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# PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE AMYLASE FROM PSYCHROPHILE

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Abstract: Soil is a complex, dynamic and living habitat for a large number of organisms. Bacteria are an important part of the soil micro-flora because of their abundance, species diversity and the multiplicity of their metabolic activities. Soil is the medium for a large variety of organisms and interacts closely with the wider biosphere; conversely, biological activity is a primary factor in the physical and chemical formation of soils. In the present study, Bacteria from soil samples were isolated and identified according to standard method. 71 isolates were isolated from different region of India. Different isolates were identified as *Klebsiella sp., Micrococcus sp., Bacillus sp., Staphylococcus sp., Enterobacter sp., Citrobacter sp., Neisseria sp., Pseudomonas sp.* and also screened for some enzymes of biotechnological importance. Isolates were screened for hydrolytic activity of amylase, protease and cellulase. All the isolates elaborated enzymatic activity which were rank as follow: amylase 40%, cellulase 30%, protease 21%. Isolates of soil samples were shown highest hydrolytic activity of amylase. This study revealed that isolates associated with soil samples and their ability to produces hydrolytic enzymes. Amylase is highly demanded industrial enzyme in various sectors such as pharmaceutical, food, textiles, detergent. WL-2 isolate was further characterized characterized by using MSA (mannitol salt agar). WL-2 show yellow colour halo on MSA. WL-2 isolate was selected as best amylase producer and identified as *Staphylococcus aureus.* WL-2 isolate was shown amylase positive results by producing clear halos in the TSA agar medium. AS-2 isolate was shown their enzyme activity at 0.879 absorbance which is corresponds to 24000 U of amylase production. Enzyme showed an optimum activity at pH 6.5 and highly stable at optimum temperature at 7.5.

Keywords: Amylase; *Bacillus sp.; Enterobacter sp.; Klebsiella sp; MRSA.*

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## INTRODUCTION

Microbes are rich source of biocatalysts (enzymes). In nature microorganisms have been endowed with vast potentials. They produce an array of enzymes, which have been exploited commercially over the year. In recent year, the potential of using microorganisms as biotechnological sources of industrial relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan 2004; Javani *et al,* 2005; Alva *et al*, 2007). Although enzymes have traditionally been extracted from plants and animals, microbial enzymes has formed the basis of commercial enzymes production due to the increasing availability of these microorganisms, their ease of improvement by the manipulation of their genes and environment great diversity of enzymes have been found that cannot be obtained from plant and animal sources and high production capability at low cost among others ( Onyeocha *et al,* 1983 ; Alves *et al,* 2002; Akinyosoye *et al,* 2003). Enzymes from fungal and bacterial sources have been increasingly applied in industrial sectors (Pandey *et al*, 2000). Amylases contribute as a class of industrial enzymes constituting approximately 25% of the enzyme market (Sindhu *et al*, 1997; Rao *et al*, 1998). It is desirable that α-amylases should be active at

the high temperatures of gelatinization (100-110°C) and liquefaction (80-90°C) to economize the processes. Therefore, there has been a need for more thermophilic and thermostable α-amylases (Sindhu *et al*, 1997). The most widely used thermostable enzymes in the starch industry are the amylases (Poonam and Dalel, 1995; Sarikaya *et al*, 2000). An extremely thermostable α-amylase is produced by *B. licheniformis* (Morgan and Priest, 1981). Amylases are enzymes, which hydrolyze starch molecules to give diverse products including dextrins, and progressively smaller polymers composed of glucose units. The α-amylase family comprises a group of enzymes with a variety of different specificities that all act on one type of substrate being glucose residues linked through an α-1-1, α-1-4, α-1-6, glycosidic bonds. Members of this family share a number of common characteristic properties (van der Maarel *et al*, 2002). Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non-reducing end successively resulting in short end products (Gupta *et al,* 2003). Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market (Sindhu *et al*, 1997; Rao *et al*, 1998). It is desirable that α- amylases should be active at the high temperatures of gelatinization (100-110°C) and liquefaction (80-90°C) to economize processes, therefore there has been a need for more thermophilic and thermostable α-amylases (Sindhu *et al,* 1997). With the availability of thermostable enzymes, a number of new possibilities for industrial processes have emerged (Haki and Rakshit, 2003). While the most widely used thermostable enzymes are the amylases in the starch industry (Poonam and Dalel, 1995; Sarikaya *et al*, 2000).

## MATERIAL AND METHODS

- Sources of media and analytical chemicals: All chemicals used were of analytical grade. Media and chemicals used in this study were purchased from HiMedia, Qualigen and SD fine chemicals, India.
- Soil sampling and analysis: Soil samples were collected at 10 cm deep from Rajasthan, Assam, Tatapani, Wular lake, Manasbal lake. Samples collection was performed by following the standard methods APHA, 1998. The soil texture varies from sandy to sandy clay. Samples were analysed for organic carbon, available phosphorus and for microbial population. The organic C in the soil sample was 1.5%. Percentage availability of total phosphorus was 65. The pH of the soil was in the range 6.62-6.83.
- Isolation of bacterial isolates based on functional screening: Cultivable bacteria were isolated using initial screening in normal saline (0.9%). Population counts of soil samples were determined by dilution plating on TSA media with vortexing at every dilution step. A volume of 100 µl of $10^{-4}$ dilution was spread plated in triplicates on TSA plates. The single colonies were selected and further streaked on same depicted media. Bacterial colonies were screened on TSA media supplemented with starch, skimmed milk, CMC for amylase, protease and cellulase respectively. All the plates were incubated at $28^0$C for 2-4 days.
- Preparation of crude enzyme extract: The pellet of 50 g wet weight of cells was suspended in 150 ml of phosphate buffer (50 mm, pH , 6.0) and disrupted by Ultrasonication (MSE Manor Roya Crawley RH 10 2QQ) at 16 Hz (30 s for five times with intermittent cooling for 5 min) and then centrifuged at 20,124 × g for 10 min at 4°C. The supernatant was then taken in a tube and stored at -20 °C for further analysis.
- Partial purification of enzyme: The supernatant obtained by centrifugation was used as crude enzyme and then fractionated with 10% to 60% saturation of ammonium sulphate. The precipitate was recovered by centrifugation (20,124 g× 10 min at 4°C), dissolved in phosphate buffer (50 Mm, pH 6.0)) and dialyzed overnight against the same buffer. The dialyzed enzyme solution was used for further studies.
- Enzyme assay: Enzyme assay was carried out by using the dinitrosalicylic acid method for the determination of reducing sugar (Miller 1959). The enzyme reaction mixture was composed of

substrate and an appropriate quantity of enzyme in 20 mM sodium phosphate buffer (pH 6.0); 500 µl of the enzyme mixture was incubated at 30°C for 10 min. The reaction was stopped by adding 500 µl of dinitrosalicylic acid solution (10.6 g of 3, 5- dinitrosalicylic acid, 19.8 g of NaOH, 30.6 g of potassium sodium tartrate, 7.6 ml of phenol, 8.3 g of sodium metabisulfate and 1,416 ml of distilled water). The reaction mixture was boiled for 5 min and cooled by placing the tubes on ice. Absorbance was measured at 575 nm in a 1 cm polystyrene cuvette by using a spectrophotometer.

- Effect of pH on amylase activity: The pH profile of AS-1 activity was determined using the following buffers for the different pH range: 4.0 to 6.0; 50 mM sodium citrate, pH 6 to7; 50 Mm sodium phosphate, pH 8 to 10; Tris- HCl.
- Effect of temperature on amylase activity: The optimum temperature for amylase activity of AS-1 was measured by determining its hydrolytic activity at different temperatures (5-85$^0$C) for 10 min at pH 8. The reaction was stopped by adding 500 µl of dinitrosalicylic acid solution (10.6 g of 3,5- dinitrosalicylic acid, 19.8 g of NaOH, 30.6 g of potassium sodium tartrate, 7.6 ml of phenol, 8.3 g of sodium metabisulfate and 1,416 ml of distilled water). The reaction mixture was boiled for 5 min and cooled by placing the tubes on ice. Absorbance was measured at 575 nm in a 1 cm polystyrene cuvette by using a spectrophotometer.

## RESULT AND DISCUSSION

Total 71 isolates were found in soil sample of Rajasthan, Assam, Tatapani, Wular lake, Manasbal lake. All the isolates were functionally screened on specific media for amylase, protease, cellulose activity. All isolates elaborated enzymatic activity which were rank as follows: amylase 40%, cellulase 30%, and protease 21%. Among all these isolates WL-1, WL-2, WL-3, ML-3, ML-7, ML-16, TP-1, TP-2, TP-3, TP-5, TP-6, TP-7, TP-8, TP-9, TP-10, TP-11, TP-12, TP-13, A-4, A-9, A-10, A-11, A-12, A-13, A-14, A-15, A-16 were found positive for amylase showing clear zone by adding 1% starch with TSA media. WL-2 showing the highest zone of clearance as compared as compared to other isolates. Morphological and biochemical characterization of WL-2 was performed and identified as *Staphylococcus auerus* species.

Table 1. Identification of bacterial species from soil

| Isolates code | Gram staining | Cellular morphology | Indol test | MR | VP | citrate | Urease | catalase | Gelatin hydrolysis | Lactose | dextrose | sucrose | $H_2S$ | Identified organism | Phylogenetic analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WL-1 | + | streptococci | - | + | + | - | - | + | + | + | - | - | - | *Staphylococcus sp.* | firmicutes |
| WL-2 | + | streptococci | - | + | + | - | + | + | + | + | + | + | + | *Staphylococcus sp.* | firmicutes |
| WL-3 | + | cocci | - | + | + | + | - | + | + | + | - | - | + | *Staphylococcus sp.* | firmicutes |
| WL-4 | – | Rod | - | + | - | - | - | + | + | + | - | - | - | *Klebsiella sp.* | Proteobacteria |
| WL-5 | – | Rod | - | - | + | + | - | + | + | + | - | - | - | *Enterobacter sp.* | Proteobacteria |
| WL-6 | – | Rod | - | + | + | - | - | + | + | + | - | - | - | *Klebsiella sp.* | Proteobacteria |
| WL-7 | – | Rod | - | - | + | - | - | + | + | + | - | - | - | *Enterobacter sp.* | Proteobacteria |
| WL-8 | – | Rod | - | - | + | + | - | + | + | + | - | - | - | *Enterobacter sp.* | Proteobacteria |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ML-1 | – | Rod | - | + | + | + | - | + | + | + | - | - | - | *Kleibsella sp.* | Proteobacteria |
| ML-2 | + | Cocci | + | + | + | + | - | + | + | + | - | - | + | *Micrococcus sp.* | Actinobacteria |
| ML-3 | + | Rod | - | + | + | + | - | + | + | + | - | - | + | *Bacillus sp.* | Firmicutes |
| ML-4 | + | Cocci | - | + | + | - | - | + | + | + | - | - | - | *Staphylococcus sp.* | Firmicutes |
| ML-5 | – | Rod | - | - | + | - | - | + | + | + | - | - | - | *Enterobacter sp.* | Proteobacteria |
| ML-6 | – | Rod bacilli | - | - | + | - | + | + | + | + | - | - | - | *Klebsiella sp.* | Proteobacteria |
| ML-7 | – | Rod bacilli | - | + | + | + | - | + | + | + | - | - | - | *Klebsiella sp.* | Proteobacteria |
| ML-8 | – | Rod bacilli | - | + | + | + | - | + | + | + | - | - | - | *Klebsiella sp.* | Proteobacteria |
| ML-9 | + | Coccobacilli | - | + | + | - | - | + | + | + | + | - | + | *Staphylococcus sp.* | Firicutes |
| ML-10 | – | Rod | - | + | + | + | - | + | + | + | - | - | + | *Klebsiella sp.* | Proteobacteria |
| ML-11 | – | Rod | - | + | + | + | - | + | + | + | - | - | + | *Citrobacter sp.* | Proteobacteria |
| ML-12 | – | Cocci | - | + | + | - | - | + | + | + | - | - | + | *Neisseria sp.* | Proteobacteria |
| ML-13 | – | Cocci | - | + | + | + | + | + | + | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| ML-14 | – | Cocci | - | + | + | - | + | + | + | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| ML-15 | – | Cocci | - | + | + | - | + | + | + | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| ML-16 | + | Cocci | - | + | + | + | - | + | + | + | - | - | - | *Staphylococcus sp.* | Firmicutes |
| TP-1 | + | Cocci | + | + | + | + | + | + | + | + | - | - | - | *Staphylococcus sp.* | Firmicutes |
| TP-2 | – | Cocci | - | - | + | - | + | + | + | + | - | + | + | *Neisseria sp.* | Proteobacteria |
| TP-3 | – | Rod bacilli | + | + | + | + | + | + | - | - | - | - | - | *Pseudomonas sp.* | Proteobacteria |
| TP-4 | – | Rod | - | + | + | - | + | + | + | + | - | - | - | *Klebsiella sp.* | Proteobacteria |
| TP-5 | – | Cocci | - | + | + | - | + | + | + | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| TP-6 | – | Cocci | - | - | + | + | - | + | + | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| TP-7 | – | Cocci | - | + | - | + | + | + | + | + | + | - | + | *Neisseria sp.* | Proteobacteria |
| TP-8 | – | Diplococci | - | + | + | + | - | + | + | + | + | - | - | *Neisseria sp.* | Proteobacteria |
| TP-9 | + | Rod bacilli | - | + | + | - | - | + | - | + | - | - | + | *Bacillus sp.* | Firmicutes |
| TP-10 | – | Rod bacilli | - | + | + | - | + | + | - | + | - | - | - | *Klebsiella sp.* | proteobacteria |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TP-11 | + | Cocci | - | + | + | - | - | + | + | + | - | - | - | *Micrococcus sp.* | Actinobacteria |
| TP-12 | + | Cocci | - | + | + | - | + | + | - | + | - | - | - | *Staphylococcus sp.* | Firmicutes |
| TP-13 | – | Rod | - | + | + | - | + | + | + | + | - | - | - | *Klebsiella sp.* | Proteobacteria |
| TP-14 | + | Cocci | - | - | + | - | + | + | + | + | - | - | + | *Staphylococcus sp.* | Firmicutes |
| TP-15 | – | Cocci | - | + | + | - | + | + | + | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| TP-16 | – | Streptococci | - | + | + | + | + | + | - | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| A-1 | + | Rod | - | + | + | + | + | + | + | - | - | - | - | *Bacillus sp.* | Firmicutes |
| A-2 | + | Rod | - | + | + | - | - | + | + | - | - | - | - | *Bacillus sp.* | Firmicutes |
| A-3 | + | Rod | + | + | + | + | + | + | - | - | - | - | - | Unidentified | --- |
| A-4 | + | Cocci | + | - | - | - | - | + | + | - | - | - | - | *Staphylococcus sp.* | Firmicutes |
| A-5 | – | Diplococci | + | + | + | + | + | + | - | - | - | - | - | *Neisseria sp.* | Proteobacteria |
| A-6 | + | Rod | + | + | + | + | + | + | + | + | - | - | - | Unidentified | --- |
| A-7 | – | Rod | - | + | - | - | + | + | - | - | - | - | - | *Pseudomonas sp.* | Proteobacteria |
| A-8 | – | Cocci | - | + | + | + | - | + | + | - | - | - | - | *Neisseria sp.* | Proteobacteria |
| A-9 | + | Rod | + | + | + | + | + | + | + | + | - | - | - | Unidentified | --- |
| A-10 | + | Streptococci | - | - | + | - | + | + | + | + | - | - | - | *Staphylococcus sp.* | Firmicutes |
| A-11 | + | Rod | - | - | + | - | + | + | + | + | - | - | - | *Bacillus sp.* | Firmicutes |
| A-12 | + | Streptococci | + | + | + | - | - | + | + | - | - | - | - | *Staphylococcus sp.* | Firmicutes |
| A-13 | – | Cocci | - | - | - | - | + | + | - | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| A-14 | – | Streptococci | - | - | + | - | + | + | + | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| A-15 | – | Cocci | - | - | + | - | - | + | - | - | - | - | - | *Neisseria sp.* | Proteobacteria |
| A-16 | – | Rod | - | + | - | + | + | + | - | - | - | - | - | *Pseudomonas sp.* | Proteobacteria |
| A-17 | – | Rod | - | + | + | - | - | + | + | - | - | - | - | *Unidentified* | --- |
| RJ-1 | + | Cocci | - | + | + | - | - | + | - | - | - | - | - | *Micrococcus sp.* | Actinobacteria |
| RJ-2 | + | Bacilli | + | + | + | + | - | + | - | + | - | - | - | Unidentified | --- |
| RJ-3 | | Rod | - | + | + | - | - | + | + | - | - | - | - | Unidentified | --- |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | – | | | | | | | | | | | | | | |
| RJ-4 | + | Cocci | - | + | + | + | - | + | + | + | - | - | - | *Staphylococcus sp.* | Firmicutes |
| RJ-5 | + | Coccobacilli | - | - | + | + | - | + | + | - | - | - | - | *Bacillus sp.* | Firmicutes |
| RJ-6 | + | Streptococci | - | + | + | - | - | + | + | - | - | - | - | *Staphylococcus sp.* | Firmicutes |
| RJ-7 | – | Cocci | - | + | + | + | - | + | - | + | - | - | - | *Neisseria sp.* | Proteobacteria |
| RJ-8 | + | Streptococci | - | + | + | + | - | + | - | + | - | - | - | *Staphulococcus sp.* | Firmicutes |
| RJ-9 | + | Cocci | - | - | + | + | - | + | - | + | - | - | - | *Staphylococcus sp.* | Firmicutes |
| RJ-10 | + | Streptococci | - | - | - | - | - | + | - | + | - | - | - | *Staphylococcus sp.* | Firmicutes |
| RJ-11 | – | Bacilli | - | - | - | + | - | + | - | - | - | - | - | *Pseudomonas sp.* | Proteobacter |
| RJ-12 | + | Coccobacilli | - | + | - | + | - | + | + | + | - | - | - | Unidentified | --- |
| RJ-13 | + | Coccobacilli | - | _ | + | + | + | + | - | + | - | - | - | Unidentified | --- |
| RJ-14 | + | Rod | - | - | + | + | + | + | - | - | - | - | - | Unidentified | --- |

Table: 2 Percentage of isolates

| ISOLATES | PERCENTAGE |
|---|---|
| *Staphylococcus sp.* | 24% |
| *Niesseria sp.* | 24% |
| *Klebsiella sp.* | 14% |
| *Bacillus sp.* | 8% |
| *Enterobacter sp.* | 6% |
| *Pseudomonas sp.* | 6% |
| *Micrococcus sp.* | 4% |
| *Citrobacter sp.* | 1% |
| Unidentified | 13% |

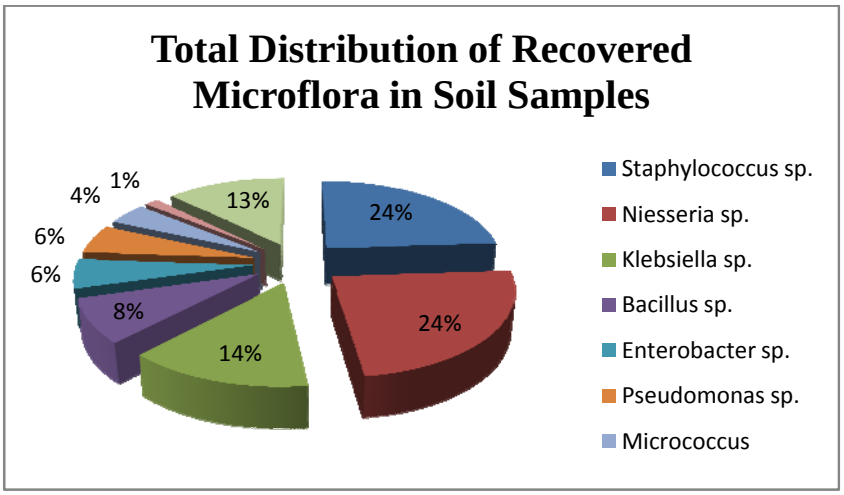


Figure 1. Total distribution of recovered isolates


Figure 2. Isolate AS-1 showed yellow colour of halo on mannitol salt agar

WL-2 isolate show enzyme activity at 0.879 absorbance which is corresponded to 24000 U of amylase production.

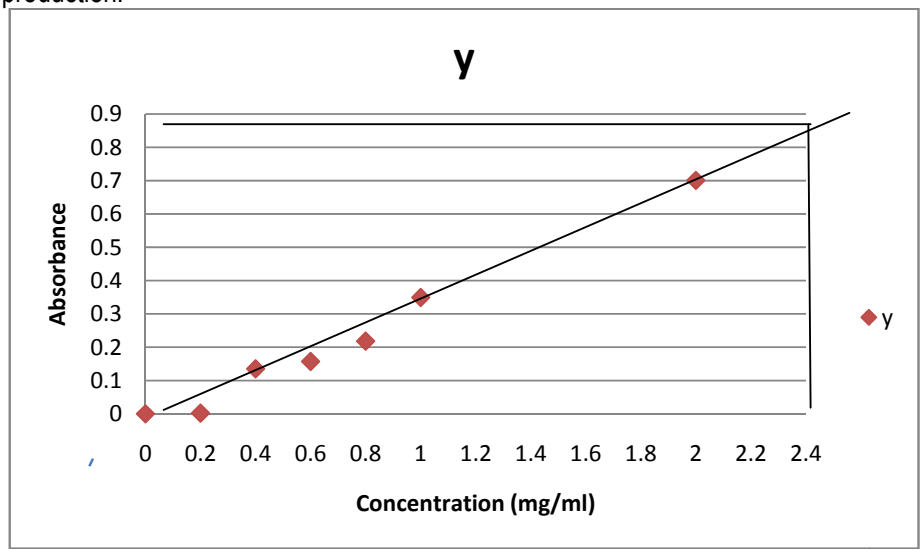


Figure 3. Standard Curve of Maltose

Saccharolytic activity of α-amylase was measured with the DNS method. Absorbance was measured at 575 nm The OD values were then converted to micrograms of maltose equivalent using a standard graph obtained from the known concentration of maltose prepared with the same buffer solutions. A unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of maltose standard per minute under the assay conditions specified.

Table 3. Purification of AS1

| Material | Total protein(mg) | Total activity(U) | Specific activity | Recovery (%) | Purification fold |
|---|---|---|---|---|---|
| Cell free extract | 632 | 565 | 1.2 | 100 | 1.0 |
| Ammonium sulphate Fraction (60%) | 214 | 224 | 2.4 | 78 | 2.0 |

The enzymatic activity of WL-2 was measured at different temperature, pH and substrates. WL-2 isolates, hydrolyze starch molecules to give diverse products including dextrins, and progressively smaller polymers composed of glucose units. The α-amylase family comprises a group of enzymes with a variety of different specificities that all act on one type of substrate being glucose residues linked through an α-1-1, α-1-4, α-1-6, glycosidic bonds (van der Maarel *et al*, 2002). The rate of amylase degradation was higher than any other substrates used in this study. WL-2 showed higher activity in the range from pH 7.0 to10.0 with a maximum observed at 8.0.

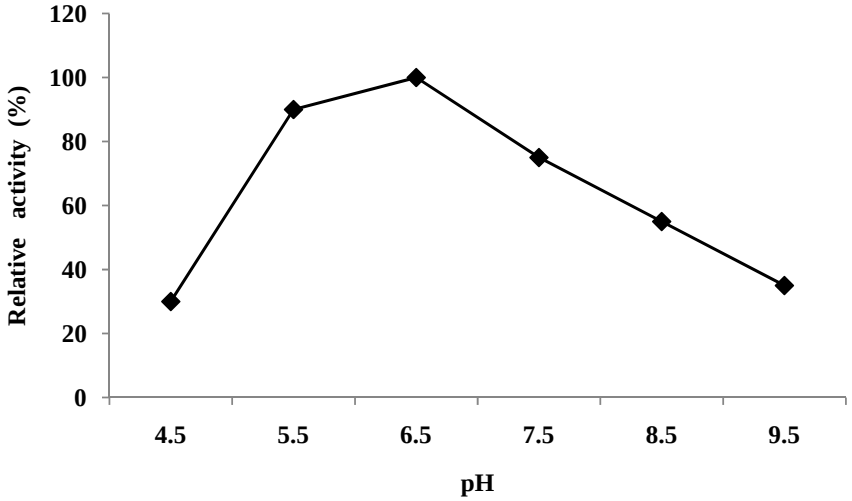


Figure 4. Effect of pH on the activity of AS-1

The reaction mixture contained 500 µl of substrate and 500µl of enzyme. The reaction mixture was incubated at 30°C for 10 minutes. The buffers were used for the different pH ranges: pH 4.0 to 6.0, 50 mM sodium citrate; pH 6.0 to 7.0, 50 mM sodium phosphate; pH 8.0 to 10, 50 mM Tris-HCl. The effect of temperature on partially purified enzyme activity was measured at pH 8.0 over a temperature range of 5-95°C and enzyme showed optimum temperature at 75°C. There are many reports on *Staphylococcus aureus* isolated from soil posses' amylase activity (Aysha Jussara *et al,* 2006).

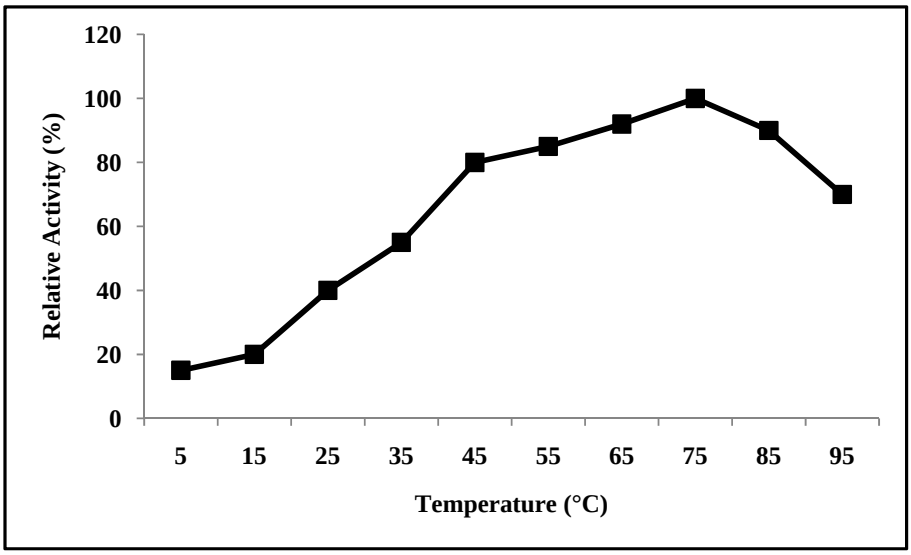


Figure 5. Effect of temperature on the activity of AS-1

The reaction mixture contained 500µl of substrate in 20mM phosphate buffer (pH 6.5) and 500µl of enzyme. The reaction mixture was incubated for 10 minutes at temperature from 10 to 60°C under standard assay conditions. The research work in present scenario to isolate thermostable amylase producer bacteria. Further characterization of amylase activity from WL-2 could prove it as a promising candidate for industrial use. In present study we also screened other bacterial isolates with the ability to produce industrially important hydrolytic enzymes. Exploration and biotechnological potential of microbial community of soil from Mansabal and Wular Lake for cold active enzymes and soil from Tatapani, Assam and Rajasthan for search of thermostable enzymes of industrial purpose is strongly suggested as a result of this study. The use of α-amylase in starch based industries has been prevalent for many decades and a number of microbial sources exist for the efficient production of this enzyme, but only a few selected strains of fungi and bacteria meet the criteria for commercial production. The search for new microorganisms that can be used for amylase production is a continuous process. More recently, many authors have presented good results in developing α-amylase purification techniques, which enable applications in pharmaceutical and clinical sectors which require high purity amylases (Paula Monteiro and Perola De, 2010)

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CONFLICT OF INTEREST: Nothing