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Co-immobilization of alpha-amylase, glucoamylase, and pullulanase by cross-linked enzyme aggregates approach for glucose syrup production from starch

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ABSTRACT

In this research, cross-linked enzyme aggregates of thermostable α-amylase (from Bacillus licheniformis), glucoamylase (from Aspergillus niger), and pullulanase (from Bacillus subtilis) were enriched by calcium and sodium ions, with BSA as a protein feeder. Initially, acetone, acetonitril, isopropanol, saturated ammonium sulfate, ethanol, and tert-butanol were used for enzyme aggregation. Among them, tert-butanol has the highest enzyme activity compared to compared to other precipitators. The optimum conditions of the immobilization process for the three enzymes and CLEAs formation were as follows: glutaraldehyde concentration of 5mM, enzyme mixture ratios (α-amylase: glucoamylase: pullulanase) of 3:1:1, Enzyme/BSA ratio 1:2, and crosslinking time 2.5 h at 2-3° C. The optimum temperature and pH for free α-amylase were 95° C, and pH 5.5, while for glucoamylase and pullulanse, they were 60-62° C, pH 5.5, respectively. The CLEAs formed using tert-butanol and a 1:2 enzyme/BSA ratio exhibited an optimum temperature of 60–62 °C and an optimum pH of 5.5. Kinetic analysis of the combi-CLEAs compared to the free enzyme mixture revealed a decreased Km, an increased Vmax, and enhanced catalytic efficiency. Moreover, the resulting multi-CLEAs retained approximately 74% of their initial activity after 10 reuse cycles. Besides, the thermal stability and half-life of the immobilized enzymes were enhanced by approximately threefold compared to the free enzymes. This increase in activity was attributed to the addition of Ca^{2+}/Na^{+} ions in a 3:1 ratio to the enzyme mixture during CLEA formation. The incorporation of these ions enhanced thermostability, functional stability, and enzyme half-life. Accordingly, the combi-CLEAs of the three amylases can be introduced as an efficient, eco-friendly, and cost-effective biocatalyst with ease of operation, offering potential for a novel integrated process design in glucose syrup production from starch.

1. Introduction

The industrial hydrolysis of starch involves several steps and the use of various enzymes including, α-amylase, glucoamylase, and pullulanase. Among the enzymes, only glucoamylase is used in immobilized form. While numerous studies have investigated the individual immobilization of starch-hydrolyzing enzymes, there are relatively few reports on the immobilization of multi-enzyme systems [1]. The development of a co-immobilized enzyme system can enhance the efficiency of the starch hydrolysis process by reducing the number of processing steps, thereby lowering both enzyme usage and overall production costs. Moreover, immobilization allows enzyme reuse and facilitates easy separation of the enzyme from starch hydrolysis products, resulting in savings on enzyme consumption, labor, and downstream processing costs [2]. The co-immobilization of enzymes may reduce the processing steps such as pH adjustment and ion exchange, potentially lowering the cost of starch hydrolysis. In this process, α-amylase first degrades starch by breaking the α-(1→4)-D-glycosidic bonds. The resulting smaller polysaccharides are then further hydrolyzed by glucoamylase to produce glucose. Distinct enzyme immobilization approaches can be selected based on enzyme changes. Physical approaches—namely entrapment, adsorption, and encapsulation—do not cause structural changes in enzymes. In contrast, chemical methods such as covalent attachment to a support, cross-linking of native enzymes, crystallized enzymes (CLECs), or physically aggregated enzyme molecules (CLEAs) may alter the protein configuration [3]. The cross-linked enzyme aggregates (CLEAs) method is

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both straightforward and efficient. In CLEAs fabrication, enzyme molecules are aggregated by modifying the dielectric constant using organic solvents, salts, or non-ionic polymers. Cross-linking primarily through the reaction between accessible lysine residues—mostly located on the enzyme surface—and the aldehyde groups of cross-linkers. This interaction typically leads to the formation of aldol condensations or Schiff base linkages. Among the main advantages of this technique over free enzymes are the ability to combine two or more enzymes in a single CLEA, enhanced structural and thermal stability due to rigidification of the tertiary structure, and the elimination of expensive carrier materials [4]. The main drawbacks of CLEAs at the industrial scale include reduced catalytic performance and limited mass transfer, which result from the formation of bulky, non-uniform particles that cause diffusional limitations. In addition, the fine particles of the CLEAs may not be retrieved during separation procedures like filtration. Consequently, controlling particle size is essential to produce uniform CLEAs and to mitigate the aforementioned drawbacks [5]. Many strategies have been recommended to dissolve diffusion challenges by improving CLEAs configuration and function. Utilizing poly-lysine and polyamine compounds and feeder proteins (e.g., soy protein isolates and bovine serum albumin (BSA)) has a favorable effect on the functional robustness of the CLEAs. Also, amino-functionalized Fe_3O_4 is widely used for magnetic immobilization of enzymes owing to the positive effect on the operational stability, specific performance, and reusability. Additionally, it is non-toxic, environmentally friendly, offers a high surface area-to-volume ratio, and allows for easy enzyme separation using an external magnetic field [6]. Co-immobilization of the enzymes within a single CLEA, also known as combi-CLEA formation, is an efficient strategy to reduce industrial production costs by minimizing processing steps and unit operations. This approach is a one-pot procedure that offers higher yield and generates less waste, while requiring less solvent and shorter processing times. It also increments overall effectiveness by minimizing the accumulation of toxic or unstable intermediates. Nevertheless, some drawbacks have also been reported. The performance and stability of the co-immobilized enzymes can be influenced by various environmental factors such as temperature, pH, and ionic strength [7]. Many research has been conducted using CLEAs for diverse enzymes. Torabizadeh et al. reported the immobilization of inulinase [8-9], Naringinase [10-11], α-amylase [12], α-amylase and maltogenic amylase [7] by the functionalized NM-CLEAs approach. Xie et al. immobilized lipase and α-amylase onto magnetic carriers, reporting substantial improvements in the enzymes' thermal stability, pH tolerance, and storage durability [13]. Blanco-L. Lamero et al. utilized a combi-CLEAs strategy incorporating Celluclast, Alcalase, and Viscozyme to catalyze the enzymatic degradation of the *Nannochloropsis gaditana* microalgal cell wall in a one-pot system [14]. Cruz-Izquierdo et al. reported the immobilization of lipase using magnetic CLEAs for the synthesis of biodiesel [15]. The present research fabricated combined CLEAs of carbohydrases, including α-amylase, glucoamylase, and pullulanase. For this purpose, all three enzymes were simultaneously aggregated in tert-butanol in the presence of a 3:1 ratio of calcium to sodium ions. Subsequently, glutaraldehyde was added as a cross-linker to form Schiff base bonds between the ε-amino (Ɛ-NH_2) groups of lysine residues—located on the enzyme surface—and the aldehyde groups of glutaraldehyde [16]. The novel biocatalyst demonstrated improvements in thermo robustness, enzyme half-life, reusability, and kinetic specifications compared to free mixed enzymes. As a consequence, based on our findings, this novel reusable biocatalyst is proposed for the simultaneous conversion of starch to glucose syrup in a single-step process under mild conditions, representing a desirable green technology for glucose syrup production.

2. Material and methods

2.1. Materials

Thermostable α-amylase (Liquozyme Supra) from *Bacillus licheniformis*, glucoamylase (Dextrozyme GA) from *Aspergillus niger,* and pullulanase from *Bacillus subtilis* were purchased from Novozymes (Bagsvaerd, Denmark). Native corn starch was dedicated by Arian Glucose company (Iran), Glutaraldehyde (25% aqueous solution), sodium potassium tartrate, 3,5-dinitro salicylic acid (DNS), D (+) glucose, potassium-sodium tartrate, sodium acetate, disodium hydrogen phosphate, sodium dihydrogen phosphate, phenol, sodium hydroxide, ammonium sulphate, acetone, acetonitrile, tert-butanol, isopropanol, and ethanol, were purchased from Merck. BSA fraction V (MW: 66,338 Da) was prepared by Fluka company. Besides, Coomassie Brilliant Blue (G-250) was supplied by GE Healthcare (Uppsala, Sweden). All other chemicals were purchased by Merck (Darmstadt, Germany).

The surface characterization was performed using a Tescan Mira II (USA) field-emission scanning electron microscope (FE-SEM) operated at 15.00 kV.

Prior to imaging, the samples were coated with a thin layer of gold via magnetron sputtering to enhance surface conductivity and image quality. UV-Vis spectroscopy was conducted using a Perkin Elmer Lambda 25 UV/VIS spectrophotometer (USA) equipped with 1 cm path length cuvettes, calibrated against a blank. All reported values represent the mean ± standard deviation of three independent replicates.

2.2. Free and immobilized enzyme performance assay

Enzyme activity assays were performed individually and in combination for the three enzymes—thermostable α-amylase, glucoamylase, and pullulanase—using the DNS method. First, a glucose standard curve (0.1–1 mg/mL) was prepared to determine product concentration based on the linear regression equation. Thereafter, the performance of enzymes was assessed by DNS (3,5-dinitrosalicylic acid) approach using starch (1.0% w/v) as substrate at pH 5.5, and temperature 60-62° C, for 0-120 min plus 10 microliters of calcium chloride (15 ppm), in sodium acetate buffer 50 mM. At intervals of 0-120 min, the reaction solution was sampled and at each sampling time, 500 µl of the reaction solution was mixed with 500 µl of DNS reagent and was heated at 85-90° C for enzyme inactivation and reaction of glucose with DNS reagent. The solution was then cooled and 167 µl of 40% potassium-sodium tartrate solution was added to it. After that, the absorbance of the released sugar was measured using a spectrophotometer at 575 nm, and the control sample contained the entire reaction mixture except for the enzyme. One international unit (IU) is the amount of enzyme that releases one µmol of glucose equivalent sugar per min under the test conditions [17].

2.3. Protein concentration estimation by Bradford approach

The Bradford micro assay at 595 nm obtained the amount of enzyme protein using a BSA[1] calibration curve. The current investigation repeated all the experiments thrice to ensure reproducibility. The standard solution of BSA 0.1-1 mg/ml concentrations was used. The samples' absorbance was measured by spectrophotometric approach at a wavelength of 595 nm [18].

2.4. Accessible surface area assessment of the enzymes by bioinformatic tools

Determining molecular weight of the enzymes is essential to estimate the kinetic and thermodynamic parameters. To achieve this, the Expasy data files for each enzyme were retrieved from the expasy website and UniProtKB, which provides detailed enzyme specifications. Bioinformatics data in UniProtKB implied that, Thermostable α-amylase from *Bacillus licheniformis*, with L0CM04 · L0CM04_BACLI code contains 512 amino acids with a molecular weight of 58,530 Da. Glucoamylase from *Aspergillus niger,* with P69328 · AMYG_ASPNG code has 640 amino acids with a molecular weight of 68,309 Da, and pullulanase from *Bacillus subtilis* with C0SPA0·PULA_BACSU code has 718 amino acids with a molecular weight of 81,077 Da.

Table 1. Molecular Weight estimation of α-amylase, Glucoamylase, and Pullulanase

Enzymes	Molecular Weight (Da)	Total amino acids
α-amylase	58,530	512
Glucoamylase	68,309	640
Pullulanase	81,077	718
Total	207,916	1870

All data was extracted from Expasy tools (Swiss Bioinformatics Resource Portal) that is located at UniProtKB section.

Estimating the solvent-accessible lysine residues on the surface of α-amylase, glucoamylase, and pullulanase is essential for determining how many lysine residues can react with glutaraldehyde during cross-linking. To achieve this, the PDB data files for each enzyme were retrieved from the Protein Data Bank (PDB) and analyzed using the GETAREA bioinformatics tool [19]. This program calculated the solvent-accessible surface areas using a water probe radius of 1.4 Å. Based on crystallography data, the PDB-format file of thermostable α-amylase from *Bacillus licheniformis* (PDB code: 1BLI; length: 512 amino acids; mass: 58,530 Da) was obtained. The structure, resolved at a 1.59 Å resolution in the monoclinic form, was deposited in the Protein Data Bank using the X-ray diffraction method. Moreover, the crystal configuration of glucoamylase from *Aspergillus niger* (PDB code: 2E8Y; length: 640 amino acids; mass: 68,309 Da) was resolved at a resolution of 2.60 Å in the monoclinic forms. Also, pullulanase from *Bacillus subtilis* (PDB code: 3EQA; length: 718 amino acids; mass: 81,077 Da) was resolved at a resolution of 1.61 Å in the monoclinic forms and deposited in the Protein Data Bank using X-RAY diffraction method.

The findings are exhibited in Table 2. GETAREA analysis indicated that 7 out of 28 lysine residues in α-amylase, 1 out of 7 in glucoamylase, and 17 out of 40 in pullulanase are accessible on the surface of combined enzymes. This analysis revealed that a total of 25 lysine residues are accessible on the enzyme

1. Bovine Serum Albumin

surfaces, which may be insufficient for effective cross-linking and Schiff base formation with glutaraldehyde. Bovine serum albumin (BSA) is a carrier protein composed of a single polypeptide chain with a molecular weight of 69,293 Da and a total of 607 amino acids. It contains 59 lysine residues, of which 30–35 possess primary amine groups capable of reacting with cross-linkers such as glutaraldehyde to form Schiff base bonds. Therefore, BSA was incorporated as an additive to enhance accessible lysine residues on the surface of the combined enzymes and covalent binding during CLEAs fabrication [19].

Table 2. Accessible Surface Area estimation of lysine residues for α-amylase, glucoamylase, and pullulanase by GETAREA and Expasy bioinformatics softwares

Enzymes	PDB code	Amino acids	Mass (Da)	Total lysine	Accessible surface lysine
Thermostable α-amylase from *Bacillus licheniformis*	1BLI	512	58,530	28	7
glucoamylase from *Aspergillus niger*	2E8Y	640	68,309	7	1
pullulanase from *Bacillus subtilis*	3EQA	718	81,077	40	17
Total	-	1870	207,916	75	25

Bioinformatics data are extracted from UniProtKB that is located at the Expasy server (https://www.expasy.org, PDB data bank at https://www.rcsb.org, and Getarea at https://curie.utmb.edu/area_man respectively.

2.5. Specifying the best enzyme mixture ratio

For this reason, distinct proportions of Liquozyme Supra, Dextrozyme GA, and pullulanase were mixed, and the performance of the enzyme mixture (100 µl of enzymes) was assessed using the DNS method for carbohydrase performance, compared to free mixed enzymes. The enzyme ratio that leads to the peak amount of performance was selected.

2.6. Selection of the best enzyme aggregator

Aggregation was achieved by mixing 100 µl of the enzyme mixture (at the optimum ratio) with 900 µl of precipitant (1:9 ratio), including acetone, acetonitrile, ethanol, isopropanol, tert-butanol, or saturated ammonium sulfate. The resulting aggregate suspension was stored in a refrigerator for 1 h to allow proper formation and precipitation of the enzyme aggregates. Subsequently, the aggregated enzymes were separated from the solvents through centrifugation at 7547×g for 15 min. Finally, the activity of aggregates (4.13 mg enzyme aggregates/100 µl sodium acetate buffer pH, 5.5) was evaluated using the DNS methods [20].

2.7. Optimum pH and temperature of the enzymes

The optimum pH of both free and immobilized enzyme mixtures was determined by incubating the reaction mixtures across a pH range of 4–9 using specific buffer systems (50 mM sodium acetate buffer for pH 4–6 and 100 mM phosphate buffer for pH 7–9) at the optimum temperature. Enzyme activity was quantified for each pH value and expressed in units ($\mu mol \cdot min^{-1} \cdot mg^{-1}$). The effect of temperature on the activity of free and immobilized enzyme mixtures was evaluated by incubating 10 µl of enzyme mixtures with 100 µl of starch (0.1% w/v) as substrate at pH 5.5 together with 10 microliters of calcium chloride (15 ppm) in a sodium acetate buffer solution (pH, 5.5) without substrate at varying temperatures (30–95°C) for 90 min. Subsequently, enzyme activity was measured using the DNS method described earlier, with results expressed in $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for each specified temperature [10-11].

2.8. Optimum glutaraldehyde concentration and holding time

In biocatalyst formation, glutaraldehyde is chiefly utilized. The ε-NH_2 groups of accessible surface lysine residues on the enzyme surface play a critical role in cross-linking. The cross-linker concentration significantly influences CLEAs performance. At inappropriate glutaraldehyde concentrations, the catalytic effectiveness of the enzyme is diminished. Therefore, various concentrations of glutaraldehyde (0–100 mM) were tested with tert-butanol as an aggregator for CLEAs preparation under storage conditions for 1–18 h. The mixture was then centrifuged at 10,867×g for 10 min, and the supernatant was removed. An enzyme activity assay was subsequently conducted to determine the optimal glutaraldehyde concentration required for achieving stable cross-linking of the enzyme mixtures. To refine the cross-linking process, the effect of holding time on the activity of fabricated CLEAs was systematically evaluated at intervals ranging from 1 to 18 h at 2–4 °C [7-12].

2.9. Effect of BSA addition

The accessible surface area assessment of α-amylase, glucoamylase, and pullulanase configurations for lysine residues demonstrated that only 25 out of 75 total lysine residues are accessible on the surface of the mixed enzymes for reaction with aldehyde groups of glutaraldehyde. Lysine residues play a pivotal role in cross-linking; a reduction in their accessibility within the enzyme configuration can result in suboptimal CLEAs formation [21]. Besides, to enhance the protein configuration of the enzymes, reduce diffusion limitations, and improve cross-linking via the reactive Ɛ-NH_2 groups of lysine with glutaraldehyde, BSA was added in varying amounts to the enzyme complex. As noted earlier, BSA contains 59 lysine residues, of which 30–35 are surface-accessible and capable of reacting with cross-linkers such as glutaraldehyde for CLEAs formation. Accordingly, varying BSA concentrations (0.5–5 mg BSA per 1 mg of combined enzyme protein) were added to the enzyme mixtures in a tert-butanol environment (as the aggregator) together with glutaraldehyde (as the cross-linker), and then held at 2-3° C for 3 h. Afterwards, the activity assay was performed to select the ideal enzyme to BSA ratio.

2.10. Assessment of the optimal amount of calcium and sodium ions in CLEAs formation

Sodium chloride and calcium chloride solutions of 20,000 ppm were prepared as stocks. After dissolving bovine serum albumin in the enzyme, the calcium and sodium salt solutions were added to the BSA enriched enzymes in the range of 100 to 1,000 ppm for sodium and 100 to 2,000 ppm for calcium, followed by thorough vortexing. Thereafter, tert-butanol was added to the combined enzymes at a 1:9 ratio. After storing it at refrigerator temperature for 30 min, 5 mM glutaraldehyde was added to it and was hold in 2-3 °C overnight. The activity of the immobilized enzymes was then determined using the DNS method.

2.11. Thermal stability and enzyme half-life assessment

Thermal robustness of the free and immobilized enzymes was measured by incubating the combined enzymes in acetate buffer (without substrate) at 85-110 °C for 0-240 min. Then, the remaining performance was evaluated by DNS method for enzymes kept at mentioned temperature separately. Finally, the thermostability and enzyme half-life for incubated CLEAs, including those formed with calcium and sodium ions, were calculated relative to the free enzyme mixture by determining the Ln % remaining activity of the enzymes over time (0-240 min). One of the essential specifications in assessing enzyme robustness is the enzyme half-life (t½). The enzyme half-life is equal to the needed time for losing 50% of the initial performance of the enzyme [22]. To estimate the enzyme's half-life, the inactivation rate constant or k_{in} was initially specified based on the linear regression slope of Ln (%) remaining activity versus holding time at the constant temperature obtained from the aforementioned curves. After that, the half-life of the enzymes (t½) was estimated using Equation 2 [23-24]:

$$t_{1/2} = ln_2/k_{in} \quad (2)$$

2.12. Kinetic specifications of free and Combi-CLEAs

The kinetics of this enzymatic reaction follows the Michaelis-Menten law and can be described using the following Equation:

$$V = (V_{max}\,[s]) / (K_m + [s]) \quad (3)$$

In this step, distinct substrate concentrations (0.25, 0.5, 1, 3, 5 mg/ml) were prepared (starch as a substrate for DNS approach). Then, an enzyme activity assay was performed using DNS method at 575 nm. Afterwards, kinetic constants V_{max} and K_m of both free and immobilized enzymes were determined by using a Lineweaver-Burk plot, which was drawn based on 1/V (reversed initial velocity) against [1/S] (inversed of substrate concentration) (Equation 4) [7-12].

$$[s]/V_0 = 1/(V_{max}.\,[s]) + K_m/V_{max} \quad (4)$$

In this Equation, [S] represents the substrate concentration (starch), V_0 denotes the initial enzyme velocity, V_{max} refers to the maximum enzyme velocity, and K_m is the Michaelis constant, defined as the substrate concentration at which V_0 equals to 1/2 V_{max}. The assay mixture included 2.07 mg of protein from free and immobilized enzymes, with varying substrate concentrations (0.25–5 $mg \cdot ml^{-1}$) at pH 5.5, and temperature 60-62 °C, for 0-120 min [7-12].

2.13. Reusability of NM-Combi-CLEAs

The reusability of the immobilized enzymes was assessed by hydrolyzing a starch suspension in water as the substrate for 60 min at pH, 5.5 and 60 °C. After incubation, NM-CLEAs was retrieved from the reaction medium by centrifuging at 10,867×g for 10 min, washed three times with phosphate buffer (pH 7.0), and then reintroduced into a fresh substrate suspension. The procedure was repeated for ten cycles. The hydrolysate suspension was assessed using DNS method throughout all ten consecutive cycles [25].

2.14. Statistical approaches

All results are introduced as mean ± standard deviation (SD) from three samples analyzed individually in triplicate. Statistical analyses were performed using Design Expert software (version 13, USA). The one-way analysis of variance (ANOVA) was used to evaluate differences among datasets, followed by a t-test to assess significant differences between groups. A p value of <0.05 was remarked statistically significant.

3. Results and discussion

3.1. Determination of free enzyme performance and protein composition

The free α-amylase, glucoamylase, and pullulanase performance were evaluated separately by DNS approach. Optimal performance of free enzymes was obtained after 1 min of substrate hydrolysis at 60° C. The results of enzyme performance and protein assay are represented in Table 3.

Table 3. Enzyme activity and protein content of free and immobilized enzymes

Enzymes	Protein (mg/ml)	Activity (µmol. min^{-1})
Free α-amylase,	9.60 ± 0.12	0.68 ± 0.15
Free glucoamylase	85.16 ± 0.26	0.72 ± 0.08
Free pullulanase	4.77 ± 0.17	0.67 ± 0.13
Combi-CLEAs	41.33 ± 0.36	4.42 ± 0.06

Values are mean ± SD, calculated as activity (µmol. min^{-1}) and protein (mg.ml^{-1}) basis for enzyme samples, analyzed individually in triplicate.

3.2. Optimum ratio of free α-amylase, glucoamylase, and pullulanase in the combined enzymes

The findings of assessing 4 distinct ratios of free enzymes demonstrated that a ratio of 3:1:1 of free α-amylase: glucoamylase: pullulanase showed the best activity notable amounts (P<0.0001), 4.43 µmol.min^{-1} performance (Table. 4). Selecting a 3:1:1 enzyme ratio is more suitable and reliable for future pilot plant or industrial-scale studies.

Table 4. Optimal enzyme mixture ratio evaluation

Enzymes	Ratios	Activity (µmol. min^{-1})
10µL α-amylase + 10µL Glucoamylase + 10µL Pullulanase	1: 1: 1	3.82 ± 0.05
20µL α-amylase + 10µL Glucoamylase + 10µL Pullulanase	2: 1: 1	4.16 ± 0.13
30µL α-amylase + 10µL Glucoamylase + 10µL Pullulanase	3: 1: 1	4.43 ± 0.07
40µL α-amylase + 10µL Glucoamylase + 10µL Pullulanase	4: 1: 1	4.26 ± 0.09

Values are mean ± SD, calculated as activity (µmol. min^{-1}) for enzyme samples, analyzed individually in triplicate.

3.3. Selection of the best precipitant

Enzymes with a protein configuration can be physically aggregated when surrounded by organic solvents, elevated ionic strength, or non-ionic polymers. This precipitation step is pivotal in forming CLEAs with appropriate robustness and performance. Hence, applying a proper precipitating agent with optimal holding time, pH, and temperature is crucial. [26]. Thus, distinct solvents and precipitants were assessed, and an activity assay was carried out on the aggregates prior to cross-linking. The findings are specified in Fig. 1. As depicted, tert-butanol was the best solvent to preserve the peak performance of the starch hydrolysis relative to other solvents (P< 0.0001), which revealed an activity of about 4.45 µmol. min^{-1}. The enzyme aggregates that were formed in the saturated ammonium sulfate showed the lowest performance, about 1.8 times lower than that observed with tert-butanol.

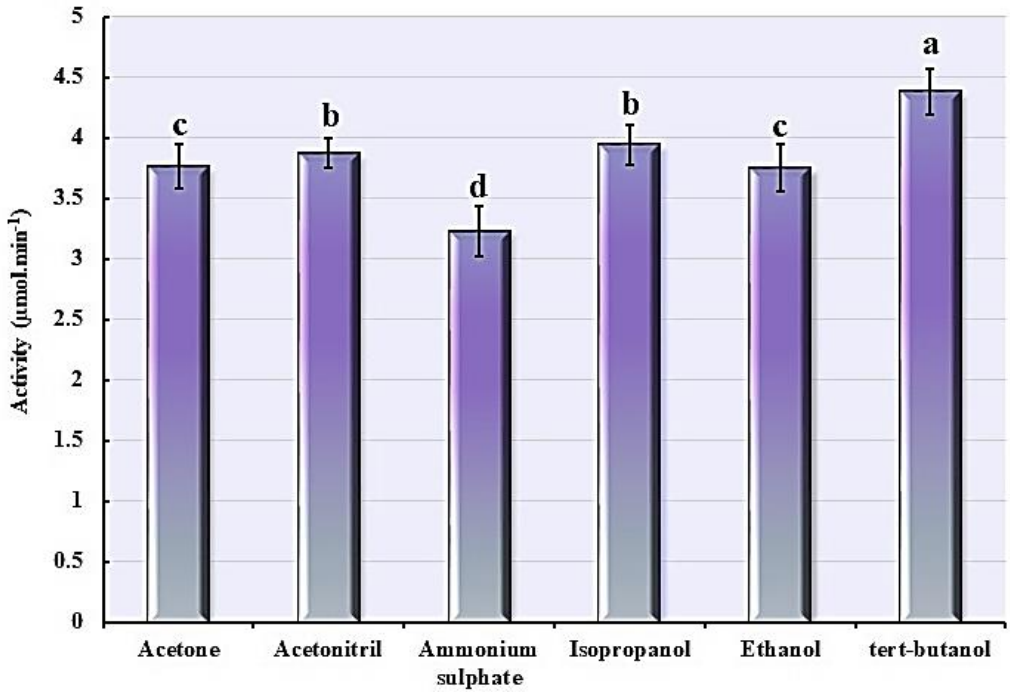
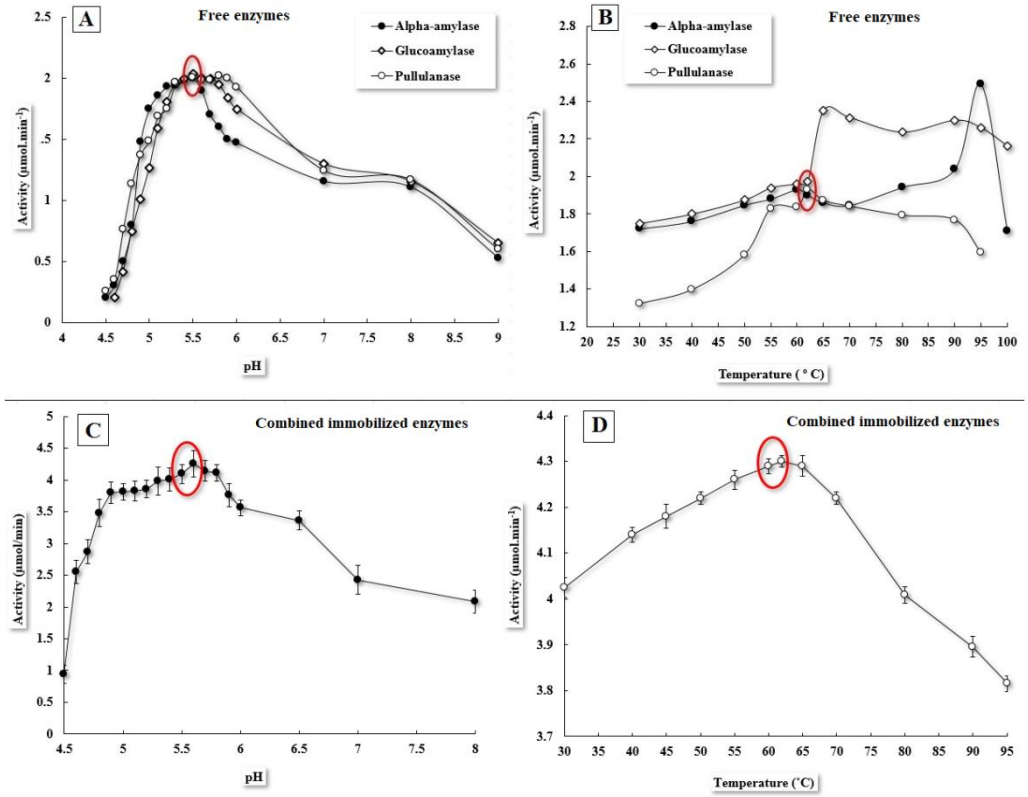


Fig 1. The best enzyme aggregators for aggregates formation
The same superscript letters are not significantly different (P > 0.05). Values are mean ± SD, analyzed individually in triplicate.

This phenomenon can be attributed to the disparity in dielectric constants between water and tert-butanol. In this procedure, the surface of the enzyme configuration surrounded by the water molecules with an elevated dielectric constant (ε = 78.3) is gradually replaced by tert-butanol molecules with a lower dielectric constant (ε = 10.90). Consequently, the reduction of the hydration layer around the enzyme molecules promotes aggregation through electrostatic attractions and dipole–dipole interactions. Therefore, tert-butanol was chosen as a suitable aggregating agent for Combi-CLEAs synthesis in subsequent experiments [26].

3.4. Optimum pH and temperature of free and immobilized enzymes

The effect of various pH (4.5-9) on the relative performance of the free and immobilized enzymes was assessed (Fig. 2). As shown in Fig. 2A, all three enzymes exhibit an intersection point at pH 5.5. The ideal pH of the combined enzymes was equal to pH 5.5 with a combined activity of 4.26 µmol. min^{-1}, showing a notable difference (P<0.0001). Furthermore, a temperature range of 30–95 °C was tested to evaluate the effect of temperature on the performance of the free and immobilized enzymes (Fig. 2B). The effect of temperature on the performance of free enzymes is shown in Fig. 2B. The results demonstrated that the optimum temperature for both free enzymes and combined CLEAs was 60 °C. Elevated temperatures can alter the protein conformation of enzymes, leading to a decline in their activity. On the other hand, lower temperatures may provide an appropriate environment for microbial contamination during the experiments. The optimal pH and temperature of the immobilized enzymes showed no substantial difference compared with those of the free enzymes (Fig. 2C and Fig. 2D).



(Fig 2A and B). **(A)**, optimal pH, **(B)**, optimum temperature assessment of free enzymes.
(Fig 2C and D). **(C)**, optimal pH, **(D)**, optimum temperature assessment of combi-CLEAs. All values are expressed as mean± standard deviation for triplicate experiments.
Fig 2. Optimal pH and temperature for free mixed enzymes and combi-CLEAs activity

3.5. The cross-linker amount and holding time

Glutaraldehyde concentration plays a critical role in CLEAs formation, influencing the catalytic efficiency and performance of immobilized enzymes. Its effect was evaluated by examining CLEAs performance at glutaraldehyde concentrations of 1, 2, 3, 5, 10, 15, 20, 30, 40, 50, and 100 mM. As shown in Fig. 3, the highest enzyme activity, approximately 1.40 µmol min^{-1}, was achieved at 5 mM glutaraldehyde. Combi-CLEAs prepared with concentrations below 5 mM exhibited unstable and fragile structures due to insufficient cross-linking, resulting in reduced performance (Fig. 3A). On the contrary, using glutaraldehyde concentrations above 5 mM reduced the flexibility of the enzyme structure and restricted mass transfer, leading to a decline in enzyme performance [10]. Moreover, an activity assessment was conducted to determine the optimal retention time for formation of CLEAs with stable and proper configurations. After incubating the enzyme cocktail for various time intervals (0, 0.25, 0.5, 0.75, 1, 2, 2.5, 3, and 18 hours), the highest enzyme activity—4.45 µmol min^{-1}—was observed at 2.5 hours, showing a significant difference ($P<0.0001$). The performance dropped dramatically before and after the mentioned time (Fig. 3B).

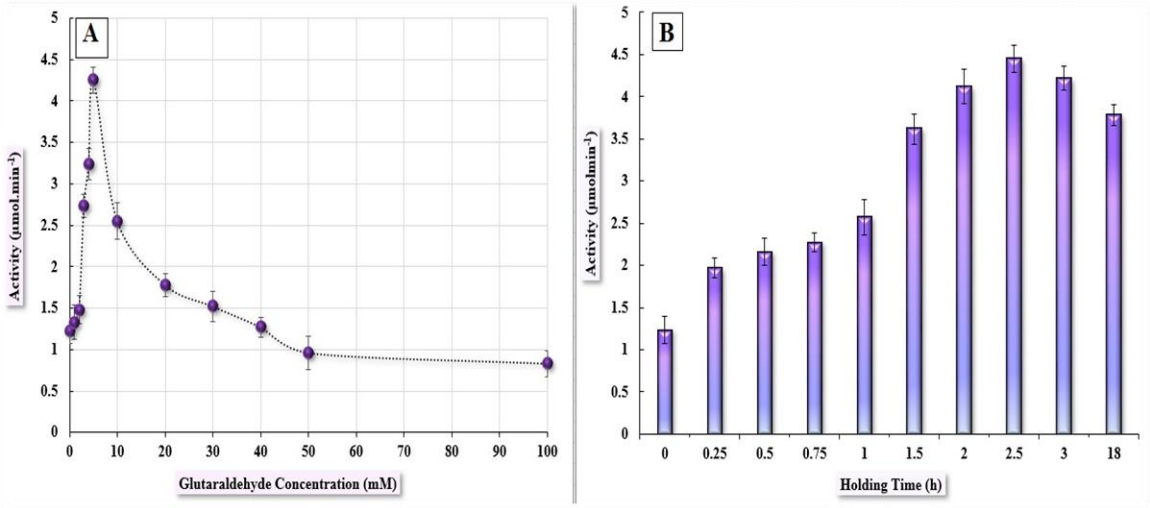


Fig 3. Assessment of the optimum glutaraldehyde concentration (mM) and the best holding time for CLEAs formation.
All values are expressed as mean± standard deviation for triplicate experiments. Optimal glutaraldehyde concentration (mM) and holding time (min). P value < 0.0001.

3.6. Optimum BSA concentration

As shown in Table 2, the accessible lysine residues (reactive Ɛ-NH_2 groups) on the surface of combined enzymes that can incorporate with aldehyde groups of cross-linker during cross-linking process, seems to be insufficient. Based on the bioinformatics analysis of accessible surface area assessment, 25 of total 75 lysine residues are accessible on the enzyme surfaces. Thus, the addition of bovine serum albumin (BSA) as a proteic feeder with 30-35 lysine residues can facilitate Schiff-base bonds formation during the cross-linking reaction. Comparative analysis of the optimal ratios of combined enzymes to BSA revealed that a 1:2 of enzymes/ BSA ratio yielded the highest enzyme activity in the prepared CLEAs. At ratios higher than 1:2, excessive cross-linking occurs, which can increase the rigidity of the enzyme structure and hinder substrate binding at the enzyme's active site. Therefore, this optimal enzyme/BSA ratio was applied in subsequent analyses (Fig. 4).

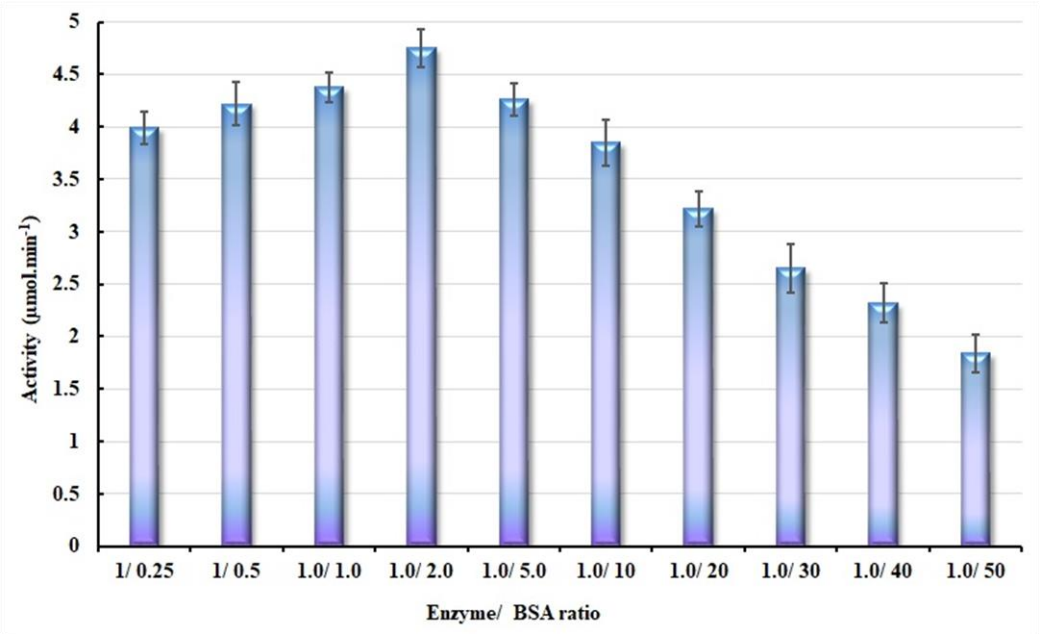


Fig 4. The optimal enzyme/BSA ratio for CLEAs fabrication
All values are expressed as mean± standard deviation for triplicate experiments

3.7. Morphology of fabricated CLEAs

The morphology and size of the synthesized combi-CLEAs was evaluated using FE-SEM (Fig. 5). The image of the free combined enzymes revealed circular droplet-like structures (Fig. 5A). Aggregated enzyme mixtures in tert-butanol displayed string-shaped configuration about 1.25 µm in mean diameter (Fig. 5B). After cross-linking with glutaraldehyde, these string-shaped aggregates transformed into star-like, branched, and more uniform configurations, with an average diameter of 3.14 µm (Fig. 5C). The fabricated Combi-CLEAs, composed of α-amylase, glucoamylase, and pullulanase, exhibited spherical to semi-spherical shapes ranging from approximately 80 nm to 2.5 µm in diameter, interconnected by filamentous structures. These string-like configurations between spherical particles can be attributed to stronger covalent bonds formed after adding glutaraldehyde at the optimal concentration (Fig. 5D).

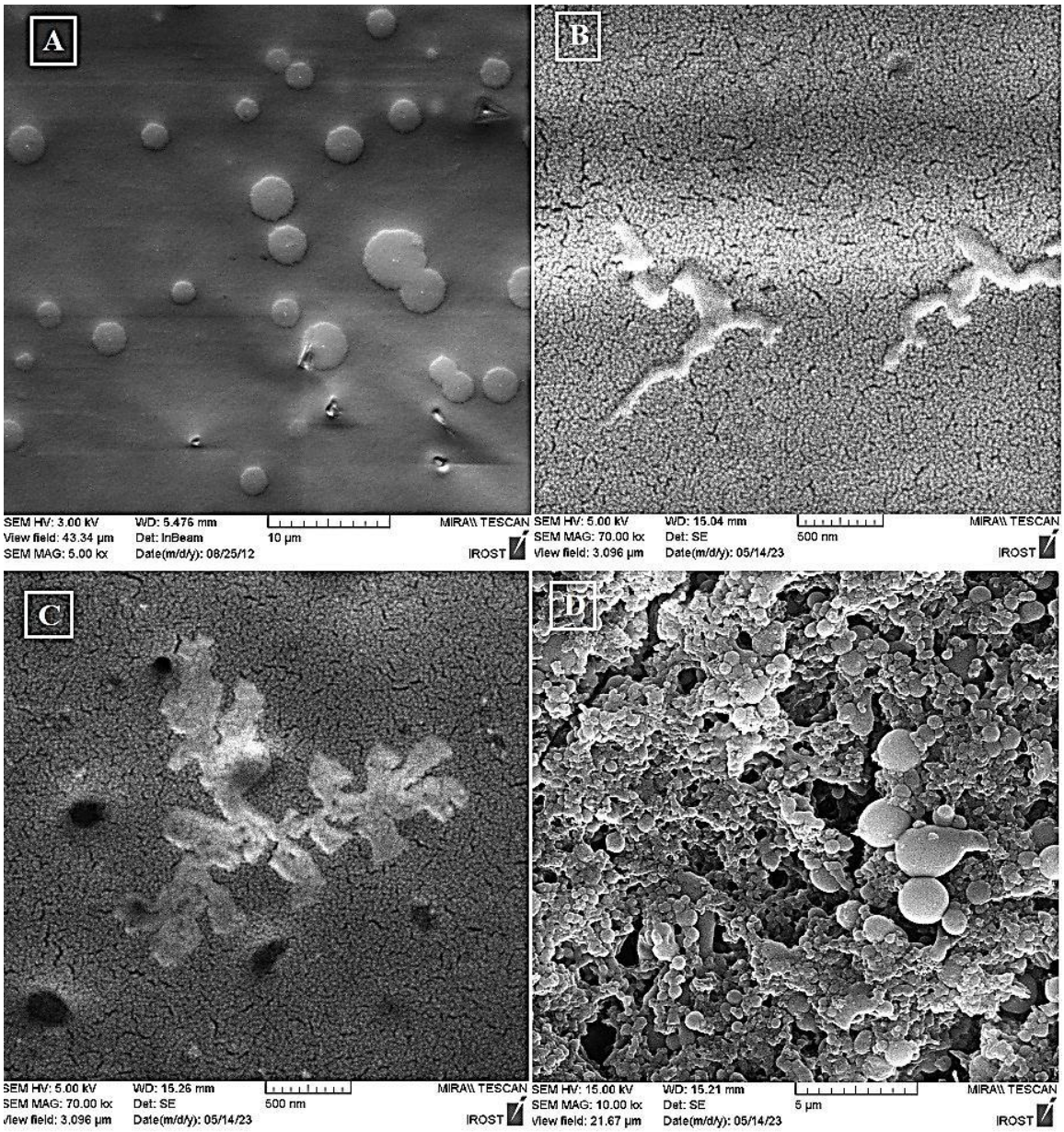


Fig 5. Scanning electron microscopy images of **(A)** free mixed enzymes, **(B)** enzyme aggregates, **(C)** cross-linked enzyme aggregates, and **(D)** final combi-CLEAs

3.8. Thermostability and enzyme half-life

Owing to the fact that, the thermal stability of the enzymes is a crucial agent in the fabrication, functional stability, and applications of enzymes, the thermal robustness of the free enzyme and combi-CLEAs were assessed at 85-110º C for up to 120 min. The inactivation rate constants (k_{in}) of carbohydrases were determined by using the DNS method (Fig. 6). The inactivation rate constant (k_{in}) at the specified temperatures was determined by plotting the Ln% of the remaining activity versus hydrolysis time in min. In this plot, the slope of the trendline represents k_{in}. Stability and maintenance of enzyme activity against temperature increases during the enzymatic process are among their most significant advantages. The findings of thermal robustness experiment at 85-110 °C is illustrated in Fig. 6.

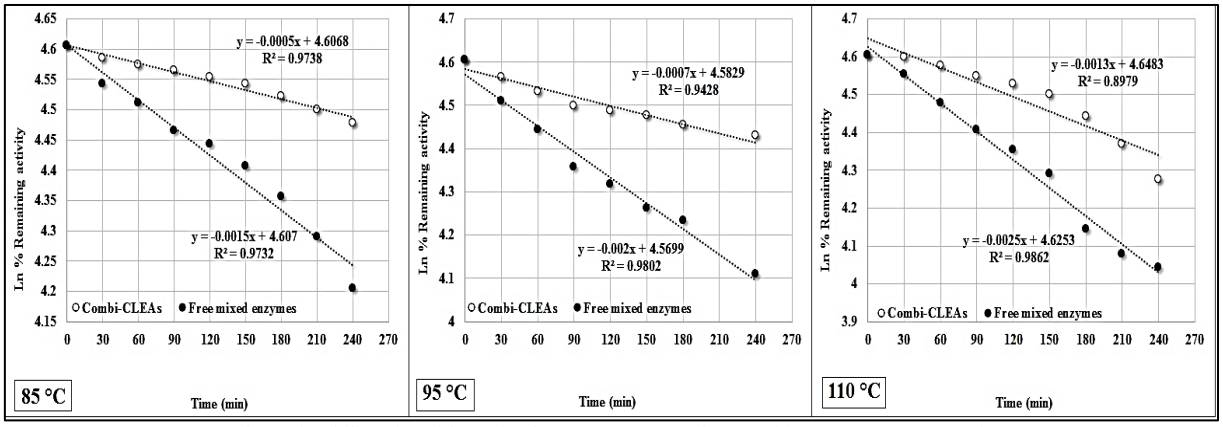


Fig 6. Thermal stability plot of free mixed enzymes compared to Combi-CLEAs at 85, 95, and 110° C

The optimal temperature for the free enzyme was identified as 60°C. Beyond this temperature, the activity of the free enzyme mixture declined, whereas the Combi-CLEAs maintained higher carbohydrase activity. Previous studies reported that immobilized enzymes exhibit greater thermal stability than free enzymes [27]. This enhanced stability is attributed to the rigid structure formed by intermolecular and intramolecular covalent cross-links, which restricts the conformational mobility of the enzyme molecules. Such thermal robustness is a significant advantage for the practical applications of immobilized enzymes. In this regard, the findings displayed 2.4-3 folds increment in the thermal stability of the immobilized mixed enzymes exhibiting carbohydrase activity at 85-110° C (Table 5).

Table 5. Kinetic parameters of free mixed enzymes and Combi-CLEAs based on DNS method.

	V_{max} (M.S^{-1})	K_m (Mol)	k_{cat} (S^{-1})	k_{cat}/ K_m (M^{-1}S^{-1})
FM-Enzs	2.62×10^{-8}	5.10×10^{-6}	1.32	0.026×10^{7}
Combi-CLEAs	2.80×10^{-8}	4.16×10^{-6}	1.41	0.034×10^{7}

Values are mean ± SD, analyzed individually in triplicate. Abbreviation means: FM-Enzs: Free Mixed Enzymes., Combi-CLEAs: Combined-Cross-linked Enzyme Aggregates.

The results exhibited an improvement in combi-CLEAs thermal robustness relative to the free mixed enzymes, indicating that the combined CLEAs of α-amylase, glucoamylase, and pullulanase were more thermostable than the free combined enzymes [27].

3.9. Kinetic specifications of the free and Combi-CLEAs

Kinetic assessments of free and immobilized enzymes using the DNS method revealed that the Km for the combined free enzymes decreased by approximately 1.2-fold, from 5.10×10^{-6} to 4.16×10^{-6} M, in the Combi-CLEAs.

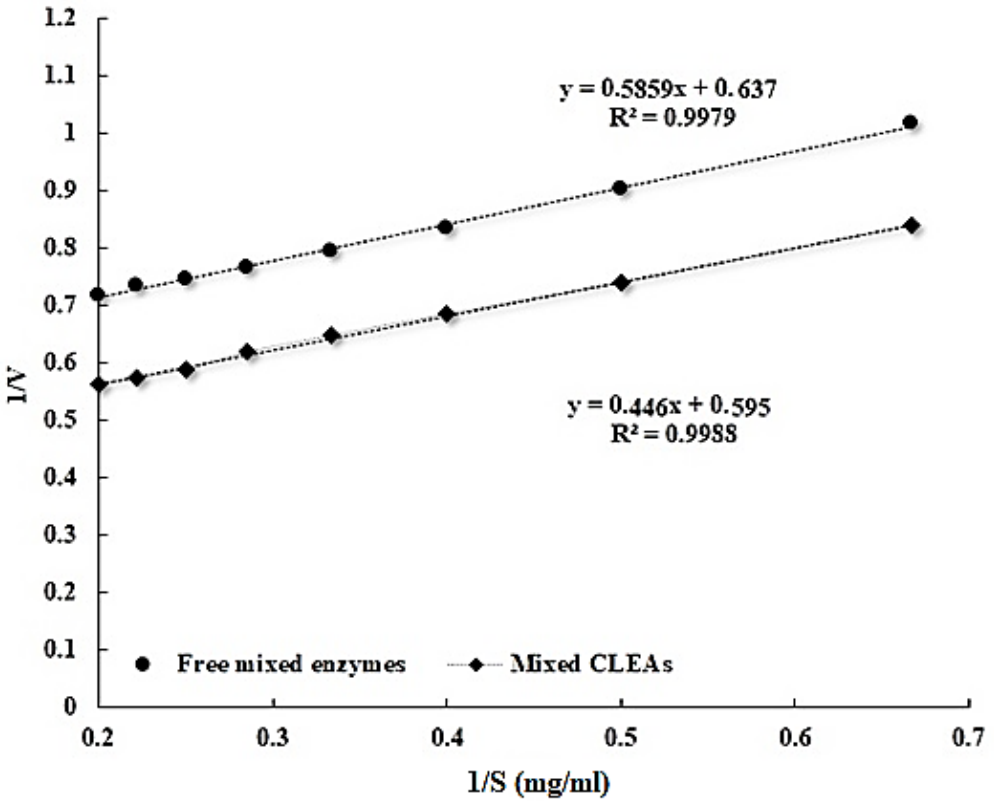


Fig 7. Lineweaver-Burk plot of free mixed enzymes compared to combi-CLEAs

At the same time, the V_{max} showed no significant change (from 2.62×10^{8} to 2.80×10^{-8} $M.S^{-1}$) after immobilization based on the DNS method. Besides, as it is exhibited, the positive effects of immobilization on Km value lead to an increment in catalytic efficiency (kcat/Km) of immobilized enzymes about 1.3 folds relative to the free enzyme. As the catalytic effectiveness of the enzymes improves, their rate constant is enhanced. In this case, the findings implied that catalytic efficiency of the free combined enzymes improved from 0.026×10^{7} to 0.034×10^{7} M^{-1} s^{-1} following immobilization as Combi-CLEAs (Table 6).

Table 6. Inactivation rate constant (kin) of free mixed enzymes and combi-CLEAs at 85-110 °C

	Kin		
	85 °C	95 °C	110 °C
Free mixed enzymes	0.0015	0.002	0.0025
Combi-CLEAs	0.0005	0.0007	0.0013

Table 7. Enzyme half-life of free mixed enzymes and combi-CLEAs

	$t_{1/2}$ (min)		
	85 °C	95 °C	110 °C
Free mixed enzymes	462 ± 1.28	346 ± 3.15	277 ± 2.12
Combi-CLEAs	1386 ± 2.23	990 ± 2.37	533 ± 3.61

Values are mean ± SD, analyzed individually in triplicate. Abbreviation means: Combi-CLEAs: Combined-Cross-linked Enzyme Aggregates.

Hence, it can be inferred that the immobilized enzymes' catalytic effectiveness and substrate specificity are ameliorated [28]. This can be explained by the increment in the affinity of Combi-CLEAs for substrate binding. This means less substrate is needed to saturate the enzyme, and the enzyme-substrate's liberation rate decelerates. It concluded that enzyme-substrate interaction is reinforced and facilitated, which can reduce mass transfer limitation and promotes the formation of an optimal enzyme configuration following the glutaraldehyde cross-linking process [29].

3.10. Optimal calcium and sodium ions

The addition of calcium and sodium ions during the aggregation process was assessed and results implied that the simultaneous addition of 1200 ppm calcium and 400 ppm sodium during CLEAs formation can enhance the thermal stability and catalytic activity of the combi-CLEAs (Fig. 8).

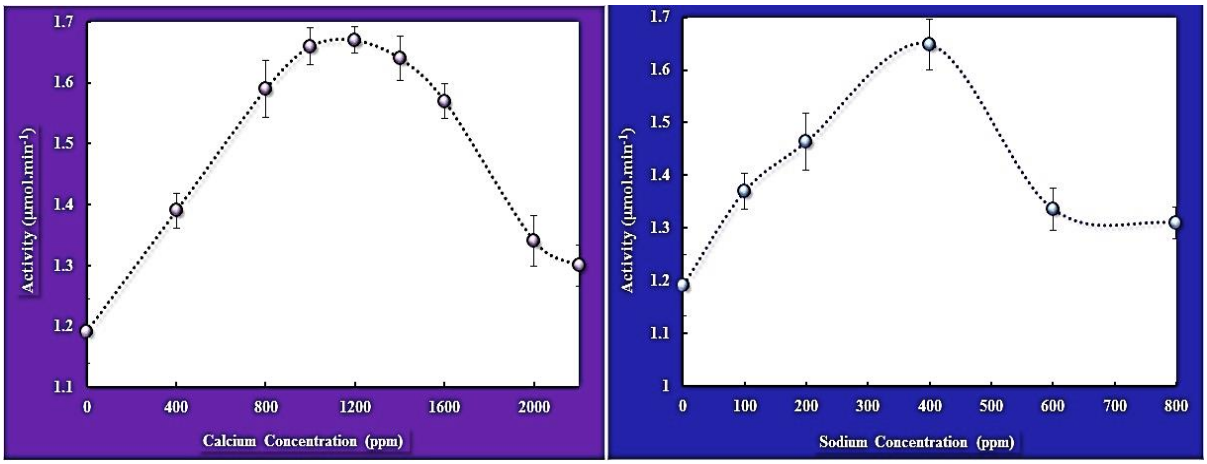


Fig 8. The optimal concentration of calcium and sodium ions for providing higher enzyme activity
All values are expressed as mean± standard deviation for triplicate experiments

The results indicated that CLEAs synthesized in the presence of cations mixture (Ca^{2+} 1200 ppm and Na^{+} 400 ppm) showed a 1.5 fold increase in activity compared to CLEAs produced without the mixed-ion addition (Fig 9). Most known α-amylases possess a conserved calcium ion located between domains A and B, which appears to be essential for maintaining the conformational and functional stability of the enzyme. Moreover, in various amylase structures, more than one calcium ion has been observed; For example, a linear Ca–Na–Ca arrangement is established in *B. licheniformis* α-amylase [30]. Interestingly, our findings revealed that the optimal Ca^{2+} to Na^{+} ratio for amylase CLEAs formation matches exactly the 3:1 ratio found in the molecular structure of *B. licheniformis* α-amylase.

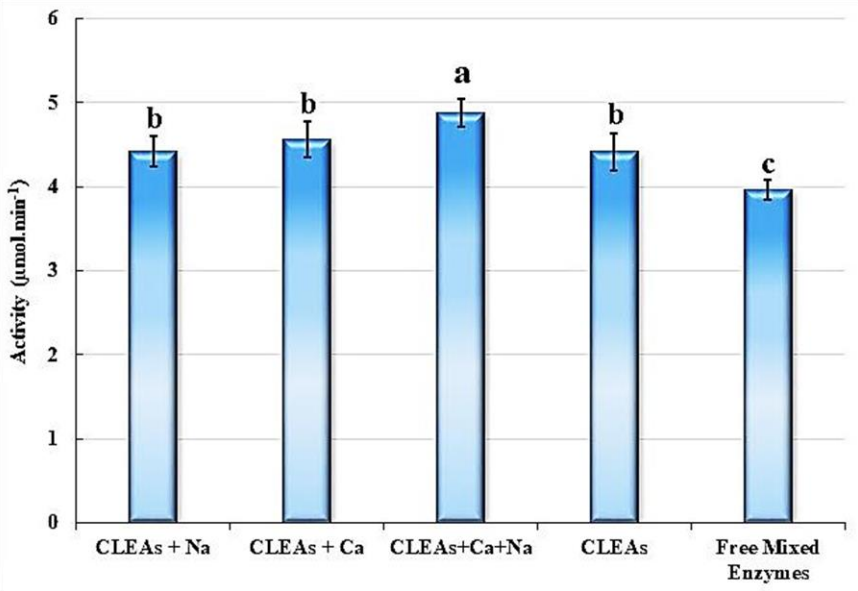


Fig 9. The effect of calcium and sodium ions separately and in combination with CLEAs compared to free mixed enzymes
The same superscript letters are not significantly different (P > 0.05). Values are mean ± SD, analyzed individually in triplicate.

3.11. Reusability of NM-Combi-CLEAs

Enzyme co-immobilization provides an attractive approach for repeated use of the same biocatalyst. In industrial applications, reusability is a key factor in determining the value and cost-effectiveness of enzymes. The primary aim of constructing combined CLEAs of α-amylase, glucoamylase, and pullulanase was to enable long-term use of the fabricated biocatalyst while maintaining enzymatic performance and facilitating its separation from the reaction medium. For this reason, Combi-CLEAs performance was estimated to be up to 10 cycles (Fig. 9). The findings indicated that the fabricated CLEAs retained 74% of their activity over 10 consecutive cycles, making the produced enzyme well-suited for industrial applications in continuous systems due to its strong operational stability and potential for reduced production costs. However, further research is required to achieve these objectives on an industrial scale [31-32].

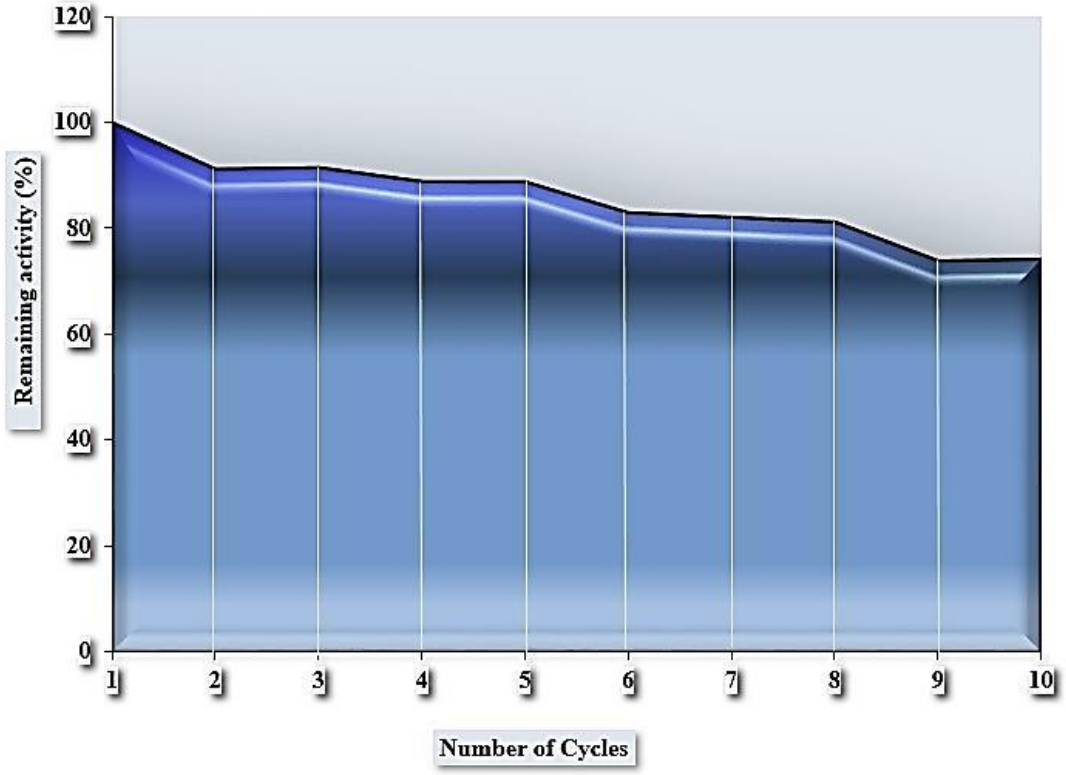


Fig 10. Reusability of the combi-CLEAs of α-amylase, glucoamylase, and pullulanase after ten cycles

4. Conclusion

Concurrent carrier-free co-immobilization of calcium- and sodium-enriched α-amylase, glucoamylase, and pullulanase was achieved for the conversion of starch to glucose syrup. The fabricated Combi-CLEAs exhibited superior catalytic activity and thermal stability compared to the free enzyme mixture. Among the six aggregators tested, tert-butanol produced the most effective precipitation of the enzyme mixture. The optimal conditions were a 3:1:1 ratio of α-amylase, glucoamylase, and pullulanase, enriched with a 1:2 enzyme-to-BSA ratio, 5 mM glutaraldehyde, and incubation at 2–3 °C overnight. The addition of Ca^{2+} and Na^{+} ions in a 3:1 ratio enhances enzyme structure and function. In a previous study, Talekar et al. (2013) prepared CLEAs from α-amylase, amyloglucosidase, and pullulanase using ammonium sulfate, reporting starch hydrolysis conversions of 100%, 60%, and 40% for Combi-CLEAs, a mixture of separate CLEAs, and a free enzyme mixture, respectively [1]. Kochane et al. (2020) compared maltogenase immobilization using various techniques, including poly(urethane-urea) (PUU) carriers and the CLEAs method, for converting wheat starch to maltose syrup. The results showed Km values of 22.46 mM for CLEA-maltogenase and 61.72 mM for maltogenase immobilized on PUU carriers. Hence, the CLEAs method was selected as the preferred technique for maltogenase immobilization [32]. Gupta et al. (2015) reported the immobilization of amyloglucosidase using the CLEAs

method for starch hydrolysis, achieving 90% residual activity after 25 reuse cycles [33].

The easy recovery and reuse of the biocatalyst offer an attractive and cost-effective approach for starch modification using carrier-free immobilized enzymes derived from inexpensive commercial enzyme preparations. Such multi-enzyme CLEAs are valuable preparations from both laboratory and industrial perspectives, suitable for various biotransformations in aqueous or non-aqueous media. The main impasses in this process are mass transfer limitation caused by the formation of large clusters and difficulties in filtration owing to nonuniform size of CLEAs particles. To overcome these disadvantages, applying a homogenizer after CLEAs formation can help achieve size uniformity. Fabricated combi-CLEAs can be applied in two arrangements; as a combined CLEAs in a submerged liquid medium or via attached to the surface of a solid medium in a continuous bioreactor. The main agents such as CLEAs particle size, enzyme concentration, holding time, and controlled pH and temperature are crucial for achieving a cost-effective process while producing high-quality glucose syrup. Based on the findings, the fabricated biocatalyst is introduced as an efficient, novel reusable, and thermostable biocatalyst for simultaneous conversion of starch to glucose syrup in a single bioreactor.

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Conflict of interest

The author declared no conflict of interest.

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فصلنامه فناوری‌های جدید در صنعت غذا

دوره ۱۲، شماره ۳، صفحه ۳۰۳-۲۸۷، بهار ۱۴۰۴

مقاله پژوهشی

تثبیت هم زمان آنزیم‌های آلفا آمیلاز، گلوکوآمیلاز و پلولاناز با استفاده از روش توده‌های تجمع یافته با اتصال جانبی برای تولید شربت گلوکز از نشاسته

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چکیده

در این تحقیق، تجمعات آنزیمی متقاطع آلفا-آمیلاز مقاوم به حرارت (از باسیلوس لیکنیفورمیس)، گلوکوآمیلاز (از آسپرژیلوس نیجر) و پولولاناز (از باسیلوس سوبتیلیس) که توسط یون‌های کلسیم و سدیم و BSA به عنوان تغذیه‌کننده پروتئینی غنی شده بودند، تهیه گردید. در ابتدا، استون، استونیتریل، ایزوپروپانول، سولفات آمونیوم اشباع، اتانول و ترت-بوتانول برای تشکیل توده‌های تجمع آنزیم استفاده شدند. در بین آنها، ترت-بوتانول بالاترین فعالیت آنزیمی را در مقایسه با سایر رسوب‌دهنده‌ها نشان داد. شرایط بهینه فرآیند تثبیت برای این سه آنزیم و تشکیل CLEAs عبارت بودند از: غلظت گلوتارآلدئید: ۵ میلی‌مولار، نسبت مخلوط آنزیم آلفا-آمیلاز: گلوکوآمیلاز: پولولاناز) ۳: ۱: ۱، نسبت آنزیم به سرم آلبومین گاوی ۲:۱ و زمان اتصال عرضی ۲/۵ ساعت، در دمای ۲-۳ درجه سانتیگراد. دما و pH بهینه برای آلفا-آمیلاز آزاد ۹۵ درجه سانتیگراد و pH 5.5 برای گلوکوآمیلاز و پولولاناز به ترتیب ۶۰-۶۲ درجه سانتیگراد و pH 5.5 تعیین گردید. CLEAs تشکیل شده توسط ترت-بوتانول و نسبت آنزیم به BSA برابر با ۱:۲ دارای دمای بهینه ۶۰-۶۲ درجه سانتیگراد و pH بهینه ۵.۵ بود. ارزیابی پارامترهای سینتیکی CLEAs ترکیبی در مقایسه با مجموعه آنزیم‌های آزاد نشان داد که K_m کاهش، V_{max} و راندمان کاتالیزوری افزایش یافته است. علاوه بر این، CLEAs ترکیبی حاصل شده، پس از ۱۰ چرخه، فعالیت آنزیمی خود را تا حدود ۷۴٪ حفظ کرده است. همچنین، ارزیابی پایداری حرارتی و نیمه عمر آنزیم‌های تثبیت‌شده حدود ۳ برابر بیشتر از آنزیم‌های آزاد بود. این افزایش فعالیت به دلیل افزودن نسبت ۳:۱ یون‌های Ca^{2+}/Na^{+} به مخلوط آنزیم در طول تشکیل CLEAs می‌باشد. افزودن یون‌ها، پایداری حرارتی، پایداری عملکردی و نیمه عمر آنزیم را بهبود بخشیده است. بر این اساس، CLEAs ترکیبی حاصل از سه نوع آنزیم آمیلاز به عنوان یک بیوکاتالیست با سهولت کارکرد، افزایش اثربخشی کاتالیستی، سازگار با محیط زیست و مقرون به صرفه برای طراحی فرآیندی یکپارچه جدید در تولید شربت گلوکز از نشاسته معرفی می‌گردد.

واژگان کلیدی: **تثبیت چندآنزیمی، CLEAs، آلفا-آمیلاز مقاوم به حرارت، گلوکوامیلاز، پولولاناز**

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