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Research Article

JACKFRUIT SEED AS A NOVEL SUBSTRATE FOR THE PRODUCTION OF AN ACIDOPHILIC AND ACID-STABLE α-AMYLASE FROM *BACILLUS* SP.4

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ABSTRACT

Objective: The objective of the current study is to do a comparative analysis of the ability of a strain of *Bacillus* to grow and produce α-amylase on various agro-residues under solid state fermentation (SSF), as amylases comprise one of the most important enzymes in industries.

Methods: Bacteria were isolated from various soil samples by serial dilution method, screened for amylase production by rapid screening method on starch agar plates and the best amylase producer was chosen. The best isolate was cultured on different agro-residues such as wheat bran, watermelon outer rind, Avarekai seed coat (*Dolichos lablab*), coconut endosperm, and jackfruit seeds for maximum amylase production. The pH and temperature optima of the enzyme were determined by culturing the bacteria under different pH and temperatures. The crude enzyme was purified by ammonium sulfate precipitation followed by ion-exchange chromatography methods.

Results: The best isolate chosen was *Bacillus* sp.4, which produced an acidophilic and acid-stable α-amylase with maximum enzyme production at the acidic pH of 5.5 and 6.5 (21.11 and 21.62 U/mg protein, respectively) and maximum stability at pH 5.5. Jackfruit seed was found to be the most suitable agro waste for α-amylase production by our isolate. Purification of the enzyme by ammonium sulfate precipitation followed by ion-exchange chromatography resulted in 23.17-fold increase in its activity (86.67 U/mg protein).

Conclusion: Considering its acid-stable and highly promising enzyme activities, the enzyme from this bacterial isolate can be further characterized for future applications in starch and other food industries.

Keywords: α-Amylase, Acidophilic, Jackfruit seed, Agro-industrial residue, *Bacillus* sp.4.

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INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* Lam.) is a favorite fruit which is widely grown and consumed in Thailand, India, and some other tropical regions of the world. The pleasantly flavored yellow-colored sweet bulbs of the fruit contain the seeds embedded in them. The bulbs which are edible in ripe jackfruit can be consumed either fresh or can be preserved into canned products. Seeds have high carbohydrate and protein contents and make-up about 10–15% of the total fruit weight [1,2]. Seeds are sometimes steamed and eaten as a snack or discarded or used in some local recipes. Proper use of this nutrient-rich agro-waste has not been attempted. The richness of the seeds, as far as carbohydrate and protein contents are concerned, inspired us to test this biomaterial as a substrate for solid-state fermentation (SSF) for α-amylase production using a newly isolated strain of *Bacillus,* namely, *Bacillus* sp.4. In an earlier report for α-amylase production, banana waste was utilized as a substrate [3].

This study is a comparative analysis of the ability of a strain of *Bacillus* to produce α-amylase on jackfruit seed and other agro-residues under SSF and we also report the partial purification and characterization of the enzyme produced.

METHODS

Bacterial isolation

Many bacteria were isolated from soil collected from diverse locations by serial dilution method. Initial screening for amylase production was performed through starch agar plate method. One of the isolates, *Bacillus* sp.4, displayed the highest zone of hydrolysis on this medium and was chosen for further studies.

Medium composition

Production media for bacteria contained 5 g of solid substrate and 8 mL of phosphate buffer (pH 6.5) in conical flasks (250-mL). The bacterial culture was allowed to grow for 2 days (48 h) at 37°C. To adjust the moisture content from 43% to 81%, sterile distilled water was used in appropriate amounts.

Evaluation of different agro-industrial wastes as substrates

Different substrates, that is, wheat bran (WB), watermelon outer rind (WM), Avarekai seed coat (*Dolichos lablab*) (AW), coconut endosperm (C), and jackfruit seeds (J), were used as solid substrates for comparing their suitability for enzyme production. All these were collected from the Bangalore marketplace, were dried under the shade and were made into a coarse powder using a mixer grinder. To compare the different substrates for enzyme production, WB and other agro-based substrates were taken in different ratios such as 1:4, 1:1, 4:1, and 5:0 along with a control of WB.

Inoculum preparation

Bacterial cultures were transferred from stock to 100 mL nutrient broth. The inoculated flasks were incubated overnight at 37°C. The broths were then centrifuged at 10,000 rpm for 10 min. Cells were harvested from the broth and their absorbance (*A*) was read at 660 nm. Accordingly, cultures with an OD of 0.5 were chosen as the standard inoculum size, which contains 4.5×10^5 cells/mL. Inoculum was prepared in sterile distilled water.

Bacterial culture condition optimization for enzyme production

pH and temperature of culture media

To determine the effect of culture conditions on enzyme production, the bacterial isolate of the current work was cultured at different

temperatures (27, 37, 40, and 50°C) and pH of the medium (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10, and 11). After an incubation period of 48 h, the enzyme activity was analyzed as per the standard methodology.

Extraction and assay of enzyme activity

To the culture flasks, 22 mL of 0.1 M phosphate buffer of pH 6.5 was added, and in a rotary shaker (200 rpm), it was mixed well at room temperature (RT) (25 + 2°C) for about 30 min. Through a muslin cloth, the mixture was filtered and the filtrate was centrifuged for 10 min at 10,000 rpm. The supernatant thus obtained was the enzyme source used for the assays.

Amylase activity of the culture supernatant was estimated by dinitrosalicylic acid method [4]. The amount of enzyme which was able to release 1 µmoL of reducing sugar as glucose per milliliter per minute was considered as one unit of enzyme activity, under the assay conditions. Enzyme activity is represented as U/mg of protein, which is the specific activity of the enzyme.

Determination of protein content

Following the standard method of Lowry *et al.* [5], the protein content of the enzyme samples was assayed, bovine serum albumin was the standard used. All of the assays were performed thrice and the standard error was estimated.

Purification of the enzyme

Salting out using ammonium sulfate

Through the standard method of salting out using ammonium sulfate, the amylase in the culture supernatant was precipitated out at different saturation percentages (0–20%, 20–40%, 40–60%, and 60–80%). Among these, at 60% saturation, the resultant supernatant did not exhibit any enzyme activity, thus confirming that it had successfully precipitated. The precipitate was mixed in 2.0 mL of phosphate buffer of pH 6.5 (0.1 M), and this was overnight dialyzed against phosphate buffer of 0.05 M [6].

DEAE column chromatography

Through a previously equilibrated (50 mM phosphate buffer of pH 6.5) DEAE-cellulose column (3 cm × 10 cm), the ammonium sulfate precipitated and dialyzed enzyme was eluted and further purified. The column was loaded with the ammonium precipitated sample (2 mg/mL in phosphate buffer) and using phosphate buffer (pH 6.5 and having 0.1–0.8 M NaCl) was eluted at a flow rate of 1 mL/min. The protein fractions (showing absorbance at 280 nm) were pooled and checked for enzyme activity, and the resulting fractions with highest enzyme activity were collected.

SDS-PAGE and molecular mass determination

To check the purity and molecular weight of the enzyme, SDS-PAGE was performed as per the standard methodology [7]. Lysozyme (14.3 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), albumin (66 kDa), and phosphorylase *b* (97 kDa) were the molecular weight markers used.

Determination of thermal and pH stability of α-Amylase

The enzyme without the substrate fractions was incubated at different temperatures ranging from 30 to 60°C for 1 h, to determine its thermal stability. Every 10 min intervals, 0.1 mL aliquots of the incubated enzyme were taken out and assayed for enzyme activity.

For checking the stability of the enzyme at different pH conditions, equal amounts of the enzyme and buffers of pH ranging from 3.5 to 9.5 were incubated at RT for 24 h. After 24 h, the residual enzyme activity was checked to understand the effect of pH on enzyme stability.

RESULTS

Strain selection

Different bacteria that were isolated from different soil sources were screened for amylase production by rapid plate method, that is, by starch hydrolysis. In a starch agar medium, when the organism is point inoculated, amylase production is indicated by the zone of clearance on flooding with iodine solution. Based on this, one of the bacterial isolate was chosen which was identified as *Bacillus* sp.4-JGI 47.

Production of amylase enzyme

Evaluation of different agricultural wastes as substrates

On comparing jackfruit seeds and WB for amylase production, jackfruit seed as substrate was found to give higher levels of α-amylase from our isolate of *Bacillus* sp. (Fig. 1). It showed specific activity of 3.7425 U/mg on WB and 6.5 U/mg on jackfruit seeds (powdered). When combination of WB and jackfruit seeds (J) was used, the combination of 1 WB:4 J gave the maximum specific activity of 6.0 U/mg when compared to other combinations. Shukla and Kar [8] reported potato peel (PP) as a better substrate than WB for α-amylase production. Mukherjee *et al*. [9] reported that PP as a solid-state substrate resulted in higher α-amylase production by *Bacillus subtilis* DM-03. For α-amylase production, another group had used banana peel through a strain of *B. subtilis* [10].

Effect of pH

The selected bacterial isolate, *Bacillus* sp.4, showed highest enzyme production at pH 5.5 and 6.5 with values 21.11 and 21.62 U/mg, respectively. The enzyme activity was higher than the controls even at the acidic pH of 3.5 and 4.5 (Fig. 2). At a pH of 8.5 and 9.5, the amylase activities were comparatively lower than that at pH 5.5 and 6.5. Nevertheless, these activities were higher than that of the control group (3.74 U/mg protein). These results were statistically highly significant

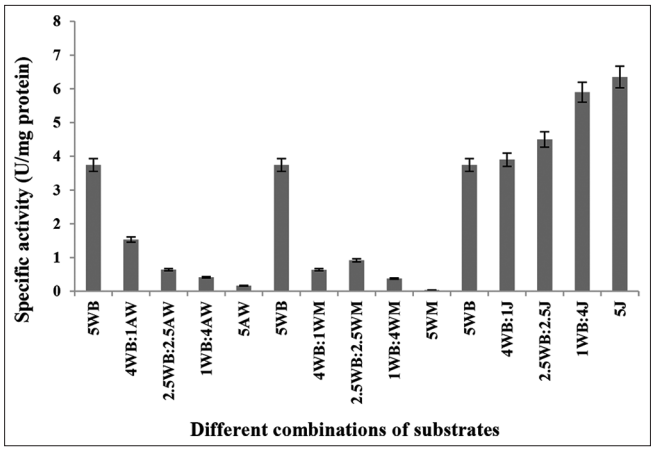

Fig. 1: Effect of different agro-residues and wheat bran on α-amylase production. WB: Wheat bran, AW: Avarekai seed coat, WM: Water melon outer rind, J: Jackfruit seed

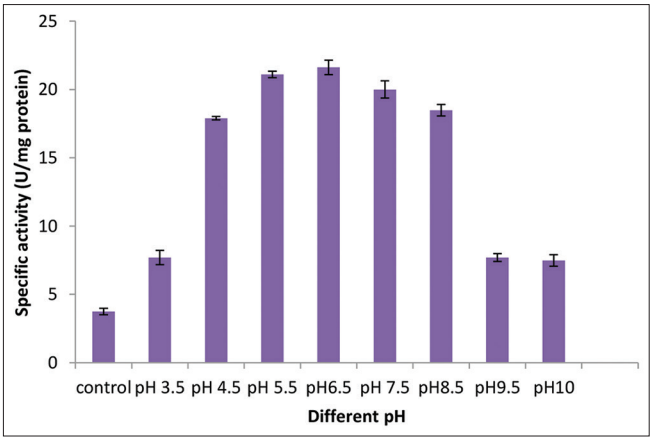

Fig. 2: Effect of pH on production of *α*-amylase by *Bacillus* sp.4

($p<0.001$). At acidic pH of 5.5 and 6.5, the enzyme production and activity were found to be 6-fold higher than that produced by the control groups. This shows that the enzyme can tolerate both acidic and alkaline pH of the production media. The enzyme production decreased with more alkaline pH. Bacteria such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *B. subtilis* required an optimal pH of 7.0 for higher enzyme productions [11-13].

Effect of temperature

Bacillus sp.4 when incubated at different temperatures, a temperature of 37°C, was observed to be the best for enzyme production. The specific activity of α-amylase at this temperature was 4.525 U/mg (Fig. 3). It was seen that enzyme production declined along with an increase of incubation temperature. Different bacteria were reported for amylase production at varied temperatures. *B. licheniformis, B. subtilis*, *B. stearothermophilus*, and *B. amyloliquefaciens* are some of the very commonly reported *Bacillus* sp. producing α-amylase at 37–60°C temperatures [12,14,15].

Enzyme characterization

Ammonium sulfate precipitation

Purification of the crude enzyme was carried out using ammonium sulfate precipitation method. The enzyme possessed highest activity at a concentration of 20–40% (8.71 U/mg) **(**Fig 4). Previous researchers reported 0–50% saturated precipitate of enzyme as having higher activity for α-amylase than that of 50–100% precipitate [16].

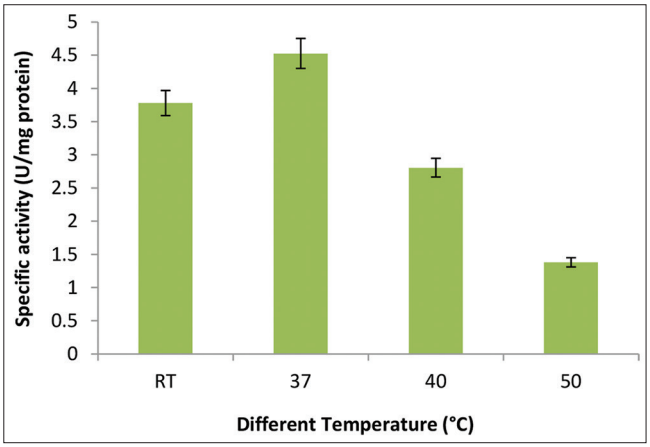

Fig. 3: Effect of temperature on production of *α*-amylase by *Bacillus* sp.4

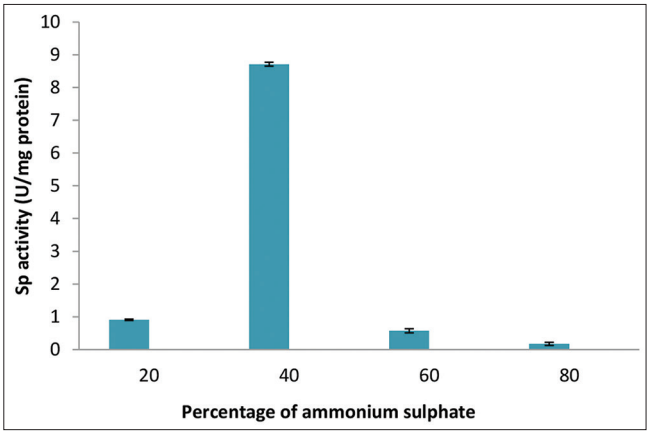

Fig. 4: Purification of *α*-amylase from *Bacillus* sp.4 by ammonium sulfate precipitation

Column chromatography using DEAE-cellulose

After DEAE-cellulose column elution of the partially purified α-amylase obtained from ammonium sulfate precipitation (Fig. 5), amylase activity was found in protein peaks 1, 2, and 3 that were in fractions 7th to 9th with highest activity in 7th fraction (693.33 U/mL) for the current isolate, *Bacillus* sp. 4. Mohammed *et al*. [17] reported 154.7 U/mg as the specific activity of the enzyme after chromatographic purification. As compared to that report, the column purification step in our isolate resulted in much higher activity.

Purification fold of the enzyme

The column purified enzyme had a specific activity of 86.67 U/mg proteins, which equals to a 23.17-fold increase in its specific activity as compared to the crude enzyme (Table 1). Mohammed *et al*. [17] reported about *Bacillus cereus* Ms6 as the enzyme purity after DEAE-cellulose chromatography to be more than 27-fold. Afifi *et al*. [18] reported a 25.14-fold purification and 23.43 mg/mL of protein as the specific activity for the α-amylase enzyme from *Penicillium olsonii* after gel filtration and ion chromatography purifications.

Effects of pH and temperature on the stability of α-amylase

The maximum stability of the enzyme recorded was at the RT of 24–28°C when incubated for 60 min, retaining 75% of its original activity, followed by at 37°C (Fig. 6). At 60°C, the lowest stability was seen after

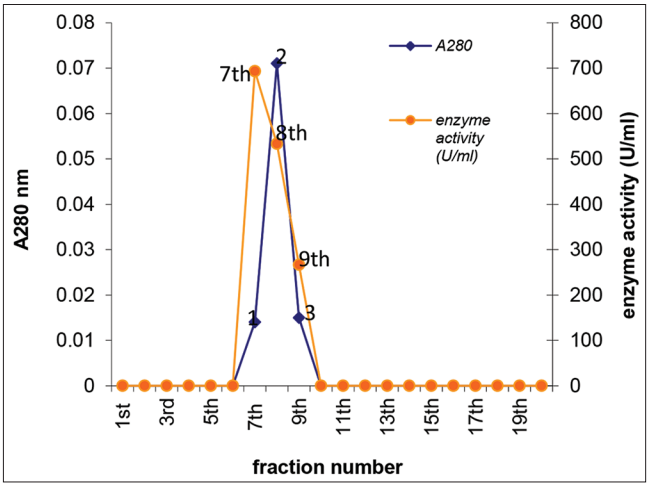

Fig. 5: DEAE-cellulose anion exchange chromatography. Purification of *α*-amylase from *Bacillus* sp.4

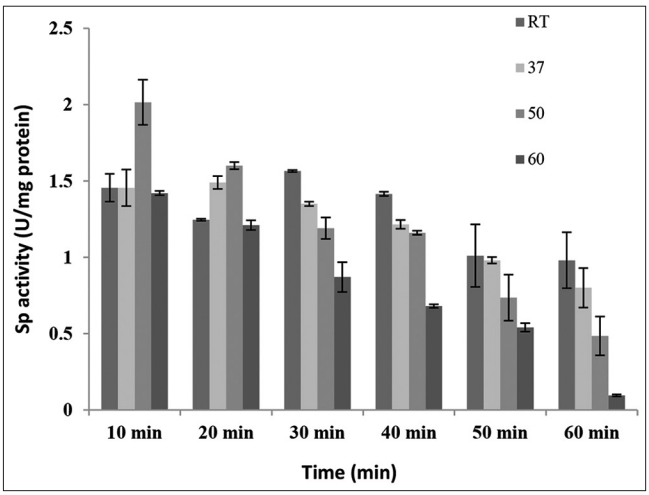

Fig. 6: Effect of temperature on the stability of *α*-amylase from *Bacillus* sp.4

Table 1: Purification summary of alpha amylase from *Bacillus* sp.4

Step	Volume (mL)	Total activity (Units/mL)	Total protein (mg/mL)	Specific activity (U/mg protein)	Yield (%)	Purification fold (%)
Crude extract	100	1870	500	3.74	100	1
Ammonium sulfate ppt.	20	224.4	10.0	22.44	12.0	6.0
DEAE-cellulose ion-exchange chromatography	1	693.33	8.00	86.67	37.07	23.17

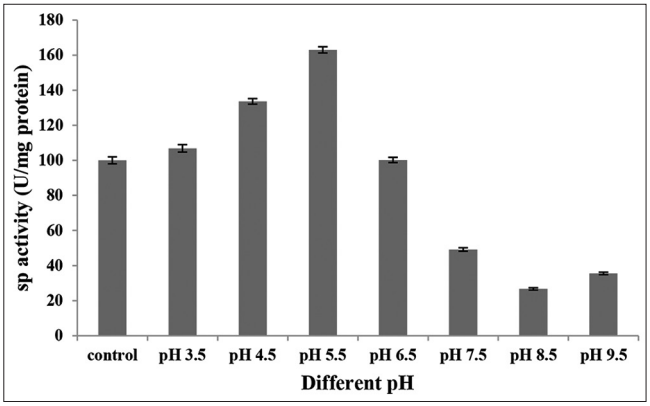

Fig. 7: Effect of pH on the stability of *α*-amylase from *Bacillus* sp.4

60 min with the enzyme retaining only <20% of its original activity. Comparable reports were shown from our previous studies [19,20].

When the enzyme was pre-incubated for 24 h with different buffers of differing pH, the acidic pH of 5.5 resulted in highest residual specific activity (6.1 U/mg of protein) as compared to various other buffers (Fig. 7). This is 1.63-fold greater than the stability of the enzyme of the control group (enzyme incubated with a buffer of pH 7.0). The enzyme showed significant stability toward acidic pH, that is, from 3.5 up to 6.5, but in alkaline pH, the stability of the enzyme gradually dropped to only 30% of that of the control value.

DISCUSSION

Jackfruit seed (JFS) and WB in different combinations (4:1 and 2.5:2.5) gave better results of enzyme activity when compared to WB as the sole substrate. Rengsutthi and Charoenrein [21] have reported that JFS has greater amylase content and its granules were much smaller than corn starch and potato starch granules. This could be the reason for obtaining higher enzyme activity value when we used jackfruit as a substrate for α-amylase production from our isolate *Bacillus* sp.4. Till now, most of the earlier workers have widely reported WB to be the best resource for enzyme production in SSF [22,23]. Gangadharan *et al*. [24] reported that among the various agro-wastes checked for SSF, the highest enzyme activity was shown by WB. They also reported that all combination of substrates containing WB resulted in giving a significant increase in enzyme activity. For the production α-amylase under SSF from *Bacillus* sp. PS-7, it was reported that WB gave maximum production when added with external supplements [25]. Our isolate could utilize jackfruit seeds more efficiently as compared to WB for α-amylase production. Since jackfruit seeds are usually discarded or used as fodder for cattle, it becomes a cheaper source for α-amylase production by industries. This can also serve as an alternative for waste disposal, reducing the burden on our environment.

Majority of the α-amylases that are thermostable show higher enzyme activity at the pH range of 6.5–7.0. These α-amylases usually demonstrate a sharp drop in their activity in acidic medium. Hence, importance should be given to α-amylases which exhibit acidic pH optima or more than 1 pH optimum. In comparison to earlier reports, our enzyme has maximum stability and activity at pH 5.5. *B. licheniformis* α-amylase [26] showed pH optima at 6.5–7 and 9.5 under SSF conditions. It was reported that α-amylase from *Bacillus* sp. PS-7 exhibited its stability within a pH range of 5.0–8.0 and at pH 6.0 had highest stability [27]. Even though our enzyme is not thermostable, it is acid stable and highly active at all the tested acidic pH, that is, 3.5, 4.5, 5.5, and 6.5. Only in the alkaline pH, the activity of the enzyme dropped. Among the substrates screened for SSF, JFS powder gave the highest enzyme activity than WB, AW, or WM by our current isolate, *Bacillus* sp.4.

CONCLUSION

Based on the above study outcomes, it can be concluded that jackfruit seeds can serve as an alternative and efficient substrate for the production of an acidic α-amylase by *Bacillus* sp.4. The broad pH range of activity and acid stability of the α-amylase from the current isolate of *Bacillus* prove that it has the potential for application in starch and food industries after further studies in this regard.

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CONFLICTS OF INTEREST

The authors state that there are no conflicts of interest.

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