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Partial Purification, Stability and Application of α Amylase Produced by *Ochrobacterium anthropi*

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Abstract

Amylases that hydrolyze starch are essential enzymes utilized in the food, textile, detergent, and pharmaceutical sectors. Although numerous fungi, yeasts, bacteria, and actinomycetes have produced alpha amylase, the majority of industrial uses have been for enzymes obtained from bacterial and fungal sources. In the present study α amylase was produced by the newly local isolate *Ochrobacterium anthropi*. Culture condition optimization showed that high amylase activity of the enzyme was characterized by expressing the optimum activity at 60ºC and pH 7.0. It showed thermal stability after 1h where relative enzyme activity was 97.1%. The pH stability covered a range starting from 6.0-9.0 for 1h. The purification studies were carried out using ammoinium sulphate. The best activity was obtained by partially purified using 80% ammonium sulphate precipitation technique and the purification fold was 1.49%. The Km value for the enzymatic hydrolysis of starch was 5. 5mM.The partially purified amylase was immobilized in sodium alginate (2%w\v) for application in detergent formulation and was proven to be much better at removing stains from cotton textiles than the other detergent that was tested. amylase was quite effective in removing chocolate stains when used with different commercial detergent (Persil, Fairy & Pril). When compared to detergent alone, the wash performance examination of chocolate stains on cotton fabric revealed a higher reflectance with detergent plus enzyme.

Keywords: Amylase, production, *Ochrobacterium anthropi*, purification, immobilization, sodium alginate

1. Introduction

Enzymes called amylases can aid in the haphazard hydrolysis of starch, glycogen and different oligosaccharides [1]. Three types of amylases have been identified including α-amylase, β-amylase and δ-amylase. An industrial enzyme called alpha amylase (EC 3.2.1.1) breaks down the alpha 1-4 glycosidic bonds in starch and other polysaccharides to create glucose and maltose, among other products [2]. These enzymes can be derived from various origins such as plants, animals and microorganisms [3]. For large scale production, microbial enzymes are the most preferred. This might be explained by the ease of isolating the enzymes, their low cost of production, their stability in a variety of challenging environments, and the simplicity of modifying bacteria to create the appropriate enzymes [4].

Bacteria is considered as an important source of α-amylase production with better enzyme properties compared to that derived from fungi [5]. Amylase produced by bacteria are more preferred due to their quick cell growth, multiplication and ease of production [5]. An enzyme's suitability for industrial applications increases with its range of heat stability. However, pH and temperature have a significant impact on enzyme stability [6].

Bacillus sp. is widely used in industry as a source of thermostable α-amylase; *B. subtilis, B. licheniformis, B. stearothermophilis* and *B. amyloliquefaciens* have been widely used for commercial α-amylase production in different applications [7]. Thermostable actinomycetes especially Thermomonespora and Thermoactinomyces are also enzyme producers. Fungi was also studied for α-amylase production including *Penicillium chrysogenum* [8], *P. solani* [9].

Regarding the wide application of α-amylase, there is a continuous need to develop the cost-effective process to produce the most stable and efficient α-amylase. These enzymes find potential application in a number of industrial processes including dish washing, laundry, food processing, food fermentation, paper and textiles [9]. Microbial amylases have been also used in medicine [10]. Amylases are the most common enzymes employed in the food business to produce processed meals including drinks, fruit juices, and sugar syrups (starch-, Maltose and fructose syrups. The biological and pharmaceutical sectors also benefit from alpha-amylases. It's interesting to note that the first enzyme to be synthesized industrially from a fungal source was amylase in 1894. It served as a prescription medication to address stomach issues. One of the oldest and biggest users of enzymes for many years has been the detergent industry. Detergents that contain enzymes like lipase, protease, and amylase prevent the need for harsh chemicals to remove stains. Alpha amylases make up the majority of the amylases found in laundry detergents. Its endo-amylolytic activity effectively hydrolyzes the starchy component of dirt and stains, improving detergent's capacity to remove stubborn stains [11].

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This paper deals with the partial purification and biochemical characterization of α-amylase produced by newly local isolate *Ochrobacterium anthropi* using starch as substrate. Application of the free and alginate immobilized enzyme as an additive in laundry was also investigated.

2.Materials and Methods

2.1. Materials

2.1.1. Microorganism

The bacterial used in the current work was isolated from sewage water samples from quolupia region and it was biochemically and genetically identified as *Ochrobacterium anthropi* cited in [12]**.**

2.1.2. Chemicals

All chemicals used in this work were of analytical grade and purchased from Sigma Aldrich and Merck companies. The solvents in this work were HPLC grade.

2.2. Methods

2.2.1. Preparation of inoculum

Erlenmeyer flask 250ml containing 100 ml of nutrient broth medium was autoclaved at 121^{o} C, 1.5 atm for 15min. It was inoculated by pure culture of the isolated bacteria and incubated for 24h at 200rpm and 35^{o} C.

2.2.2. Fermentation process and amylase production

The chosen basal medium, which included the following components (g\l): magnesium sulfate 0.25, ferrous sulfate 0.01; manganese chloride 0.015; calcium chloride 0.05; potassium dihydrogen phosphate 0.05; starch 10; peptone 10; yeast extract 20, was maintained at 6.5 pH. Autoclaving the manufacturing medium for 15 minutes at 121C^{0} and 1.5 bar of pressure disinfected it. Inoculation with 2ml of the previously prepared inoculum of the used isolate and incubated at 40^{o}C±2^{o}C for 48h. At the end of production process, the fermentation broth was subjected to centrifugation at 10000 rpm for 10 min at 4^{o} C [13,14]. The clear supernatant was separated and used for testing enzyme activity.

2.2.3. Extracting α-amylase

In order to isolate the enzyme, bacterial cells were separated from the enzyme using a cold centrifuge running at 10,000 rpm for 10 minutes at 4^{o}C. The acquired crude extract was kept in the freezer in order to partially purify it later.

2.2.4. Partial purification of amylase using the precipitation with ammonium sulphate salt

The enzyme was gradually precipitated (fractionated) by adding ammonium sulphate to a 100 ml crude extract of the enzyme in accordance with the target saturation level of the enzyme. For fifteen minutes, the mixture was agitated at 4^{o} using a magnetic stirrer. The fractionation pattern was determined in five fractions: (0-20), (20-40), (40-60), (60-80), and (80-100) %. This was done in phases. The percentage of total enzyme deposition was identified as the one that produced higher enzyme precipitates and high specific enzyme activity. We redissolved the enzyme precipitate in two milliliters of sodium phosphate buffer (pH 6.4) [15].

2.2.5. Enzyme assay

The release of reducing sugar was measured using the DNS technique to determine the amylase activity of the crude enzyme that was produced following centrifugation. The reaction mixture (1 ml of soluble starch solution combined with 1 ml of pH 6.7 buffer) and 1 ml of crude enzyme were combined in a test tube, and it was then incubated at 35 °C for 15 minutes in a water bath. Next, 2.0 milliliters of DNS reagent were added to each tube, and the reaction was halted by boiling the reaction mixture for ten minutes in a water bath. Following room temperature cooling, the released sugar was calculated using the glucose standard curve and the absorbance (O.D.) at 540 nm was measured using a spectrophotometer. Under the tested circumstances, one unit of amylase activity was defined as the release of one millimole of reducing sugar per minute. [13].

2.2.6. Protein determination

The protein content of the culture extract was determined according to Lowry [15].

2.2.7. Enzyme characterization conditions

Estimation of optimum pH and temperature for amylase activity

By adjusting the pH of 0.1M phosphate buffer, the impact of pH on the activity of the amylases was ascertained (6, 7, 8 & 9). The typical assay conditions were followed in measuring the activity. The reaction was carried out at several temperature values, specifically 35, 40, 50, and 60^{o}C at their optimal pH, in order to identify the ideal temperature. The typical assay conditions were followed in measuring the activity.

2.2.8. Effect of thermal stability and pH stability on amylase activity

After aliquots of the enzyme were incubated at (40, 50, and 60^{o}C) for 1, 2, and 3 hours, the residual activity was measured to

determine the temperature stability of α-amylase. The enzyme was incubated without a substrate at the specified temperature, and residual activity was assessed under ideal circumstances [6].

After liquots of the partly purified enzyme were preincubated at pH ranges of 6 to 9, the residual activity was measured to assess the pH stability of the enzyme for 1, 2, and 3 hours. After the enzyme was incubated at the pH that was evaluated without substrate, the residual activity was assessed at the ideal pH and temperature.

2.2.9. Determination of kinetic parameters

The line weaver Burk Plot (1934) was utilized to compute the kinetic parameters for the hydrolysis of α amylase, with starch serving as the substrate at values ranging from 0.5% to 3%. All of the reactions were carried out after the maximum rate, Vmax (um/mg/min), and Michaelis-Menten constituent, Km (mg/ml), were calculated.

2.2.10. Enzyme immobilization

Preparation of alginate beads

Alginate gel beads were made in compliance with [16]**.** After dissolving sodium alginate in distilled water, an enzyme solution volume and sodium alginate solution were combined to produce a final concentration of 2%, 3%, and 4%wv. The process of forming alginate gel beads involved dumping a combination of alginate solution via a 300 mm nozzle from a height of about 20 cm into a 100 ml solution containing 2% CaCl2 (w\v) using a syringe while stirring continuously. The beads were immersed in the mixture for a whole day. After a thorough distilled water wash, the alginate beads containing the partly purified enzyme were set aside for additional research.

2.2.11. Application of partially purified immobilized amylase in detergents

Evaluation of calcium alginate immobilized amylase for use in detergent

The use of calcium alginate immobilized amylase as a detergent additive was examined by using some commercial detergents as Persil, Pril and Fairy. The detergent solution (1% v/v) was pre heated for 15min to un activate the enzyme. Both free amylase and immobilized amylase from *Ochrobacterium anthropi* were incubated with detergent solution for 10 min and residual activity was determined in comparison to control (without any detergent) [17]**.**

2.2.12. Wash performance analysis of amylase immobilized in calcium alginate beads

Evaluating the wash performance study of an enzyme generated by a local strain immobilized by entrapment involved calculating the cotton fabric's capacity to release chocolate stains. Cotton cloth (3 cm by 4 cm) was dyed with 300 milliliters of liquefied chocolate after it had set at 70 degrees Celsius, and it was then left to dry overnight in a hot oven. Every stained piece of fabric was submerged in one of the following:

A) 25ml tap water control
B) 25ml tap water + 1ml immobilized enzyme
C) 20ml tap water + 1%(v\v) of commercial detergent (Pril, Persil, Fairy)
D) 20ml tap water + 1%(v\v) of commercial detergent (Pril, Persil, Fairy) containing 1ml immobilized enzyme

Flasks were shirred at 200rpm, 40^{o}C for 1h. chocolate stain removal by the calcium alginate amylase was examined visually by looking at the pieces of dried cloth. The chocolate stained cloth washed with tap water was considered as the control [18]**.**

3. Results and discussion

3.1. Partial purification of α amylase using ammonium sulphate

Centrifugation was used to separate the enzyme from the culture filtrate at 10,000 rpm for 15 minutes at 4^{o}C. The recovered supernatant was subsequently used as crude enzyme prior to ammonium sulfate precipitation (Table 1). The fractions precipitated at 80% saturation level showed the greatest α amylase activity. The partly purified amylase showed specific activity of 965 U/mg protein, which was higher than the crude extract's 646 U/mg protein with an approximately purification factor of 1.49. Several co-workers used ammonium sulphate for enzyme precipitation as it increases the enzyme stability. In addition, it is more soluble, less expensive and remains chemically unaffected in different pH [19]**.** Furthermore, [13,20] reported previously that 80% saturation was the best percentage for α amylase precipitation from *B. subtilis* MTCC9447 and *B.methylotrophicus* P11-2**. Fincan [21]** precipitated α amylase from *B. subtilis* using 70% saturation of ammonium sulphate. The concentration of ammonium sulphate depends on the equilibrium of charges present on the protein surface and disruption of water layer surrounding the enzyme [13]**.**

Table 1: Partial purification of α amylase extracted from *O. anthropi*

Fractions	Total protein mg\ml	Total activity U\ml	Specific activity U\mg	Purification fold
Crude extract	0.41±0.01	265±0.15	646±1	1±0
20	0.19±0.01	32.7±0.1	172.1±0.6	0.26±0.01
40	0.16±0.005	41.8±0.1	261.2±0.1	0.40±0.1
60	0.12±0.005	66.9±0.1	557.5±0.1	0.86±0.005
80	0.08±0.01	77.2±0.1	965±0.5	1.49±0.005

3.2. Enzyme constants of α amylase

Using a line weaver-Burk plot, the linear regression approach was used to determine the kinetic constants of K_m and $V_{max.}$ Thus, the concentration (S) of the substrate (starch) was plotted against the amylase activity (v). The kinetic constants (K_m and V_{max}) of the enzyme were evaluated. The k_m and V_{max} which showed the affinity between enzyme and substrate were found to be 5.5mM and 0.58 U/ml protein respectively (Fig 1). The value of k_m was 5.5mM. **Muniasamy and Rathnasaamy, [22]** previously reported that K_m value with a high V_{max} value showed affinity between the enzyme and the substrate. Similary, a smaller k_m value of 0.55mM was obtained during solid state fermentation of amylase from *Monascus sanguineus* **[23].** **Aguilar [24]** determined the k_m value of purified α amylase3.44mg/ml using *Lactobacillus manihotivorans* LMG18010T

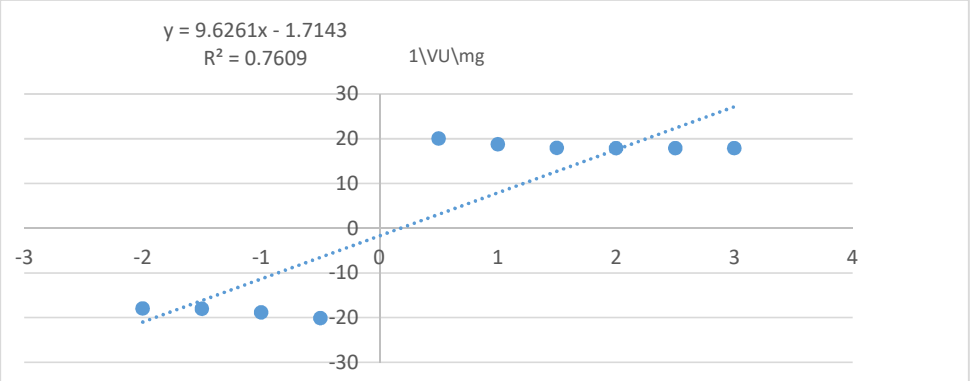


Figure 1: Linear weaver-Burk plot for partially purified α amylase produced by *O. anthropic*

3.3. Characterization of partially purified enzyme

The characterization of partially purified amylase of *O. anthropi* was done by determing the optimum pH and optimum temperature at which the enzyme activity was maximum.

3.3. Optimum temperature and thermal stability of partially purified amylase

Temperature is an important factor that affects amylase activity and temperature profile was investigated in the range of 35 to 70°C. (Fig 2). As the amylase activity reached maximum at 60°C whereby the activity was recorded to be 25.2U\ml after which the enzyme activity dropped. This decrease in enzyme activity recorded at 70°C may be due to the denaturation of the enzyme this leads to an evidence that amylase of *O. anthropi* is thermostable.

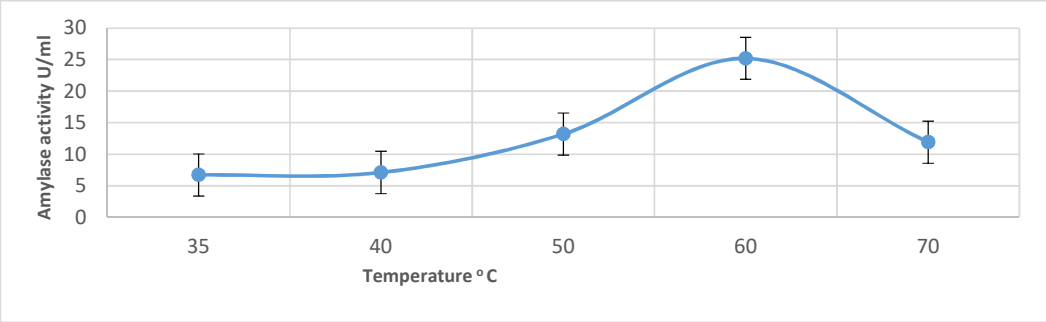


Figure 2: Effect of different temperature on amylase activity

For one hour, the thermal stability of amylase was assessed at 40°C, 50°C and 60°C for 1h, 2h and 3h (Fig3). The enzyme was quite stable at 60°C since it retained 97.1% of the initial activity after 1h. At 40 or 50°C incubations the enzyme activity decreased to 55.5 and 66.8% respectively. Thus, the present study indicates that the activity of α amylase produced by *Ochrobacterium anthropi* was more stable at high temperature (60°C) for 1h. **Finore [25] and Ravindran [26]** have been reported that amylase activity with temperature optima of 70°C from *B. stearothermophilus* and *Geobacillus thermoleovorans* respectively. This is in line with [27] who showed that the optimum temperature for amylase production of *B. subtilus* was 60°C. **Muniasamy and Rathnasaamy, [22]** who reported that amylase activity using *Bacillus simplex* remains active between 40 and 60°C for 60min. similar results were reported for purified α amylase from *Bacillus subtilis* y25 which exhibited appreciable thermal stability at temperatures up to 60°C for 50 min [28]**. Cordeiro [29]** have determined that α amylase activity was stable at 50°C over 2h and it lost 4, 13 and 38% of its activity at 60, 70 and 90°C respectively. Also, [30] determined that α amylase from *Bacillus sp.* AB04 was stable between 50 and 80°C. [31] also reported that the best

temperature of amylase stability for *Bacillus licheniformis* was 70°C. Its activity was decreased at 70°C (70% after 4 h), 80°C (65% after 4 h) and 90°C (50% after 1 h). Varied between 10 to 50° C. The thermostability values and the inhibition period of enzyme namely vary according to the molecular weight of the enzyme, type of substrate, ionic strength of buffer and the source of enzyme [32].

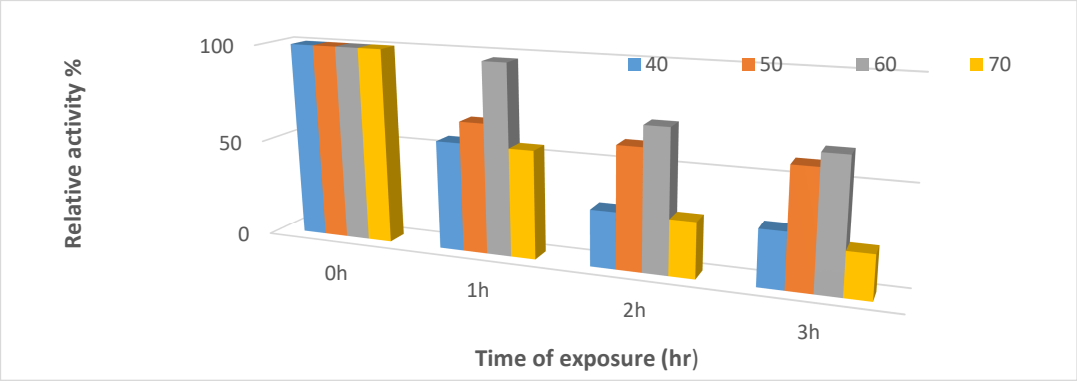


Figure 3: Thermal stability of the partially purified α amylase of *O. anthropi*

3.4. Optimum pH of partially and stability of purified amylase

By employing phosphate buffer 0.1 M, the ideal pH of *O. anthropi* amylase has been determined across a broad pH range, ranging from 5.5 to 9.0.

(Fig4). The pH profile showed that enzyme exhibited a very broad pH activity profile ranging from 6 to 8.0 with optimum pH 7.0. The pH profile shows that the enzyme activity was active and nearly stable in alkaline conditions while when subjected to acidic pH, the activity was greatly reduced. Bacterial α-amylases have different pH profiles some of which have sharp pH profile with rapid decrease in the enzyme activity at the higher and lower pH [33]. However, several α-amylases exhibit a very broad pH range [34]. In fact, the change in the pH of the reaction mixture leads to a change in the ionic nature of the carboxylic and amino acid groups of the enzyme which affects both the catalytic site and the conformational status of the enzyme and thus in turn alters the enzyme activity [35].

The α-amylase when exposed to extreme pH (5.0 and 10) resulted in great loss of activity. The change in pH affects the enzyme stability as it changes the ionic state or denaturation of the enzyme molecule. In addition, these changes in pH also affect the secondary and tertiary enzyme structure which lead to decrease in enzyme activity. Similar results were obtained by [36] using *Escherichia coli* where the enzyme was stable with pH values 7-8 and maximum activity was shown at pH 7.0.

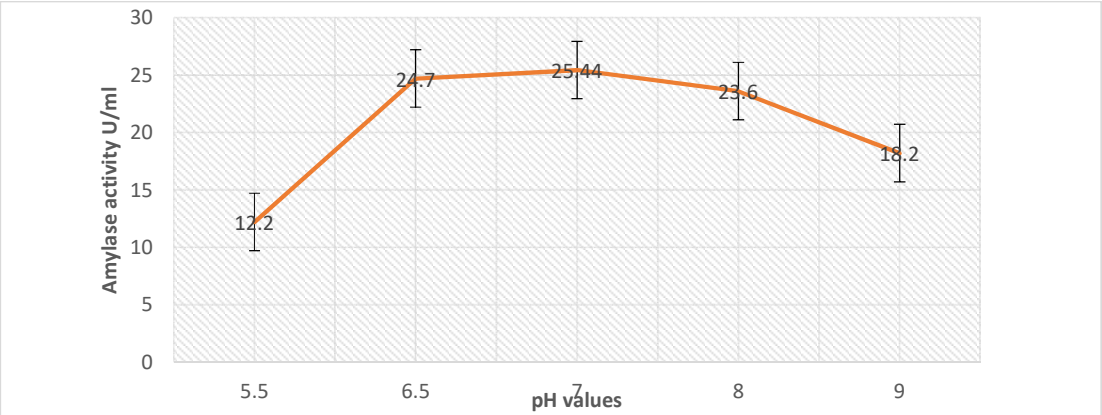


Figure 4: Effect of different pH values on amylase activity

To study the effect of optimum pH for amylase stability, the amylase solution was incubated with different buffer pH values ranging between (5-10) for 1,2 and 3h at room temperature, then the relative activity was calculated (Fig 5). The best pH for amylase stability was ranging between 7 -8 pH values for 1h and it decreased in acidic pH (6.0) and alkaline pH (9.0) showing relative activity 60.7% and 62.2% after 3h respectively.

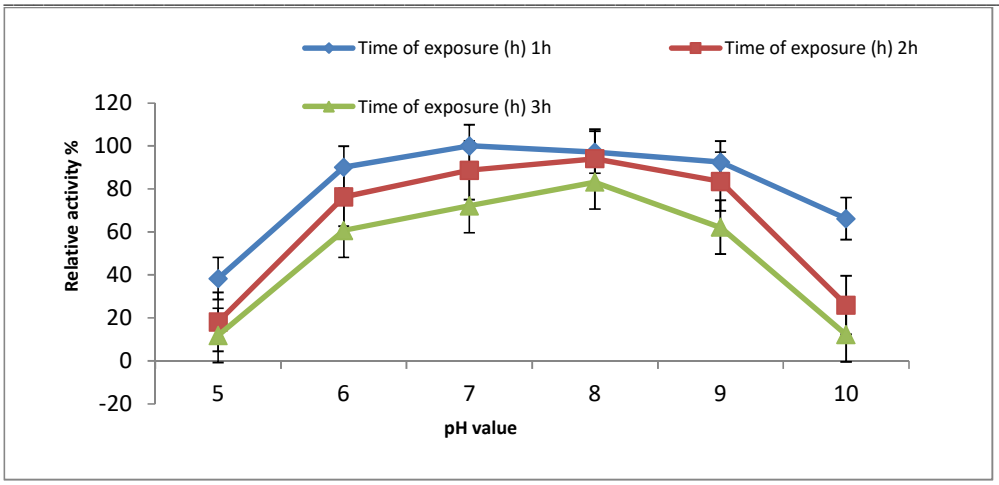


Figure 5: Effect of pH on the stability of partially purified α-amylase from *O. anthropi*

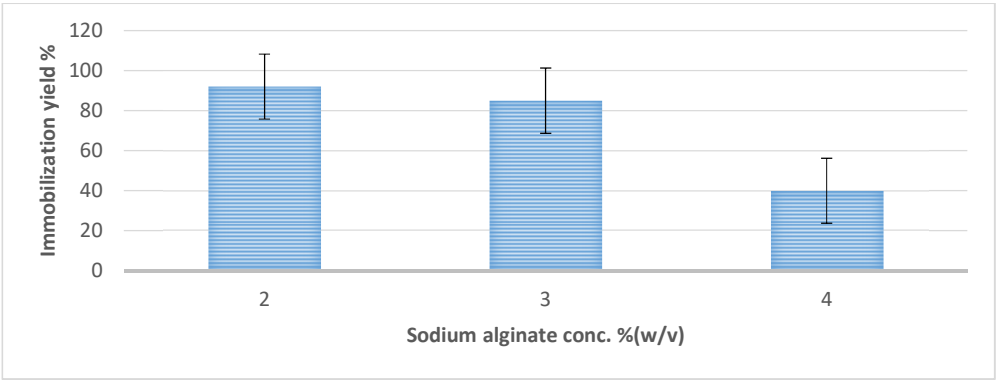


Figure 6: Immobilization yield % with different sodium alginate concentrations
Immobilization yield= activity of immobilized enzymex100/A-B
A= activity of free enzyme
B= activity of remaining enzyme washed water and filter by calcium chloride solution of un bound enzyme.

3.5. Amylase immobilization

3.5.1. Effect of different concentration of sodium alginate on immobilized yield

The immobilized enzyme yield was studied on changing the sodium alginate concentration from 2-4%(w/v). The results shown in (Fig 6) indicated that the immobilization yield of partially purified amylase produced by *Ochrobacterium anthropi* was maximum (92%) on using 2% (w/v) sodium alginate, while it decreased until it reached 40% with 4%(w/v) sodium alginate. Both the results we obtained were consistent with the findings of [36], who reported that immobilized enzyme was prepared using varying doses of sodium alginate (1.5–5.0%). The greatest immobilization yield was recorded at 2% (w/v). The ease with which the substrate and product may diffuse into and out of the alginate gel could be the cause of the sodium alginate concentration. The pore size of the beads will shrink as the concentration of sodium alginate increases, which will also result in a decrease in enzyme escape from the beads [37]. The amylase activity increased while using immobilized enzyme (27.2 u/ml) compared to free enzyme where the activity was lower (25.44 u/ml). This may be due to direct contact of biocatalyst with the substrate increases the amylase activity [38].

3.5.2. Evaluation of the calcium alginate immobilized α-amylase for using in detergent formulations

The potential of amylase, either free or immobilized in sodium alginate beads, as an addition for commercial detergents was evaluated (Fig 7). When the immobilized amylase was present, Peril detergent was shown to have the highest level of enzyme activity. This might be explained by the fact that some detergents have favourable effects on the hydrophobic contacts and enzyme confirmation that stabilize the protein molecule's buccal structure [18]. Conversely, with fairy detergent, the immobilized and free enzymes had relative activities of 9.04% and 8.45%, respectively. There was a correlation seen between a potential decline in protein confirmation and a drop in enzyme activity in specific detergents. **[39].**

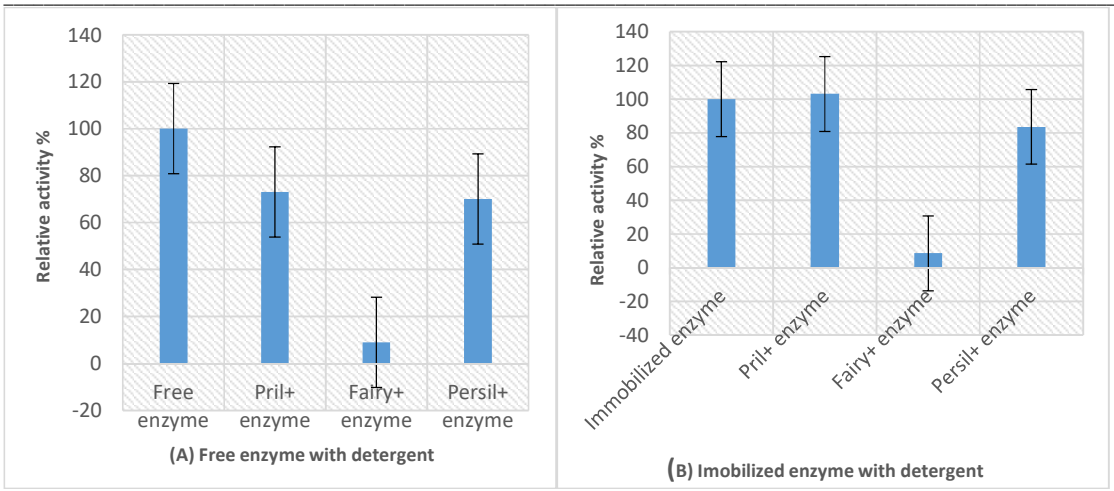
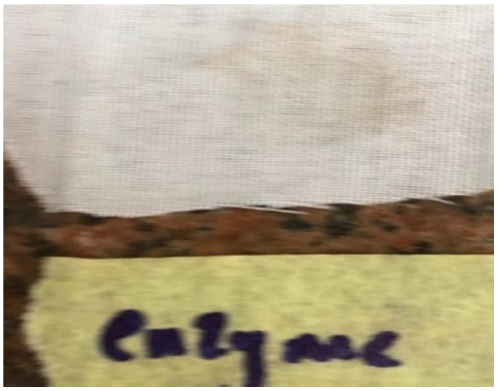


Figure 7: Testing compatibility of *Ochrobacterium anthropi* amylase with various commercial detergents with a) free enzyme & b) immobilized enzyme

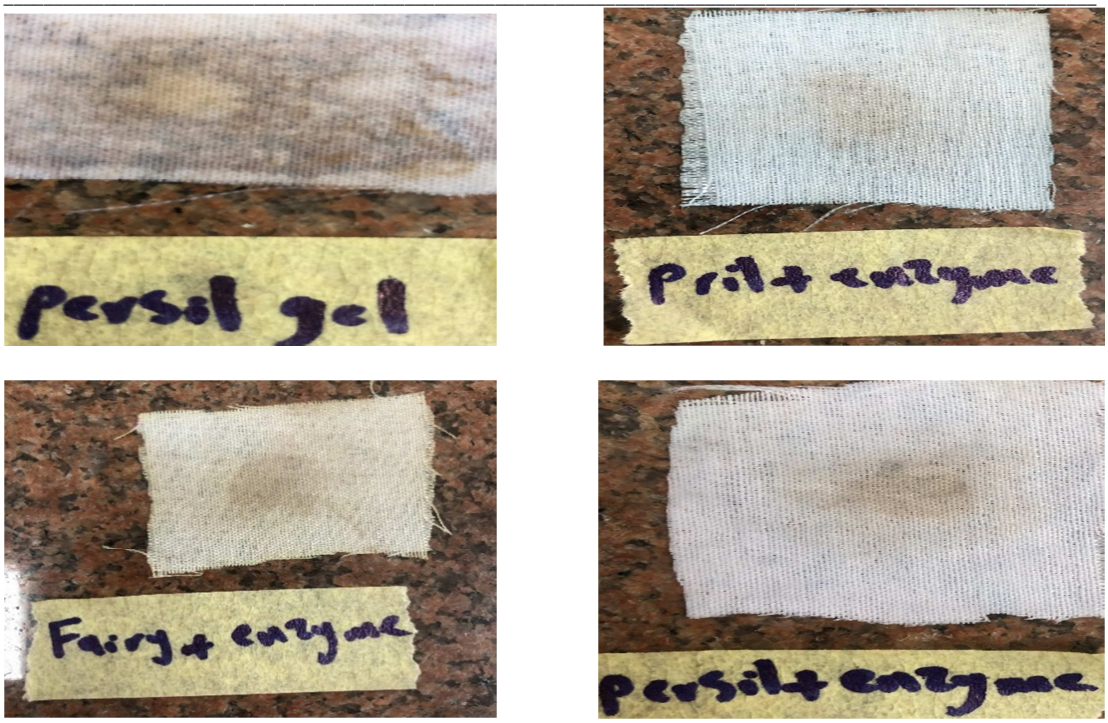
*The amount of Enzyme activity was measured in the presence of 1% liquid detergents at 60°C at pH 7.0. The activity of free enzyme in absence of detergent (25.44 U/ml) & for immobilized was (27.2 u/ml) both of them were taken as 100%.

a) Cloth stained with chocolate washed with hot water



b) Chocolate stained washed with partially purified enzyme

C) Chocolate stained cloth washed with water and detergents (Fairy, Peril, Persil)



d) Chocolate stained cloth washed with detergents and enzyme

Figure 8: Washing performance evaluation of the amylase from *O. anthropi* strain in the presence of the commercial detergents

The results presented in figure (8) showed that using hot water only has low capability for stain removing. The best stain removal was obtained by a mixture of enzyme and pril. While the other detergents with amylase enzyme revealed considerable low activity. it is evident that treatment with *O. anthropi* amylase enhanced the cleaning efficiency of the boiled detergent. The enhancement of fabric cleansing was reported by [5] **using** bacterial amylase from *Bacillus licheniformis* MTCC1483 and [40] by amylase from *Paenibacillus lactis* strain. This finding highlights the future application of *Ochrobacterium anthropic* amylase as an additive in laundry detergent formulations

4. Conclusion

The results revealed to characterize partially purified amylase obtained from newly local isolate *O. anthropi*. The biochemical characteristics of amylase showed a promising range of pH stability and interesting thermal stability at 60°C. It was found that the immobilized α-amylase on calcium alginate beads improved the washing performance of detergents and removed chocolate stain from clothes which was difficult to remove under normal conditions. Therefore, it is possible to investigate the immobilized enzyme further for potential usage in laundry detergents and to utilize it as a cleaning component.

Conflicts of interest

"The authors have no conflicts to declare".

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