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Effect of temperature, pH and metal ions on amylase produced from selected indigenous extremophile bacteria in Pakistan

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Abstract

There is a burgeoning demand for amylase production due to wide range applications of amylase in different industrial processes like saccharification of starchy materials, food, detergents and textile industries. But high cost of fermentation media is one of the technical barriers in amylase production from microbial sources. Extremophiles microorganisms (thermophilic and halophilic) could be potential source for thermostable amylase. Present study deals with isolation of extremophilic amylase-producing bacteria from soil samples in starch agar medium and their subsequent identification through 16sRNA analysis. Different parameters like pH, temperature, and metal ions concentration (Ca^{+2}, Mn^{+2}, Fe^{+2}, Zn^{+2}) were optimized for amylase production. Five thermophilic strains and one halophilic strain were found positive for amylase production. Phylogenetic analysis showed that the amylase producing thermophilic strains includes *Bacillus spp., Rheinheimera spp., Alishewanella spp., Pseudomonas spp., Microbacterium spp.* while halophilic strain includes *Bacillus spp*. Thermophilic strains showed optimum amylase production at pH of 8 & 60°C, while maximum amylase activity for halophilic strains was observed at pH 7 and 40°C. Divalent ions Ca^{+2} and Mn^{+2} enhanced the amylase production while Zn and Fe did not a have any significant effect. Current research revealed that use of extremophile bacteria could be an important step towards the development of environmental friendly and cost effective process for thermostable amylase production.

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Introduction

Starch is a main part of our diet and is being used in food as well as in industry as gelling agent, bulking agent, staining agent, thickener and colloidal stabilizer (Jaspreet *et al.*, 2007). Soil inhabiting microorganisms are the sources of many enzymes including amylase. Amylase breaks down starch and glycogen into smaller molecules. It contributes about 25% in total sale of industrially used enzymes and has many potential industrial applications. It is also extensively used in treatment of digestive disorders (Burhan *et al.*, 2003). Starch comprises of amylase and amylopectin. Alpha-amylase is able to cleave α-1,4 glycosidic bonds present in the inner part of the amylose or amylopectin chain. Alpha-amylase belongs to a family of endo-amylases that catalyze the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glyosidic bonds (Stamford *et al.*, 2001; Whitcomb and Lowe, 2007).

Chemical technologies are becoming popular due to the generation of dangerous by-products that contaminate our environment and require high cost for energy input at large scale manufacturing (Gomes *et al.*, 2003). Microorganisms using their diverse enzyme system efficiently perform their metabolic processes with higher specificity under ambient conditions. Due to the high catalytic efficiency and more specificity, enzymes produced from microorganisms are good alternative to harsh chemical technologies and therefore the research in the field led to explore microbial diversity to discover enzymes for pollution free "dream technology" in the future (Gomes *et al.*, 2003;Prakash and Jaiswal, 2009).

Extremophile microorganisms are structurally adapted at the molecular level to withstand harsh conditions (Stamford *et al.*, 2001) and are capable of producing different thermostable enzymes. Such organism have been isolated and characterized from various environments such as soil, spring, food material and wastes containing carbohydrate in different studies (Gupta *et al.*, 2003). They have modified themselves to flourish in extreme environments. These properties make them to survive in harsh conditions and in addition, help them to grow in bio-industrial processes designed basically on the optimal conditions of these biomolecules (Kamekura, 2011). Moreover, thermostable amylases produced from these microorganism enzymes have been currently investigated to improve industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins (Prakash and Jaiswal, 2009).

The main objective of the study was to isolate and identify amylase producing bacteria from Halophilic sites, their partial characterization for enzyme production and properties in regard to the effect of temperature, metal ion-dependency and pH.

Materials and methods

Isolation and bio characterization of Thermophilic Bacterial Strains

For isolation of thermophiles bacterial strain, soil samples (collected from D.I.Khan desert soil) were placed on nutrient agar plates and incubated at 40°C for 24 hrs. Isolated strains were identified and characterized according to Bergey's manual of determinative bacteriology. The gram straining reactions and colony morphology results were noted.

Isolation and bio characterization of Halophilic bacterial isolates

Soil samples, collected from Khewra region of Pakistan were serially diluted and spread on nutrient agar plates (having extra added NaCl to favor the halophiles growth), and incubated at 3°C for 24 hrs. The isolated strains were further identified using Gram staining and biochemical tests (Triple sugar iron, motility test, Urease test, catalase test, H_2S test, Citrate utilization test). Protease production was checked by casein hydrolysis test.

Starch Iodine Test

Starch iodine test was performed for screening of starch producing bacteria isolated from soil samples.

Isolated colonies were picked up from each plate containing pure culture and streaked on starch agar plates containing starch as a carbon source. After incubation at 37°C for 24hr, individual plates were flooded with Gram's iodine to observe the zone of hydrolysis for observation of Amylase activity.

Molecular Characterization and phylogenetic analysis of amylase producing strains

The selected amylase producing strains were further preceded for DNA extraction and DNA was extracted and purified DNA. Their band images were visualized by using Gel Doc system. The 16S rRNA gene sequence was used for sequencing and the extracted DNA was sequenced at Macrogen and for their classification into groups phylogenetic tree was built. The sequences obtained were then blasted in NCBI database, deposited into Gene Bank and sequence analysis was done by Bio Edit software. The phylogenetic tree was constructed by MEGA 4.0 software using NJ model after downloading similar sequences.

Production of crude enzyme

For crude production of enzyme, the pure culture of isolated strains (strains positive for starch iodine test) was enriched in nutrient broth and incubated at 37°C for 24hrs. They were inoculated in production medium and allowed to ferment for 24, 48, 72, 96 hrs under shaking condition at 37°C. A suitable volume of fermentation broth was centrifuged at 10,000 rpm for 20 min at 4°C. Cell free supernatant recovered by centrifugation was used for amylase activity and considered equivalent to crude enzyme. The assay medium constitutes 1% soluble starch in 0.02M phosphate buffer at pH 6.9.

Amylase activity assay

Amylase activity was measured by the release of glucose from starch by DNS reagent. Reducing sugar assay was done by using Bernfeld method. The DNS reagent was prepared by modified method of Miller. During the reaction, color development was stabilized by Rochelle salt and enhanced by phenol while sulphite protects the reagent and reducing sugars from oxidation.

For determining amylase activity, 1mL of crude enzyme was taken and was added in a mixture of 1 mL of starch solution (1% W/V starch solution).This mixture was mixed and kept in a water bath at 50°C for 30 minutes. After incubation, the mixture was brought to the room temperature and reaction was stopped by adding 2 mL of DNS reagent, then mixture was kept in a pre-heated boiling water bath at 99.9°C for 5-10 min. This mixture was cooled to room temperature and absorbance of solution was measured against water blank and control (which does not contain enzyme extract) at 540nm. One unit of amylase is the amount of enzyme in 1 mL of filtrate which releases 1.0 µg of reducing sugar from 1% starch solution in 30 minutes at 37°C at pH 6.9.

Procedure for Standard Curve

250 mg of Maltose was dissolved in 100 mL of distilled water and was autoclaved. From this stock solution 00 µL, 100µL, 200µL, 400µL, 600µL, 800µl and 1000µL volumes were taken in glass tubes which were labeled as A, B, C, D, E, F, and G, respectively. Volume was balanced 1mL by taking distilled water in respective tubes as 1000µL 900µL 800µL 600µL 400µL 200µL 00µL. 3.0 mL of DNS (Dinitrosalisylic acid) was added in each tube. All tubes were placed in boiling water bath for 15 min and O.D was taken at 540 nm. The reducing sugar values were read

and used to draw a standard curve of absorbance as related µg of maltose/ mL or mg of maltose/mL. The amount of reducing sugar released by 1mL of the enzyme extract 1% starch assay medium was calculated from this curve.

Optimization of Various Parameters for the Amylase activity of Isolates

Effect of pH & temperature

The isolated strains were assessed at different pH (5, 6, 7, 8, 9) and various temperature (30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C) for maximum amylase activity. The results were noted after 24 hrs at optical density of 540 nm.

Effect of metal ions on amylase activity

Different metal ions ($CaCl_2.H_2O$, $MnCl_2.H_2O$, $FeSO_4$ & $ZnSO_4$.) with 2 g/L concentration effect were measured for all isolated strains by adding the in production medium along with other medium ingredients. Then OD was measured for amylase production after 24 hrs and 48 hrs.

Results

Isolation, screening and characterization of amylase producing bacterial strains

A total of nine thermophilic bacterial strains (named TS-1, TS-2, TS-3, TS-4, TS-5, TS-6, TS-7, TS-8, TS-9) were isolated from two thermophilic soil samples (Shadra spring) when incubated at higher temperature of 55°C. While seven pure isolates were obtained from high salinity soil sample named as HS-1, HS-2, HS-3, HS-4, HS-5, HS-6 and HS-7.

Table 1. Results of isolates for colony morphology and Gram staining.

Bacterial strain	Color	Size	Pigmentation	Margin	Form	Gram staining reaction
TS-1	Transparent	Moderate	Nil	Entire	Circular	G –ve
TS-2	Opaque	Small	Nil	Regular	Circular	G +ve
TS-3	Mucoid	Large	Nil	Regular	Circular	G +ve
TS-5	Mucoid	Large	Nil	Regular	Circular	G +ve
TS-9	Slightly opaque	Moderate	Nil	Regular	Circular	G–ve
HS-7	Orange	Small	Nil	Smooth	Circular	G+ve

Growth of bacterial isolates on starch

Total six bacterial strains (five thermophilic and one halophilic) showed positive qualitative test for amylase production when plates were flooded with iodine solution on the cultures grown in starch medium plates (Fig. 1 a). A clear zone around growth indicated hydrolysis of starch.

The positive isolates for amylase production were HS-7 and TS-1, TS-2, TS-3, TS-5, TS-9 from halophilic and thermophile sources, respectively. Different concentrations of starch in culture plates ranging from 1 % to 5 % were added.

With increase in concentration of starch, a gradual decline in zone of hydrolysis for amylase production was observed (Fig. 1 b).

Identification and biochemical Characterization of amylase producing strains

All the isolates positive for amylase production were identified and characterized according to Bergey's manual of determinative bacteriology. TS-1 and TS-9 were found to be Gram negative, while TS-2, TS-3, TS-5 and HS-7 were Gram positive. All isolates were negative for pigment formation (Table 1).

Except HS-7 halophilic strain, all thermophilic strains were negative for triple sugar iron test (TSI), citrate utilization, indole and H_2S production. Protease activity was observed for TS-3, TS-5, TS-9 and HS-7, while TS-1 and TS-2 were negative for protease test. Catalase reaction was positive for TS-1 and HS-7 while all other strains were negative for catalase activity (Table 2).

Table 2. Biochemical characterization of isolates.

Bacterial Strains	Triple sugar iron test	Indole production test	Citrate utilization test	Catalase test	H2S production test	Protease test	Urease test	Starch hydrolysis test
TS-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
TS-2	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
TS-3	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
TS-5	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
TS-9	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
HS-7	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve

-ve= isolate negative for test, +ve= islate positive for test.

Molecular Characterization and Phylogenetic analysis of selected strains:

Amylase producing bacterial strains were further proceeded for DNA extraction. Extracted DNA bands were visualized by using Gel Doc system. For phylogenetic analysis, 16S rRNA gene sequencing was done for amylase positive bacterial isolates.

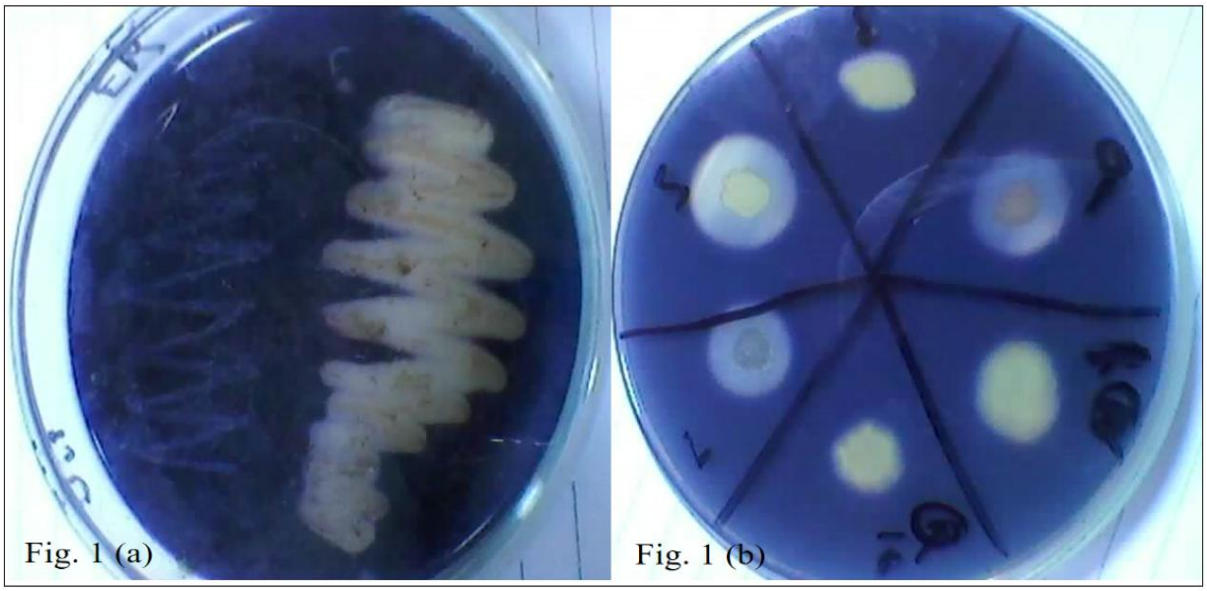

Fig. 1. (a) Represents Iodine plate assay for qualitative analysis of amylase production. Starch in the medium was hydrolyzed by production of Amylase enzyme from selected bacteria isolated from inoculation of different soil samples on starch containing medium. After 24 hrs incubation at 37°C temperature upon hydrolysis of starch, a clearance area was observed after starch consumption. Left side having negative strain for starch hydrolysis while right side a bacterial isolate showing positive reaction for starch consumption. (b) Shows selected isolates from soil samples of different areas, giving zone of clearance after starch hydrolysis. All positive strains with less or high quantity of Amylase produced from isolated bacteria named accordingly.

The sequences obtained were blasted in NCBI database, deposited into Gene Bank. Sequence analysis was done by Bio Edit software.

The phylogenetic tree was constructed by MEGA 4.0 software using NJ model after downloading similar sequences. Fig. 2 shows the phylogeny of the studied samples with the similar sequences downloaded from the NCBI. Tree shows six different clusters from cluster I to VI. Cluster I is composed of *Rheinheinomera spp*. and our isolate TS-5 was 99% similar to it.TS-3 was greatly resembled to cluster II of *Alishewanella spp*. Cluster III having *Pseudomonas spp*. was observed very close to strain TS-2 with about 99%. Cluster IV with *Bacillus spp*. was in close resemblance to isolated strain HS-7 having 98%. TS-9 showed very much similarity to *Bacillus spp*. of cluster V and TS-1 was showing 99% resemblance to *Micobacterium* species.

All strains were identified for amylase production and could be used for amylase application where starch hydrolytic activity required.

Effect of pH on halophilic strain

Halophilic *Bacillus spp*. When grown under different pH i.e. from pH 5 to pH 9) in starch yeast extract medium. Activity of amylase at individual pH value for *Bacillus spp*. was measured. Enzyme activity under various pH values after 24hrs, 48hrs and 96 hrs is shown in Fig. 3(a).

An increase in amylase activity was observed with gradual increase in pH, while peak amylase activity was observed at pH of 7 after 24 hrs. At lower pH no such good amylase activity was observed.

Effect of pH on thermophilic strains

When provided various pH conditions, both *Micobacterium spp.* and *Rheinheinomera spp.* gave optimum amylase activity at slightly alkaline pH of 8, represented in Fig. 3 (b) and Fig.3 (c), respectively. High amylase activity was noted after 24 hrs when O.D was taken at 540 nm.

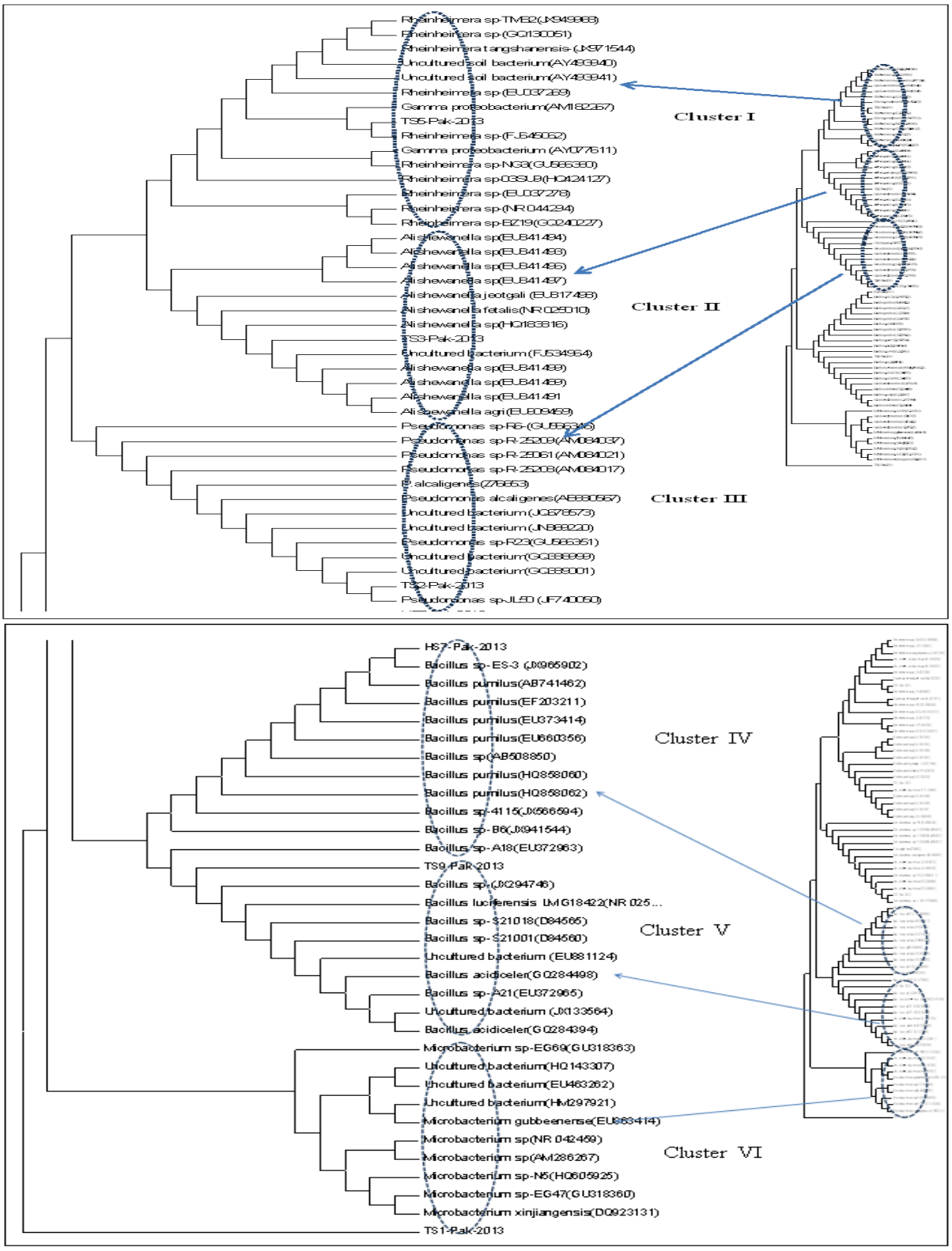

Fig. 2. Phylogenetic relationship of the study samples with identical sequences downloaded from NCBI.

Effect of temperature on halophilic strain

Amylase activity from halophilic *Bacillus spp*.at different temperatures i.e.30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C was measured as described in Fig. 4 (a). *Bacillus spp*. Showed highest activity at 40°C after 72 hrs of inoculation on medium.

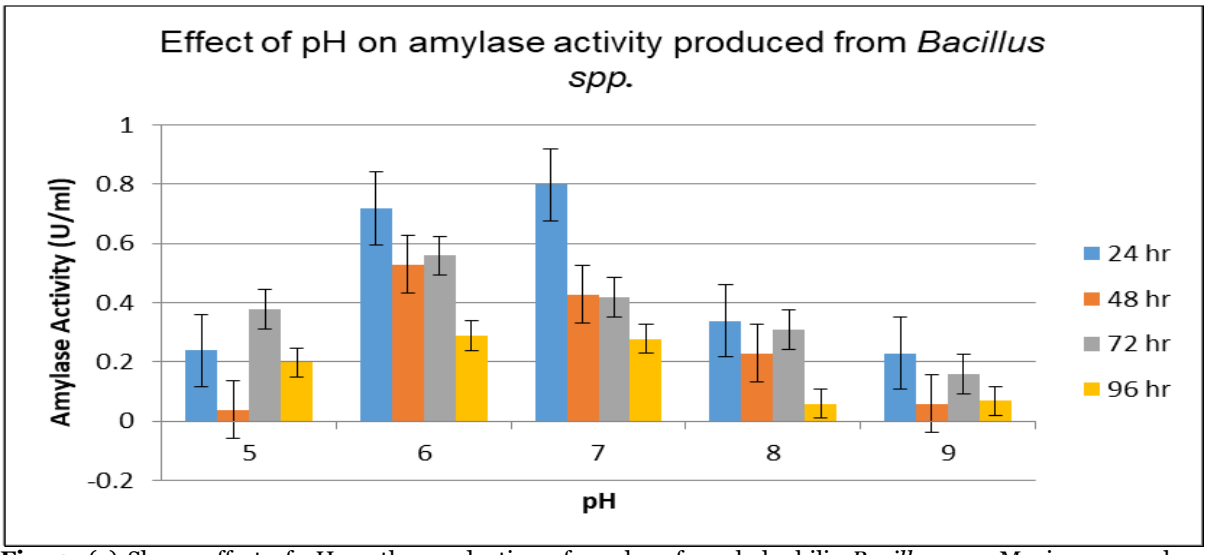

Fig. 3. (a) Shows effect of pH on the production of amylase from halophilic *Bacillus spp*. Maximum amylase activity was observed at pH 7 after 24 hrs incubation of Bacillus strain in starch medium. A decline in activity was measured at acidic and basic pH.

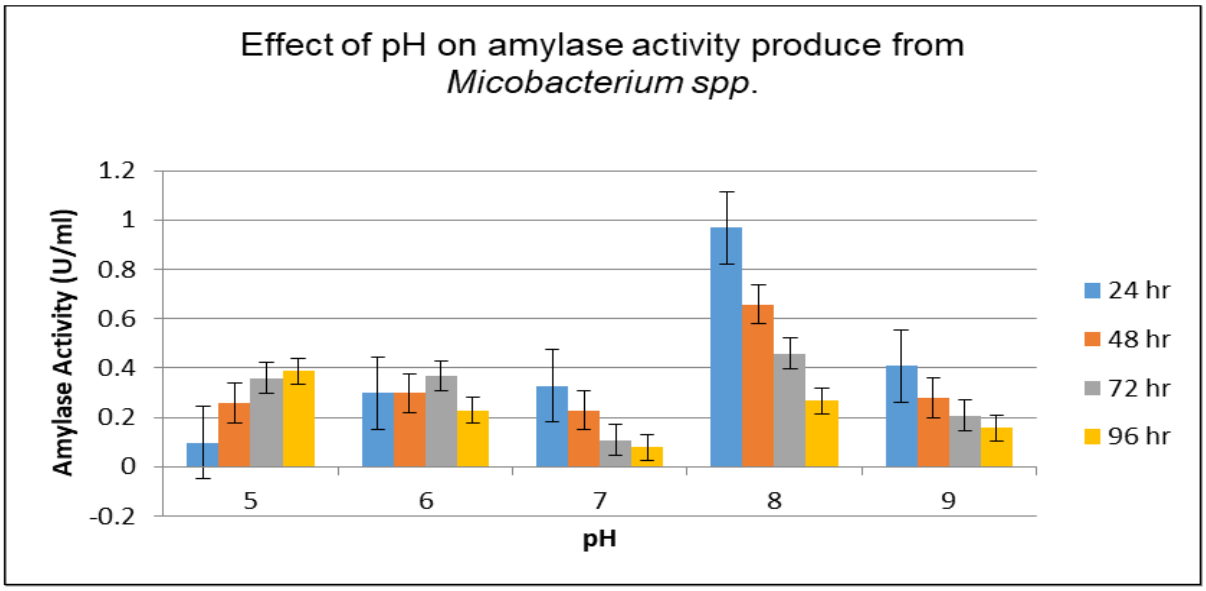

Fig. 3. (b) Describes the effect of pH on the production of amylase from thermophilic *Micobacterium spp*. An increase in amylase activity was observed with increase in pH after 24 hrs. While peak value for amylase activity was measured at pH 8.

Effect of temperature on thermophilic strains

Effect of various temperatures (30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C) on amylase activity from two thermophilic strains (*Micobacterium spp*. and *Rheinheinomera spp*.) was evaluated. Each isolate exhibited individual temperatures for optimum amylase activity, when provided with a range of temperatures as mentioned above. *Micobacterium spp*. showed high yield at the temperature of 50°C after 48 hrs. *Rheinheinomera spp*. showed peak yield at 60°C after 48 hrs of inoculation. Effect of varying degrees of temperature on amylase activity produced

from *Micobacterium spp.* and *Rheinheinomera spp.* are described in Fig.4 (b) and Fig. 4 (c), respectively.

Effect of $CaCl_2.H_2O$ on amylase activity

All amylase producing strains were evaluated for amylase production in the presence of $CaCl_2.H_2O$ at 40 °C and optical density was measured after 24 hrs and 48 hrs. All selected strains were observed for enhanced production of amylase after 24 hrs suggesting that Ca^+ ions have augmented production of amylase enzyme as described in Fig. 5(a).

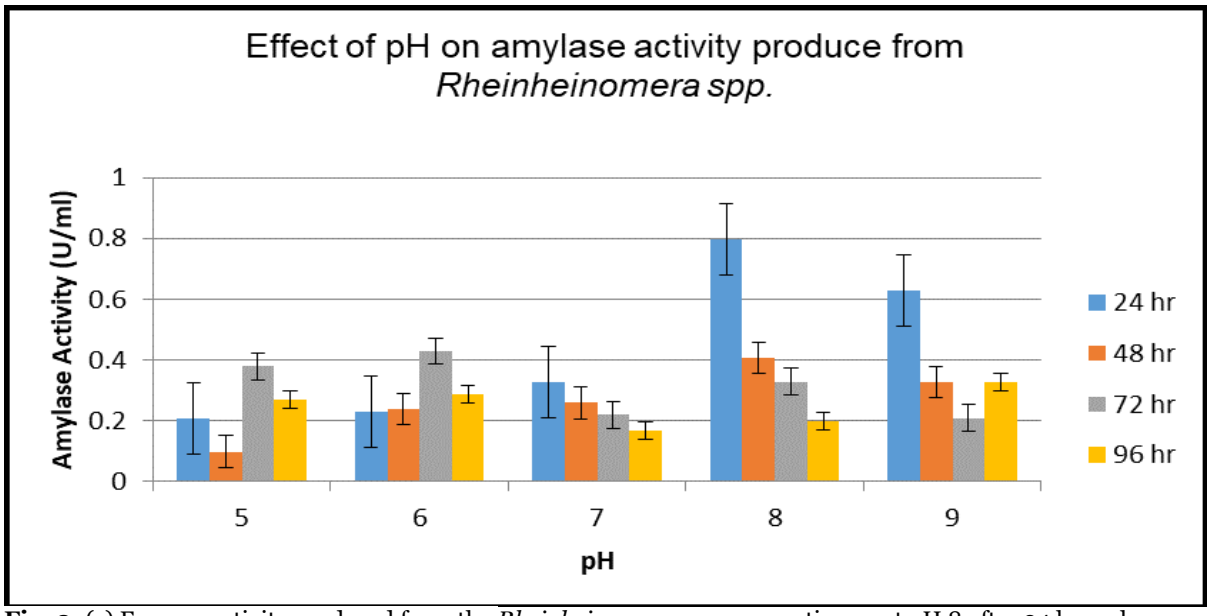

Fig. 3. (c) Enzyme activity produced from the *Rheinheinomera spp.* was optimum at pH 8 after 24 hrs, whereas a decreased amylase activity was observed at acidic and higher alkaline pH.

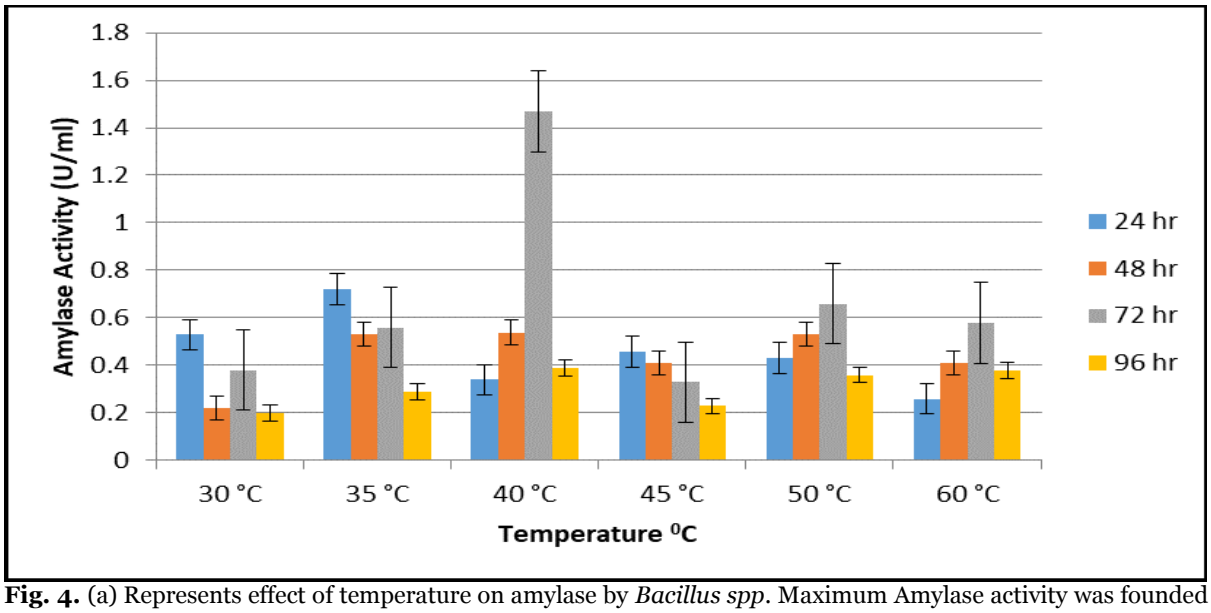

Fig. 4. (a) Represents effect of temperature on amylase by *Bacillus spp.* Maximum Amylase activity was founded on temperature 40°C after 72 hrs incubation.

Effect of $MnCl_2.H_2O$ on amylase activity

$MnCl_2.H_2O$ was added in the production medium to observe its effect on amylase production. Absorbance was taken at540 nm for 24 hrs and 48 hrs after incubating cultures at 40°C. Fig. 5(b) shows that in the presence of $MnCl_2.H_2O$, there was an increase in amylase activity after 24 hrs incubation.

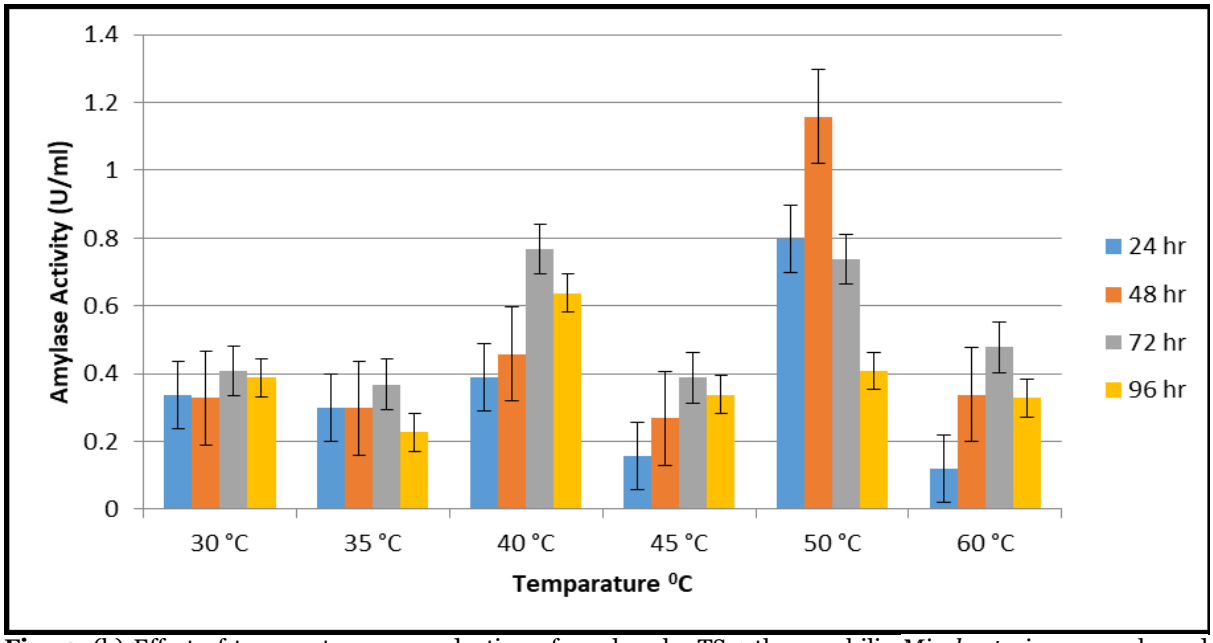

Fig. 4. (b) Effect of temperature on production of amylase by TS-1 thermophilic *Micobacterium spp.* showed maximum Amylase activity on 50°C after 48 hrs incubation.

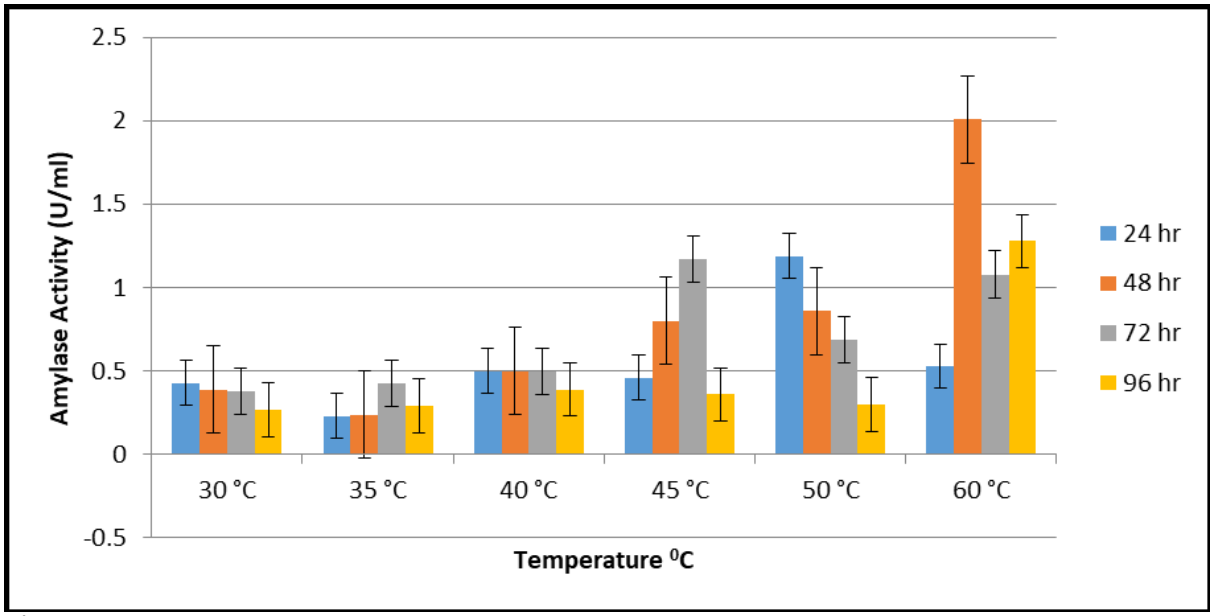

Fig. 4. (c) Effect of temperature on production of amylase by TS-5 (*Rheinheinomera spp.*). Thermophilic strain showed peak amylase activity at 60°C after 48 hrs incubation.

Effect of $FeSO_4$ and $ZnSO_4$ on amylase activity

Effect of $FeSO_4$ and$ZnSO_4$ metal ions on amylase activity in production medium is shown in Fig5 (c) and Fig. 5 (d), respectively. $FeSO_4$ did not make a much difference on enzyme activity production, while in case $ZnSO_4$, only a small increase in amylase activity was observed when compared to other metal ions at conditions of temperature 40 C and time was 24 and 48 hrs incubation. Best amylase activity was obtained after 24 hrs incubation.

Discussion

In the present study, five thermophilic bacterial species (*Micobacterium spp., Pseudomonas spp., Alishewanella spp., Rheinheinomera spp.* and *Bacillus spp.*), while one halophilic bacteria (*Bacillus spp.*) were isolated for amylase production. Other

studies also reported the use of *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* as good producers of thermostable α-amylase (Prakash and Jaiswal, 2009). Out of these amylase positive strains, a few were selected for optimization of various parameters for maximum amylase production.

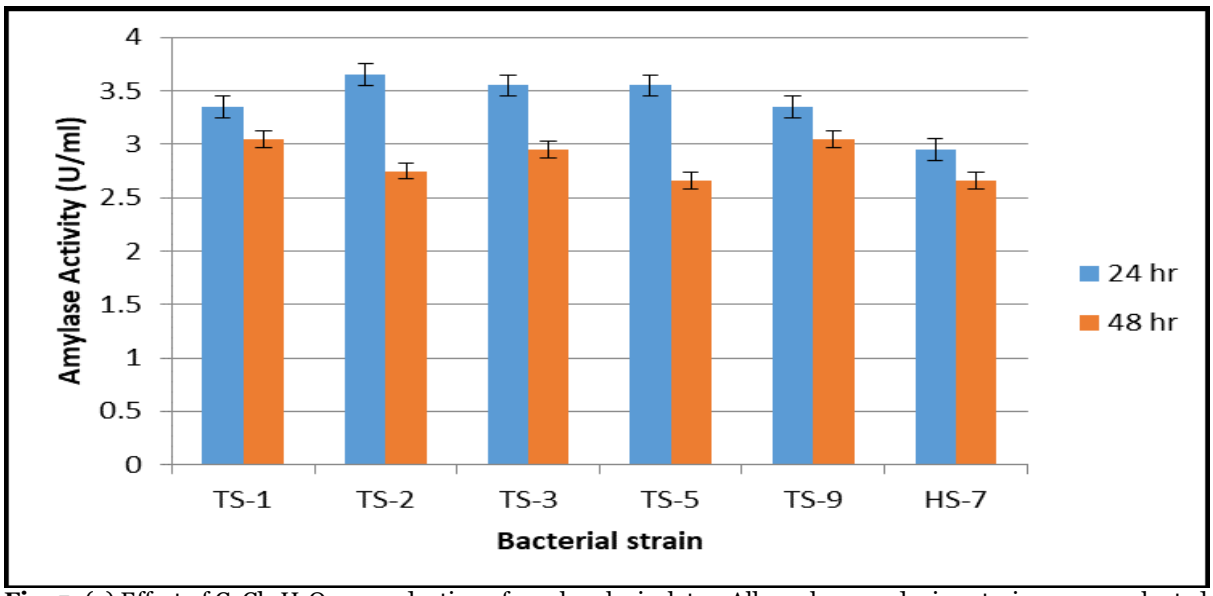

Fig. 5. (a) Effect of $CaCl_2.H_2O$ on production of amylase by isolates. All amylase producing strains were evaluated for amylase production in the presence of $CaCl_2.H_2O$ at optimum temperature 40°C for all selected isolates and optical density was measured after 24 hrs and 48 hrs. All selected strains were observed for enhanced production of amylase after 24 hrs.

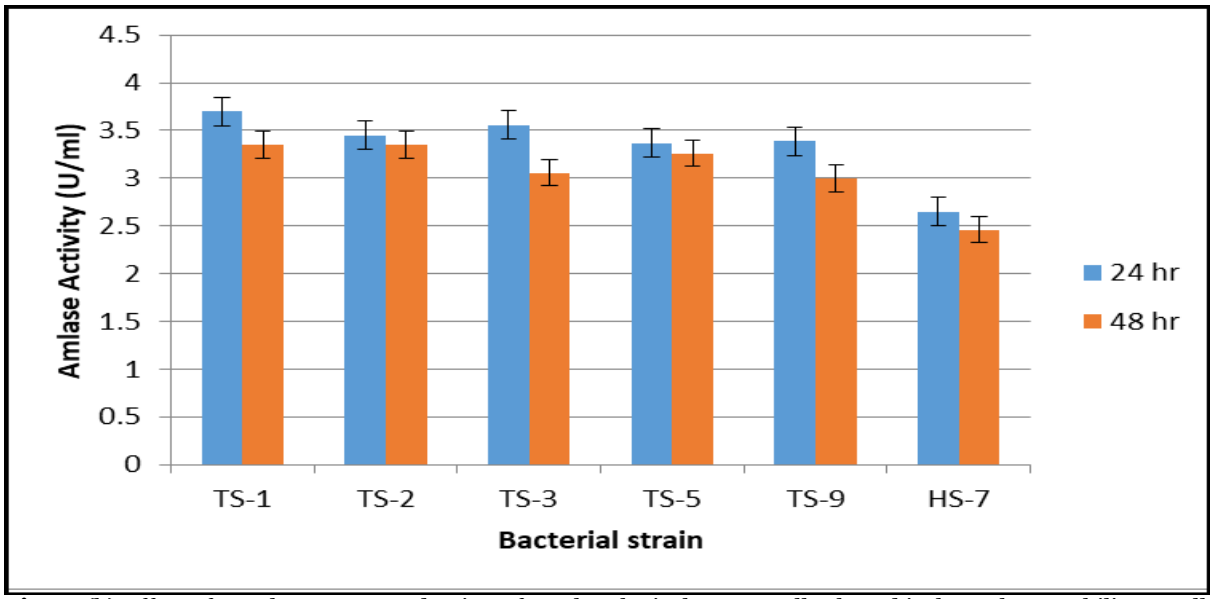

Fig. 5. (b) Effect of $MnCl_2.H_2O$ on production of amylase by isolates. On all selected isolates thermophilic as well as halophilic, $MnCl_2.H_2O$ effect on amylase activity was best after 24 hrs incubation when temperature range was 40°C.

Production of Amylase

Extracellular amylase production was done by inoculating serial dilution of samples into medium containing starch as carbon source. Clear zone around colonies indicated starch hydrolysis when flooded with iodine solution (Sterner and Liebl, 2001). The

area of clearance was as a result of starch utilization by microorganism. Other than starch, glucose, galactose, maltose, date syrup and molasses can also be used as carbon source for amylase production. Previous studies have revealed significant effect of carbon source on enzyme production (Qureshi and Dahot, 2009; Qureshi *et al*., 2010; Qureshi *et al*., 2012). Simair *et al*, 2017 compared the effect of different carbon sources on enzyme production and observed that amylase production was maximum when molasses was used as carbon source.

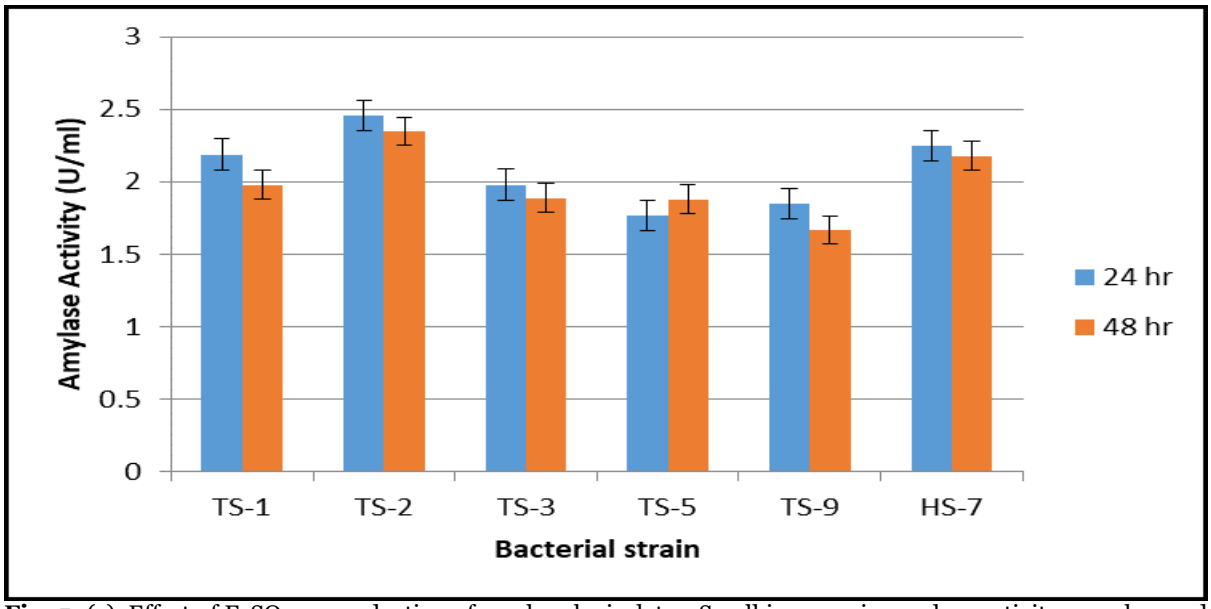

Fig. 5. (c). Effect of $FeSO_4$ on production of amylase by isolates. Small increase in amylase activity was observed for TS-5 after 48 hrs when compared to other isolates at conditions of temperature 40°C after 24 hrs of incubation. Best Amylase activity was increasingly obtained after 24 hrs incubation for all other strains.

Effect of temperature on Amylase activity:

Amylase production from bacteria is dependent on variety of factors including temperature, salt concentration, pH of the production medium and metals ion concentration (Muralikrishna and Nirmala, 2005). The pH and temperature are the most critical factors that have to be optimized for enzyme production. Temperature is related to the metabolic activities of the microorganisms as it effects on growth and metabolic activities (Ramesh and Lonsane, 1989). In this study, thermophilic strains TS-1 (*Micobacterium spp.),* TS-2 (*Pseudomonas spp.*), TS-3 (*Alishewanella spp.*), TS-5 (*Rheinheinomera spp*.) and TS-9 (*Bacillus spp.)* and halophilic strains HS-7 (*Bacillus spp*.) produced significant level of amylase over a range of temperature between 40-60°C, with an optimum yield at 40°C. Asad described the highest amylase production from *Bacillus spp*. at 45°C while using starch agar medium as carbon source (Asad *et al*., 2011). Božićalso obtained maximum amylase production at temperature of 37°C (Božic *et al*., 2011). Nouadri also reported similar optimum temperature conditions for amylase production. It was observed that enzyme has reflected its activity over a broad temperature range between (20-60°C) with maximum activity at 30°C (Nouadri *et al*., 2010). As the temperature was increased from 40°C, amylase titer decreases.

This might be due to decreased microbial growth and denaturation of enzyme at higher temperature. Apparently the activity of α-amylase was affected largely by exposing to temperature above 60°C. The enzyme lost about 50% of its activity at temperature ranging between 50 - 60°C for two hours (Afifi *et al*., 2008). A wide range of temperatures (35-80°C) has been reported for optimum amylase production from bacteria by Burhan *et al*., 2003. Here in our study, all thermophilic strains showed best amylase production

at50°C and 60°C.According to their salt requirements, halophiles are classified as extreme, moderate and medium halophiles. Moderately halophilic bacteria are extremophiles which can grow in a medium with 3-15% NaCl (Leveque *et al.*, 2000). In our study, *Bacillus spp*. was grouped under moderate halophile as it can tolerate salt concentration from 5-15%. Our results were quite comparable to Enache who reported similar kind of findings (Enache and Kamekura, 2010).

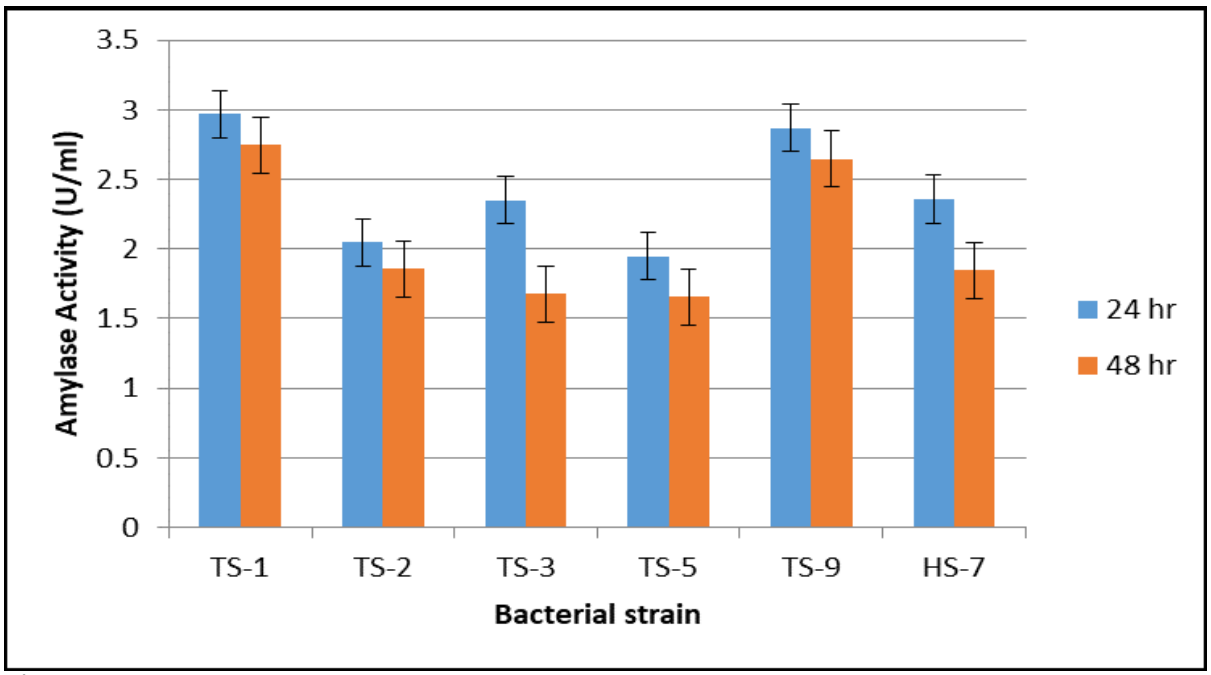

Fig. 5. (d). Effect of $ZnSO_4$ on production of amylase by isolates. Increase amylase activity was observed for all isolates after 24hr at 40°C.

Effect of temperature on Amylase activity:

The pH of medium plays an important role in amylase production due to its effects on growth and enzyme synthesis by microorganisms (Tapan *et al.*, 2006). Optimization studies of growth condition showed that maximum amylase activity of halophilic *Bacillus spp*. was observed at pH 7.0. Above and below the optimum pH, *Bacillus* showed poor amylase activity. Coronado *et al,* reported an optimum pH of 7.0 for alpha amylase activity by a moderate halophilic strain; *Halomonas meridian* (Coronado *et al.*, 2000). Our results were also quite comparable to Goyal *et al*, who achieved optimum enzyme yield at pH 7 (Goyal *et al.*, 2005). In contrast to our results, Dar *et al*, observed highest yield at acidic pH of 6 (Dar *et al.*, 2015).

Effect of metal ions on Amylase activity:

Most amylases are known to be metal ions dependent (Autha and Priya, 2011). Increased amylase activity was observed in the presence of Ca^{2+}, Mn^{2+}metal ions, while Fe^{2+}, and Zn^{2+} have a little effect on amylase activity. These results were comparable to other studies reported by (Tapan *et al.*, 2006; Yasser *et al.*, 2013).

Conclusion

Current research revealed that extremophile bacteria could be an important step towards the development of environmental friendly and cost effective process for thermostable amylase production. These microorganisms could be excellent candidates for enzymes production at industrial level due to high thermo-stability.

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