PAPER NAME

Yandri and Hadi_2022_1305-4241-1-PB.pdf

AUTHOR

Bambang Irawan

WORD COUNT

6853 Words

CHARACTER COUNT

36800 Characters

PAGE COUNT

13 Pages

FILE SIZE

1.3MB

SUBMISSION DATE

May 8, 2023 1:48 PM GMT+7

REPORT DATE

May 8, 2023 1:49 PM GMT+7

23% Overall Similarity

The combined total of all matches, including overlapping sources, for each database.

- 20% Internet database
- 14% Publications database
- Crossref database
- Crossref Posted Content database
- 8% Submitted Works database

Excluded from Similarity Report

- Bibliographic material
- Quoted material
- Cited material
- Small Matches (Less then 10 words)
- Manually excluded sources
- Manually excluded text blocks

Available online at www.ijournalse.org

Emerging Science Journal

(ISSN: 2610-9182)

Vol. 7, No. 1, February, 2023

Immobilization and Stabilization of *Aspergillus Fumigatus* α-Amylase by Adsorption on a Chitin

Yandri Yandri [1*], Ezra Rheinsky Tiarsa [1], Tati Suhartati [1], Bambang Irawan [2], Sutopo Hadi [1]

[1] *Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Lampung, Lampung 35145, Indonesia.*

[2] *Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung, 35145, Indonesia.*

Abstract

In this research, immobilization of *A. fumigatus* α-amylase on chitin was studied with the main purpose to improve the characteristics of the enzyme. A series of experiments were carried out to study stability improvement, thermodynamic parameters, include k_i, ΔG_i, and $t_{½}$, and reusability of the immobilized enzyme. The experimental results indicate that significant thermal stability was achieved, as indicates by the ability of the enzyme to retain its relative activity above 39% after 80 min of incubation at 60ºC. Thermodynamic parameters, include k_i, ΔG_i, and $t_{½}$, indicate that the immobilized enzyme is more rigid, stable, and less flexible in the water, resulting in increased stability up to 1.5 times compared to that of the native enzyme. Furthermore, the immobilized enzyme was able to retain over 46% of its initial activity after six consecutive applications for starch hydrolysis, confirming the potential of chitin for the production of immobilized enzymes on an industrial scale.

Keywords:

α-Amylase; Chitin;
Aspergillus Fumigatus;
Immobilization; Stabilization.

Article History:

Received:	28	June	2022
Revised:	18	August	2022
Accepted:	09	September	2022
Available online:	12	October	2022

1- Introduction

Microbial α-amylase is one of the prime enzymes applied in very diverse industries, such as detergents, syrups, bread, dairy products, starch processing, animal feed, textiles, paper, candy, sugar, bioethanol, pharmaceuticals, and waste treatment [1, 2]. This enzyme is well known for its good performance in increasing the production yield of enzymatic reactions, such as starch hydrolysis, resulting in a significant reduction in production costs. In addition, this enzyme exhibits the ability to control starch de-polymerization, working at physiological pH and temperature conditions, preventing the formation of side products such as 5-hydroxy-2-methylfurfuraldehyde. The ability to fix the color and taste of the product, reduce activation energy, and unnecessary purification process are other advantageous features of α-amylase [3-5].

Apart from its various valuable characteristics, α-amylase still has a number of practical obstacles that need to be overcome in order to improve the performance of the enzyme. Relatively low thermal stability and denaturation at high temperatures, together with solubility in water, make this enzyme unsuitable for high temperature reactions in aqueous systems, such as starch hydrolysis [2]. Previous workers reported a method to improve the stability of α-amylase isolated from *Aspergillus* sp. using an enzyme mobilization method, which means improvement of α-amylase stability is possible [5]. The *A. oryzae* α-amylase was reported to have optimum temperature of 45ºC and its activity decreased significantly at 60 ºC [4]. Slightly higher optimum temperature (50ºC) was reported for the *A. fumigatus* α-amylase but it showed a significant decrease of the activity to only 21.50% when applied at 60°C for 80 min [5].

* **CONTACT**: yandri.as@fmipa.unila.ac.id

DOI: http://dx.doi.org/10.28991/ESJ-2023-07-01-06

The easiest method to improve the stability of an enzyme is the immobilization technique. This technique involves physical adsorption or non-covalent binding of enzyme molecules on the surface of a water-insoluble supporting matrix [6]. This approach is generally considered the most cost-effective and easiest procedure to improve the stability, and therefore offers the opportunity to reuse the enzyme. In addition to improved stability, immobilized enzymes are attractive since they provide the industry with the opportunity to recover the enzyme, simplify the reactor design, run the reaction in a continuous process, and prevent contamination of the product [2, 7, 8]. For example, the immobilized pectinase on an activated montmorillonite support can be reused for up to 6 cycles, retaining 60% of its initial activity. This immobilized enzyme has been approved to increase the clarification rate in pineapple juice production [9].

The improvement of the enzyme stability can be investigated by determination of half-life and thermodynamic parameters, namely the thermal inactivation rate constant (k_i) and the change of free energy due to denaturation (ΔG_i) [10, 11]. In previous research, the immobilization method was reported to significantly improve the α-amylase stability, reusability, and some other properties using metal oxide nano-composites as a matrix. The immobilized enzyme lost only 35% of its activity when the temperature reached 70°C after 60 min of pre-incubation and its optimum pH increased from 5.5 to 7.0. After immobilization, the enzyme was found to become more resistant to thermal denaturation, as indicated by increased energy required for the thermal denaturation from 108.27 to 111.21 kJ $mole^{-1}$ [12]. In another study, significant improvement of thermal stability of the *A. fumigatus* α-amylase immobilized on zeolite/chitosan hybrid was reported, as indicated by increased of ΔG_i from 104.35 to 108.03 kJ $mole^{-1}$, and extension of half-life from 38.75 to 180.03 min [5].

Although immobilization has resulted in significant improvements in the enzyme performance, this method is acknowledged to involve a weak binding of the enzyme on a matrix. This weak binding may result in the release of some enzyme from the matrix during the reaction progression, leading to limited reusability of the enzyme [2]. In this regard, the selection of suitable support is very important to optimize the performance of the immobilized enzyme.

In this research, the *A. fumigatus* α-amylase was immobilized on chitin as an effort to increase the stability and reusability of the enzyme. *A. fumigatus* is a very promising source of enzyme on an industrial scale, since this fungus does not require special nutrients [13]. Chitin has been reported as an effective organic support for enzyme immobilization, with significant enhancement of the performance, efficiency, and stability of the immobilized enzymes. As a result, better reusability of the enzymes can be achieved which makes them more cost-effective when applied in industrial-scale processes [14]. Chitin (poly-(β(1,4)-2-acetamide-2-deoxy-D-glucopironase)) is a linear polysaccharide formed by N–acetyl–D–glucosamine monomer in –β linkage position, and can be isolated from the exoskeleton of crustaceans, insects, and invertebrates [15, 16]. Chitin is insoluble in water, chemically inert and thermally stable, abundantly available and cheap, and environmental friendly [17, 18]. The amine, amide, or hydroxyl groups in a chitin structure may undergo the non-covalent interaction with the enzyme by forming the hydrogen bonds [19]. Physical interaction between the enzyme and chitin offers several benefits, such as: does not affect to the enzyme conformation, the enzyme is easily separated from the matrix, and the matrix can be regenerated [20]. According to a previous research, the α-glucosidase enzyme from *Thermatoga maritime* which immobilized on a chitin can be used repeatedly up to 10 times and retained 66% of its initial activity [21]. Likewise, the *A. fumigatus* α-amylase immobilized on a chitin/bentonite hybrid matrix has greater thermal stability about 3.8 times than the native form, and maintained 72% of its initial activity after incubating at 60ºC for 80 min [22].

With respect to the previous findings by others, immobilization of *A. fumigatus* α-amylase on chitin was carried out in this study to evaluate the effect of immobilization on the stability and reusability of the immobilized enzyme. The results obtained in present study are valuable basis for further investigations to optimize the performance of the *A. fumigatus* α-amylase in order to satisfy industrial needs.

2- Materials and Methods

Local isolate of *A. fumigatus* was obtained from the Laboratory of Microbiology, Department of Biology, Lampung University. Chitin and all other chemicals reagents in analytical grade were purchased from Sigma-Aldrich™ and were used as received.

2-1- Research Procedure

The experiments were carried out in accordance with procedures applied in previous study [22], and briefly consists of production, isolation, partial purification, immobilization, and characterization of the native and immobilized enzymes. Production, isolation, and partial purification of native enzyme by precipitation using ammonium sulphate and dialysis has been done in our previous research. The protein content was determined based on the Lowry method to estimate the specific activity and purity of the enzyme [23]. The native and immobilized enzymes were analyzed to determine optimum temperature, steady-state kinetics (K_M and V_{max}), thermal stability, thermodynamic parameters ($t_{½}$, k_i, and ΔG_i), and reusability study. The flowchart of the research is presented in Figure 1.

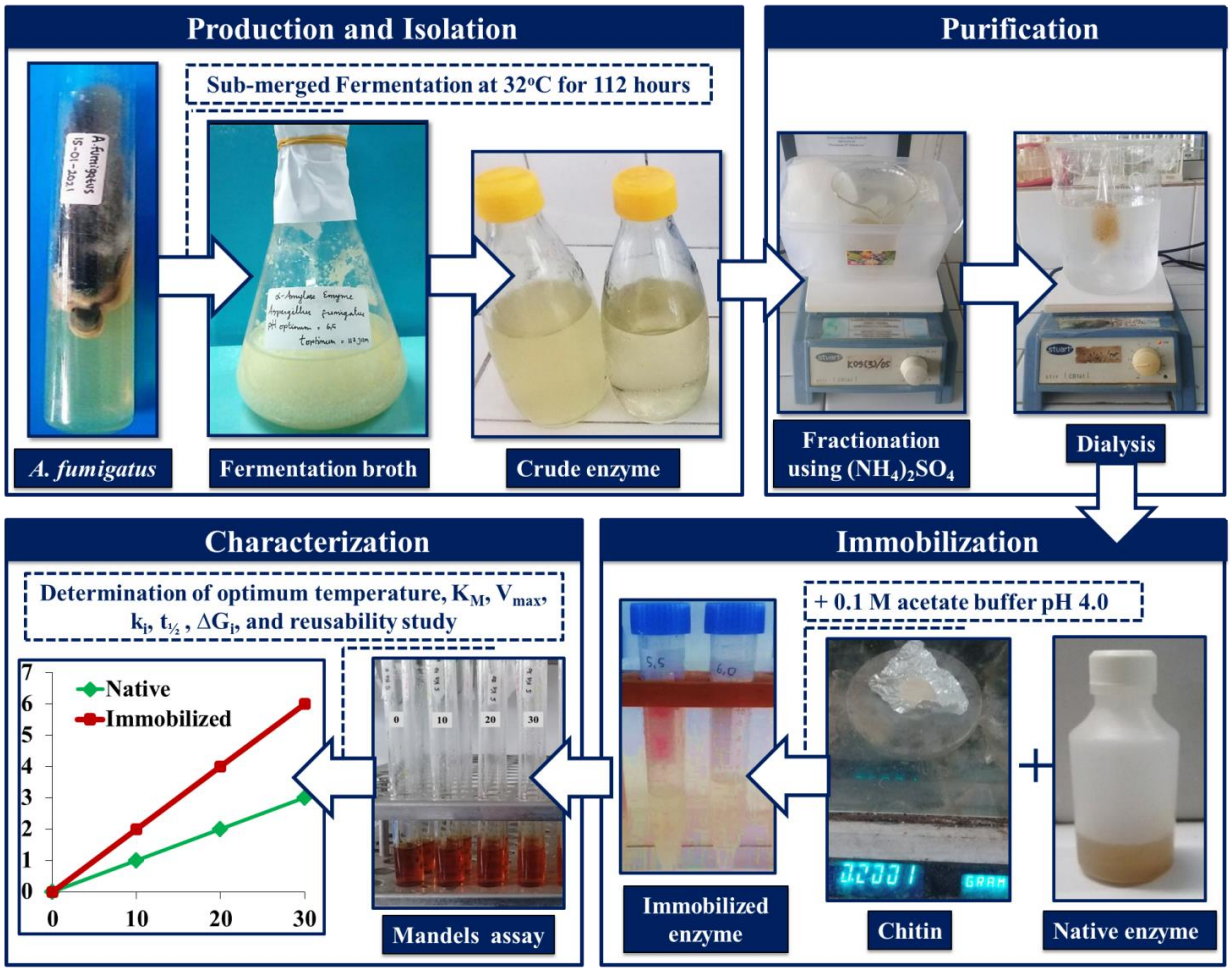

Figure 1. The flowchart of the research

2-2- Determination of Initial Buffer pH

This procedure was based on previous researches [22, 24]. The native enzyme (0.5 mL) was immobilized onto 0.20 g of chitin using 0.1 M acetate/phosphate buffer with a pH variation of 4.0; 4.5; 5.0; 5.5; and 6.0. Acetate buffer was particularly used for pH 4.0 and 4.5. An aliquot of 0.25 mL of the supernatant of the immobilized enzyme was taken as a "binding" sample, then the enzyme activity was determined by the Fuwa method using iodine reagent and defined based on the amount of substrate reduced after 10 min incubation [25]. The immobilized enzyme was then eluted from the matrix using 1.0 mL of a mixture of 0.1 M phosphate buffer pH 8.0 and 1 M NaCl (1:1). The supernatant was separated and 0.25 mL of the supernatant was taken as an "eluted" sample, then the enzyme activity was assayed. From this experiment, the initial pH was determined and used for further immobilization experiment.

2-3- Immobilization of α-Amylase onto Chitin

Immobilization was conducted following the method as described in pervious works [22, 24, 26]. The native enzyme (0.5 mL) was immobilized onto 0.20 g of chitin using 0.5 mL of the buffer with the predetermined initial pH. The enzyme was incubated at 4°C for 30 min, then centrifuged for 10 min. An aliquot of 0.25 mL of the supernatant was taken to be used as a control. Then, 0.75 mL of starch substrate was added to the immobilized enzyme, followed by incubation at 60°C for 30 min, and eventually centrifuged for 15 min. The amount of reducing sugar in the supernatant was determined by Mandel's method using dinitrosalicylic acid reagent, and used as a base to determine the activity of the enzyme [27].

2-4- Determination of Optimum Temperature

This variable was determined since each enzyme has a temperature range, known as optimum temperature, for achieving the maximum rate of reaction [2]. According to the previous findings, the optimum temperature of the *A. fumigatus* α-amylase was in the range of 50-55°C, but higher range was reported after immobilization [5, 22, 24]. In this present study, determination of optimum temperature was carried out by performing the experiments at different incubation temperatures as specified in Mandel's assay, which are 50, 55, 60, 65, 70, and 75°C. The experiments were run for 30 min, and the incubation temperature at which the highest enzyme activity was achieved was taken as the optimum temperature. As a common rule, it is acknowledged that the higher the relative activity at extreme temperature, the more thermo-stable the enzyme is [20].

2-5- Steady-state Kinetics

In enzymatic reaction, the rate of product formation depends on the availability of substrate. The relationship between substrate concentration and the rate of an enzyme-catalyzed reaction is described by the Lineweaver–Burk equation (Equation 1). This equation is used to calculate the kinetic parameters of the reaction, namely Michaelis constant (K_M) and the maximum reaction rate (V_{max}). The K_M constant indicates the amount of substrate in order to become saturated by the enzyme and generally refer to K_M as a measure of the affinity of the enzyme for its substrate (The K_M is a constant that is equivalent to the concentration of substrate at which the reaction takes place at one half its maximum rate), while the V_{max} measures the extent of the activity [20]. According to previous works, the optimum substrate concentration for the *A. fumigatus* α-amylase was in the range of 0.2-0.4% and become higher 2-4 times after immobilization [5, 22, 24]. In present study, the Lineweaver–Burk graph was constructed by plotting the enzyme activity against the concentrations of starch (0.2, 0.4, 0.6, 0.8, and 1.0%).

$$\frac{1}{v} = \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

where, K_M= Michaelis constant; V_{max} is maximum reaction rate; v = initial reaction rate; and [S] is substrate concentration.

2-6- Determination of Thermal Stability

Thermal stability of an enzyme can be determined by first exposing the protein to a range of temperatures for a fixed period of time, and subsequently measuring its activity at one favourable temperature [20]. The method used in current investigation was based on previous studies [5, 22, 24]. The thermal stability of the *A. fumigatus* α-amylase was estimated from the remaining or residual activity after inactivation at 60 oC for varied inactivation time (ti) of 0, 10, 20, 30, 40, 50, 60, 70, and 80 min [28, 29]. The enzyme activity was assayed by Mandel's method, and the data obtained were used to determine k_i, $t_{½}$, and ΔG_i. The residual activity was determined using Equation 2.

$$\text{Residual activity (\%)} = \frac{E_i}{E_o} \times 100\% \quad (2)$$

where E_o is the residual activity at to; and E_i is the residual activity at ti [25]. The higher the residual activity after incubation, the more thermo-stable the enzyme is [20].

2-7- Determination of $t_{½}$, k_i, and ΔG_i

The enzyme stability improvement is indicated by its half-life and thermodynamic parameters, namely the thermal inactivation rate constant (k_i) and the change of free energy due to denaturation (ΔG_i) [10, 11]. The half-life ($t_{½}$) is the time required for the enzyme to break down substrate until the enzyme loses half of its activity. Therefore, the longer the half-life, the more stable the enzyme is against denaturation [11]. Thermal inactivation rate constant (k_i) and half-life ($t_{½}$) were calculated using the first-order enzyme inactivation rate Equation 3.

$$\ln\left(\frac{E_i}{E_o}\right) = -k_i \times t_i \quad (3)$$

where k_i is the thermal inactivation rate constant; E_o is the residual activity at t_o; E_i is the residual activity at t_i; and t_i is the thermal inactivation time [11]. The slope of the graph is calculated using Equation 4.

$\ln\left(\frac{E_i}{E_o}\right)$ against t_i is k_i. (4)

The free energy change due to denaturation (ΔG_i) is the energy required to change the enzyme from the initial state to denaturated state. The value of ΔG_i was calculated from thermodynamic Equation 5.

$$\Delta G_i = -RT \ln \frac{k_i.h}{k_B.T} \quad (5)$$

where ΔG_i is the free energy change due to denaturation; R is the ideal gas constant; T is the thermal inactivation temperature (K); k_i is the thermal inactivation rate constant; h is the Planck constant, and k_B is the Boltzman constant [11]. The lower the k_i. and the higher the ΔG_i, the more stable and rigid the enzyme is [11].

2-8- Reusability Study

One important benefits offered by immobilized enzyme is reusability which enables continuous process to be conducted [30]. In present study, the previously used immobilized enzyme was rewashed using the initial buffer. The enzyme was then reacted with a new starch substrate (0.75 mL) and assayed by Mandel's method, as described on previous literatures [22, 24]. In this study, the reusability test was performed in sixth cycles.

2-9- Statistical Analysis

All measurements were done in duplicate (n = 2), and data were reported as mean ± standard deviation (SD). Analysis of variance (ANOVA) together with the student t-test (Paired Two Sample for Means) were conducted to identify the significant differences between two replicate samples. In this analysis, the level of significance was set at $p < 0.05$.

3- Results and Discussions

3-1- Determination of Initial Buffer pH

The experimental results showing the activities of immobilized (binding) enzyme and eluted enzyme at different *pH*s are presented in Figure 2.

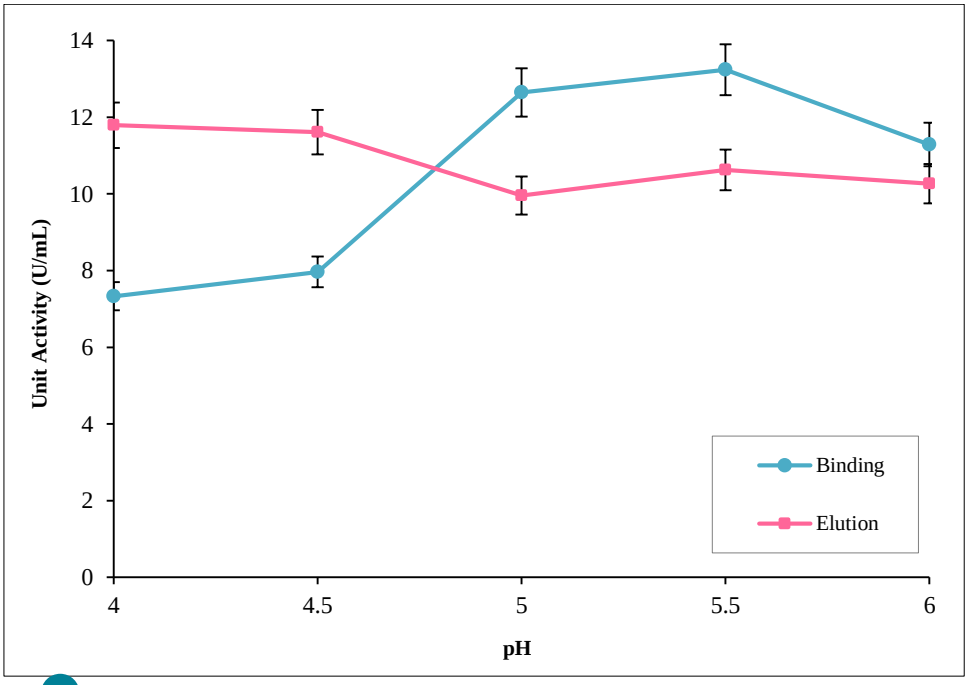

Figure 2. Initial buffer pH for enzyme immobilization (mean ± SD; n = 2; p < 0.05)

The results in Figure 2 suggest that the α-amylase molecules were successfully bound onto chitin at the acidic pH range of 4.0-4.5 which were achieved using 0.1 M acetate buffer. The results also indicate that the activity at pH 4.0 is lightly higher than that of pH 4.5, therefore 0.1 M acetate buffer pH 4.0 was selected as the initial buffer. In previous studies [31, 32], it was suggested that at the pH in range of 4.0-4.5, the enzyme cationic form exists due to the protonation of the amine group, while the presence of carbonyl and hydroxyl groups makes the surface of the chitin becomes negatively charged. As a result, immobilization of the enzyme takes place due to electrostatic interaction between positively charged enzyme molecules and negatively charged chitin molecules [33]. Since the interaction between enzyme and chitin molecules involves hydrogen bond, this interaction is influenced by the pH [19, 34], implying that increased pH will result in decreased activity of the enzyme. The results of the current study are in agreement with this trend as shown by decreased activity of the immobilized enzyme due to increase the pH. The results obtained are also in agreement with the pervious findings by others [5], which show that s immobilized α-amylase on a chitosan/zeolite matrix exhibited maximum activity at the acidic pH [5]. Other workers reported that the optimum pH for immobilization β-glucosidase on a chitin is at pH 6.5 [21]. In the study, phosphate buffer was used for pH adjustment and in this case it was suggested that immobilization involves interaction between the negatively charged enzyme and positively charged amino groups of the chitin [21]. Illustration of α-amylase immobilization on a chitin via physical adsorption is shown in Figure 3.

3-2- Determination of Optimum Temperature

At the optimum temperature, the substrate and enzyme molecules will have an ideal kinetic energy to optimize the collisions, resulting maximum rate of reaction. At such temperature, the active sites of the enzyme are properly open to interact with the substrate to form the enzyme-substrate complex [35]. The effect of temperature on the activity of the native and immobilized enzymes can be seen in Figure 4.

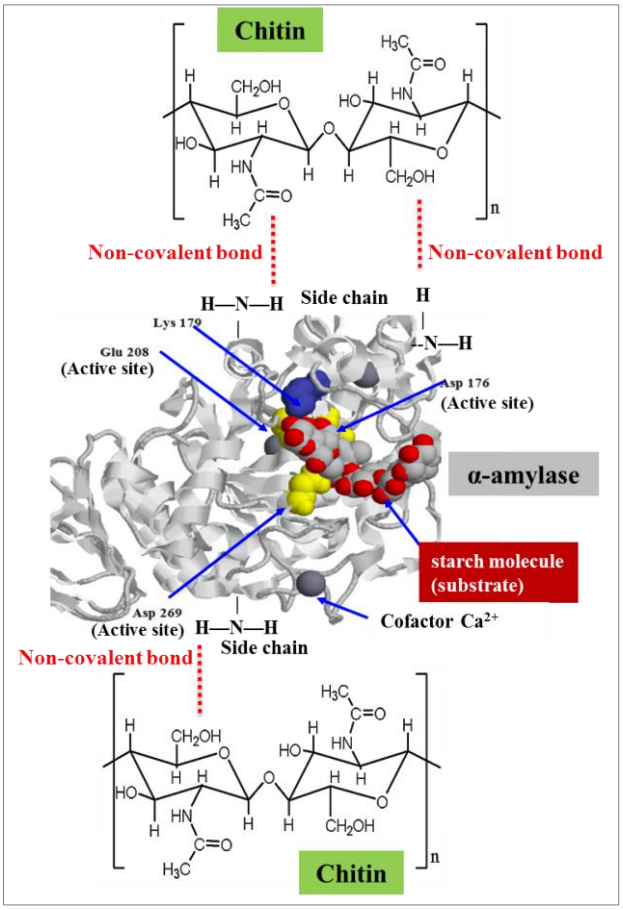

Figure 3. The illustration of α-amylase immobilization on a chitin via physical adsorption

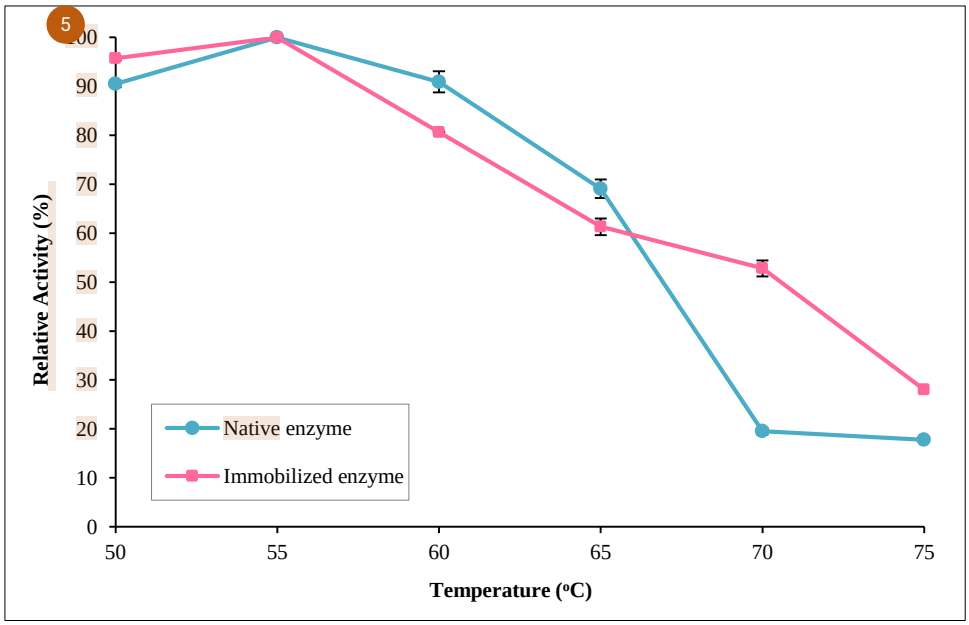

Figure 4. Optimum temperature of the native and immobilized enzymes (mean ± SD; n = 2; p < 0.05)

As presented in Figure 4, both the native and immobilized enzymes have the same optimum temperature of 55°C. The same optimum temperature was also reported by others [5] for the α-amylase immobilized on a zeolite/chitosan hybrid. The experimental results in Figure 4 also indicate quite significant decrease of the activity at higher temperatures. However, the average activity of the immobilized enzyme at extreme temperatures (70 and 75 °C) was higher, justifying better thermo-stability of the immobilized enzyme, which also means that the immobilized enzyme is more resistant to thermal denaturation. Hence, chitin is a promising option as a supporting matrix for immobilization of the *A. fumigatus* α-amylase, with the potential application such as batch process using a stirred-tank reactor, although further research to increase stability of the enzyme at higher temperatures is still needed.

As a comparison, optimum temperature for the native and immobilized *A. fumigatus* α-amylase obtained in this study and the results reported by others in the latest experiments are summarized in Table 1.

Table 1. Optimum temperature of the *A. fumigatus* α-amylase in the latest experiments

Matrix Type	Optimum Temperature (°C)		Ref.
	Native enzyme	Immobilized enzyme	
Zeolite/chitosan hybrid	50	55	[5]
Bentonite	55	70	[22]
Chitin/bentonite hybrid	55	60	[24]
Chitin	55	55	Present Study

The results presented in Table 1 imply that although slightly differences with the use of different supports should be acknowledged, in overall the results are comparable.

3-3- Steady-State Kinetics

The Lineweaver-Burk plots for native and immobilized enzyme, constructed from the experimental results conducted at optimum temperature and the starch concentrations from 2.0 to 10.0 mg/mL are shown in Figure 5, and the kinetic parameters obtained together with the findings reported by others are compiled in Table 2.

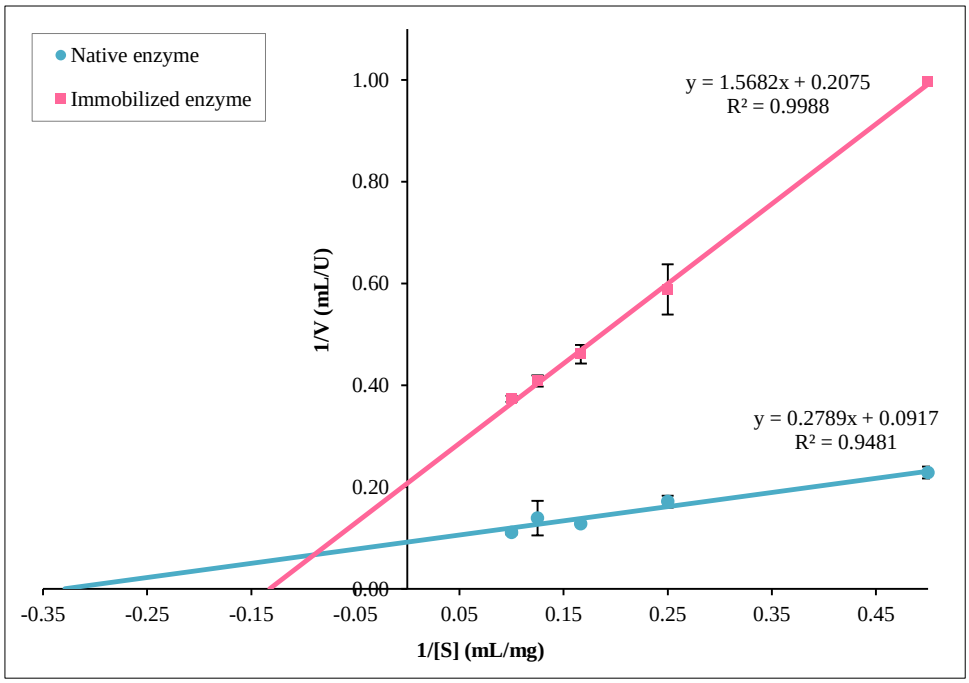

Figure 5. The Lineweaver-Burk plots for native and immobilized enzyme, constructed from the experimental results conducted at optimum temperature and the starch concentrations from 2.0 to 10.0 mg/mL

Table 2. Kinetic parameters for the native and immobilized *A. fumigatus* α-amylase on different matrices

Matrix Type	K_M (mg mL^{-1})		V_{max} (µmole mL^{-1} min^{-1})		Ref.
	Native enzyme	Immobilized enzyme	Native enzyme	Immobilized enzyme	
Zeolite/chitosan hybrid	3.48 ± 0.27	12.05 ± 4.95	2.21 ± 0.10	1.60 ± 0.58	[5]
Bentonite	3.04 ± 1.04	8.31 ± 2.67	10.90 ± 1.89	1.44 ± 0.30	[22]
Chitin/bentonite hybrid	3.04 ± 1.04	11.57 ± 0.76	10.90 ± 1.89	3.37 ± 0.12	[24]
Chitin	3.04 ± 1.04	7.56 ± 0.77	10.90 ± 1.89	4.82 ± 0.40	Present Study

(All data values were shown as mean ± SD, n = 2)

The results of present study indicate that the immobilized enzyme has higher K_M and lower V_{max} than the native enzyme. These results imply that the immobilization of the enzyme leads to a lower affinity and accessibility to the substrate, and requires a greater substrate concentration to reach V_{max}. Consequently, the immobilized enzyme showed a significant decrease in reaction rate compared to that of the native enzyme. Other workers suggest that decreased

activity of enzyme is caused by structural changes, thereby reducing the V_{max} of the reaction [20, 36]. Chitin matrix entrapped the enzyme molecules and prevented the entrance of substrate onto the active site of the enzyme [6]. Compared to the results by others as presented in Table 2, it can be seen that the K_M observed in present study is relatively smaller, most likely due to a lack of functional groups in the chitin surface strengthened the non-covalent bonds between the enzyme and the matrix, as suggested by others [19]. Unlike K_M, lower V_{max} in present study as compared to the native enzyme and other matrices was observed, implying that the reaction by immobilized α-amylase on a chitin was faster and produced more products.

3-4- Determination of Thermal Stability

The results of thermal stability assay of the native and immobilized enzymes can be seen in Figure 6, showing gradual decrease of residual activity following an increase in inactivation time and comparison with other studies are presented in Table 3.

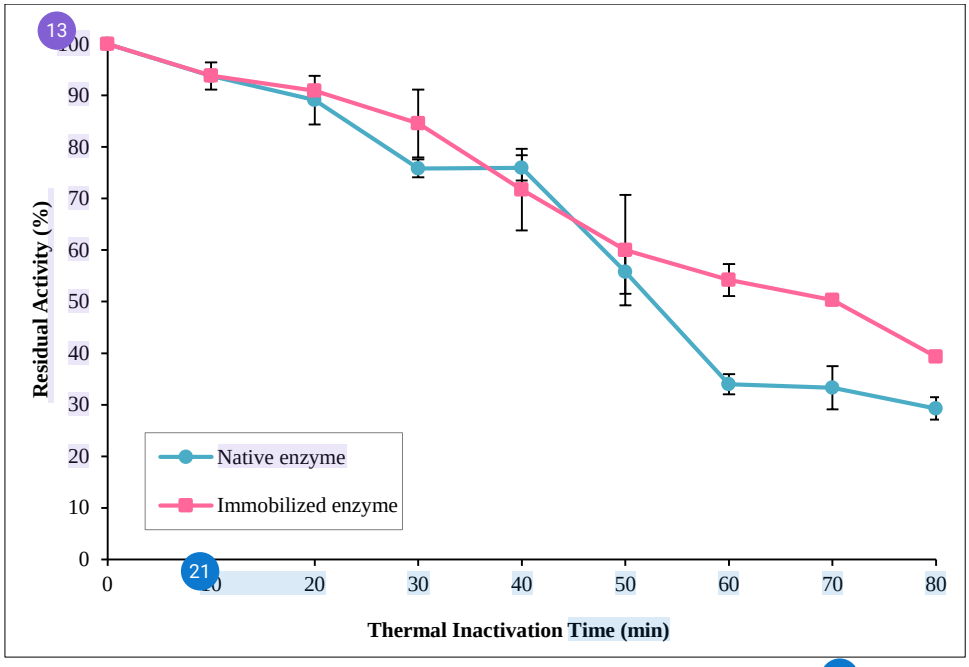

Figure 6. Thermal stability of the native and immobilized enzymes (mean ± SD; n = 2; p < 0.05). The data were determined at optimum temperature and starch concentration. The initial enzyme activity was assuming 100%

Table 3. Thermal stability of *A. fumigatus* α-amylase immobilized on different matrices

Matrix	Residual activity (%)		Thermal inactivation rate constant (k_i) (min^{-1})		Ref.
	Native enzyme	Immobilized enzyme	Native enzyme	Immobilized enzyme	
Bentonite	28	60	0.0171 ± 0.0008*	0.0060 ± 0.0003*	[22]
Bentonite/Chitin hybrid	28	71	0.0171 ± 0.0008*	0.0045 ± 0.0004*	[24]
Zeolite/Chitosan hybrid	21	72	0.0179	0.0037	[5]
Chitin	28	39	0.0171 ± 0.0008*	0.0117 ± 0.0002*	Present Study

* Values were shown as mean ± SD, n = 2. All data were determined after 80 min of incubation at 60°C.

As a comparison, thermal stability of *A. fumigatus* α-amylase immobilized at different matrices are presented in Table 3. The results in Table 3 showed a significant increase in thermal stability for the immobilized enzyme compared to that of the native enzyme, as demonstrated by the higher initial activity of the immobilized enzyme. The results also indicate that the residual activity of the native enzyme after inactivation at 60°C for 80 min was only 29% while the immobilized enzyme maintained residual activity of 39%, implying the ability of chitin to protect the enzyme molecules from heat denaturation. At higher temperatures, the native enzyme is degraded more easily leading to shorter half-life, due to thermal degradation and denaturation of the enzyme, while the immobilized enzyme could maintain its activity and stability from denaturation effect as a result of protection by chitin, as has also been suggested by others [2, 22].

Thermal stability of the immobilized enzyme is related to the ability of chitin to provide non-covalent linkages to the enzyme molecules by forming the hydrogen bonds between amine, amide, or hydroxyl groups in a chitin structure and amine groups in side chains of the enzyme [19]. These linkages prevented conformational changes that might occur at

higher temperatures and helped to retain the proper tertiary structure of the enzyme [37]. With reference to adsorption, the increase in thermal stability was attributed to the increase of enzyme rigidity due to the strong electrostatic interaction [37]. In addition, thermal stability has relationship to the k_i value which is a measure of the enzyme rigidity, stability, and less flexibility in the water. The enzyme adsorption on a chitin prevents the entrance water molecules to active sites of the enzyme.

3-5- Determination of $t_{½}$, k_i, and ΔG_i

The enzyme stability improvement can be investigated by determination of half-life and thermodynamic parameters, include thermal inactivation rate constant (k_i) and the change of free energy due to denaturation (ΔG_i) [10, 11]. The residual activities of native and immobilized enzymes from thermal stability assay were plotted to the first-order enzyme inactivation rate graph shown in Figure 7. The slope of the graph is thermal inactivation rate constant (k_i), and this constant was used to calculate $t_{½}$ and ΔG_i, as summarized in Table 4.

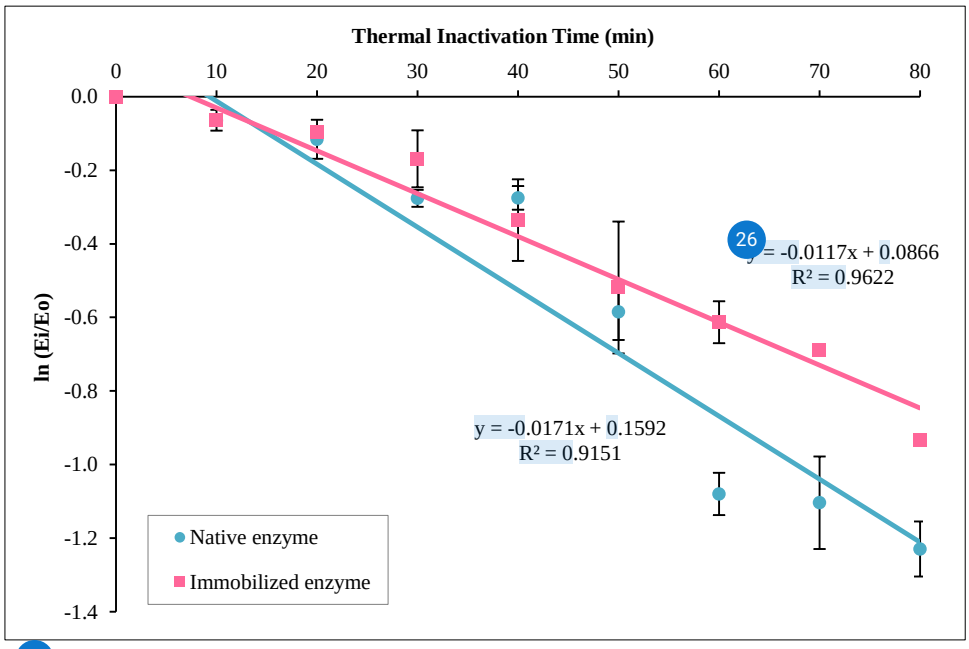

Figure 7. First-order inactivation rate plot of native and immobilized enzymes (mean ± SD; n = 2; p < 0.05)

Table 4. $t_{½}$ and ΔG_i values for native and immobilized *A. fumigatus* α-amylase on different matrices

Matrix	Half life ($t_{1/2}$) (min)		The transformation of free energy because denaturation (ΔG_i) (kJ mole^{-1})		Stability Improvement	Ref.
	Native enzyme	Immobilized enzyme	Native enzyme	Immobilized enzyme		
Bentonite	40.53 ± 1.90	115.50 ± 5.79	104.47 ± 0.13	107.37 ± 0.14	2.9	[22]
Bentonite/Chitin hybrid	40.53 ± 1.90	154.00 ± 13.40	104.47 ± 0.13	108.17 ± 0.24	3.8	[24]
Zeolite/Chitosan hybrid	38.75 ± 1.53	180.03 ± 3.31	104.35 ± 1.09	108.03 ± 0.05	4.6	[5]
Chitin	40.53 ± 1.90	59.23 ± 0.78	104.47 ± 0.13	105.52 ± 0.04	1.5	Present Study

(All data values were shown as mean ± SD, n = 2. The data were determined after 80 min of incubation at 60ºC.)

The results in Table 4, indicate that thermal stabilization of the immobilized enzyme was successfully improved, as demonstrated by a significant decrease of k_i, the increase of half-life ($t_{½}$), and increase of ΔG_i, compared to those of native enzyme The decrease in k_i for immobilized enzyme is associated with the decrease of denaturation rate due to the less flexibility in the water. Therefore, the folding conformation in the immobilized enzyme structure will increase, resulting in higher stability [38-40]. Comparing to the results by others as presented in Table 3, it can be seen that higher k_i was obtained in present study, suggesting that the rigidity of the immobilized enzyme on a chitin is slightly weaker than that of the immobilized enzyme on other matrices. This weaker rigidity might ease the entrance of the water to the active sites of enzyme and obstructed the formation enzyme-substrate complex, leading to lower stability [37].

3-6- Reusability Study

The results of reusability experiment are presented in Figure 8, showing gradual decrease of the activity from initial activity of 100% to 46% after six reuse.

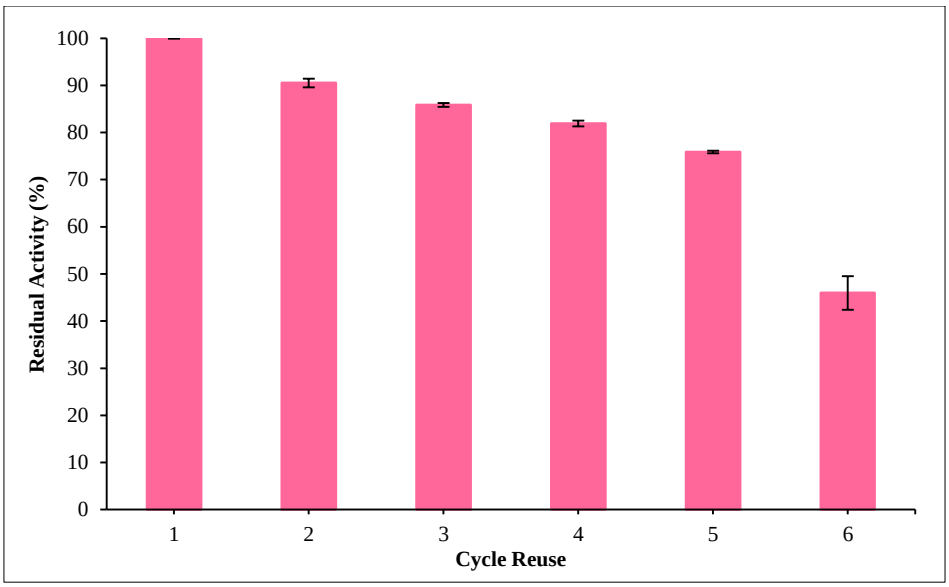

Figure 8. Residual activities of the immobilized enzyme (mean ± SD; n = 2; p < 0.05) for six subsequent uses. Assuming the initial enzyme activity was 100%

As shown by the graph, the reuse of immobilized enzyme led to a gradual decrease of the activity, from an initial activity of 100% to 91, 86, 82, 76, and 46%, after the sixth application, suggesting the gradual release of the enzyme from the matrix due to rewashing treatment as has been suggested by previous workers [22]. As can be seen, the residual activity after sixth application is 46%, which is considered relatively high, suggests that the immobilized enzyme has the potential to be reused more than sixth cycles. In addition, these results also imply that the linkage between matrix and the enzyme does not disturb the active site so that the enzyme retains catalytic activity for a longer time.

Compared to results of other reusability studies, as complied in Table 5, the result of this current study is higher than those reported by others, particularly compared to that of immobilized enzyme on zeolite/chitosan hybrid.

Table 5. Reusability study for immobilized *A. fumigatus* α-amylase in the latest experiments

Matrix Type	Reuse Cycles	Residual Activity (%)	Ref.
Bentonite	6	42	[22]
Bentonite/chitin hybrid	6	36	[24]
Zeolite/chitosan hybrid	5	11	[5]
Chitin	6	46	Present Study

The results in Table 5 clearly indicate the promising potential of chitin as a supporting matrix for development of immobilized α-amylase for commercial applications, although further research is still need. In addition, chitin is environmentally friendly, naturally available, and the adsorbed can be easily separated by centrifugation.

4- Conclusion

This research demonstrates that chitin is a promising matrix for immobilization of *A. fumigatus* α-amylase. A series of experiments carried out revealed appreciable improvement in the performance of the immobilized enzyme, including improved thermal stability, as indicated by the ability of the enzyme to retain relative activity above 39% after 80 min of incubation at 60°C. Thermodynamic parameters, including k_i, ΔG_i , and $t_{½}$, indicate that the immobilized enzyme is more rigid, stable, and less flexible in the water, resulting in increased stability up to 1.5 times compared to that of the native enzyme. Furthermore, the immobilized enzyme was able to retain over 46% of its initial activity after six consecutive applications for starch hydrolysis, confirming the potential of chitin for the production of immobilized enzymes on an industrial scale.

5- Declarations

5-1- Author Contributions

Conceptualization, Y.Y. and E.R.T; methodology, Y.Y.; software, S.H.; validation, Y.Y., T.S. and H.S.; formal analysis, S.H.; investigation, S.H.; resources, B.I.; data curation, Y.Y.; writing—original draft preparation, E.R.T.; writing—review and editing, S.H.; visualization, E.R.T.; supervision, T.S.; project administration, S.H.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript.

5-2- Data Availability Statement

The data presented in this study are available on request from the corresponding author.

5-3- Funding

The authors are grateful to the Ministry of Education, Culture, Research and Technology for funding this project in the form of Basic Research 2022 with contract numbers 114/E5/PG.02.00.PT/2022 on 10th May 2022 and 2147/UN26.21/PN/2022 on 13th May 2022.

5-4- Institutional Review Board Statement

Not applicable.

5-5- Informed Consent Statement

Not applicable.

5-6- Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

6- References

[1] Far, B. E., Ahmadi, Y., Khosroushahi, A. Y., & Dilmaghani, A. (2020). Microbial alpha-amylase production: Progress, challenges and perspectives. Advanced Pharmaceutical Bulletin, 10(3), 350–358. doi:10.34172/apb.2020.043.

[2] Vogel, A., & May, O. (2019). Industrial Enzyme Applications (1stEd.). Wiley-VCH, Hoboken, New Jersey, United States. doi:10.1002/9783527813780.

[3] Vitolo, M. (2001). Enzymatic Modification of starch. World Journal of Pharmacy and Pharmaceutical Sciences, 9(4), 1341–1358. doi:10.20959/wjpps/20204-15958.

[4] Ebrahimi, M., Akhavan, T., & Hekmat, A. (2020). Structural evidence for kinetic and thermal stability changes of α-amylase due to exposure to [emim][lactate] ionic liquid. Turkish Journal of Biochemistry, 45(6), 785–791. doi:10.1515/tjb-2019-0270.

[5] Yandri, Y., Ropingi, H., Suhartati, T., Hendri, J., Irawan, B., & Hadi, S. (2022). The Effect of Zeolite/Chitosan Hybrid Matrix for Thermal-stabilization Enhancement on the Immobilization of Aspergillus fumigatus α-Amylase. Emerging Science Journal, 6(3), 505–518. doi:10.28991/ESJ-2022-06-03-06.

[6] Cheetham, P. (1981). Immobilized enzymes: An introduction and applications in biotechnology. Biochemical Education, 9(3), 119. doi:10.1016/0307-4412(81)90251-x.

[7] Zhang, Y., Ge, J., & Liu, Z. (2015). Enhanced Activity of Immobilized or Chemically Modified Enzymes. ACS Catalysis, 5(8), 4503–4513. doi:10.1021/acscatal.5b00996.

[8] Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme and Microbial Technology, 40(6), 1451–1463. doi:10.1016/j.enzmictec.2007.01.018.

[9] Mohammadi, M., Rezaei Mokarram, R., Shahvalizadeh, R., Sarabandi, K., Lim, L. T., & Hamishehkar, H. (2020). Immobilization and stabilization of pectinase on an activated montmorillonite support and its application in pineapple juice clarification. Food Bioscience, 36, 1–8. doi:10.1016/j.fbio.2020.100625.

[10] Ikegaya, K. (2005). Kinetic analysis about the effects of neutral salts on the thermal stability of yeast alcohol dehydrogenase. Journal of Biochemistry, 137(3), 349–354. doi:10.1093/jb/mvi037.

[11] Kazan, D., Ertan, H., & Erarslan, A. (1997). Stabilization of Escherichia coli penicillin G acylase against thermal inactivation by cross-linking with dextran dialdehyde polymers. Applied Microbiology and Biotechnology, 48(2), 191–197. doi:10.1007/s002530051037.

[12] Dp, S., Vu, B., Geetha G, & Pv, M. (2019). Improvement in the Properties of α-Amylase Enzyme by Immobilization using Metal Oxide Nanocomposites as Carriers. Advances in Nanomedicine and Nanotechnology Research, 2(1), 77–88.

[13] Van De Veerdonk, F. L., Gresnigt, M. S., Romani, L., Netea, M. G., & Latgé, J. P. (2017). Aspergillus fumigatus morphology and dynamic host interactions. Nature Reviews Microbiology, 15(11), 661–674. doi:10.1038/nrmicro.2017.90.

[14] Anu Bhushani, J., & Anandharamakrishnan, C. (2014). Electrospinning and electrospraying techniques: Potential food based applications. Trends in Food Science and Technology, 38(1), 21–33. doi:10.1016/j.tifs.2014.03.004.

[15] Mei, S., Han, P., Wu, H., Shi, J., Tang, L., & Jiang, Z. (2018). One-pot fabrication of chitin-shellac composite microspheres for efficient enzyme immobilization. Journal of Biotechnology, 266(1), 1–8. doi:10.1016/j.jbiotec.2017.11.015.

[16] Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. Reactive and Functional Polymers, 46(1), 1–27. doi:10.1016/S1381-5148(00)00038-9.

[17] Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. Marine Drugs, 13(3), 1133–1174. doi:10.3390/md13031133.

[18] Zdarta, J., Meyer, A. S., Jesionowski, T., & Pinelo, M. (2018). A general overview of support materials for enzyme immobilization: Characteristics, properties, practical utility. Catalysts, 8(2), 1–27. doi:10.3390/catal8020092.

[19] Saravanan, D., Hemalatha, R., & Sudha, P. N. (2011). Synthesis and characterization of cross linked chitin/bentonite polymer blend and adsorption studies of Cu (II) and Cr (VI) on chitin. Der Pharma Chemica, 3(6), 406–424.

[20] Robinson, P. K. (2015). Enzymes: principles and biotechnological applications. Essays in Biochemistry, 59(1), 1–41. doi:10.1042/BSE0590001.

[21] Alnadari, F., Xue, Y., Zhou, L., Hamed, Y. S., Taha, M., & Foda, M. F. (2020). Immobilization of β-Glucosidase from Thermatoga maritima on Chitin-functionalized Magnetic Nanoparticle via a Novel Thermostable Chitin-binding Domain. Scientific Reports, 10(1), 1–12. doi:10.1038/s41598-019-57165-5.

[22] Yandri, Y., Tiarsa, E. R., Suhartati, T., Satria, H., Irawan, B., & Hadi, S. (2022). The Stability Improvement of α -Amylase Enzyme from Aspergillus fumigatus by Immobilization on a Bentonite Matrix. Biochemistry Research International, 2022(1), 1–7. doi:10.1155/2022/3797629.

[23] Classics Lowry, O., Rosebrough, N., Farr, A., & Randall, R.. (1951). Protein Measurement with the Folin Phenol Reagent. Journal of Biological Chemistry, 193(1), 265–275. doi:10.1016/s0021-9258(19)52451-6.

[24] Tiarsa, E. R., Yandri, Y., Suhartati, T., Satria, H., Irawan, B., & Hadi, S. (2022). The Stability Improvement of Aspergillus fumigatus α -Amylase by Immobilization onto Chitin-Bentonite Hybrid. Biochemistry Research International, 2022(1), 1–9. doi:10.1155/2022/5692438.

[25] Fuwa, H. (1954). A new method for microdetermination of amylase activity by the use of amylose as the substrate. Journal of Biochemistry, 41(5), 583–603. doi:10.1093/oxfordjournals.jbchem.a126476.

[26] Yandri, Suhartati, T., Yuwono, S. D., Qudus, H. I., Tiarsa, E. R., & Hadi, S. (2018). Immobilization of α-amylase from bacillus subtilis ITBCCB148 using bentonit. Asian Journal of Microbiology, Biotechnology and Environmental Sciences, 20(2), 487–492.

[27] Eveleigh, D. E., Mandels, M., Andreotti, R., & Roche, C. (2009). Measurement of Saccharifying Cellulase. Biotechnology for Biofuels, 2(1), 21. doi:10.1186/1754-6834-2-21.

[28] Ahmed, N. E., El Shamy, A. R., & Awad, H. M. (2020). Optimization and immobilization of amylase produced by Aspergillus terreus using pomegranate peel waste. Bulletin of the National Research Centre, 44(1), 1–12. doi:10.1186/s42269-020-00363-3.

[29] Yang, Z., Domach, M., Auger, R., Yang, F. X., & Russell, A. J. (1996). Polyethylene glycol-induced stabilization of subtilisin. Enzyme and Microbial Technology, 18(2), 82–89. doi:10.1016/0141-0229(95)00073-9.

[30] Sirisha, V. L., Jain, A., & Jain, A. (2016). Enzyme Immobilization: An Overview on Methods, Support Material, and Applications of Immobilized Enzymes. Advances in Food and Nutrition Research, 79, 179–211. doi:10.1016/bs.afnr.2016.07.004.

[31] Emmerich, K., Madsen, F. T., & Kahr, G. (1999). Dehydroxylation behavior of heat-treated and steam-treated homoionic cis-vacant montmorillonites. Clays and Clay Minerals, 47(5), 591–604. doi:10.1346/CCMN.1999.0470506.

[32] Kumari, A., & Kayastha, A. M. (2011). Immobilization of soybean (Glycine max) α-amylase onto Chitosan and Amberlite MB-150 beads: Optimization and characterization. Journal of Molecular Catalysis B: Enzymatic, 69(1–2), 8–14. doi:10.1016/j.molcatb.2010.12.003.

[33] Genç, N., Dogan, E. C., & Yurtsever, M. (2013). Bentonite for ciprofloxacin removal from aqueous solution. Water Science and Technology, 68(4), 848–855. doi:10.2166/wst.2013.313.

[34] Fujimoto, Z., Takase, K., Doui, N., Momma, M., Matsumoto, T., & Mizuno, H. (1998). Crystal structure of a catalytic-site mutant α-amylase from Bacillus subtilis complexed with maltopentaose. Journal of Molecular Biology, 277(2), 393–407. doi:10.1006/jmbi.1997.1599.

[35] Fernandez-Lafuente, R. (2009). Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. Enzyme and Microbial Technology, 45(6–7), 405–418. doi:10.1016/j.enzmictec.2009.08.009.

[36] Abdel-Mageed, H. M., El-Laithy, H. M., Mahran, L. G., Fahmy, A. S., Mäder, K., & Mohamed, S. A. (2012). Development of novel flexible sugar ester vesicles as carrier systems for the antioxidant enzyme catalase for wound healing applications. Process Biochemistry, 47(7), 1155–1162. doi:10.1016/j.procbio.2012.04.008.

[37] Secundo, F. (2013). Conformational changes of enzymes upon immobilisation. Chemical Society Reviews, 42(15), 6250–6261. doi:10.1039/c3cs35495d.

[38] Yang, X. Y., Tian, G., Jiang, N., & Su, B. L. (2012). Immobilization technology: A sustainable solution for biofuel cell design. Energy and Environmental Science, 5(2), 5540–5563. doi:10.1039/c1ee02391h.

[39] Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., & Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnology & Biotechnological Equipment, 29(2), 205–220. doi:10.1080/13102818.2015.1008192.

[40] Almulaiky, Y. Q., El-Shishtawy, R. M., Aldhahri, M., Mohamed, S. A., Afifi, M., Abdulaal, W. H., & Mahyoub, J. A. (2019). Amidrazone modified acrylic fabric activated with cyanuric chloride: A novel and efficient support for horseradish peroxidase immobilization and phenol removal. International Journal of Biological Macromolecules, 140(78), 949–958. doi:10.1016/j.ijbiomac.2019.08.179.

23% Overall Similarity

Top sources found in the following databases:

- 20% Internet database
- Crossref database
- 8% Submitted Works database
- 14% Publications database
- Crossref Posted Content database

TOP SOURCES

The sources with the highest number of matches within the submission. Overlapping sources will not be displayed.

1	hindawi.com Internet	6%
2	researchgate.net Internet	5%
3	ijournalse.org Internet	2%
4	University of Central Oklahoma on 2022-12-07 Submitted works	1%
5	mdpi.com Internet	1%
6	Yandri Yandri, Hendri Ropingi, Tati Suhartati, Bambang Irawan, Sutopo ... Crossref	<1%
7	downloads.hindawi.com Internet	<1%
8	ncbi.nlm.nih.gov Internet	<1%

9	eprints.uad.ac.id Internet	<1%
10	Universitas Muhammadiyah Sinjai on 2022-10-05 Submitted works	<1%
11	scribd.com Internet	<1%
12	"Effect of Immobilization Towards Thermal Stability of a-Amylase Isola... Crossref	<1%
13	revroum.getion.ro Internet	<1%
14	East Tennessee State University on 2009-06-22 Submitted works	<1%
15	Linqiu Cao. "Carrier-bound Immobilized Enzymes", Wiley, 2005 Crossref	<1%
16	repository.lppm.unila.ac.id Internet	<1%
17	Higher Education Commission Pakistan on 2015-10-28 Submitted works	<1%
18	Mahidol University on 2022-12-15 Submitted works	<1%
19	Maryam Mohammadi, Reza Rezaei Mokarram, Rahim Shahvalizadeh, K... Crossref	<1%
20	Yandri Yandri, Nurmala Nurmala, Tati Suhartati, Heri Satria, Sutopo Ha... Crossref	<1%

21	mpge.ou.edu Internet	<1%
22	Bayramoglu, Gulay, Aydin Akbulut, and M. Yakup Arica. "Immobilizatio... Crossref	<1%
23	Kovalenko, G.A.. "Immobilization of glucoamylase by adsorption on car... Crossref	<1%
24	Pennsylvania State System of Higher Education on 2016-03-29 Submitted works	<1%
25	University of Malaya on 2022-06-19 Submitted works	<1%
26	Yeditepe University on 2022-05-15 Submitted works	<1%
27	dyuthi.cusat.ac.in Internet	<1%

Excluded from Similarity Report

- Bibliographic material
- Cited material
- Manually excluded sources
- Quoted material
- Small Matches (Less then 10 words)
- Manually excluded text blocks

EXCLUDED SOURCES

ijournalse.org

Internet

58%

EXCLUDED TEXT BLOCKS

In this research, immobilization of A. fumigatus α-amylase on chitin was studied w...

www.researchgate.net