Pak. J. Bot., 57(6): DOI: http://dx.doi.org/10.30848/PJB2025-6(19)

ALPHA AMYLASE PRODUCTION, PURIFICATION AND CHARACTERIZATION USING INDIGENOUS *BACILLUS SUBTILIS*

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Abstract

The α-amylase enzyme hydrolyzes starch and glycogen into maltose, isomaltose and glucose. It is produced from plants, animals and microbes but microbial α-amylase has the highest commercial and industrial value. *Bacillus subtilis* was identified on the basis of 16S rRNA. Further, characterization of *Bacillus subtilis* was done using BLAST tool and phylogenetic tree with MEGA X. Different carbon and nitrogen sources were supplemented to the LB medium. The supplemented LB medium was sterilized and used for the growth of *Bacillus subtilis.* High yield of α-amylase was produced at 3% molasses and 3% peptone. Alpha Amylase was precipitated at 60% ammonium sulphate concentration then partially purified α-amylase was further purified through column chromatography. The optimum pH and temperature for alpha amylase was found to be 9 and 55°C respectively. Kinetic studies of alpha amylase were performed to check Km and Vmax. values. Impact of different metal ions were observed on α-amylase activity and high α-amylase yield was recorded in the presence of Fe^{2+} ions. SDS-PAGE technique revealed the molecular weight of α-amylase which was ~46kDa. The purified α-amylase utilized for characterization studies. The role of α-amylase was analyzed in detergent industry with different company detergents. Enzyme activity was estimated by checking release of reducing sugars which had application in textile industry. The enzyme capability with detergents and release of reducing sugars in de-sizing step in textile industry makes alpha amylase suitable for local industries in Pakistan.

Key words: Alpha Amylase, *Bacillus subtilis*, Optimization, Characterization, Detergent industry, Metal catalysts, Phylogenetic tree.

Introduction

The α-amylase (E.C. 3.2.1.1) belongs to hydrolase class which is important for starch digestion into reducing sugars. Linear chain 1,4-α-D-glycosidic in homopolysaccharides like glycogen, starch and oligosaccharides are broken down by α-amylases (Rameshkumar & Sivasudha, 2011). The enzyme α-amylase hydrolyzes starch easily than β-amylase which is obtained from sprouted grains. Starch acts as a primary substrate for all alpha amylases, which results in tiny molecules of maltose, iso-maltose, dextrin and glucose production (Gopinath *et al.*, 2017). Amylase are classified into two major category gluco-amylase and alpha-amylase, in which α-amylase is extensively used in different commercial applications (Singh *et al.*, 2022). To fulfil the increasing industrial demands of alpha amylase, there is a need to enhance alpha amylase production with most economical process (Langyan *et al.*, 2022). Amylase production through submerge fermentation that is using the synthetic media is uneconomical and inexpensive (Balakrishnan *et al.*, 2021). So, there is a need of hour to replace expensive processes of enzyme production (food ingredients) with less expensive processes for alpha amylase production (agricultural wastes). For this purpose, scientists use agriculture waste materials for the amylase production through solid state fermentation which has many benefits like economical, simple technique, better recovery of any product and low level of catabolic inhibition (Singh *et al.*, 2022). Different bacterial strains are used for alpha amylase production like *Bacillus subtilis, Bacillus cereus, Bacillus megaterium* and *Bacillus amyloloquefaciens*. Many other microbes are also used like fungi, yeast and bacteria for alpha amylase production (Raju *et al.*, 2013). In the textile industry, enzymes like α-amylases can be used as alternative biocatalyst instead of chemicals, as chemicals can harm the fabrics (Allan *et al.,* 1997). Various enzymes play important to improve the quality of textiles. α-amylases can be used to remove the starch in de-sizing process while catalase and cellulase improve the whiteness and softness (Ashraf *et al.,* 2003). Enzymes are ecofriendly chemicals so their amalgamation in detergent making can enhance the activity of detergent to remove the stain, also make detergent safe for environment. To meet the growing demands of α-amylase in textile and detergent industry, the current study was designed for production of α-amylase from *Bacillus subtilis* by optimizing the levels of carbon & nitrogen sources concentration, its purification and characterization of various parameters like temperature, pH, metal ions and substrate concentration.

Material and Methods

The present study was conducted in the laboratories of Institute of Biochemistry and Biotechnology (IBBT), University of Veterinary and Animal Sciences, Lahore, Pakistan. All the solvents and chemicals were analytical scale and were purchased from Sigma-Aldrich (Germany).

Microorganism collection and culture: The culture of *Bacillus subtilis* was available in Microbiological Institute, University of Veterinary and Animal Sciences, Lahore, Pakistan. The culture of *Bacillus subtilis* was

streaked on LB agar plates, for this purpose sterilized Luria Broth agar was used (Sigma-Aldrich, Germany) and stored in freezer at 4°C. Glycerol stock of overnight grown culture of *Bacillus subtilis* was prepared by mixing 800µl cells (10^6 to 10^8 CFU/mL) and 200µl sterilized glycerol. The glycerol stock was stored at -20°C (Naramchimeg *et al.,* 2019).

16 S rRNA identification of bacteria

Extraction of bacterial DNA: DNA of bacteria was extracted and processed as method explained by Imran *et al*., (2018).

Agarose gel electrophoresis of PCR amplified product: 1% agarose gel was used to examine the PCR amplified 16 S rRNA gene. The 3 µL 16 S rRNA gene was added with 1 µL loading dye and lo was mixed with 1µl loading dye and run on gel after loading. Ladder of 1 KB size was loaded on gel to confirm the size of gene of interest. The gel running time was 40 minutes with continuous voltage supply (100 V). The bands of gene of interest and ladder were visualized on UV Trans illuminator.

Phylogenetic tree and sequencing of 16 S rRNA gene: The amplified 16 S rRNA gene was used for sequencing for identification of bacterial strain (Lu *et al.*, 2018). The 16 S rRNA gene product was sent to Macrogen Company in Korea for sequencing. The sequence of gene (16 S rRNA) was aligned and compared in various tools of GenBank like NCBI, BLAST for homology analysis (Altschul *et al.*, 1997). On the basis of closely related DNA sequences available in GenBank phylogenetic tree was constructed through MEGA 11.

α-Amylase production through *Bacillus subtilis*: Bacterial single colony from freshly prepared LB agar plate was cultivated on LB Broth to assess the species ability to produce α-amylase. LB media was placed in incubator and incubated in incubator shaker at 37°C (120 rpm) for 24 hours. After incubation, LB culture was taken, shifted to falcon tubes, and agitated at 6000rmp for 15 minutes. The supernatant was used for further analysis while pellet was discarded (Naramchimeg *et al*., 2019).

Influence of carbon sources on alpha amylase activity: Different carbon sources like rice bran, molasses and wheat bran were added in concentrations from 0% to 3% in the Luria Bertani broth before sterilization. Freshly prepared inoculum of *Bacillus subtilis* from overnight grown culture was added in Luria broth medium using different carbon sources at 37°C for 24 hours in incubator shaker (124 rpm). After centrifugation, supernatant was used for alpha amylase activity while pellet was discarded (Ahmad *et al.*, 2020).

Influence of nitrogen sources on alpha amylase activity: Freshly prepared inoculum of *Bacillus subtilis* from overnight grown culture was added in Luria broth medium using different nitrogen sources at 37°C for 24 hours in incubator shaker (124 rpm). After this incubation, samples shifted in to sterilized falcon tubes, placed in centrifuge and agitated at 6000 rpm for 15 minutes. The supernatant was used for alpha amylase activity while pellet was discarded (Ahmad *et al.*, 2020).

Purification of α-amylase after optimized conditions of carbon and nitrogen sources: After the optimization of different nitrogen and carbon sources, alpha amylase was produced in the optimized concentration of molasses and peptones sources. In 300ml LB broth, 3% molasses and peptone were added and autoclaved. After centrifugation, supernatant was used for amylase activity assay and pellet was discarded.

Activity assay: Starch is used as substrate to check alpha amylase activity. A 50 mM phosphate buffer (pH 7) was utilized to examine the activity of α-amylase using 1% starch solution. The reaction mixture containing 1ml enzyme and 1ml of 1% starch solution in phosphate buffer of pH 7. This mixture was placed in incubator for 15 minutes at 40°C. The 2ml of DNS (3, 5 Dinitrosalisylic acid) was added in reaction mix and kept in boiling water bath at 100°C for 10 minutes (Shah *et al*., 2014). Once the tube was cooled, optical activity was checked on Spectrophotometer at 540 nm. An identical composition with blank was also prepared, but without enzyme. The released sugars were determined by maltose standard curve (Imran *et al.*, 2018).

Ammonium sulphate precipitation: For the partial purification of α-amylase, overnight grown culture of *Bacillus subtilis* under optimized conditions was centrifuged and supernatant was used for enzyme precipitation. Alpha Amylase was partially purified using ammonium sulphate precipitation from 20 to 80%. For alpha amylase precipitation, ammonium sulphate was gradually added and mixed well, after precipitation sample was centrifuged at 6000rpm for 15 minutes. After centrifugation, each pellet was dissolved in 10ml of 50 mM Tris buffer (pH 8). Alpha amylase activity was performed for each pellet that was obtained after 20, 40, 60 and 80% precipitation. For this reaction mixture was prepared containing 0.1ml of re-suspended pellet sample and 1ml of 1% starch-buffer solution. Reaction mixture was incubated in water bath at 55°C for 15 minutes. After incubation, 2ml of DNS reagent was added and boiled in water bath for 10 minutes. After boiling, mixture was cooled at room temperature and absorbance was measured at 540nm in spectrophotometer (Imran *et al.*, 2018).

Dialysis: After partial purification of α-amylase, the sample which showed maximum activity was dialyzed to eliminate the salt. For this purpose, dialysis membrane was dipped into buffer to open the membrane. When the membrane was opened sample was placed in membrane and membrane was tied from both ends and placed in buffer of pH 8. The buffer was replaced with fresh buffer after every 4 hours until salt was completely removed from the sample. To check either salt is removed or not $BaCl_2$ test was performed (Imran *et al*., 2018).

Enzyme purification: Dialyzed sample was purified through column chromatography. Sephadex G-50 resin was used to pack the column. The Column of gel filtration chromatography was equilibrated with buffer of pH 8 (Tris-HCl). In pre-equilibrated column, 1mL sample was applied and eluted with same buffer of pH 8. Different fractions (16) of crude alpha amylase were collected each of 3mL in labeled tubes and enzyme activity assay was performed.

Characterization of alpha amylase

Influence of temperature on alpha amylase activity: A purified enzyme was used to assess optimum temperature for enzyme activity; alpha amylase activity was checked at various temperatures from 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C. Reaction mixture was prepared containing 1ml starch solution in buffer alongside 0.1 purified enzyme was added in the labeled test tubes and placed at their respective temperatures for 15 minutes. After incubation 2 ml DNS reagent was added and boiled for 10 minutes. After cooling absorbance was measured at 540nm (Abdel-Fattah *et al.*, 2013).

Influence of pH on alpha amylase activity: To determine the optimum pH for enzyme activity, assay was performed at different buffer of pH, 50mM each of Tris-HCl buffer (7.0-9.0), Phosphate buffer (5.0-7.0) and acetate buffer (3.0-5.0) were used. Reaction mixture in the labeled tubes was prepared using 1 ml of starch prepared in different buffers, 0.1ml enzyme. Reaction mixture was incubated at 55°C for 15 minutes. After 15 minutes, 2ml DNS reagent was added and boiled for 10 minutes, absorbance was measured at 540nm (Abdel-Fattah *et al.*, 2013).

Impact of metal ions on α-amylase activity: Enzyme activity was determined in the presence of different metal ions. The metal ions (0.25 mL of 1 mM) Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Co^{2+} (mM) were used to determine the effect on enzyme activity. For this purpose, reaction mixture was prepared in the labeled test tubes with metal ions. Activity assay was performed and absorbance was measured at 540nm (Abdel-Fattah *et al.*, 2013).

Molecular weight determination: SDS PAGE technique was used to determine the molecular weight of alpha amylase. SDS PAGE was prepared using resolving gel (12%) and stacking gel (5%). SDS PAGE apparatus was adjusted and resolving gel was dispensed between the plates. When resolving gel was polymerized the sample loading stacking gel and comb was placed in it. After solidification of stacking gel, stacking comb was removed very carefully and solidified gel plate was transferred to electrophoretic tank. 1X tris-glycine buffer was poured into tank and well was washed with same buffer. Partially purified enzyme and protein marker were loaded into wells. After loading the enzyme and protein marker, it was allowed to run. After completion of electrophoresis, gel was removed between the plates and stained with the staining solution for 20 minutes. After staining the gel with dye, the gel was placed in de-staining solution for overnight and band was visualized (Imran *et al.*, 2018).

Kinetics studies of α-amylase: Alpha amylase activity was estimated using different concentrations of substrate from 1 to 10 mM and the Line weaver-Burk plot was obtained after plotting double reciprocal graph between velocity of reaction and substrate concentrations for the assessment of Km and Vmax values (Imran *et al.*, 2018).

Comparative analysis with standard enzyme: Activity of α-amylase that was studied compared with standard α-amylase by Sigma-Aldrich, Germany. For this purpose, the solution of standard alpha amylase was prepared. The reaction mixture was prepared for both α-amylases. Blanks were also prepared in same concentration without amylase. Alpha amylase activity was measured under standard assay conditions.

Alpha Amylase industrial applications:

Study in textile industry: A piece of cotton fabric (10cm×10cm) was incubated with 1% starch for 15 min at 60°C. After being air dried, the cloth was cleaved into two equal parts. One part of the paper was incubated with alpha amylase for 15 minutes at 55°C in 50mM tris buffer of pH 9. Level of sugars produced by alpha amylase was monitored under standard assay conditions. The second part of the paper was used as negative control and was given the same treatment but without alpha amylase. The absorbance was measured at 540 nm wavelength (Zafar *et al.*, 2019).

Compatibility with various commercial detergents: Different commercial detergents available in local market namely Brite (Company Colgate-Palmolive, Pakistan), surf excel (Unilever Pakistan limited), Ariel (P & G European technology center), sunlight (Company Unilever, Pakistan limited), express power (Company Colgate-Palmolive, Pakistan limited) and Aspin was used to examine the compatibility of α-amylase. Commercial detergents at concentrations of 0.5 and 1% were utilized. Before adding the enzyme, the detergent's natural enzymes were inactivated by heating the diluted detergent for an hour at 70 degrees centigrade. Reaction mixture was prepared using 0.5ml enzyme, 1ml detergent solution and 1ml of starch prepared in tris-HCl pH 9. Total reaction volume was placed in incubator at 55°C for 15 minutes and the remaining activity was examined under standard assay conditions. Reaction mixture without any detergent solution was also prepared which used as a control. The absorbance was determined at the wavelength of 540nm (Spectrophotometer Cecil, CE2042).

Data Analysis Tools: All the data of this research was analyzed using different statistical tools like ANOVA through SPSS software and the level of significance was set at $p<0.05$.

Results and Discussions

Bacteria characterization: Genomic DNA was extracted, and bands of extracted DNA were visualized in gel electrophoresis under UV light. PCR amplification of 16S rRNA gene was done and amplified product was visualized on agarose gel electrophoresis under UV light with 1 kb DNA ladder to ensure the size of amplified product as shown in Fig. 1. The results showed efficient amplification; a single band of DNA amplified product 1500bp was observed. The polymerase chain reaction amplified product was used for 16 S rRNA gene sequencing. The gene sequence determined for *Bacillus strain* was 1114bp nucleotides long and deposited in Gen bank under the accession number (PP064568.1). According to the blast results *B. subtilis* was showed 94.9% similarity with *Bacillus subtilis* strain accession number (KT362178.1). The 16S rRNA sequence was aligned using CLUSTALW program with other genus *Bacillus* sequences that were obtained from GenBank. Analysis of phylogenetic tree was performed through software MEGA11 (Molecular Evolutionary Genetic Analysis) based on neighbor joining. The homology analysis of *Bacillus subtilis* strain (Fig. 2) was showed that was related to bacterial strain *Bacillus subtilis* (KT362178.1). Molecular identification using bacterial 16 S rRNA gene sequence was very important for correct identification of bacterial species as compared to morphological and biochemical characterization. Conversely, low similarity indicated that species might represent a new taxon (Drancourt *et al.*, 2000).

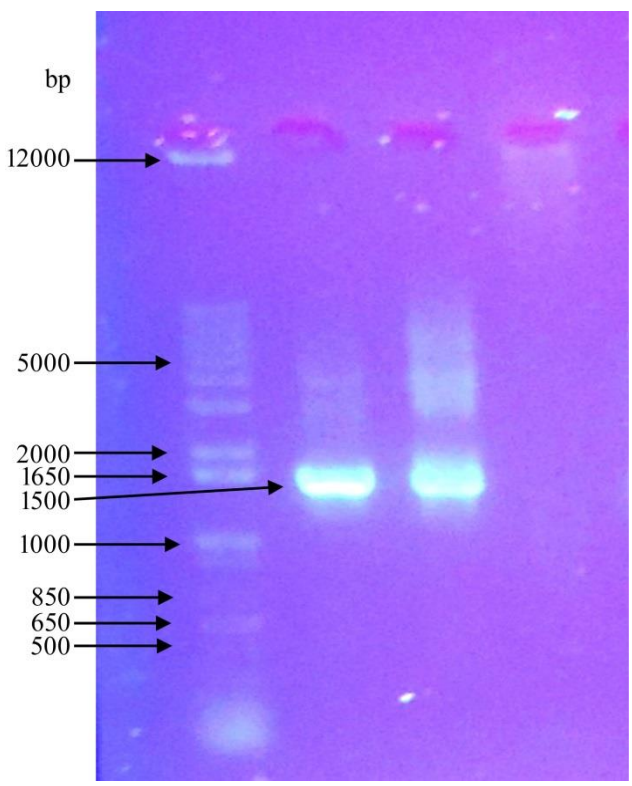

Fig. 1. Agarose gel electrophoresis results of PCR product of 16S rRNA gene of isolated bacteria showing band equal to 1500bp. First well is showing ladder.

Optimization the conditions for alpha amylase production

Influence of carbon sources on alpha amylase yield: Different carbon sources supplemented effects were examined on the yield of alpha amylase. For this purpose, LB medium was used supplemented with 0 to 3% carbon sources. Enzyme activity results demonstrated that with increase the concentration of wheat bran from 0 to 3%, yield of alpha amylase was increased and its optimum activity was recorded at 3% (115±1.53^{a} U/mL) of wheat bran. ANOVA test revealed that wheat bean concentrations were significantly different ($p<0.05$) on yield of alpha amylase (Table 1). Enzyme activity results demonstrated that with the increased concentration of molasses from 0 to 3%, the yield of alpha amylase increased and optimum yield was obtained at 3 % (192±1.86^{a} U/mL) of molasses. ANOVA test revealed that molasses concentrations showed significant different ($p<0.05$) on alpha amylase yield (Table 1). Alpha amylase activity results demonstrated that with the increased concentration of rice bran from 0 to 2% production of α-amylase was increased and decreased at 2 to 3%, and its maximum activity was recorded at 2% (182±1.86^{a} U/mL) of rice brane. ANOVA test revealed that rice bran concentrations demonstrated significant results for alpha amylase yield ($p<0.05$) (Table 1).

The results were in agreement with Simair *et al*., (2017). They reported that molasses showed best results for alpha amylase yield from *Bacillus* sp., BCC01-50. Other research findings revealed that different organisms had different capacity to utilize carbon sources for high yield of alpha amylase from different bacterial strains. Niyonzima & More (2014) reported that potato peels powder was the best carbon source for high yield of alpha amylase. Molasses was the most inexpensive source of carbon (carbohydrates) as it contained a lot of reducing and non-reducing sugars, vitamins and minerals, molasses influences the α-amylase production (Rajagopalan & Krishnan, 2008.). Carbon sources are considered important factors for alpha amylase production and microbial growth. In recent study, yield of alpha amylase was optimized using different carbohydrates sources like rice bran, wheat bran and molasses from bacterial strain (*Bacillus subtilis*). Luria broth medium was complemented with different carbohydrate sources and high yield of alpha amylase was obtained with molasses after 24 hours on incubation which was 192 U/mL as contrast to wheat bran and rice bran. This high yield of alpha amylase was due to rich nutrients and growth promoters in molasses. Raul *et al*., (2014) said that starch and dextrin were the good sources for high yield of alpha amylase using *Bacillus altitudinis* which gave the 5.5U/mL amylase yield. In the prevoius study, α-amylas production was also reported from *Bacillus amyloliquifacians* by utilization of rice brane, potato peels and wheat brane. Maximum yield of alpha amylase was obtained in the growth medium containing combination of potato peels and wheat bran (Mojumdar & Deka, 2019).

Yield of alpha amylase with different nitrogen sources: Alpha amylase yield was checked using different levels of nitrogen sources. For this purpose, LB medium was used supplemented with 0 to 3% nitrogen sources. Enzyme activity results demonstrated that with changing level of nitrogen sources from 0-3%, high yield of alpha amylase was obtained at 3% yeast extract (88.3 ± 0.882^{a} U/mL). ANOVA test revealed that alpha amylase yield showed significant differences ($p<0.05$) with different levels of yeast extract (Table 2).

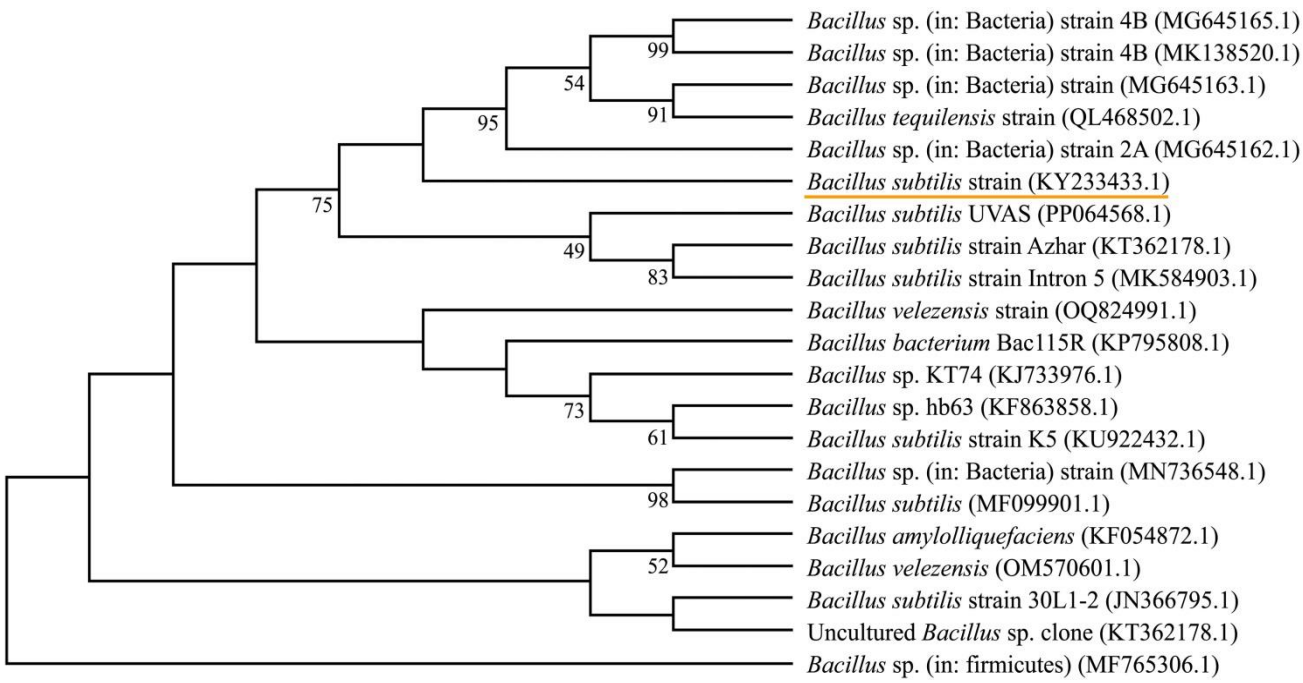

Fig. 2. Phylogenetic relationship of 16S rRNA gene sequence of *Bacillus subtilis* (PP064568) using the neighbor joining method.

Table 1. Effect of Carbon sources on α-amylase production.

Carbon source type	**Wheat bran**			
Concentration (%)	Control	1%	2%	3%
α-amylase activity (U/mL)	49.3 ± 1.86^{c}	90.3 ± 1.2^{b}	111 ± 1.45^{a}	115 ± 1.53^{a}
Carbon source type	**Molasses**			
Concentration (%)	Control	1%	2%	3%
α-amylase activity (U/mL)	49.3 ± 1.86^{c}	130 ± 1^{c}	156 ± 2.19^{b}	192 ± 1.86^{a}
Carbon source type	**Rice bran**			
Concentration (%)	Control	1%	2%	3%
α-amylase activity (U/mL)	49.3 ± 1.86^{c}	112 ± 1.45^{b}	182 ± 1.45^{a}	178 ± 1.45^{a}

Table 2. Effect of Nitrogen sources on α-amylase production.

Nitrogen source type	**Yeast extract**			
Concentration (%)	Control	1%	2%	3%
α-amylase activity (U/mL)	49.3 ± 1.86^{c}	55.7 ±2.03^{bc}	60.3 ± 1.45^{b}	88.3 ± 0.882^{a}
Carbon source type	**Peptone**			
Concentration (%)	Control	1%	2%	3%
α-amylase activity (U/mL)	49.3 ± 1.86^{d}	114 ± 1.2^{c}	123 ± 1.53^{b}	130 ± 1^{c}

Enzyme activity results demonstrated that with the increased concentration of peptone from 0 to 3%, maximum yield of alpha amylase was obtained at 3% peptone level (130 U/mL). ANOVA test revealed that peptone revealed significant yield for alpha amylase ($p<0.05$). Error bars showed means ±SD (Fig. 3). Nitrogen played important role in the prolifiration of microrganisms, considered to be secondry energy source and influenced yield of alpha amylase. In recent study, yeast extract and peptone were supmlemeted to the LB medium from 1 to 3% while high yield of alpha amylase was obtained at 3% peptone level. The result was highly compareable with Abo-Kamer *et al*., (2023) who used different nitrogen sources and found that petone gave high yield of alpha amylase (124U/mL) form *Bacillus cereus*. Hallol *et al*., (2022) stated that petone revealed significant impact on alpha amylase yield. Moreover, Simair *et al*., (2017) reported that high alpha amylase yield was obtained with beef extract from different species of *Bacillus.* It was seen that different microorganism had different growth and nutrient requirements.

Comparative analysis of different carbon and nitrogen sources: Comparative analysis of various nitrogenous and carbon sources was checked for alpha amylase high yield. When growth media supplemented with various levels of peptone and yeast extract, highest yield of alpha amylase was obtained at 3% peptone and 3% yeast extract levels as shown in Fig. 3.

α-amylase purification

Ammonium sulphate precipitation: Ammonium sulphate precipitation results showed that maximum activity of α-amylase was noted in 60% precipitated sample. After partial purification additional salt was removed from the alpha amylase protein through dialysis.

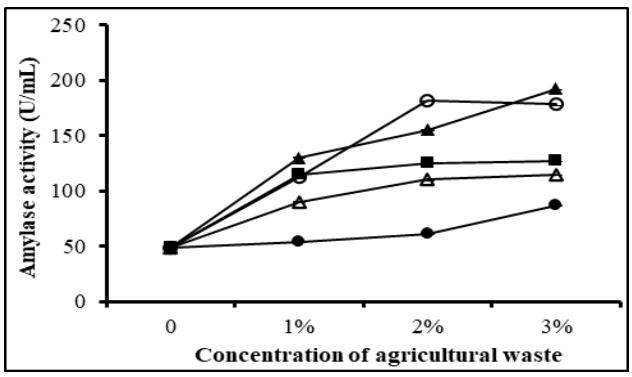

Fig. 3. Use of different carbon and nitrogen sources for α-amylase production. (○) rice bran; (Δ) wheat; (▲) molasses; (●) yeast extract; (■) peptone on α-amylase production.

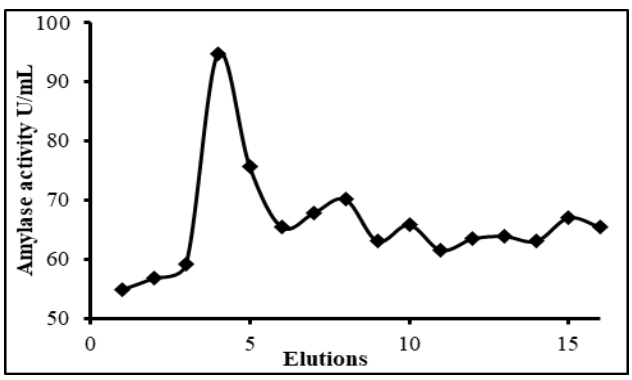

Fig. 4. Eluted fractions from Sephadex G-50 using 50 m*M* Tris-HCl buffer.

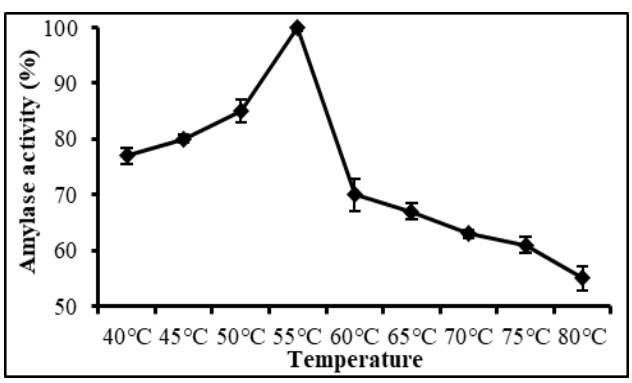

Fig. 5. Effect of temperature on α-amylase activity.

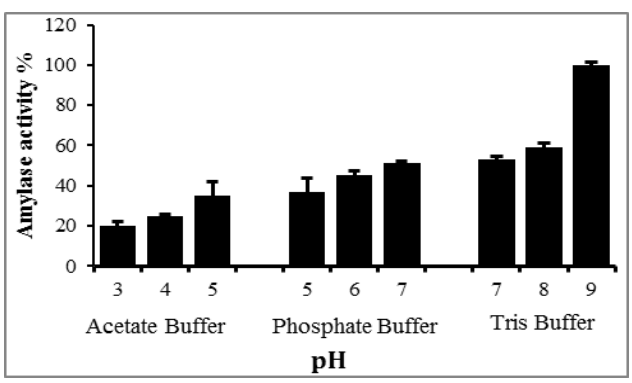

Fig. 6. Effect of pH on α-amylase activity. α-amylase activity was recorded using 50m*M* of each of sodium acetate buffer (3-5), phosphate buffer (5-7), Tris HCl buffer at 55◦C.

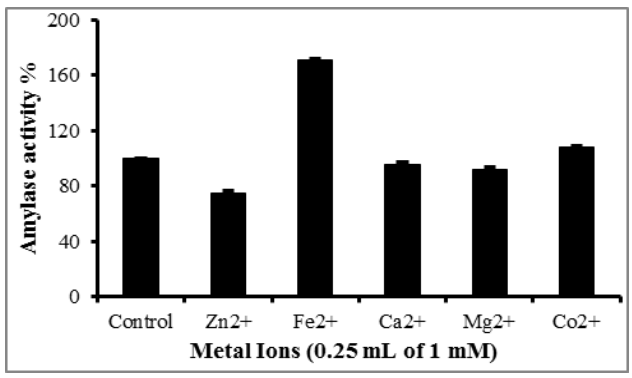

Fig. 7. Effect of metal ions on α-amylase activity.

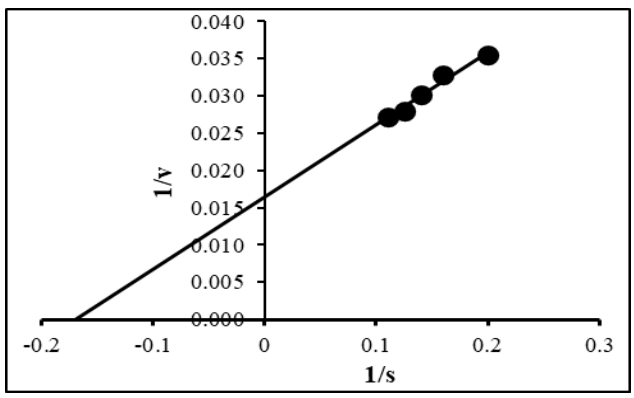

Fig. 8. Lineweaver Burk plot: α-amylase activity was performed by varying starch concentrations from 0 to 10 mg/mL.

Gel filtration chromatography: Dialyzed sample was purified through gel filtration chromatography. Colum was packed with sephadex G-50 as filtration resin. Sixteen fractions (3mL) were collected and used for amylase activity assay. Maximum activity was observed in fraction 4 as compared to other fractions (Fig. 4).

Characterization of α-amylase

Influence of temperature on alpha amylase yield: Alpha amylase yield was increased with increasing temperature from 40 to 55°C produced from *Bacillus subtilis*. The optimum temperature for high alpha amylase yield was 55°C and alpha amylase yield was decreased with the increasing temperature from 55°C (Fig. 5). Effect of temperature exhibited significant results ($p<0.05$) for alpha amylase yield. The extracted alpha amylase was test against various temperature from 40 to 80°C for optimum activity. The optimum temprature for the activity of α-amylase was found at 55°C. A study on *Bacillus velezensis KB 2216* showed that optimum alpha amylase yied was obtained at 55°C which was similar with this study (Bhatt *et al.*, 2020). Yassin *et al*., (2021) was found optimum temprature for α-amylase activity at 55°C. Simair *et al*., (2017) reported that alpha amylase produced from *Bacillus* sp., *BCC 01-50* showed maximal activity at 65°C.

Alpha amylase yield and effect of pH: Alpha amylase activity was checked at different pH levels from 3-9 using different buffers. The activity of α-amylase increased from 3 to 9 pH and optimum alpha amylase activity was

obtained at pH 9 (Fig. 6). Changing pH level revealed significant impact ($p<0.05$) on alpha amylase activity. Alpha amylase activity values were similar with the result of Simair *et al*., (2017) with change in pH. *Bacillus* sp., BCC 01-50 showed high alpha amylase activity at alkaline pH (pH 9). Alpha amylase optimum activity was also observed at pH 8 (Febriani *et al.*, 2019). Similar results were also reported by Pancha *et al*., (2010). Pancha *et al*., (2010) results were also matched with recent study and Simair *et al*., (2017).

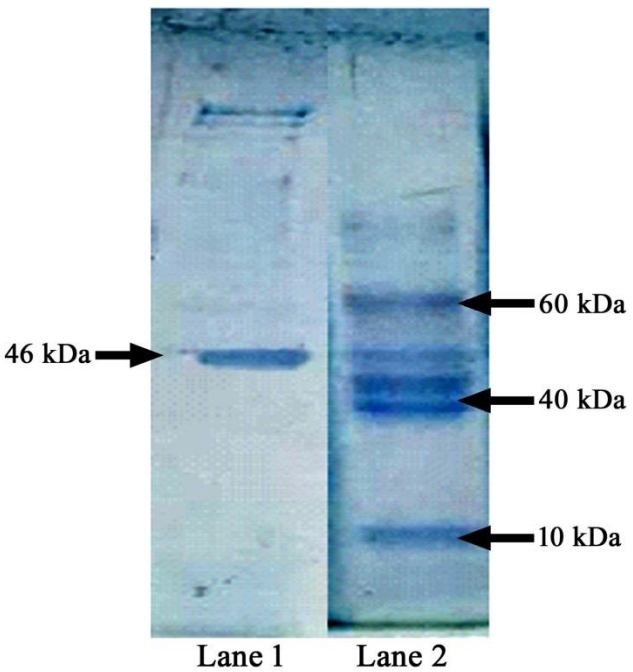

Fig. 9. Molecular weight analysis. Lane 2 shows thermoscientific pre-stained protein ladder marker 26616, Lane 1 shows ~46 kDa band of partially purified protease after column chromatography.

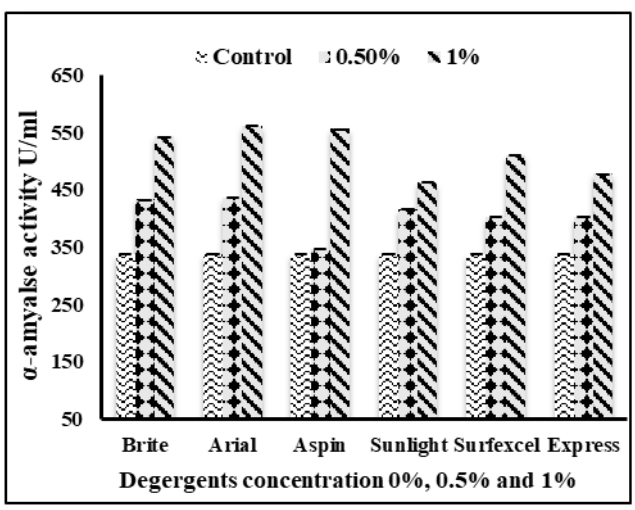

Fig. 10. Compatibility of commercial detergent with α-amylase.

Alpha amylase activity and impact of metal ions: Impact of metal ions were checked against alpha amylase to find out suitable cofactor. Alpha amylase activity was found to be less affected with different metal ions. It was found that alpha amylase activity was increased in the presence of Co^{2+} and Fe^{2+} while maximum alpha amylase activity was seen in the presence of Fe^{2+} (Fig 7). Effect of metal ions showed significant different ($p<0.05$) on alpha amylase activity. Fe^{2+} and Co^{2+} revealed combined effect on activity of alpha amylase. Fe^{2+} and Co^{2} showed 170% and 108% enzyme activity, respectively. The result was in agreement with Raza & Rehman (2016). The increase in alpha amylase activity was observed because iron ions interact with negatively charged amino acids in the active site (Hossain & Uddin, 2011). Although, in most cases it was observed that Ca^{2+} ion enhanced alpha amylase activity but it relied on alpha amylase source. Alpha amylase activity was significantly decreased in the presence of Zn^{2+} but less decrease in alpha amylase activity was observed in the presence of Mg^{2+} and Ca^{2+} ions. It was found that alpha amylase activity was increased in the presence of Fe^{2+} and Ba^{2+} (Febriani *et al.*, 209). Simair *et al*., (2017) reported that alpha amylase activity was significantly decreased in the presence of Mg^{2+} and Zn^{2+} ions. Alpha amylase activity was inhibited with EDTA and Zn^{2+} produced by *Bacillus* species (Ahmed *et al.*, 2019).

Kinetics studies of alpha amylase: Alpha amylase activity was increased with increase in substrate concentration from 1-10mg/ml. By increasing the substrate concentration, a linear increase in enzyme velocity was recorded. Double reciprocal graph between amylase activity and substrate concentrations (Fig. 8) of alpha amylase revealed than Vmax and Km values were 61 U/mL and 6 mg/mL Kinetic studies of alpha amylase showed that apha amylase activity was linearly increased with increase in substratre concentration starch from 1-10mg/mL. Kinetic parametres K_m 6 mg/mL and V_{max} 61 U/mL were calculated through lineweaver burk plot. Singh *et al*., (2022) determined Vmax and Km values at 0.5-6.5mg/mL substrate concentration for α-amylase was 6.2 U/mL and 1.4 mg/mL, respectivity for different *Bacillus* strains B-10. Kizhakedathil (2021) was used Lineweaver–Burk plot to calculate the maximum velocity (Vmax) and Michalis-Menten constant Vamx and Km values were found to be 20.83 U/mL and 45 mM, respectively.

Alpha amylase molecular weight: Molecular characterization of partially purified alpha amylase was carried out after purification using Sephadex G-50 gel. SDS PAGE technique was used with molecular marker of protein of different segments. The typical molecular weight of α-amylases generally falls within the range of 50-60kD, while variations from 10 to 210kDa have been reported in literature. Alpha amylase molecular weight was found to be 46 kD as shown in Fig. 9. Alpha amylase molecular weight obtained from *Bacillus* strains was fond in range from 22 kD to 68 kD (Gupta *et al*., 2003). During this study, alpha amylase molecular weight was found ~46 kD through SDS-PAGE. The nearest molecular weight of alpha amylase was found to 53.11 kD which was produced by *Bacillus cereus* (Abo-Kamer *et al.*, 2023). Febriani *et al*., (2019) determined alpha amylase molecular weight which was 12.2kD produced from *Geobacillus* sp. According to study of Ataallahi *et al*., (2021), the molecular weight of alpha amylase was 50kD produced from *Bacillus subtilis.*

Comparative analysis with standard enzyme: The studied α-amylase activity was compared with standard α-amylase by Sigma, Germany. The studied α-amylase showed more activity (748 U/mL) compared to standard α-amylase (449 U/mL). Standard alpha amylase has less activity may be due storage effect or structural changes.

α-amylase industrial applications

Use in textile industry: The use of α-amylase as de-sizing in textile industry was observed at laboratory scale. When starch containing clothe was incubated with α-amylase and enzyme activity assay was observed under standard assay conditions, alpha amylase activity was seen 841 U/mL as compared to control which showed that more reducing sugars were released in sample compared to control and starch present in the fabric piece that was not treated with enzyme. The role of α-amylase in textile industry as de-sizing was observed at laboratory scale. The starch containing cotton fabric was treated with alpha amylase formed from *Bacillus subtilis* and amount of reducing sugars was found to be 4468mg/mL compared to control 732mg/mL (Zafar *et al.*, 2019).

Compatibility with various commercial detergent: Alpha amylase activity was detected in the presence of various detergents. Detergents in the concentration at 0.5% and 1% were used. Alpha amylase velocity of reaction was enhanced from 0.5-1% detergent concentration. However, maximum alpha amylase activity was detected in the presence of 0.5 and 1% Arial detergent as shown in Fig. 10. The compatibility of α-amylase from *Bacillus subtilis* was studied with different detergents available in local market. Detergents were utilized at the concentrations of 0.5% and 1%. The endogenous enzyme of detergents was previously deactivated. Alpha amylase activity was increased from 0.5 to 1% in case of all detergents but highest activity was found in case of aspin at 0.5% and Arial at 1%. The results were comparable with Singh *et al*., (2022) who determined the compatibilty of detergents with α-amylase and found that the alpha amylase activity was increased in the existence of various detergents.

Conclusion

In recent study, alpha amylase produced, purified & characterized from *Bacillus subtilis*, while, optimization studies revealed that maximum activity of alpha amylase was in the presence of 3% molasses and peptone. Molasses has the potential to serve as highly cost-effective energy source for industrial alpha amylase production. In detergents, alpha amylase showed high compatibility with detergents and could be used in detergent industry to remove starch stain. Moreover, enzyme is appropriate in textile industry as de-sizing agent of fabrics. This locally produced enzyme will be biodegradable and ecofriendly.

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(Received for publication 21 June 2024)