# Production, purification and characterization of the α-amylase from local bacteria isolate *Bacillus subtilis* ITBCCB148

# Production, purification, and characterization of the α-amylase from local bacteria isolate *Bacillus subtilis* ITBCCB148

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**Abstract.** Production, purification and characterization of the α-amylase from local bacteria isolate *Bacillus subtilis* ITBCCB148 has been successfully carried out. The purified enzyme increased 149.9 times with specific activity of 115,500 U/mg compared to enzyme crude extracts. This enzyme has an optimum pH of 5.5 and an optimum temperature of 60°C and can maintain its stability at a temperature of 60-80°C, thus this enzyme is categorized as a thermostable enzyme.

**Keywords**: α-amylase, *B. subtilis* ITBCCB148, characterization, purification

## 1. Introduction

Amylase is an enzyme that can catalyze the breakdown of starch, glycogen and various oligosaccharides randomly. This enzyme can be found in plants, animals and microorganisms. Microbial enzymes are generally preferred in industrial applications because mass production capacities are more economical and microbes are easily manipulated to obtain enzymes with desirable characteristics [1]. α-Amylase (EC 3.2.1.1) is a group of endoamylase that catalyzes the breakdown of starch from the center of the starch molecule in the α-1.4 glucosidic bond. Various industries that have utilized the α-amylase from microbia are the industried of food, beverage, textile, detergent, brewing and pharmaceutical. The starch producing industry requires amylolytic enzymes that are resistant at high temperatures. Therefore, it is very important to obtain a thermostable enzyme, an enzyme that can work over a wide temperature range by isolating directly from nature that lives in these conditions [2].

Bacteria have become an important source for producing thermostable α-amylase with better properties than fungi [3]. Among the bacteria, *Bacillus* sp. is widely used to produce thermostable α-amylase which is required in industry. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* are known as good α-amylase producers and these have been widely used for commercial enzyme production in various applications [4]. Several studies on bacillus α-amylase have

found that they have different optimum abilities. Abdel-Fattah *et al.* [1] reported that *B. licheniformis* worked optimally at 60-80$^{o}$C and pH 6-7.5; Raul *et al.* [5] reported *B. subtilis* MTCC 121 optimum at 40$^{o}$C and pH 7.1; and Annamalai *et al*.[2] reported *B. cereus* optimum at 65$^{o}$C and pH 8.0.

The production of enzymes for industrial applications must also consider the economic value. The utilization of local microbia sources to produce α-amylase can reduce production costs compared to the use of imported enzymes, thus to support the use of enzyme locally isolated, it is necessary to develop the isolation and purification techniques for enzymes to obtain enzymes with high activity and purity levels.

In this study, the isolation and purification of the α-amylase from the local bacteria isolate *B. subtilis* ITBCCB148 was carried out through several stages, namely: fractionation with ammonium sulfate salt, dialysis, ion exchange cromatography and molecular filtering. The purified enzyme was then characterized, including: determination of pH, optimum temperature and enzyme stability. Measurement of enzyme activity was carried out using the Mandels method [6] and the protein content was determined by the Lowry method [7].

## 2. Experimental

### *2.1 Research procedures*

#### *2.1.1 Production of α-amylase*

Enzyme production was carried out by growing bacteria in media containing 0.5% starch, 0.5% yeast extract, 0.05% $KH_2PO_4$, 0.02% $MgSO_4.7H_2O$ and 0.01% $CaCl_2 \cdot 2H_2O$ which were dissolved in phosphate buffer pH 6.5. The fermentation time was 72 hours at 32$^{o}$C [8]. This medium is similar to that used for *Saccharomycopsis fibuligera* in producing α-amylase [9].

#### *2.1.2 Isolation of α-amylase*

Enzyme isolation was carried out by separating the enzyme from bacterial cells using a cold centrifuge at a rate of 6000 rpm for 30 min [8]. The pellets stuck to the tube walls were removed and the supernatants (crude extract of the enzyme) were then filtered with filter paper to completely separate from the bacterial cells. The crude extract obtained was stored in the freezer for further purification.

#### *2.1.3 Enzyme purification*

##### *2.1.3.1 Precipitation with ammonium sulfate salt*

The precipitation of the enzyme was carried out in a gradual manner (fractionation) by adding an amount of ammonium sulfate according to the desired saturation level of the enzyme into 100 mL of crude extract of the enzyme. The mixture was stirred with a magnetic stirer at 4$^{o}$C for 15 minutes. The determination of the fractionation pattern was carried out in stages in 5 fractions, namely (0-20), (20-40), (40-60), (60-80) and (80-100)%. The fraction which gave more enzyme precipitates and high specific activity was determined as the fraction of enzyme deposition as a whole. The enzyme precipitate was redissolved in 2 mL of phosphate buffer [5].

##### *2.1.3.2 Dialysis*

The removal of residual salts from enzyme molecules is essential if further purification steps are to be carried out. This is done is by dialysis. The enzyme solution was placed in a selectively permeable membrane bag (cellophane bag) then the bag was immersed in a large volume buffer then stirred with a magnetic stirer and the temperature was kept at 4$^{o}$C.

The semipermeable membrane has pores that allows small molecules such as ammonium and sulfate ions to pass through until there is a balance of volume outside and inside the bag, while proteins with large molecules cannot pass through the pores. Dialysis will increase the volume of the enzyme solution [10].

*2.1.3.3 Purification of enzyme resulted from dialysis by ion-exchange chromatography*
*DEAE-cellulose column chromatography*
Purification of enzyme with the DEAE-cellulose column begins with the determination of the appropriate buffer pH for the anion exchange column.

*CM-cellulose column chromatography*
Enzyme purification by CM-cellulose column chromatography begins with determination of the pH of the buffer suitable for the cation exchanger.

*2.1.3.4 Purification of enzyme resulted from dialysis by chromatography Sephadex G 100 column*
Enzyme purification by column chromatography Sephadex G-100 molecular filtering using 0.05 M phosphate buffer, pH 6.0 as elution buffer.

*2.2 Testing of enzyme activity and protein content*
The enzyme activity test was determined by the Mandels method based on the glucose formed [6] while the protein content was determined by the Lowry method [7].

*2.3 Characterization of the purified α-amylase*
Characterization of the purified α-amylase was carried out by determination of several variables such as the optimum pH, optimum temperature, and thermal stability test.

*2.3.1 Determination of optimum pH and temperature*
Determination of the optimum pH was done by varying the pH of the substrate in 0.1 M phosphate buffer, namely 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 7.5 and 8.0. The temperature was kept at the specified optimum pH. Determination of the optimum temperature of the enzyme was carried out by varying the incubation temperature, namely 50; 55; 60; 65; 70; 75 and 80$^{o}$C at their optimum pH. Enzyme activity was measured by the Mandels method.

*2.3.2 Determination of the thermal stability of the purified enzyme*
It was done by measuring the enzyme activity every 10 minutes interval for 80 minutes of heating at the optimum temperature and pH. The residual activity was measured by comparing the enzyme activity at 10 minute intervals with the initial activity of the enzyme (without heating). The initial activity of the enzyme is given a value of 100% [8, 11, 12].

## 3. Results and Discussion

*3.1 Enzyme purification*
*3.1.1 Fractionation with ammonium sulfate*
The α-amylase was successfully purified by precipitation using ammonium sulfate and produced the highest specific activity in the fraction (60-80)% of 3847.250 U/mg. Based on the fractionation pattern in Figure 1 it can be seen that in fractions other than (60-80) % there are still many precipitated enzyme with high specific activity. Therefore the whole fractionation was carried out in two stages of fractionation, namely (0-20) and (20-85) % to obtain enzyme in high yield. Graph of enzyme precipitation at two saturation levels is shown in Figure 2.The activity of enzyme units in a fraction of 20 - 85% is 1,358.25 U/mL with a specific activity of 3880.7 U/mg, an increase of 5.0 times compared to crude extract of the enzyme and the yield of 46.2%.

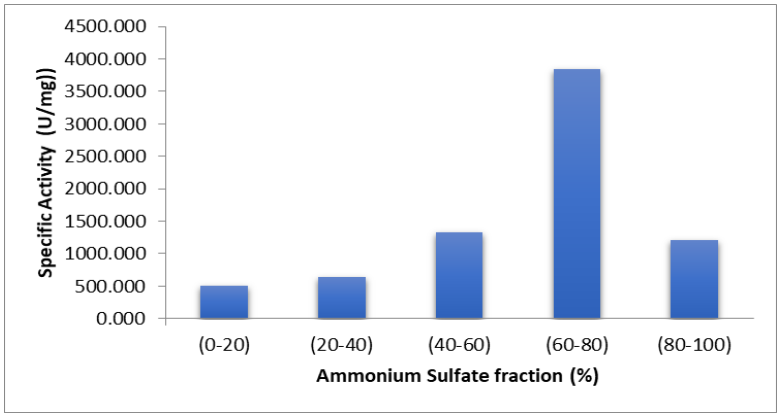


**Figure 1**. The relationship between enzyme fractions at various saturation levels of ammonium sulfate with enzyme specific activity of α-amylase from *B. subtilis* ITBCCB148

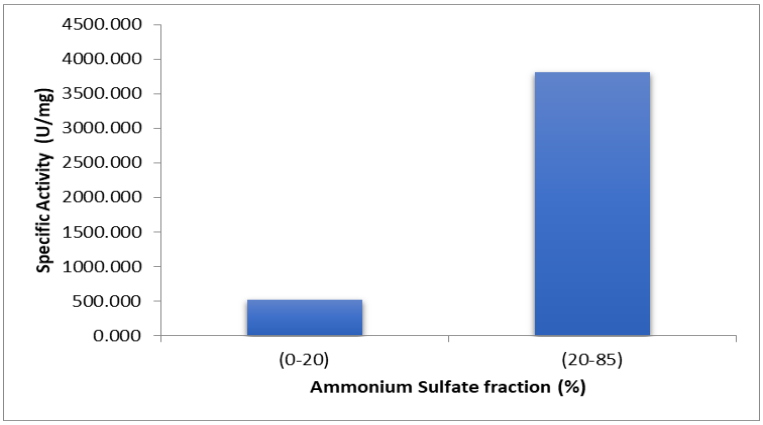


**Figure 2**. Relationship between enzyme fractions at two levels saturation of ammonium sulfate with enzyme specific activity of α-amylase from *B. subtilis* ITBCCB148

*3.1.2 Dialysis*

Enzyme purification by dialysis is carried out to separate the remaining salt ions from the enzyme mixture. The unit activity of the enzyme from dialysis was 1,096.53 U/mL with a specific activity of 5482.6 U/mg, an increase of 7.1 times compared to crude extract of the enzyme, with a gain of 45.8%.

*3.1.3 DEAE-cellulose column chromatography*

Enzyme purification with the DEAE-cellulose column begins with the determination of the appropriate buffer pH for the anion exchange column. The results showed the enzyme did not exchange the ion counter at a pH between 5.0-8.8. Enzymes can exchange the counter ion well above pH 8.8, that is, pH 9.0, so a tris buffer of 0.025 M pH 9.0 is used as the initial buffer. While the buffer for elution was used Tris buffer 0.025 M pH 7.0. The pattern of protein ($A_{280}$) and activity (u/mL) of the α-amylase enzyme resulted from DEAE-cellulose column chromatography can be seen in Figure 3. Purification of the DEAE-cellulose anion exchange chromatography enzyme produced three protein peaks (Figure 3) with α-amylase activity located at the peak of protein number 3, namely in fraction 34 to fraction 36 with the highest activity at fraction number 35. Specific activity of protein peak number 3 was 28935 $U.mg^{-1}$, an increase of 37.6 times compared to crude extract of the enzyme and a gain of 35.8 %.

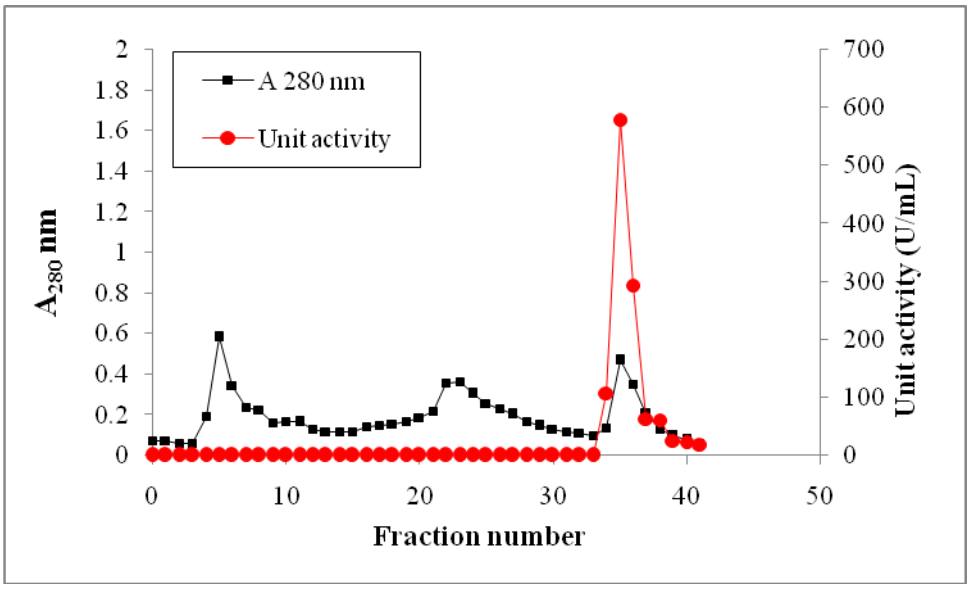


**Figure 3**. Chromatogram of the α-amylase from *B. subtilis* ITBCCB148 on the DEAE-cellulose column

*3.1.4 CM-cellulose column chromatography*

Enzyme purification by CM-cellulose column chromatography begins with determining the pH of the buffer suitable for the cation exchanger. The results show that the enzyme cannot exchange the counter ion above pH 5.0. Enzymes can exchange the counter ion well at pH 5.0, then a phosphate buffer 0.05 M, pH 5.0 as was used as the initial buffer. The 0.05 M phosphate buffer pH 8.5 is used as elution buffer. The pattern of protein ($A_{280}$ nm) and α-amylase activity resulted from CM-cellulose column chromatography can be seen in Figure 4. From this figure, it can be seen that the fraction that shows the presence of α-amylase activity is at the peak of protein number 3, namely the 26$^{th}$ fraction. up to the 30$^{th}$ fraction. α-amylase activity is at peak number 3 with the highest activity at fraction number 28, with unit activity of 1,683.1 U/mL and specific activity of 42,077.5 U/mg, an increase of 54.6 times compared to crude extract of the enzyme and a gain of 34.6 %.

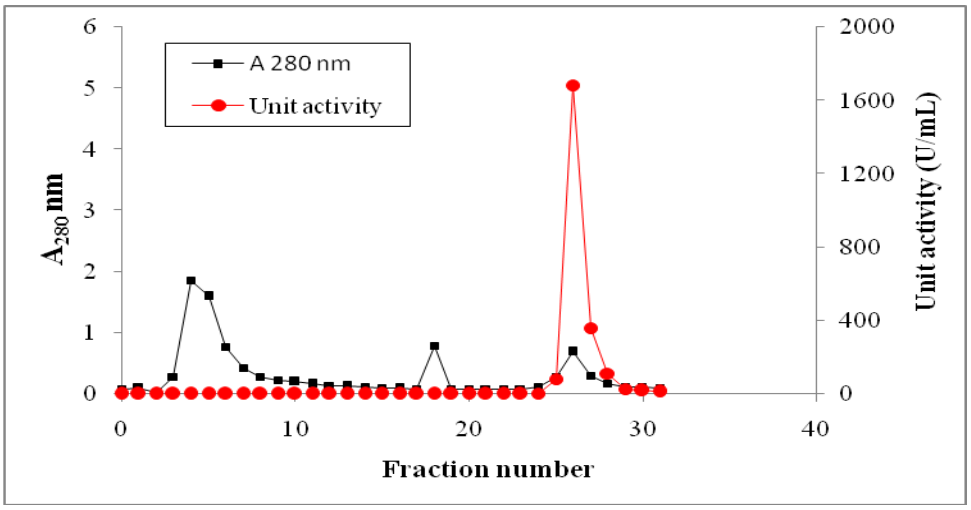


**Figure 4**. Chromatogram of the α-amylase from *B. subtilis* ITBCCB148 on the CM-cellulose column

*3.1.5 Purification of the enzyme from dialysis using Sephadex G 100 column chromatography*
Enzyme purification by column chromatography Sephadex G-100 molecular filtering using 0.05 M phosphate buffer, pH 6.0 as elution buffer. The protein pattern ($A_{280}$) and activity of α-amylase obtained by the Sephadex G-100 column chromatography can be seen in Figure 5. Figure 5 shows that of the 3 protein peaks, only one protein peak number 2 has α-amylase activity, namely the fraction 9 to the 16$^{th}$ fraction with the highest activity was in the 10$^{th}$ fraction. The specific activity of the enzyme in the highest fraction was 115,500 U/mg with a purity level of 149.9 times compared to crude extract of the enzyme and a gain of 9.5%.

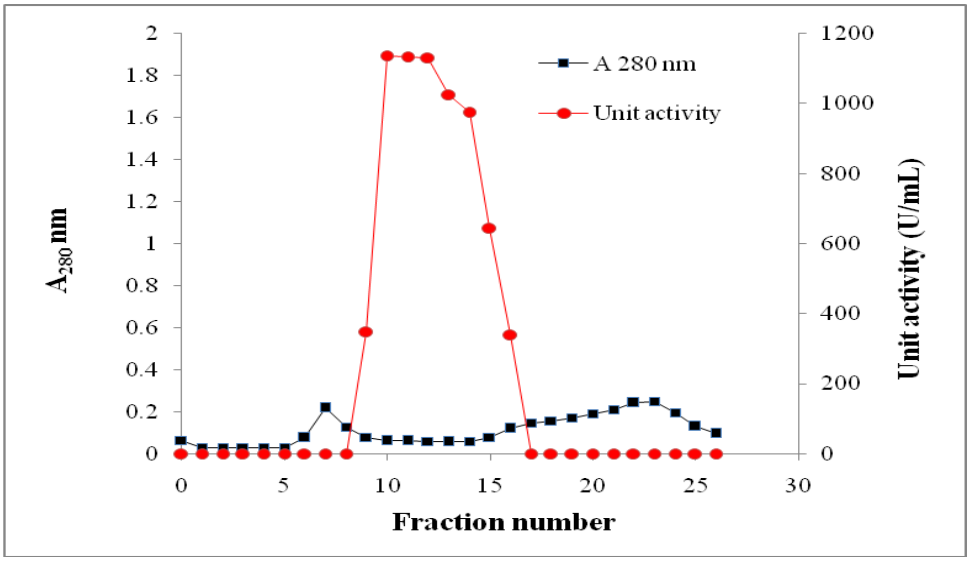


**Figure 5**. Chromatogram of α-amylase from *B. subtilis* ITBCCB148 on Sephadex G-100 column filtration

A summary of the entire purification process of α-amylase can be seen in Table 1.

Table 1. Schematic of α-amylase purification from *B. subtilis* ITBCCB148

| Stage | Enzyme Volume (mL) | Unit Activity (U/mL) | Total Activity (U) | Protein content (mg/mL) | Specific Activity (U/mg) | Purity Level (times) | Recovery (%) |
|---|---|---|---|---|---|---|---|
| Crude extract | 3,500 | 138.65 | 485,275 | 0.18 | 770.3 | 1 | 100 |
| Fractionation (20-85%) ammonium sulfate | 165 | 1,358.25 | 224,111.25 | 0.35 | 3,880.7 | 5.0 | 46.2 |
| Dialysis Result | 203 | 1,096.53 | 222,595.59 | 0.2 | 5,482.6 | 7.1 | 45.8 |
| DEAE | 300 | 578.7 | 173,610 | 0.02 | 28,935 | 37.6 | 35.8 |
| CMC | 100 | 1,683.1 | 168,310 | 0.04 | 42,077.5 | 54.6 | 34.6 |
| Sephadex G-100 | 50 | 924 | 46,200 | 0.008 | 115,500 | 149.9 | 9.5 |

The α-amylase purification scheme of *B. subtilis* ITBCCB148 in Table 1 shows a high increase in enzyme specific activity for each purification step, especially the results of the Sephadex G-100 column chromatography. The increase in enzyme specific activity for each purification step was 5.0 x for the ammonium sulfate fraction of 20 - 85%, 7.1 x for dialysis results, 37.6 DEAE-cellulose column chromatography, 54.6 x for CM-cellulose column chromatography; and 149.9 x for Sephadex G-100 column chromatography. This high increase in specific activity indicates that the enzyme purification stage carried out is quite good. The total enzyme activity for each purification stage experienced a high decrease. This high decrease in total activity is probably due to the enzyme loss of activity during the process, especially when deposited with ammonium sulfate, which is because the solution used is very dilute. Likewise, the enzyme from the Sephadex G-100 column chromatography will experience a decrease in activity if it is not concentrated as quickly as possible. The results obtained were compared with those obtained by Yandri *et al.* [8] and Srivastava [13], who purified the α-amylase from *B. stearothermophilus*. The α-amylase enzyme obtained showed a high increase in specific activity (153 x), and the loss of activity during the purification process was not so large (gain 34.9%).

*3.2 Characterization of purified enzyme*

*3.2.1 Determination of the optimum pH of the purified enzyme*

Determination of the optimum pH of the enzyme has been carried out at variations in the pH of the substrate from 5.0 to 8.0. Graph of the optimum pH of the enzyme in the pH range of 5.0 - 8.0 can be seen in Figure 6.

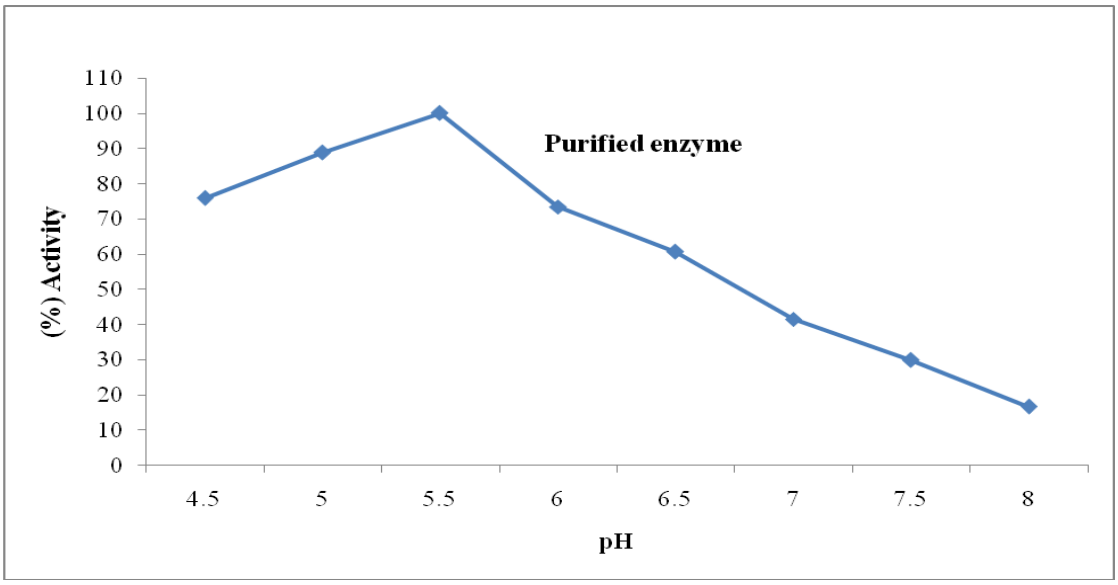


**Figure 6**. The optimum pH of the purified α-amylase enzyme

Figure 6 shows the enzyme resulting from dialysis has an optimum pH of 5.5. In the more acidic condition (pH 4.5-5.0) the purified enzyme still has a large residual activity. However, at pH 6.5-8.0 the activity decreased significantly.

*3.2.2 Determination of the optimum temperature of the purified enzyme*

Figure 7 shows the optimum temperature of the purified enzyme is 60°C. At a temperature of 70°C, the residual activity decreases but it can maintain the residual activity up to 80°C. At a temperature of 85°C, the purified enzyme decreased, and the residual activity left was 33.5%. These results indicate

that the purified enzyme is able to maintain its stability at 60-80°C, thus, this enzyme belongs to the enzyme that is thermostable.

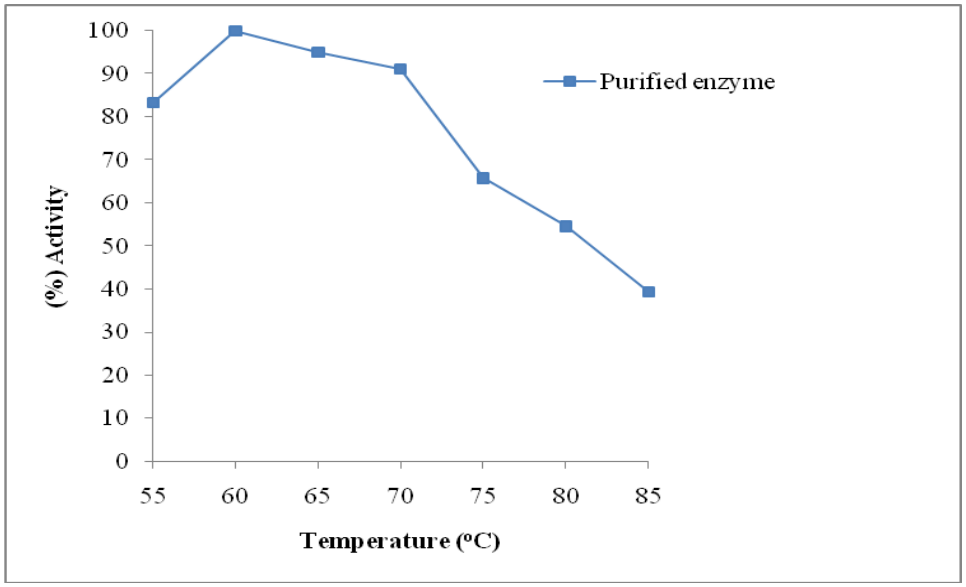


**Figure 7**. The optimum temperature of the purified α-amylase

*3.3 Thermal stability of the purified enzyme*

Based on the graph in Figure 8, it is known that the residual activity of the purified enzyme after 100 min of incubation is 8%. This indicates that the purified enzyme does not have a good enough stability under conditions of thermal inactivation for a longer time, it still needs to be increased its stability so that it is applicable to be used in industry.

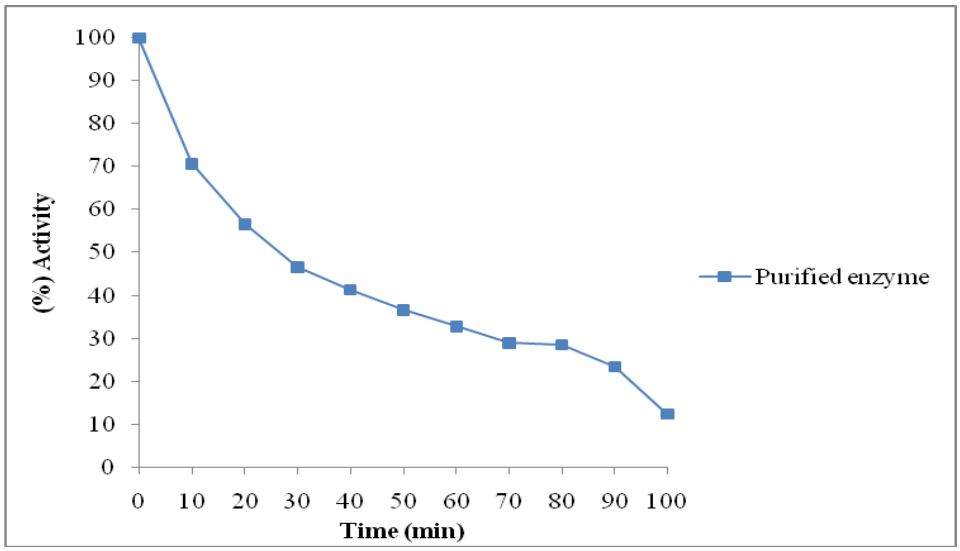


**Figure 8**. Thermal stability of the purified α-amylase

## 4. Conclusions

Based on the results of this study, it can be seen that the purified α-amylase from the local bacteria isolate ITBCCB148 increased its specific activity 149.9 times compared to the crude extract of the enzyme. The purified enzyme has an optimum pH of 5.5 and an optimum temperature of 60$^{o}$C and is stable at 60-80$^{o}$C.

## 5. Acknowledgments

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