Fungal α-amylase entrapped in agar-agar organic matrix "beads" enhances fabric starch-desizing potentials and α-amylase-detergent compatibility

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Abstract

Aureobasidium pullulans α-amylase (*ApAmy*) mixed with melted agar-agar solution and drop-wisely added to a mixture of organic solvent solution allowed for the entrapment of the α-amylase in the agar-agar organic matrix as beads. The immobilized *ApAmy*'s characteristics and wash performance were elucidated in comparison with the soluble *ApAmy*. Agar-agar at 2.0 % (w/v) and toluene: chloroform at 3:1 resulted in the highest immobilization yield retaining about 98% residual activity after ten catalytic cycles. The optimum temperature and pH for the immobilized enzyme were 60°C and 6.5 respectively. The immobilized *ApAmy* hydrolysed branched and linear substrates thus establishing its broad substrate specificity. Relatively, the immobilized *ApAmy* (*iApAmy*) was more tolerant to organic solvents than the free enzyme. The *iApAmy* was mildly inhibited by cobalt but metals such as zinc, manganese, calcium and sodium enhanced the free and immobilized *ApAmy* activity. The *iApAmy* had a higher washing efficiency (77%) in the presence of detergents than the free enzyme (68%) and control (36%).

The *iApAmy* showed good potentials as a detergent additive and from its characteristics, it could be useful in other industrial applications.

Introduction

Alpha-amylase is an enzyme that hydrolyses starch to yield mostly maltose and glucose residues. They perform this feat via the hydrolysis of the α-1, 4- glycosidic bonds present in starch (Khuma and Khare et al., 2015). This enzyme is ubiquitously expressed in numerous plants, bacteria, fungi and higher animals. α-Amylases have several industrial applications, as they are involved in several industrial processes leading to the production of pulp and paper, glucose syrup, cyclodextrins, and detergent production (Hashemi et al., 2013). Industrially, amylases serve in the breakdown of starch (liquefaction and saccharification) to simple sugars such as maltose and glucose. A major drawback in the utilization of free/soluble enzymes in most industrial processes is the non-reusability and reduced stability, hence, the focus on immobilized enzymes (Husain, 2017). Immobilized enzymes tend to have improved biocatalytic properties and thermostability in comparison with the soluble enzymes (Mesbah and Singh, 2018; Ademakinwa et al., 2019). Several techniques exist for enzyme immobilization but entrapment (despite its minor drawbacks such as enzyme leakage) offers a cheap and readily available approach (Mesbah and Singh, 2018). A major advantage of this entrapment technique is that it does not interact with the enzymes active site (Bilal et al. 2017). This allows the enzyme to retain its native conformation. The choice of support for entrapment varies depending on a host of factors but oftentimes these supports must be cheap, stable and inert. Some common support reported for amylase immobilization are chitosan Uzun and Akatin (2019), alginate (Ademakinwa et al. 2019), agar-agar (Mesbah and Singh, 2018) etc. The choice of agar-agar stems from its easy availability, non-toxicity and overall stability (Sattar et al., 2018); hence, these properties were considered for the immobilization of α-amylase in this study. In this study, a novel approach for the entrapment of α-amylase from the fungus, *Aureobasidium pullulans,* was investigated. This particular fungus had been reported to secrete a variety of useful industrial enzymes such as laccase (Ademakinwa and Agboola, 2016; Ademakinwa 2021; Ademakinwa

and Fashakin 2021) amylase (Ademakinwa et al., 2019; Ademakinwa and Agboola, 2019), rhodanese (Ademakinwa et al., 2021); fructosyltransferase (Ademakinwa et al., 2017, Ademakinwa et al., 2018; Ademakinwa and Agboola, 2020). Previous reports on using agar-agar for amylase entrapment focused on mixing the enzyme solution with the agar-agar solution, allowing it to solidify in glass slides and cutting the solidified enzyme: agar mixture and hardening in glutaraldehyde (Mesbah and Wiegel, 2018). The modified approach utilized in this study focused on forming 'beads' in which the enzyme was entrapped in the agar-agar organic matrix with the aid of organic solvents such as toluene and chloroform. This approach was less clumsy and easily adaptable. The beads were used for starch hydrolysis for several catalytic cycles. Thereafter, the immobilized α-amylase was characterized and used in starch removal from fabrics.

Methodology

2.1 Fungal α-amylase production via submerged fermentation and assay

The fungus, *A. pullulans*, was grown under submerged fermentation. The α-amylase submerged fermentation production medium was carried out as previously described by Ademakinwa et al. (2019) and it contained (in g/L): potassium dihydrogen phosphate (2), sodium chloride (2), potato starch (20), calcium chloride (0.1), peptone (10) and magnesium sulphate (1.0). The medium was inoculated with the fungus (10 mm agar plug) and incubation was carried out for seven days. Thereafter, the crude enzyme was obtained by centrifugation of the grown culture medium at 4000 x g for 20 min. The cell free supernatant served as the enzyme source and it was stored at 4°C.

α-amylase was assayed using starch (1% v/v) as the substrate as described by Ademakinwa and Agboola (2019). The reducing sugar released (maltose) was determined by the dinitrosalicyclic acid method (Miller, 1959). One unit of α-amylase activity was described as amount of enzyme that allowed for the release of maltose from starch under standard assay conditions. Protein concentration was determined via the method of Bradford (1979).

2.2 Immobilization in agar: agar organic matrix and its operational conditions

Agar-agar solutions at different concentrations (0-4% w/v) were melted, cooled and added to alpha-amylase solutions (1:1) with a specific activity of 343 U/mg. The enzyme-agar mixture was then drop-wisely added (using a sterile syringe) to a solution containing chilled chloroform and toluene (1:3) and enzyme beads were immediately formed. The beads were separated via decantation, washed in 5 mM acetate buffer (pH 6.0) and then stored at 4°C.   The immobilized enzyme at the different agar-agar concentration were used in starch hydrolysis. The activity of the free enzyme was regarded as the control i.e. 100% relative to the amylolytic activities of the immobilized enzyme at the different agar-agar concentration

Reusability of the immobilized amylase

The immobilized beads containing the entrapped enzyme (100 mg) was applied in starch hydrolysis (1% v/v) starch for several cycles and the immobilization yield was determined using the equation [1] **See equation in 1 in the supplementary files.**

2.3 Characterization of the immobilized α-amylase

2.3.1 Effect of pH and determination of pH stability on α-amylase

The effect of pH on the free and immobilized α-amylase were determined at pH ranging from 3.0 to 8.0. The following buffers were used: 5 mM glycine-HCl (pH 3.0); 5 mM sodium acetate (pH 4 to 5) and 5 mM potassium phosphate (pH 6.0 to 8.0). Amylase activities were estimated using 1% starch as the substrate. The pH corresponding to the highest amylase activity was regarded as 100% and the others were reported as the relative activities.

2.3.2 Effect of temperature and determination of temperature stability on α-amylase

The effect of temperature on the free and immobilized α-amylase was determined at 20 to 80°C. This involves the incubation of the reaction mixture at the designated temperature. *α-amylase* activities were determined with starch as the substrate as described earlier. The temperature corresponding to the highest amylase activity was designated as 100% and the others were reported as relative activities.

2.3.3 Influence of detergents on α-amylase

The effects of different detergents such as Triton X-100, local detergents (Sunlight, Waw, Rana), sodium dodecylsulphate, Tween-80 andTween-20 on the free and immobilized α-amylase were determined at concentrations ranging from 5 and 10% (w/v or v/v) respectively. The detergents were incubated with the free and immobilized enzymes for 2 h at 60°C. The control experiment was conducted such that the reaction mixture did not contain any of the aforementioned detergents and the amylase activity was regarded as 100%. The experiment was conducted in triplicate measurements

2.3.4 Substrate specificity of α-amylase

The potentials of the free and immobilized α-amylase in the hydrolysis of numerous substrates (1% w/v) such as soluble corn starch, glycogen, maltotriose, p-nitrophenyl-α-D-glucopyranoside, sucrose, maltose and β-cyclodextrin was investigated. The α-amylase activities of the soluble corn-starch served as the control and it was regarded as 100%. The experiments were conducted in triplicate measurements

2.3.5 Effect of metals on α-amylase

The influence of several metal chlorides on the amylolytic potentials of the free and immobilized α-amylase was determined using 1 and 5 mM chloride salts of sodium, lithium, magnesium, calcium, cobalt and Zinc. The solutions containing the appropriate concentration of the metal was incubated for 2 h. The control experiment was designed such that no metal was included in the assay mixture. The

amylase activities in the control was regarded as 100%. The experiment was conducted in triplicate measurements.

2.3.6. Effect of organic solvent on alpha-amylase

The effects of organic solvents (water miscible and immiscible) on the free and immobilized α-amylase were investigated at final concentrations of 5 and 10% v/v at pH 6.0 and a temperature of 60ºC for 2 h. The organic solvents used were acetone, ethanol, methanol, isopropanol, diethyl ether, n-hexane and dimethyl formamide. For the water immiscible solvents, the reaction mixture was agitated for the duration of the assay. The enzyme was incubated with the reaction mixture to start the reaction. Control experiments contained no organic solvent and the alpha-amylase activities were regarded as 100%.

2.4 Starch desizing from cotton fabrics by the free and immobilized *α-amylase*

The clean cotton cloth (5 cm × 5 cm) was stained with 1.0 ml of 1% (w/v) corn starch solution and dried to constant weight at 60ºC for 40 min. The dried starch- stained cloth and 1.0 g of the immobilized enzyme was incubated in a 50 ml conical flask containing 10 ml sterilized tap water. The reaction mixture was incubated at 60ºC for 1 h under constant agitation (200 rpm). Thereafter, the piece of cloth/immobilized enzyme were removed and the washout was used to estimate the reducing sugar using Anthrone's method (Shukla and Singh, 2015) with slight modification.

To determine the compatibility of detergents with the immobilized enzyme, another set of similar experiment were conducted such that, instead of the immobilized enzyme and tap water as described above, it was replaced with (i.) detergents (1% w/v) + immobilized enzyme (ii.) detergents only (iii.) soluble enzyme + tap water (iv.) soluble enzyme + detergents

The starch concentration in the washout was estimated using the method described by Prakash and Jaiswal (2011). The washing efficiency (☐) was determined via the equation below: **see equation 2 in the supplementary files.**

The immobilized enzyme was reused in starch desizing and detergent compatibility experiments. After each washout, the immobilized enzyme was rinsed with acetate buffer and then reused. The washout yield was determined using the equation below: **see equation 3 in the supplementary files.**

Results And Discussion

3.1. Immobilization of α-amylase

A. pullulans α-amylase was successfully entrapped in the agar-agar organic matrix as beads. The optimum agar-agar concentration resulting in the optimum activity was 2.0% w/v (Fig.1) with a relative activity of 140%. A noticeable observation was that at agar-agar concentration below the optimum, the beads were fragile and the observed reduced relative activity was due to the inability of the agar-agar support to entrap the enzyme effectively. At concentrations above the optimum, the relatively reduced

activity observed in this study might be due to the inability of the starch to reach the active site of the entrapped enzyme (Mesbah and Weigel, 2018). Recently, Mesbah and Weigel (2018) reported that agar-agar at 1% was adequate for the immobilization of *Alkalimnicola* amylase and attempts at increasing the concentration negatively impacted the activity of the enzyme. Meanwhile, some authors such as Prakash and Jaiswal (2011) have reported immobilization amylase using 4% agar-agar. The differences observed in terms of the optimum polymer concentration for amylase entrapment might be associated with the nature/source of isolation the enzyme. As obtained in this study, the agar-agar concentration of 2.0% was adopted for the immobilization of the enzyme thereafter. The immobilized enzyme retained about 140% of its initial activity after seven catalytic cycles that later decreased to 96% on reaching ten cycles (Fig.2). The observed decrease might be due to factors such as the enzyme leaking from the beads or being denatured (Prakash and Jaiswal, 2011). The cycles of catalysis observed in our study for the immobilized amylase was relatively lower than what was obtained for amylase immobilized on agar-agar organic matrix blocks. The amylase immobilized on agar-agar block retained about 58% of its initial activity after 16 catalytic cycles (Mesbah and Wiegel, 2018).

3.2. Effect of temperature and pH on free and immobilized α-amylase

The optimum pH observed for the free and immobilized amylase was 5.5 and 6.5 respectively. Immobilization altered the pH from 5.5 to 6.5 (Fig.3). This observation was relatively similar to the observation of Mesbah and Wiegel (2018) where the immobilized amylase pH profile was altered from 10.5 (for the soluble enzyme) to 10.0 after immobilization. Immobilization of the enzyme resulted in a minor increase in the optimum temperature from 50 (for the soluble) to 60ºC (Fig 4). Further experiments for the immobilized amylase was not conducted above 70ºC due to the disintegration of the beads as the temperature was increased above 70ºC. Similar observations were reported by Prakash and Jaiswal (2011) and Mesbah and Wiegel (2018).

3.3 Effect of organic solvent, metal ions and detergents on free and immobilized α-amylase

Tables 1 and 2 provides the summary for the relative activities obtained for the effect of organic solvents, metals and detergent on free and immobilized amylase respectively. At 10% v/v of the organic solvents used in this study, the free enzyme was inhibited as it lost about 40, 12 23, 36, 6, 36 and 26% activities at this concentration (Table 1). Further increase in the organic solvent concentration to 20% v/v resulted in significant decrease in the activities of the enzyme. The immobilized enzyme offered some measure of resistance to the organic solvents even at 20% v/v (Table 2). The relative increased stability of the immobilized amylase in organic solvents when compared to the free enzyme allows for its utilization in numerous industrial biocatalytic process such as the synthesis of oligosaccharides in a medium devoid of water (Doukyu et al. 2007).

Metal ions such as Li^+, Co^{++}, Mg^{++}, and Zn^{++} to a lesser degree inhibited the free amylase while only Ca^{++} and Na^+ enhanced the amylolytic activity (Table 1). In contrast to the immobilized amylase, all metals except Co enhanced the amylolytic activity (Table 2). It can be deduced that since both the free and

immobilized amylase were activated by Ca^{++}, A. pullulans amylase can be designated Ca^{++} dependent. Most amylases are Ca^{++} dependent with a few exceptions (Mesbah and Wiegel, 2018).

Stability in detergents/surfactants is a desirable property for amylases to be utilized in pharmaceutical, fabrics and detergent-allied industries. In this study, the immobilized amylase was stable to numerous detergents such as Tween-80, Tween-20, Triton X-100 with a relative activity of 134, 121 and 114% respectively at 1% v/v concentration. An increase of the surfactant concentration to 5% leads to a minor inhibition (18% loss of activity) of the immobilized amylase. Sodium dodecyl sulphate (SDS) inhibited the immobilized amylase at 5% v/v of the detergent resulting in 26% inhibition. Local detergents such as Waw and Rana inhibited the immobilized amylase at 5% w/v while Sunlight enhanced the amylolytic activity with a relative activity of 104% at the same concentration. When the local detergent concentration was increased to 10% w/v, they all inhibited the immobilized enzyme. The inhibition of the immobilized amylase by SDS was also reported by Mesbah and Wiegel (2018). All detergents inhibited the free enzyme except for 1% v/v SDS. At 1% SDS, the relative activity obtained was 102% which later decreased to 66% when the concentration of SDS was increased to 5% w/v.

3.4 Substrate specificity of free and immobilized *α*-amylase

The pattern of hydrolysis of amylolytic substrates by the free and immobilized amylase was quite similar only that in most cases, the relative activities of the immobilized amylase is higher than that obtained for the free enzyme (Table 3). Both the free and immobilized *α*-amylase hydrolysed linear and branched substrates and did not hydrolyse certain substrates like cyclodextrins and p-PNPG. The inability of the free and immobilized amylase to hydrolyse substrates like cyclodextrins and α-PNPG might be due to the presence of cyclic bonds in this substrate (Uzun and Atakin, 2019) and lack of α-glycosidase activities (Pasin et al., 2017) respectively. Substrates such as soluble starch, maltose and amylopectin were hydrolysed to a greater degree than others such as glycogen, sucrose and maltotriose (Table 3).

3.5 Wash performance of free and immobilized *α*-amylase

The washing efficiency (⬜) obtained in this study indicated that the immobilized amylase had a greater amount of starch in the washout than the free enzyme and the control. When only tap water was used, the ⬜ was very low (19%) but with the addition of detergents, the efficiency increased to about 57%. When the free enzyme was utilized without the addition of detergents, the ⬜ obtained was about 43% inclusion of the detergents enhanced the release of starch in the washout leading to a ⬜ of 68%. The use of immobilized enzymes only resulted in a washing efficiency of approximately 59% meanwhile, the combination of detergents with the immobilized amylase further increased the ⬜ to 74%. The use of free (Shukla and Singh 2015) and immobilized (Prakash and Jaiswal 2011) amylases in the release of starch from fabrics have been reported in the literature. Shukla and Singh (2015) reported that about 76% of starch in the washout after the free amylase from *Laceyella sacchari* was used in combination with detergent. The use of detergents in combination with immobilized enzymes is quite useful in the removal

of starch from fabrics as observed in this study and others (Prakash and Jaiswal 2011, Shukla and Singh, 2015, Uzun and Akatin 2019)

Conclusion

The α-amylase from *A. pullulans* was immobilized in agar-agar organic matrix as beads at 2.0% w/v agar-agar concentration and chilled toluene: chloroform solution (3:1). The immobilized enzyme retained about 96% of its initial activity after 10 catalytic cycles. The immobilized amylase displayed some level of increased stability in organic solvents and surfactants when compared to the free enzyme. The immobilized enzyme was metal-activated and only hydrolysed linear and branched substrates with no preference for cyclic substrates. When used in combination with some detergents, the immobilized amylase improved the release of starch from starch-stained fabrics. The properties of the immobilized amylase make it a good candidate for other starch-based industrial applications.

Declarations

Declaration of competing interest(s): The author declares no conflict of interest

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Tables

Table 1 Effect of organic solvents, detergents and metal ions on free alpha amylase from *A. pullulans*

	Organic Solvent	Relative Activity (%)	Detergents	Relative Activity (%)	Metal Ions	Relative Activity (%)
• i.	Control	100	Control	100	Control	100
• ii.	Acetone 10% 20%	60±6 24±3	SDS 1% 5%	102±5 66±2	Li^+ 1% 5%	94±6 88±5
• iii.	Methanol 10% 20%	88±5 34±5	Tween-80 1% 5%	86±7 40±5	Na^+ 1% 5%	101±8 95±6
• iv.	Isopropanol 10% 20%	57±6 26±4	Triton-X-100 1% 5%	88±5 23±3	Ca^{2+} 1% 5%	119±10 100±6
• v.	Diethyl ether 10% 20%	64±5 17±5	Tween-20 1% 5%	76±5 64±5	Zn^{2+} 1% 5%	98±8 69±5
• vi.	Ethanol 10% 20%	94±6 58±5	*Sunlight™ 5% 10%	84±5 26±3	Mg^{2+} 1% 5%	78±7 49±5
1.	n-Hexane 10% 20%	64±5 40±3	*WaW™ 5% 10%	79±3 56±5	Co^{2+} 1% 5%	67±8 39±4
1.	Dichloromethane 10% 20%	60±6 33±5	*Rana™ 5% 10%	80±7 36±4		

Table 2 Effect of organic solvents, detergents and metal ions on immobilized alpha amylase from *A. pullulans*

	Organic Solvent (v/v)	Relative Activity (%)	Detergents	Relative Activity (%)	Metal Ions	Relative Activity (%)
• i.	Control	100	Control	100	Control	100
• ii.	Acetone 10% 20%	94±5 66±3	SDS 1% 5%	110±6 76±3	Li$^+$ 1% 5%	109±4 100±2
• iii.	Methanol 10% 20%	99±6 72±6	Tween-80 1% 5%	134±7 111±5	Na$^+$ 1% 5%	164±12 130±8
• iv.	Isopropanol 10% 20%	96±7 70±4	Triton-X-100 1% 5%	114±10 82±8	Ca^{2+} 1% 5%	174±13 136±8
• v.	Diethyl ether 10% 20%	80±5 52±8	Tween-20 1% 5%	121±11 106±8	Zn^{2+} 1% 5%	124±5 94±3
• vi.	Ethanol 10% 20%	107±8 92±5	*Rana^{TM} 5% 10%	104±8 76±5	Mg^{2+} 1% 5%	110±6 100±4
1.	n-Hexane 10% 20%	96±7 48±5	*Waw^{TM} 5% 10%	94±7 66±4	Co^{2+} 1% 5%	89±7 65±6
1.	Dichloromethane 10% 20%	98±5 65±3	*Rana^{TM} 5% 10%	89±6 55±5		

Table 3 Substrate specificity of free and immobilized alpha amylase from *A. pullulans*

	Substrate (% w/v)	Relative Activity (%)
i.	Soluble starch (control)	100
ii.	Glycogen	
	Free	16±4
	Immobilized	28±5
iii.	Maltotriose	
	Free	23±6
	Immobilized	48±8
iv.	α-PNPG	
	Free	0
	Immobilized	0
v.	Sucrose	
	Free	2±1
	Immobilized	4±1
vi.	Maltose	
	Free	68±3
	Immobilized	94±5
vii.	α-cyclodextrin	
	Free	0
	Immobilized	0
viii.	β-cyclodextrin	
	Free	0
	Immobilized	0
1.	Amylopectin	
	Free	89±8
	Immobilized	98±9
24.	Amylose	
	Free	74±5
	Immobilized	90±6

Table 4 Wash performance of free and immobilized α-amylase

		%Washing Efficiency ($\square$)
i.	Control (Distilled water only)	19.57±4.9
ii.	Detergent A-only (D_1)	55.97±5.8
iii.	Detergent B-only (D_2)	56.87±6.8
iv.	Detergent C-only(D_3)	61.23±7.3
v.	Soluble enzyme-only (S)	43.49±5.2
vi	S+D_1	67.67±6.1
vii.	S+D_2	69.43±6.6
viii.	S+D_3	69.45±6.9
x.	Immobilized enzyme (I)	59.33±6.7
xi.	I+D_1	73.34±8.2
xii.	I+D_2	76.88±5.5
xiii.	I+D_3	75.57±6.3

D1- Sunlight

D2-Waw

D3-Rana

Figures
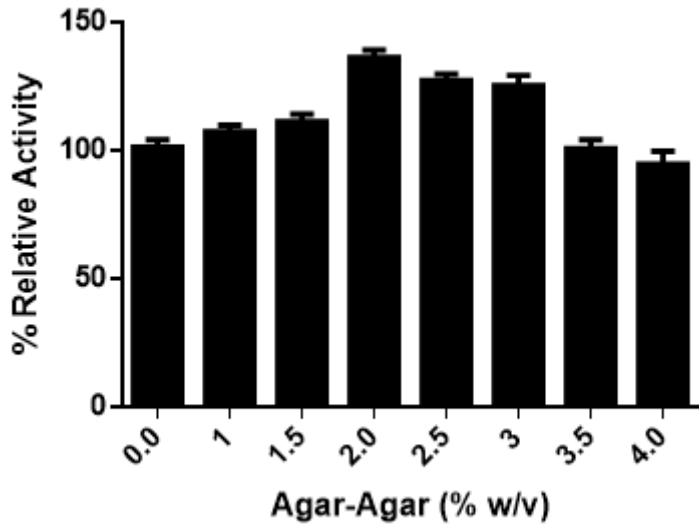
Figure 1

Effect of agar-agar concentration on the relative activities of immobilized Aureobasidium pullulans amylase
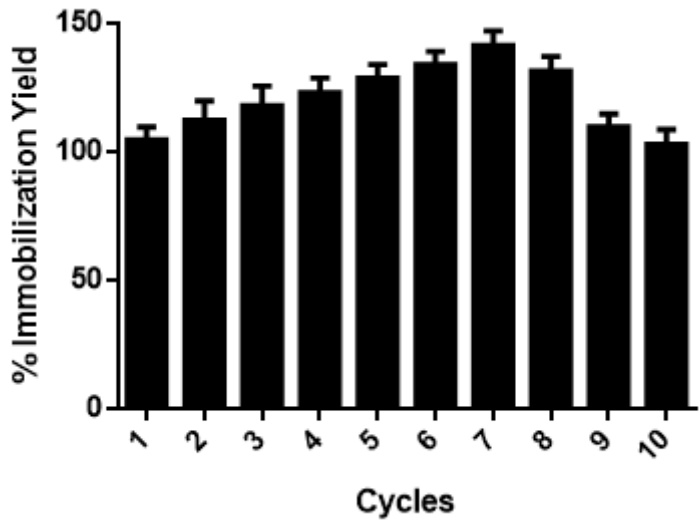

Figure 2

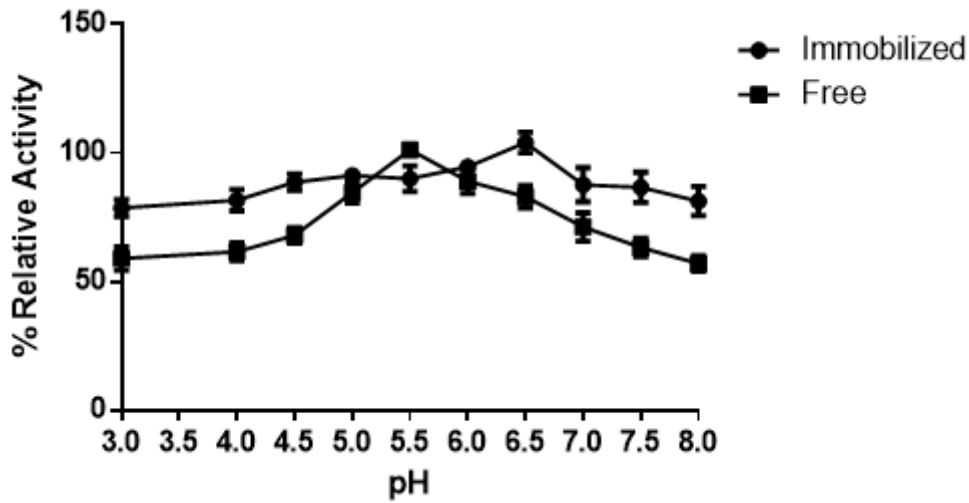Reusability of amylase entrapped in agar-agar organic matrix beads

Figure 3

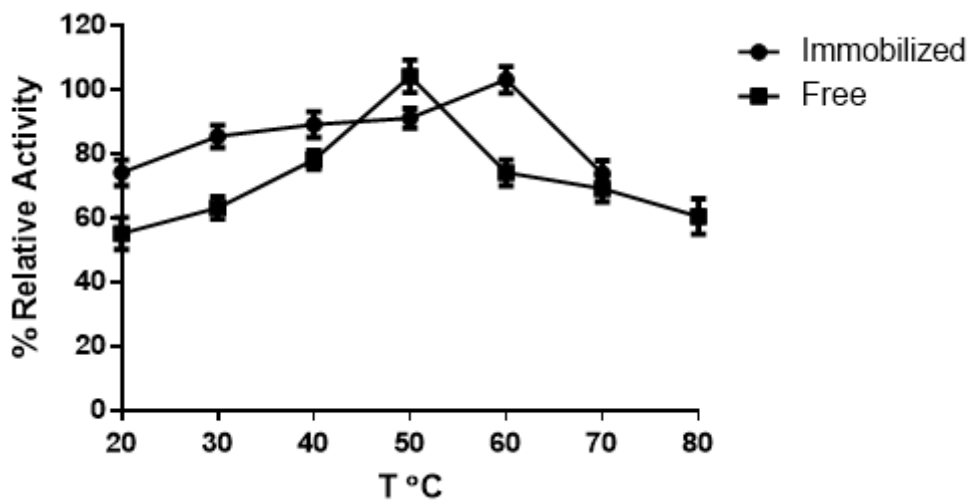Effect of pH and temperature on free and immobilized amylase

Figure 4

Effect of temperature on the free and immobilized A. pullulans amylase

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- formula.docx