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RESEARCH PAPER

Modification of chitosan-bead support materials with L-lysine and L-asparagine for α-amylase immobilization

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Abstract
Maltose syrups have got wide-range utilizations in a variety of applications from bakery to drug-development. α-Amylases are among the most widely utilized industrial enzymes due to their high specificity in production of maltose syrup from starch. However, enzymes are not stable in ex vivo conditions towards alteration in pH, temperature, and such other parameters as high salt concentrations and impurities, where immobilization is required to advance the stability of the enzyme with which approach the requirement of isolation of the enzyme from media is eliminated as well. In this study, Termamyl® α-amylase was immobilized on the none-modified chitosan beads (NMCB), L-lysine-modified chitosan beads (LMCB), and L-asparagine-modified chitosan beads (AMCB) to assess effects of the support material on optimum conditions and kinetic parameters of the α-amylase activity in production of maltose from starch. Immobilization on NMCB, LMCB, and AMCB puts a strong influence on optimum pH, optimum temperature, stability, and kinetic parameters of α-amylase. Modification of chitosan beads with L-lysine and L-asparagine dramatically altered the overall immobilization yield, and enzyme's response to pH and temperature variations and the kinetic parameters. AMCB provided the best immobilization yield (49%), while LMCB only improved the yield by 2% from 22 to 24%.

Keywords Immobilization · *Bacillus licheniformis* α-amylases · Chitosan beads · Maltose syrup

Introduction

Maltose syrups are of great interest in the brewing, baking, confectionery, canned food, and pharmaceutical industries, where enzymatic processing is preferred because of its superiority over chemical methods [1]. Since enzymes are the main catalysts in the production of high maltose syrups, enzyme engineering at the gene level such as mutagenesis [2] and at the protein level such as immobilization, which is essential in improving the yield and preventing possible enzyme leakage into the product [3], have been widely studied. α-Amylases (EC 3.2.1.1) are the family of endo-amylases that catalyze the hydrolysis of α-D-(1,4) glycosidic linkages in starch and its analogous carbohydrates into oligo- and mono-saccharides (i.e. maltose and glucose). Currently, α-amylases take the place of chemical methods in the starch industry [4]. Among the α-amylases, *Bacillus licheniformis* α-amylases (BLAs) are effectively thermostable, and are used in the alcohol, sugar, and brewing industries for the initial hydrolysis of starch [5]. To enhance its usability and reach maximum benefit, its immobilization onto different support materials has been studied. Since starch is a large molecule, the immobilization technique must be convenient for substrate and product diffusion, where the support materials are required to be insoluble [6].

Immobilization advances the utilization of the enzymes through increasing ex vivo operational stability, improving adaptability towards lower and higher pHs, raising temperature, increasing inhibitors in the working media, and providing reusability and recovery from the media after use

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[7, 8]. The success of the immobilization depends on the enzyme itself and the support. The support should possess hydrophilicity, mechanical stability, rigidity, regenerability, high affinity to enzymes, non-toxicity, include free reactive functional groups for chemical modification, and ease of preparation in different geometrical configurations. Even though there is no individual support possessing all of these properties, chitosan meets many of the requirements. Thus, chitosan has found a place as a flexible support material in enzyme immobilization applications [7, 9, 10].

Chitosan is mainly obtained by at least 50% *N*-deacetylation of chitin, which is the second most abundant biopolymer [11, 12]. The general structure of chitosan is a copolymer of poly [β-(1 → 4)-(2-amino-2-deoxy-D-glucopyranose) + (2-acetamido-2-deoxy-D-glucopyranose)] [12]. Biocompatibility, biodegradability, hydrophilicity, and its abundance of functional groups make chitosan a good candidate for bio-applications, where it mostly undergoes chemical modifications [13]. The most common route to modify chitosan is through either its primary amino group or the first and second hydroxyl groups [11, 14].

In this comparative study, chitosan beads in its L-lysine and L-asparagine-modified versions were utilized as Termamyl® α-amylase immobilization supports in the production of maltose from starch. The findings revealed that the L-lysine and L-asparagine modification had a strong impact on the optimum operation conditions, stability and kinetic parameters. Particularly, L-asparagine modification advanced the overall immobilization yield more than two times. To the best of our knowledge, this is the first study reporting on the modification of chitosan beads with L-lysine and L-asparagine as α-amylase immobilization support for starch processing.

Materials and methods

Materials

α-Amylase (Termamyl® 120 L, type L) were purchased from Novozymes A/S (Bagsvaerd, Denmark). Chitosan flakes (medium molecular weight), L-asparagine and L-lysine, bovine serum albumine (BSA), ethanol, glutaraldehyde, EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride], and NHS (*n*-hydroxysuccinimide) were purchased from Sigma-Aldrich (MO, USA). All other chemicals used in the study were of analytical grade and were used without further purification. Jenway 6400 Spectrophotometer, Memmert water bath, Heidolph MR Hei-Standard heater, Precisa XB 220A balance, and Easy-load masterflex model 7518-00 peristaltic pump were used as equipment throughout the study.

Preparation of chitosan beads

Chitosan beads were initially prepared by two methods; (1) utilization of sodium tripolyphosphate in the presence of 0.1–1.0 M sodium hydroxide as solvent and (2) 1 M sodium hydroxide–ethanol solution. The formed chitosan beads in tripolyphosphate solution did not provide a good surface for the amino acid loading, which caused poor enzyme immobilization in comparison to the beads obtained via the aqueous sodium hydroxide–ethanol bath. Therefore, the chitosan beads were prepared according to neutralization method throughout the study using a peristaltic pump combined to a 0.1 mm (ID) flamed glass transfer pipette, which was simply designed by our group. 2 g of chitosan flakes were dissolved in 100 mL of 5% (v/v) aqueous acetic acid solution by continuous stirring overnight to total dissolution. The chitosan solution (2%, w/v) from the tip of the glass pipette was allowed to fall into aqueous 400 mL of 1.0 M sodium hydroxide solution containing 100 mL ethanol. The peristaltic pump system had constant speed to achieve uniformly shaped and sized beads. The beads took their ideal spherical form as soon as dropping into the solution. The formed beads were then incubated at 200 rpm in 4 °C coldroom overnight. At the end of the incubation, the beads were rinsed with pH 7.0 (100 mM) phosphate buffer and were then used for α-amylase immobilization, or chemical modification before the immobilization.

Modification of the chitosan beads with L-lysine, L-asparagine, and L-cysteine

L-Asparagine and L-lysine possess two primary amino groups as α/θ and α/ε amino groups, respectively [15]. In the case of lysine immobilization on to the chitosan beads, glutaraldehyde most probably will bind to α-amino of the lysine because of ~ 10^2 difference of association constants between α- and ε-amino groups. This will lead α-amylase to bind glutaraldehyde activated ε-amino groups of lysine on lysine-modified chitosan beads. In the case of asparagine, α-amylase will most possibly prefer to bind glutaraldehyde activated θ-amino groups of asparagine on asparagine-modified chitosan beads. The α-amylase enzyme will be away from carboxyl group of lysine and 4-carbon chain aliphatic group of lysine will be interacting with the enzyme. In contrast to this, in the case of asparagine, charged groups will be at proximity of the enzyme. As stated in literature, the small changes in the microenvironment of enzyme support material cause overall performance of the immobilized enzymes [16].

Modification of the chitosan beads was performed in two steps. The first step was the activation of the chitosan

beads through a 1–3% glutaraldehyde treatment for 2 h at room temperature. After, the activated chitosan beads were washed four times with 100 mM pH 7.0 phosphate buffer. In the second step, the activated chitosan beads were put into 100 mL of 100 mM pH 7.0 phosphate buffer solution containing 1 g of L-lysine or L-asparagine, and were then incubated for up to 4 h at room temperature. A 1 mL sample was taken from the media and stored for determination of unbound amino acids. The chitosan beads were rinsed four times with pH 7.0 (100 mM) phosphate buffer, and a 1 mL sample was taken after every rinsing step to determine the concentration of the non-chemically bound amino acids. Finally, the rinsed chitosan beads were used for the immobilization of α-amylase. Unbound amino acids were assayed by the modified Ninhydrin method [17].

The amino acid bonding yield (%) was calculated according to the equation given below:

$$\text{Bonding yield (\%)} = (A - B)/A \times 100,$$

where A is the total amino acid added in the initial modification solution and B is the unbound amino acid which remains free after modification.

Immobilization of α-amylase on the chitosan beads

The procedure of α-amylase immobilization on both the modified and unmodified chitosan beads was kept the same. The beads were activated by treating them with the desired glutaraldehyde concentration for 30–60 min at room temperature. Following the activation, the beads were rinsed four times with the same buffer. Then, the activated beads were treated with the desired protein concentration in the same buffer at 4 °C overnight, or 1 h at room temperature. At the end of the incubation, a 1 mL sample was taken to determine the unbound protein concentration. α-Amylase immobilized beads were then rinsed three times pH 7.0 (100 mM) phosphate buffer. After every rinsing, 1 mL sample was taken to determine the non-chemically bound protein concentration. Then, the beads were put into 1 mg/mL BSA in pH 7.0 (100 mM) phosphate buffer (PB) for 1 h at room temperature to deactivate residual GA (glutaraldehyde) molecules. Finally, the immobilized α-amylase chitosan beads were stored at 50 mM pH 5.5 acetate buffer in a 4 °C fridge.

The immobilization yield (%) was also calculated with the following equation:

$$\text{Immobilization yield (\%)} = (A - B)/A \times 100,$$

where A is the total protein of enzyme added in the initial immobilization solution and B is the unbound protein of enzyme which remains free after immobilization.

The activity yield (%) was defined using the following equation:

$$\text{Activity yield (\%)} = \text{Ba}/\text{Aa} \times 100,$$

where Aa is the activity of enzyme added in the immobilization solution and Ba is the activity of the immobilized enzyme.

Protein determination

Protein quantification was assayed by the Bradford method [18]. The amount of immobilized protein on the beads was calculated by subtracting of the amount of protein determined in supernatant and rinsing solution from the total protein concentration that was used for immobilization.

α-Amylase activity assay

Both the soluble and the immobilized α-amylase activities were assayed by the DNS method [19, 20]. The activity was identified as that 1 mg enzyme produced 1 μmol of reducing sugar per minute. Three beads were used for the routine assay of the activity of the immobilized enzymes. Both the soluble and the immobilized α-amylase were first incubated for 2 min with 0.5% (w/v) starch in 100 mM pH 7.0 phosphate buffer in a 20 mL glass tube. To show that the immobilized enzymes can work under high ionic strength conditions, PB buffer was used at 100 mM.

Properties of the free and immobilized α-amylases

Effect of temperature on both the free and immobilized α-amylases

The optimum temperature found for the free and immobilized α-amylases was between 25 and 60 °C. Therefore, thermal stability of both the free and the immobilized α-amylase was determined by measuring the residual activity of the enzymes incubated at 25 and 50 °C in the phosphate buffer (100 mM pH 7.0). Temperature stability was performed for the free and immobilized enzymes kept with and without starch 0.1% (w/v) at 25 and 50 °C. The former case was applied dynamic stability, while the latter case was applied for static stability.

Effect of pH on both the free and immobilized α-amylases

Optimum pH for the free and the immobilized α-amylases was determined by varying the pH of assay buffers from 4.0 to 9.0. The used buffers were 100 mM sodium acetate buffer (pH 4.0–5.5), 100 mM phosphate buffer (pH 6.0–8.0), and 100 mM glycine buffer (pH 9.0). All the activity assays were performed with 0.5 mg/mL in 100 mM pH 7.0 PB at room temperature.

Effect of substrate concentration on both the free and immobilized α-amylases

K_m and V_{max} values of the free and the immobilized α-amylase were determined through the Lineweaver–Burk plot by varying starch concentration in a range of 0.125–0.750 mg/mL. The enzyme concentration was kept at minimum concentration to prevent substrate limitation The reactions were performed in 100 mL pH 7.0 phosphate buffer at room temperature.

Reusability

The immobilized α-amylase activities were assayed in 100 mM pH 7.0 phosphate buffer with 0.5 mg/mL starch at room temperature. This operation was performed ten times.

Storage stability

Both the free and the immobilized enzymes were kept for 100 days at 4 °C to determine the storage stability. The storage stability determination was based on the residual activity measurement on different days.

Operational stability

Operational stability of both the free and immobilized α-amylases was carried out in 100 mM pH 7.0 phosphate buffer containing 0.5 mg/mL starch at room temperature. The reaction period was 11 h.

Results and discussion

Modification of chitosan beads with L-lysine and L-asparagine

Chitosan (CS) concentration was chosen to be 2% (w/v) to obtain optimum rigid beads; higher concentrations made chitosan difficult to obtain stable and spherical beads. In contrast to this, lower concentrations of CS (i.e., 1%) did not provide rigid and size-controlled CS-beads. The GA concentration and incubation time were standardized at 2% and 2 h. Higher concentrations of GA resulted in brittle bead formation, while lower concentrations did not provide long-term stable beads. Displacing GA with EDC/NHS chemistry did not provide long-term stable CS-beads. Therefore, it is understood that GA not only worked to provide free carbonyl groups for L-lysine or L-asparagine loading, but also it worked to crosslink CS polymers on CS-beads, as well.

The loading amounts of each amino acid and coupling yields were calculated by subtracting the amount of unbound and non-chemically bound amino acids from the total amount of the amino acids used for the modification of the chitosan beads. The bonding ratios of L-lysine and L-asparagine were 55 and 62%, respectively. Non-chemically bound amino acids were negligible in comparison to unbound amino acids. The obtained coupling yields were slightly higher than those previously reported (i.e., 30–57%) elsewhere [16].

α-Amylase immobilization on both the modified and unmodified chitosan beads

Immobilization of α-amylase on both the modified and unmodified chitosan beads were performed in pH 7.0, 100 mM phosphate buffer, where glutaraldehyde works well to bind the amino group selectively and the occurrence of the multipoint covalent attachment is at a minimum [21]. The optimization of the glutaraldehyde concentration and the activation time were carried out for α-amylase immobilized unmodified chitosan beads, and these values were used for both the unmodified and modified ones. Briefly, glutaraldehyde concentration was standardized at 2% (w/v) for a 30-min treatment, since the α-amylase immobilization yield was obtained at 35% for 1% glutaraldehyde concentration, and the chitosan beads became breakable at 4% glutaraldehyde concentration. At this level, utilization of EDC/NHS chemistry provided higher yield (i.e., 60% immobilization at 1% EDC/NHS treatment), but in terms of flexibility GA was preferred, since it adds a spacer between CS-beads and the enzyme. The enzyme concentration was chosen as 1 mg/mL for immobilization, because utilization of high protein concentrations during immobilization might cause loss of activity in relation to intense protein–protein interaction on the support [22, 23]. Table 1 demonstrates the results of the α-amylase immobilization on the chitosan beads. The maximum immobilization yield achieved was 75.7% on L-lysine bound chitosan beads. This yield is slightly higher than that of Tripathi et al.'s report [4] where 69% immobilization yield at 4 mg/mL initial α-amylase concentration from *Vigna radiata* was reported.

Table 1 Activity and immobilization yields of α-amylase on the chitosan beads

Support material	Immobilization yield (%)	Activity yield (%)	Overall immobilization yield (%)
NMCB	51.2	11.1	22
LMCB	75.7	17.8	24
AMCB	60.3	29.3	49

Overall immobilization yield refers to % activity yield/immobilization yield

NMCB none-modified chitosan beads, *LMCB* L-lysine-modified chitosan beads, *AMCB* L-asparagine-modified chitosan beads

Even though the immobilization yield was higher for all supports, activity yield was only obtained reasonably for AMCB. The ratio of activity yield over immobilization yield was calculated, which might be used to evaluate overall yield of immobilization, where the AMCB support provided the best overall yield. NMCB and LMCB supports gave similar overall immobilization yield.

General properties of the free and immobilized α-amylases

Effect of temperature

The relative activities of both the free and immobilized α-amylases are presented in Fig. 1a as a function of temperature. The optimum temperature of α-amylase shifted through immobilization. The free α-amylase showed its maximum activity at 40 °C, while immobilized α-amylases on NMCB, and LMCB/AMCB showed their maximum activities at 45 and 55 °C, respectively. While chitosan beads alone caused only 5 °C increase in the optimum temperature, further modifications on chitosan beads shifted the optimum temperature values by 15 °C. Increase in optimum temperature, commonly, is one of the natural results of immobilization [4, 21, 24–26]. Bayramoglu et al. [21] reported optimum temperatures for both the free and immobilized *B. licheniformis* α-amylases as 50 and 60 °C, respectively, which can be attributed to the different formulation and test conditions.

To give a better understanding for the observed changes in optimum temperature, the activation energies of the both free and immobilized α-amylases were calculated according to Arrhenius diagram, where 41.7, 87.1, 235.25, and 474 kJ/mol activation energy requirements were obtained for the free and the immobilized α-amylases on NMCB, LMCB, and AMCB, respectively.

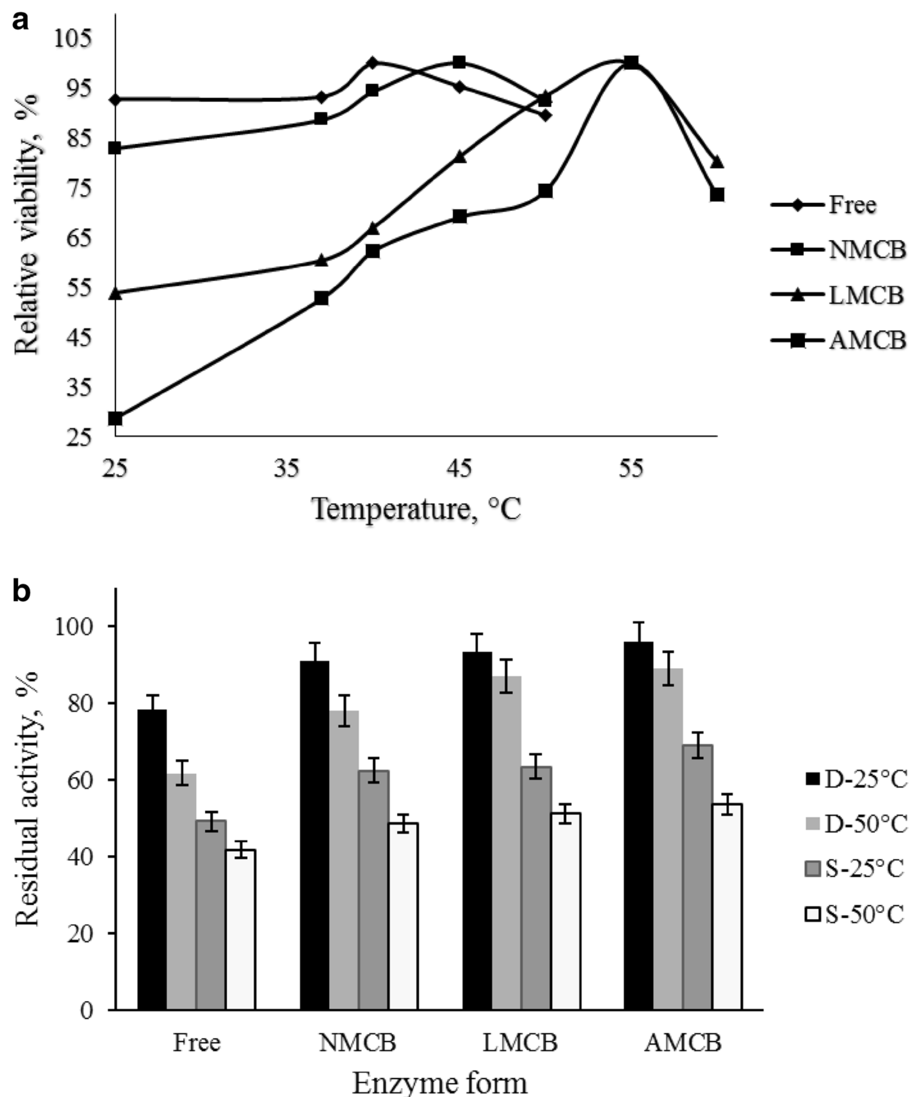

Fig. 1 **a** Effect of temperature on the activity of the free and the immobilized α-amylase. **b** Thermal stabilities of the free and immobilized α-amylases

All activities were assayed at mentioned temperatures using 0.5 mg/mL soluble starch in 100 mM pH 7.0 phosphate buffer.

The properties of the industrial enzymes must be improved to use them effectively on a large scale. Improving thermal stability is one of the main reasons of the enzyme immobilization [27]. The effect of the temperature on stability of both the immobilized and free α-amylases, shown in Fig. 1b, was determined by incubating them with/without substrate at 25 and 50 °C for 6 h. 50 and 25 °C was chosen to evaluate the possible improvement with immobilization and amino acid coating.

Thermal stabilities of the free α-amylase, α-amylase immobilized on NMCB, LMCB and AMCB for 6 h in the presence of 0.5 mg/mL soluble starch in 100 mM pH 7.0. D-refers to dynamic stability, while S-refers to static stability.

After incubation at indicated temperatures, activities were assayed at 25 °C using 0.5 mg/mL soluble starch in 100 mM pH 7.0 phosphate buffer. 6 h incubation at 25 and 50 °C did cause loss of activity for the free and immobilized α-amylases, where the highest activity loss was observed for 50 °C as expected. Residual activities (%) of α-amylase immobilized on AMCB, LMBC, and NMCB and the free α-amylase for incubation at 25 and 50 °C were obtained as 69/54, 64/51, 62/49, and 49/42 for static stability, respectively. Dynamic thermal residual activities (%) for incubation at 25 and 50 °C were obtained as 96/89, 93/87, 91/78, and 79/62, respectively. In all cases, dynamic thermal stabilities were obtained higher than static stabilities what could be related to that the presence of substrate and product might protected the 3D of the α-amylase. When the incubation for dynamic stabilities was extended up to 16 h at 50 °C incubation, the remaining activities were obtained as 17, 37, 48, and 52 (%). α-Amylase immobilized on L-asparagine-modified chitosan beads (AMCB) showed the highest stability against heat treatment under the tested conditions, whereas its relative activities were lower at 25 and 50 °C. Besides, the sharpest activity changes in response to change in assay temperature were obtained for LMCB.

Effect of pH

The study of optimum pH was carried out for the free and immobilized α-amylase types under the same conditions. pH is an important factor that influences the dissociation of the side groups of the amino acids within the enzyme and stability of the insoluble support. The interaction between protein and the support, and surface properties of the enzyme are affected by varying the pH [4]. The optimum values of pH of free and immobilized α-amylases are shown in Fig. 2a. The optimum pH of free α-amylase was observed at pH 6.0, while it was shifted to pH 7.5, pH 7.0, and pH 8.0 through immobilization on NMCB, LMCB, and AMCB, respectively. In the literature, the optimum pH of *B. licheniformis* α-amylase was reported between 5.5 and 7.5 [21, 25, 26, 28], while commonly immobilization of α-amylases on chitosan beads led to shift the optimum pH to more basic regions [4]. Adaptability of the immobilized α-amylases to different pH values showed differences, as well, (Fig. 2b–d).

Free α-amylase did not show activity change over 20% in response to pH alteration from 4.0 to 9.0, while α-amylase immobilized on LMCB and AMCM beads showed dramatic changes. α-Amylase immobilized on LMCB beads showed the best activity at pH 7.0, while at lower and higher pH values, the activity tremendously decreased down to 24 and 37.5% for pH 4.0 and 9.0 values. However, in contrast to this, α-amylase immobilized on AMCB beads showed its minimum activities between 6.0 and 7.0.

All the beads were initially stored in 50 mM at pH 5.5 acetate buffer at 4 °C, which were directly used or incubated at room temperature right after being taken out from the 4 °C without the presence of starch. α-Amylase immobilized on NMCB showed their maximum relative activity at 60–90 min interval other than pH 5.5 acetate buffer, where 30–60 min interval was observed the highest relative activity period (Fig. 2b). In contrast to this, α-amylase immobilized on (Fig. 2c) AMCB and (Fig. 2d) LMCB showed varying trends depending on the assay pH. For pH 4.0–6.5 range and pH 8.0, 30–60 min interval was observed the highest relative activity, while for pH 7.0, 7.5 and 9.0 showed their highest activity at 60–90 min interval (Fig. 2c). The highest relative activity for pH 6.0, 6.5, and 9.0 was observed at 30–60 min interval (Fig. 2d). In contrast to this, other than pH 5.5, 60–90 min interval was determined for the highest relative activity period. At pH 5.5, no incubation and/or 0–30 min incubation were enough to obtain highest relative activity. Amino acid coating of chitosan beads altered the enzyme's behavior in addition to the effect came with the immobilization itself. The immobilized enzymes taken out from +4 °C required prior incubation at room temperature to show their proper activity at the dedicated pH values, while free α-amylase did not show this requirement. The other important observation was that pH 5.5 and pH 5.0 provided the best condition to keep the relative activity stable for α-amylase immobilized on AMCB/LMCB and NMCB, respectively. In response to increase in incubation time at room temperature, decreases in relative activity were probably related to both pH and temperature-dependent denaturation. However, running a batch reactor with 0.5 mg/mL for 150 min showed in pH 7.0 PB, in all cases (free and the immobilized α-amylases), 0–30 min time interval was enough to reach 100% relative activity; less than 5% decreases in relative activity for 90–120 min interval was observed, which was higher than those of incubated at room temperature up to the run time (e.g., 30, 60, 90,120, and

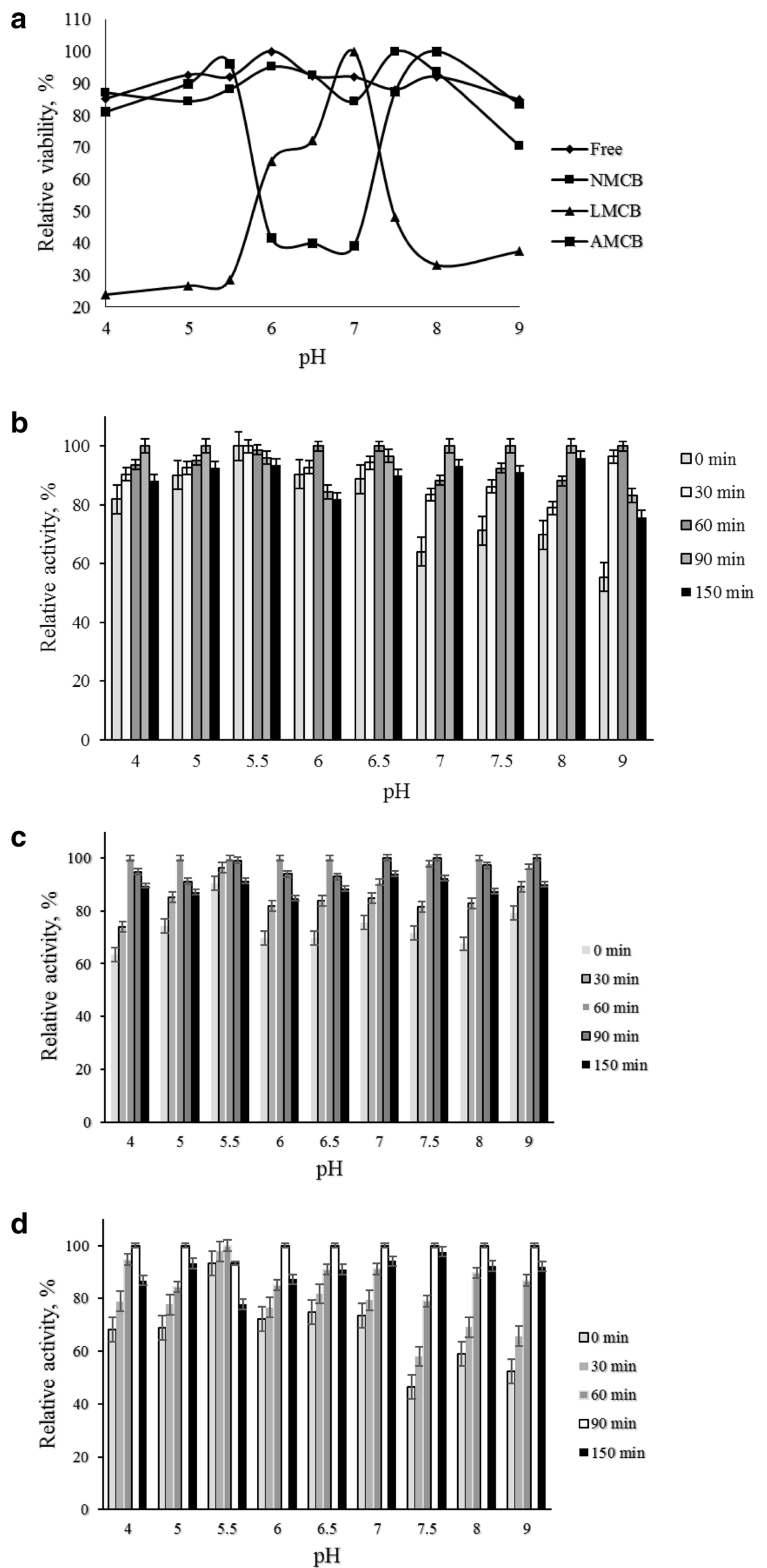

Fig. 2 **a** Optimum pHs of the free and immobilized α-amylases. **b** pH adaptation and stability of α-amylases on NMCB. **c** pH adaptation and stability of α-amylases on LMCB. **d** pH adaptation and stability of α-amylases on AMCB

150 min). This can be resulted from two possible reasons as (1) presence of starch triggered α-amylase to show its activity which might helped the immobilized enzyme to adapt new conditions faster. The other possible reason (2) could be presence of the substrate caused dynamic stability conditions, which helps enzymes to show better stability in comparison to static stability [29]. Differences between dynamic and static stabilities were clearly shown temperature stabilities.

Reusability

Immobilization provides reusability of the enzymes, which refers to the immobilized enzyme carrying its activity up to its final use, and this leads to reduce the overall cost of the industrial enzymes [30]. Thus, reusability is the most important advantage of the immobilized enzymes [24]. The stability of the immobilized α-amylases in the aspect of reusability was, therefore, searched by measuring the activity for the hydrolyses of starch at 25 °C (Fig. 3).

The activities were assayed 25 °C using 0.5 mg/mL in 100 mM pH 7.0 phosphate buffer.

After each run, the beads were rinsed with cold 50 mM pH 5.5 acetate buffer, since the buffer was utilized as the storage buffer. The reason of using cold buffer is to mimicking storing condition other than the pH. Up to 5th run, α-amylase immobilized on LMCB protected its activity relatively higher, while at 6th run, a dramatic decrease was observed; after taking out the beads, remaining media were incubated 10 min longer to see if any enzyme leakage caused the decreases, where no recognizable increase was observed. Similarly, α-amylase immobilized on NMCB showed dramatic changes at 8th run. In contrast to these, α-amylase immobilized on AMCB showed a continuous stable activity losses, whose final activity was obtained relatively higher in comparison to α-amylase immobilized on NMCB and LMCB. The results showed that the immobilized enzymes could be used effectively more than ten times as shown elsewhere [4, 24, 30].

Storage stability

Improved storage stability of industrial enzymes is the one of the most important parameters to using them effectively and economically [30], for which immobilization has been shown as one of the approaches to improve the storage stability [7]. Thus, both the free and immobilized Thermamyl® were stored at 4 °C for 100 days in 50 mM pH 5.5 acetate buffer. At the end of 100 days, the free enzymes NMCB, LMCB, and AMCB lost their activities as 84, 52, 41, and 37%, respectively (Fig. 4). It should be noted that free enzymes were diluted in 50 mM pH 5.5 acetate buffer, whose activity decrease was less than 10% when it was kept its own preservative containing media. As seen from Fig. 4, both LMCB and AMCB showed higher storage stability than NMCB, meaning that the modifications provided positive effect on the immobilized enzyme stabilization. Tanyolaç et al. [31] reported that the immobilized Thermamyl® lost its 44% activity in 90 days. Tripathi et al. [4] reported that immobilized α-amylase on chitosan beads lost its 55% activity in 100 days, and Kahraman et al. [24] reported that immobilized α-amylase on glass beads lost its 20% activity in 25 days. Therefore, it can be speculated that even for immobilized enzymes, activity losses during storage are natural process. Dynamic storage stabilities were not tested.

The storage was in 50 mM pH 5.5 acetate buffer in 4 °C fridge. Storage stabilities of the free and immobilized α-amylase activities were assayed 25 °C using 0.5 mg/mL in 100 mM pH 7.0 phosphate buffer.

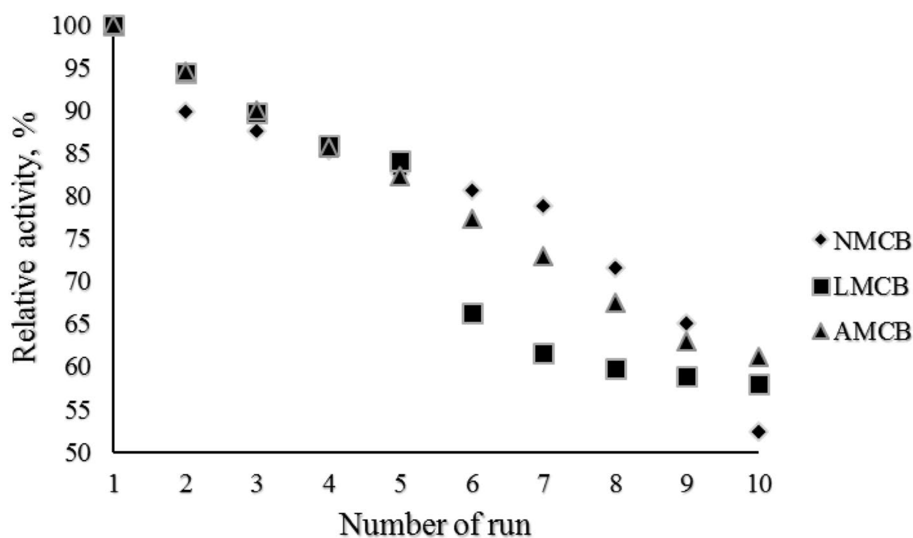

Fig. 3 Reusability of the immobilized α-amylases

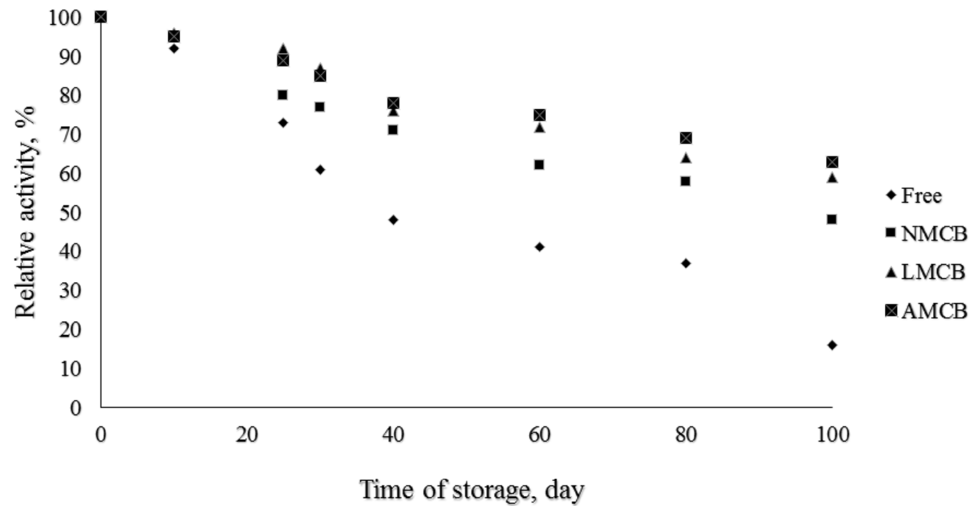

Fig. 4 Storage stability of the free and immobilized α-amylases

Table 2 Kinetic parameters of both the free and immobilized α-amylases

Parameter	Free	NMCB	LMCB	AMCB
K_m (mg/mL)	0.702	1.814	0.802	2.355
V_{max} (U/mg)	0.479	1.112	0.384	1.709

NMCB none-modified chitosan beads, *LMCB* L-lysine-modified chitosan beads, *AMCB* L-asparagine-modified chitosan beads

Kinetic parameters

Immobilization could alter the enzyme behavior and change K_m and V_{max} due to changes in the microenvironment and the enzyme structure. These changes mainly depend upon the reaction conditions, the support character, and the reactor design [7].

Kinetic parameters of the free and immobilized α-amylase activities were assayed 25 °C using 0.5 mg/mL in 100 mM pH 7.0 phosphate buffer.

Thermamyl® α-amylase immobilized on the chitosan beads showed different K_m and V_{max} from each other and the free form (Table 2). K_m and V_{max} values were calculated with Lineweaver–Burk diagram (Fig. 5). The immobilized Thermamyl® α-amylase on LMCB showed similar K_m to free Thermamyl® α-amylase, 0.802, and 0.702 mg/mL, respectively. The immobilized Thermamyl® on the other chitosan beads showed as much as 2.5 times higher (NMCB) and 3.3 times higher (AMCB) than the free enzyme. The increase in K_m, in addition to changes in enzyme structure and optimum working conditions, might be caused by “Nerst Layer” [4], where mass transfer is driven by diffusion. Similar results were obtained by that Bayramoğlu et al. [21] reported that immobilized BLA onto reactive membranes showed 2.3 times higher K_m value that the free BLA. Tripathi et al. [4] also reported

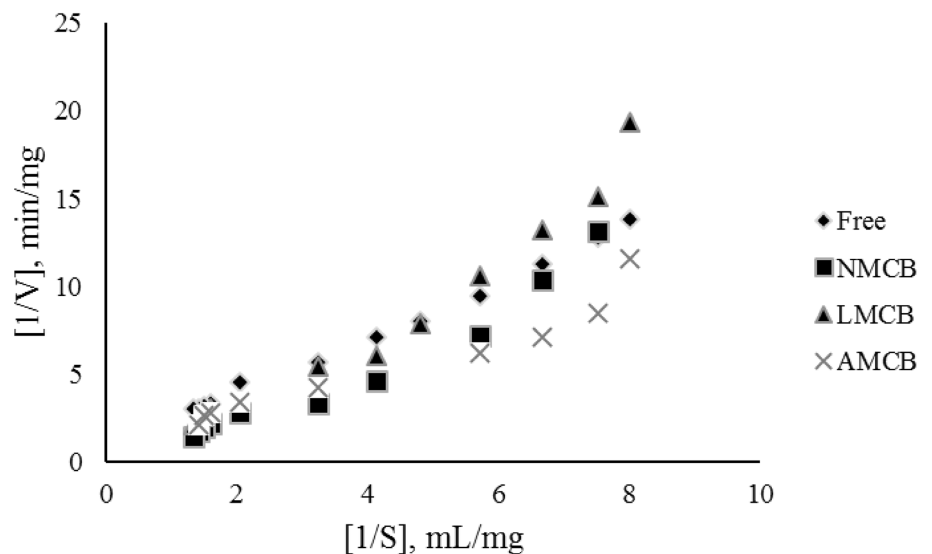

Fig. 5 Lineweaver–Burk plots of the free and immobilized α-amylases

that the Km value increased from 2.77 to 5 mg/mL through immobilization of α-amylase on chitosan beads. The immobilized Thermamyl® α-amylase on LMCB showed a minimum V_{max}, and this value was also lower than the free Thermamyl®, 0.384 U/mg and 0.479 U/mg, respectively. In contrast to this, α-amylase on NMCB and AMCB gave 1.112 and 1.709 U/mg V_{max} values. Increase in V_{max} values of enzymes is expected upon immobilization, while the decrease has been reported as rare cases [22]. It is not clear to us how L-lysine and L-asparagine modification of chitosan beads resulted in this much distinct effect on the kinetic parameters. One of the major differences observed for α-amylase immobilization on LMCB and AMCB was that the amount of immobilized α-amylase, so it can be speculated that enzyme loading could affect the kinetic parameters.

Operational stability of the immobilized α-amylases

Operational stability of an enzyme is an important parameter of industrial enzymes [30]. The operational stabilities of both the free and immobilized Thermamyl® α-amylase were monitored for 11-h continuous runs as batch process; the results are depicted in Fig. 6. The relative activities decreased with increasing time, gradually. This could be attributed to enzyme inactivation. The operational half-life ($t_{1/2}$) of both the free and immobilized Thermamyl® α-amylase were calculated using the following equation:

$$t_{1/2} = \frac{\ln 2}{k_D}, \quad k_D = \frac{\ln\left(\frac{A_0}{A}\right)}{t},$$

where t is the operation time, k_D is decay constant, A_0 and A are the enzymatic activities at the beginning and at t time, respectively [30].

The assays were performed 25 °C using 0.5 mg/mL in 100 mM pH 7.0 phosphate buffer.

As seen from Fig. 6, the immobilization helped to improve operational stability. The free enzyme lost about 20% activity within an hour, while the immobilized ones protected their activity up to 97%. A dramatic decrease was observed for α-amylase immobilized on NMCB within 3 h of operation, while no sudden decrease was observed for α-amylase immobilized on LMCB and AMCB within the tested periods, for which residual decrease was observed. 11 h of operation resulted in loss of 80% activity of the free enzyme, while immobilized enzymes showed 40–51% residual activity end of 11 h, where the highest residual activity was recorded for α-amylase immobilized on AMCB.

Conclusion

Chitosan beads and their L-lysine and L-asparagine-modified versions were utilized to improve efficiency of Thermamyl® α-amylase immobilization. Glutaraldehyde was utilized to introduce amino acids and the enzyme, where its concentrations were minimized to prevent any possible hydrophobic–hydrophobic or ionic interactions between the activated chitosan-bead surface and the amino acids. The interactions were also minimized to prevent amino acid modified chitosan-bead surfaces and α-amylase, since excess GA utilization adds more possible interactions besides covalent binding [32]. This could be one of the reasons that at longer incubation periods (i.e. 4 h at room temperature) for 2% GA treatment, enzyme leakage was observed within 4 months.

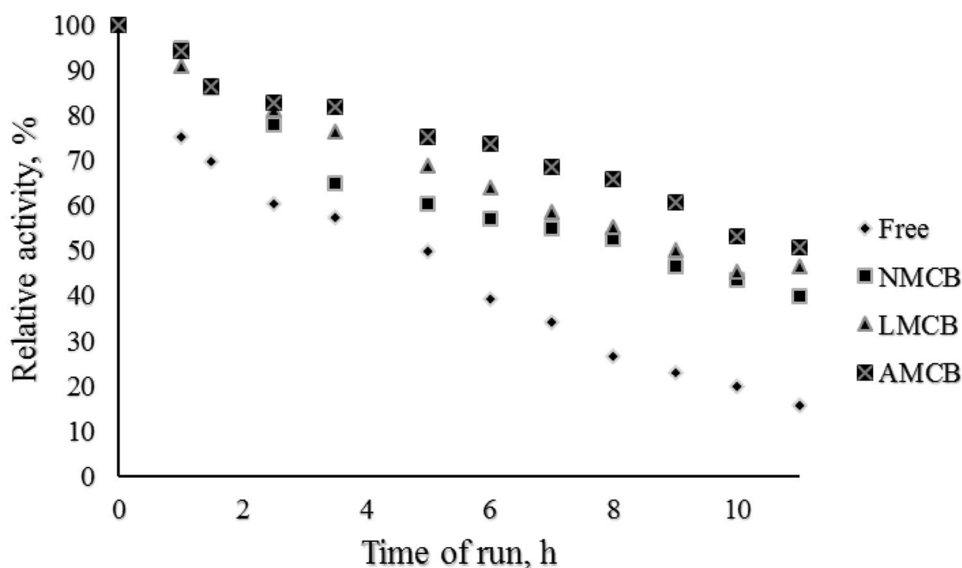

Fig. 6 Operational stability of the free and immobilized α-amylase activities

The selected GA concentration provided long-term stable immobilization under storage conditions and increased immobilized enzymes robustness in working conditions, where decreases in activity yield were natural phenomenon of immobilization.

Modification of chitosan-bead surface with L-lysine and L-asparagine brought great impact on overall yield of α-amylase immobilization. Even though lysine and asparagine are quite similar for their functional groups, carboxamide groups on asparagine in contrast to primary amino group on lysine could be the reason of that GA crosslinking took place differently and bound α-amylase oriented at different orientation, where small changes on chitosan could bring major changes in enzyme immobilization [23]. Asparagine modification of chitosan beads increased the overall immobilization yield from 22 to 49%, while lysine modification only provided 2% difference. Similarly, asparagine modification brought stability of temperature, pH, and operational and storage conditions. In contrast to this, lysine modification helped to increase the enzyme's affinity towards starch in contrast to non-modified and lysine-modified chitosan beads, while the V_{max} gave a lower value in comparison to free α-amylase which was observed for chitosan-based support materials [22]. Alteration in kinetic parameters was expected, since poly-ionic support materials alter K_m and V_{max} values in addition to immobilization phenomenon itself [32].

In conclusion, Termamyl® α-amylase showed distinct characteristics in response to modification of chitosan beads with L-lysine and L-asparagine in the production of maltose from starch.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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