al. (2010).

The effect of inhibitors on amylolytic enzyme activity is also varying. It was found that the present enzyme was inhibited by EDTA, PMSF, β-mercaptoethanol and H_2O_2. Generally, an amylase inhibited by EDTA requires metal ions for its activity (Kumar et al. 2010).

Metal ions play a vital role in the activity of an amylolytic enzyme. Some amylases require metal ions to get stimulated (Najafi et al. 2005). Bano et al. (2009) reported that metal ion, such as Ca^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+} accelerated the amylase activity, whereas the same enzyme was inhibited by Na^+ and Ba^{2+}. However, Vijayabaskar et al. (2012) reported a positive influence of Na^+ ion on amylase activity. Among the tested metal ions of this study it was observed that amylolytic enzyme activity was enhanced with Ca^{2+}, Na^+ and Fe^{2+} at 1 mM and 5 mM concentration each. It indicates that this enzyme requires these metal ions as co-factors for its activity. However, Ba^{2+}, Mg^{2+} and Mn^{2+} decreased the amylolytic enzyme activity, which could be due to the destruction or blockage of the enzyme active site (Bano et al. 2009; Karmakar & Ray 2011). The probable insight to this is the competition between the exogenous cation and the enzyme-associated cation of the metalloenzymes like amylase, resulting in decreased enzyme activity (Leveque et al. 2000).

There are several reports available of α-amylase production by various *Bacillus* sp. whose molecular weight ranged from 42 to 150 kDa (Kumar et al. 2010). The difference in molecular weight of amylase is the result of corresponding gene that codes for the amylase (Sidhu et al. 1997). The molecular weight of amylolytic enzyme of *B. subtilis* subsp. *inaquosorum* PR-1 is quite similar to the 94.5 kDa amylase produced by *Alicyclobacillus acidocaldarius* (Kumar et al. 2010).

Previously, many studies have been done on amylolytic enzyme capability to degrade various sources of raw starch granules. For example, corn, wheat and potato starch by *Bacillus* sp.YX-1 (Liu and Xu 2008), potato starch by *Bacillus* sp. (Goyal et al. 2005), sago starch by *Aspergillus awamori* (Matsubara et al. 2004), rice starch by *Aspergillus awamori* (Matsubara et al. 2004), cassava and corn starch by *Bacillus aquimaris* MKSC 6.2 (Puspasari et al. 2012), tuber starches of white yam, cassava, sweet potato and cocoyam by *Aspergillus niger* AM07 (Omemu et al. 2005), cassava starch by *Aspergillus fumigatus* KIBGE-IB33 (Pervez et al. 2014). But *Dioscorea alata* containing 75–84% of starch (Riley et al. 2006) was not explored previously for its starch conversion process using an amylolytic enzyme. Generally, the substrate used in starch processing industry contains only 15% starch (Goyal et al. 2005). Due to the inhibitory effect of the substrates or products on the enzyme activity, only few reported amylases can be able to draw the industrial importance in case of raw starch hydrolysis. Hence there is an urgent need to explore amylolytic enzyme, which can increase the direct hydrolysis of raw starches under high concentration (Liu & Xu 2007). The amylolytic enzyme of *B. subtilis* subsp. *inaquosorum* PR-1 can efficiently hydrolyze different concentration of raw starch of *Dioscorea* within a short period of time, it will be economically attractive for starch processing industry.

From the present research work, it can be concluded that endophytic *B. subtilis* subsp. *inaquosorum* PR-1 obtained from *Potentilla fulgens* of Meghalaya is a potential source of amylolytic enzyme with starch as a substrate. Additionally, the enzyme was found to show optimum activity under moderate temperature (50 °C) and broad range of pH stability with metal dependence. Its capacity to digest raw starch makes the amylolytic

enzyme of the organism suitable for various industrial applications.

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References

Annamalai N., Thavasi R., Vijayalakshmi S. & Balasubramanian T. 2011. Extraction, purification and characterization of thermostable, alkaline tolerant α-amylase from *Bacillus cereus*. Indian J. Microbiol. **51:** 424–429.

Babu K.R. & Satyanarayana T. 1993. Parametric optimization for extracellular α-amylase production by thermophilic *Bacillus coagulans* B49. Folia Microbiol. **38:** 77–80.

Baks T., Janssen A.E.M. & Boom R.M. 2006. The effect of carbohydrates on α-amylase activity measurements. Enzyme Microb. Technol. **39:** 114–119.

Bano S., Qader S.A.U., Aman A. & Azhar A. 2009. Partial purification and some properties of α-amylase from *Bacillus subtilis* KIBGE-HAS. Indian J. Biochem. Biophys. **46:** 401–404.

Bano S., Qader S.A.U., Aman A., Syed M.N. & Azhar A. 2011. Purification and characterization of novel α-amylase from *Bacillus subtilis* KIBGE HAS. AAPS PharmSciTech **12:** 255–261.

Benson D.A., Cavanaugh M., Clark K., Karsch-Mizrachi I., Lipman D.J., Ostell J. & Sayers E.W. 2013. GenBank. Nucleic Acids Res. **41:** D36–D42.

Bhattacharjee K., Banerjee S. & Joshi S.R. 2012. Diversity of *Streptomyces* spp. in Eastern Himalayan region-computational RNomics approach to phylogeny. Bioinformation **8:** 548–554.

Bhore S.J., Ravichantar N. & Loh C.Y. 2010. Screening of endophytic bacteria isolated from leaves of *Sambung Nyawa* [*Gynura procumbens* (Lour.) Merr.] for cytokinin-like compounds. Bioinformation **5:** 191–197.

Bhore S. & Tiong O.K. 2012. Bacterial endophytes of therapeutically important *Strobilanthes crispa* (L.) Bremek and *Vernonia amygdalina* Del. J. Pharm. Biomed. Sci. **14:** 1–3.

Burhan A., Nisa U., Gokhan C., Omer C., Ashabil A. & Osman G. 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant α-amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. Process Biochem. **38:** 1397–1403.

Castro R.A., Quecine M.C., Lacava P.T., Batista B.D., Luvizotto D.M., Marcon J., Ferreira A., Melo I.S. & Azevedo J.L. 2014. Isolation and enzyme bioprospection of endophytic bacteria associated with plants of Brazilian mangrove ecosystem. SpringerPlus **3**: 382.

Cordeiro C.A.M., Martins M.L.L. & Luciano A.B. 2002. Production and properties of α-amylase from thermophilic *Bacillus* sp. Braz. J. Microbiol. **33:** 57–61.

Dalvi P. & Anthappan P. 2007. Amylase and pectinase from single source for simultaneous desizing and scouring. Indian J. Fibre Text. Res. **32:** 459–465.

Davis P.E., Cohen D.L. & Whitaker A. 1980. The production of α-amylase in batch and chemostatic culture. Antonie van Leenwenhoek **46:** 391–398.

El-Deeb B., Fayez K. & Gherbawy Y. 2013. Isolation and characterization of endophytic bacteria from *Plectranthus tenuiflorus* medicinal plant in Saudi Arabia desert and their antimicrobial activities. J. Plant Interact. **8:** 56–64.

Fisher P.J., Petrini O. & Lappin-Scott H.M. 1992. The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). New Phytol. **122:** 299–305.

Goyal N., Gupta J.K. & Soni S.K. 2005. A novel raw starch digesting thermostable α-amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch. Enzyme Microb. Technol. **37:** 723–734.

Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. & Williams S.T. 1994. Bergey's Manual of Determinative Bacteriology, 9th Ed., Baltimore, Lippincott Williams & Wilkins.

Hynniewta S.R. & Kumar Y. 2008. Herbal remedies among the Khasi traditional healers and village folks in Meghalaya. Indian Journal Traditional Knowledge **7:** 581–586.

Jalgaonwala R.E. & Mahajan R.T. 2011. Evaluation of hydrolytic enzyme activities of endophytes from some indigenous medicinal plants. J. Agric. Technol. **7:** 1733–1741.

Janardhan B.S. & Vijayan K. 2012. Types of endophytic bacteria associated with traditional medicinal plant *Lantana camara* Linn. Pharmacognosy Journal **4:** 20–23.

Ingle S.S. 2011. Isolation and characterization of endophytes from medicinal plant, *Withania somnifera* (Ashwagandha). IUP J. Biotechnol. **5:** 7–13.

Jasim B., Joseph A.A., John C.J., Mathew J. & Radhakrishnan E.K. 2014. Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of *Zingiber officinale*. 3 Biotech **4:** 197–204.

Jin H., Yang X.Y., Yan Z.Q., Liu Q., Li X.Z., Chen J.X., Zhang D.H., Zeng L.M. & Qin B. 2014. Characterization of rhizosphere and endophytic bacterial communities from leaves stems and roots of medicinal *Stellera chamaejasme* L. Syst. Appl. Microbiol. **37:** 376–385.

Karmakar M. & Ray R.R. 2011. A maltotriose producing thermostable amylase from *Bacillus* sp KR11. J. Microbiol. Biotech. Res. **1:** 91–99.

Kar S. & Ray R.C. 2008. Partial characterization and optimization of extracellular thermostable Ca^{2+} inhibited α-amylase by *Streptomyces erumpens* MTCC 7317. J. Sci. Ind. Res. **67:** 58–64.

Kim T.U., Gu B.G., Jeong J.Y., Byun S.M. & Chin Y.C. 1995. Purification and characterization of a maltotetraose-forming alkaline α-amylase from an alkalophilic *Bacillus* strain, GM8901. Appl. Environ. Microbiol. **61:** 3105–3112.

Kumar G.S., Chandra M.S., Mallaiah K.V., Sreenivasulu, P. & Choi Y.L. 2010. Purification and characterization of highly thermostable α-amylase from thermophilic *Alicyclobacillus acidocaldarius*. Biotechnol. Bioprocess Eng. **15:** 435–440.

Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J. & Higgins D.G. 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23:** 2947–2948.

Leveque E., Janecek S., Haye B. & Belarbi A. 2000. Thermophilic archaeal amylolytic enzymes. Enzyme Microb. Technol. **26:** 3–14.

Liu X.D. & Xu Y. 2008. A novel raw starch digesting α-amylase from a newly isolated *Bacillus* sp. YX-1: purification and characterization. Bioresour. Technol. **99:** 4315–4320.

Lodewyckx C., Vangronsveld J., Porteous F., Moore E.R.B., Taghavi S. & Lelie V.D. 2002. Endophytic bacteria and their potential applications. Crit. Rev. Plant Sci. **21:** 583–606.

Lowry O.H., Rosebrough N.J., Farr A. & Randall R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193:** 265–275.

Marlida Y., Saari N., Hassan Z. & Radu S. 2000. Raw starch degrading enzyme from isolated strains of endophytic fungi. World J. Microbiol. Biotechnol. **16:** 573–578.

Martinez T.F., Alarcorn F.J., Lopez M.D. & Moyano F.J. 2000. Improved detection of amylase activity by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with copolymerized starch. Electrophoresis **21:** 2940–2943.

Matsubara T., Ammar Y.B., Anindyawati T., Yamamoto S., Ito K., Iizuka M. & Minamiura N. 2004. Degradation of raw starch granules by α-amylase purified from culture of *Aspergillus awamori* KT-11. J. Biochem. Mol. Biol. **37:** 422–428.

Miller G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31:** 426–428.

Mishra S. & Behera N. 2008. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. Afr. J. Biotechnol. **7:** 3326–3331.

Najafi M.F., Deobagkar D. & Deobagkar D. 2005. Purification and characterization of an extracellular α-amylase from *Bacillus subtilis* AX20. Protein Expr. Purif. **41:** 349–354.

Nielsen J.E., Borchert T.V. & Vriend G. 2001. The determinants of α-amylase pH-activity profiles. Protein Eng. **14:** 505–512.

Omemu A.M., Akpan I., Bankole M.O. & Teniola O.D. 2005. Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil. Afr. J. Biotechnol. **4:** 19–25.

Pandey A., Nigam P., Soccol C.R., Soccol V.T. Singh D. & Mohan R. 2000. Advances in microbial amylases. Biotechnol. Appl. Biochem. **31:** 135–152.

Pervez S., Aman A., Iqbal S., Siddiqui N.N. & Qader S.A.U. 2014. Saccharification and liquefaction of cassava starch: an alternative source for the production of bioethanol using amylolytic enzymes by double fermentation process. BMC Biotechnol. **14:** 49.

Pimentel M.R., Molina G., Dionisio A.P., Marostica Jr. M.R. & Pastore G.M. 2011. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. Biotechnol. Res. Int. **2011:** 576286.

Prakash O., Nimonkar Y., Munot H., Sharma A., Vemuluri V.R., Chavadar M.S. & Shouche Y.S. 2014. Description of *Micrococcus aloeverae* sp. nov., an endophytic actinobacterium isolated from *Aloe vera*. Int. J. Syst. Evol. Microbiol **64:** 3427–3433.

Preveena J. & Bhore S.J. 2013. Identification of bacterial endophytes associated with traditional medicinal plant *Tridax procumbens* Linn. Anc. Sci. Life **32:** 173–177.

Puspasari F., Radjasa O.K., Noer A.S., Nurachman Z., Syah Y.M., van der Maarel M., Dijkhuizen L., Janecek S. & Natalia D. 2013. Raw starch-degrading α-amylase from *Bacillus aquimaris* MKSC 6.2: isolation and expression of the gene, bioinformatics and biochemical characterization of the recombinant enzyme. J. Appl. Microbiol. **114:** 108–120.

Ram P.C., Srivastava K.N., Lodha M.L. & Mehta S.L. 1986. Purification and characterization of proteolytic enzymes from normal and opaque-2 *Zea mays* L. developing endosperms. J. Biosci. **10:** 257–266.

Rasiah I.A. & Rehm B.H. 2009. One-step production of immobilized α-amylase in recombinant *Escherichia coli*. Appl. Environ. Microbiol. **75:** 2012–6.

Riaz A., Qadar S., Anwar A., Iqbal S. & Bano S. 2008. Production and characterization of thermostable α-amylase from a newly isolated strain of *Bacillus subtilis* KIBGE-HAR. Internet J. Microbiol. **6 (1):** https://ispub.com/IJMB/6/1/7538.

Riley C.K., Wheatley A.O. & Asemota H.N. 2006. Isolation and characterization of starches from eight *Dioscorea alata* cultivars grown in Jamaica. Afr. J. Biotechnol. **5:** 1528–1536.

Ronquist F. & Huelsenbeck J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:** 1572–1574.

Roy B., Swargiary A., Syiem D. & Tandon V. 2010. *Potentilla fulgens* (family Rosaceae), a medicinal plant of northeast India: a natural antihelmintic? J. Parasit. Dis. **34:** 83–88.

Sajedi R.H., Manesh H.N., Khajeh K., Ahmadvand R., Ranjbar B., Asoodeh A. & Moradian F. 2005. A Ca-independent α-amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104. Enzyme Microb. Technol. **36:** 666–671.

Sambrook J., Fritsch E.F. & Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schulz B., Wanke U., Draeger S. & Aust H.J. 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycol. Res. **97:** 1447–1450.

Shafiei M., Ziaee A.A. & Amoozegar M.A. 2011. Purification and characterization of an organic-solvent-tolerant halophilic α-amylase from the moderately halophilic *Nesterenkonia* sp. strain F. J. Ind. Microbiol. Biotechnol. **38:** 275–281.

Sheikh N., Kumar Y., Misra A.K. & Pfoze L. 2013. Phytochemical screening to validate the ethnobotanical importance of root tubers of *Dioscorea* species of Meghalaya, North East India. J. Med. Plants Stud. **1:** 62–69.

Sidhu G.S., Sharma P., Chakrabarti T. & Gupta J.K. 1997. Strain improvement for the production of a thermostable α-amylase. Enzyme Microb. Technol. **21:** 525–530.

Singh N.S. & Singh D.K. 2011. Biodegradation of endosulfan and endosulfan sulfate by *Achromobacter xylosoxidans* strain C8B in broth medium. Biodegradation **22:** 845–857.

Sivaramakrishnan S., Gangadharan D., Nampoothiri K.M., Soccol C.R. & Pandey A. 2007. α-Amylase production by *Aspergillus oryzae* employing solid-state fermentation. J. Sci. Ind. Res. **66:** 621–626.

Song G.C., Yasir M., Bibi F., Chung E.J., Jeon C.O. & Chung Y.R. 2011. *Nocardioides caricicola* sp. nov., an endophytic bacterium isolated from a halophyte, *Carex scabrifolia* Steud. Int. J. Syst. Evol. Microbiol. **61:** 105–109.

Srivastava R.A.K. & Baruah J.N. 1986. Culture conditions for production of thermostable amylase by *Bacillus stearothermophilus*. Appl. Environ. Microbiol. **52:** 179–184.

Stamford T.L.M., Stamford N.P., Coelho L.C.B.B. & Araujo J.M. 2001. Production and characterization of a thermostable α-amylase from *Nocardiopsis* sp. endophyte of yam bean. Bioresour. Technol. **76:** 137–141.

Stamford T.L.M., Stamford N.P., Coelho, L.C.B.B. & Araujo J.M. 2002. Production and characterization of a thermostable glucoamylase from *Streptosporangium* sp. endophyte of maize leaves. Bioresour. Technol. **83:** 105–109.

Strobel G., Daisy B., Castillo U. & Harper J. 2004. Natural products from endophytic microorganisms. J. Nat. Prod. **67:** 257–268.

Sunitha V.H., Ramesha A., Savitha J. & Srinivas C. 2012. Amylase production by endophytic fungi *Cylindrocephalum* sp. isolated from medicinal plant *Alpinia calcarata* (Haw.) Roscoe. Braz. J. Microbiol. **43:** 1213–1221.

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. **30:** 2725-2729.

Tao X., Jang M.S., Kim K.S., Yu Z. & Lee Y.C. 2008. Molecular cloning, expression and characterization of α-amylase gene from a marine bacterium *Pseudoalteromonas* sp. MY-1. Indian J. Biochem. Biophys. **45:** 305–309.

Thippeswamy S., Girigowda K. & Mulimani V.H. 2006. Isolation and identification of amylase from dhal industry waste. Indian J. Biochem. Biophys. **43:** 295–298.

Thomas P. & Reddy K.M. 2013. Microscopic elucidation of abundant endophytic bacteria colonizing the cell wall-plasma membrane peri-space in the shoot-tip tissue of banana. AoB Plants **5:** plt011.

Vijayabaskar P., Jayalakshmi D. & Shankar T. 2012. Amylase production by moderately halophilic *Bacillus cereus* in solid state fermentation. Afr. J. Microbiol. Res. **6:** 4918–4926.

Whitehead T.R. & Cotta M.A. 1995. Identification of intracellular amylase activity in *Streptococcus bovis* and *Streptococcus saliuarills*. Curr. Microbiol. **30:** 143–148.

Zaferanloo B., Bhattacharjee S., Ghorbani M.M., Mahon P.J. & Palombo E.A. 2014. Amylase production by *Preussia minima*, a fungus of endophytic origin: optimization of fermentation conditions and analysis of fungal secretome by LC-MS. BMC Microbiol. **14:** 55.

Zaferanloo B., Virkar A., Mahon P.J. & Palombo E.A. 2013. Endophytes from an Australian native plant are a promising source of industrially useful enzymes. World J. Microbiol. Biotechnol. **29**: 335–345.

Zinniel D.K., Lambrecht P., Harris N.B., Feng Z., Kuczmarski D., Higley P., Ishimaru C.A., Arunakumari A., Barletta R.G. & Vidaver A.K. 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and Prairie plants. Appl. Environ. Microbiol. **68:** 2198–2208.

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