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Characterization of Amylase Produced by Thermophilic *Bacillus* sp. TS9

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Abstract

Amylases constitute the second largest group of enzymes in the world enzyme market and are produced by many bacterial and fungal strains. The potent producer of amylase is Bacillus due to the production of thermostable enzymes that can withstand harsh conditions in industrial bioprocess. The α-amylase produced by Bacillus sp. TS9 was purified through gel filtration chromatography and then crude, partially purified and purified α-amylase was characterized. The crude, partially purified and purified amylase showed stability to a wide range of temperature (35-80°C) and pH (6-9) with optimum temperature and pH is 55°C and 9 respectively. The purified amylase also retained 70% of its activity at 100°C after incubation of 3 hours. The crude, partially purified and purified amylase showed stability to $Na^{+1}$and Mg^{+2}, methanol, and commercial detergents, less affected by Zn^{+2}, $(NH_4)_2SO_4$, Triton-X-100, Tween-80 and SDS and some solvents but its activity was reduced by Ca^{+2} and Hg^{+2}. The amylase obtained from Bacillus sp. TS9 is Ca^{+2} independent as don't require Ca^{+2} ions for its activity, but still the purified amylase was sensitive to EDTA to some extent. Its activity was completely inhibited by mercapto-ethanol revealing that histidine residues are present at the active site of an enzyme. As the amylase obtained from thermophilic Bacillus sp. TS9 showed stability to high temperature and pH, solvents, metal ions, detergents, and surfactants, so can be utilized in starch processing, detergent, textile, and food industries.

Keywords: Alpha amylase, Starch, Thermostable, Metals, Surfactants, Inhibitors, Solvents.

Introduction

Amylase are starch degrading enzyme and is the family of hydrolysing enzyme, classified into three subtypes (alpha amylase or endo-1, 4-α-D glucohydrolase; beta amylase or β-1,4-glucan maltohydrolase; glucoamylase or amyloglucosidase) on the basis of substrate specificity and bond cleavage. The mechanism of hydrolysing bonds in starch by three types of amylases

are variable such as alpha amylase targets internal α-1,4- glucosidic bonds in starch thus producing glucose, maltose and dextrin as end products. The beta amylase act as exoamylase and hydrolyse α-1,4- glucosidic bonds by removing two glucose molecules at the terminal regions (non-reducing ends) of the starch releasing maltose. Furthermore, the glucoamylase yields glucose and maltose by cleaving both α-1, 4 and α-1, 6 glucosidic bonds **(Okpo and Andy, 2019)**. Starch is a polysaccharide composed of two types of glycose polymers designated as amylose and amylopectin. Both amylose and amylopectin are composed of glucose units, which are linked to one another by glycosidic bonds. The amylose is a linear polymer composed of about 6000 glucose units that are connected through α-1,4- glucosidic bonds, however, amylopectin is composed of both linear chain (containing 10-60 glucose units linking through α-1,4- glucosidic bonds) and branched chain (containing 15-45 glucose units linking through α-1,6- glucosidic bonds) **(Gopinath *et al.*, 2019)**.

Several studies reported that amylase is a metallo-enzyme that require metal ions (Calcium) as a co-factor for catalytic activity, structural conformation and stability **(Saboury, 2002)**. Moreover, further investigations have revealed that four β-strands of amylases contains conserved sequences depicted in arrangement (I-IV) and appeared in the loop region of 3, 4, 5 β-strands that connects β-strand 7 to the α-helix 7 **(Nielson and Borchert, 2000)**. Amylases can be produced by many living organisms including plants, animals and microorganisms. But among all living organisms microbial amylases are usually preferred due to high thermal stability and generally regarded as safe status. The potent producers of amylases includes bacteria (*Bacillus, Clostridium thermosulfurogenes, Aeromonas caviae, Pseudomonas sp.* (aerobic)), Molds (*Aspergillus oryzae, A. kawachii, A. niger, A. awamori, Rhizopus oligosporus, Rhizopus japonicas*) and yeast (*Saccharomycopsis capsularia, Amylomyces rouxii*). The first amylase was isolated from fungal source in 1984 for the treatment of digestive disorders by adding the enzymes as a supplement in pharmaceutical product. Among all microbial strains the reported potent bacterial producers are *Bacillus subtilis, B. amyloliquefaciens and B. licheniformis.* Moreover, the *Aspergillus* and *Rhizopus* are the preferred fungal strains for the industrial production of thermostable amylases **(Rao *et al.*, 2007)**.

As microorganisms are ubiquitous in nature and through several methods the potent bacterial and fungal enzymes producers can be screened. The most potential and efficient enzyme producing microbial strains can be isolated from the environments that are rich in substrate. The enzyme producer strains can be isolated through serial dilution or through substrate selection on the basis of their affinity and specificity to the particular substrate **(Anbu *et al.*, 2004; Gopinath *et al.*, 2005)**. The concentration of amylase among different genus, species and strains are variable and even the habitat of microorganism can influence the amount of enzyme such as the bacterial and fungal strains isolated from amylose or starch rich environment will have capability of comparatively high enzyme yield. Moreover, physical (temperature, pH, aeration) and chemical (carbon, nitrogen) factors also play pivotal role in the enzyme production. Furthermore, the fermentation process (submerged or solid state) can also influence the yield of enzyme. Several studies have reported that the microorganism can be genetically engineered to further augment the

production of enzyme at commercial scale **(Sundarram and Murthy, 2014)**.

Amylases constitute about 25-30% of world enzyme market and is considered as second largest group of enzyme. According to data provided by research associations, the alpha amylase shares in 2010 was 1 $ billion dollars, which was increased to 1.5$ billion dollars in 2015 with 6.6% increase in CAGR (Compound annual growth rate). The amylase share is on continuous increase and is 7.5 $ billion with 5 years CAGR of 8.2% in the year 2020 due to the increasing demands. About 12 major and 400 minor suppliers are contributing worldwide to fullfill the increasing demands of amylase in industrial applications **(Paul *et al.*, 2020)**. Amylases are used in the production of maltooligomer or oligosaccharide mixture obtained through corn starch digestion, fructose and maltose syrup suspension and ethanol by direct starch fermentation. Moreover, amylases are also employed in textile industries for desizing, bread production, pulp and paper industries industrial liquefaction, bioethanol production and removal of starchy strains **(Ahmad *et al.*, 2019)**.

The aim of the study is to characterize the crude, partially purified and purified thermostable α-amylase obtained from *Bacillus* sp. TS9 in order to determine the thermal stability of enzyme and to determine that either ammonium sulphate precipitation and gel filtration chromatography has any influence on the catalytic activity of the enzyme.

Material Methods

Characterization of Crude α-Amylase

The characterization of crude enzyme isolated from *Bacillus* sp. TS9 was carried out. After inoculation of production media for amylase, it was incubated for 3 days for maximum production of α-amylase at 40°C and 150 rpm. Then production medium was centrifuged at 10,000 rpm for 30 minutes to remove bacterial cells from the medium. Afterwards, cell free supernatant containing crude α-amylase was further processed.

Effect of Temperature on Crude α-Amylase

The effect of temperature on crude α-amylase was determined by incubating the crude α-amylase at different temperatures. Mixed 1 ml of crude enzyme with 1 ml of substrate buffer (1 g of starch dissolved in 100 ml of potassium phosphate buffer having pH 6.9) and incubated at 5, 15, 25, 35, 45, 55, 65 and 80°C for one hour and then residual activity was determined by DNS method **(Jawad *et al.*, 2020)**. The control was incubated at 40°C under standard assay conditions.

Temperature Stability of Crude α-Amylase

The temperature stability of crude α-amylase was determined by incubating the crude enzyme at 40°C for 0-3 hours and at 100°C for 3 hours. Then the sample was withdrawn from the sample after every hour and its residual activity was determined by DNS method **(Carvalho et., 2008)**. The control was incubated at 40°C for 1 hour under standard assay conditions.

Effect of pH on Crude α-Amylase

The effect of pH on crude α-amylase was determined by incubating the crude enzyme with buffers of different pH at 40°C for 1 hour. 1 ml of crude enzyme produced by *Bacillus* sp. TS9 was mixed with equal amount of buffers of different pH ranges from 4 to 10 and with substrate buffer and then incubated at 40°C for 1 hour. In

control, only substrate buffer was added and incubated at 40°C under standard assay conditions. Then residual activity was determined by DNS method **(Kalsoom *et al.*, 2018)**. The 0.5 M buffers used were sodium acetate buffer (pH 4 and 5), potassium phosphate buffer (pH 6 and 7), Tris HCL buffer (pH 8 and 9) and sodium hydro-oxide/ di-sodium hydrogen phosphate buffer (pH 10).

pH Stability of α-Amylase

The pH stability of crude α-amylase was determined by incubating the crude enzyme with equal amount of potassium phosphate buffer having pH 6.9 and with substrate buffer (1gm starch dissolved in potassium phosphate buffer having pH 6.9) for 0-4 hours at 40°C. The sample was withdrawn after every hour and residual activity was determined. The control was incubated at 40°C for 1 hour under standard assay conditions **(Kalsoom *et al.*, 2018)**.

Effect of Metal ions on Crude α-Amylase

The effect of metal ions on crude α-amylase was determined by incubating the crude enzyme with 100 mM metal ions solutions. 1 ml of crude enzyme produced by *Bacillus* sp. TS9 was mixed with equal volume of metal ions solutions and with substrate buffer at 40°C for 3 hours. Then its residual activity was determined. In control, 1 ml of distilled water was added in place of metal ions solutions **(Li *et al.*, 2020)**. The metal ions solutions used were Na, K, Mg, $(NH_4)_2SO_4$, Zn and Hg in the form of NaCl, $MgCl_2$, $(NH_4)_2SO_4$, $ZnCl_2$ and $HgCl_2$.

Effect of Substrates on Crude α-Amylase

The effect of different substrates on crude α-amylase was determined by incubating the crude enzyme with 1% different substrates dissolved in 0.02 M potassium phosphate buffer having pH of 6.9. 1 ml of crude enzyme was mixed with equal volume of different substrate buffers and incubated at 40°C for 1 hour and then residual activity was determined through DNS method **(Alonazi *et al.*, 2021).** The control was incubated with starch buffer (1 g starch dissolved in 0.02 M potassium phosphate buffer) and incubated at 40°C under standard assay conditions. The different substrates used were sucrose, maltose, lactose and dextrose.

Effect of Detergents on Crude α-Amylase

The effect of detergents on crude α-amylase was determined by incubating the crude enzyme with 1% detergents solution. 1 ml of crude enzyme was mixed with equal amount of 1% detergents solution and with substrate buffer and incubated at 40°C for 3 hours and then residual activity was determined. In control, 1 ml of distilled water was added in place of detergent solution and then incubated at 40°C under standard assay conditions. The detergents used were Ariel, Surfexel, Bonus, bright and express power **(Correa *et al.*, 2011)**.

Effect of Solvents on α-Amylase

The effect of solvents on crude α-amylase was determined by incubating the crude enzyme with 1% solvent solutions. 1 ml of crude enzyme was mixed with equal amount of 1% solvents and with substrate buffer and incubated at 40°C for 3 hours and then residual activity was determined. In control, 1 ml of distilled water was added in place of solvents and then incubated at 40°C under standard assay conditions **(Tiwari *et al.*, 2014)**. The solvents used were methanol, acetone, chloroform, benzene, nitrobenzene, xylene, di-methyl sulphoxide and formaldehyde.

Effect of Surfactants on Crude α-Amylase

The effect of surfactants on crude α-amylase was determined by incubating the crude α-amylase with 1% surfactant solutions. About 1 ml of crude enzyme was mixed with equal volume of 1% surfactant solution and substrate (1g of starch dissolved in 0.02 M potassium phosphate buffer) and then incubated at 40°C for 3 hours. Then residual activity was determined by DNS method **(Tiwari *et al.*, 2014)**. In control, 1 ml of distilled water was added in place of surfactant solution and incubated at 40°C under standard assay conditions. The surfactants used were SDS, Tween-80 and Triton-X-100.

Effect of Inhibitors on Crude α-Amylase

The effect of inhibitors on crude α-amylase was determined by incubating the crude enzyme with 1% inhibitors. 1 ml of crude enzyme was mixed with equal volume of inhibitor solution and substrate containing buffer and then incubated at 40°C for 3 hours. Then residual activity was determined by DNS method **(Mahato *et al.*, 2021)**. Control contained 1 ml of distilled water in place of inhibitors solution and incubated at 40°C under standard assay conditions. The chelating agents used were EDTA and tri-sodium citrate, PMSF, β-Mercepto-ethanol and Phenyl-acetyldehyde.

Results

Characterization of Crude Amylase Produced by Thermophilic *Bacillus* sp. TS9

Effect of Temperature on Crude α-Amylase: Crude α-amylase showed stability over a wide of temperature ranging from 5°C to 80°C. At 5°C and 15°C, the residual activity of crude amylase was 69% and 86.6% respectively. However, temperature stability of the crude amylase increases with increase in temperature. The residual activities of crude amylase at temperature range from 25°C to 80°C was 96.6%, 90%, 105.8%, 96.6%, 89.4% and 84.6%, respectively. Maximum residual activity was obtained at 45°C (Figure 1).

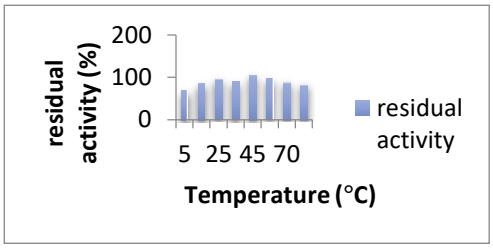

Figure 1. Effect of temperature on crude amylase.

Effect of pH on Crude α-Amylase: At acidic pH, the crude amylase showed less amylolytic activity, such as, at pH 4, 5 and 6 the residual activity of the crude amylase was 24.07%, 40% and 40.75, respectively. The residual activity of crude amylase at pH 7 and 8 was 59.25% and 75.92%, respectively. At basic pH, the amylolytic activity of crude amylase increased with maximum residual activity of 88.33% was observed at pH 9. At pH 10, the activity of the purified amylase was decreased by 53.71% (46.29%) (Figure 2).

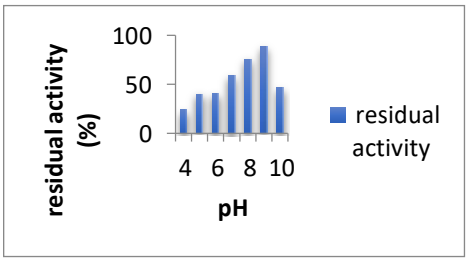

Figure 2. Effect of pH on crude amylase.

Effect of Metal Ions on Crude α-Amylase: The crude amylase showed much stability to Na^{+1} and Mg^{+2} ions and residual activity of 89% and 99.8% was observed,

respectively. $(NH_4)_2SO_4$, Zn^{+2} ions affected the activity of crude amylase to some extent and showed 31.25% decrease in activity (68.75% residual activity) and 42.86% (57.14% residual activity), respectively. However, Hg^{+2} and Ca^{+2} affected the activity of crude amylase and a decrease in activity of 34.64% (65.36% residual activity), 38.57% (61.43% residual activity) was observed, respectively (Figure. 3).

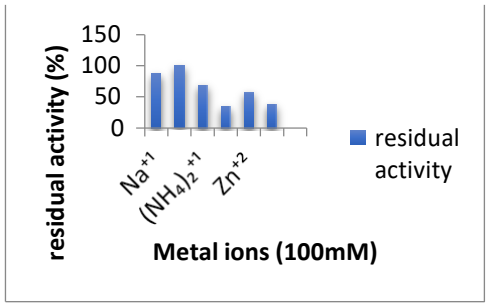

Figure 3. Effect of metal ions on crude amylase.

Effect of Substrates on Crude α-Amylase: Maximum residual activity of 89.9% and 77.7% was shown by maltose and lactose, respectively. However, decrease in activity of 40% (60% residual activity) and 40.5% (59.5% residual activity) was observed by using dextrose and sucrose, respectively, as the substrates (Figure 4).

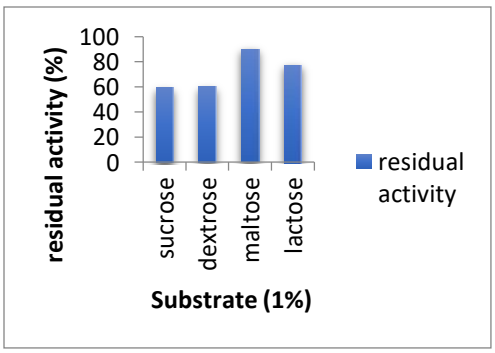

Figure 4. Effect of different substrates on crude amylase.

Effect of Solvents on Crude α-Amylase: The solvents were found to affect the amylolytic activity of crude amylase. Some solvents such as xylene, benzene, DMSO, acetone, formaldehyde and methanol reduced the activity of crude amylase to about its half, i.e., decrease in activity of 45.5% (54.5% residual activity), 55.5% (44.4% residual activity), 44.5% (55.5% residual activity), 47.5% (52.5% residual activity), 49.5% (50.5% residual activity) and 50.6% (49.4% residual activity), respectively was observed. However, in case of nitro-benzene and chloroform decrease in the activity of 63.7% (36.3% residual activity) and 72.8% (27.2% residual activity) was observed, respectively (Figure 5).

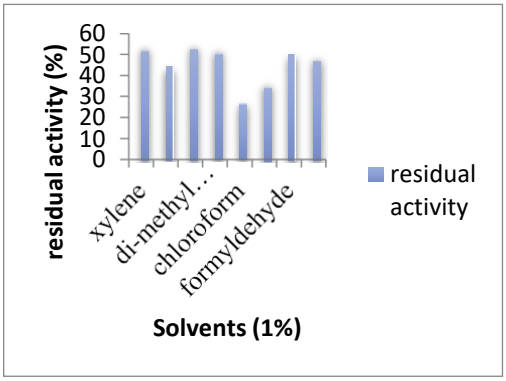

Figure 5. Effect of solvents on crude amylase.

Effect of Detergents on Crude α-Amylase: Crude amylase showed high stability to "Ariel" and its residual activity was 90.89. Also the "Express-power" showed less effect on the activity of crude enzyme and it possess 82.07% residual activity. However, "Surf-excel" and "Bonus" reduced the residual activity to 64.75% and 58.4%, respectively. 81.2% (18.2% residual activity) decrease in activity was observed in case of "Bright" (Figure 6).

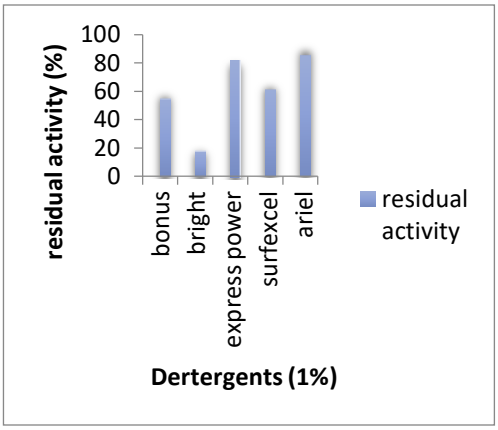

Figure 6. Effect of detergents on crude amylase.

Effect of Surfactants on Crude α-Amylase: Triton-X-100 reduced the activity of crude amylase to its half and showed 51.51% residual activity. However, Tween-80 and SDS affected the amylolytic activity of crude α-amylase and decrease in activity of 52.6% (47.4% residual activity) and 52.4% (47.6% residual activity) was observed, respectively (Figure 7).

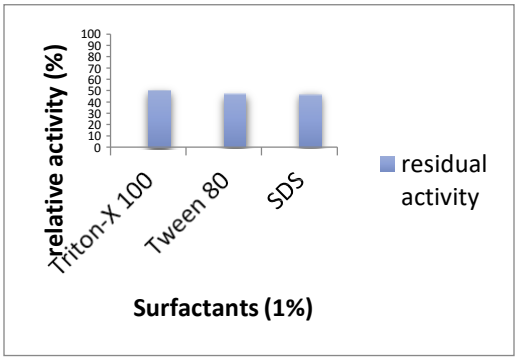

Figure 7. Effect of surfactants on crude amylase

Effect of Inhibitors on Crude α-Amylase: Inhibitors such as Tri-sodium citrate and EDTA reduced the activity of crude amylase to 60.6% and 57.57% respectively. Decrease in amylolytic activity to 51.4% (48.6% residual activity) and 65.8% (34.28% residual activity) of crude amylase was obtained when treated with phenyl-acetaldehyde and PMSF respectively. However, β-mercapto-ethanol almost inhibited the activity of crude amylase, indicating that, the amylase produced by TS9 contains "Histidine" residues. A decrease in activity of 82.6% (17.4% residual activity) was observed when treated with β-mercapto-ethanol (Figure 8).

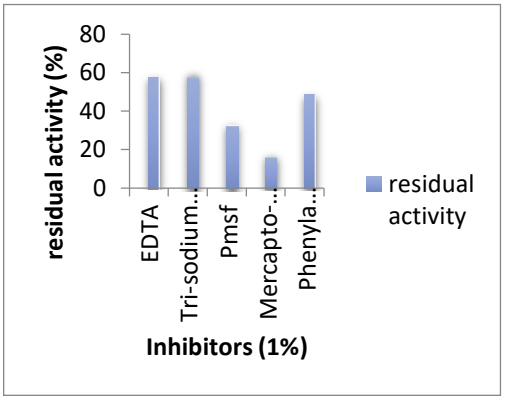

Figure 8. Effect of inhibitors on crude amylase

Characterization of Partially Purified Amylase

Effect of Temperature on Partially Purified α-Amylase: The partially purified α-amylase (precipitates dissolved in potassium phosphate buffer) showed stability at wide range of temperature from 5°C to 80°C. At 5°C the partially purified amylase possess 67.15% residual activity. Increase in temperature also favours increase in the amylolytic activity. At 15, 25, 35 and 45°C, the residual activity of amylase was 82.4%, 87.61%, 94.77% and 103.45%, respectively. Maximum residual activity 109.85%was observed at 55°C. At

70°C and 80°C, the residual activity of amylase was reduced and it was 88.55% and 86.87%, respectively (Figure 9).

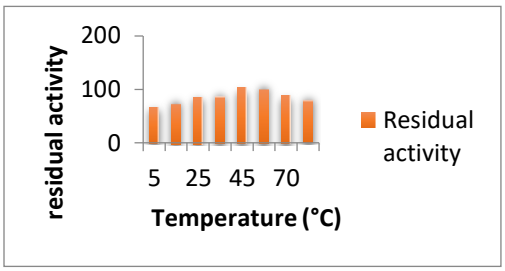

Figure 9. Effect of temperature on partially purified amylase.

Effect of pH Purified α-Amylase: Partially purified amylase showed stability at wide range of pH from 4-10. The residual activity of partially purified amylase at pH 4, 5 and 6 was 60.66%, 61.32% and 63.14%, respectively. However, partially purified amylase showed stability at pH 7- 10 with maximum residual activity of 96.64% at pH 8. The residual activity of partially purified amylase reduced at pH 9 and 10 showed 85.67% and 77.19% residual activity, respectively (Figure 10).

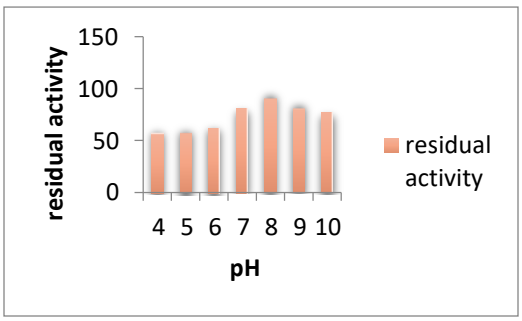

Figure 10. Effect of pH on partially purified amylase.

Effect of Metal Ions on Partially Purified α-Amylase: Metal ions such as Ca^{+2} and Zn^{+2} affected the activity of partially purified amylase and decrease in activity of 60.4% (39.6% residual activity), 63.68% (36.32% residual activity). and 85.68% (14.32% residual activity) was obtained, respectively. $(NH_4)_2^{+1}$ and Hg^{+2} affected the residual activity to some extent and decrease in activity of 39.03% (60.97% residual activity) and 44.68% (55.32% residual activity) was observed, respectively. However, partially purified amylase was comparatively stable to Na^{+1} and Mg^{+2}, and residual activities were 92.89% and 99.79%, respectively (Figure 11).

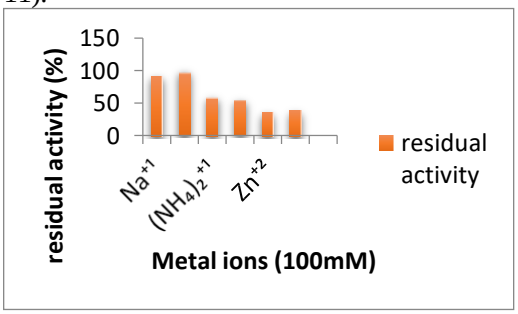

Figure 11. Effect of metal ions on partially purified amylase.

Effect of Substrates on Partially Purified α-Amylase: Maximum residual activity of 151.31% and 152.2% was observed when maltose and lactose were used as substrates, respectively. Decrease in activity of 14.14% (85.86% residual activity) and 6.11% (93.89% residual activity) was observed when sucrose and dextrose were used as substrates, respectively (Figure 12).

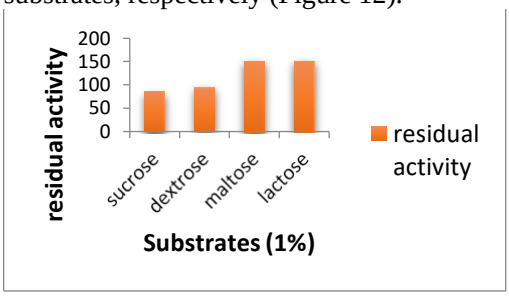

Figure 12. Effect of different substrates on partially purified amylase.

Effect of Solvents on Partially Purified α-Amylase: Solvents such as benzene, DMSO and acetone affected the activity of partially purified amylase and decrease in activity of 59.4% (40.6% residual activity), 50.9% (49.1% residual activity), and 50.97 (49.03% residual activity) was observed, respectively. Chloroform, nitro-benzene and formaldehyde and xylene affected the activity of partially purified amylase to some extent and decrease in activity of 39.97% (60.03% residual activity), 29.28% (70.72% residual activity), 21.48% (78.52% residual activity) and 21.21% (78.11%) was observed, respectively. The partially purified amylase showed stability towards methanol and residual activity of 87.56% was observed (Figure 13).

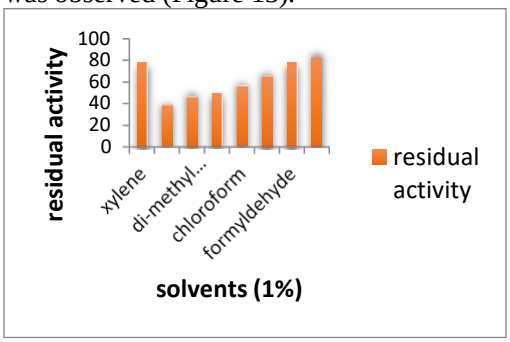

Figure 13. Effect of solvents on partially purified amylase.

Effect of Detergents on Partially Purified α-Amylase: The partially purified amylase showed stability to "Surf-excel" and "Ariel" and the residual activities were 155.05% and 155.17%, respectively. However, "bright", "Bonus" and "Express power" affected the activity of partially purified amylase to some extent and decrease in activity of 39.04% (60.96% residual activity), 28.64% (71.36% residual activity) and 26.93% (73.07% residual activity) was observed (Figure 14).

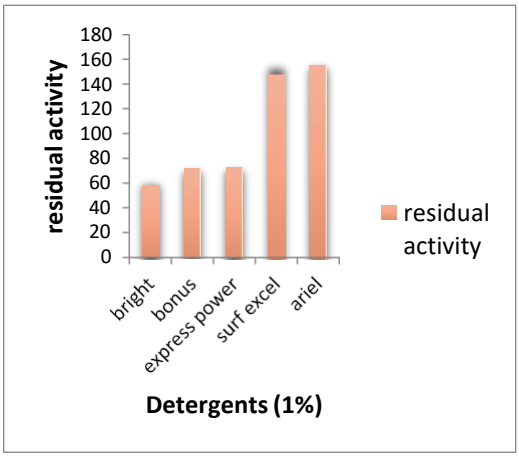

Figure 14. Effect of detergents on partially purified amylase

Effect of Surfactants on Partially Purified α-Amylase: Triton-X-100 affected the activity of partially purified amylase and decrease in activity of 48.77% (31.23% residual activity) was observed. However, Tween-80 and SDS showed less effect on the activity of partially purified amylase and decrease in activity of 40.65% (59.35% residual activity) and 41.6% (58.4% residual activity) was observed, respectively (Figure 15).

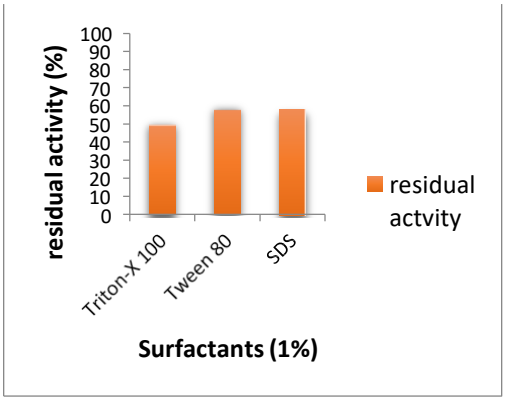

Figure 15. Effect of surfactants on partially purified amylase.

Effect of Inhibitors on Partially Purified α-Amylase: PMSF and Phenyl-acetaldehyde showed very low effect on the activity of partially purified amylase and a decrease in activity of (77.35% residual activity) and (66.03% residual activity) was observed, respectively. EDTA and Tri-sodium citrate inhibited the activity of partially purified amylase to some extent and residual activities of 31.12% and 43.84% observed respectively. β-Mercapto-ethanol almost inhibited the activity of α-amylase and showed 72.76% decrease in activity (27.24% residual activity) (Figure 16).

Figure 16. Effect of inhibitors on partially purified amylase.

Characterization of Purified α-Amylase

Effect of Temperature on Purified α-Amylase: The purified α-amylase showed stability over a wide range of temperatures from 5°C to 80°C. It was noticed that amylolytic activity increases with increase in temperature at certain limit. At 5°C, the residual activity of the purified amylase was 73.1% and at 15°C and 25°C, the activity of the purified amylase increased and it was 97.5% and 100%, respectively. However, incubation at high temperature favoured the increase in amylolytic activity of purified amylase with maximum residual activity of 121.7% was observed at 55°C. At 35°C and 45°C, the activity of purified amylase increased by 19% (119% residual activity) and 17% (117% residual activity), respectively. Further at 70°C, the activity of purified amylase decreased by 10% (90% residual activity) and at 80°C, the activity of purified amylase was 96.8% i.e. the 3.2% decrease in activity was observed (Figure 17).

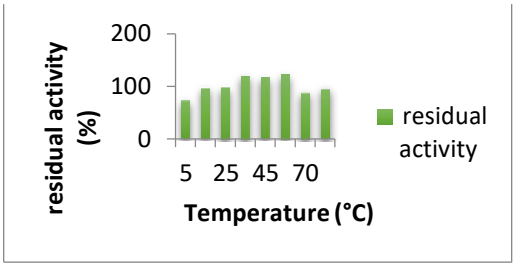

Figure 17. Effect of temperature on purified amylase.

Effect of pH on the Purified α-Amylase: Purified α-amylase showed stability over a wide range of pH from 4-10. At pH 4, the residual activity of amylase was 61.09%. However, the residual activity of Purified amylase increases with increase in pH from 5-10 with the maximum residual activity obtained at pH 9 which is 105.38 % (Figure 18).

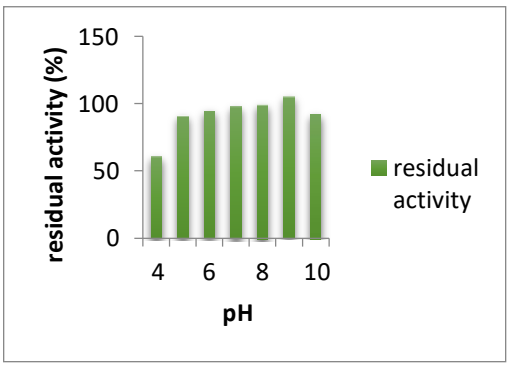

Figure 18. Effect of pH on purified amylase.

Effect of Metal Ions on Purified α-Amylase: Hg^{+2} and Ca^{+2} reduced the activity of purified amylase to 44.04% and 39.78% respectively. However, Zn^{+2} and $(NH_4)_2SO_4$ also reduced the activity of purified amylase to some extent and the activity was decreased by 25.6% (74.4% residual activity) and 31.92% (68.08% residual activity) was observed. Purified amylase showed stability to Mg^{+2} and Na^{+1}; Na^{+1} do not affect the activity of purified amylase and 99.78%residual activity was observed, while in case of Mg^{+2}, the residual activity was increased to 109.78% (Figure 19).

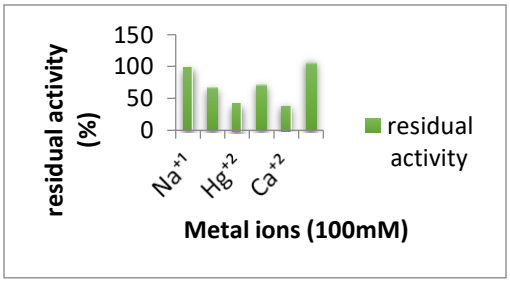

Figure 19. Effect of metal ions on purified amylase.

Effect of Substrates on the Purified α-Amylase: When incubated with sucrose, the purified amylase showed very low activity of 40.37%. However, incubation with dextrose, maltose and lactose substrates significantly increased the residual activity i.e., 87.7%, 83.3% and 80.37%, respectively (Figure 20).

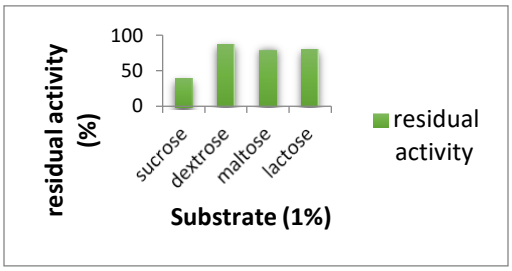

Figure 20. Effect of substrates on purified amylase.

Effect of Solvents on Purified α-Amylase: Formaldehyde and acetone reduced the activity of purified amylase by 46.18% (53.19% residual activity) and 38.3% (61.7 % residual activity), respectively. Benzene, DMSO, nitrobenzene and chloroform don't affect the activity of purified amylase and the residual activities of 85.1%, 87.23%. 87.2% and 78.72% was observed, respectively (Figure 21).

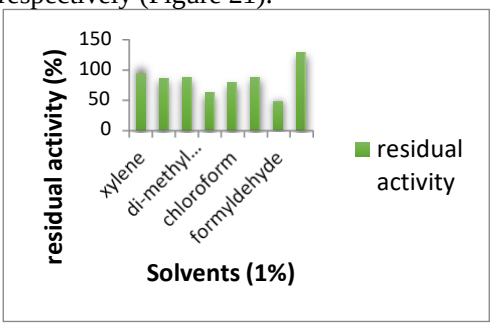

Figure 21. Effect of solvents on purified amylase.

Effect of Detergents on Purified α-Amylase: Express power reduced the activity of purified amylase to its half and residual activity of 51.06% was observed. However, other commercial detergents such as Bonus, Bright, Surf-excel and Ariel also affect the activity of purified amylase and decreased the activity by 38.3% (61.7% residual activity), 27.6% (72.34% residual activity), 35.6% (64.04% residual activity) and 32.56% (67.44% residual activity) was observed, respectively (Figure 22).

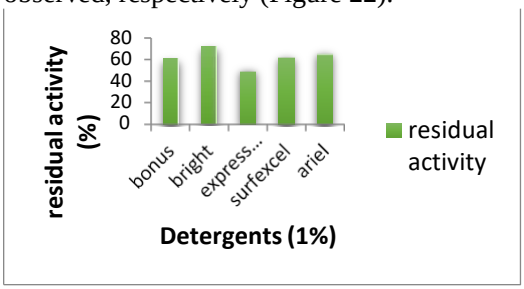

Figure 22. Effect of detergents on purified amylase.

Effect of Surfactants on Purified α-Amylase: Both triton-X-100 and Tween 80 reduced the activity of purified amylase by 40.43% (59.57% residual activity). However, SDS decreased the activity of purified amylase by 38.3% (61.7% residual activity) (Figure 23).

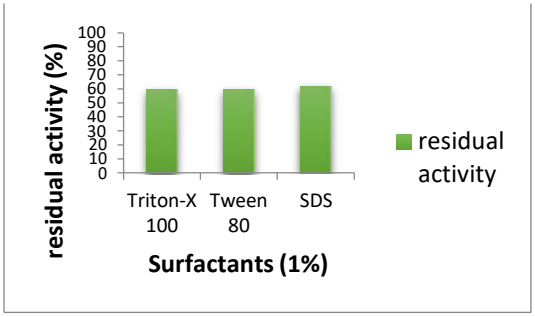

Figure 23. Effect of surfactants on purified amylase.

Effect of Inhibitors on Purified α-Amylase: Tri-sodium citrate, PMSF and phenyl-acetaldehyde reduced the activity of amylase to some extent i.e. 76.5%, 74.4% and 72.34%, respectively. However, EDTA and β-mercapto-ethanol affected the activity of purified amylase and decreased the activity by 53.2% (46.85 residual activity) and 55.4% (44.6% residual activity), respectively (Figure 24).

Figure 24. Effect of inhibitors on purified amylase.

Temperature Stability and pH Stability of Crude, Partially Purified and Purified Amylase

Temperature Stability at 40°C: At zero time the activity of crude and partially purified amylase was 22.5% and 25.9%, while purified amylase showed comparatively high activity of 48.7%. After incubation of 1 hour at 40°C, the activity of crude and precipitates was 100% and 99% respectively, while the activity of purified amylase was 101.3%. After incubation of 2 hours, the activity of crude, partially purified and purified increased to 88.2%, 108% and 147%, respectively. After incubation of 3 hours, the activity of both crude and partially purified amylase was reduced to 74% and 98%, respectively, but the activity of purified amylase was further increased to 155.6%. It was clear (Figure 25) that purified amylase was more stable than crude amylase.

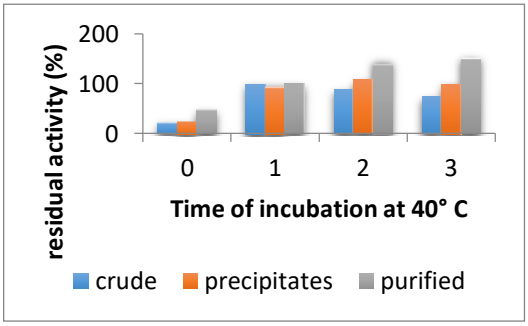

Figure 25. Stability of crude, partially purified and purified amylase at 40°C.

Temperature Stability at 100°C: When incubated for 1 hour at 100°C the amylase obtained from TS9 showed stability in all forms (crude, partially purified and purified form) and the activity of crude, partially purified and purified amylase was 77%,

80% and 78% respectively. After incubation at 100°C for 2 hours, the activity of both crude and partially purified amylase reduced to 56% and 57%, respectively, while the purified amylase still retained 77% of its activity. After incubation of 3 hours at 100°C, the activities of both crude and partially purified amylase was reduced to 13% and 11%, while the purified amylase showed stability and retained 70% of its activity (Figure 26).

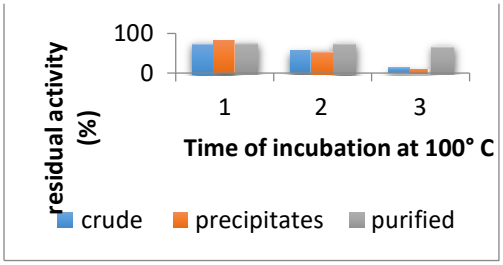

Figure 26. Stability of crude, partially purified and purified amylase at 100°C

pH Stability: At zero-time the activity of crude and partially purified amylase was 20% and 17% respectively, while the activity of purified amylase was comparatively higher (46%). After incubation of 1 hour the activity of crude, partially purified amylase and purified amylase was 100%. 98% and 100%, respectively. After incubation of 2 hours, the activity of crude and partially purified amylase was reduced to 67% and 65%, while the activity of purified amylase increased by 10% (110% residual activity) showing more stability. After incubation of 3 hours, the activity of crude and partially purified amylase was reduced to 33% and 54%, while the purified amylase still retained 101% residual activity. After incubation of 4 hours, the crude and partially purified amylase reduced to 17% and 49% while the purified amylase showed higher stability and retained 73% of its activity (Figure 27).

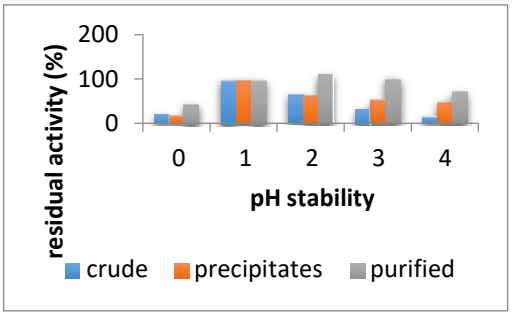

Figure 27. pH stability of crude, partially purified and purified amylase.

Discussion

The crude α-amylase isolated from *Bacillus* sp. TS9 after 10% ammonium sulphate saturation was purified through a single purification step (gel filtration chromatography) and the crude, partially purified and

Effect of Temperature

It is evident from the current study that the crude, partially purified and purified amylase produced by thermophilic *Bacillus* sp. TS9 showed stability to a wide range of temperatures (35°C-80°C) and the maximum activity was shown at temperature range of 45°C-55°C. Even the activity of purified amylase was increased by 21% (121% residual activity) at 55°C. The purified amylase was stable and its activity was increased when incubated for 3 hours at 40°C and the crude, partially purified and purified amylase retained its activity when heated at 100°C. **Vijayalakshmi *et al.*, (2012)** also characterized the crude amylase isolated from *Bacillus subtilis* and the maximum activity was found at 50°C. **Mahdavi *et al.*, (2010)** determined the effect of temperature on the purified amylase isolated from thermophilic *Bacillus spp.* And it showed stability at the wide range of temperature

i.e. from 10℃-70℃ and the maximum activity was obtained at 50°C. **Kumar *et al.*, (2013)** also determined the stability of purified amylase isolated from *Bacillus laterosporus* and the maximum activity of amylase was observed at 60°C.

Effect of pH

The effect of pH and the pH stability of crude, partially purified and purified amylase was determined and characterized. The crude, partially purified and purified amylase produced by thermophilic *Bacillus* sp. TS9 showed stability at the pH range of 5-9. The maximum activity of purified amylase was obtained at pH 9. The purified amylase was stable and its activity was increased by 10% of its original value when incubated for 2 hours at 40°C with potassium phosphate buffer (pH7). **Shafaat *et al.*, (2011)** also reported the crude extracellular amylase obtained from *Bacillus subtilis* and it showed stability at the pH range of 5-10, with the maximum activity obtained at pH 7. **Pancha *et al.*, (2010**) also characterized the crude amylase obtained from thermophilic *Bacillus* sp. and the maximum activity was obtained at pH 8. **Krishnan and Chandra (1983)**, characterized the purified amylase obtained from *Bacillus licheniformis* and the maximum activity of purified amylase was observed at pH 9. **Verma *et al.*, (2011)** determined the optimum pH for purified amylase isolated from *Bacillus spp.* and it showed stability at pH range from 7.5- 8.5. All these investigations supports our study, that amylase showed maximum activity at pH 7-9.

Effect of Metal ions

The crude partially purified amylase and purified amylase isolated from thermophilic *Bacillus* sp. TS9 showed stability to Na^{+1}, K^{+1} and Mg^{+2} ions to large extent and metal ion, Zn^{+2} affected the activity to some extent, while Ca^{+2} and Hg^{+2} reduced the activity to large extent. The extracellular amylase require Ca^{+2} ions for their activity, and the activity of amylase enhanced in the presence of Ca^{+2} ions but in our study the activity of amylase both in crude and purified form was reduced, revealed that the amylase obtained from thermophilic *bacillus spp.* was Ca^{+2} independent. However, the activity of both crude and purified amylase isolated from TS9 enhanced in the presence of Mg^{+2} ions to indicating that the enzyme require Mg^{+2} ions for its activity. Kim *et al* in 2012 reported that the enzyme activity of amylase obtained from thermophilic *Bacillus* sp. was not affected by Ca^{+2} ions **(Kim *et al.*, 2012).** Most of α-amylase contain Ca^{+2} binding site, but structural analysis revealed that some amylase has no Ca^{+2} binding site because of amino acid substitution. Malhotra *et al* in 2000 also reported that the activity of amylase obtained from *Bacillus thermooleovorans* don't require Ca^{+2} ions for its activity **(Malhotra *et al.*, 2000). Najafi *et al.*, (2005)** determined that the activity of purified amylase obtained from *Bacillus subtilis* was inhibited by Hg^{+2}, Cu^{+2} and Ag^{+2} ions. **Asgher *et al.*, (2005)** determined the effect of metal ions on the crude extracellular amylase obtained from thermophilic *Bacillus subtilis* and it was observed that Hg^{+2}, Cu^{+2} and Co^{+2} strongly inhibited the activity of amylase and some metal ions such as Mg^{+2}, Fe^{+2}, Mn^{+2} and Ni^{+2} affect the activity of amylase to some extent. Similarly, **Kumar *et al.*, (2013)** also reported that activity of amylase obtained from *Bacillus laterosporus* was not increased in the presence of Ca^{+2} ions, but also its activity was reduced in the presence of Mg^{+2} ions. All these reports revealed that metal ions play key role in the

stabilization of metal ions and some metal ions can affect the activity of amylase. The inhibition by some metal ions is because of the competition between exogenous cations and inhibition by Hg^{+2} is demonstrating that sulphydrl groups are present at the catalytic site of amylase **(Leveque *et al.,* 2006).** Amylases showing greater stability to metal ions, indicates that these metal ions play key role in the structural stability or may take part in the catalysis.

Effect of Inhibitors

The effects of various inhibitors such as EDTA, pmsf, phenyl-acetaldehyde, tri-sodium citrate and mercapto-ethanol were also checked on the activity of crude, partially purified and purified amylase isolated from thermophilic *Bacillus* sp TS9. All inhibitors showed different effects on the activity of crude and purified amylase. The class of enzyme and the active site that participate in reaction with substrate can be determined through inhibitors.

EDTA

EDTA is the chelating agent that can inhibit the activity of amylase by binding to the metal ions present at the active site of the enzyme and that metal ions catalyse the reaction when bind to the substrate. So, EDTA binds to the metal ions, consequently inhibit the activity of enzymes. When EDTA inhibits the activity of enzymes, it shows that the enzyme is metallo-enzyme. In our study, the activity crude enzyme isolated from *Bacillus* sp. TS9 was reduced to about its half by EDTA i.e. 57% activity of crude amylase was observed. **Mahdavi *et al.,* (2010)** reported that extracellular amylase produced by *Bacillus cereus* was affected by EDTA and its activity was inhibited partially by EDTA, which also supports our study. In the current study it was observed that the activity of the purified amylase obtained from *Bacillus* sp. TS9 was affected by EDTA and 53.2% decrease in its activity from its original value was observed. Similarly, **Hmidet *et al.,* (2010)** reported that the activity of purified amylase obtained from *Bacillus mojavensis* was not enhanced in the presence of Ca^{+2} ions but are still inhibited by EDTA, which supports our study. Mercapto-ethanol is another chelating agent that inhibits the Histidine residues and di-sulphide bonds present at the active site of amylase and take part in the reaction when binds to the substrate and mercapto-ethanol targets that histidine residues consequently inhibiting the activity of amylase **(Li & Yu, 2012)**. In current study, the mercapto-ethanol inhibits the activity of both crude and purified amylase obtained from *Bacillus* sp. TS9. **Asoodeh *et al.,* (2010)** also reported that the purified amylase produced by *Bacillus spp.* was affected by marcepto-ethanol and reduced its activity. **Kumar *et al.,* (2013)** reported that the activity of amylase obtained from thermophilic *Bacillus laterosporus* was also inhibited by β-mercapto-ethanol. All these investigations support our study.

PMSF

Another chelating agent phenylmethylsulfonyl fluoride (PMSF) effect was also checked on the crude, partially purified and purified amylase produced by *Bacillus* sp. TS9 and it was found that it reduced the activity of crude amylase to 34% of its original value. The pmsf inhibit the activity of amylase by binding sulfonyl groups to the serine residues present at the active site of amylase and thus inhibit their activity. Actually, these serine residues participate in the reaction when substrate and enzyme binds **(Adinarayana *et al.,* 2003)**. Some other inhibitors such as tri-sodium

citrate and phenyl-acetaldehyde showed no effect on effect on the activity on the crude, partially purified and purified amylase obtained from TS9.

Effect of Substrates

The effect of various substrates on crude, partially purified and purified amylase produced by *Bacillus* sp. TS9 was also characterized and results showed that along with starch some other substrates such as lactose, maltose and dextrose can also be used. However, the sucrose showed no significant results and the activity of amylase both in crude and purified form was reduced to 59% and 40% respectively. **Vijayalakshmi *et al.*, (2012)** also reported the effect of various substrates such as maltose, glucose, corn starch and Barley starch on amylase activity produced from thermophilic *Bacillus* sp. Similarly, **Kim *et al.*, (2012)** also reported the effect of various substrates on the amylase produced by *Bacillus spp.* and significant effect was observed by using amylose and cyclo-dextrin substrates, and activity was reduced when glycogen and amylo-pectin was used as the substrates. All these investigations revealed that, different substrates can be used and it supports our study indicating that amylases showed maximum activity on the certain substrates as it may contain sufficient amount of nutrients and remain loose in moist conditions.

Effect of Commercial Detergents, Surfactants and Solvents

The stability of crude, partially purified and purified amylase was also determined towards the commercial detergents such as Ariel, Surf-excel, Express-power, Bright and Bonus. Bright reduced the activity of crude amylase produced by *Bacillus* sp. TS9 to 18% of its original value, and bonus reduced the activity of crude amylase to its half, while it showed stability to other commercial detergents. However, the purified amylase obtained from *Bacillus* sp. TS9 showed stability towards commercial detergents, and its activity was not too much affected. Similarly, the effect of surfactants such as Triton-X-100, Tween-80 and SDS was also determined on the crude, partially purified and purified amylase obtained from *Bacillus* sp. TS9. The surfactants affect the activity of both crude and purified amylase. About 50%-60% activity of crude amylase was reduced in the presence of surfactants while 30%-40% decrease in activity of purified amylase was observed when treated with surfactants. Similarly, **Shafaat *et al.*, (2011)** reported that the activity of amylase obtained from *Bacillus spp*. was reduced by SDS. In one another study, **Fattah *et al.*, (2012)** also reported that the activity of amylase produced by thermophilic *Bacillus spp.* was reduced by SDS and other commercial detergents. Some studies also reported the increase in activity of amylase when treated with surfactants and detergents. **Kim *et al.*, (2012)** reported that activity of amylase increased in the presence of SDS, Triton-X-100, Tween 80 and Tween 20.

Conclusion

The study can be concluded that the alpha amylase produced by *Bacillus* sp. TS9 was highly thermostable and showed stability at wide range of pH, therefore, the enzyme can be used in different industrial applications as it can withstand the harsh conditions in industrial bioprocesses. Moreover, the resistance of alpha amylase to surfactants, solvents and commercial detergents also augmented the enzyme usage to industrial scale and can be used in

detergents, textile, leather, paper and pulp industries.

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