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Alpha-amylase immobilization; methods and challenges

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Abstract

Alpha-amylase is one of the most widely used enzymes in the starch industry. However, industrial application of soluble alpha-amylase is hampered by changes in pH and temperature (adverse effects on enzyme stability) and activity loss, leading to higher costs. Immobilization of alpha-amylase is an efficient strategy to reduce the enzyme losing and subsequently reduces costs in this regard. Alpha-amylases are immobilized by adsorption, entrapment, covalent attachment, and cross-linking. A barrier in alpha-amylase immobilization is the large size of its substrate, namely amylose and amylopectin. Most of these immobilization methods decrease the affinity of the enzyme for its substrate as well as the maximum rate of reaction (V_{max}). This review aims to study different aspects of alpha-amylase including enzyme activity, applications, structure, starch, immobilization methods,

and immobilization's obstacles to improve alpha-amylase efficiency in the industry and also lowering the costs related to providing this enzyme.

Keywords: Alpha-amylase, Starch, Immobilization, V_{max}, K_m

1. Introduction

Enzymes are known as biocatalysts carrying out specific chemical reactions [1]. Several types of enzymes are used on the industrial scale, such as in food and beverage, pharmaceutical, laundry detergent, motor-fuel industry (bioethanol), and leather industry [2,3]. The biocatalysts used in industry are disposable and so impose extra costs of providing new enzymes [4,5].

Immobilization of alpha-amylase is the most widespread strategy to increase the number of cycles using this enzyme. Immobilization saves enzyme, providing the possibility of more sophisticated and modern processes (such as fed-batch, continuous or fixed-bed processes), and a more convenient handling and longer storage period [6-8].

The starch industry is the major industry consuming alpha-amylase. Although acid can be used for the digestion of starch, enzymes are more applicable to process starch due to mild reaction conditions and lack of secondary reactions [9].

Several types of amylases, including alpha-amylases, β-amylases, and glycoamylases are used in industry [10]. Alpha-amylases hydrolysis α-1,4 bonds between glucose subunits, thereby cleaving branched/unbranched starch. Among alpha-amylases, microbial alpha-amylases are more popular owing to advantages such as higher stability in harsh process conditions, easy genetic manipulation, and an inexpensive production [11,12]. Therefore, alpha-amylases produced by different bacteria such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Aspergillus oryzae*, and *Rhizopus* sp. are widely used in different industrial sectors, including manufacturing detergents, paper, textile, and food industries [13,14].

To increase the number of application cycles and improving their stability, alpha-amylases need to be immobilized, whereas immobilization imposes several limitations on the activity of enzymes and preparation processes. Also, immobilization influences kinetics parameters such as maximum reaction rate (V_{max}) and Michaelis constant (K_m). The K_m value shows the affinity of enzymes for substrates, and the low values highlight the higher affinity of the

enzymes for substrates [15,16]. Given the growing importance of alpha-amylase in the starch industry, this review provides an overview of literature focusing on alpha-amylase immobilization and its difficulties.

2- Amylase and polymer therapeutics

There are several applications of amylase in the pharmaceutical industry. Dextrin and dextran as substrates of amylase are antithrombotic (antiplatelet) agents used to decrease blood viscosity and as a volume expander in patients with hypovolaemia [17]. Also, amylase and its associated polymer substrates are highly interested in polymer therapeutics, particularly the science of drug delivery science. Conjugation of these polymers with bioactive molecules protects other tissues from possible adverse effects of them, as well as protects biomolecules from being degraded, immunological interactions, or renal uptake. However, applying biodegradable polymer offers a strategy to release the active payload at the target site. Then, in a predictable and safe method, amylase breaks down these polymers [18]. The first utilization of dextrin as a protective polymer was to improve the enhanced permeability and retention (EPR*)* effect of recombinant human epidermal growth factor; this complex (EGF-dextrin) would localize to the wound site, thereby accelerating wound healing [19,20].

An affordable, selective, and controlled release of bioactive molecules to the site of infection may be achieved by Dextrincolistin. There was a successful outcome in preclinical studies, while clinically less successful [21,22].

To increase the targeted delivery, receptors on the surface of the cells can be exploited by adding tags to the polymers in use that recognize these receptors. Tilmanocept is an example; it is a mannosylated tagged dextran-based polymer providing an innovative therapeutic strategy for melanoma and breast cancer patients. Tilmanocept binds tightly to CD206 mannose receptors of the reticuloendothelial cells in lymph nodes [23].

Also, alpha-amylase-replacement therapy is a proper therapeutic method in pancreatic insufficiency disorders like cystic fibrosis; in these patients, alpha-amylase is not secreted to the normal site of action. Thus, they are in urgent need of amylase replacement therapy [24]. On the contrary, alpha-amylase inhibitors (acarbose) are prescribed in type 2 diabetic patients, which mostly reduce the digestion of carbohydrates by inhibiting amylase and reducing blood glucose [25,26]. Similarly, Phaseolamin is an enteric amylase inhibitor used with the aim of

weight loss [27]. Phaseolamine also was reported can be used to control hyperglycemia in diabetes [28].

Finally, glucose, as the final product of amylase, has been supposed to underlie the effective inhibition of the production of the toxins related to gas gangrene [29].

3. Safety evaluation of alpha-amylases

Emergence in enzyme preparation technologies results from the advancement of science in protein engineering and molecular biology techniques so that microbially-derived enzymes are being used throughout the world in the food industry. After publishing considerations for evaluating human food safety of enzyme preparations in 1983, it was updated by developing recombinant DNA technology and adopted for its applications in animal feed. Moreover, its use in the Generally Recognized as Safe (GRAS) process for enzymes is peer-reviewed and clarified. According to these guidelines, the safety of enzyme preparations used in human and animal food was widely evaluated by peer-reviewing based on published scientific studies concerning the history of safe use of enzymes, the establishment of Safe Strain Lineages to serve as their production strains, and well-known strain improvement methods [30-35].

The majority of the toxicity evaluations of the alpha-amylase enzyme preparations were based on three methods:

1- 90-day oral toxicity testing in a rodent (generally in rat)

2- Ames test (*Salmonella Typhimurium* and *Escherichia coli* strains)

3- Chromosomal aberration test

Previously safety evaluation was conducted for C16F alpha-amylase enzyme preparations derived from *Bacillus licheniformis* (whole broth or WB and clarified preparation or UFC). Oral toxicity testing for Whole Broth (WB) and Ultra-filtered Concentrate (UFC) were done in rat according to OECD (The Organisation for Economic Co-operation and Development), and the systemic toxicity of C16F alpha-amylase preparations was evaluated in 90 days toxicological study. Moreover, *Salmonella typhimurium* strains TA98 and TA100 were used for the evaluation of the mutagenic effects of the preparations according to the Ames test. After 13 weeks of oral gavage, the safety of the alpha-amylase in both preparations doesn't

induce systemic toxicity and is not mutagen [36]. In another study, *Salmonella Typhimurium* strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2uvrA, were used for the Ames test to evaluate mutagenic effects of alpha-amylase from the genetically modified *Bacillus licheniformis* strain DP-Dzb52. 90-day oral toxicity testing for the preparation was done in rats and revealed that the preparation can be considered safe under the intended conditions of use [37].

4. Alpha-amylase structure

Alpha-amylase hydrolyzes starch via internal a-1, 4-glycosidic bonds and produces maltotriose and maltose (from amylose) or glucose, maltose, and limit dextrins (from amylopectin). The molecular weight of most alpha-amylases is around 45-60 kDa [38].

Alpha-amylases mainly belong to the family of GH13; also, the enzyme is classified in GH57, GH119, and GH126 families. [39]. The family GH13 alpha-amylases possess catalytic machinery, retaining reaction mechanism, 4–7 conserved sequence regions (CSRs), and type of $(\beta/\alpha)_8$-barrel catalytic domain. The alpha-amylases classified in family GH57, as well as GH13, use the same retaining mechanism. Moreover, it has specific catalytic machinery, five CSRs, and a $(\beta/\alpha)_7$-barrel fold [39].

The GH13 alpha-amylases generally have three domains consisting of domain A (which Contains $(\beta/\alpha)_8$-barrel domain as catalytic part), domain B, which is located between the strand β3 and helix α3 of the $(\beta/\alpha)_8$-barrel domain, and domain C has a β-sheet structure attached to domain B via simple loop [40,41]. The active site of alpha-amylase locates in a cleft between domains A and B, and it consists of three acidic amino acid residues, one glutamate (Glu), and two aspartate (Asp) residues. In alpha-amylase from *Aspergillus oryzae* (TAA), Glu230, as a general acid/base catalyst, donates a proton to the leaving glycosidic oxygen group and provides a nucleophilic species for the dislocation of the glycoside. Asp206 (as the catalytic nucleophile) and Asp297 form a covalent intermediate and stabilization of the transition state, respectively [42,43]. Figure 1 shows the 3D structure of alpha-amylase from *A. oryzae* (PDB code: 2TAA).

5. Starch, the most widely used substrate of alpha-amylase

Starch is a well-known carbohydrate resource in plants and a significant energy source. Starch is produced in the amyloplast of plants. The most important starch sources are tubers, roots, cereals, and rhizomes [44]. Starch consists of two parts, amylose and amylopectin.

Amylose contains glucose monomers which are attached via α (1-4) glycosidic bonds and amylopectin is polymerized via α (1-4) glycosidic bands and is branched by α (1-6) glycosidic bands [45-47]. Starch normally contains 15-30% amylose and 70–75% amylopectin; however, waxy starch have slight amount of amylose [48,49]. According to digestibility, starches are classified in three groups of the rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) [50]. Starch forms semi-crystalline granules having distinct morphology and size for in different plant species [44,51].

The granule size is a determining factor of digestibility by alpha-amylase. Generally, due to the diffusion of amylase through the grain fragment, small starch granule is hydrolyzed faster than large granule [52,53]. Another important physical characteristic is molecular size. The amylopectin molecule contains an average of 2000000 glucose units, making it one of the most significant natural molecules; however, this enormous size makes immobilization of enzymes difficult [54,55]. Also, molecular size varies according to the starch sources. The largest and smallest types of amylose are from potato and cereal, respectively [51,56]. Table 1 shows the physical characteristics of different starch from various sources.

Table 1: Some physical characteristics of different starch sources			Ref.
Starch source	**Granule size (µm)**	**Amylose (%)**	[51,52,57-62]
Potato	5–100	25–31	
Sweet potato	7-28	19–20	
Maize	2–30	20–28	
Rice	3–8	17–29	
Wheat	15–35 (A granules), 2–10 (B granules)	17–34	
Barley	15–25 (A granules), 2–5 (B granules)	22–27	
Triticale	3–33	23–27	
Sorghum	5–20	22–30	
Oat	3–10	18–29	
Rye	10–40 (A granules), 5–10 (B granules)	26–30	

Arrowroot	8-42	19–21	
Bean	8-55	23-39	
Sago	20-40	24-31	

6. Immobilization of alpha-amylase

The greatest hindrance in applying alpha-amylase is its wasting during large-scale reactions causing the high cost of providing new enzymes [63]. Immobilization methods, such as entrapment, adsorption, cross-linking, and covalent attachment, are known to retrieve enzyme [64]. Furthermore, immobilization allows enzymatic material recovery and multiple reusing, lowering production costs and improving catalytic activity retention and enzyme stability [65]. The advantages and disadvantages of immobilization methods are mentioned in table 2.

Table 2: advantage and disadvantage of immobilization methods

Method	Advantage	Disadvantage	Ref
Adsorption method	The weak interactions keep native structure of the enzyme and its activity	Diffusional limitations and conformational changes	66-71
Entrapment method	Affordable and fast method requiring mild conditions, and protects the enzyme from mechanical shear, hydrophobic solvents, and gas bubbles	Loss of enzyme activity and limitation in mass transfer and low-level enzyme loading.	72-75
Cross-linking method	Increased specific activity, greater volumetric activity per biocatalyst mass, more simple production, higher purity, less production costs, and less contaminations by the support	Low mechanical stability, low activity, poor reproducibility, and difficulties with handling	75-77

Covalent attachment method	Strong attachment of enzyme with carrier	Rigorous preparation condition and loss of enzyme activity due to reaction with toxic cross-linking reagents	,7873

6.1. Adsorption method

Among several enzyme immobilization methods, adsorption to solid carriers seems to be widely applicable. The physical interactions, including van der Waals forces, hydrogen bonding, and ionic interactions, are formed between the enzyme and carrier. The weak interactions keep the native structure of the enzyme and its activity [66,67].

Notably, selecting a carrier with reasonable cost, availability, stability, and reactivity is essential for establishing a good affinity between enzyme and carrier. Moreover, the physicochemical parameters of the carriers, including particle size, type of functional groups placed on the surface, surface area, and pore structure, should be regarded. The surface of carriers should provide the specific active groups causing the enzyme-carrier interactions. Also, it can be applied by intermediate agents (carrier modifiers) when specific active groups are absent [79].

Carriers are classified into two groups, organic (such as chitosan, cellulose, chitin, and alginate) and inorganic (such as silica, hydroxyapatite, and titania) [79].

Silicas are one of the most common inorganic carriers used to immobilize alpha-amylase. Table 3 shows several carriers used in the immobilization of enzymes by adsorption.

Table 3: Effects of Immobilization by adsorption on the kinetic of alpha-amylase

Alpha-amylase	**Carrier**	**Carrier modifier**	**K_m**	**V_{max}**	**Ref**
Diastase alpha amylase from malt	Polypyrrole (PPy) particles	_	1.49 ± 0.05 mg/ml (Soluble enzyme, 0.50 ± 0.04)	3.44 ± 0.02 mg/ml/min (Soluble enzyme, 7.40 ± 0.05)	69
Bacillus	Mesoporous	_	_	_	80

subtilis	silica SBA-15				
Bacillus species	Mesoporous silica thin film	_	_	_	[81]
Bacillus subtilis	Zirconia	_	AZ-1: 9.53 $*10^{-4}$ mol/ml and AZ-2: 7.07 $*10^{-4}$ mol/ml (Soluble enzyme, 2.51)	AZ-1: 0.15 $*10^{-4}$ mol/ml/min and AZ-2: 0.86 $*10^{-4}$ mol/ml/min (Soluble enzyme, 1.02)	[70]
Bacillus subtilis	Alumina	_	AA-1: 4.67 $*10^{-4}$ mol/ml and AA-2: 7.09 $*10^{-4}$ mol/ml (Soluble enzyme, 2.51)	AA-1: 0.99 $*10^{-4}$ mol/ml/min and AA-2: 0.83 99 $*10^{-4}$ mol/ml/min (Soluble enzyme, 1.02)	[71]

In the absorption method, carriers and enzymes need specific functional groups on their surface to achieve a successful enzyme immobilization [82-84]. Also, modifying agents have two reactive groups, one of them chemically interacts with the carrier, and the second one physically attaches to the enzyme. Bifunctional carbonyl compounds, such as glutaraldehyde, are widely used to immobilize enzymes by adsorption [85,86]. R. Reshmi *et al.* immobilized alpha-amylase by adsorption method using alumina. Figure 2 shows immobilized alpha-amylase onto alumina surface [71].

Most studies of immobilization by adsorption indicated an increased K_m and decreased V_{max} values compared to soluble alpha-amylase, probably due to diffusional limitations and conformational changes (see table 3) [68-71].

6.2. Entrapment method

In this method, the enzyme is entrapped in the synthetic or natural polymeric porous membrane (such as gel and microencapsulation); substrates and the products freely diffuse through it (see figure 3). This approach is an affordable and fast method requiring mild conditions and protects the enzyme from mechanical shear, hydrophobic solvents, and gas bubbles; however, it has a limitation in mass transfer and low-level enzyme loading [72,73]. A drawback of this method is the loss of enzyme activity; to tackle this problem, polymer porosity, surface functionality, network structure, and particle size needs to be modified [74,75].

Sidra Pervez *et al.* prepared immobilized alpha-amylase by agar-agar matrix support (table 4) [87]. The studies on immobilization of alpha-amylase by entrapment approach revealed that the approach decreases V_{max} and affinity for the substrate [87,88]. Diffusional substrate limitation in the entrapment approach causes a decreased affinity for the substrate [89,90]. Also, diffusional resistance is the main hindrance in decreasing of V_{max} immobilization process [87,91].

Table 4: Effects of Immobilization by entrapment on the kinetic of alpha-amylase

Alpha-amylase	**Carrier**	**Immobilization yield [%]**	**K_m**	**V_{max}**	**Ref**
Pennisetum typhoides	Calcium alginate beads	69	-	-	90
Aspergillus fumigatus	Agar-Agar	80	3.39 mg ml^{-1} (Soluble enzyme: 1.41)	698 kU mg^{-1} (Soluble enzyme: 947 kU mg^{-1})	87
Bacillus subtilis	Calcium alginate /Cellulosic residue	64.46	-	-	92

Bacillus circulans GRS 313	Calcium alginate beads	75	Bead size 4, 3 and 2 mm were 31.2, 28.2, and 23.75, respectively.	Bead size 4, 3 and 2 mm were 30.03, 33.08, and 36.23, respectively.	[93]
Fusarium solani	Calcium alginate beads	81	18.52 (mg ml^{-1} (Soluble enzyme 27.47)	1.23 mole min^{-1} ml^{-1} (Soluble enzyme 5.28)	[88]
Aspergillus oryzae	Sol–gel entrapment	–	–	–	[94]
Bacillus amyloliquefaciens	Drop-wise addition of an aqueous mixture of sodium alginate	89	–	–	[95]
Bacterial isolate (MW2)	Sodium alginate and agar, chitosan	72.18% (sodium alginate, agar), 66.45% (chitosan)	-	-	[96]

6.3. Cross-linking method

In the cross-linking method, for linking two enzymes and conducting carrier-free immobilization, bifunctional cross-linking agents or simply cross-linkers are used [97]. To form cross-linking between enzymes, flocculating agents such as glutaraldehyde, polyethyleneimine, polyamines, and polystyrene sulfonates are extensively used [98].

The cross-linking method has several advantages, including increased specific activity, more significant volumetric activity per biocatalyst mass, more straightforward production, higher purity, less production costs, and less contamination by the support [77,99]. However, cross-

linking methods have some drawbacks, including low mechanical stability, low activity, poor reproducibility, and difficulties with handling. To overcome these, cross-linked enzyme aggregates (CLEAs), cross-linked enzyme crystals (CLECs), and combi-CLEAs have been developed [75,76].

CLECs are prepared by crystallizing the enzyme at an optimum pH range (from an aqueous buffer solution) and treating it with glutaraldehyde [75]. Despite simple preparation, the CLECs method is expensive because it requires pure enzyme [100].

CLEA, via non-covalently binding, is prepared from non-purified precipitated enzymes that remain permanently insoluble while covalent binding with a cross-linking agent (such as glutaraldehyde) [101,102]. Alpha-amylase from *Bacillus amyloliquefaciens* was immobilized by the CLEA method and used glutaraldehyde to make the covalent binding. The immobilized alpha-amylase kept 65% activity following four-time reuses (Figure 4) [103].

When CLEAs immobilize more than one enzyme, it is called combi-CLEA [74]. combi-CLEA has an advantage compared to CLEA due to the proximity of the first active site of the enzyme to the second active site, which causes the quickly transferring of the first product to the second step [75].

In a study, alpha-amylase was immobilized with glucoamylase and pullulanase by combi-CLEA. The activity of enzymes in combi-CLEAs was almost kept up to 5 cycles [104]. Table 5 shows the effects of immobilization by cross-linking on alpha-amylase kinetic. The values of K_m and V_{max} were shown to decrease [103-107]. In the cross-linking immobilization method, V_{max} values have decreased due to the substrate's diffusion limitation [105,108]. Low K_m values of the immobilized alpha-amylase demonstrated that the conformational changes of the alpha-amylase following cross-linking process caused proper orientation of the active sites towards the starch (substrate) [109,110].

Table 5: effects of immobilization by cross-linking on the kinetic of alpha-amylase

Alpha amylase	**Type of cross-linking**	**cross-linking agent**	**K_m**	**V_{max}**	**Ref**
Bacillus amyloliquefaciens NCIM	CLEA	Glutaraldehyde	0.3245 ± 0.013 mg/ml (Soluble enzyme,	0.179 ± 0.023 µmole/min (Soluble enzyme,	103

2829			2.748 ± 0.027)	0.174 ± 0.011)	
Bacillus lehensis G1	CLEA	Chitosan	Mag1-p-CLEAs and Mag1-CLEAs were 2.02 and 1.26 mM, respectively. (Soluble enzyme, 3.82)	Mag1-p-CLEAs and Mag1-CLEAs were 4.57 and 2.72 µmol ml^{-1} min^{-1}, respectively. (Soluble enzyme, 4.59)	105
Bacillus licheniformis	CLEA	Glutaraldehyde	CLEAs–BSA–CN and CLEAs–BSA were 5.26 and 3.12 mg/mL, respectively. (Soluble enzyme, 5.35)	CLEAs–BSA–CN and CLEAs–BSA were 1.12 and 1.09 µmol min^{-1}, respectively. (Soluble enzyme, 1.34)	106
Bacillus sp.	CLEA	Glutaraldehyde	Magnetic CLEAs and CLEAs were 0.21 ± 0.019 and 0.21 ± 0.023 mg/mL, respectively. (Soluble enzyme, 0.93 ± 0.014)	Magnetic CLEAs and CLEAs were 81 ± 0.27 and 83 ± 0.13 µmol/min, respectively. (Soluble enzyme, 85 ± 0.١١)	107
Alpha amylase, glucoamylase, pullulanase	combi-CLEA	Glutaraldehyde	-	-	111
Alpha amylase (*Aspergillus oryzae)* and	combi-CLEA	Glutaraldehyde	3.33 × 10-4±0.000017 M (Soluble enzyme, 4.86 × 10-	9.98 ± 0.057 µmol.min^{-1} (Soluble enzyme, 10.95 ± 0.042)	104

maltogenic amylase			4±0.000021)		

6.4. Covalent attachment method

The covalent attachment approach improves enzyme stability via covalent binding of the enzyme with carrier [78,112]; electrons are shared between the surface of the carrier and the amine functional group of the enzyme [74]. Several reactions, such as diazo linkage, iso-urea linkage, peptide binding, and alkylation, contribute to covalent interaction between enzyme molecules and support [98]. By immobilization, the enzymes remain attached to the carrier even in harsh conditions [73]. The disadvantage of the method is the rigorous preparation condition and loss of enzyme activity due to reaction with toxic cross-linking reagents [78].

S Demir *et al.* modified nano $CaCO_3$ particles with 3-aminopropyl triethoxysilane to provide a functional group on the surface. Then, glutaraldehyde was added to make a covalent attachment between the enzyme reactive functional groups ($–NH_2$) and the modified nano $CaCO_3$ particles (figure 5) [113].

Most studies of immobilization by covalent attachment showed high K_m values (reduced affinity for substrate) and variable V_{max} values (table 6) [65,113-116]. The increased values of K_m may result from changes in the accessibility of the substrate, steric effects, structural changes, and changes in the affinity of the substrate during immobilization. Also, the most likely reason for the increase in V_{max} values is conformational changes of alpha-amylase [114,117]. However, a study indicated that the reduced V_{max} of the immobilized alpha-amylase was due to the multiple linkages between the enzyme and carrier, which consequently caused a decrease in the rate of reaction [118].

Table 6: effects of immobilization by covalent attachment on the kinetic of alpha-amylase

Alpha amylase	**Carrier**	**Carrier modifier**	**K_m**	**V_{max}**	**Ref**
porcine pancreas	$CaCO_3$	3-aminopropyl triethoxysilane	0.55 mg/mL (0.45 mg/mL for soluble enzyme)	0.35 mg/mL/min (10 for soluble enzyme)	113

Bacillus licheneformis	Cellulose fibers	periodic acid	-	-	119
porcine pancreas	Glass beads	3-aminopropyl-triethoxysilane and triethylamine	-	-	120
Bacillus subtilis	Magnetic Nanoparticles (MNPs)	tetra methyl ammonium hydroxide	Increase 28.9-fold (0.5 mg mL^{-1} for soluble enzyme)	Increase 40.5-fold (10 mg mL^{-1} for soluble enzyme)	114
Anoxybacillus sp. SK3-4	Amino-epoxide	-	-	-	121
Aspergillus Oryzae	Chitosan-montmorillonite nanocomposite beads	Glutaraldehyde	9.12 µmol/ml (Soluble enzyme, 6.80)	0.629 µmol/mg.min (Soluble enzyme, 1.30)	115
Aspergillus Oryzae	Magnetic nanoparticles coated with silica and gold	3-phosphono propionic acid (3-PPA)	8.054 mg/mL (Soluble enzyme, 5.8)	1.851 lmole/min (Soluble enzyme, 1.811)	65
Aspergillus Oryzae	TiO_2	poly-L-lysine	15.03 mM (Soluble enzyme, 11.04)	855 U/mg (Soluble enzyme, 920)	116
Bacillus Subtilis	Chitosan bead	Glutaraldehyde	0.431 mg/ml (Soluble	227 U/mg Enzyme (Soluble	68

			enzyme, 0.208)	enzyme, 416.67)	

7. Conclusion

Alpha-amylase, due to specificity, high catalytic efficiency, and mild operation conditions, has gained remarkable popularity in the industry. Microorganisms are the primary source of alpha-amylase with different characteristics. The soluble enzyme has limited optimization activity due to harsh conditions and production costs. In order to optimize alpha-amylase catalytic properties and lower production costs, the enzyme's immobilization has been developed. The amylose and amylopectin molecule size is the greatest obstacle to alpha-amylase immobilization. Four main methods are used to prepare alpha-amylase immobilization, including adsorption, entrapment, covalent attachment, and cross-linking. Apart from the cross-linking method, all these methods use various carriers. Each method has several advantages and drawbacks, causing changes in the K_m and V_{max} values. Immobilization using adsorption, cross-linking, entrapment, and covalent attachment causes an increase in Km and a decrease in V_{max} values. The most important reasons for decreasing V_{max} and affinity for the substrate have been attributed to the diffusional limitations due to the large size of the substrate. Also, the reasons for decreasing the affinity and V_{max} by covalent attachment immobilization have been due to changes in the accessibility of the substrate for the active site, steric effects, structural changes, and changes in affinity during immobilization. A recently developed method removes all these drawbacks. This method allows immobilization of silica particles (SPs) in a thin organosilica layer and makes large substrates accessible to the enzyme active site. Therefore, partial shielding is a promising strategy to improve stability and preserve activity for several industry use cycles. The method is a good candidate for the immobilization of alpha-amylase to solve the problem.

Conflict of interest:

None

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Author contribution:

Design of the this study and supervision of the team was performed by Babak Elyasifar; collecting data and writing of the manuscript was performed by Ladan Mafakher, Javad Khalili Fard, and Sajjad Yazdansetad:; Critical revision was done by Yasin Ahmadi:

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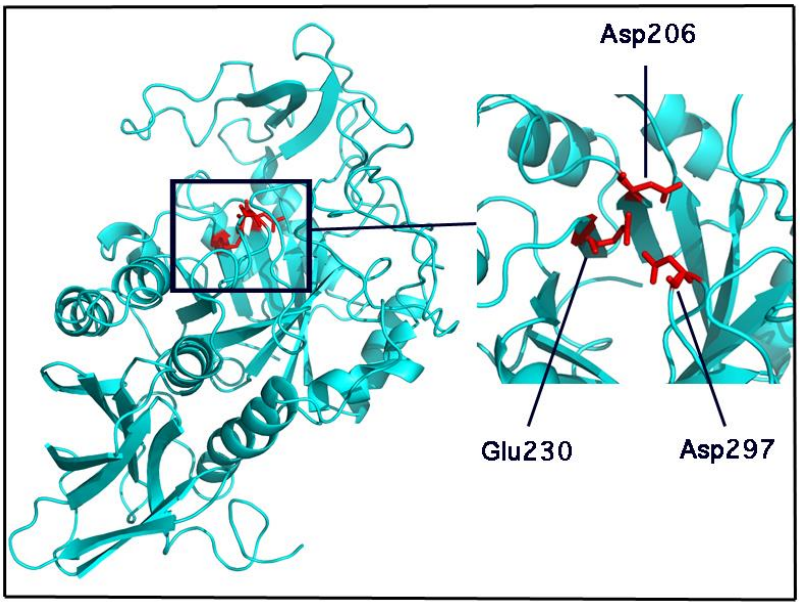

Figure 1: 3D structure of alpha-amylase from *A. oryzae* (TAA). The active site is depicted in red color Asp206, Glu230, and Asp297. The *N*- and the *C*-terminal are shown in blue and red, respectively.

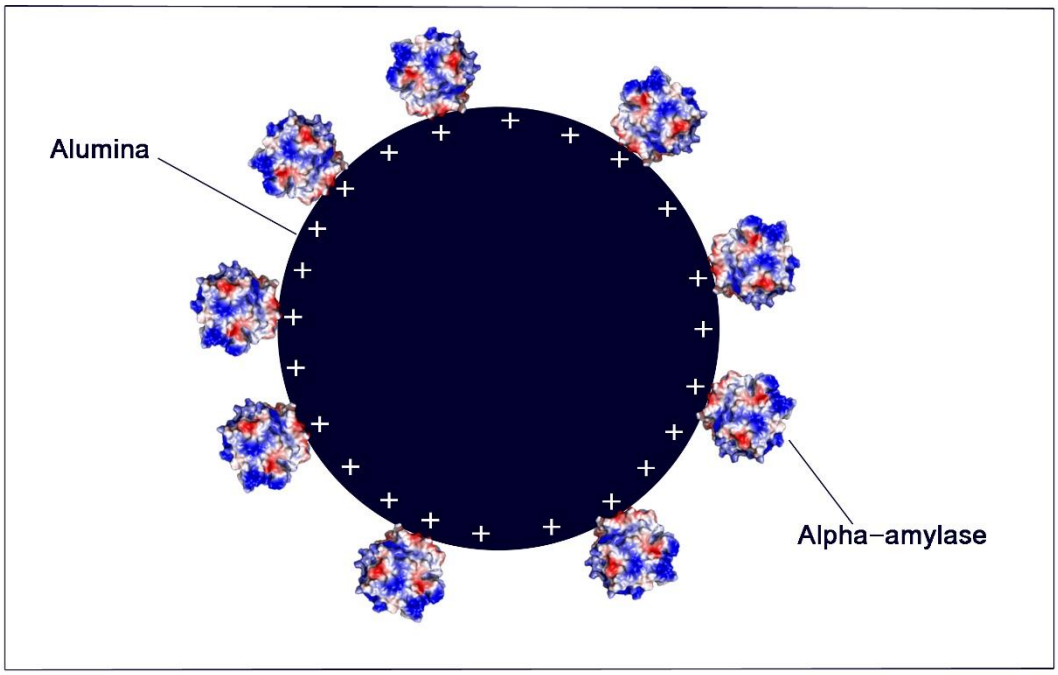

Figure 2: Schematic of alpha-amylase immobilization by adsorption alumina surface

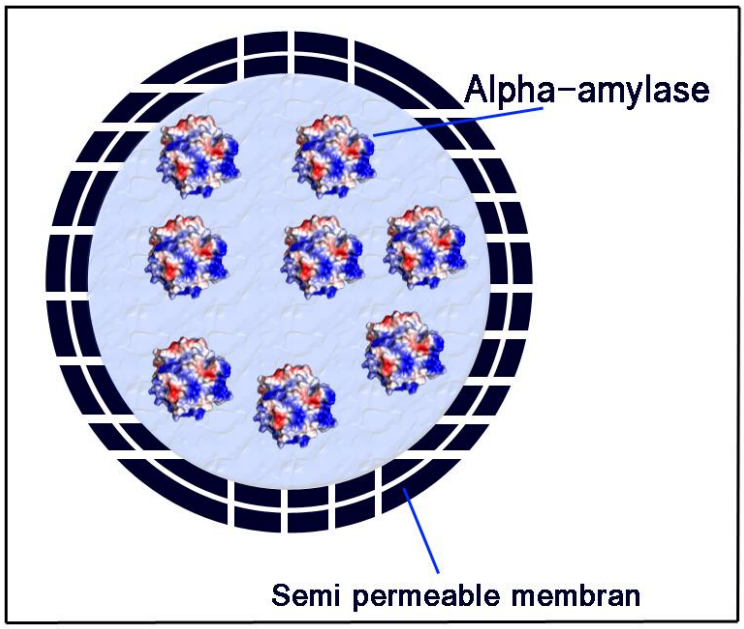

Figure 3: Schematic of entrapment of alpha-amylase

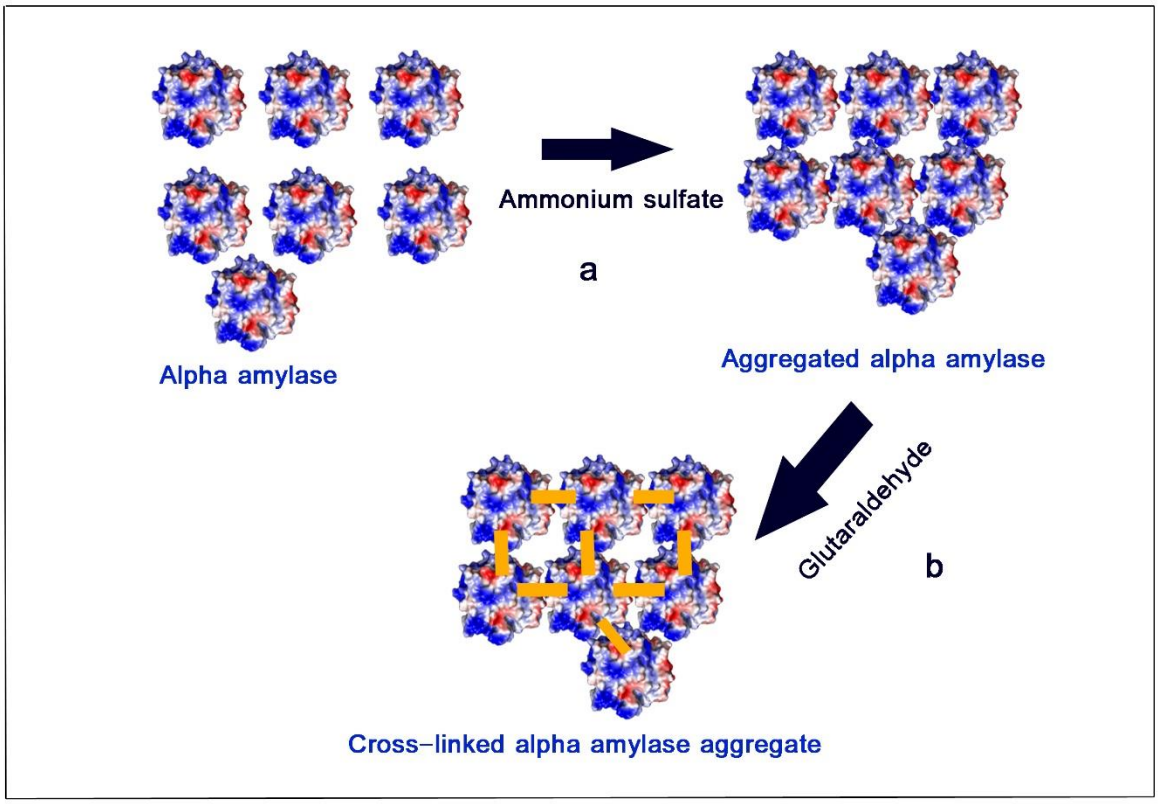

Figure 4: Preparation of cross-linked alpha-amylase aggregate. (a) Aggregation of alpha-amylase; (b) Covalent binding with a cross-linking agent.

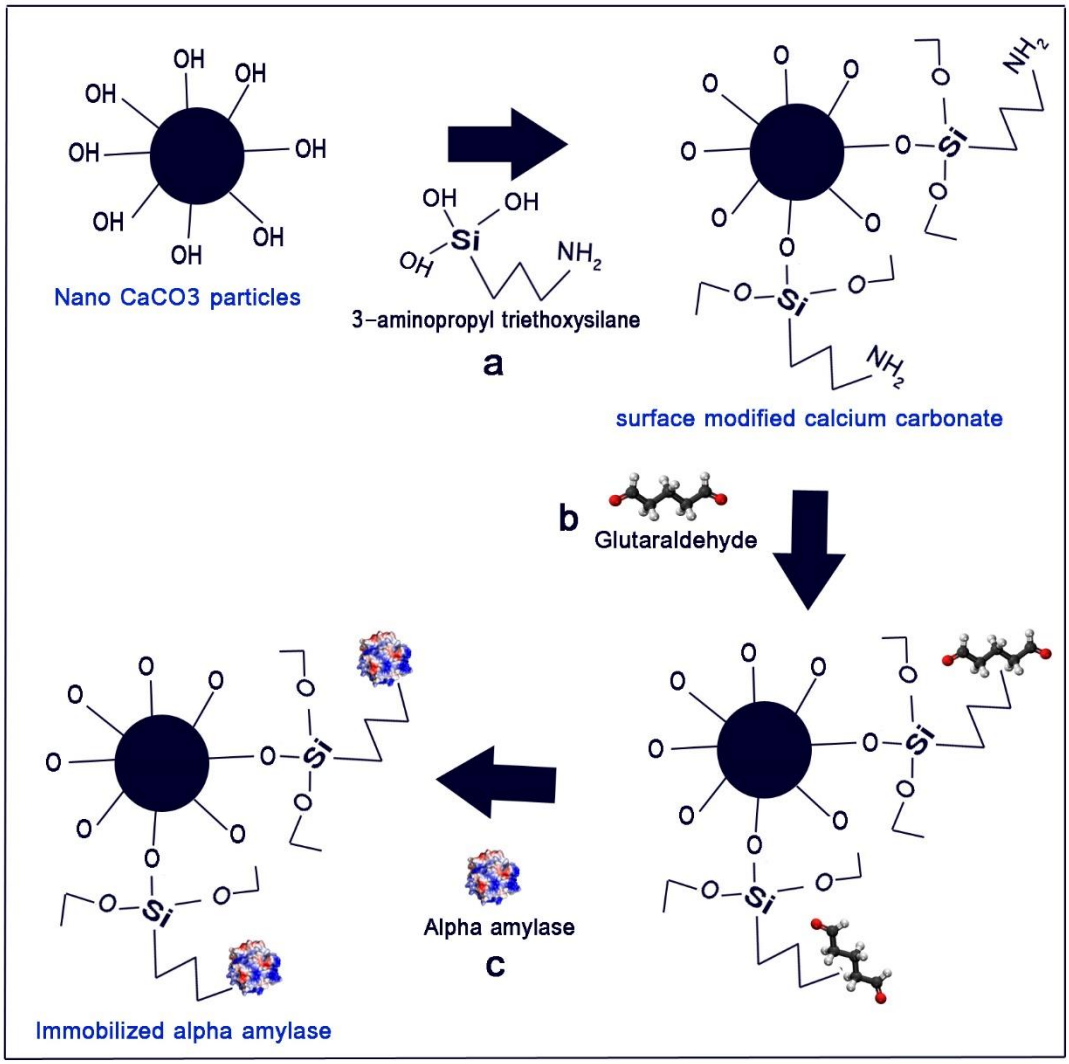

Figure 5: Procedure involved in immobilization of alpha-amylase by covalent attachment method. (a) preparation of functional group on the surface of nano $CaCO_3$ particles; (b) Modified nano $CaCO_3$ particles preparation by glutaraldehyde; (c) covalent attachment of the enzyme reactive functional groups (–NH2) and the modified nano $CaCO_3$ particles.