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Simultaneous Optimization of Factors Affecting Native Starch Pretreatment and Enzymatic Hydrolysis Using Magnetic Covalent Immobilized α-Amylase

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Abstract

In this study, for the first time the effects of different factors on hydrolysis of low concentration of corn starch were investigated in two steps. In the first step, thermal hydrolysis with pH variation and sonication treatment and finally enzymatic hydrolysis of starch were investigated by one factor at a time method. The best conditions of maximum reducing sugar production and maximum releasing of amylose were starch with pH=4.5, sonication time 30 min and 24 hours solution storage in 4 °C with enzymatic hydrolysis by 335 ppm immobilized enzyme for 15 min. In the second step, the effects of three variables on starch hydrolysis to determine the optimal conditions were investigated by 2^3 factorial design with multiple responses desirability method. Starch concentration 0.5% (w/v), initial starch pH of 4.5 and enzymatic hydrolysis temperature 50°C were determined as optimum conditions for achieving maximum changes in reducing sugar concentration 92.48 (mg/g starch) and ratio of final and initial soluble amylose content of hydrolysate solution 0.62 (by more than 75% separation of immobilized enzyme on MNPs after the process by external magnetic field. Also, experiment showed the possibility of immobilized enzyme reusing, which can retain 40% of its ability to produce reducing sugar and retain magnetic features of nanoparticles after 6 cycles.

What is "already known":	• α-amylase was immobilized on the carriers for enzymatic hydrolysis of soluble starch. • Hydrolysis of native starch using immobilized enzyme requires pretreatment. • Solubility of native starch is increased by thermal hydrolysis and/or sonication.
What this article adds:	• Covalently immobilized α-amylase on MNPs for enzymatic hydrolysis of native starch • Simultaneous optimization of thermal and enzymatic hydrolysis operating conditions • Enhancing enzymatic hydrolysis efficiency using sonication at natural starch pH and 50°C

1. Introduction

The first step for starch digestion and use in industrial processes is starch hydrolysis. Alpha-amylase can be used for enzymatic hydrolysis of starch and it catalyzes the α-1,4-glucosidic bonds and does not break the α-1,6-glucosidic bonds of starch's structure [1]. The activity of alpha-amylase enzyme on starch's amylose occurs in two stages. First, rapid and complete hydrolysis of amylose is performed and converted it to maltose and maltotriose. Typically, this degradation causes rapid loss of viscosity and iodine staining ability. The second stage, which is much slower than the first stage, involves the slow hydrolysis of oligosaccharides and the formation of glucose and maltose as the final product. The maximum activity of alpha-amylase is in the acidic suspension with a pH between 4.5 and 7 [2]. By immobilizing the enzymes, the re-extraction problems can be solved and it can be more cost-effective [3]. For catalytic applications, immobilization with covalent method is suggested because the bond formed in this method is much stronger than other immobilization methods and the amount of enzyme released from the carrier during chemical processes is minimal [4].

Magnetic nanoparticles (MNPs) of iron oxide (Fe_3O_4) can be good candidates as carriers for enzyme immobilization due to their special properties such as non-toxicity, high biocompatibility, paramagnetic properties, high surface-to-volume ratio and reusability [4]. These nanoparticles can be used in environments with low viscosity in which adsorption of materials on the MNPs does not reduce the magnetic properties. Therefore, it is necessary to select low concentration of starch and modify the starch granules before hydrolysis with an immobilized enzyme on Fe_3O_4 MNPs. As a result, these MNPs can be mainly separated by magnetic fields. Starch without modification or high concentration cover the nanoparticle's surface and decrease their recovery by external magnetic field. Li et al. reported that high concentration of starch had negative effects on liquefaction of corn starch catalyzed by alpha amylase [5].

Using native starch has some limitations such as non-solubility in cold water, high resistance to enzymatic hydrolysis, high resistance to temperature, pressure and shear stress and a strong tendency to form colloids that are not always suitable for specific industrial applications. These natural limitations can be significantly improved through starch modification methods such as physical (swelling, thermal treatment, annealing, sonication, etc.) and chemical (cross-linking, acidic hydrolysis, etc.) methods. Through starch modification processes, desirable properties such as high thermal stability, softer texture and solubility in water can be achieved [6].

Due to rigid structure of starch, chemical modification like acid hydrolysis needs harsh conditions such as high concentration of acid to set pH around 1.65 and high temperature around 120 °C [7]. Also, the probability of in the final production can restrict some application of these modified starches in special industries such as food and pharmaceutical. These methods are cost effective and use lots of energy; as a result, different alternatives should be used to overcome those restrictions [8].

Physical modification methods can be safely applied as a modification process in food products and bioethanol production [9]. In thermal treatment, the gelatinization phase starts at a temperature of approximately 45 °C and continues until a temperature of 75 °C. At temperatures above 75 °C, all amylopectin double helixes are broken and the amylose chains release from starch particles, but the granular and swollen structure of the starch is maintained until high temperatures and shear stresses are reached. At temperatures above 95 °C, the starch particles were completely destroyed, joined together, no independent granule maintained and amorphous gels form [10]. Also, in this phase, the starch with low concentration should be selected because high concentration of starch would make difficulties in gelatinization process, inhibit swelling and disruption of starch particles. Thus, it causes retention of starch crystallinity after thermal treatment [5]. In this process, the concentration of the reducing sugar was lower than the released amylose because it causes the loosening of amylose chains, gelatinizes and increases the starch solubility and has little or no effects on the production of reducing sugar [7, 11].

The sonication process accelerates the deploy-merization of the gelatinized starch by breaking down the amylopectin units and releasing the amylose units. As a result, by destructing the polymer network, the viscosity of the solution decreases. Sonication is an inexpensive, simple and quick process do not widely change the chemical structure and starch properties. Previous studies showed that the factors such as sonication time and starch concentration can affect the amylose concentration and viscosity significantly [12, 13]. In the study of Wang et al. they aimed to convert amylopectin to reducing sugar and they concluded that combining the sonication and enzymatic hydrolysis with glucoamylase showed even greater effects both on reducing sugar yield and starch molecular degradation than the single enzymatic hydrolysis and sonication treatments [14]. Also, Wang et al. reported that sonication of starch before enzymatic hydrolysis with glucoamylase led to the furtherance of starch hydrolysis degree [15].

In many industries amylopectin is used for different purposes like food packaging, energy storing or converting to reducing sugar [16]. As a result, scientists prefer to isolate amylopectin from amylose for easier usage of amylopectin. Modification of starch with thermal hydrolysis and sonication before enzymatic hydrolysis with amylase can decrease the amylose concentration and increase the amylopectin purity for different purposes. Moreover, in this processes, low concentration of starch needs modifications to achieve higher purity of amylopectin. Wee et al. obtained 60% sugar yield by enzymatic hydrolysis of soluble starch (0.4% w/v) by glucomylase at 61 °C and pH=4.5 [17]. To the best of our knowledge, study on the hydrolysis of dilute native starch using the immobilized α-amylase onto MNPs along with pretreatment at the same time has not been investigated.

In present study, the thermal hydrolysis of low concentration of starch at gelatinization temperature followed by using sonication process was investigated. Moreover, in the thermal hydrolysis the effect of pH on starch structure was investigated. In the second step, the enzymatic hydrolysis of starch using covalent immobilized alpha-amylase on the surface of modified MNPs was studied. In the process of thermal hydrolysis, initial suspension pH value and sonication time were studied and in the enzymatic hydrolysis process, immobilized enzyme concentration, enzymatic hydrolysis time and solution storage time were investigated to explore their effects on starch morphology using one factor at a time (OFAT). Finally, three factors of starch concentration, thermal hydrolysis pH and temperature of enzymatic hydrolysis were simultaneously optimized with factorial design. In order to evaluate the efficiency of the selected method, the amounts of ratio of final and initial soluble amylose content of hydrolysate solution, reducing sugar and percentage of decreasing immobilized enzymes were measured and statistical analysis were carried out on multiple responses and desirability function.

2. Material and method

2.1. Materials

Ferrous chloride ($FeCl_2.4H_2O$), ferric chloride ($FeCl_3.6H_2O$), glucose, sodium hydroxide, iodine, potassium iodide, ethanol (96% (v/v)), hydrochloric acid and ammonia solution (25% (v/v)) were products of Merck. Amylase enzyme from *Aspergillus oryzae*

(EC 3.2.1.15), (3-aminopropyl) triethoxysilane (APTES), 3,5-dinitrosalicylic acid (DNS) and glutaraldehyde were obtained from Sigma-Aldrich. Corn starch were bought from Sahra Company. Double distilled water was used in the preparation of all solutions.

2.2. Preparation of functionalized MNPs

The synthesis, Coating and surface activation of MNPs followed by immobilization of alpha amylase on MNPs were performed by the procedure reported in Eslamipour et al. study [18].

2.2.1. Synthesis of Fe_3O_4 MNPs

Fe_3O_4 MNPs were synthesized from chemical co-precipitation method. 0.146 g of $FeCl_2.4H_2O$ and 0.4 g of $FeCl_3.6H_2O$ salts were dissolved in 15 mL ethanol (96%) and 35 mL heated double distilled water. Next, the solution was placed in a 70°C water bath and stirred under nitrogen atmosphere for 10 min. Next, ammonia solution was added and black deposit of MNPs were observed. The solution was stirred for 1 h. Finally, the synthesized magnetic nanoparticles were collected by a magnet and washed with ethanol and double distilled water for several times.

2.2.2. Coating MNPs using APTES

10 mL of 40 °C heated double distilled water and 10 mL ethanol (96%) were added to 0.2 g MNPs synthesized in previous step. The solution was sonicated using a sonifier (model Bandelin 3200, process frequency 20 kHz) with output energy of 65W. Next, the solution was placed in 40°C water bath with stirring under nitrogen atmosphere for 10 min. 2 mL ammonia solution (25%) and 2 mL APTES were added to the solution and the solution were stirred for 2 h. Finally, the coated MNPs were collected and washed with ethanol and double distilled water.

2.2.3. Surface activation of MNPs using glutaraldehyde

The aminated MNPs were dispersed in 0.5% glutaraldehyde and 0.1 M citrate-phosphate buffer with pH=7 and then shaken for 1 h in room temperature at 200 rpm. Finally, the activated magnetic nanoparticles were washed with double distilled water and citrate-phosphate buffer.

2.3. Enzyme immobilization

MNPs solution with concentration of 1000 ppm was prepared, then the solution was mixed with amylase solution (1000 ppm) and 0.1 M citrate-phosphate buffer solution with pH of 6 (volume ratio 1:1:1). Finally, the prepared solution was shaken in shaker incubator (150 rpm, 30 °C) for 4 h. The concentration of immobilized enzyme was measured and by subtraction of remaining amylase concentration from initial enzyme concentration.

2.4. Evaluating sonication time and initial pH in starch thermal hydrolysis

Based on our studies the gelatinization temperature of 90 °C for present corn starch was obtained. In this step the influence of initial pH in thermal hydrolysis of starch at gelatinization temperature (90 °C) and sonication time were observed. Based on the study of Sirohi et al., HCl was selected for exploring the effect of pH in thermal hydrolysis step [19].

2.4.1. Sonication time

The starch solution without pH adjustment (natural pH=4.5) was heated in 90 °C water bath for 60 min. Then the solution was sonicated for 15 and 30 min using a sonifier with the output energy of 75 W. Finally, the concentration of amylose and reducing sugar were measured.

2.4.2. Starch solution pH

The starch solution pH was adjusted on 3.5, 4.5 (natural pH) and 5.5 with HCl (0.5 M) and sodium hydroxide (0.25 M). Then the solution was heated in 90 °C water bath for 60 min. Afterward, the solution was sonicated for 30 min. Finally, the concentration of amylose and reducing sugar were measured.

2.5. Evaluating factors in immobilized enzymatic hydrolysis of starch using OFAT method

In this step, enzymatic hydrolysis of starch with immobilized amylase on MNPs was studied. The starch solution pH from thermal hydrolysis step (pH=4.5) was adjusted on 6 with 0.25 M sodium hydroxide. Then in different stages of the experiment 2 mL thermal-acidic hydrolysis starch solution (0.5% w/v) with 1 mL immobilized enzyme in citrate-phosphate buffer (0.1 M, pH=6) was shaken for 5 min at 40 °C under rotation speed of 200 rpm.

Three factors of immobilized enzyme concentration (35, 100, 165 and 335 ppm), enzymatic hydrolysis time (10, 15 and 20 min), storage time of thermal hydrolysis starch solution in refrigerator for 24 h or immediately usage for next stage in different levels were investigated in three stages using OFAT method. In each stage, the effect of a single factor was estimated at fixed conditions of the other factors and the optimum factor condition were determined and then the next stage was done.

After each of the above experiments, the immobilized enzyme on magnetic nanoparticles were separated using external magnetic field and the concentration of amylose and reducing sugar were measured.

2.6. Factorial experimental design of starch hydrolysis by immobilized alpha-amylase

In this step, the main aim was to optimize the factors that influence both thermal and enzymatic hydrolysis stages at the same time. In addition to amylose and reducing sugar concentration changes, the percentage of remaining immobilized enzyme in the solution were considered as multiple responses.

In primary experiments, the magnetic nanoparticles were not completely isolated from starch concentration of 1% (w/v) by external magnetic field but the magnetic nanoparticles were completely isolated from starch concentration of 0.5 and 0.7% (w/v). Therefore, the rest of the experiments were continued with starch concentration of 0.5 and 0.7% (w/v).

The effect of starch concentration (A), thermal hydrolysis pH (B) and temperature of enzymatic hydrolysis (C) were simultaneously investigated with 2^3 factorial design.

In the process of thermal hydrolysis of starch, the starch solutions 0.5 and 0.7% (w/v) each with pH 3.5 and 4.5 were heated in 90 °C water bath for 60 min. Then the solution was sonicated for 30 minutes with the output energy of 75 W. In the enzymatic hydrolysis of starch process, the pH of obtained starch solution from each condition of thermal hydrolysis step were adjusted on 6 with HCl (0.5 M) and sodium hydroxide (0.25 M). Then 2 mL of starch solutions with immobilized amylase 350 ppm in citrate-phosphate buffer (0.1 M, pH=6) was shaken for 15 min at 40 and 50 °C under rotation speed of 200 rpm.

Then the multi-responses of ratio of final and initial process soluble amylose content and reducing sugar concentration changes and percentage of remaining immobilized enzyme dry weight were statistically examined by Design expert 11.0.3 software

2.7. Reusing of immobilized enzyme

The magnetic immobilized enzymes were collected and then ratio of soluble amylose content of final and initial of each cycle and concentration of reducing sugar were measured and the preservation of magnetic properties of nanoparticles were determined by using external magnetic field. Finally, the magnetic nanoparticles were washed with ethanol and water for several times and above procedure repeated several times.

2.8. Analysis methods

Bradford method has been used to measure the amount of enzyme immobilized on MNPs [20]. The activity of free and immobilized alpha-amylase enzyme was measured by Miller method [21]. The specific activity of free alpha-amylase was determined to be 51.21 (μmol glucose/mg enzyme. min).

The amount of produced reducing sugar (or one tenth of dextrose equivalent) were determined by Miller method [21] using DNS reagent. The initial soluble reducing sugar concentration of native corn starch was 8.25 (mg reducing sugar/g starch).

In order to measure the released amylose content in the environment during the pre-treatment process and enzymatic hydrolysis of starch, the iodometric method of starch was applied with some modification [22].

2.9. Methods of Characterization

In order to evaluate the starch functional groups, starch solutions (0.5% (w/v)) and pH = 4.5 were first dried by a freeze dryer and then Fourier-transform infrared spectroscopy (FTIR) spectrum of the starch particles was taken. By identifying the functional groups, it is possible to determine the changes at each stage of the experiment. Scanning electron microscopy (SEM) images from platinum coated samples were taken to investigate the structural changes of starch particles and magnetic nanoparticles in each of the test steps.

3. Results and discussion

3.1. Thermal hydrolysis of starch

3.1.1. Effect of sonication time

As shown in Fig. 1(a & b) after heating starch suspension for 1 h in boiling water bath and gelatinization of starch particles, the amylose and reducing sugar concentration were increased.

In addition after using the ultrasonic waves, the amylose and reducing sugar concentration were increased more than the gelatinization step which is corresponded to Iida et al. [13].

Thermal hydrolysis did not have much effect on increasing the amylose and reducing sugar concentration due to the lower temperature compared to other researches and use less energy [7, 11]. However, in this process by heating the starch suspension in boiling water bath, the starch particles were gelatinized and amylose was permeated out of the starch particles; thus, the ratio of soluble amylose concentration was increased about 1.5 times. Subsequently, by using ultrasonic waves the amylopectin crosslinks in starch particle are broken and more amylose was released into the reaction medium (about 2 times of the gelatinization stage and 3 times of the process start), but there was no significant difference between the amount of released amylose at 15 and 30 min using the ultrasound waves (p-value=0.3579). Ineffective ultrasonic process for 30 minutes indicated that ultrasonic waves promoted the crystallization of starch particles to a certain extent [23]. Also, in this stage, the concentration of reducing sugar was increased slightly. Therefore, the ultrasonic process is more efficient for 30 minutes than for 15 minutes because the starch structure was collapsed and also, more reducing sugar were achieved. Falsafi, et al., reported that sonication of starch for 20 min with ultrasonic probe 350W increased the amylose content to 25.26% and also, increased the starch solubility. [12]. Chan, et al., concluded that sonication of corn, potato, sago and mungbean starch for 10 min with ultrasonic bath increased the amylose content by 5% for corn and 2% for the other kinds due to depolymerization of amylose and increase the linear chains [24].

3.1.2. Effect of starch solution pH

As shown in Fig. 1(c & d) and statistical analysis after heating for 60 min in a boiling water bath and starch gelatinization, the amount of amylose and reducing sugar concentrations were increased, but pH variable was not significantly effective on amylose and reducing sugar concentrations due to having *p*-value greater than 0.05. In addition, after the use of ultrasound waves the amylose and reducing sugar concentration also highly were increased. By using ultrasound waves, the amylopectin cross-links and branches in starch structure were broke down and more amylose were released in reaction medium. In addition, in statistical analysis of changes in amylose and reducing sugar concentration in sonication step pH variable was not significantly effective (*p*-value > 0.05). It can be concluded that the duration of thermal hydrolysis in current research was short comparing to other studies [25] and existing acid has not been able to play a major role in releasing amylose and producing reducing sugar in this short period of pretreatment .In this stage, with low starch concentration, the acid played a major role in loosening the amylose chains and did not have significant impact on producing reducing sugar.

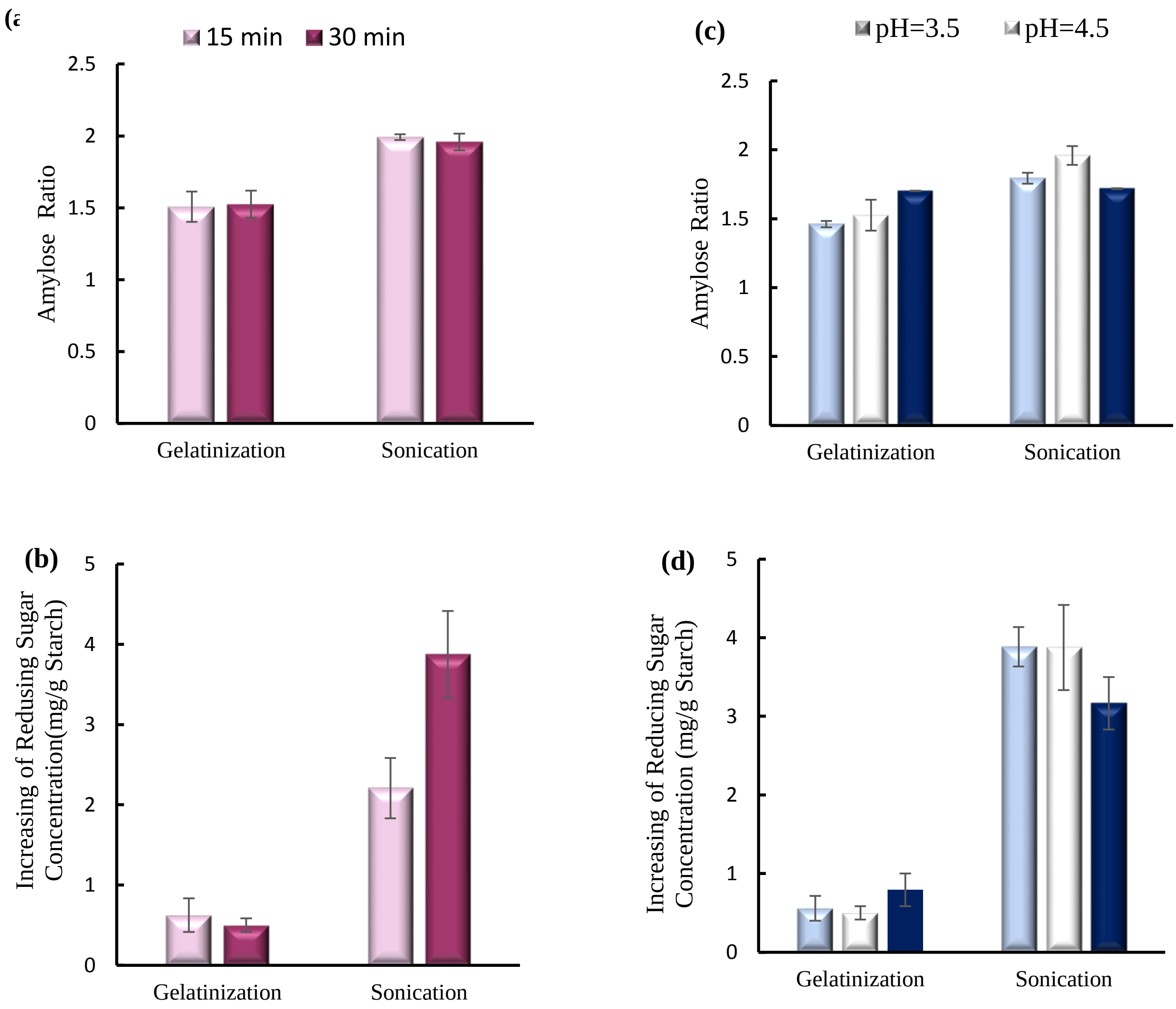

Fig. 1 Changing in amylose concentration (a, c) and reducing sugar concentration (b, d) at gelatinization point and after ultrasound process time (left side) and at three pHs (right Side)

In various studies, it has been observed that in short periods of acidic hydrolysis of high-concentration starch, acidic hydrolysis has not been effective in increasing the concentration of amylose, but by increasing the time of this process, a higher efficiency can be achieved in the release of constructive amylose [25-28]. The optimal pH was obtained 4.2 by Li et al. in 2014, who performed the thermal hydrolysis of corn starch for 30 minutes at 40 to 90°C followed by enzymatic hydrolysis with free alpha-amylase [29]. In another research, the highest reducing sugar production at pH of 3 and 4 of acidic hydrolysis of corn starch at 95 °C for 105 minutes was yielded [30]. So, the initial starch solution pH 4.5 was considered for the rest of experiments.

3.1.3. Evaluating the ability of the thermal hydrolysis method using free enzyme

In this study, 2 mL of hydrolyzed starch solution (0.5%, pH= 4.5) and free enzyme with the concentration of 35 ppm was mixed for 5 min and the decreasing changes in amylose concentration were obtain 0.28 (amylose ratio). The reducing sugar concentration was 122.38 (mg/g starch) compared to 8.25 (mg/g starch) initial reducing sugar concentration. Therefore, it can be concluded that the thermal hydrolysis pretreatment and subsequent use of ultrasound waves have been effective in reducing the starch particles hardness.

3.2. Characteristic of the thermal hydrolyzed starch and enzymatic hydrolysis

As shown in Fig. 2(a), native starch particles had spherical shapes and smooth surfaces. After thermal hydrolysis, the starch particles were gelatinized and the amylose chains were released (Fig. 2(b)). Then, by using ultrasound waves, the cross-linking bonds and branches of amylopectin in starch structure were broken and more amylose chains were released in the reaction medium (Fig. 2(c)). Also, Tian et al. in 2018, reported similar morphologies for starch heated at 90°C for 20 min [31].

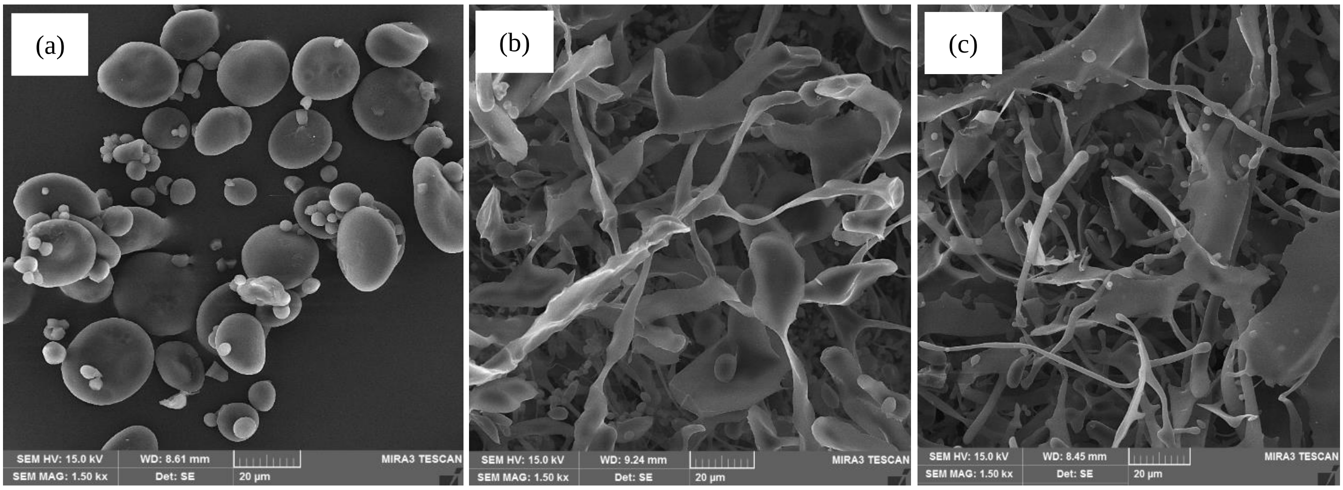

Fig. 2 SEM images of (a) Native starch (b) Gelatinized starch (c) sonicated starch

As shown in Fig. 3, when the alpha-amylase enzymes were immobilized on magnetic nanoparticles, the MNPs were covered by this enzyme and their diameter has increased. After hydrolysis process, some nanoparticles have a reduced diameter due to enzyme leakage, and some are coated with non-hydrolyzed starch (Fig. 3(c)).

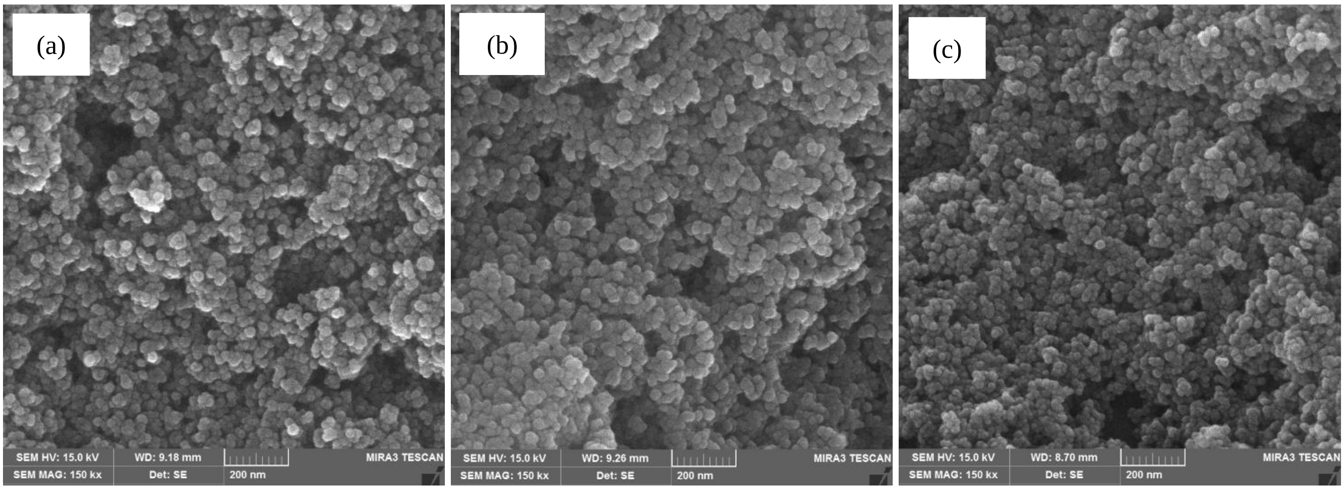

Fig. 3 SEM images of (a) MNPs, (b) Immobilized alpha-amylase on MNPs, (c) MNPs after enzymatic hydrolysis

As shown in Fig. 4, FTIR spectrum in the wavenumbers of 3423.61, 3419.81 and 3440.97 (cm^{-1}) indicate the stretching mode of the O-H groups. The wavenumbers of 1648.74, 1647.05 and 1647.25 (cm^{-1}) show the first overtone of the O–H bending vibration and 1158.91, 1154.06 and 1153.57 (cm^{-1}) indicate the C-O stretching. In addition, 2927.41, 2925.04 and 2924.57 (cm^{-1}) show C-H stretching and 998.40, 1080.94 (and 1023.65) and 1024.18 (cm^{-1}) represent the CH_2–O–CH_2 stretching vibrations for native starch, gelatinized and sonicated starch, respectively. In sonication phase, as in the gelatinization phase, all peaks indicating O-H, C-O, C-H, and CH_2-CH_2 stretching bonds have less intensity than native starch and gelatinized starch. It indicates the breaking of these bonds during the process and the reduction of their number. However, the amount of O-H bending vibration transmittance relative to gelatinized starch remained unchanged [32, 33].

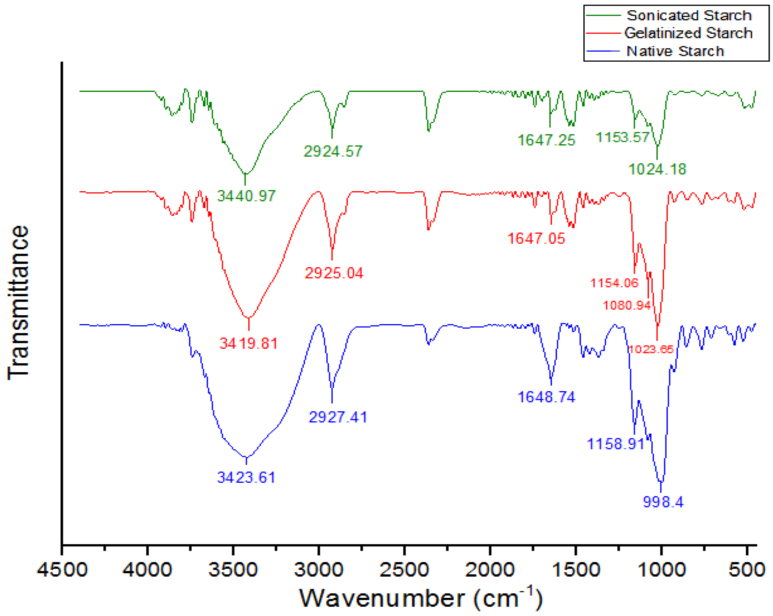

Fig. 4 FTIR spectrum of the starch particles from top to bottom: sonicated starches, gelatinized and native

3.3. Influence of enzymatic hydrolysis conditions on starch using OFAT Design

In this step the thermal hydrolysis and sonication process was followed by enzymatic hydrolysis to achieve more reducing sugar in a shorter time. For this purpose, after immobilization of the alpha-amylase on the magnetic nanoparticles the amount of immobilized alpha-amylase and its specific activity was determined to be 352 (mg enzyme/ g MNPs) and 12.5 (µmol glucose/mg enzyme. min). As mentioned in Section 2.5, in this experiment the influence of three factors was examined.

3.3.1. Effect of immobilized alpha-amylase concentration

As shown in Fig. 5(a), the maximum changes in decreasing amylose concentration and the increasing in reducing sugar concentration were related to immobilized enzyme with the concentration of 335 ppm (enzyme to starch ratio of 33.5 mg/g). It is obvious, when immobilized enzymes with higher concentration were used, more amyloses were attacked by immobilized enzymes and hydrolyzed to reducing sugar. As a result, more reducing sugar were produced.

3.3.2. Effect of enzymatic hydrolysis time

As shown in Fig. 5(b), as the enzymatic hydrolysis time increased, the decreasing changes in amylose concentration and the increasing changes in reducing sugar concentration were increased. Therefore, the enzymes were still active and were able to hydrolyze starch for up to 20 minutes.

Due to the near decreasing changes between the time of 15 and 20 minutes in amylose concentration, a more detailed statistical analysis was performed and did not show a significant increase in amylose concentration at these two times because the P-value was greater than 0.05 (*p*-value = 0.27).

3.3.3. Effect of storage time of thermal hydrolyzed starch

Starch samples with thermal hydrolysis should be stored for a long time in order to bring the test conditions closer to industrial conditions. Thus, in food industry it is necessary to store the specimens at low temperatures in order to minimize the potential for microorganisms to grow. For this purpose, two starch samples of 0.5% (w/v) with thermal hydrolyzed at pH=4.5 were prepared. One specimen was kept in the refrigerator for 24 h and the other was immediately subjected to enzymatic hydrolysis for 15 min at 40 °C.

As shown in Fig. 5(c), the decreasing amylose ratio and the increasing changes in reducing sugar concentration in the sample kept at 4 °C for 24 hours were higher than the sample without storage. It seems, the sample stored in the refrigerator was continued to have slightly hydrolysis due to having a pH=6 and remaining at this pH for a long time, resulting in continued decreasing changes in amylose concentration (*p*-value=0.04). However, it didn't affect the reducing sugar production (*p*-value=0.0582).

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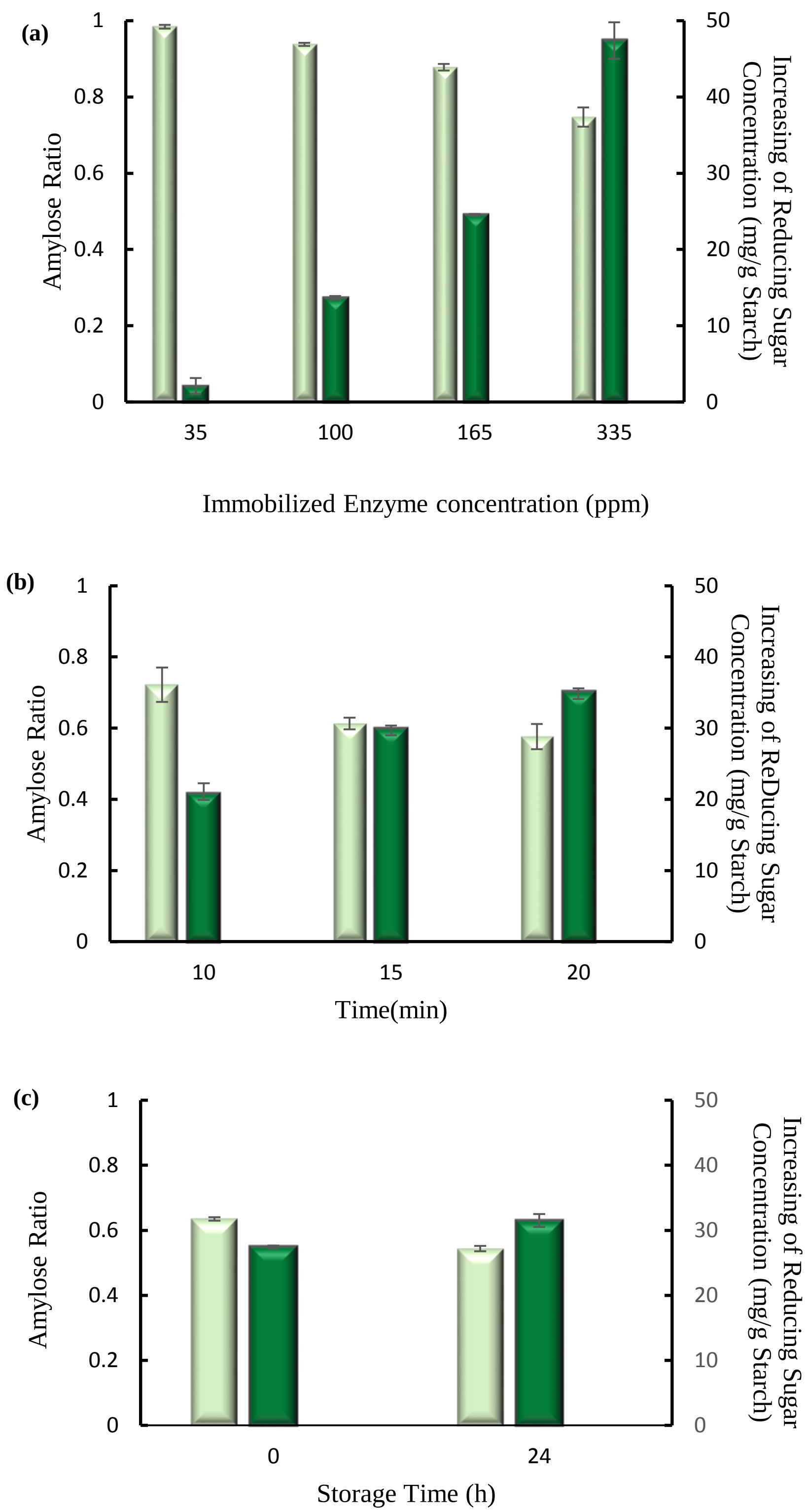

Fig. 5 Decreasing changes in amylose concentration ( ) and increasing reducing sugar concentration ( )

For this purpose, two starch samples of 0.5% (w/v) with thermal hydrolyzed at pH=4.5 were prepared. One specimen was kept in the refrigerator for 24 h and the other was immediately subjected to enzymatic hydrolysis for 15 min at 40 °C.

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3.4. Results and statistical analysis of factorial experimental design

In this step, the immobilized enzyme with the concentration of 335 ppm and the enzymatic hydrolysis time for 15 min were considered. The results were reported in Table 1 and analyzed using Deign Expert statistical software.

As shown in the first trial of Table 1, if the experiment was performed at the first trial condition, maximum efficiencies were obtained in increasing the concentration of reducing sugar and decreasing in amylose concentration. During the thermal hydrolysis and sonication, the amylose chains are released in the solution and a small part of them are converted to reducing sugar. As a result, the amylose concentration increases. However, in the enzymatic hydrolysis, amylose chains are converted to reducing sugar; hence, the amylose concentration decreases and a high concentration of reducing sugar will be achieved [34]. As a result, starch characteristics like solubility and resistance can be improved. In starch hydrolysis at a concentration of 0.7% (w/v), probably due to the increase in starch concentration and constant enzymatic hydrolysis time of the starch, the MNPs surface were covered by starch particles and the enzyme activity were decreased.

Table 1 Optimization of multi-response in 2^3 Factorial Design for thermal-acidic and enzymatic hydrolysis of starch

Trial	Variable Level			Results		
	A	B	C	Reducing sugar concentration changes (mg/g starch)	Soluble amylose ratio (g final /g initial)	Separated MNPs dry weight % (g/g initial MNPs)
1	0.5	4.5	50	92.48±2.10	0.63±0.01	77.5±1.1
2	0.5	3.5	50	90.14±1.30	0.64±0.03	71.9±1.3
3	0.7	3.5	50	62.55±0.51	0.77±0.02	93.1±1.1
4	0.5	3.5	40	76.38±7.91	0.67±0.001	69.8±0.8
5	0.5	4.5	40	75.95±7.30	0.68±0.07	67.7±0.6
6	0.7	4.5	50	56.32±1.42	0.75±0.005	81.8±1.8
7	0.7	3.5	40	56.27±6.26	0.80±0.05	86.8±1.4
8	0.7	4.5	40	56.27±2.04	0.84±0.09	65.6±1.4

In addition, in all experiments more than 65% of the initial MNPs dry weight was retained and able to preserve their own magnetic. According to Table 1 the remaining nanoparticles dry weight for starch concentration of 0.7% (w/v) was higher than starch concentration of 0.5% (w/v). It seems, for starch with low concentration, the interaction between temperature, starch concentration, and pH probably affects the viscosity of the solution, causing the highest nanoparticles recovery to occur at 0.7% (w/v). The effective variables and interactions were shown in Table 2. It can be concluded from the ANOVA table that starch concentration and temperature were affecting variables on increasing reducing sugar concentration and decreasing amylose concentration but the pH variable and all binary and tertiary interactions between the variables were not effective. No affection of the pH variable can be attributed to the short time of thermal hydrolysis and slight difference between the two pH levels. Also, it can be concluded that all the main variables and the two-way interaction of starch concentration-pH were affected separated MNPs dry weight changes. The pH values were adjusted to 3.5 and 4.5. pH 4.5 is the pH of native starch, and considering a pH lower than 3.5 would cause enzyme denaturation and oxidation of nanoparticles. In the OFAT method, pH 3.5 and 4.5 had the maximum reducing sugar in sonication stage, so these two were selected for pH levels in factorial design method.

The optimum condition was obtained at starch concentration of 0.5% (w/v) (enzyme to starch ratio of 33.5 mg/g), thermal hydrolysis pH 4.5 and hydrolysis temperature 50 °C based on criteria of maximizing changes in the reducing sugar and amylose concentrations and in range of the percentage of maintaining immobilized enzyme dry weight (first trial of Table 1) and with desirability of 0.944. The optimum pH of 4.5 was the natural starch solution pH and used less chemical substances.

Table 2 ANOVA of the effects of three responses in 2^3 Factorial Design for acidic and enzymatic hydrolysis of starch

Source	DF	Changes in reducing sugar concentration			Changes in soluble amylose concentration			Separated Nanoparticles dry weight		
		SS	MS	P-value	SS	MS	P-value	SS	MS	P-value
model	7	3203.04	457.58	0.0015	0.081	0.027	0.0013*	1342.27	191.75	<0.0001
A	1	2679.87	2679.87	<0.0001	0.072	0.072	0.0002	407.33	407.33	<0.0001
B	1	4.68	4.68	0.7468	5.6E-05	5.6E-05	0.8865***	209.89	209.89	<0.0001
C	1	335.35	335.35	0.0221	9.5E-03	9.5E-03	0.0825**	294.72	294.72	<0.0001
AB	1	16.54	16.54	0.5470				324.27	324.27	<0.0001
AC	1	143.34	143.34	0.1014	POOLED†			27.01	27.01	0.0171
BC	1	2.98	2.98	0.7962				77.92	77.92	0.0009
ABC	1	20.27	20.27	0.5061				1.12	1.12	0.5585
Pure error	8	334.79	41.58		0.029	3.6E-03		24.00	3.00	

The optimum temperature 50°C is similar to the optimum temperature reported by Gangadharan in 2009 that enzymatically hydrolyze potato starch at 50°C [35]

3.5. Reusability of immobilized enzyme

An important feature of immobilized enzymes on magnetic nanoparticles is the possibility of their separation by the external magnetic field and their multiple use. In the present study, the enzymatic hydrolysis of 0.5% (w/v) of starch with thermal-hydrolysis at pH=4.5 was repeated 13 times.

As shown in Fig. 6, immobilized alpha-amylase has been able to maintain 40% of its ability to reduce the concentration of amylose after 4 cycles and in the production of reducing sugar after 6 cycles of native corn starch. In addition, after usage in 6 consecutive cycles, the magnetic properties of the nanoparticles gradually began to decrease.

The results are almost identical to study of Tuzman et al. in 2012, who were able to use 5 cycles of magnetic polymeric bead for hydrolysis of 1% of soluble starch without losing their magnetic properties at 35 °C and pH=8 [36]. Dhavale, et. al, reported that the activity immobilized α-amylase on chitosan coated Fe_3O_4 MNP remained unchanged up to five cycles and a slight decrease in the activity was observed afterward. The immobilized α-amylase were reused 20 times without significant loss of activity for hydrolysis of 0.5% (w/v) of soluble starch [37]. It seems that chitosan coated particles caused

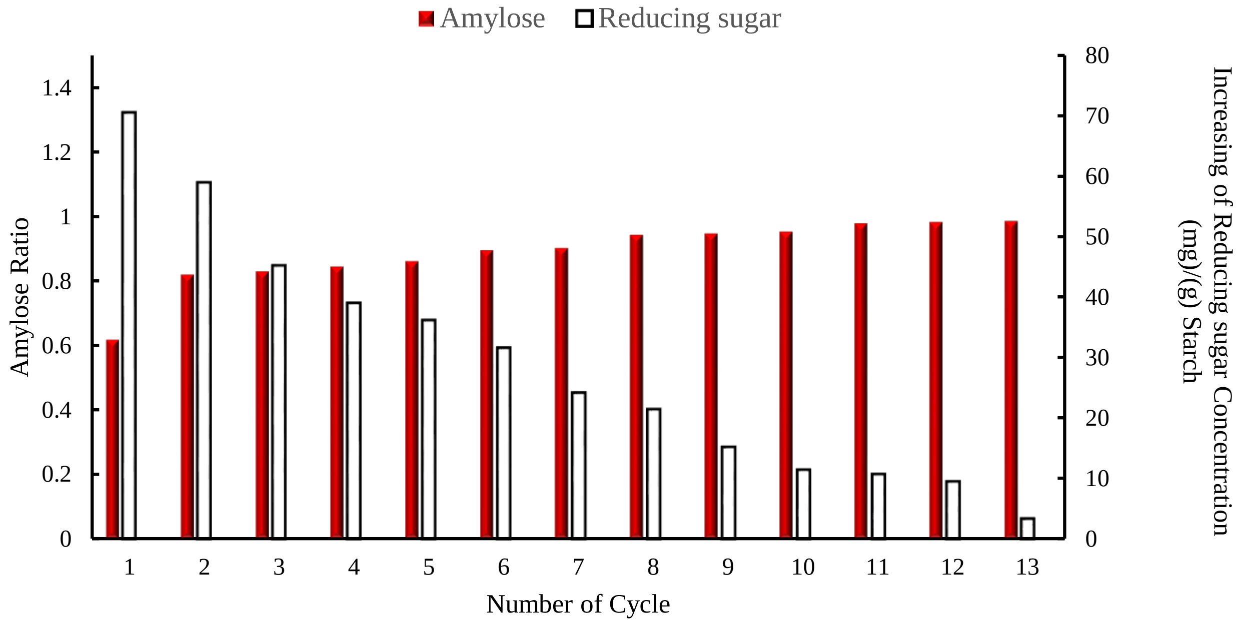

Fig. 6 Changes in amylose ratio and reducing sugar concentration in reusability of immobilized alpha-amylase

4. Conclusion

In present study, the thermal hydrolysis of native corn starch at higher gelatinization temperatures was performed followed by sonication process for 30 min. The enzymatic hydrolysis of starch with immobilized alpha-amylase on magnetic nanoparticles was further discussed. By immobilizing the alpha-amylase on MNPs, they can be separated by an external magnetic field from the reaction medium, used continuously, and achieved high economic efficiency. Thermal hydrolysis at the natural starch pH and sonication in a short time can be used as a suitable pretreatment with lowest energy consumption to reduce the hardness of starch particles before enzymatic hydrolysis with a immobilized enzyme. In this process, about 10 g of reducing sugar per 100 g of primary starch has been produced after 15 min enzymatic hydrolysis at 50 °C, which is about 50% of the production efficiency of reducing sugar from amylose dissolved during the process. Dilute starch solution with reduced amylose amount without adding chemicals can be used to investigate residual amylopectin in biomedical issues. In the present study, the hydrolyzed starch can be used in the food, pharmaceutical, and cosmetic industries, overcoming the obstacles of using native starch due to its hard structure and insolubility in water.

5. Acknowledgements

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6. Declaration of competing interest

The authors report no conflicts of interest.

7. Authors' Contributions

Designate each author's contribution using their initials. "Conceptualization, S.K. and P.H.; methodology, validation, formal analysis, investigation, resources, data curation, writing—original draft preparation, S.K; writing—review and editing, visualization, supervision, project administration, P.H.".

8. Using Artificial Intelligent Chatbots

The authors declare no artificial intelligent chatbot use.

9. Ethical Consideration

This study did not involve human participants or animals. The research complied with institutional guidelines for laboratory safety and good scientific practice.

Conflict of interest

The authors declare no conflict of interest.

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