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Isolation and characterization of amylase producing thermophilic bacteria from Sita kund in Munger district of Bihar

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Abstract*—Thermophiles are the microorganisms that live and grow in extremely hot environment that would kill most other microorganisms. The present study was conducted to isolate eight thermophilic bacteria from hot spring and its nearby water source at Sita kund, Munger, India and designated HP1, HP2, HP3, HP4, K1, K2, K3 and K4. The isolated bacteria showed their optimum growth at pH 6.8 and grew maximally at 55°C temperature. Isolated bacteria were mostly Gram negative. All samples were ampicillin resistant showing that they had modified the ampicillin binding proteins involved in cell synthesis. The enzymes from microbial sources are more stable and obtained cheaply. Amylases are among the most important enzymes and are of great significance in present day industry. The bacterial sample HP2, K1, K3 and K4 showed amylase production on starch agar plate. Among the four bacterial samples, K4 showed maximum amylase production as compared to others thus encouraging future studies to explore further its industrial and environmental applications. Further molecular study is required for identification and taxonomic characterization of strains.

***Keywords*—Thermophilic bacteria, Sitakund, Amylase, ampicillin resistance**

I. INTRODUCTION

Temperature is a vital parameter for microbial growth and microorganisms may prefer different ranges of temperature to survive. Accordingly, they have been categorized as psychrophiles, mesophiles, thermophiles and hyperthermophiles. Temperature as low as 45°C and as high as 113°C is known to sustain bacterial growth. Thermophiles are organisms with optimum growth temperature above 45°C. Such organisms have attracted microbiologists and biochemists for a long time. The main reason for this attention has originated from more stable protein structure and resistance to various chemical reagents. These microbes belong to two phylogenetically distinct domains of life, Bacteria and Archaea [1]. It is generally agreed that bacteria dominate the microbial community in most hydrothermal environments at temperatures between 50 and 90°C. Identification and characterization of these microbes has been of prime interest because of their economic importance and M. Argano [2] has classified thermophilic prokaryotes into several groups depending on their optimum temperature. The main reason for making it possible to thrive at high temperatures is the stability of their enzymes and proteins as they are more stable at high temperatures because of slight difference in amino acid sequences in proteins as compared with mesophilic bacteria. The stability of proteins is a result of increased number of ion pairs i.e. ionic bonds, between the positive and negative charges of various amino acids. Not only enzymes and proteins have adapted to the high temperature but also the cytoplasmic membrane is accordingly modified [3]. Investigations have shown that they have high growth rates and generation time as short as 1 h [3, 4, 5,].Several microbial enzymes are of immense industrial importance; especially the ones isolated from thermophiles as they are capable of withstanding high temperatures employed in industrial reactor. Amylase is one such enzyme. It is a digestive enzyme that aids in the breakdown of carbohydrates by breaking the bonds between sugar molecules in polysaccharides through a hydrolysis reaction. It is found in animals, plants, and bacteria.
α amylases (E.C.3.2.1.1) are enzymes that catalyze the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such as glucose, maltose and maltotriose units

[6]. Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market [7] (Fig 1). Currently, an effort has been made to characterize amylase activity in some locally isolated thermophilic bacteria. The site of this work comes under Sitakund Dih Nawagarhi Panchayath in Munger Block in Munger District of Bihar, India

II. MATERIALS AND METHODS

A. Sample collection

Samples were collected from the hot spring and its nearby hand pumps of Munger district (East Bihar) region popularly known as "Sita kund". Samples were collected in sterile falcon tubes and temperature and pH were recorded.

B. Isolation of pure colony

Samples were isolated in Thermus agar media (pH 6.8) [8]. and purification was done repeatedly by streaking procedure until a single pure colony was observed.

C. Characterization of culture conditions

Characterization was done for different isolated pure colonies of kund samples and hand pump samples at different

sets of temperature viz. 45ºC, 50 ºC, 55 ºC, 60 ºC and 65 ºC and pH range of 6.4, 6.6, 6.8, 7 and 7.2.

D. Gram staining

Gram staining was performed following the protocol of J.W Bartholomeu [9]. Briefly, crystal violet was applied for 2 min at room temperature in a heat-fixed smear slides and were briefly rinsed under running tap water to remove excess crystal violet. Gram iodine mordant was applied for 1 min and briefly washed in tap water. To remove any non-specific crystal violet staining, a Gram decolorizing agent (Acetone-alcohol decolorizer) was applied to the slides for 30 s then quickly rinsed under running tap water until the water ran clear. The slides were stained with Gram Safranin for 2 min followed by washing to remove excess stain. Samples were then viewed under bright field microscope.

E. Antibiotic sensitivity test

Sensitivity of isolated bacterial strains towards four antibiotics viz. kanamycin (K), neomycin (N), ampicillin (A) and streptomycin (S) was observed by allowing the cultures to grow on plates having above mentioned antiobiotics for 24 hours.

F. Screening for amylase production

The plate containing single isolated colony was incubated for 24 to 48 hours. After proper incubation, Gram's iodine solution was added. Results were recorded immediately as the blue colour formed with starch may fade giving a false-negative result. Appearance of a clear zone surrounding the bacterial growth indicates starch hydrolysis (+) by the organism due to its production of the extracellular enzymes.

G. α amylase assay

0.5 ml of (crude enzyme) culture supernatant was incubated at 25°C for 3 min. Starch solution was further added and incubated for 5 min at room temperature. The reaction was stopped by adding 1 ml DNS reagent. The absorbance was measured at 540 nm using UV-Vis Spectrophotometer.

The enzyme activity was calculated as:

$$\text{Enzyme activity} = \frac{\text{OD (test) x concentration of standard (µmoles) x dilution of enzyme/OD (standard) x incubation time (3 min)}}{}$$

III. RESULTS

A. Sample characteristics

Two different samples were collected from two different sites viz. kund samples – K1, K2, K3, K4 and hand pump samples – HP1, HP2, HP3, HP4. The temperature was found to be 56º and 62º C respectively. The pH of the samples was 6.7 and 7.1 respectively (Fig 2 and Fig 3).

B. Isolation and purification of bacteria

The samples were spread on Thermus agar plates and incubated at 55°C temperature for 8 hours. Both samples showed luxurious growth of four different colonies. Pure cultures were obtained by streaking (Fig 4 and Fig 5).

TABLE 1

	HP 1	HP 2	HP 3	HP 4
Cell shape	Rod	Rod	Rod	Rod
Colony colour	White	Yellow	Yellow	Pale yellow
	K 1	K 2	K 3	K 4
Cell shape	Rod	Rod	Rod	Rod
Colony Colour	Orange	Yellow	Yellow	Pale yellow

C. Characterization of culture condition

Effect of temperature

Different cultures showed different temperature for optimum growth. Mostly best growth occurred at 55°C except for the samples HP4 and K1 with highest growth monitored at 65°C and sample HP1 with 60°C as its optimum temperature. Minimum temperature required was 45°C confirming thermophilic nature of the isolated bacteria (Fig 6).

Effect of pH

All the bacterial samples except HP3 and K4 showed highest growth at pH 6.8. Samples HP1, HP2, K1 and K3 showed 1.5 times luxurious growth while slightly improved growth of sample HP4 and K2 was monitored at pH 6.8 as compared to growth at pH 6.6. Sample HP3 and K4 exhibited optimum growth at pH 7.0 and 6.6 respectively (Fig 7).

D. *Morphological and cellular characterization of samples*

All pure bacterial cultures isolated from hand pump sample were rod shaped. Two of the isolated cultures, namely HP1 and HP2 were Gram negative while HP3 and HP4 were found to be Gram positive. All bacterial cultures from Kund sample were rod shaped and three were found to be Gram negative (K1, K2 and K4) while one was gram positive (K3) (Fig 8 and Fig 9).

E. *Antibiotic sensitivity test*

The growth of isolated bacterial samples was monitored in presence of different antibiotics. Sensitivity of isolated bacterial strains towards four antibiotics viz. kanamycin (K), neomycin (N), ampicillin (A) and streptomycin (S) was tested. All the Hand pump samples except HP1and Kund samples K1 and K3 showed antibiotic sensitivity in the order S > K > N > A while HP1 showed K > S > N > A , K2 showed K > N > S > A and K4 showed N > K > S > A. All samples were ampicillin resistant (Fig 10 and Fig 11).

F. *Amylase production in isolated bacteria*

Bacterial colonies were grown on starch agar plates. 24 hours after inoculation iodine solution was added and left for 50 seconds. Clear zone surrounding bacterial colonies indicated starch hydrolysis and dark blue or black colour surrounding the bacterial colony indicated the absence of starch hydrolysis. Only one bacterial culture isolate from hand pump sample (HP2) and three from kund (K1, K3 and K4) showed positive result indicating amylase production. (Fig 12)

G. *Amylase assay*

The enzymatic activity of amylase producing thermophilic bacteria was monitored. Sample K4 showed the highest activity, while sample K1 showed moderate production of amylase as compared to the samples K3 and HP2. The specific activity of α amylase in HP2, K1, K3 and K4 was found to be 0.597 ± 0.098, 0.69 3± 0.0264 0.692 ± 0.0864 and 0.804 ± 0.043 respectively (Fig 13).

IV. DISCUSSION

Samples were collected from two sites, one from a hot water pond named Sita kund and other from a nearby hand pump. Four pure strains of bacteria were isolated from each sample. Three out of four samples isolated from kund were Gram positive, while two of the hand pump samples were Gram negative. The acidity or alkalinity as indicated by pH is a factor that profoundly affects all living microbial cells. A number of authors have examined the effects of pH on the growth kinetics of microorganisms [3,10,11,12,13,14]. Large proteins, such as enzymes, are affected by pH. Their conformation changes and this very often brings about an alteration of the ionic charges on the molecule. Usually, the catalytic properties of the enzymes are lost and metabolism is halted. Most bacteria grow best around neutral pH values (6.5 - 7.0). Temperature is another major factor determining the kinetics of food deterioration reactions.

All the isolated bacteria grow differently at different pH and temperature. Five bacterial samples out of eight bacterial samples, showed high growth rate at 55°C temperature. Six bacterial samples showed high growth rate at pH 6.8. This indicates that all the isolated bacterial samples are thermophilc with best growth shown at 55°C.

Senstivity of isolated bacterial strains towards four antibiotics kanamycin, neomycin, ampicillin and streptomycin was tested. Clear zone in bacterial lawn in the presence of antibiotic is known as the zone of inhibition. Ampicillin inhibits cell wall synthesis by inhibiting formation of the peptidoglycan cross link, Neomycin inhibits prokaryotic translocation through the ribosome large subunit., Streptomycin inhibits prokaryotic peptide chain initiation and induces mRNA misreading while tetracycline inhibits prokaryotic aminoacyl-tRNA binding to the ribosome small subunit. Differential sensitivity of strains indicates that different bacteria employ different mechanisms for survival in adverse conditions.

All samples were ampicillin resistant showing that they had modified the ampicillin binding protein involved in cell synthesis. Amylases are one of the integral parts of food, paper, pulp and textile industries. With increase in its application spectrum, the demand of the enzyme has also been increased In the present study, total four isolated bacterial samples showed the presence of starch hydrolysis. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. Amylase has been derived from several fungi, yeasts and bacteria. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors [6]. α-Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. However, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries [14]. Fungal and bacterial amylases could be potentially useful in several industries and their thermophilic resistance would be an added advantage.

V. CONCLUSION

The findings of our experiment suggested that the isolated bacteria could withstand high temperature. The isolated organisms showed significant amylases producing ability. This ability makes them an important biotechnological model for thermostable amylase production. The investigations clearly indicate that the Sita kund hot spring is a rich source of many thermophilic bacteria producing metabolites that remain functional even at high temperature. Further, molecular characterization and identification of these strains is required for their mass production and isolation of enzymes.

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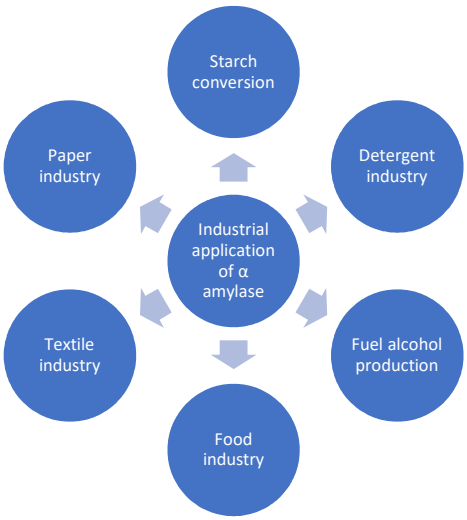


Fig 1

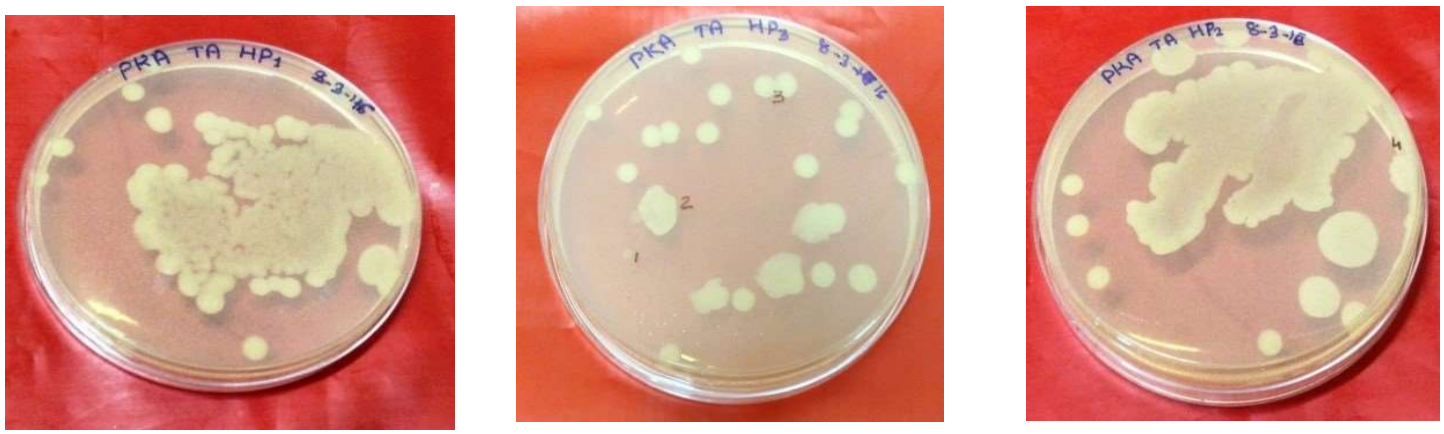


Fig 2 A B C

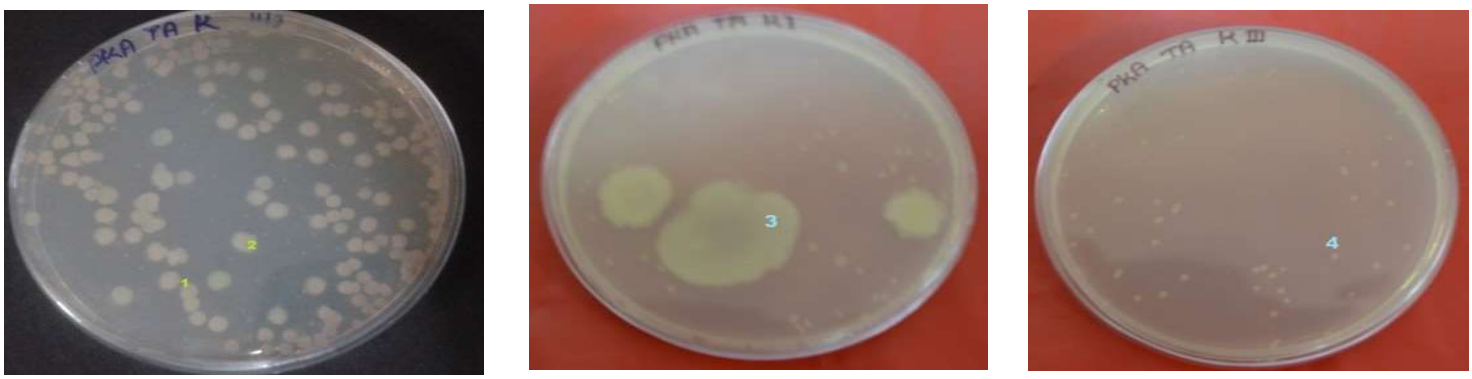


Fig 3 A B C

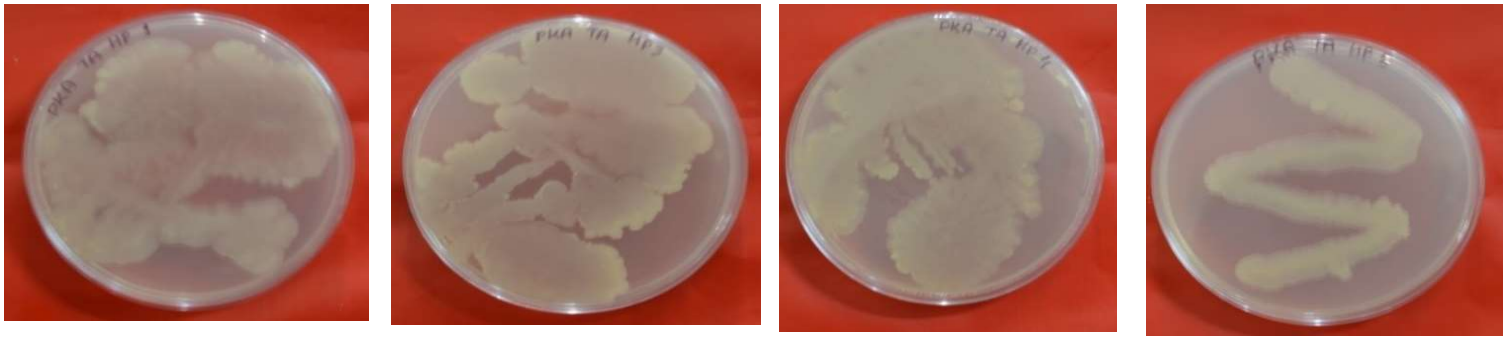


Fig 4 HP 1 HP 2 HP 3 HP 4

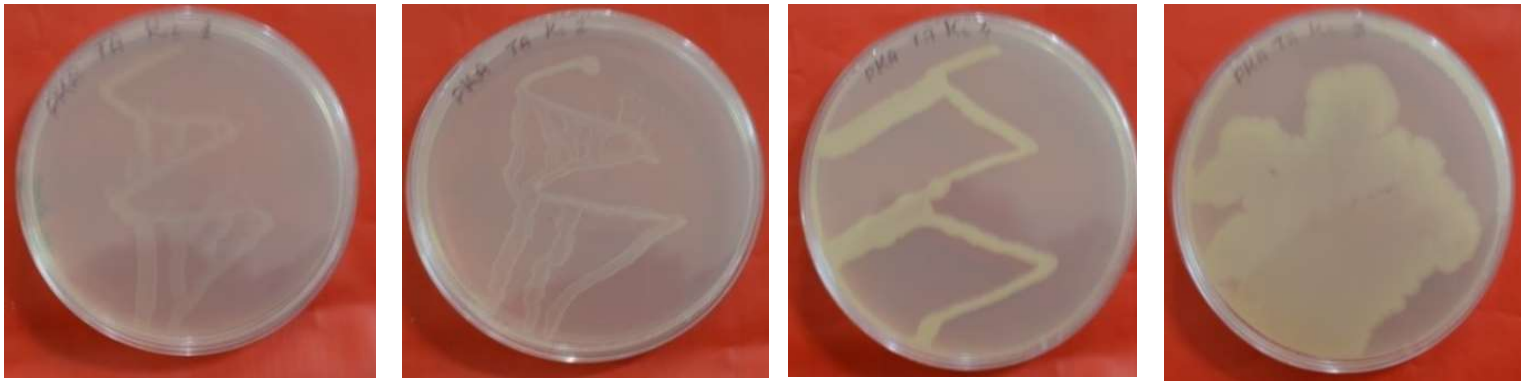


Fig 5 K 1 K 2 K 3 K 4

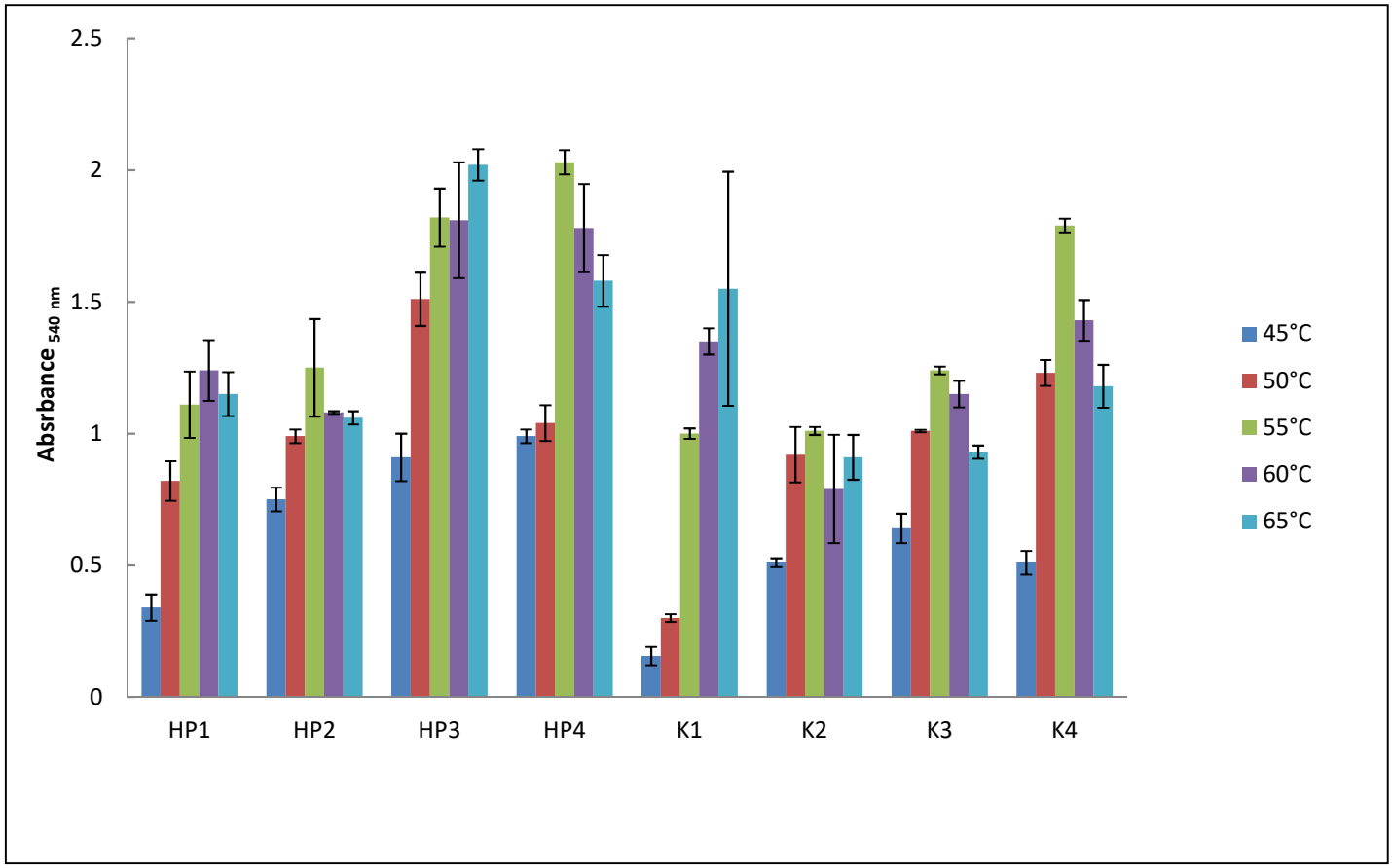

Absrbance 540 nm
2.5
2
1.5
1
0.5
0
HP1
HP2
HP3
HP4
K1
K2
K3
K4
45°C
50°C
55°C
60°C
65°C

Fig. 6

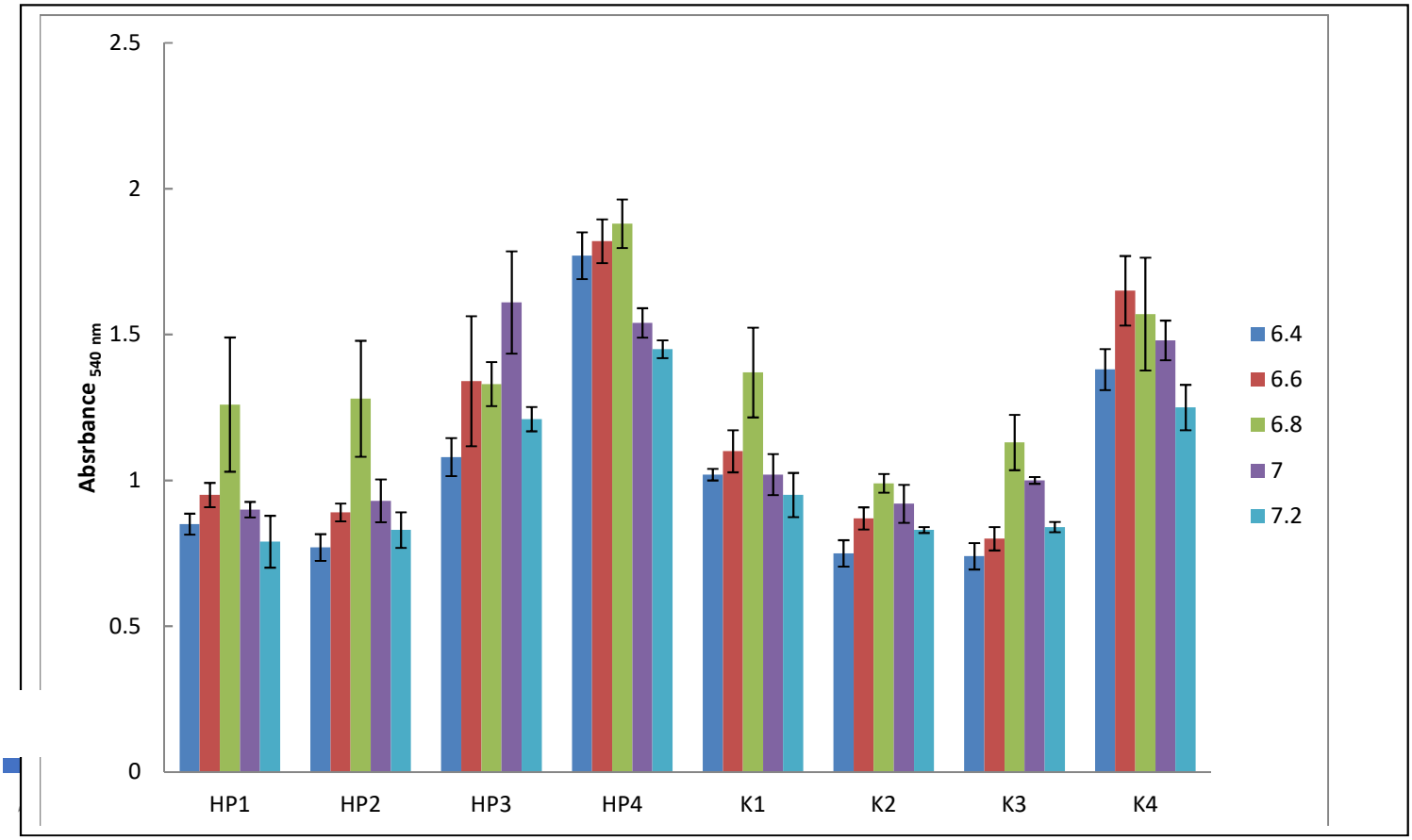

Absrbance 540 nm
2.5
2
1.5
1
0.5
0
HP1
HP2
HP3
HP4
K1
K2
K3
K4
6.4
6.6
6.8
7
7.2

Fig 7

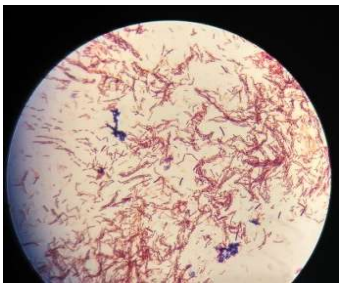
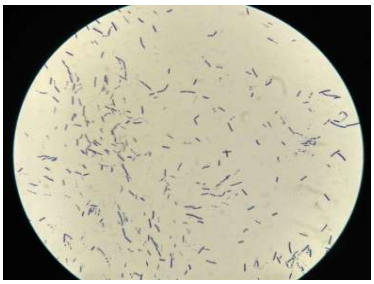
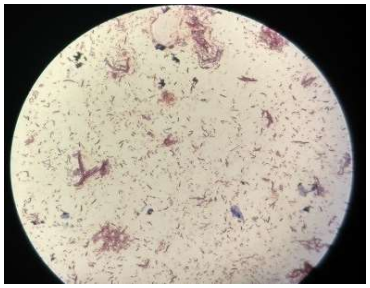
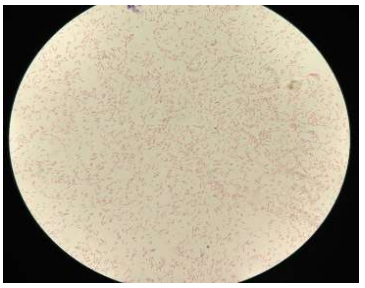
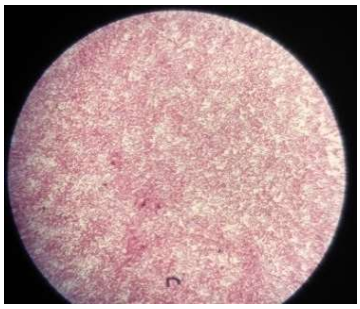





Fig 8 HP 1 HP 2 HP 3 HP 4



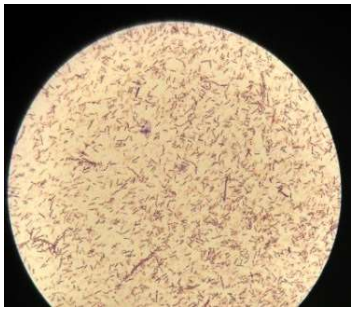
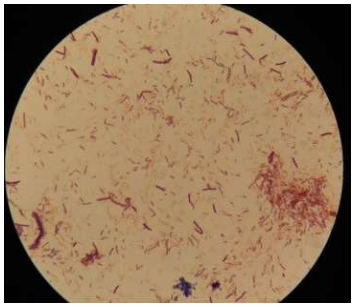
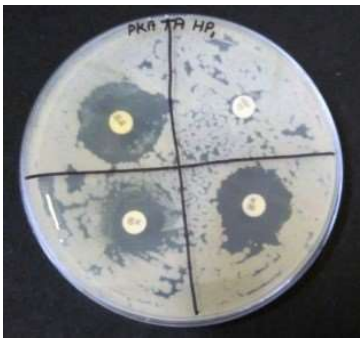
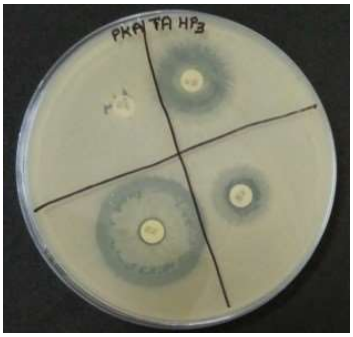
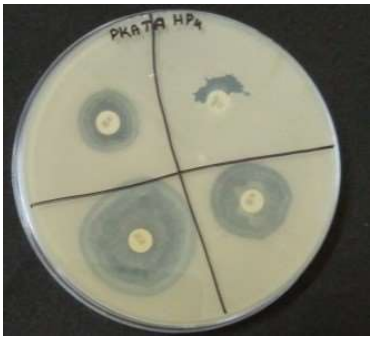



Fig 9 K 1 K 2 K 3 K 4







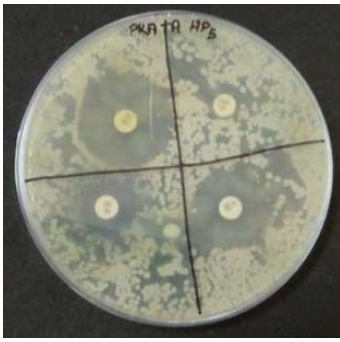
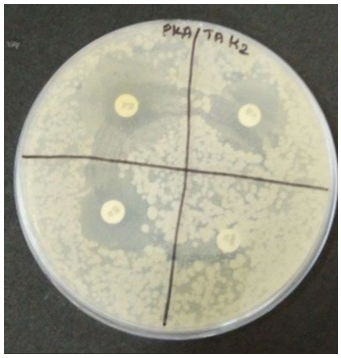
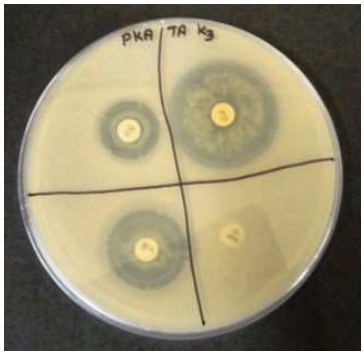


Fig 10 HP 1 HP 2 HP 3 HP 4





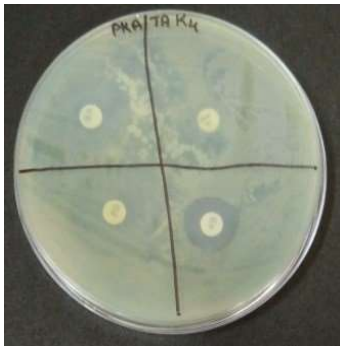


Fig 11 K 1 K 2 K 3 K 4

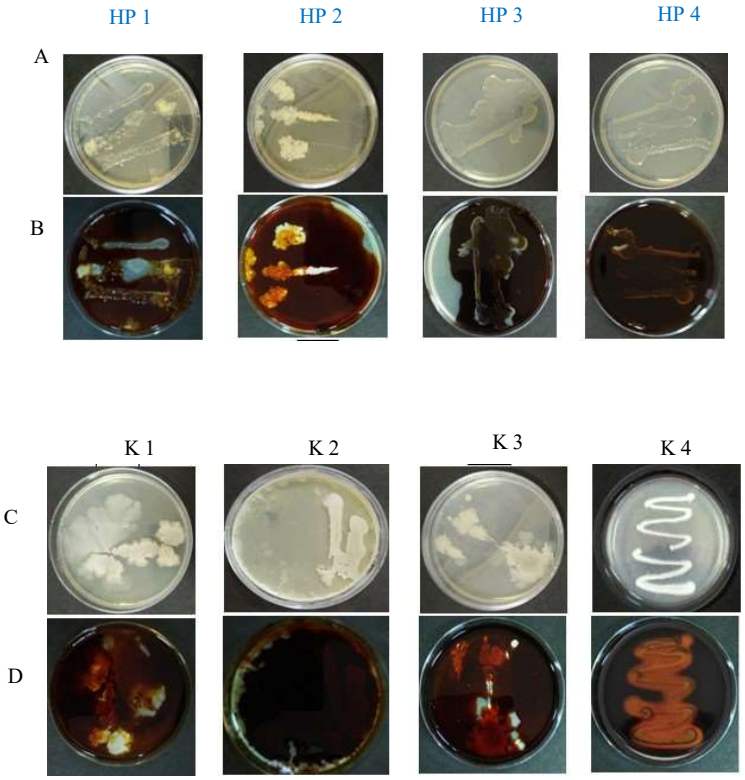

HP 1
HP 2
HP 3
HP 4
A
B
K 1
K 2
K 3
K 4
C
D

Fig.12

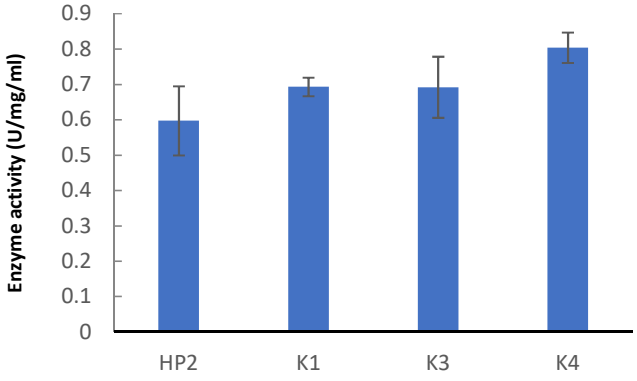

Enzyme activity (U/mg/ml)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
HP2
K1
K3
K4

Fig 13

Table Head

Table 1: Characteristics of isolated bacterial samples isolated from hand pump and Sita kund

Figure Caption

Fig 1: Industrial applications of α amylase

Fig 2: Bacterial colonies isolated from hand pump sample. Colonies of samples A and B are circular and yellowish. Colonies obtained in sample Care irregular, smooth and yellowish in nature.

Fig 3: Bacterial colonies isolated from Sita kund samples. All the samples are circular and yellowish in nature

Fig 4: Purification of four different bacterial strains from Hand Pump sample by streaking

Fig 5: Purification of four different bacterial strains from Sita kund sample by streaking

Fig 6: Growth of bacterial strains at different temperatures

Fig 7: Growth of bacterial strains at different pH

Fig 8: Microphotograph (10X) of Gram stained bacterial strains isolated from hand pump

Fig 9: Microphotograph (10X) of Gram stained bacterial strains from isolated from Sita kund

Fig 10: Effect of antibiotics on bacterial strains isolated from hand pump

Fig 11: Effect of antibiotics on bacterial strains isolated from Sita kund

Fig 12: Screening for amylase production by bacterial strains isolated from hand pump (A- bacterial colonies, B- change in colour after addition of iodine)

and kund (C-bacterial colonies, D-change in colour after addition of iodine) samples

Fig 13: Enzymatic activity of α-amylase positive bacterial strains