

Production Optimization and Industrial Applications of Amylase From Indigenous Bacterial Species Using Banana Peels

Madiha Muhammad Fazil, Iqra Javed, Kiran Ali, Humera Waheed*, Nida Dastagir

Dow College of Biotechnology, Dow Research Institute of Biotechnology and Biomedical Sciences, Dow University of Health Sciences, Ojha Campus Karachi-75270, Pakistan.

Abstract: Introduction: Alpha amylases are starch hydrolyzing enzymes that possess high industrial demand. Various strategies have been adopted to enhance cost-effective enzyme production of which utilization of agro-industrial waste is very promising. **Methodology:** In this study two amylase producers *Bacillus megaterium* and *Exiguobacterium auranticum* were isolated and identified by 16sRNA sequencing their growth conditions were optimized via submerged fermentation using banana peels as carbon source. *Bacillus megaterium* found to be mesophilic alkaline (37°C, pH of 9) strain giving 472 U/ml while *Exiguobacterium auranticum* found to be acidic thermophilic (50°C, pH 5) that gave 391 U/ml. **Results and Conclusion:** Production of amylase by acidic thermophilic *E. auranticum* is reported here for the first time. The crude enzyme also showed better chocolate and curry stain removal capacity when combined with commercial detergent. Also juice clarification assay showed promising results which indicates that the enzymes could be potentially used in detergent and food industry.

Keywords: Alpha-amylase; *Bacillus megaterium*; *Exiguobacterium auranticum*; submerged state fermentation; banana peels.

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***Correspondence:** Humera Waheed, Dow College of Biotechnology, Dow Research Institute of Biotechnology and Biomedical Sciences, Dow University of Health Sciences, KDA Scheme 33, Gulzar-e-Hijri, Suparco Road Ojha Campus Karachi-75270, Pakistan, Tel: 0092- 334-3206688 Email: humera.waheed@duhs.edu.pk

Introduction

Enzymes (biocatalyst) have numerous advantages compared to chemical catalysts and therefore possess profound applications in various biological and industrial processes. Microorganisms are considered as the primary source of industrially important enzyme's production largely due to their stability and specificity, cost-effective and rapid growth cycles, easy nutrient requirement, ease of optimization and production, non-toxic, and eco-friendly nature [1]. Among all the industrial important enzymes, amylases mark up to 25% to 30% share in the world enzyme market [2]. Secretory microbial amylases are potentially useful in starch saccharification processes in food, textile and brewing industries along with applications in detergent, medicinal, pharmaceutical and other chemical industries [3]. There are mainly three types of amylases (α -, β - and γ -types), of which α -amylases are ubiquitously found in animals, plants and microbes. The α -amylase is a fast-acting hydrolase enzyme that breakdown carbohydrates mainly starch to liberate products like glucose and maltose [4]. Bacterial amylases are preferred over fungal types as they can be produced under varying pH, temperature, organic solvents and salt conditions both by submerged and solid-state fermentation. Moreover, bacterial strains can be manipulated easily to meet up desired industrial needs and their growth cycles are less time consuming than fungi [5]. Submerged state fermentation is sometimes preferred due to easy sterilization process, better product recovery and easy control over process [6].

Enzymes are in tangible demand in many industries but they are expensive due to the high cost of synthetic media used to produce them [7]. Recent advances in biotechnology leads towards the use of agricultural and industrial by-products for value-added products production including enzymes. Increased industrialization of agricultural sector generates tremendous waste that is either dumped untreated or burned to generate green-house gases [8]. According to United Nations Food and Agriculture Organization (FAO), the processing of horticultural crops generates up to 30% mass as waste composed mainly of peels, rind, seeds *etc.* [9]. Organic agro-residues such as husk, bran, bagasse, fruits and vegetable peels can serve as potential raw material that support microbial growth and provide essential nutrients [8]. Pakistan is an agro-based economy and its crop sub-sector contributes 8.27% of its 20.9% agricultural GDP. The major crops grown here are cotton, rice, maize, wheat, sugar-cane on around 22 million hectares of cultivated land [10]. Sindh province is the major banana producer that accounts for 82% production of the country [11]. Also, potatoes are one of the main staple foods grown and consumed in curries, chips and salads throughout the year. Around 15 to 40% part of both of these is lost as waste in form of peels that is rich in starch content [9]. The aim of our research is economic utilization of these waste for production of α -amylase which is an industrially important enzyme in Pakistan particularly in detergent and food industry.

Material and Methods

Chemical and Reagents:

Starch and DNS were purchased from Sigma-Aldrich. All other chemicals and reagents were mainly obtained from Oxoid, and Merck. All solutions were prepared with distilled water.

Sample Collection and Screening:

Natural starch rich waste (potato peels, banana peels, stale rice, stale bread, corn flour, organic matter enriched soil) was used for the isolation of starch reducing bacteria. Sample (1:10) dissolved in distilled water, serially diluted (10^{-5}) followed by spread plate method for bacterial growth on nutrient agar plates. After pure culturing all the isolates were incubated on 1% starch agar plates for 24hr at 37°C and presence of clear zone around the colonies upon flooding with Lugol's iodine solution were considered as the amylase producers. The 2 isolates giving highest zones were selected for further investigation.

Morphological and Biochemical Characterization:

Morphological analysis (surface, color, shape, Gram's nature *etc.*) along with several biochemical tests were performed for the identification of the strains. The biochemical tests include: catalase, hemolytic, mannitol, gelatinase, oxidase, urease, citrate, indole, MR-VP (Methyl Red / Voges-Proskauer), carbohydrate fermentation tests (glucose, fructose, lactose, maltose, galactose, xylose, sucrose). Bergey's manual was used for strain identification [12].

16S RNA Sequencing:

The identification of isolates was further confirmed by 16S RNA sequencing by sending freshly cultured bacterial isolates to Macrogen (USA). Briefly, genomic DNA was extracted and amplified by using universal forward (27F) and reverse (1492R) primers. The pure PCR amplicon was purified and sequencing was performed by using universal forward (785F) and reverse primers (907R). The obtained DNA sequences were compared with available nucleotide database for homology using Basic Local Alignment Search Tool (BLAST) provided by National Center for Biotechnology Information (NCBI). Sequence alignment was performed with ClustalW program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and phylogenetic tree was constructed with neighbor-joining and maximum-likelihood method with 1000 bootstrap values using MEGA X software [13].

Substrate Preparation:

The stale bread, banana and potato peels were bought from local shops in Karachi. They were chopped into small pieces and were dried for 48 h at 70°C in drying oven. Finally, the dried samples were grinded to fine powder and stored in airtight containers at room temperature.

Inoculum and Fermentation Medium:

The inoculum was prepared by introducing a loop-full of selected strains in sterile nutrient broth for 24h to obtain O.D. of 0.3 at 600 nm at 37°C. Fermentation media (25 ml) was prepared in 100ml Erlenmeyer flask containing 3% of total substrate, 0.5% nitrogen source, 0.1% K₂HPO₄, 0.05% each of NaCl and MgSO₄. The pH was adjusted to 7.0 and the volume was made up to 25 ml with distilled water and autoclaved at 121°C.

Submerged-State Fermentation and Enzyme Extraction:

For fermentation, 2% of the pure culture (O.D. 0.3 at 600 nm) was inoculated aseptically in the autoclaved media (25 ml). The flasks were kept on shaking incubator for 24 h at 120 rpm at 37°C. After incubation, the media was harvested by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was collected and subjected to amylase activity analysis by DNS method.

Estimation of Amylase Activity:

Amylase activity was determined by DNS method by using maltose as standard. Briefly, 50 µl of crude enzyme (supernatant) was incubated with 50 µl of 1% starch solution prepared in 100 mM potassium phosphate buffer (pH 7.0) for 5 min at 50°C. After addition of 100 µl of DNS reagent the tubes were incubated in boiling water bath for 10 min. After boiling, 1 ml of distilled water was added and the absorbance of liberated reducing sugar was measured at 546 nm using substrate and enzyme blanks. Each sample was analyzed in triplicates. One unit of enzyme activity is defined as amount of enzyme required to release 1 µmol of reducing sugar in a minute under specific assay conditions.

The formula used for unit calculation is:

$$\frac{\mu\text{mol of product} \times \text{total volume of reaction} \times \text{dilution factor}}{\text{volume of enzyme} \times \text{assay time}}$$

Optimization of Parameters in Shake Flask:

The effect of various parameters for production of amylase in bulk quantity under suitable experimental condition was investigated. Different carbon sources (banana peels, potato peels, stale bread, corn flour and soluble starch) were tested as substrate for maximum enzyme production and supernatant were analyzed by DNS assay. The carbon source that showed maximum activity was selected for further parameters optimization. The effect of different inorganic nitrogen source was then optimized using 0.5% ammonium sulphate, ammonium nitrate and sodium citrate. Further, effect of temperature on enzyme production was determined by incubating fermentation flasks at 30°C, 37°C, 50°C, 60°C and 70°C. These conditions with maximum enzyme production were then selected to further determine optimum pH for bacterial growth. It was obtained by adjusting pH of the fermentation medium at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 by using 6 M HCl or 1 M NaOH. Finally, the selected parameters (carbon source, nitrogen source, temperature and pH) were used to test incubation time (24, 48 and 72 h), inoculum size (2%, 4%, 6%, 8% and 10%). Each sample was analyzed for amylase activity by DNS method in triplicates and the units were calculated according to the given formula. Also,

three independent experiments for each parameter optimization were performed for each of the strain.

Qualitative analysis:

A. Quick Tube Test:

Clear 1% starch solution (1 ml) was taken in test tubes and 1 drop of Lugol's iodine solution was added to give solution a dark blue color indicating the presence of starch. Crude enzyme (100 µl) was added in the test tube, shaken for few seconds to observe for color change. Loss of blue color indicates starch hydrolyzation by presence of active enzyme. The tube with 100 µl water instead of crude enzyme was taken as control.

B. Plate Assay:

The starch assay plate was prepared with 1% soluble starch and 2% agar-agar to check the presence of activity in isolates or crude enzyme (supernatant). The wells (where necessary) in starch plates were punched with sterile tips and 100 µl of the supernatant was added in it. The plates were incubated for 24 h at 37°C to let the crude enzyme hydrolyze the starch and form zone of hydrolysis. After incubation the plates were flooded with iodine solution to observe the enzyme activity.

C. Wash performance assay:

Due to the growing interest for amylases in detergent industry, the stability of our crude enzyme was analyzed with the detergent. For this, small white cotton clothes were stained with beetroot gravy and chocolate separately and were left overnight. Next day the clothes were washed with water and treated with crude enzyme and detergent as follows:

1. Flask 1: 100 ml distilled water with both the stained clothes in separate flasks.
2. Flask 2: 98 ml distilled water + 2 ml of 1% Surf excel detergent with stained cloths.
3. Flask 3: 96 ml distilled water + 2 ml of 1% Surf excel detergent + 2 ml crude enzyme with the stained cloths.

All the flask were then incubated for 30 min at 37°C in the shaking incubator. After incubation time, the cloth pieces were rinsed with tap water, dried and were compared for stain removal capacity with cloth from flask 1 as control [4].

D. Juice Clarification:

Freshly prepared grape juice was mixed with different concentrations of crude enzyme and incubated for 24h at room temperature. Total soluble solids (TSS) by filter paper assay method and clarification of juice were observed. Briefly 20ml of the sample was passed through pre-weight Whatman filter paper. The filter paper was dried at 100°C for 20 min and was re-weighted. The TSS was calculated using following formula [14]:

$$\frac{\text{Weight (final)} - \text{Weight (initial)}}{\text{Sample volume}} \times 10^6$$

Results

Screening of Amylase Producers:

A total of 14 isolates were obtained from the samples that were individually screened for starch hydrolyzing property on starch agar plates. Out of these 14, 7 did not form any zone of hydrolysis around them after incubation. This indicates them as non-amylolytic bacteria. Two best producers, isolate 1 (MIK1) from enriched soil and isolate 2 (MIK2) from bread with 21.7- and 17.5-mm zone of hydrolysis were selected for further studies (Table 1).

Characterization and identification of amylase producers: Morphological and biochemical characterization was performed to identify and characterize best positive isolates. Both the isolates were found to be Gram positive and had shown circular, smooth, convex colonies of white and orange color respectively (Table 2). Microscopic analysis showed isolate MIK1 cells are arranged as rods in chain and MIK2 appeared as cocci in pairs or tetrads (figure 1). On the basis of biochemical test (Table 3) and 16s RNA sequencing the MIK1 is identified as *Bacillus megaterium* and MIK2 as *Exiguobacterium auranticum*.

Table 1: Amylolytic isolates from different sample sources with their zone of hydrolysis.

Isolate Number	Sample Source	Hydrolysis Zone (mm)
1*	Enriched Soil	21.7
2*	Bread	17.5
3	Enriched soil	16.5
4	Rice	14.5
5	Banana	14.0
6	Corn flour	12.5
7	Banana	4.0

Note: * Represents isolates selected for further screening.

Table 2: Microscopic and cultural characteristics of best amylolytic isolates.

Morphology	MIK1	MIK2
Colony form	Circular	Circular
Colony color	White	Orange
Colony surface	Smooth	Smooth
Elevation	Convex	Convex
Gram's nature	Positive (purple)	Positive (purple)
Cell shape	Rods	Cocci
Arrangement	In chains	Pairs and tetrads

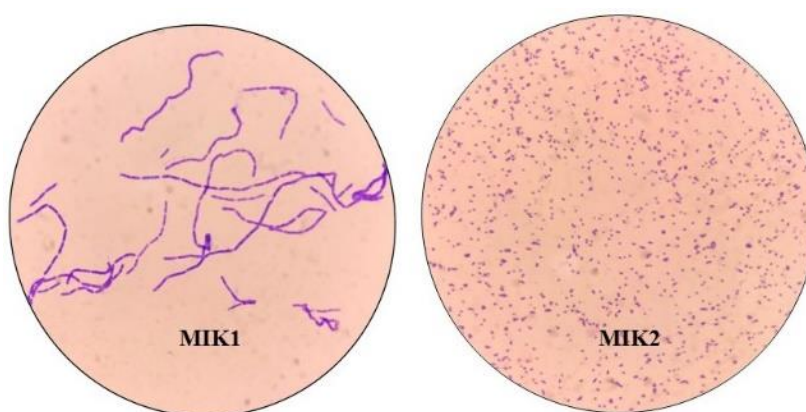


Figure 1: Microscopic images of gram staining of best amylolytic isolates.

Table 3: Biochemical characteristics of best amylolytic isolates.

Biochemical Tests	MIK1	MIK2
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Catalase test	+	+
Gelatinase	+	+
Mannitol salt	+	-
Oxidase	-	-
Urease	+	-
Citrate	-	-
Indole	-	-
Hemolytic	-	-
MR (Methyl Red)	+	+
VP (Voges-Proskauer)	+	-
Glucose	+	-
Sucrose	+	+
Fructose	-	-
Maltose	+	+
Xylose	+	-
Galactose	-	-
Lactose	+	-

Effect of different agro-waste as carbon source: The starch rich wastes (banana peel, potato peel, stale bread, corn flour) were used as carbon source for amylase production taking soluble starch as control. The wastes were used alone or in combination to see the synergistic effect of these waste on enzyme production. Both the strains showed highest amylase production by utilizing banana peels as the carbon source with 230 U/ml and 257 U/ml for *B. megaterium* and *E. auranticum* respectively compared to control (figure 2A).

Effect of different nitrogen source: Three inorganic nitrogen sources namely; ammonium sulfate, ammonium nitrate and sodium nitrate were tested to find out the best nitrogen source for amylase production. Out of these three, ammonium sulphate gives the best amylase enzyme unit in both the strains. The units for *B. megaterium* were found to be 209 U/ml while for *E. auranticum*, 255 U/ml were observed (figure 2B).

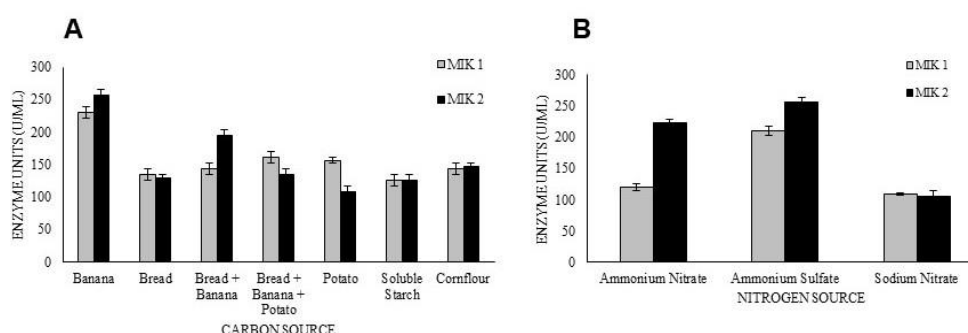


Figure 2: Amylase activity units (A) Carbon sources (B) Nitrogen sources. (MIK1) *Bacillus megaterium*, (MIK2) *Exiguobacterium auranticum*

Effect of temperature: Both the strains were grown under different temperatures ranging from 30-70°C to determine the best temperature for production of enzyme. *B. megaterium* showed highest enzyme production at 37°C with 446 U/ml. Its enzyme production decreases gradually at all the other temperatures suggesting the strain to be mesophilic. *E. auranticum* showed 189 U/ml

at 30°C which increases up to highest unit 295 U/ml at 50°C. Further rise in the temperature resulted in decrease in its enzyme units (figure 3A).

Effect of pH: To analyze the optimum pH for enzyme production by the selected strains, pH of the fermentation media was set to 4.0 - 10.0 before autoclaving by using 6 M HCl or 1 M NaOH. The *B. megaterium* showed good enzyme production in neutral to alkaline range (pH 7.0 to 9.0) with highest units (389 U/ml) observed at pH 9.0. However, enzyme activity drastically declined as the pH reaches at 10.0 reflecting that the strain could not survive extreme alkaline environment. The *E. auranticum* gave notable units of 395 U/ml at acidic pH of 5.0 that decreases gradually at other pH suggesting its acidic nature (figure 3B).

Effect of incubation period: To determine the best incubation time, 100 ml of the inoculated media was incubated at respective temperature and 25 ml was drawn aseptically at 24, 48 and 72 h and the enzyme units were calculated by DNS assay. Both the strains showed same enzyme production pattern i.e., highest enzyme units were observed within 24 h of incubation at the optimized parameters. The *B. megaterium* showed 360 U/ml while *E. auranticum* showed 375 U/ml within 24 h of incubation (figure 3C).

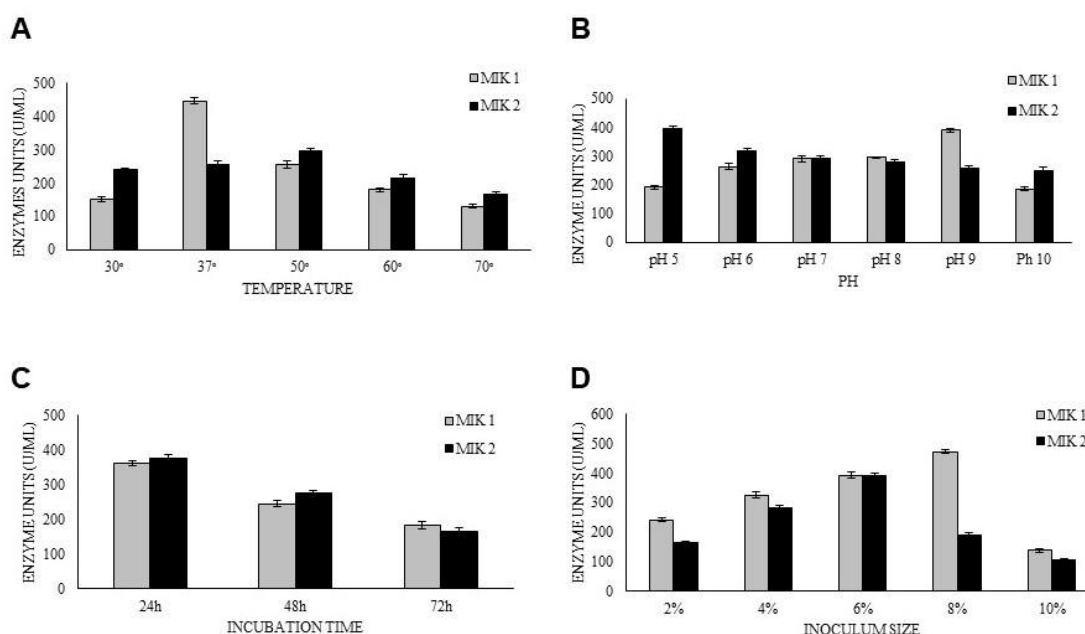


Figure 3: Amylase activity units (A) Temperature (B) pH (C) Incubation time (D) Inoculum size. (MIK1) *Bacillus megaterium*, (MIK2) *Exiguobacterium auranticum*

Effect of inoculum size: For determination of maximum inoculum size for enzyme production, sterile nutrient broth was inoculated with 2-3 colonies from fresh agar plates. The test tubes were then incubated for 24 h until it reaches O.D. of 0.3 at 600 nm. From that, 2%-10% of the broth was transferred into respective flasks and after incubation time, enzyme units were calculated. The *B. megaterium* enzyme production was increased with increasing inoculum size until the size reaches to 8% with an enzyme unit of 472 U/ml. The *E. auranticum* showed increasing units until inoculum size of 6% with enzyme units of 391 U/ml. Further increase in inoculum size for both the strains resulted in sharp decline in enzyme productions units (figure 3D).

Qualitative analysis: The efficiency of the crude enzyme was analyzed by several qualitative tests such as quick tube test, starch plate assay, wash performance, juice clarification and DNS method. In quick tube test, 1% starch in test tubes with drop of iodine was added to give solution

dark blue color. After that 100 μ l of crude enzyme was added upon which the color loss rapidly indicating its strong starch hydrolyzing activity (figure 4A). For wash performance assay, stained cotton clothes (chocolate and beetroot stains) were washed with detergent (2%) alone and with (1%) enzyme solution. The detergent in presence of amylase from our strains showed better stain removal capacity as compared to detergent alone (figure 4C). Juice clarification was visibly observed after 24 h and the TSS content decreased with increasing concentration of amylase of both strains suggesting that the enzyme has degraded various polysaccharides found in the juice.

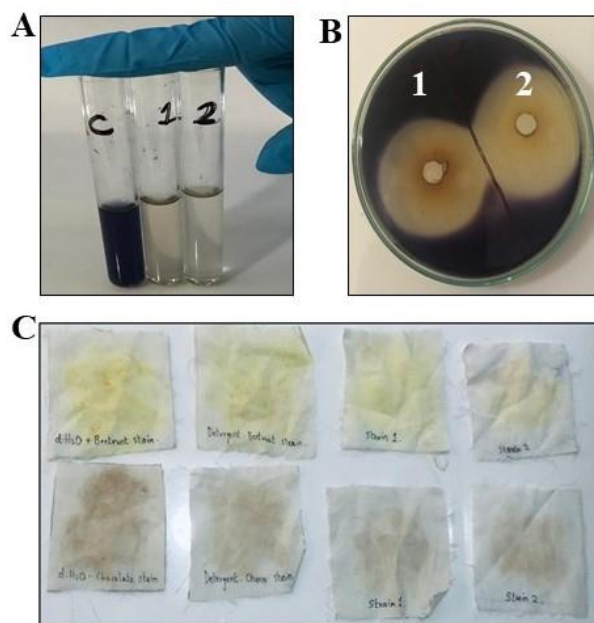


Figure 4: Qualitative tests for Amylase activity (A) Quick tube test (B) Starch agar plate assay (C) Wash performance assay. Upper lane cloth with beet root stain and lower lane cloth with chocolate stain. (Strain1) *Bacillus megaterium*, (Strain 2) *Exiguobacterium auranticum*.

Discussion

Amylases useful for various industrial applications were previously isolated from *Bacillus* strains that can utilize agro-waste carbon sources and showed activity at wide range of temperature and pH [15]. In this study, we have identified two amylase producing bacterial strains, *B. megaterium* and *E. auranticum* from enriched soil. Both the strains give highest units in the presence of banana peels as a carbon source compared to starch and other starch-rich substrates. The selection of organic and inorganic nitrogen sources in fermentation medium increases microbial metabolism and growth [6]. Both the isolated strains gave best enzyme unit with ammonium sulphate as nitrogen source followed by ammonium nitrate. Similar findings were reported where ammonium sulphate showed highest amylase titers [16]. The temperature of media directly influence growth of organism and pH affects transport of various metabolites across cell membrane including enzymes and their stability along with microbial physiology [17]. Bacterial amylases are known to be best produced at temperatures ranges between 30-60°C [3]. Alpha-amylases from salt tolerant and thermostable, and mesophilic strains of *B. megaterium* had been reported which showed optimum temperature for the amylase production at 35-37°C. [18]. The enzyme produced by *B. megaterium* identified in our studies showed optimal activity units at 37°C and pH of 7-9 indicating it to be mesophilic alkaline in nature. The production of amylases at the alkaline pH 8.0-9.5 had already been observed in many *Bacillus* species [19, 20]. On the other hand, minimal literature is available for amylase production by *Exiguobacterium* with no report by *E. auranticum* using banana peels. Previously, a novel, psychotropic to mesophilic (34 kDa) α -

amylase was identified from *Exiguobacterium* sp. that showed activity at 0, 10 and 37°C with optimum activity at pH 7 [21]. Also, a 54 kDa thermostable α -amylase active at 45°C, pH 8.5 has been isolated from hot spring isolated *Exiguobacterium* sp. The crude enzyme showed better stain removal capacity in presence of soapnut extract that is known for its cleaning abilities [22]. The isolated *E. auranticum* gives active enzyme production between 30-70°C with optimal enzyme production at 50°C, pH 5. This is the first report of α -amylase production from thermostable *E. auranticum* in acidic environment. Thermo-stable enzymes possess high commercial value due to higher rate of catalysis, stability and stain removal capacity in detergent formulations whereas fermentations carried out for shorter or longer period of time than the optimum duration results in low yield due to low microbial growth, exhaustion of nutrients, release of toxic substances and proteases [23]. Both the strains showed highest units for the alpha amylase production at 24 h of incubation. Lower inoculum size also results in lower enzyme production as there are low number of cells input in the fermentation media. These cells take longer time to reach at the optimum number for formation of the required product [24]. Both the isolated strains, *B. megaterium* and *E. auranticum* showed maximum enzyme production at 6 to 8% inoculum size. A similar trend was observed with *B. subtilis* where the highest enzyme activity was reported with the inoculum size of 7.5% [16]. After optimizing all the parameters, *B. megaterium* and *E. auranticum* produced 472 and 391 U/ml respectively. The amylases produced by them showed potent starch hydrolyzing activity as evident by qualitative tests. Enzymes such as amylases are in high use in detergents as “green chemicals” due to their tough stains removing capacity under mild conditions and contributes in safer environment. Amylases are most commonly used in liquid and laundry detergents [1,25]. Amylase produced by both the isolated strains were able to remove tough stains in combination with detergent and could be potentially useful in detergent formulations due to their temperature and pH stability. Enhanced juice clarification with reduction in total suspended solids shows that amylase treatment resulted in decrease in juice cloudiness which is an important factor in packaged juices production. The produced amylases inherently degrade carbohydrates very promisingly so they could be employed to achieve clarity and bright color of juices in food industry.

Conclusion

Two extracellular amylase producing gram positive strains; *Bacillus megaterium* and *Exiguobacterium auranticum* were isolated from soil and bread samples respectively. Both the strains showed maximum enzyme production in presence of banana peel and ammonium sulfate as carbon and inorganic nitrogen source respectively at 24 h of incubation and 6 to 8% initial inoculum size. The *B. megaterium* is found to be mesophilic alkaline amylase producing strain. On the other hand, *E. auranticum* found to be acidic thermophilic in nature which is the first report according to the literature survey and to the best of our knowledge. The maximum enzyme units after optimization of growth conditions are found to be 472 and 391 U/ml by *B. megaterium* and *E. auranticum* respectively. The wash performance and TSS assay revealed the potential of using these amylases in the detergent and food industry. However, purification of the amylases from these strains would be more beneficial in order for their utilization in these industries.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. The study on humans was conducted in accordance with the ethical rules of the Helsinki Declaration and Good Clinical Practice.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

None.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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