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Research Article

Optimization, Production, and Partial Purification of Thermostable α-Amylase Produced by Marine Bacterium *Bacillus sp*. NRC12017

Ebtsam M El-Kady[1], Mohsen S Asker[1], Saadia, M Hassanein[2], Eman A Elmansy[1], Fawkia M El-Beih[2]

[1]Microbial Biotechnology Department, National Research Centre, El-Tahreer Street, Dokki, Cairo, Egypt
[2]Microbiology Department, Faculty of Science, Ain Shams University, Cairo, Egypt

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ABSTRACT

α-Amylase biosynthesis was illustrated under stress circumstances of high temperature and high salinity in aerobically cultivated culture of a newly isolated moderately thermophilic bacterium of spore-forming *Bacillus* sp. NRC12017 in medium containing starch, peptone and yeast extract. Maximum yield (18.41 U/ml) was took place at pH 6.5 with 200 µl inoculum size at 45°C and an incubation time of 3 days. The ideal volume of the fermentation broth was found to be 15 ml in 100 ml Erlenmeyer flask, supplementation of starch at 2.5% and peptone plus yeast extract at 0.7% brought about the greatest production of α-amylase. Two fractions of α-amylase activities, designated FIII and FIV, were refined from culture filtrate utilizing ammonium sulfate (60 and 80%) and showed the main band at 30 KDa. Both fractions had the highest activity at 250 µl starch in a reaction mixture and pH 6.0-7.0. FIII and FIV showed that a temperature of 50 and 55°C and a reaction time of 20 and 30 min were the best available conditions for their activities, respectively, both fractions were stable up to 65°C and the activity was decreased drastically to 3.22 and 3.26 % when heated at 70°C. Concerning pH stability, a broad range of pH stability (5.0-11.0) was obtained by FIII and FIV.

Keywords: α-Amylase, partial purification, *Bacillus* sp. NRC12017, optimum condition.

INTRODUCTION

Enzymes are used vastly in industry as they can accelerate various kinds of chemical reactions. Amylases are a category of enzymes that used widely in industrial process and represent approximately 25-33% of the global market of enzymes[1]. These enzymes belong to glycoside hydrolases family (GHs) and enhance hydrolysis of α-(1,4) - and/or β-(1,6)-linkage in starch molecules and related polymers to give different products of low molecular weight including dextrins and other smaller polymers composed of glucose monomers[2]. Amylases are assorted into four different classes, *i.e.* exoamylases, endoamylases, transferases, and debranching enzymes. α-Amylase (EC 3.2.1.1) is a kind of endo-amylase that mainly breaks the inner α-(1, 4)-D-glucan bonds of starch[3]. α-Amylase is the most intensively studied amylolytic enzymes, which fractures starch and is applied broadly in several branches of the food, pharmaceutical, and chemical industries[4]. α-Amylases can be originated from diverse sources like plants, animals, and microorganisms[5]. Microbial α-amylases generally considered to be well suited for industrial demands as they have higher yield and are definitely more thermostable[6]. Thermo-stability is an essential feature of almost all of the enzymes that used in industry. Thermo-stable amylases are obtainable from the mesophile *Bacillus licheniformis*[7], *Bacillus* sp. ANT-6[6] and *Bacillus* sp. ASMIA-2[8]. A probable source for obtaining α-amylase that is stable under hard conditions is halo-tolerant or halophilic bacteria from marine habitat that are also exposed to high temperatures as well as an extreme salt concentration[9]. The most significant feature of halophilic enzymes that permits them to surely be a novel alternate for use in the biotechnological sectors is their capability to be thermostable, resist a wide range of pH and survive in high salt concentrations[10]. A lot of halophilic or halotolerant bacteria were informed as prospective amylase creator, that includes *B. dipsosauri*, *Halobacillus* sp, *B. halodurans, Halothermothrix orenii*, *Bacillus* sp strain TSCVKK, *Chromohalobacter* sp. TVSP101 and *Rheinheimera aquimaris*[11-17]. The present study was aimed to isolate halophilic bacteria from different local marine environments, optimize the fermentation conditions for the maximum production of α-amylase, and to characterize the enzyme produced in the culture supernatant.

MATERIALS AND METHODS

Sample collection

Samples were collected from water and sediment of marine and salterns at different locations for isolation of bacteria. The samples were taken from Rashid, Sidi Bisher beach at Alexandria, Safaga, Hurghada, El-Ain Elsokhna beach, Marsa Matrouh and Wadi El-Natron.

**Author for Correspondence: mohsenmsa@yahoo.com:*

The sediment and water samples were collected in sterile bottles and brought to the lab, stored in the refrigerator at 4°C until it was used.

Isolation and purification of α-amylase producing bacteria

Samples were suspended into 90 ml sterilized saline solution (0.85%, NaCl) and diluted using the dilution method[18]. Also, collected marine water samples were diluted by 10-fold dilution technique. 100 µl of each diluted sample (10^{-4} -10^{-6}) concentrations were placed on the starch agar plate and spreads with a sterile L-shaped glass rod[19]. The plates were incubated at 50°C for 24 and 48 h, single colonies of different sizes were selected. The colonies were subjected to purification to obtain single pure colonies.

Screening of isolated samples for α-amylase production

All pure isolates were inoculated on starch plates to test for α-amylase secretion, incubated at 50°C for 3 days and stains with an iodine solution (0.5%). Amylase positive stains were determined by the presence of a clear zone of starch hydrolysis around the colony on the starch plates[20]. The diameter of these clear zones, which was an indication of α-amylase activity, was measured. Colonies having a clear zone around them were selected for further investigation.

Identification of potent amylase producer

Biochemical, morphological, and physiological characteristics of the potential producer (isolate number 16) was determined by adopting standard methods[21]. The identification was confirmed with phylogenetic analysis. Briefly, genomic DNA of bacteria was extracted and universal primer 5'-TCCGTAGGTGAACTTTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' primers was used for the amplification of DNA[22]. A single discrete PCR amplicon band was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Sequencing products were resolved on an Applied Bio-systems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). Data were submitted to GenBank database. The DNA sequence was compared to the GenBank database in the national Center for Biotechnology Information (https://www.ncbi.nlm. nih.gov/) using the BLAST program[23].

Growth conditions for α-amylase production

To choose a proper culture medium for growth of the isolated strain and α-amylase production, primarily five different fermentation media were examined: **Medium 1** (g/l): Na_2HPO_4 6.0, KH_2PO_4 3.0, NaCl 0.5, $MgSO_4$ 0.24, $CaCl_2$ 0.01, peptone 3.0, Starch 10.0[6]. **Medium 2** (g/l): Starch 10.0, yeast extract 2.0, peptone 5.0, $MgSO_4$ 0.5, NaCl 0.5, $CaCl_2$ 0.15[24]. **Medium 3** (g/l): Starch 20.0, Peptone 0.5, $MgSO_4.7H_2O$ 0.5, NaH_2PO_4 0.1, $(NH_4)_2SO_4$ 0.1, KCl 0.1[25]. **Medium 4** (g/l): Peptone 10.0, Starch 5.0, Beef extract 10.0[12]. **Medium 5** (g/l): Starch 20, peptone 10, yeast extract 4, $MgSO_4$ 0.5, $CaCl_2$ 0.2[19]. The pH of the media was adjusted to 7 with 0.1N HCl and 0.1N NaOH. The media were sterilized and Erlenmeyer flasks of 100 ml capacity containing 20 ml of culture medium were inoculated with 1 ml of previously prepared inoculum and incubated at 50°C in a rotary shaker at 140 rpm for 72 h. The samples were harvested after 72 h and the cells were separated by centrifugation (5000 rpm for 15 min at 4°C) in centrifuge (SIGMA 3-18 KS). The cell dry weight (CDW) of culture broth was measured by harvesting the cells after centrifugation and drying them at 105°C to a constant weight.

Enzyme assay

α-Amylase activity was determined by measuring the reduction in blue color intensity resulting from enzyme hydrolysis of starch[26]. The reaction mixture consisted of 200 µl cell-free supernatant, 250 µl of soluble starch (1% w/v) and 500 µl phosphate buffer (0.2 M pH 7) incubated at 50°C for 30 min. The reaction was stopped by adding 250 µl of 0.1 N HCl and color was developed by adding 250 µl of iodine solution. The optical density (OD) of the blue color solution was determined at 660 nm using (JASCO V-630) spectrophotometer. One unit (U) of enzyme activity is defined as the amount of enzyme that hydrolyzes 0.5 mg of starch per minute under assay optimum conditions. Specific activity was expressed as units of enzyme activity per mg of protein.

Protein determination

Protein content was determined by Bradford's method using bovine serum albumin as standard[27]. All measurements were performed three times and the average value was taken.

Factors affecting α-amylase production from Bacillus sp NRC12017

Flask cultures were performed in 100 ml Erlenmeyer flasks containing 20 ml of the medium 5. Factors affecting cell growth and α-amylase production were investigated using one factor at a time method. The optimized parameters were incubation time (1, 2, 3, 4 and 5 days) at 50°C under shaking conditions at 140 rpm, inoculum size (100, 200, 300, 400 and 500 µl), medium volume (10, 15, 20, and 25 ml), temperature (40, 45, 50 and 55°C) and initial pH of the medium (5.5, 6.0, 6.5, 7.0, 7.5, and 8.0). Studies were also performed to evaluate the influence of different carbon sources (maltose, sucrose, xylose, fructose, and starch 2% w/v) and different concentrations of starch (0.5, 1.0, 1.5, 2.0 and 2.5%, w/v). Also different nitrogen sources were used (peptone, yeast extract, peptone plus yeast extract, yeast plus meat extract and peptone plus meat extract 1.4% w/v) and different concentrations (0.35, 0.70, 1.05, and 1.40% w/v) of peptone plus yeast extract were tested to get the best one for α-amylase production. The enzyme activity and protein concentration were determined.

Partial purification of α-amylase

Ammonium sulfate was added to the cell-free supernatant to bring the saturation to 20–80% at room temperature with constant stirring then allowed to stand overnight at 4°C. The precipitate was collected by centrifugation at 5000 rpm for 15 min in a refrigerated centrifuge. The precipitate was collected, re-dissolved in the minimum volume of 0.2 M phosphate buffer (pH 7) and dialyzed using a dialysis membrane with a 10-KDa cut-off against distilled water at 4°C. The supernatant was analyzed for enzyme activity and protein concentration.

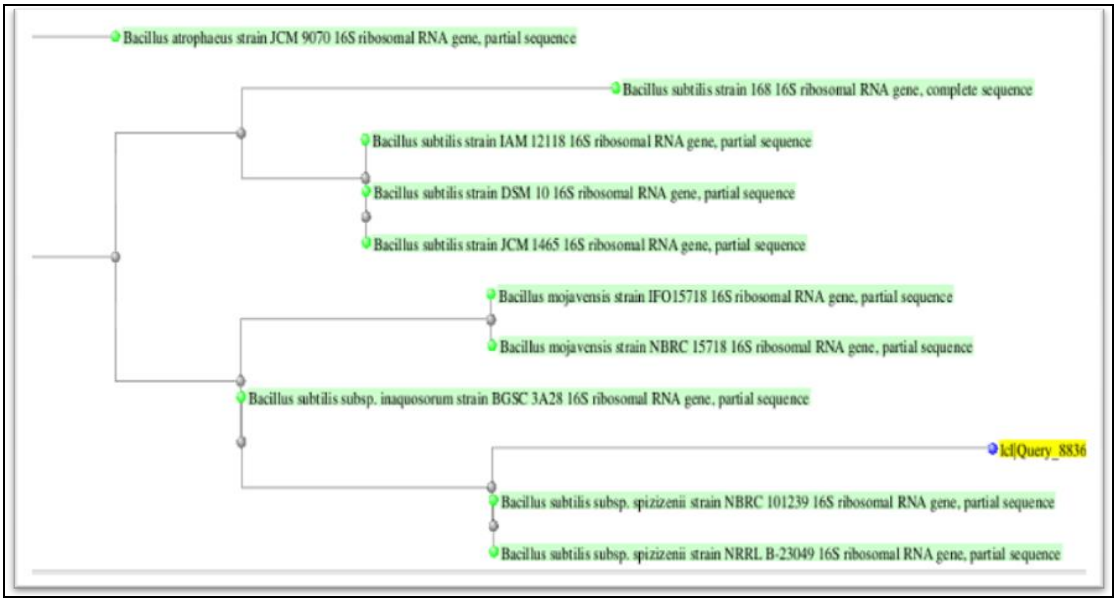


Figure 1: Phylogenetic tree based on the 16S rRNA sequences of isolate and its closest *Bacillus* sp.

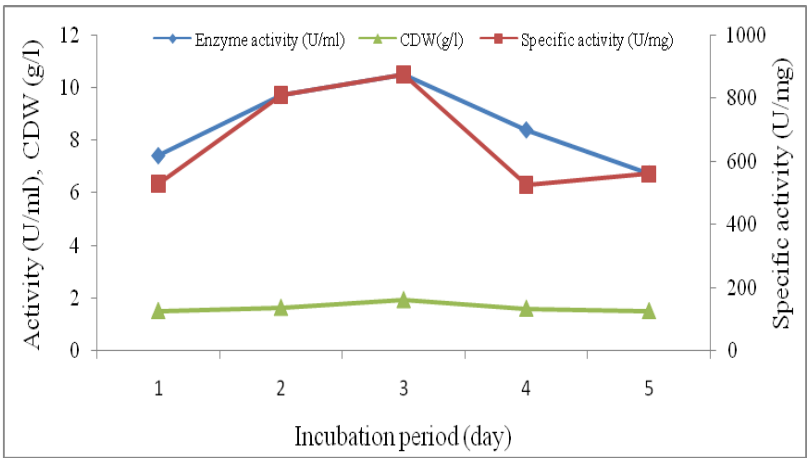


Figure 2: Effect of incubation period on α-amylase production by *Bacillus* sp NRC12017.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 12% polyacrylamide gel as described by method of Laemmli[28] and protein bands were detected by Coomassie brilliant blue R-250.

Characterization of partially purified α-amylase

The α-amylase activity was assayed at different incubation times (10, 20, 30, 40 and 50 min). Effect of starch concentration on α-amylase activity was measured at different concentrations of starch in the reaction mixture (50, 150, 250 and 350 µl). The optimum temperature and pH was evaluated by performing the enzyme assay at different temperatures (40, 45, 50, 55, 65, and 75°C) and different pH (5, 6, 7, and 8). The effect of pH on α-amylase stability was determined in a pH range (5–11) using different buffers (citrate-phosphate buffer, phosphate buffer and glycine-NaOH buffer). Also, the effect of temperature on α-amylase stability was tested by incubating the enzyme without substrate at different temperatures (40, 45, 50, 55, 60, 65, and 70°C) for 30 min.

RESULTS AND DISCUSSION

Total 50 bacterial strains were isolated from marine sediment and water samples on starch agar plates. These isolates were further inspected for the excretion of α-amylase using a starch agar plate. Only 38 isolates could produce α-amylase as pointed out by an area of clearance around them. Among these 38 positive isolates, only two isolates showed high α-amylase productivity thus selected as the best α-amylase manufacturer for further studies. One potential isolate was identified basing on its microscopic, morphological and biochemical characterization and it was proven to be *Bacillus* sp. These studies revealed *Bacillus* sp. as gram positive bacilli and spore forming bacterium. Biochemical activities showed that *Bacillus* sp was positive to starch hydrolysis, catalase, nitrate reduction, oxidase, and Voges-Proskauer, while negative for citrate utilization. A molecular technique was used to prove and further confirm the identification of the isolate to the species level. The partial 16S rDNA sequence was determined and was compared to the GenBank databases. The isolate

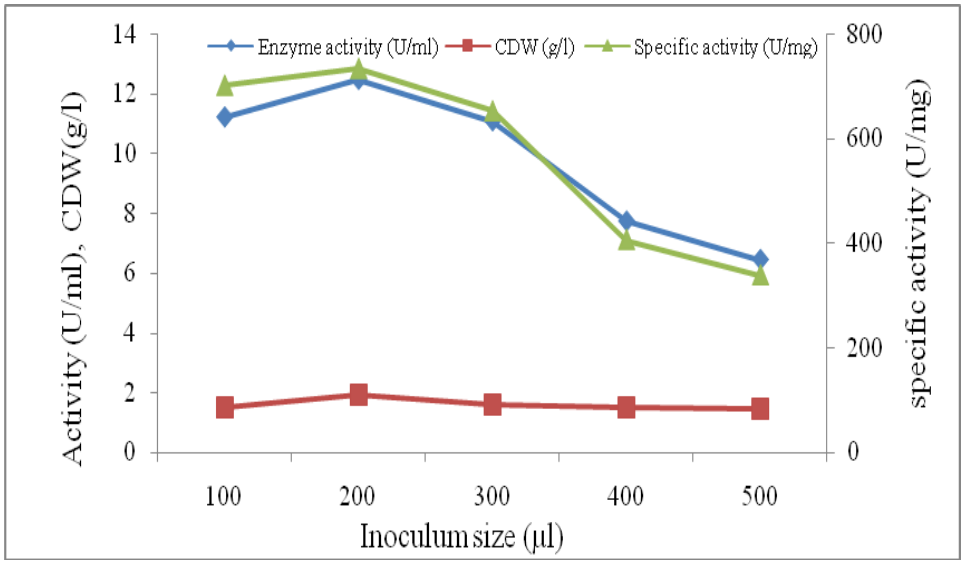


Figure 3: Effect of inoculum size on α-amylase production by *Bacillus* sp NRC12017.

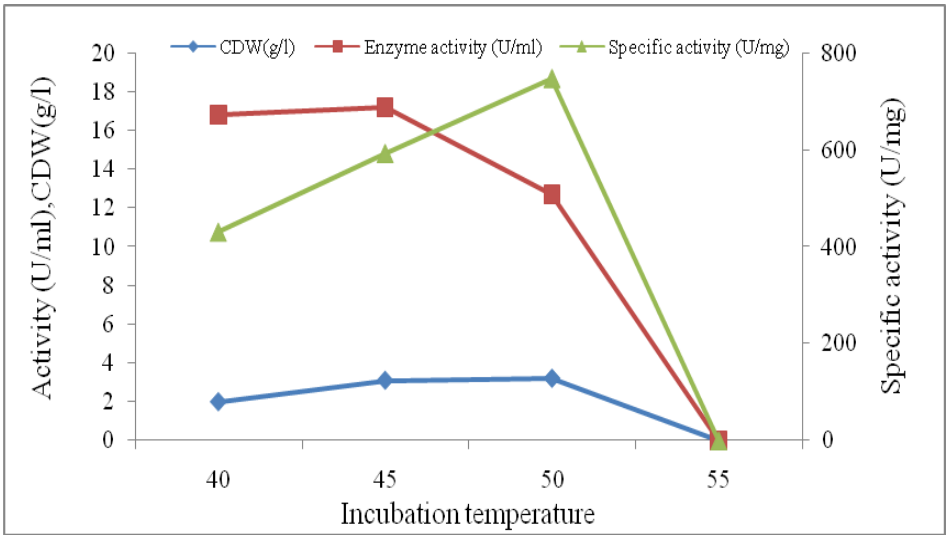


Figure 4: Effect of incubation temperature on α-amylase production by *Bacillus* sp NRC12017.

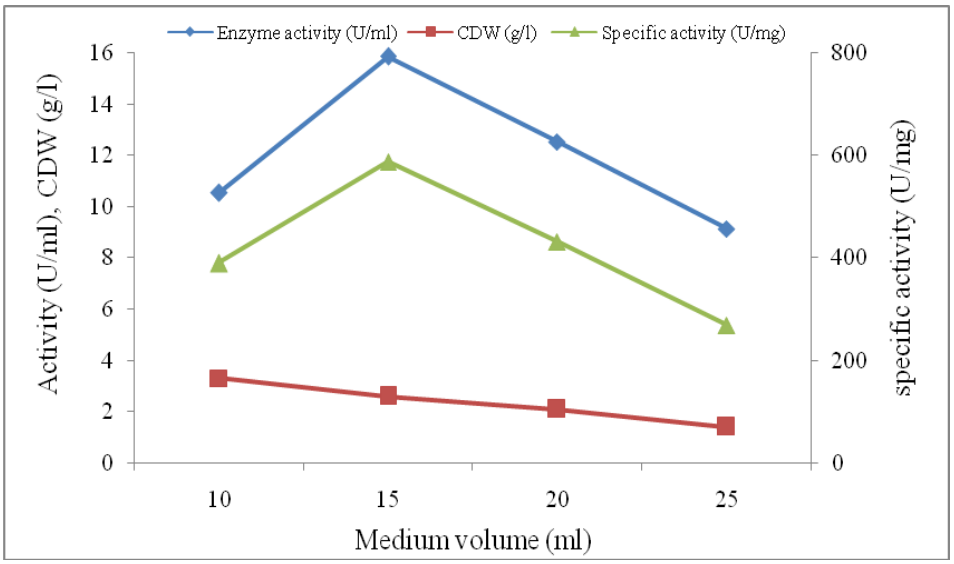


Figure 5: Effect of medium volume: flask volume on α-amylase production by *Bacillus* sp NRC12017.

was identified as *Bacillus* sp NRC12017. The sequence was submitted to GenBank in NCBI (http://www.ncbi.nlm.nih.gov/nuccore/ JQ425073) with the accession number **KY614073** (Figure 1).

The *Bacillus* species are industrially important microorganisms because they grow quickly; produce a lot

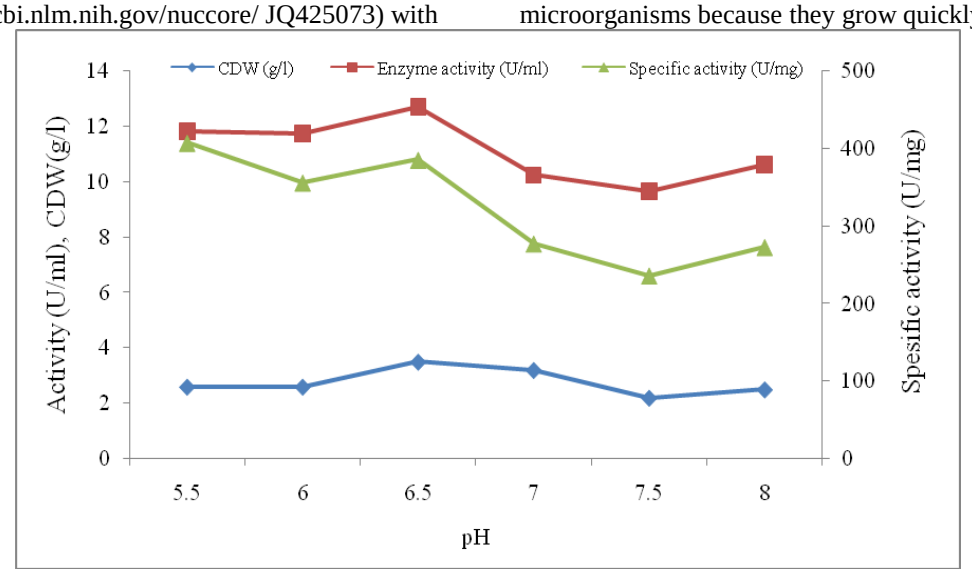


Figure 6: Effect of initial pH on α-amylase production by *Bacillus* sp NRC12017.

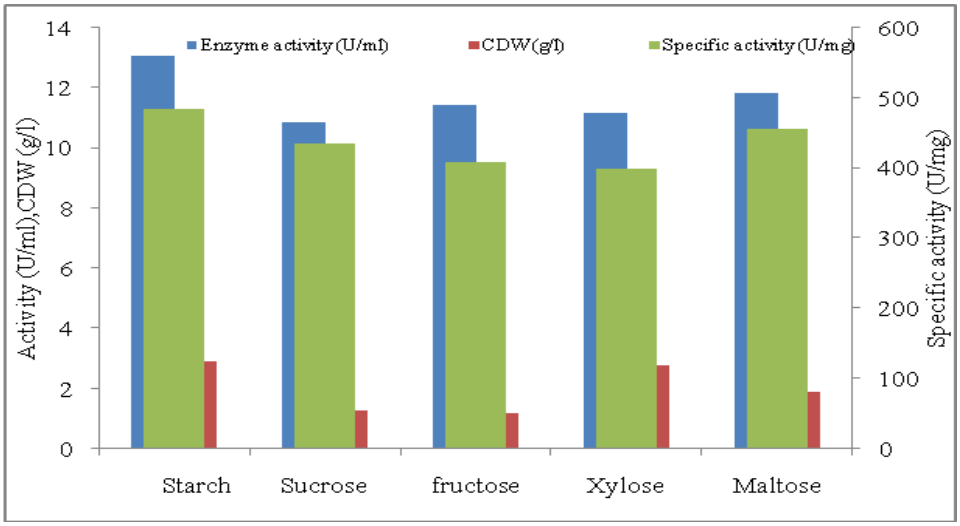


Figure 7: Effect of different carbon sources on α-amylase production by *Bacillus* sp NRC12017.

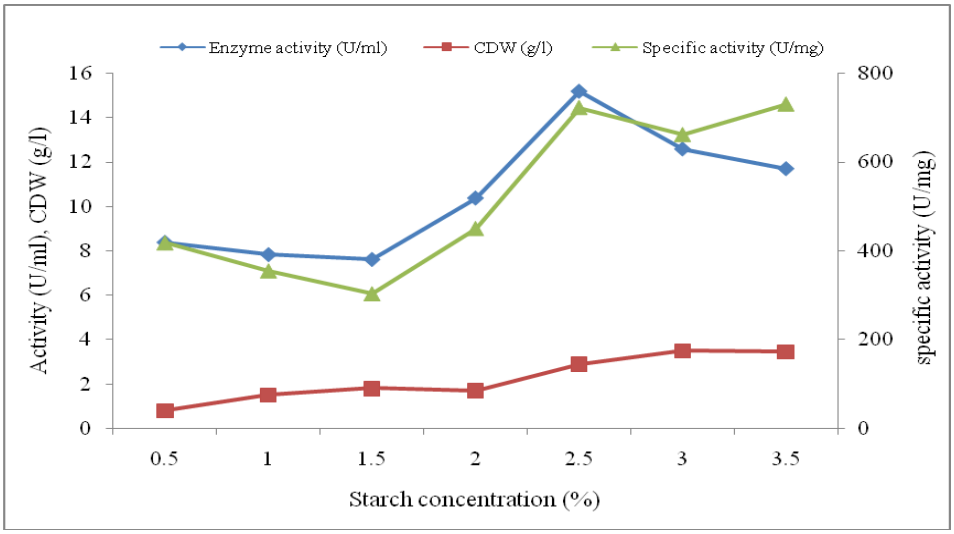


Figure 8: Effect of different starch concentrations on α-amylase production by *Bacillus* sp NRC12017.

of extracellular enzymes into the growth medium of which amylases are of especially extensive modern industrial importance and not harmful during handling[29].

The growth of *Bacillus* sp. NRC12017 and the creation of α-amylase rely upon the sort of production medium. Therefore, additional studies on enzyme production from

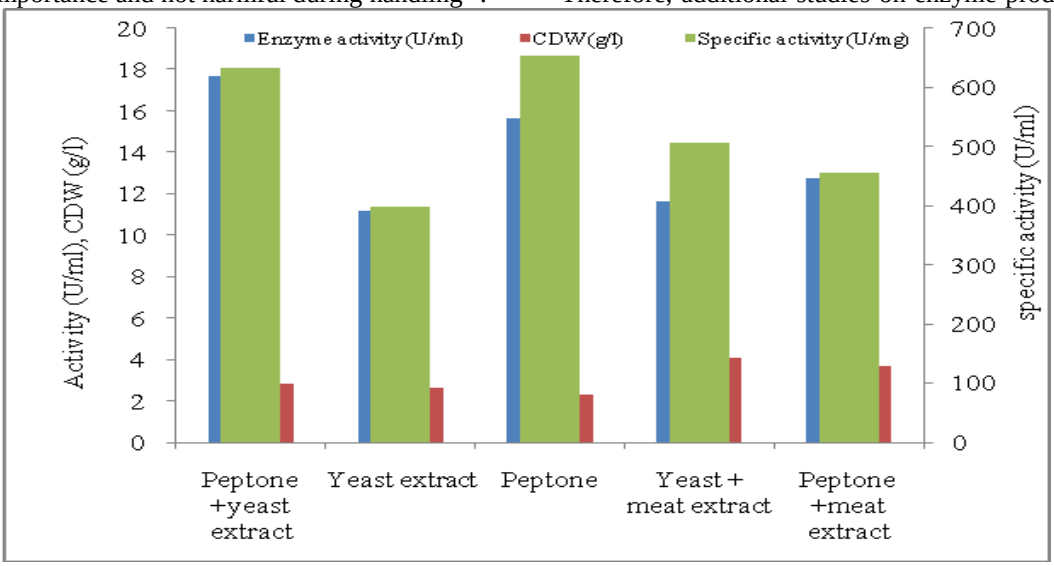


Figure 9: Effect of different nitrogen sources on α-amylase production by *Bacillus* sp NRC12017.

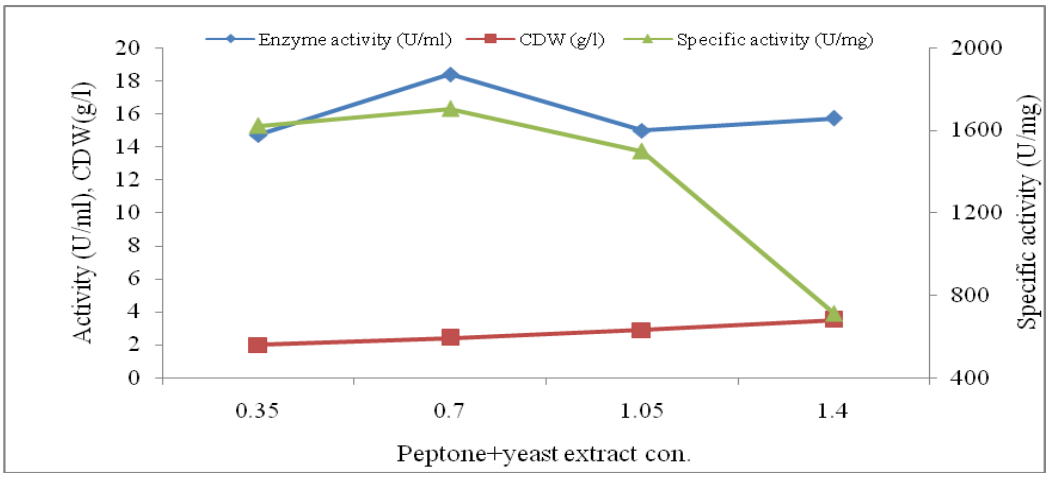


Figure 10: Effect of different concentrations of peptone plus yeast extract on α- amylase production by *Bacillus* sp NRC12017.

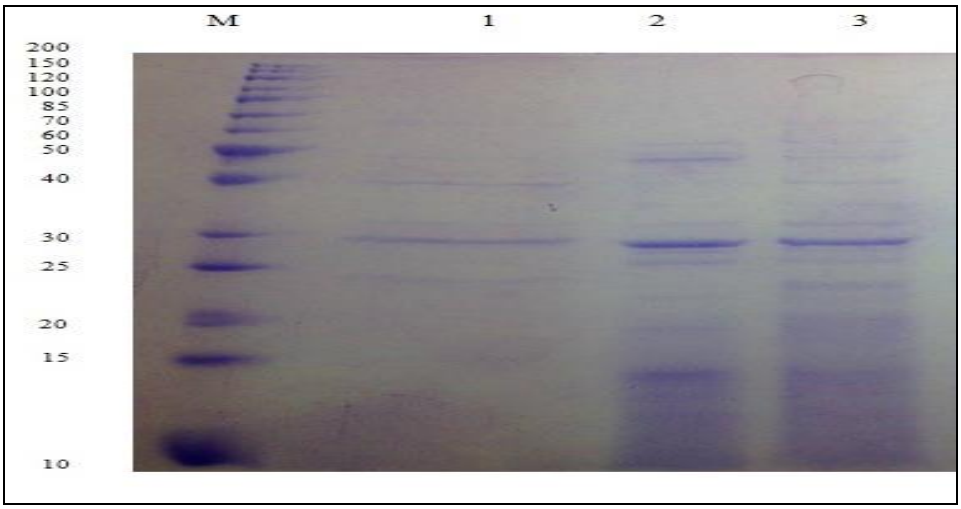


Figure 11: Polyacrylamide gel electrophoresis analysis of the partially purified α-amylase from *Bacillus* sp NRC12017. SDS-PAGE: M marker, Lane 1 crude enzyme, Lane 2 FIII, Lane 3 FIV.

Table 1: Summary of the partial purification of α-amylase isolated from *Bacillus* sp NRC12017.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude enzyme	10.260	11046	1076.60	100	1
FI	0.176	0.00	0.00	0.00	0.00
FII	0.221	0.00	0.00	0.00	0.00
FIII	0.616	3093.7	5022.24	28.00	4.66
FIV	0.631	3063.6	4854.43	27.73	4.51

Bacillus sp. NRC12017 in shake-flask cultures were carried out using five different media at pH 7 and incubated for 72 h of incubation at 50°C. The α-amylase activity of *Bacillus* sp. NRC12017 was measured in all media. Among the culture media, the medium 5 exhibited a significant effect on the microorganism development and enzyme creation (10.5 U/ml). In medium 2, the generation of α-amylase enzyme is similar to that in medium 4 (8.5 and 8.8 U/ml, respectively), however lower than the medium 5. Additionally results demonstrate that the medium 3 showed the most reduced values for α-amylase production (5.45 U/ml) and the growth of the microorganism and this might be due to the very low nitrogen concentration in the medium. Lower levels of nitrogen and also over abundance of nitrogen are equally detrimental causing enzyme inhibition[30].

From the time course study in shake culture, α-amylase productivity was found low (7.41 U/ml) at 1 day of incubation, likely because of the lag phase that bacteria entered during the growth, where they adapted with the outside growth conditions. It was gradually increased with increase in the incubation period and incubation for 3 days was the best for both enzyme productivity (10.51 U/ml) and bacterial growth (Figure 2). Hmidet et al.[31] reported that the enzyme productivity was maximal when bacteria entered in the late exponential phase of growth. Further increment in the incubation time decreased the activity. At 5 days of incubation, it was extremely reduced possibly due to the exhaustion of nutrients, death of microorganisms, gathering of byproducts in the medium such as toxins, inhibitors, proteolysis of α-amylase by proteases enzymes and also the cells may attained the decline phase and showed diminished amylase synthesis[8,32]. These findings are similar to results of *B. licheniformis* ATCC 12759[33] and *Bacillus* isolates[19]. While, Kaur and Vyas[34]; Deb et al.[35]; Singh et al.[36] and paul et al.[37] found that an extended period of incubation beyond 48 h did not increase the enzyme production from *B. licheniformis* ATCC 12759; *Bacillus* isolates; *Bacillus* sp. DLB 9, *B. amyloliquefaciens* P-001 and *Bacillus* sp. strain B-10.

The highest productivity of α-amylase was recorded at 200 µl of inoculum size (11.98 U/ml) (Figure 3). Moreover, the lowest output was observed at 500 µl (6.27 U/ml) of inoculum size. The increase in inoculum level above 200 µl declines the growth of microbe and enzyme synthesis. This might be attributed to a higher concentration of inoculum that lead to an increased competition for nutrients as carbon source, which might lead to nutrients consumption. Furthermore, lower inoculum size would have lead to diminished enzyme output as a longer period is needed for bacteria to grow and reach the optimum number to consume substrate and synthesize the required product[38]. Our findings are in a good agreement with Riaz et al.[39] in *B. Subtilis* and Deb et al.[35] in *B. amyloliquefaciens* P-001 as they obtained the maximum yield of the enzyme at 4 and 1% v/v inoculum size, respectively. Choubane et al.[40] found that there is an increase in amylase productivity, from inoculum size of 1 - 4% where the activity reaches its highest level in case of *Bacillus* sp. R2. It is noteworthy that there is no precise bacterial inoculum volume suitable for amylase production. It can vary from 0.5% for *B. amyloliquefaciens*[41] to 2.95%[42] for *Bacillus* sp. and 8% for *B. cereus*[43].

The incubation temperature influence on α-amylase production was shown in Figure (4). A bacterial growth and α-amylase production were reported at a large scale of temperature (35–80°C)[16]. In the current study, development of bacteria and enzyme synthesis happened at temperatures lower than 55°C. The bacterium could grow satisfactorily at all temperatures tested except 55°C but the maximal α-amylase activity in the fermentation medium was accomplished at 45°C (17.20 U/ml). Followed by this, 40°C was the second best temperature on α-amylase production (16.79 U/ml). Moreover, the minimum amount of α-amylase production was recorded at 50°C (12.71 U/ml). These results are in harmony with Liu and Xu[44] as they obtained maximum α-amylase activity (53 U/ ml) at 45°C. Asgher et al.[45] informed that productivity of α-amylase was maximum at 50°C by *B. subtilis* Js 2004. Also, Deb et al.[35] informed a progressive raise in α-amylase production with increasing temperature and maximum enzyme production was observed at 42°C.

The highest productivity of m α-amylase was recorded at 15 ml broth medium in 100 ml conical flask (15.85 U/ml) and the minimum production was reported at 25 ml broth medium in a Erlenmeyer flask of 100 ml capacity (9.13 U/ml) (Figure 5). The biosynthesis of α-amylase was diminished with the increase in the broth volume, and this behavior might probably because of a reduction in air supply as a result of the drooping in the agitation rate of medium that happened with high volume of fermentation medium[46]. Riaz et al.[39] and Dash et al.[47] conveyed that the maximum yield of α-amylase from *B. subtilis* GCBUCM-25 and *B. subtilis* BI19 was gained at 25 and 50 ml of enzyme production medium in 250 ml Erlenmeyer flask, respectively.

The initial pH impact on the production of α-amylase by *Bacillus* sp. NRC12017 was investigated at different pH (5.0-8.0) (Figure 6). The maximum synthesis of α-

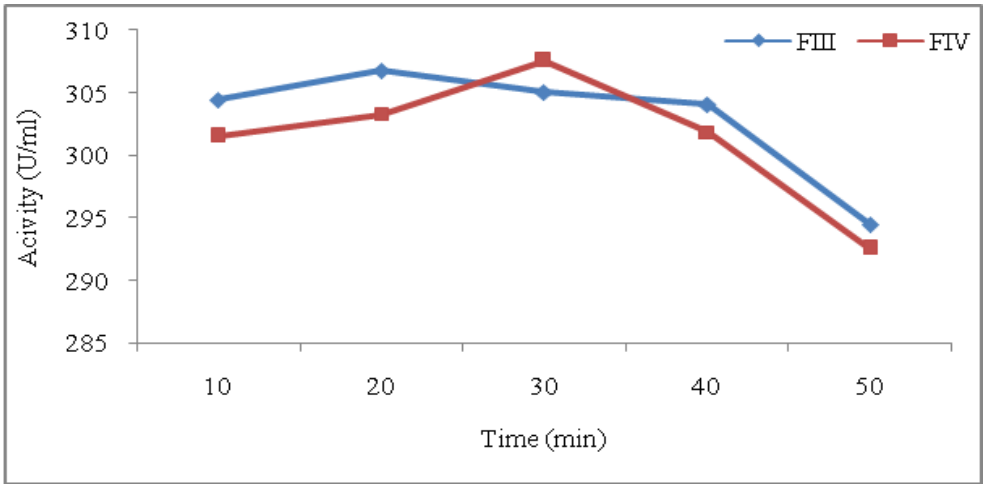


Figure 12: Effect of incubation time on activity of partially purified α amylase.

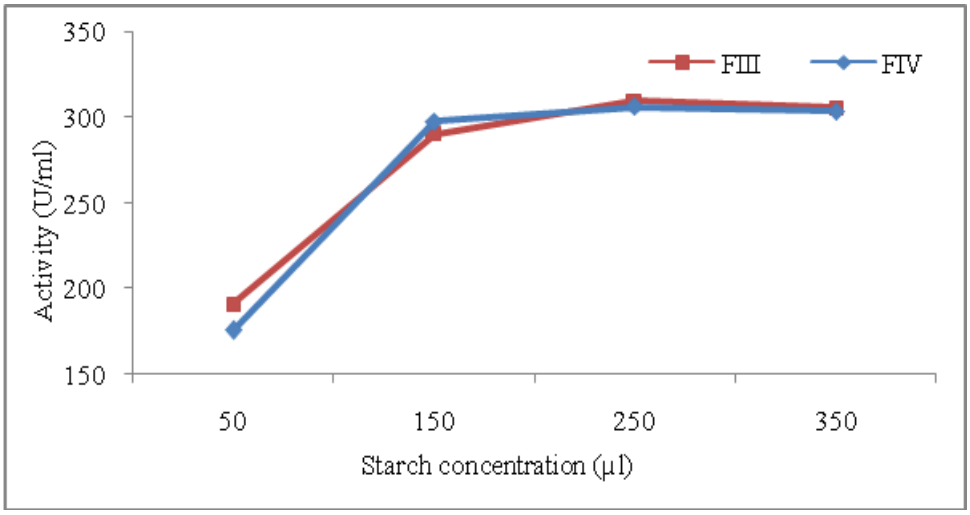


Figure 13: Effect of starch concentration on the activity of partially purified α-amylase.

Optimal temperature

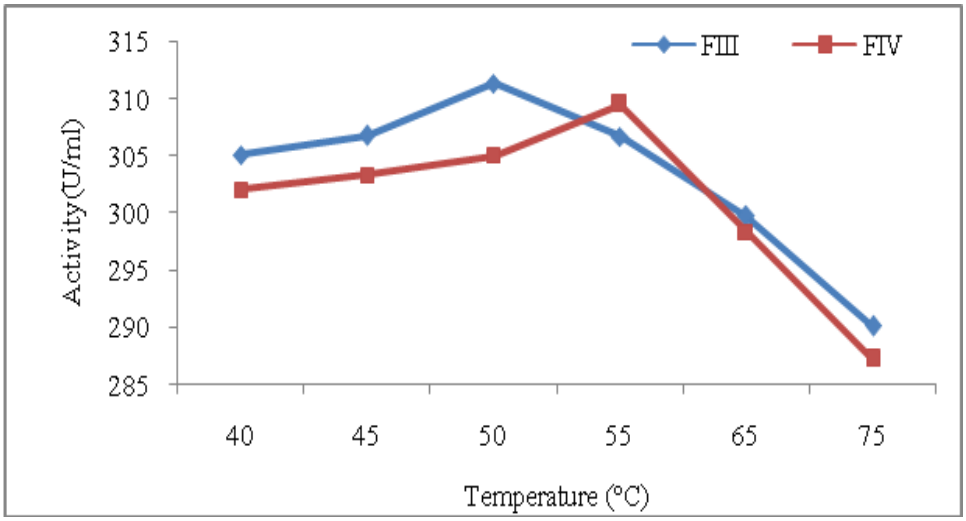


Figure 14: Effect of incubation temperature on the activity of partially purified α-amylase.

amylase enzyme was observed at pH 6.5 (12.71 U/ml) and this may be a result of enhanced bacterial growth, while the minimum amount of yield of α-amylase was noticed at pH 7.5 (9.65 U/ml). The pH variation observed during the growth of the microorganism also affects stability of the product in the medium. Many of the previous researchers informed an ideal range of pH between 6.0 and 7.0 for both microbial growth and

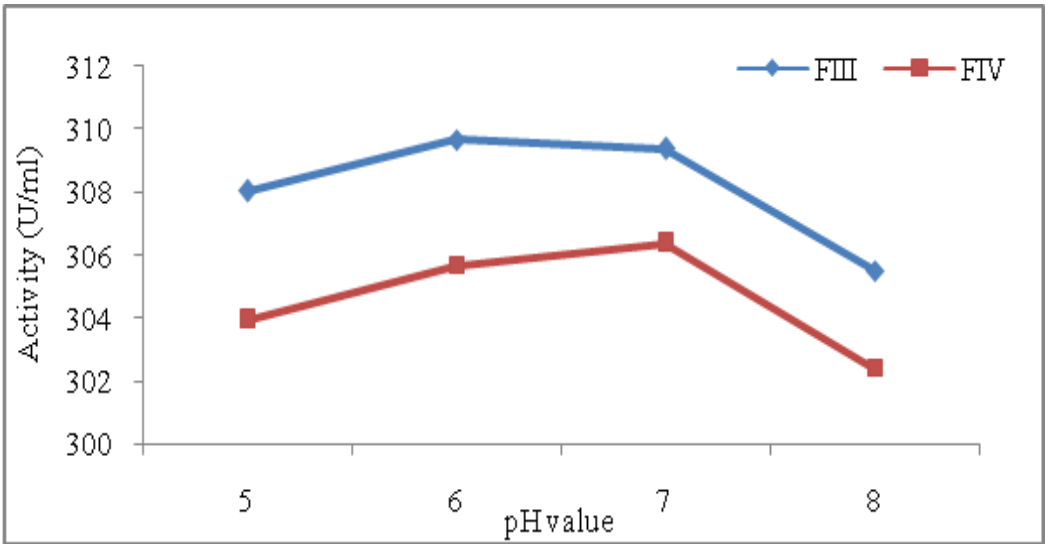


Figure 15: Effect of pH on the activity of partially purified α-amylase.

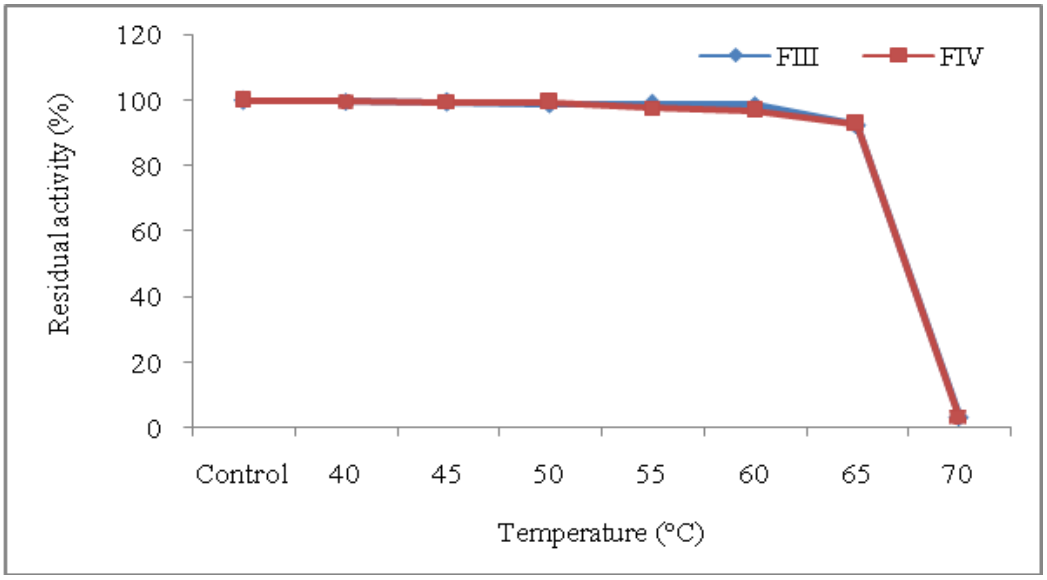
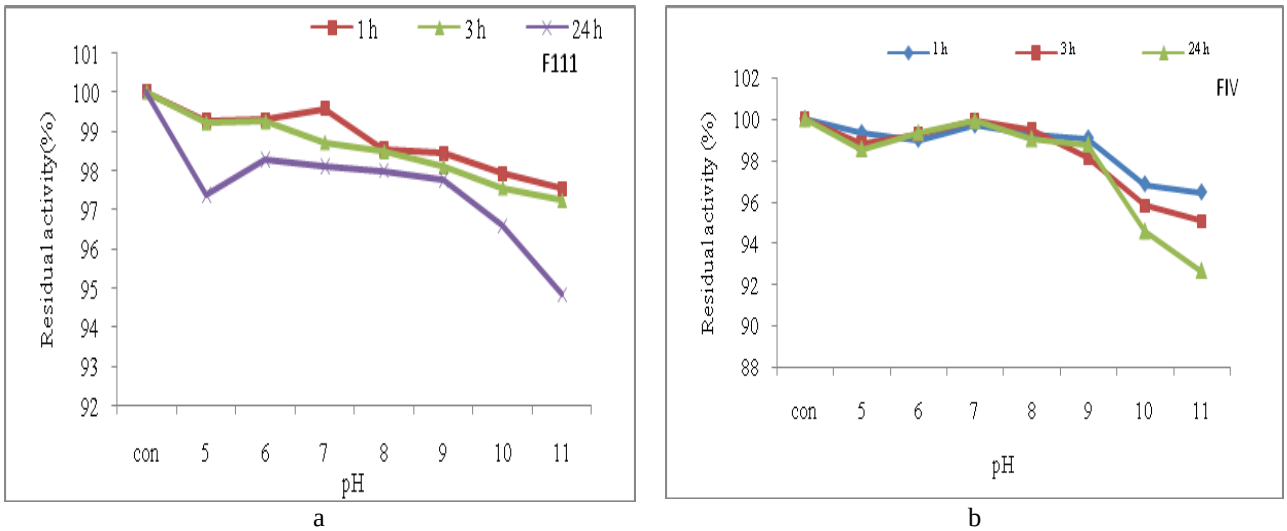


Figure 16: Thermal stability of partially purified α-amylase from *Bacillus* sp NRC12017.



a b

Figure 17: pH stability of FIII (a) FIV (b) from *Bacillus* sp NRC12017.

amylase production[48]. α-Amylase production was maximum at pH 7.0 by the *B. amyloliquefaciens* and *B. subtilis* RSKK96[49,50].

Results showed in Figure (7) indicated the effect of various carbon sources on production of α-amylase by *Bacillus* sp. NRC12017. The starch effectiveness on α-amylase production (13.06 U/ml) was more than the other tested carbon sources. Furthermore, α-amylase yield was comparable in carbon sources such as fructose, maltose, and xylose. These results indicated that α-amylase production by this strain was constitutive since biosynthesis of the enzyme occurred not only in the existence of starch but also with other sources of carbon. The results were in conformity with the studies proceeded by Vijayalakshmi et al.[29] in the case of *B. subtilis* KC3 and Salman et al.[48] in the case of *B. subtilis* RM16 where diversified carbon sources were supplemented for α-amylase production and the maximum productivity was noticed with starch as the source of carbon.

The influence of starch concentration on α-amylase production was clarified in Figure (8). As starch concentration increase, the amount of α-amylase increase till it reaches the highest level at a starch concentration of 2.5% then it starts to decrease. There was a decrease in enzyme production at excessive starch concentration and this might be imputed to the rapid consumption of starch leading to the release of toxic metabolic wastes which suppress the growth of bacteria and α-amylase production. Also, high starch concentrations caused the broth culture to be more viscous, so interferes with O_2 transfer resulting in restriction of dissolved O_2 required for the microbial growth. Mishra and Behera[25] found that raising the starch concentration increased both growth and α-amylase production by *Bacillus* strain from kitchen wastes and the maximum yield of the enzyme was reached at a starch concentration of 2%.

Among the tested organic nitrogen sources, the highest amount of α-amylase production was registered in peptone plus yeast extract containing medium (17.71 U/ml) followed by a medium containing peptone only as a sole nitrogen source (15.68 U/ml) (Figure 9). Kaur and Vyas[34] reported that the maximum yield of α-amylase by *Bacillus* sp. DLB 9 was attained when a combination of yeast extract plus peptone (0.5% was used. Viswanathan et al.[51] studied the effect of different nitrogen sources viz; yeast extract, meat extract, beef extract, and peptone on α-amylase productivity from *B. megaterium* and peptone was reported to be the most effective nitrogen source for α-amylase output followed by meat extract.

Nitrogen source concentration in the growth medium is important in the production of α-amylase. Both lower and higher nitrogen sources levels are equally detrimental and cause repression of the enzyme[30]. Figure (10) demonstrated that *Bacillus* sp. NRC12017 showed enhanced growth and α-amylase yield at 0.7% concentrations of yeast extract plus peptone (18.41 U/ml). Dettori et al.[52] declared that the strains of *B. stearothermophilus* and *B. amylolyticus* secreted maximum α-amylase in a medium provided with 1% peptone plus 0.5% yeast extract. Also, Kaur and Vyas[34] attained a maximum production of α-amylase from *Bacillus* sp. DLB9 in a medium containing a combination of yeast extract and peptone (38.4 U/ml) at 0.5%. While Salman et al.[48] reported 1.5% yeast extract as the best concentration for the maximal productivity of α-amylase by *B. subtilis* RM16.

Partial purification and characterization of α-amylase

α-Amylase was partially purified using ammonium sulfate precipitation and the results were summarized in Table (1). Both FI and FII didn't show any α-amylase activity while both FIII and FIV showed α-amylase activities. FIII and FIV were purified 4.66 and 4.51 fold with the yield of 28.00 and 27.73%, respectively. Partial purification of α-amylase was also done by other researchers. A study carried out by Sajedi et al.[53] showed 3.4-fold with ammonium sulfate precipitation. Asoodeh et al.[54] obtained 1.8-fold of enzyme after purification. In our finding, the enzyme was more purified than that given by the above researchers and the purification fold also increased.

SDS-PAGE

Partially purified proteins FIII and F IV obtained by 60 and 80% ammonium sulfate precipitation were subjected to SDS-PAGE. Both fractions showed a main band at 30 KDa (Figure 11).

Characterization of partially purified α-amylase

Effect of incubation period

For determination of optimum reaction time, enzyme assay was carried out at different reaction time ranging from 10–50 min at constant temperature and pH. Data in Figure (12) revealed that the best incubation time for FIII and FIV was 20 and 30 min, respectively. After 40 min of incubation at 45°C, the enzyme activity decrease by about 4.0% of its activity in both FIII and FIV. Paul et al.[37] reported a reaction time of 30 min was the best available time for the activity of α-amylase obtained from *Bacillus* sp. MB6. But Deb et al.[35] reported 40 min as the optimum incubation time for α- amylase activity from *B. amyloliquefaciens* P-001.

Effect of starch concentration

The activity of FIII and FIV was evaluated at various substrate concentrations (50, 150, 250, and 350 µl) from 1% starch solution. Initially, the activity of α-amylase increased with increasing substrate concentration up to 250 µl. Further increase in substrate concentration decreased α-amylase activity (Figure 14). Singh et al.[36] evaluated α-amylase activity at various starch concentrations (0.5-6.5 mg/ml) and they found that the α-amylase activity increased with increasing substrate concentration up to 3.5 mg/ml starch. Further increase in substrate concentration had no effect on enzyme activity.

The activity of FIII and FIV increased with increase in temperature and reached to the maximum at 50 and 55°C, respectively. Above 55°C, the activity of both fractions decreased slightly and showed only 6.8% and 7.1% activity reduction, respectively at 75°C (Figure 13). Amoozegar et al.[12] and Aygan et al.[55] reported that α-amylase obtained from *Halobacillus* sp. MA-2 and *Bacillus* sp. AB68, respectively with an optimum temperature of 50°C. But, Paul et al.[37] found that the

Bacillus sp. MB6 produced α-amylase with maximum activity at 55 °C.

Optimal pH

Most enzymes are active only over a narrow pH range and have specific pH at which the activity is at its maximum. An increase or decrease in pH also causes denaturation in enzymes, there by affecting their activity. A pH range from 5.0-8.0 was used to study the effect of pH on α-amylase activity (Figure 15). Optimum pH of both FIII and FIV was 6.0-7.0. Demirkan et al.[56] and Paul et al.[37] reported pH 6.0 as optimum for activity of α-amylase from *B. amyloliquefaciens* and *Bacillus* sp. MB6, respectively. While, Singh et al.[36] found that α-amylase purified from *Bacillus sp*. strain B-10 showed maximum activity at pH 7.0.

Temperature and pH stability

Stability is of great importance for the economy of their industrial application. In the case of thermal stability, the enzyme was pre incubated at different temperatures (40, 45, 50, 55, 60, 65, and 70°C) for 30 min and then enzyme activity was assayed. The results showed that 100% of the original activity of α-amylase produced by *Bacillus* sp.NRC12017 was reduced to about 92 % after heating at 65°C (Figure 16). After this temperature, the activity was decreased drastically and the enzyme was completely inactivated when heated at 70°C. The enzyme was stable up to 65°C. Thus, the results concluded that the crude enzyme is moderately temperature stable. α-Amylase that was partially purified from *B. amyloliquefaciens* P-001 was found to retain about 73% of its activity after heating at 50°C for 30 min[35].

The pH stability of α-amylase (FIII and FIV) obtained from the *Bacillus* sp NRC12017 was shown in Figure (17 a, b). The pH stability study revealed that both fractions of the enzyme (FIII and FIV) were stable at a wide range of pH when incubated for different times of 1, 3 and 24 h. In this investigation, both fractions exhibited nearly or over 97% of the original activity over a wide range of pH (5.0-9.0). After 24 h of pre-incubation, both FIII and FIV fractions lost about 5.14 and 7.33%, respectively of their original activities at pH 11. For most *Bacillus* sp, the pH optimum and stability of α-amylase have been reported in the range of pH 5.0 to 9.0. The results are similar to the previous findings that showed pH optimum and stability of α-amylase in the range of pH 5.0 to 9.0 for most *Bacillus* sp. [57, 45, 58, 59] . A wide range of pH stability might have a significant advantage of handling the enzyme for the commercial and industrial process.

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