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Original article

## Identification and characterization of thermophilic amylase producing bacterial isolates from the brick kiln soil

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## ABSTRACT

The present experiment was designed to isolate bacterial strains from the brick kiln soil and to check the activity and enzyme kinetics of amylase from these isolates. The bacterial colonies were isolated from soil samples through the serial dilution method. The bacterial isolates were identified through morphological, electron microscopic and molecular analysis. The 16S ribosomal RNA sequences of the isolates IR-1, IR-2, IR-3, IR-8, and IR-9 showed high similarities with *Bacillus tequilensis*, *Bacillus paramycooides*, *Proteus alimmentorium*, *Bacillus wiedmannii*, and *Pseudomonas aeruginosa*, respectively. All of the bacterial isolates showed a positive catalase activity except IR-9. Furthermore, the isolates showed variable antagonistic effects against different bacterial pathogens. All of the strains produced indole acetic acid (IAA), and the concentrations increased in the presence of tryptophan application. The isolates showed the amylase enzyme activity and maximum activity of isolates was achieved in 4% starch concentration. The IR-9 isolate showed the highest amylase activity of 5.9 U/ml. The  $V_{max}$  values of the extracellular amylase from different bacterial isolates ranged between 12.90 and 50.00 IU ml<sup>-1</sup>. The lowest  $K_m$  value of 6.33 mg starch was recorded for IR-8 and the maximum  $K_{cat}$  value of 2.50 min<sup>-1</sup> was observed for IR-3. The amylase activity of the isolates was significantly affected by a range of different incubation time, temperature, and pH values. Further tests are required before the potential utilization of these isolates for amylase production, and in the biopesticide and biofertilizer applications.

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## 1. Introduction

Thermally adapted enzymes are of special interest for their applications in industries due to their stability at a wide pH range and high temperatures under typical operating conditions. Enzymes isolated from the thermostable organisms (psychrophiles and thermophiles) are a large resource and are under investigation and research for their biotechnological applications. The thermostable organisms, such as *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Bacillus pumilus*, and *Thermus aquaticus*,

produce several extracellular thermostable enzymes (De Carvalho et al., 2008). Extreme temperatures-adapted organisms are enzymes rich resources having widespread thermal properties. However, the thermophilic and psychrophilic enzymes need various chemical and genetic modifications to further improve their characteristics to fulfill various industrial applications (Siddiqui, 2015).

The production of various extracellular enzymes by different microbes is a research topic of prime focus these days. Among different microbes, numerous bacteria produce a variety of commercially important enzymes. Optimized fermentation of the microbes can be used to produce enzymes from thermostable microorganisms or through the mesophiles cloning that grows fast by the recombinant DNA technology (Haki and Rakshit, 2003). The *Bacillus* species such as *Bacillus amyloliquefaciens* and *Bacillus subtilis* are the organisms of choice for amylase production (Ikram-ul-Haq et al., 2005). The alpha-amylases have a wide range of applications in textile, brewing, detergent, backing, and starch industries

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(BravoRodriguez et al., 2006; Gupta et al., 2003; Liu and Xu, 2008; Sarikaya et al., 2000).

The thermostable bacteria have unusually thermostable enzymes, which are similar to those enzymes found in mesophilic bacteria (Battestin and Macedo, 2007). The thermophilic alpha-amylases have widespread industrial applications in the production of sugar, brewing, and processing starch (Lévêque et al., 2000). The most important characteristic of the industrial enzymes is their ability of thermostability. The *Bacillus* sp. ANT-6 was reported to produce thermally stable alpha-amylases (Burhan et al., 2003). The *Bacillus subtilis* JS-2004 strain produces high amounts of thermostable alpha-amylases and is highly suitable for applications in the starch processing and food industries.

The amylases can be categorized into three types, i.e. alpha-amylase, beta-amylase, and gamma amylase. The alpha-amylase is one of the most important industrial *endo*-amylases that is capable of hydrolyzing the internal 1,4-glycosidic linkages to glucose, maltose, and dextrin (Gupta et al., 2003). The alpha-amylases are calcium metalloenzymes, which are completely unable to function in the calcium absence, and carbohydrates of long chains are broken down into sugar components to provide energy. The beta-amylases are slower acting than that of alpha-amylases. In the physiology of humans, both pancreatic and salivary amylases are alpha-amylases, also found in bacteria, plants, and fungi. The beta-amylase secretes succeeding units of maltose from the non-reducing end of a polysaccharide chain by alpha 1, 4-glucan linkage hydrolysis. The beta amylases can be synthesized by bacteria, fungi, and plants. The gamma amylase is different from other forms of amylases. It is most effective in acidic environments with an optimum pH of 3.0.

So far, limited numbers of enzymes have been isolated from extremophiles that showed activities at both lower and high temperatures. The  $\beta$ -galactosidase, isolated from *H. lacusprofundi*, showed activities at a broad range of temperatures i.e. –5 to 60 °C (Karan et al., 2013). Some cellular components with a high level of thermostability reveal that surviving may approach close to 250 °C on their upper end (Fuchida et al., 2014). For example, the most thermally stable protein rubredoxin from *Pyrococcus furiosus* found to date has a melting temperature of 200 °C (Hiller et al., 1997). While the psychrophilic organisms living at an extremely lower temperature in brine films between –15 °C to 250 °C (Mykytczuk et al., 2013). Hyperthermophilic organisms are metabolically active organisms that live in boiling mud pots, geysers, hydrothermal vents, and hot springs at 120 °C (Dalmaso et al. (2015).

The time–temperature integrator (TTI) is a critical application of extremophilic enzymes for the confirmation of the sterilization process in the packing and processing of food. An alpha-amylase from the hyperthermophilic *P. furiosus* with a  $T_m$  around 110 °C has been recommended recently to be used in the food industry as a TTI (Brown et al., 2013). In various fluids of the human body, the activity level of alpha-amylase have clinical importance e.g. in cancer, pancreatic problems, and diabetes (Abou-Seif and Youssef, 2004), while plant and microbial alpha-amylases are used as industrial enzymes (Lin et al., 1998). The amylolytic enzymes, which degrade starch, are of great importance in different biotechnological applications such as fermentation, food, paper, and textile. The starch hydrolysis by chemical means in the starch industries has been fully replaced by microbial amylases (Pandey et al., 2000). One of the limitations of the thermophilic enzymes is their negligible activity at the lower temperature. This limitation can be converted into an advantage with lignocellulose degrading enzyme through designer plant construction. The thermophilic enzymes are inactive at ambient temperatures during the growth phase of the mesophilic plants but are activated to promote hydrolysis of the cellular biomass for biofuel production at a higher

temperature of the post-harvest phase (Guerrero et al., 2016; Klose et al., 2012).

Although several studies have demonstrated isolation and characterization of thermophilic bacteria and their enzymes from the extreme environments, the present study was designed to isolate extremophilic bacteria from the brick kiln soil and to investigate their potential of producing enzymes possessing high biotechnological and environmental applications.

## 2. Materials and methods

### 2.1. Sample collection and culture initiation

The soil samples were collected from a brick kiln at Harichand Charsadda, Khyber Pakhtunkhwa, Pakistan. The bacterial colonies were isolated through the serial dilution method. Briefly, one gram of soil was suspended in 9 mL of double distilled water and was further diluted five times. The samples were then spread on Luria Bertani (LB) agar plates [1% NaCl (w/v), 0.5% Yeast Extract (w/v), 1% Trypton (w/v), pH 7.0], solidified with 1.5% Agar and incubated at 32 °C for 24–48 hrs. The plates were examined after every 12 hrs to select and inoculate the bacterial colonies on new LB agar plates to get pure colonies. The bacterial colonies were further preserved as liquid cultures in LB media at 4 °C and as glycerol stocks at –80 °C.

### 2.2. Morphological and gram-staining analysis

The morphology of the colonies or colonial characteristics of the isolated bacterial strains such as size, shape, margin, elevation, opacity, and color were observed macroscopically. The bacterial colonies were streaked on LB agar and were observed for different morphological characteristics after 24 hr of incubation. The gram staining was performed by the Potassium Hydroxide (KOH) technique using fresh cultures after 18–24 hrs of growth following the standard procedures (Buck, 1982). Briefly, a 20  $\mu$ l aliquot of 3% KOH was placed on a slide and was mixed with a visible amount of bacterial culture transferred through a sterile loop. The cells and KOH solution were thoroughly mixed on the slide for 40–60 s. The appearance of a marked viscosity or gelling indicated the isolate to be a gram-negative. The absence of gelling indicated the isolate to be gram-positive.

### 2.3. Scanning electron microscopic analysis

SEM analysis of the isolates was conducted using S-3400 N field-emission scanning electron microscope (SEM, Hitachi, Japan). The overnight cultures of the isolates were centrifuged at 8000 rpm for 5 min. The cell pellets were then washed with 0.2 M Phosphate buffer (PBS) (pH 7.2–7.4) followed by fixation with 2.5% glutaraldehyde for 3 hr. Cell pellets were then washed twice with PBS followed by dehydration by a concentration gradient of 50%, 70%, 80% and 90% and 100% ethanol for 15 min at each step. The accelerating voltage was kept at 5 kV, and images were collected digitally from the emitted secondary electron signal.

### 2.4. Molecular identification and phylogenetic analysis

For genomic DNA isolation, the bacterial strains were cultured overnight in LB media at 37 °C with 150 rpm shaking. The genomic DNA of bacterial cultures was isolated through the CTAB method (Wilson, 1997). The amplification of the 16S rRNA gene was performed with universal primer 27F1 (5'-AGAGTTTGATCATGGCT CAG-3') and 1494Rc (5'-TACGGCTACCTTGTTACGAC-3'). The 25  $\mu$ l reaction mixture contained 2.5  $\mu$ l of 10 X buffer (with 2.5 mM

MgCl<sub>2</sub>), 2.0 µl of 20 pmole reverse and forward primer each, 3.0 µl (2.5 mM each) of dNTP mixture, 0.5 µl (2.5 U) Taq DNA polymerase, and 50 ng of DNA template. The DNA thermal cycler (T100, BioRad, USA) was used for the amplification of DNA samples. The conditions used in the PCR reaction were as follows; initial denaturation at 94 °C for 3 min, 30 cycles each cycle consisted of denaturation at 94 °C, annealing at 54 °C and primer extension at 72 °C for 1 min each and a final extension at 72 °C for 10 min. The PCR products were then visualized under the UV light in a gel documentation system (Vilber Lourmat). The resulting amplicons were gel-purified through QIAquick purification kit (Qiagen, Germany) and were sequenced (Macrogen, Korea). The sequences were BLAST searched in the NCBI GeneBank database against the known homologous bacterial 16S ribosomal RNA sequences. The identified sequences were aligned using ClustalW and the phylogenetic tree was constructed based on the Neighbor-Joining (NJ) algorithm (Saitou and Nei, 1987) using the MEGA 7 software (Kumar et al., 2018). The sequences were then submitted to GenBank for the acquisition of accession numbers.

### 2.5. Catalase test

The catalase test was carried out according to the method described by (Chandra et al., 2016). Briefly, the cell culture plates were kept at 37 °C for an hour to make the bacterial colonies functional, then a small number of bacterial colonies were transferred to a sterilized glass slide and a drop of 3% H<sub>2</sub>O<sub>2</sub> was mixed with it. The positive results were evidenced by the formation of bubbles while colonies with no bubbles or only a few scattered bubbles were considered negative for the catalase activity.

### 2.6. Indole acetic acid (IAA) assay

The indole acetic acid (IAA) was measured following the method of Gordon and Weber (1951). Briefly, the isolated strains in LB medium were grown overnight at 37 °C with 100 rpm shaking. A 2 mL cell culture of each isolate was mixed with a few drops of Orthophosphoric acid and an equal amount of Salkowski's reagent (Patten and Glick, 2002). The samples were kept at dark conditions for an hr. After incubation, the appearance of pink color indicated the production of IAA. The Optical Density (OD) of the samples was measured at 535 nm with UV-Vis Spectrophotometer and the IAA concentrations were determined based on the standard curve for IAA.

### 2.7. Determination of amylase activity

The individual bacterial strains were grown in LB media at 32 °C for 12–24 hrs to release the amylase enzyme in the extracellular medium. A 2 mL of the overnight bacterial culture was centrifuged at 12,000 rpm. The pellet was discarded and the supernatant that contained the crude amylase enzyme was used for the assay (Abdel-Fattah et al., 2013). The supernatant was supplemented with 1 mL of 2% starch; the pH of the reaction mixture was adjusted to 7.0 and was incubated at 37 °C for 3 hrs. After incubation, 0.5 mL of iodine was added and the OD was measured at 660 nm using the spectrophotometer (Mishra and Behera, 2008).

### 2.8. Antimicrobial activity test

The isolated bacterial strains were screened for their antimicrobial activity against several bacterial strains such as *Citrobacter* sp., *Calciobacter* sp., *Bacillus* sp., *Escherichia coli*, and *Xanthomonas* sp. The disc diffusion method was used for measuring the zones of inhibition of the test strains. The agar plates with sterile filter paper discs were inoculated with the test microbes. The agar plates were then

incubated at 37 °C for 24–48 hrs and the zones of inhibition were measured.

### 2.9. Formation of a standard curve for starch

To find the amount of starch reduced in the medium by amylase enzyme from the bacterial isolates, a standard curve for starch was formulated. Different concentrations of the starch solution were added to separate test tubes. From each concentration of starch, 1 mL was added to 2 mL of LB and incubated for 3 hrs at 37 °C. After incubation, 0.5 mL (500 µl) of iodine was added and the OD was measured at 660 nm using a spectrophotometer, and a standard curve was made from these values.

### 2.10. Kinetic analysis of amylase from the isolates

The kinetic analysis of the extra-cellular amylase of the different isolates was measured after incubating the supernatant with different concentrations of starch (0.5, 1, 2, 3, and 4%) and measuring a decrease in starch concentration as mentioned above. The enzyme activity was expressed as International Units (IU) with one unit equivalent to hydrolysis of 10 mg Starch 10 min<sup>-1</sup>ml<sup>-1</sup>. Lineweaver-Burk plot was then employed to obtain Michaelis-Menten kinetics results (Miao et al., 2015).

### 2.11. Effect of time, temperature, and pH of the reaction on amylase activity

The inoculums of all the isolated strains were grown in LB medium as previously described. The cells were harvested and the supernatant was used as crude extra-cellular amylase enzyme. The crude extra-cellular amylase from each strain was incubated with 2% starch and incubated for different periods or at different temperatures (35 °C to 80 °C) and pH (4.0–10.0) to determine the effect of culture conditions on the enzyme activity.

### 2.12. Statistical analysis

Means and standard deviations were calculated through Microsoft Excel Ver. 2010. The data were statistically analyzed through analysis of variance (ANOVA). Means were compared through the LSD test at a probability of  $\alpha = 0.05$ .

## 3. Results

### 3.1. Isolation and morphological identification

The incubation of serially diluted brick kiln soil in the LB agar plates for 48 hrs at 32 °C resulted in the isolation of 5 distinct bacterial colonies. The pure cultures of these isolates were further tested for macroscopic observations. Morphological traits such as colony color, shape, margins, elevation, and opacity were carefully observed to characterize the bacteria into 5 distinct isotypes (Table S1). The different bacterial isolates were named as IR-1, IR-2, IR-3, IR-8, and IR-9. The colonies of the IR-1 and IR-8 isolates were found with creamy whitish. The IR-3 isolate produced creamy yellow colonies, while the IR-2 and IR-9 produced white and yellowish colonies. The colonies of all strains were circular except those of IR-1 that were irregular in shape. The data regarding the colony margins revealed that the colonies of IR-1, IR-3, and IR-8 were entire while those of IR-2 and IR-9 had undulate margins. The colony elevation of IR-1, IR-2, and IR-3 was recorded as flat, while colonies of IR-8 and IR-9 were raised in elevation. The opacity was recorded for the strains and all were found to be translucent. Further characterization and identification were conducted

through scanning electron microscopic (SEM) analysis (Fig. 1). The SEM analysis revealed the cellular morphological features of the isolates.

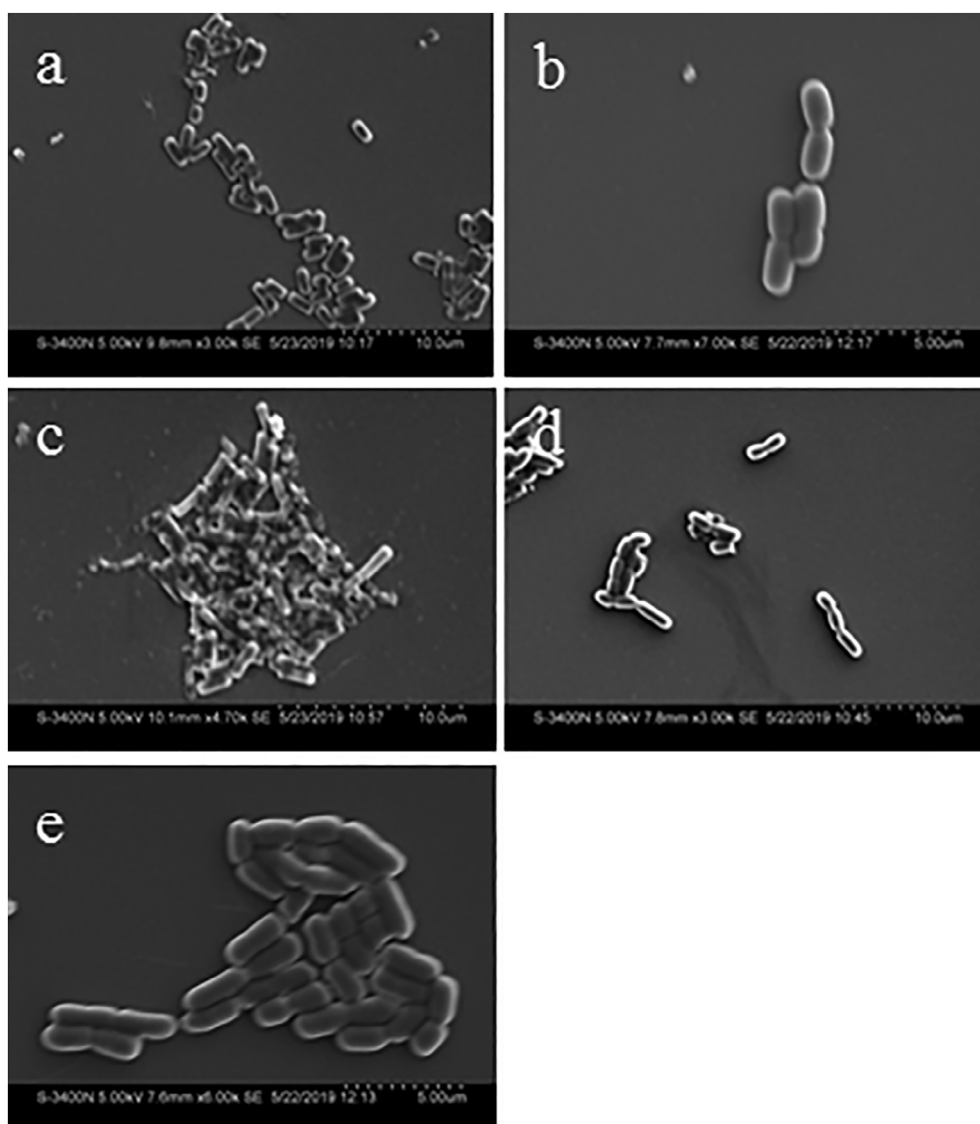
### 3.2. Molecular and phylogenetic analysis

About 1400 bp sequences of all isolates were amplified and sequenced. The sequences were BLAST searched against the homologous 16S ribosomal RNA sequences. A phylogenetic tree was constructed based on the Neighbor-Joining algorithm that showed the close homologous sequences of all the bacterial isolates (Fig. 2). According to the BLAST search in the NCBI database and the phylogenetic tree, the isolates IR-1, IR-2, IR-3, IR-8, and IR-9 showed close homologies with *Bacillus tequilensis*, *Bacillus paramycooides*, *Proteus alimentorum*, *Bacillus wiedmannii*, and *Pseudomonas aeruginosa*, respectively. The top hit strains with accession numbers and the percent identities are given (Table 1). The sequences of all the bacterial isolates were submitted to the GeneBank for accession numbers. The 16S ribosomal RNA sequences of

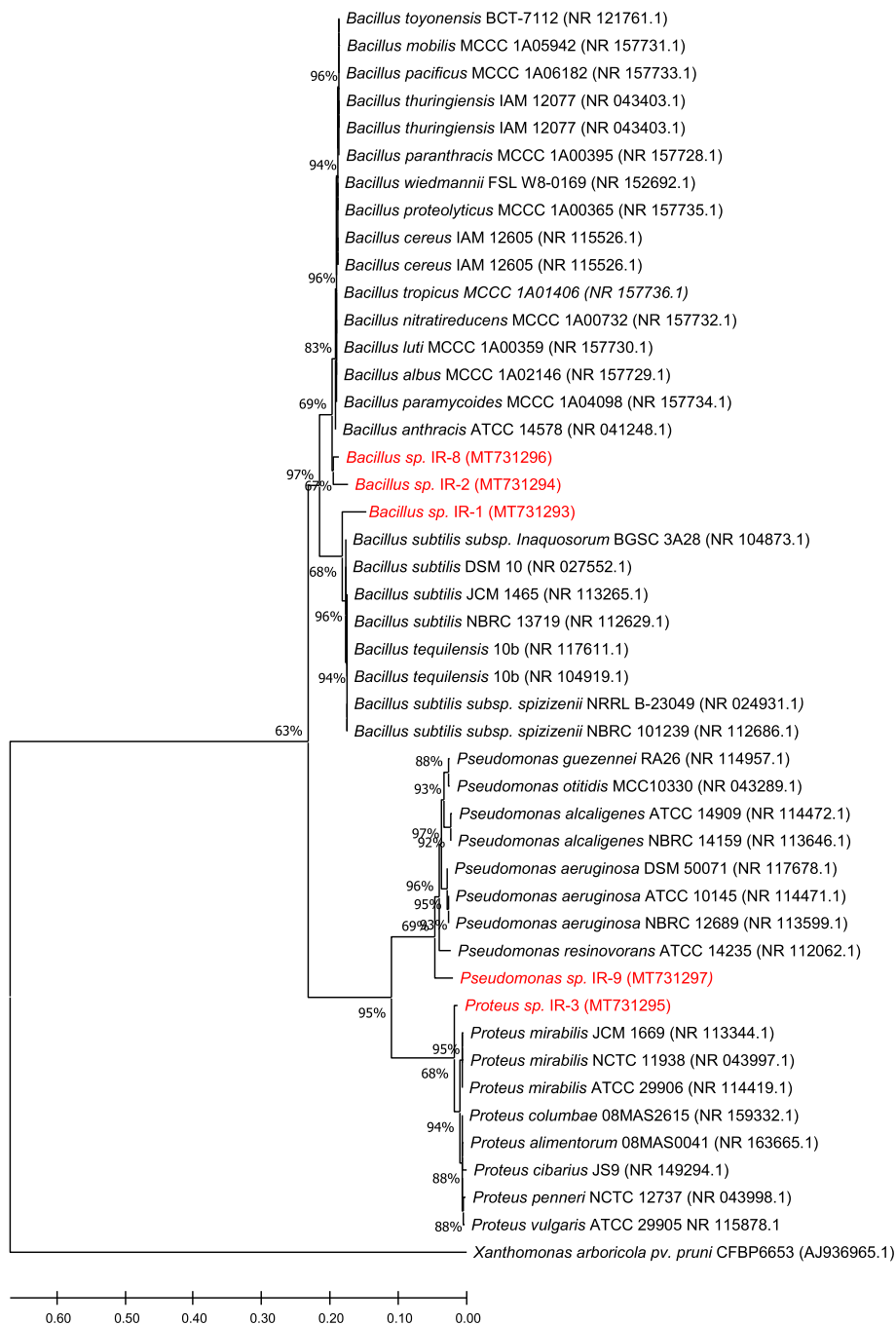
IR-1, IR-2, IR-3, IR-8, and IR-9 were assigned accession numbers as MT731293, MT731294, MT731295, MT731296, MT731297, respectively.

### 3.3. Biochemical characterization of the bacterial isolates

The isolated strains were subjected to different biochemical tests i.e. catalase, gram staining, IAA production, and amylase activity (Table S2). The presence of effervescence was observed as an indication that the isolated strains were capable of producing catalase enzyme. The effervescence is observed due to the catalase activity of breaking down the  $H_2O_2$  into water and oxygen molecules. All the strains were found to be positive for catalase activity except for the IR-9. Using the KOH method, all of the isolates were differentiated into gram-positive and gram-negative. Out of the 5 isolates, the IR-3 and IR-9 were gram-negative, while IR-1, IR-2, and IR-8 were gram-positive. The production of the IAA was observed for all the strains and it was found that except IR-8, all strains were able to produce IAA. Furthermore, all the strains were



**Fig. 1.** Scanning electron microscope (SEM) analysis of the bacterial isolates. SEM images showed small linked rods of *Bacillus tequilensis* (a), *Bacillus paramycooides* (b), *Proteus alimentorum* (c), *Bacillus wiedmannii* (d), and *Pseudomonas aeruginosa* (e).



**Fig. 2.** The phylogenetic analysis using the 16S rRNA gene sequences of the bacterial isolates. The sequences were aligned through “Clustal W” using MEGA 7. A phylogenetic tree was constructed using the Neighbor-Joining method. The bootstrap values are shown as percentages of 1000 replicates. *Xanthomonas arboricola* pv. *Pruni* CFBP6653 (AJ936965.1) was used as an out-group sequence.

found to hydrolyze starch to produce sugars using extracellular amylases.

### 3.4. Antagonistic activity analysis

The strains were screened for their antagonistic activity against five different test bacterial strains; *Citrobacter* sp., *Calcibacter* sp., *Bacillus* sp., *Escherichia coli*, and *Xanthomonas* sp. (Table 2). Streptomycin was used as a positive control antibiotic. The IR-1 strain

showed antagonistic activity against all the test bacterial strains and the highest activities were recorded against *Calcibacter* and *E. coli*. The IR-2 strain exhibited high activities against *Citrobacter*, *Calcibacter*, and *Xanthomonas*, while no activity was observed against *E. coli*. The IR-3 strain showed high antagonistic activities against *Citrobacter* and *Calcibacter* and moderate activities against the other test strains. The IR-8 strain showed moderate activities against all the tested bacterial strains. Likewise, high and moderate activities were also recorded for IR-9.

**Table 1**  
The 16S rDNA sequence analysis of bacterial isolates from the brick kiln soil.

| Bacterial isolates | NCBI accession # | Sequence analysis<br>Closest NCBI taxon, strain, with<br>accession number | Percent<br>identity |
|--------------------|------------------|---|---------------------|
| IR-1               | MT731293         | <i>Bacillus tequilensis</i> 10b<br>(NR_117611.1)                          | 98.41               |
| IR-2               | MT731294         | <i>Bacillus paramycoides</i> MCCC<br>1A04098 (NR_157734.1)                | 97.46               |
| IR3                | MT731295         | <i>Proteus alimenterum</i> 08MAS0041<br>(NR_163665.1)                     | 98.67               |
| IR8                | MT731296         | <i>Bacillus wiedmannii</i> FSL W8-0169<br>(NR_152692.1)                   | 98.10               |
| IR-9               | MT731297         | <i>Pseudomonas aeruginosa</i> DSM<br>50,071 (NR_117678.1)                 | 97.54               |

### 3.5. Indole acetic acid (IAA) production

The IAA was quantitatively measured in all bacterial isolates. The IAA production was determined at various tryptophan concentrations supplemented in the culture medium (Fig. 3). Among all the bacterial isolates, the IR-3 showed the highest production of IAA i.e.  $1.1 \pm 0.06 \mu\text{g ml}^{-1}$  as compared to the other isolates in the absence of exogenous tryptophan concentration. The strains IR-1, IR-2, and IR-9 produced  $0.86 \pm 0.07 \mu\text{g ml}^{-1}$ ,  $0.79 \pm 0.05 \mu\text{g ml}^{-1}$ , and  $0.5 \pm 0.3 \mu\text{g ml}^{-1}$  IAA, respectively at  $0 \text{ mg ml}^{-1}$  tryptophan. The application of exogenous tryptophan in the culture medium increased the IAA production in all isolates. Considerable increases were observed in the IR-1, IR-2, and IR-9 isolates as compared to IR-3, and IR-8 isolates. The lowest IAA concentrations were observed in the IR-8 isolate. These results revealed a positive correlation between the exogenous tryptophan concentrations and the IAA production in the bacterial isolates.

### 3.6. Characterization of amylase enzyme

The collected absorbance data of the samples were converted to the concentration of starch by using the calibration curve for starch (Figure S1). The initial velocity of amylase degradation of the amylase of the starch reaction was calculated from the slope of the initial linear region of the starch concentration versus the absorbance curve. The absorbance data collected were converted to starch concentration by using the starch calibration curve. As many data points as possible were used to construct the linear region such that the sum of error squared generated was a value greater than 0.91.

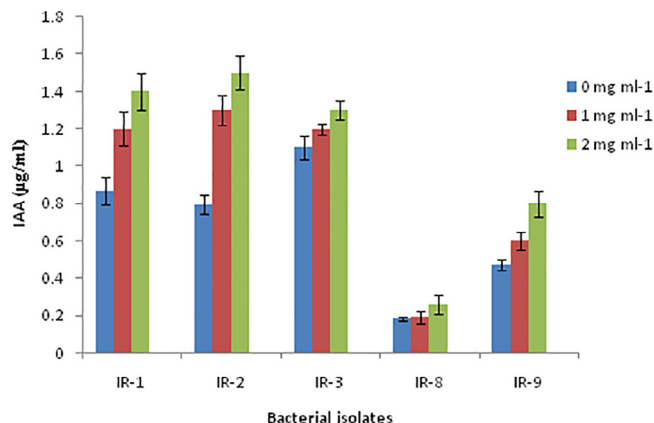
### 3.7. Starch utilization activity of the isolates

The amylase enzyme activities of the strains were calculated at various concentrations of starch (0.5–4%) (Fig. 4). The maximum activities of the strains were achieved in 4% starch concentration. As the concentration of the starch gradually increased, the amylase

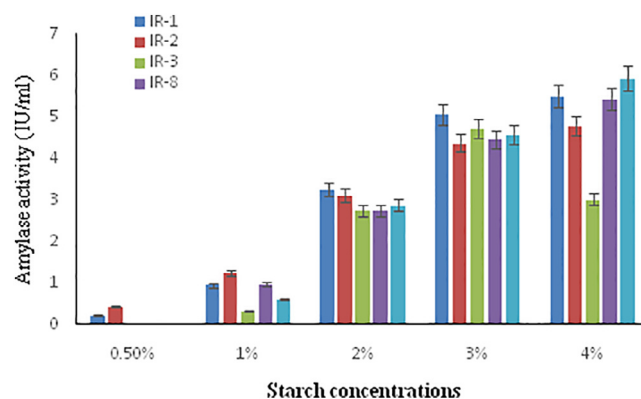
**Table 2**  
The antagonistic activities of the isolated strains against different bacteria.

| Isolated bacterial strains<br>Test isolates | IR-1 | IR-2 | IR-3 | IR-8 | IR-9 | Streptomycin |
|---|------|------|------|------|------|--------------|
| <i>Citrobacter</i> sp.                      | +    | ++   | ++   | +    | ++   | +++          |
| <i>Calcibacter</i> sp.                      | ++   | ++   | ++   | +    | +    | +++          |
| <i>Bacillus</i> sp.                         | +    | +    | +    | +    | +    | +++          |
| <i>E. coli</i>                              | ++   | -    | +    | +    | ++   | +++          |
| <i>Xanthomonas</i> sp.                      | +    | ++   | +    | +    | +    | +++          |

(-) sign shows no activity, (+) sign shows zone of inhibition below 10 mm and (++) shows zones of inhibition above 10 mm.



**Fig. 3.** The effect of various tryptophan concentrations on the production of indole acetic acid (IAA) in the bacterial isolates.



**Fig. 4.** The amylase activity of the bacterial isolates in the presence of various starch concentrations. Data are averages ± SD.

activity of isolates also increased. Among the isolates, the IR-9 showed the highest activity of 5.9 U/ml, followed by 5.5 U/ml, and 5.4 U/ml by IR-1, and IR-8, respectively. The bacterial strains IR-2, and IR-3 showed 4.9 U/ml and 4.7 U/ml optimum activities in the presence of 3% starch concentration. It implies that the 4% starch concentration was favorable at 37 °C for the optimum amylase activity. While the lower starch concentration showed less or no activity.

### 3.8. Kinetic analysis of the amylase enzyme

The Lineweaver-Burk or double reciprocal plot kinetic model of the amylase enzyme activity of different bacterial strains was

expressed using starch as a limited substrate of the reaction using the Michaelis and Menten equation. The plot resulted has a slope that was equal to  $K_m/V_{max}$  and an intercept which was equal to  $1/V_{max}$  (Figure S2). The kinetic constants  $V_{max}$ ,  $K_m$ , and  $K_{cat}$  for the amylase were determined after a fixed period of 3 hrs incubation (Table 3). The  $V_{max}$  values of the extracellular amylase from the different bacterial strains ranged between 12.90 and 50.00 IU ml<sup>-1</sup>. The highest  $V_{max}$  value of 50.00 IU ml<sup>-1</sup> was recorded for IR-3, followed by IR-2 and IR-9 having  $V_{max}$  values of 28.57 and 25.00 IU ml<sup>-1</sup>, respectively. In contrast, the lowest value of  $V_{max}$  12.90 IU.mL<sup>-1</sup> was recorded for IR-8. Similarly, the highest  $K_m$  value of 125 mg was observed for IR-3, followed by 66.67 and 36.36 mg for IR-2, and IR-9, respectively. On the other hand, the lowest  $K_m$  value of 6.33 mg was recorded for IR-8. The maximum  $K_{cat}$  value of 2.50 min<sup>-1</sup> was observed for IR-3, followed by 2.33 min<sup>-1</sup> for IR-2. The lowest value of  $K_{cat}$  i.e. 0.49 min<sup>-1</sup>, on the other hand, was recorded for IR-8.

### 3.9. Effect of temperature

After incubation for three hrs at different temperatures, the enzyme activity was observed. The maximum amylase activity was observed by most of the strains at a range of 45 °C to 55 °C (Table 4). The amylase activity increased in all strains with increasing the temperature from 35 °C to 55 °C. All bacterial strains showed maximum amylase activities at 50 °C. The amylase activities were 8.61, 8.48, 8.47, and 7.84 U/ml in IR-1, IR-3, IR-9, and IR-2, respectively at 50 °C. The maximum amylase activity i.e. 7.57 U/ml was observed in IR-8 strain at 45 °C. On the other hand, the minimum amount of amylase activity was observed at temperatures lower than 45 °C and higher than 55 °C. Among different incubation temperatures, the 45 °C to 55 °C was found to be the favorable temperature range for maximum enzyme activities. Overall, the temperature had a significant impact on the amylase enzyme activities.

### 3.10. Effect of pH

The enzyme was incubated for three hrs with different pH values ranging from 4 to 10 (Table 5). The maximum amylase activity of 5.83 U/ml was recorded in IR-2 at pH 7, followed by 3.64 U/ml in IR-3 at pH 9. While IR-8 and IR-9 showed maximum activities of 3.39 and 2.52 U/ml at pH 6, respectively. Except for the IR-1, the other strains showed maximum enzyme activities at a pH range of 5 to 8. Lower and higher pH values than this range decreased the enzyme activities in all strains.

### 3.11. Effect of incubation time

The crude amylase enzyme obtained from the bacterial strains was subjected to different incubation times at a fixed temperature of 37 °C (Table 6). The amylase activities of the bacterial strains were increased with increasing the incubation time and the maximum activity was recorded for different strains within the incubation time observed. The maximum amylase activity (1.021 U/ml)

**Table 3**  
The enzymatic characteristics of the amylase enzyme of the bacterial isolates.

| Bacterial isolates | $V_{max}$ (IU ml <sup>-1</sup> ) | $K_m$ (mg)   | $K_{cat}$ (min <sup>-1</sup> ) |
|--------------------|----------------------------------|--------------|--------------------------------|
| IR-1               | 16.67 ± 1.3                      | 18.18 ± 1.4  | 1.09 ± 0.1                     |
| IR-2               | 28.57 ± 2.5                      | 66.67 ± 4.1  | 2.33 ± 0.2                     |
| IR-3               | 50.00 ± 3.2                      | 125.00 ± 6.2 | 2.50 ± 0.3                     |
| IR-8               | 12.90 ± 0.7                      | 6.33 ± 0.3   | 0.49 ± 0.02                    |
| IR-9               | 25.00 ± 1.7                      | 36.36 ± 2.8  | 1.45 ± 0.1                     |

was recorded in the IR-1 at 24 hrs of incubation time, followed by 0.91, 0.91, 0.81, and 0.79 IU/ml in IR-2, IR-8, IR-3, and IR-9, respectively.

## 4. Discussion

The brick kiln soil provides a unique environment with high temperatures conducive to the presence of thermophilic bacteria. Such an environment contains a high concentration of heavy metals and toxic gases that make the survival of biological organisms very difficult. The present study was aimed to isolate and identify the thermophilic bacteria with the potential to produce the thermostable amylase enzyme. The bacterial strains were isolated from the brick kiln soil through the serial dilution and spread plate techniques. Similar methods were previously used for the isolation of thermophilic bacteria from the soil samples (Abe et al., 1988). At least five different bacterial strains (IR-1, IR-2, IR-3, IR, 8, and IR-9) were isolated and identified through morphological features and molecular analysis. The electron microscopic and phylogenetic analysis of the 16S ribosomal RNA sequences confirmed these strains belonged to the genus *Bacillus*, *Proteus*, and *Pseudomonas*.

The isolated strains showed variable antagonistic activities against a number of the test bacterial strains. Moreover, the isolated strains were subjected to various biochemical tests such as the gram staining, catalase activity, and the production of the indole acetic acid (IAA) and amylase enzymes. The production of IAA was tested at various concentrations of exogenously applied tryptophan in the medium. All strains showed the production of IAA except the IR-8 that produced very low IAA concentrations. The presence of the tryptophan concentrations increased the IAA production in all strains. These results revealed that exogenous tryptophan had no negative impact on IAA production. Rather, a positive correlation was found between the increasing tryptophan concentrations and IAA production. These results are supported by previous studies where a positive impact of the exogenous tryptophan was found on the IAA production by the bacterial isolates (Idris et al., 2007; Chagas Junior et al., 2015).

The isolated strains were tested for the production of the amylase enzyme. Different starch concentrations were applied, in which higher activity of amylase enzyme was observed at 4% starch concentration in all bacterial isolates except the IR-3 that showed the higher enzyme activity at 3% starch concentration. These results are supported by a previous study. Gebreselema (2015) observed the highest activity of the amylase enzyme at 4% starch concentration. The isolated crude amylase enzyme was incubated at a wide range of different temperatures. Most of the bacterial isolates showed high enzyme activity at a temperature range of 45 °C to 55 °C. Among the five bacterial isolates, four isolates showed maximum enzyme activity at 50 °C, while the IR-8 isolate showed maximum amylase activity at 45 °C. A similar study was also conducted by Mohamed et al. (2009). They reported that some of the alpha-amylases were stable up to 50 °C and some of them at 40 °C after incubation for 15 min. Igarashi et al. (1998) reported that the maximum temperature required for the optimum growth and enzyme activity in *Bacillus sp.* was 30 °C and 55 °C, respectively. Aguilar et al. (2000) conducted characterization and purification of extracellular alpha-amylase from the *Lactobacillus manihotivorans* LMG 18010 T. They concluded that the enzyme activity in the isolate was maximum at a pH of 5.5 and a temperature of 55 °C. They further concluded that the stability of the alpha-amylase was good at a pH range of 5 to 6 coupled with incubation of the enzyme in the presence of soluble starch. The growth of the microorganisms, as well as the amylase production, is affected by slight changes in the temperature and pH (Declerck et al., 2003). In the present study, most of the bacterial isolates

**Table 4**The effect of temperature on the amylase enzyme activities ( $\text{mg ml}^{-1}$ ) in the isolated bacterial strains.

| Temperature | Bacterial isolates      |                         |                         |                         |                          |
|-------------|-------------------------|-------------------------|-------------------------|-------------------------|--------------------------|
|             | IR1                     | IR2                     | IR3                     | IR8                     | IR9                      |
| 35 °C       | 3.20 ± 0.2 <sup>a</sup> | 3.69 ± 0.3 <sup>a</sup> | 3.75 ± 0.2 <sup>a</sup> | 2.03 ± 0.1 <sup>a</sup> | 0.11 ± 0.02 <sup>c</sup> |
| 40 °C       | 5.42 ± 0.6 <sup>a</sup> | 5.44 ± 0.5 <sup>b</sup> | 4.61 ± 0.3 <sup>a</sup> | 5.09 ± .4 <sup>b</sup>  | 3.45 ± 0.2 <sup>a</sup>  |
| 45 °C       | 6.95 ± 0.5 <sup>b</sup> | 6.79 ± 0.4 <sup>b</sup> | 5.21 ± 0.3 <sup>b</sup> | 7.57 ± 0.5 <sup>b</sup> | 7.91 ± 0.5 <sup>b</sup>  |
| 50 °C       | 8.61 ± 0.6 <sup>b</sup> | 7.84 ± 0.5 <sup>b</sup> | 8.48 ± 0.7 <sup>b</sup> | 5.64 ± 0.4 <sup>b</sup> | 8.47 ± 0.7 <sup>b</sup>  |
| 55 °C       | 6.92 ± .4 <sup>b</sup>  | 6.99 ± 0.3 <sup>b</sup> | 5.79 ± 0.2 <sup>b</sup> | 5.17 ± 0.4 <sup>b</sup> | 6.74 ± 0.3 <sup>b</sup>  |
| 60 °C       | 1.12 ± 0.1 <sup>c</sup> | 2.74 ± 0.1 <sup>a</sup> | 5.17 ± 0.3 <sup>b</sup> | 4.71 ± 0.2 <sup>b</sup> | 5.52 ± 0.4 <sup>b</sup>  |
| 65 °C       | 0.74 ± 0.1 <sup>c</sup> | 4.54 ± 0.3 <sup>a</sup> | 2.46 ± 0.5 <sup>a</sup> | 2.79 ± 0.6 <sup>a</sup> | 2.49 ± 0.5 <sup>a</sup>  |
| 70 °C       | 1.01 ± 0.1 <sup>c</sup> | 4.08 ± 0.3 <sup>a</sup> | 4.35 ± 0.2 <sup>a</sup> | 3.01 ± 0.3 <sup>a</sup> | 2.24 ± 0.2 <sup>a</sup>  |
| 75 °C       | 1.83 ± 0.1 <sup>c</sup> | 4.11 ± 0.2 <sup>a</sup> | 3.99 ± 0.3 <sup>a</sup> | 3.04 ± 0.2 <sup>a</sup> | 2.52 ± 0.2 <sup>a</sup>  |
| 80 °C       | 2.66 ± 0.2 <sup>a</sup> | 3.46 ± 0.3 <sup>a</sup> | 4.38 ± 0.2 <sup>a</sup> | 4.71 ± 0.3 <sup>b</sup> | 4.41 ± 0.1 <sup>a</sup>  |

Data are averages ± SD. Values with different letters are significantly different based on LSD test ( $p < 0.05$ ).**Table 5**The effect of pH on the amylase activities ( $\text{IU ml}^{-1}$ ) of the isolated bacterial strains.

| Isolates | pH                       |                          |                         |                         |                          |                          |                          |
|----------|--------------------------|--------------------------|-------------------------|-------------------------|--------------------------|--------------------------|--------------------------|
|          | pH 4                     | pH 5                     | pH 6                    | pH 7                    | pH 8                     | pH 9                     | pH 10                    |
| IR-1     | 0.28 ± 0.02 <sup>b</sup> | 0.14 ± 0.01 <sup>a</sup> | 0.00                    | 2.43 ± 0.2 <sup>d</sup> | 0.98 ± 0.06 <sup>c</sup> | 0.00                     | 0.00                     |
| IR-2     | 0.53 ± 0.03 <sup>a</sup> | 1.12 ± 0.1 <sup>b</sup>  | 3.88 ± 0.3 <sup>c</sup> | 5.83 ± 0.3 <sup>d</sup> | 4.76 ± 0.4 <sup>d</sup>  | 0.00                     | 0.00                     |
| IR-3     | 0.52 ± 0.02 <sup>a</sup> | 0.52 ± 0.09 <sup>a</sup> | 2.05 ± 0.1 <sup>b</sup> | 2.87 ± 0.2 <sup>b</sup> | 2.33 ± 0.3 <sup>b</sup>  | 3.64 ± 0.2 <sup>c</sup>  | 1.81 ± 0.03 <sup>b</sup> |
| IR-8     | 0.99 ± 0.05 <sup>a</sup> | 2.08 ± 0.2 <sup>b</sup>  | 3.39 ± 0.3 <sup>c</sup> | 2.60 ± 0.1 <sup>b</sup> | 3.09 ± 0.1 <sup>c</sup>  | 2.98 ± 0.1 <sup>c</sup>  | 1.83 ± 0.01 <sup>b</sup> |
| IR-9     | 0.20 ± 0.01 <sup>a</sup> | 2.30 ± 0.3 <sup>c</sup>  | 2.52 ± 0.2 <sup>c</sup> | 2.33 ± 0.2 <sup>c</sup> | 1.31 ± 0.02 <sup>b</sup> | 0.41 ± 0.02 <sup>a</sup> | 0.00                     |

Data are averages ± SD. Values with different letters in a row are significantly different based on LSD test ( $p < 0.05$ ).**Table 6**The effect of different incubation times on the amylase activities ( $\text{IU ml}^{-1}$ ) in the isolated bacterial strains.

| Incubation time<br>Strains | Incubation time          |                          |                          |                          |
|----------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
|                            | 3 hrs                    | 6 hrs                    | 12 hrs                   | 24 hrs                   |
| IR-1                       | 0.47 ± 0.02 <sup>a</sup> | 0.57 ± 0.03 <sup>b</sup> | 0.88 ± 0.06 <sup>c</sup> | 1.02 ± 0.1 <sup>c</sup>  |
| IR-2                       | 0.45 ± 0.03 <sup>a</sup> | 0.54 ± 0.05 <sup>b</sup> | 0.68 ± 0.42 <sup>b</sup> | 0.91 ± 0.07 <sup>c</sup> |
| IR-3                       | 0.59 ± 0.04 <sup>a</sup> | 0.62 ± 0.04 <sup>a</sup> | 0.71 ± 0.05 <sup>b</sup> | 0.80 ± 0.08 <sup>c</sup> |
| IR-8                       | 0.51 ± 0.04 <sup>a</sup> | 0.57 ± 0.06 <sup>a</sup> | 0.71 ± 0.06 <sup>b</sup> | 0.91 ± 0.07 <sup>c</sup> |
| IR-9                       | 0.50 ± 0.05 <sup>a</sup> | 0.51 ± 0.03 <sup>a</sup> | 0.60 ± 0.08 <sup>a</sup> | 0.79 ± 0.06 <sup>b</sup> |

Data are averages ± SD. Values with different letters in a row are significantly different based on LSD test ( $p < 0.05$ ).

were found to have maximum amylase activity at pH 7. The IR-3 isolate showed maximum amylase activity at pH 9. While the IR-8 was found with maximum enzyme activity at pH 6. Previous studies showed that the *Bacillus* species produced thermally stable amylases and the enzyme activity was found optimum at pH 8 (Behal et al., 2006; Parmar and Pandya, 2012). In a similar study, the amylase activity was determined at different pH levels from 5 to 10, and it was found that the activity was maximum at pH 7, while the activity decreased at acidic pH of 5 (Singh and Kumari, 2016). Goyal et al. (2005) reported that the alpha-amylase obtained from *Bacillus* sp. I-3 was stable at a pH range of 5–5.5 and had maximum activity at pH 7.

In enzyme kinetics, the  $K_m$  and  $V_{max}$  are the important coefficients. Smaller  $K_m$  value shows a greater affinity of the enzyme towards the substrate (Hamilton et al., 1992). The enzyme followed the Michaelis-Menten kinetics of catalysis. The kinetic constants  $K_m$  and  $K_{cat}$  of the crude amylase were determined using the corn starch. In this study, the  $V_{max}$ ,  $K_m$ , and  $K_{cat}$  values for the amylase enzyme were recorded. For the bacterial isolates IR-2, and IR-3, the  $V_{max}$ ,  $K_m$ , and  $K_{cat}$  values were higher as compared to the other isolates. In IR-2 and IR-3, the  $V_{max}$  values were 50  $\text{IU.mL}^{-1}$  and 28  $\text{IU.mL}^{-1}$ , the  $K_m$  values were 125  $\text{mg ml}^{-1}$  and 66.67  $\text{mg.mL}^{-1}$ , and the  $K_{cat}$  values were 2.50 and 2.33, respectively. In a similar study, Gangadharan et al. (2009) reported  $V_{max}$  and  $K_m$  values for alpha-amylase from *Bacillus amyloliquefaciens*, which were

found to be 3.07  $\text{mg ml}^{-1}$  and 4.11  $\text{mg ml}^{-1} \text{min}^{-1}$ , respectively at 50 °C. Goyal et al. (2005) observed  $V_{max}$  and  $K_m$  values for alpha-amylase from *Lactobacillus manihotivorans*, which were 0.45  $\text{mg ml}^{-1} \text{min}^{-1}$  and 3.44  $\text{mg ml}^{-1}$ , respectively, at 50 °C. Further, the  $K_m$  and  $V_{max}$  values of alpha-amylase from *Bacillus licheniformis* were reported as 8.3  $\text{mg ml}^{-1}$  and 2778  $\text{U mg}^{-1} \text{min}^{-1}$ , respectively, at 60 °C (Haq et al., 2010).

## 5. Conclusion

In the present study, five different bacterial isolates were identified and characterized from the brick kiln soil based on morphological and molecular analysis. The isolates belonged to the genus of *Bacillus*, *Proteus*, and *Pseudomonas*. The bacterial isolates showed variable antagonistic effects against the tested bacterial strains. Preliminary evaluation and characterization of the amylase enzyme were conducted in these isolates. Different growth conditions such as temperature, pH, and incubation time had a significant impact on the production of amylase enzyme in the bacterial isolates. In conclusion, the bacterial isolates with variable antagonistic activities, and the production of IAA and amylase enzymes should be further evaluated for their potential utilization in the biopesticide and biofertilizer industries as well as higher and faster production of industrially important enzymes.



## Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2020.11.017>.

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