

Salt-Assisted Mesostructured Cellular Foam (MCF) Silica Synthesis from Bagasse Bottom Ash for Enzymatic Starch Hydrolysis

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Abstract

Synthesis of MCF silica is presently conducted solely using TEOS and TMB, with the purpose of immobilizing amylolytic enzymes. Utilizing BBA and KCl to create the salt-assisted MCF silica present a viable option for converting a natural waste into an effective enzyme carrier, given its substantial silica content. The objectives were to produce the MCF silica, to employ the MCF silica as the glucoamylase carrier, and to know characteristics of the immobilized enzyme by conducting hydrolysis of starches. The carrier had surface area of $45.5 \text{ m}^2 \cdot \text{g}^{-1}$, pore volume of $0.12 \text{ cm}^3 \cdot \text{g}^{-1}$, pore size of 9.3 nm, and mesoporous silica type IV. Reduction in the carrier pore diameter and the medium to strong FTIR vibrations indicated free glucoamylase immobilization on carrier. The immobilization reached 88.5% efficiency, influenced by factors such as initial enzyme concentration, PO_4 buffer pH, and temperature, with agitation speed having a minor impact. This optimum value was obtained at the initial enzyme concentration of $9.0 \text{ mg} \cdot \text{mL}^{-1}$, agitation speed of 120 rpm, buffer pH of 5.5, and temperature of 30°C . Hydrolysis of starches (tapioca, wheat, potato, corn) resulted in Dextrose Equivalent (DE) values ranging from 5.1% to 63.9%, with the immobilized glucoamylase showing better performance in potato starch hydrolysis (DE of 63.9%) and corn starch (DE of 45.6%). The use of BBA in synthesis of the salt-assisted MCF silica proved to be a viable and sustainable alternative for enzyme immobilization, with potential applications in industrial starch hydrolysis.

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Keywords: bagasse bottom ash; salt-assisted synthesis; dextrose equivalent; amylolytic enzyme; enzyme transporter efficiency

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1. Introduction

Immobilizations of amylolytic enzymes have been made on natural and synthetic nanoparticles, however, their limited surface areas result in suboptimal immobilization efficiency. In contrast, mesoporous silicas having extensive surface areas provide a more effective way of attaching amylases compared to nanoparticles. Numerous mesoporous silica

carriers either native, polymeric, or functionalized synthetic type, have immobilized glucoamylase, α -amylase, and pullulanase enzyme.

Mesoporous cellular foam (MCF) silica synthesized from tetraethyl orthosilicate (TEOS) using 1,3,5-trimethylbenzene (TMB) as the swelling agent demonstrated high adsorption capacity for free SQzyme AGP glucoamylase [1-3] to yield the dextrose equivalent values of 1.74-76.30% (w/w). Adsorption of *Aspergillus niger* glucoamylase onto mesoporous silicas (MPS) prepared from TEOS and *n*-decane (the swelling agent) resulted in the immobilization efficiency of

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79.05%, but the maximum reaction rate was slower than free glucoamylase during soluble starch hydrolysis [4]. Entrapment of *Aspergillus niger* glucoamylase within mono-polymeric mesoporous silica matrix made from TEOS and phenyltriethoxysilane in alcoholic environment, was successfully conducted where the immobilized enzyme exhibited an optimum unit activity comparable to that of free glucoamylase in saccharification of soluble starch [5]. Functionalized MPS (synthesized from TEOS and *n*-decane) significantly enhanced performance of *Aspergillus niger* glucoamylase where immobilization of free enzyme in phosphate buffer supplemented with phthalate resulted in recovery of 90-95% of free enzyme's activity [4,6]. Functionalized MCF silica (synthesized from TEOS and TMB modified with organosilanes) facilitated *Aspergillus niger* glucoamylase immobilization [7], achieving the immobilization efficiencies of 3.3-74.7% where the MCF-based glucoamylase demonstrated high performances. As described, the mesoporous silicas are mainly developed from TEOS, which is not easily found, hence a method to develop the mesoporous silicas from easily obtained materials is needed.

Bagasse bottom ash (BBA) presents a viable alternative source for the MCF silica synthesis. This worthless natural material is considered invaluable and readily available abundantly in sugarcane plants. The waste contains over 50% (w/w) SiO₂ [8-11], hence it must undergo the extraction and transformation process to give the MCF silica. Extraction of silica from BBA presents both economic and ecological benefits, being sustainable and environmentally friendly, while also offering the potential to decrease waste production [12-14]. This is in contrast to the silica derived from chemical precursors such as TEOS, sodium silicate, and silicic alkoxides [12]. Hermanto *et al.* [15] recommended enhancing process sustainability to mitigate environmental impacts, following an analysis of the life cycle assessment for a silicon production plant operating at 1 kg/h. Furthermore, transformation of the waste into amorphous nanosilica yielded a life cycle assessment indicating a global warming potential of CO₂ equivalent of 7.51-12.59 kg [16]. In recent investigations, mesoporous silicas were synthesized from BBA-based Na₂SiO₃, Pluronic P123, and surfactant in an acidic environment to adsorb dyes [17,18]. Rahman *et al.* [19], who compared different synthesis conditions for the mesoporous silicas derived from BBA-based Na₂SiO₃, revealed that the polyethylene glycol-based method produced the product having high surface area. These efforts proved the prospect of BBA to substitute TEOS.

Given the absence of methods for preparing the MCF silica from BBA, our study was conducted to explore this opportunity. In this

study, potassium chloride (KCl) was employed as the expansion agent instead of TMB, aiming to lower costs associated with the MCF preparation as price of KCl is cheaper than TMB. To address the gap in research regarding immobilization of amylases on the MCF carrier as none of the amylolytic enzymes immobilizations is related to the BBA-based MCF silica as discussed previously [20], the developed salt-assisted MCF silica was used to immobilize glucoamylase. Functionality of the immobilized glucoamylase was evaluated through its application in the hydrolysis of starches.

2. Materials and Methods

2.1 Materials

BBA was collected from a sugarcane processing facility located in Lampung Province, Indonesia. Other materials were purchased from local suppliers: dextrose (Merck, >99%), dinitrosalicylic acid (Merck, >98%), hydrochloric acid (HCl, Merck, 37%), monobasic potassium phosphate (Merck, >99%) and monobasic sodium phosphate (Merck, >99%), sodium hydroxide (NaOH, Merck, >97%), ammonium fluoride (NH₄F, Merck, >98%), potassium chloride (KCl, Merck, >99%), sodium acetate salt (Sigma, >98%), Pluronic P123 (Sigma, 435465), sulfuric acid (H₂SO₄, 98%), Pierce™ BCA protein assay kit (Thermo Scientific), and glucoamylase. Tapioca, potato, wheat, and corn starch were bought from local supermarkets. All materials were used directly without pretreatment.

2.2 Conversion of BBA to Salt-based MCF Silica

Initially, silica was obtained from the raw material. BBA was grounded and sieved to achieve the particle size of 200 mesh. 50 g of the sieved BBA was placed into a glass beaker containing 500 mL of reverse osmosis (RO) water and 5 mL of sulfuric acid. The mixture was stirred at 100 rpm for 30 minutes and then filtered. The residue was rinsed with RO water until pH of filtrate reached 7.0. The wet residue was dried at 105 °C for four hours and then calcined at 550 °C for six hours. The dried residue was transformed into sodium silicate by placing 11 g of the residue into an Erlenmeyer flask containing 50 mL of 3 M NaOH. The mixture was heated in a water bath to 80 °C for four hours, stirred at 100 rpm for 30 minutes, and then filtered to obtain the sodium silicate solution. Finally, to synthesize the BBA-based MCF silica, 4 g of Pluronic P123 was dissolved in 70 mL of 1.6 M HCl at room temperature. After incorporating 10 g of KCl into the Pluronic solution, the mixture was heated to 40 °C with vigorous stirring for two hours. Then, the sodium silicate solution was added into the mixture while maintaining high stirring speed.

The solution mixture was subsequently placed in water bath set at temperature of 40 °C for 20 hours. Following this, the mixture was transferred to a magnetic stirrer operating at low speed, where 10 mL of ammonium fluoride at concentration of 0.92 mg.mL⁻¹ was added gradually. An aging process was performed in water bath at 80 °C for three days. After filtration, the residue was washed with RO water until pH of filtrate was 7.0. The wet solids were dried at 100 °C for 12 h and then calcined at 300 °C for 30 minutes, followed by six hours at 500 °C to yield the BBA-based MCF silica.

2.3 Enzymatic Hydrolysis

To optimize adsorption of free glucoamylase on the BBA-derived MCF silica, different parameters were experimented, as detailed in Table 1. Initially, free glucoamylase was introduced into 30 mL of 0.1 M Sorensen phosphate buffer. Then, 0.5 g carrier was added. The mixture was put into water bath shaker, maintained at certain speed and temperature. After five hours, the mixture was filtered. The residue was washed with 0.1 M Sorensen buffer (3×50 mL) in accordance with pH of the immobilization process. Filtrates from the separation and washing stage were stored in refrigerator prior to protein analysis. The wet residue was dried overnight in oven set at 40 °C and then allowed to cool. The dried product was kept in refrigerator before used. Efficiency of the immobilization process was based-on the difference between the initial and final concentration of free enzyme as described in the previous literatures [1-3].

Performance of the immobilized glucoamylase in hydrolysis of starches was assessed based-on the operating conditions as shown in Table 1. The procedure began with addition of 30 mL of a 0.1 M sodium acetate buffer into a 100 mL Erlenmeyer flask. After that, a specific concentration of starch was introduced into the medium. The immobilized glucoamylase was mixed with the starch solution. The mixture was agitated in a water bath shaker at a certain speed and temperature for eight hours. Samples were collected at the beginning and end of the process. Hydrolysis using free

glucoamylase was also performed under identical conditions to facilitate comparison of the enzyme activity.

2.4 Reusability Study

The immobilized glucoamylase was used repeatedly in a batch reactor where the operating conditions were based-on the optimum hydrolysis operational factors. It was set as follow: the optimum amount of starch was dissolved in a 100 mL Erlenmeyer flask containing 30 mL of 0.1 M sodium acetate buffer at its optimum pH. Then, the immobilized glucoamylase was added according to the optimum initial enzyme concentration. The mixture was placed in a water bath shaker set at its optimum agitation speed for 8 hours. The initial and final samples were collected for DE analysis. After the hydrolysis process, the immobilized enzyme was filtered-off and washed with 0.1 M sodium acetate buffer at the optimum pH (3×50 mL). The residue was reintroduced into a fresh reaction medium and its performance checked. The immobilized enzyme activity was determined to be 100% in the 1st trial. Activities obtained in other runs were compared with that of the 1st trial.

2.5 Characterizations and Analysis

The nitrogen adsorption-desorption isotherm data were collected using a Quanta-chrome NOVA touch LX-4 automated gas sorption analyzer operated at liquid nitrogen temperature, to estimate the average cell pore size, average window pore size, specific pore volume, and specific surface area using the Barrett-Joyner-Halenda (BJH) method and the Brunauer-Emmett-Teller (BET) method. Samples of the carrier and immobilized enzyme were also analyzed through the Agilent FTIR spectroscopy. Protein content was determined using Pierce™ BCA protein assay kit, while hydrolysis of starches was assessed by the DNS method. A Shimadzu UV-VIS 1800 spectrometer was employed to quantify the protein and glucose levels.

Table 1. The operational factors of immobilization and hydrolysis process.

Experimental Factor	Enzyme Immobilization ²	Starch Hydrolysis ²
Enzyme concentration	2-10 mg.mL ⁻¹	2,500-10,000 U
Agitation speed	80-140 rpm	120-160 rpm
Sorensen buffer pH	5.0-7.0	4.1-5.1
Temperature	30-50 °C	40-80 °C
Starch concentration	-----	3-8% (w/v)

3. Results and Discussion

The immobilization of free glucoamylase to the MCF silica carrier derived from BBA was achieved through adsorption. The process involved submerging the carrier in an enzyme solution for a certain time under conditions that maintain the activity of glucoamylase, as described in Figure 1. The physical attachment of the enzyme to the carrier was facilitated by non-covalent interactions, such as the electrostatic attraction between oppositely charged entities or hydrogen bonding. Although this method is straightforward and economical, it results in enzyme leaching due to the relatively weak interactions between the enzyme and the carrier [21-23].

The MCF silica is synthesized from inorganic silica species through formation of hydrogen bonds, as indicated in reference [24]. This process involves the addition of an expansion agent, which facilitates the transition from a highly ordered hexagonal symmetry to the MCF structure [25]. In our experimental procedure, KCl was employed as the expansion agent, following recommendation by Choi *et al.* [26]. The synthesis was conducted under acidic conditions using HCl, where assembly of the hydrophilic group and positively charged silica species occurred through electrostatic interactions, which were mediated by negatively charged chloride ions.

The surface area, pore volume, and pore size after the BJH desorption analysis are presented in Table 2. The BBA-MCF silica carrier exhibited a surface area of $48.5 \text{ m}^2 \cdot \text{g}^{-1}$. The diameters of the

carrier pores were distributed between 3.05 and 26.32 nm, with a peak diameter observed at 9.33 nm. The BJH desorption cumulative micropore volume measured $0.1214 \text{ cm}^3 \cdot \text{g}^{-1}$. The distribution of the carrier pore was identified within the range of $0.003\text{--}0.017 \text{ cm}^3 \cdot \text{nm}^{-1} \cdot \text{g}^{-1}$, where 46.24% of the pore volume consisted of the carrier pore diameters exceeding 6 nm. These characteristics are smaller than the TEOS-TMB-derived MCF silica, which, for example, has BET surface area of $378 \text{ m}^2 \cdot \text{g}^{-1}$, pore volume of $2.12 \text{ cm}^3 \cdot \text{g}^{-1}$, and pore diameter size of 15.8 nm [1,2,27]. Additionally, the MCF silica developed by Choi *et al.* [26] from KCl salt and Aldrich sodium silicate solution had high surface area ($309\text{--}387 \text{ m}^2 \cdot \text{g}^{-1}$) and pore volume ($1.28\text{--}1.56 \text{ cm}^3 \cdot \text{g}^{-1}$), but with similar pore diameter sizes (9.07–13.7 nm). It seems that the MCF silica prepared using KCl salt resulted in smaller pore sizes compared to the one derived from trimethylbenzene (TMB). TMB is a bulky organic compound and known as a pore expanding/swelling agent capable of enlarging micelles or creating significant voids within the precursor structure [28,29], while KCl is a crystalline inorganic salt that typically produces small and less adaptable templates, leading to the development of small pores [30,31]. In the preparation stage, our research used 10 g of KCl along with diluted HCl, contrasting notably with the approach taken by Choi *et al.* [26] who used 5 g of KCl and diluted acetic acid for formation of the carrier from Aldrich sodium silicate. Hence, further observations are required in development of salt-assisted MCF silica from BBA to increase

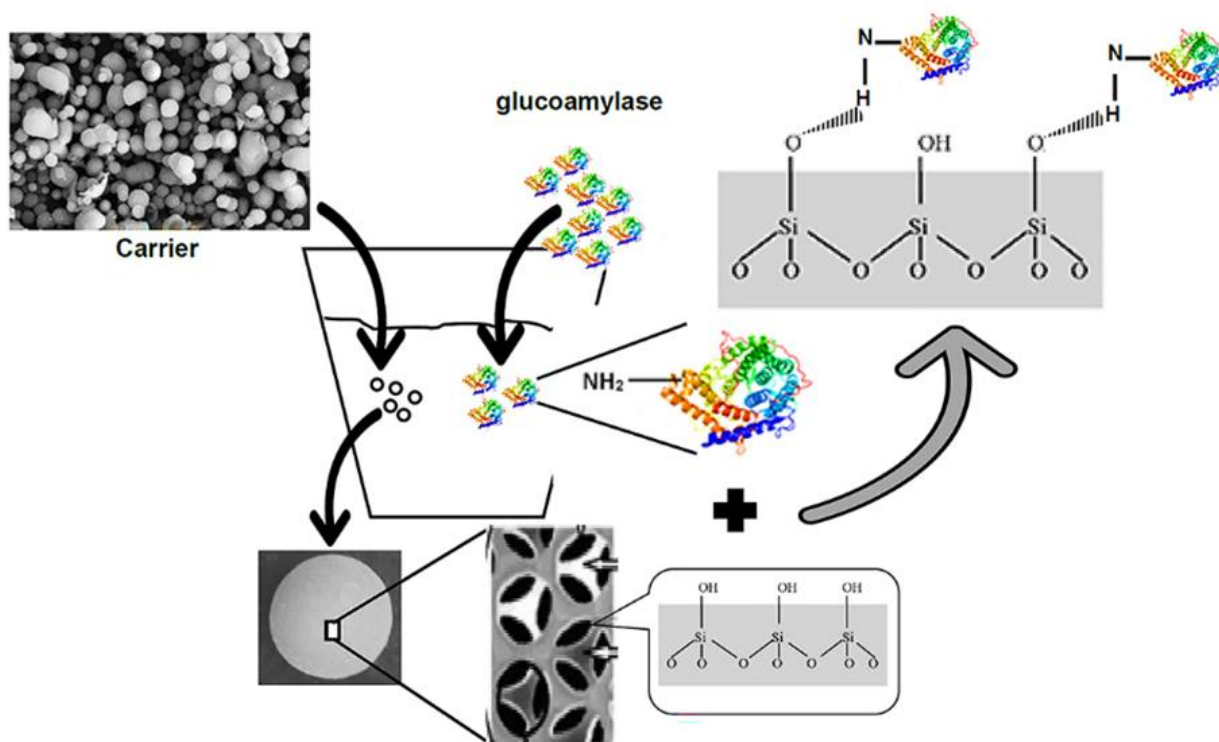


Figure 1. Adsorption of glucoamylase to the BBA-derived MCF silica.

surface area of the product, such as optimization of the salt concentration and revision of the preparation method. Choi *et al.* [26] who prepared the S-MCF from Aldrich sodium silicate concluded that the surface area and pore volume of the silica decreased in the presence of higher concentrations of salts in the synthetic solution.

Mesoporous properties of the BBA-derived MCF silica were evaluated through the N₂ adsorption-desorption isotherms and pore-size distribution, as shown in Figure 2. The observed hysteresis loop was characteristic of mesoporous silica materials, classified as type IV [32,33], typical of materials with mesoporous channels and cavities. The hysteresis was noted in the relative pressure (P/P_0) range of 0.5 to 0.94, confirming presence of mesostructured pores. As described previously, the pore sizes of the carrier were distributed between 3.05 and 26.32 nm, with peak diameter of 9.33 nm. After glucoamylase immobilization, there was a significant reduction in pore volume and average diameter to 3.4 nm, indicating that the enzyme occupied the pores. This reduction can be attributed to three main factors: size of the enzyme, which blocks the larger pores; electrostatic interactions between the functional groups of the enzyme and the silica pore walls, favoring immobilization; and the synthesis conditions, such as presence of KCl, which may have influenced the final structure of the material, reducing the available surface area after immobilization. Thus, the observed reduction in surface area and pore volume reflects the efficient immobilization of glucoamylase, as confirmed by the isotherms and pore distribution.

The FTIR analysis is shown in Figure 3. Strong vibrations occurred in the wavelength range of 1,300-850 cm⁻¹, peaking at 1,013.8 cm⁻¹. Literatures indicated that these frequencies related with the silicone-oxy compounds (Si-O-Si group) [32,33]. A medium peak was observed at 1,640 cm⁻¹. This peak belongs to the double bond region [32] and could associate with the following functional groups: C=C stretching, primary amine (N-H bend), secondary amine (>N-H bend, C=O stretching), amide (NH₂ bending, C=O stretch), and organic nitrates [34-37]. A strong vibration peak was found at 3,347.1 cm⁻¹. The peak existed in the single bond region [33] and related to the following functional groups: hydroxyl group (H-bonded, OH stretch), primary amide (NH₂ stretch), secondary amine (>N-NH-stretch), Si-OH stretch [34-37]. These vibrations were related

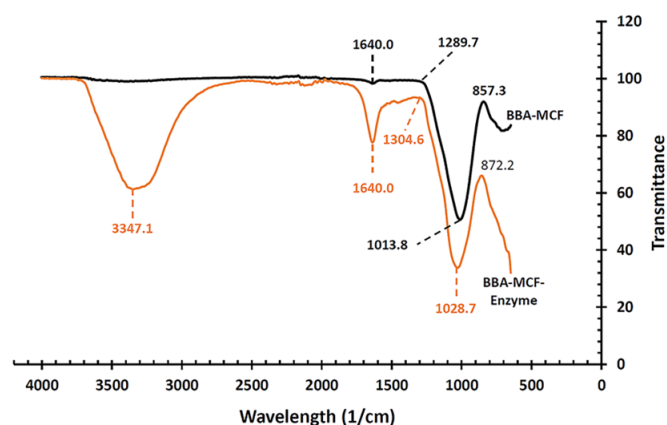


Figure 3. FTIR spectrum of the carrier and immobilized enzyme

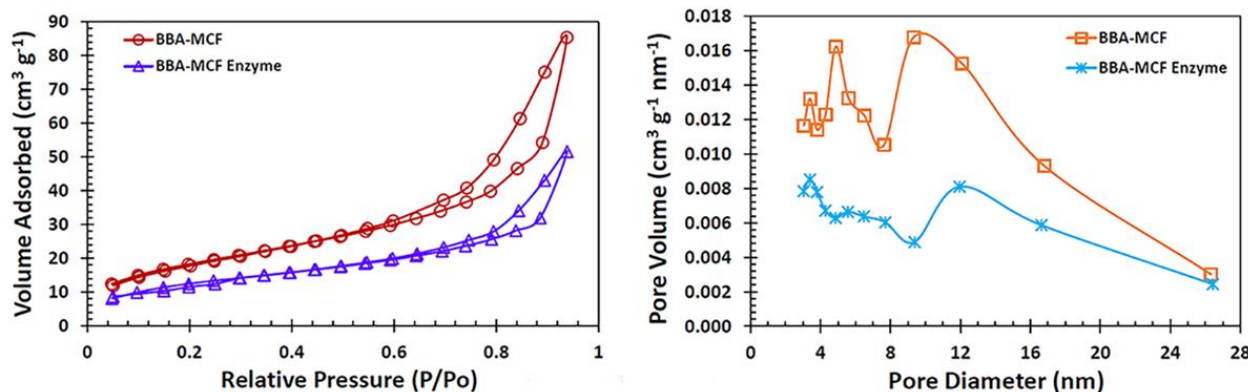


Figure 2. N₂ adsorption-desorption isotherms (left) and pore size distribution (right) of the BBA-based MCF silica and immobilized glucoamylase.

Table 2. BJH desorption of the carrier and immobilized enzyme.

Compound	Surface Area (m ² .g ⁻¹)	Micropore Volume (cm ³ .g ⁻¹)	Pore Diameter (nm)
BBA-MCF Silica	48.5	0.12	9.33
BBA-MCF Silica-Enzyme	26.3	0.07	3.4

with glucoamylase since the enzyme is composed by amino acid compounds, which basically contain amino groups (-NH_2) and carboxyl groups (-COOH). The observed frequencies were slightly lower than those observed in the TEOS-based MCF silica as described previously [1-3].

Figure 4 indicates that the BBA-derived MCF silica contains a significant amount of silicon (Si) compound even though the initial silica (SiO_2) content in the BBA extract was quite high (96.46% (w/w)) found after the gravimetric analysis. However, following the pre-treatment process, the extracted BBA retained only trace amounts of aluminum (Al). The SEM-EDX analysis suggests that the minimal presence of aluminum could contribute to the aggregation of BBA-based MCF silica. Furthermore, presence of carbon (C), oxygen (O), and silicon (Si) elements in the immobilized enzyme sample confirms that these elements are associated with both the carrier material and the chemical composition of the enzyme. Carbon, oxygen, and nitrogen, in particular, are indicators of the organic components contributed by the glucoamylase enzyme.

Adsorption of free glucoamylase onto the carrier was further confirmed by reduction in pore diameter (Table 2). The pore size decreased to 3.4 nm, suggesting that the enzyme molecules occupied the carrier's surfaces. The salt-assisted BBA-based MCF silica proved to be effective for glucoamylase adsorption, accommodating enzymes with molecular weights of less than 200 kDa ($\sim 80 \text{ \AA}$), such as *Aspergillus terreus*

glucoamylase (86 kDa) [38]. *Aspergillus niger* glucoamylase (46-118 kDa) [39,40], *Thermomyces lanuginosus* glucoamylase (52-72 kDa) [41,42], and *Amylomyces* sp. glucoamylase [43]. George *et al.* [6] noted that the mesoporous silica channels were specifically designed to accommodate enzyme molecules with dimensions similar to glucoamylase's.

The N_2 adsorption-desorption isotherm (Figure 2) confirmed the immobilization, as the hysteresis loop for the immobilized enzyme shifted between 0.64-0.94. The significant decrease in the pore volume after the enzyme attachment confirmed the reduction in available pores, further supporting efficient adsorption onto the salt-assisted BBA-based MCF silica. Despite the reduction in pore volume, the overall mesoporous structure of the material remained intact after enzyme immobilization, as evidenced by the preserved adsorption-desorption profile. Additionally, the FTIR analysis (Figure 2) provided further validation for glucoamylase adsorption. The peaks related to Si-O-Si stretching at $1,028.7 \text{ cm}^{-1}$ remained present, while new peaks, especially at $1,640.0 \text{ cm}^{-1}$ and $3,347.1 \text{ cm}^{-1}$, were identified, corresponding to amide and amine bonds from the enzyme's polypeptide chain [2,36,44].

Results of the enzyme adsorption onto carrier are illustrated in Figure 5. Four factors, i.e. free enzyme concentration, agitation speed, Sorensen buffer pH, and temperature, were analyzed using the OFAT approach. Overall, the immobilization efficiencies ranged from 78.0% to 88.5%, with the

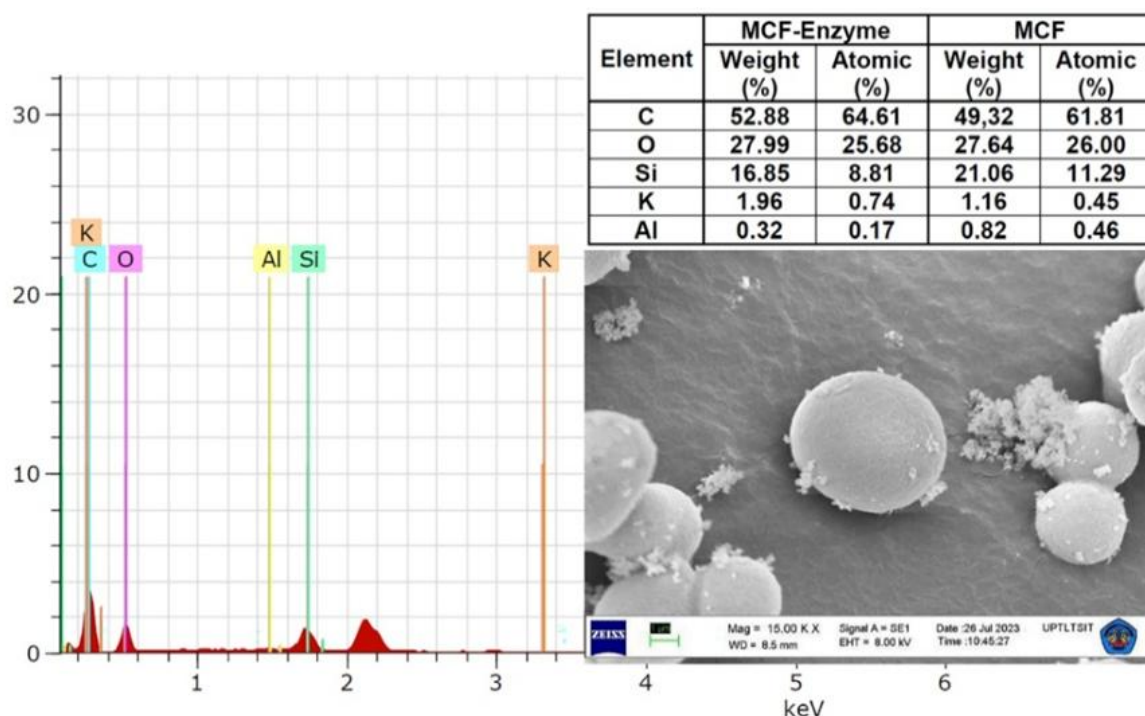


Figure 4. SEM-EDX on the immobilized glucoamylase (Carbon (C), Oxygen (O), Silicone (Si), Aluminum (Al), Potassium (K)).

highest efficiency achieved at the optimal operational temperature, while the lowest enzyme concentration yielded the least favorable results. The optimal parameters identified were an enzyme concentration of 9.0 mg.mL^{-1} , an agitation speed of 120 rpm, a buffer pH of 5.5, and an operational temperature of 30°C .

The immobilization temperature was examined in the range of $30\text{--}50^\circ\text{C}$. The highest efficiency, approximately 88.5%, was recorded at 30°C , while the lowest result, around 82.0%, was observed at 50°C (uncertainty analysis: average value = 85.6%, confidence interval at 95% confidence level = 2.00). At 35°C , the immobilization efficiency approached the optimal level, with only a minor difference in efficiency (< 2%), suggesting that the immobilization temperature could be fine-tuned within the $30\text{--}35^\circ\text{C}$ range. Beyond 45°C , a significant decline in immobilization efficiency was noted, exceeding 3%. The factor demonstrated a significant impact on the immobilization process, as indicated by the regression analysis, which yielded a coefficient of determination (R^2) of 0.93 along with a low significance F and p -value (< 0.05). The optimal temperature identified in these experiments aligned with findings from a previous study that utilized TEOS-based MCF silica as a carrier for glucoamylase [2]. However, the immobilization of glucoamylase on the salt-assisted BBA-based MCF silica yielded a slightly higher efficiency (approximately 3%). Previous studies reported achieving the optimal immobilization efficiencies at 30°C [6,45]. Conversely, *Aspergillus niger* glucoamylase was immobilized on various mesoporous silicas primarily derived from TEOS conducted at the ambient temperature [4].

pH of a 0.1 M Sorensen phosphate buffer was adjusted between 5.0 and 7.0, found significantly influencing the process. Generally, an increase in

the pH of the medium results in a decrease in immobilization efficiency. The highest immobilization efficiency was recorded at pH of 5.5, with approximately 88.5% of the free enzyme being immobilized, while the lowest efficiency, around 78%, was observed at pH of 7.0 (uncertainty analysis: average value = 83.3%, confidence interval at 95% confidence level = 4.01). The substantial difference between these two conditions underscores the significant impact of Sorensen buffer pH on glucoamylase immobilization. Similar to the preceding factor, the pH of buffer exhibited a significant impact, as indicated by the R^2 value of 0.90, with both the significance F and p -value were below 0.05. The optimal buffer pH aligns with previous findings regarding the immobilization of glucoamylase on TEOS-based MCF silica [2].

Impact of free glucoamylase concentration was examined in the range of 5–10 mg of enzyme per mL of buffer. The highest efficiency achieved was approximately 88.5% at the concentration of 9.0 mg.mL^{-1} (equivalent to 270 g of free enzyme), while the lowest efficiency recorded was 78% at the minimum concentration of 5.0 mg.mL^{-1} (uncertainty analysis: average value = 83.8 %, confidence interval at 95% confidence level = 3.22). The initial enzyme concentration was found to have a substantial impact on the immobilization process, as evidenced by the significant disparity between the maximum and minimum immobilization efficiencies. Examination of the efficiency data via regression analysis revealed a notably high R^2 value of 0.761, accompanied by a low significance F and p -value, both of which are less than 0.05. In comparison to TEOS-based MCF silica [2,7], a greater quantity of glucoamylase was immobilized on the BBA-based MCF silica, indicating a higher availability of enzymes on the surfaces of the BBA-based

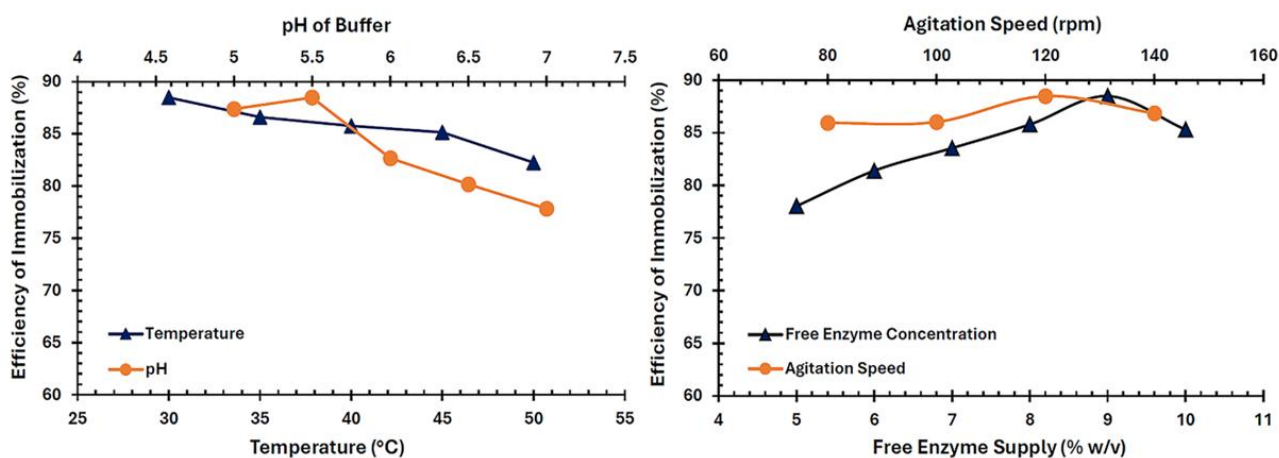


Figure 5. Effects of the operational factors on immobilization ([factors constant at temperature variation: 120 rpm, 270 mg free enzyme; pH of 5.5]; [factors constant at buffer pH variation: 120 rpm, 270 mg free enzyme, 30°C]; [factors constant at enzyme concentration variation: 120 rpm, pH of 5.5, 30°C]; [factors constant at agitation speed variation: 270 mg free enzyme, 30°C , pH of 5.5])

carrier. Bayne *et al.* [46] noted that carriers with pore sizes of 10-100 nm exhibited reduced protein loads as pore size increased.

Agitation speed of the mixture was varied between 80 and 140 rpm. The maximum efficiency recorded was 88.5% at 120 rpm, while the minimum efficiency of 86% occurred at 80 rpm (uncertainty analysis: average = 86.8%, confidence interval at 95% confidence level = 1.16). This parameter had a negligible impact on the immobilization process, given the small difference between the highest and lowest efficiencies. The regression analysis showed a low R^2 of 0.32; however, the significance F was notably high at 0.43, despite the p -value was less than 0.05. Previous studies indicated that a lower agitation speed of 100 rpm achieved a glucoamylase immobilization efficiency of 84.8% on TEOS-based MCF silica, despite utilizing the same amount of MCF silica [2]. The immobilization of free *Aspergillus niger* glucoamylase on amino-functionalized TEOS-based large ordered mesoporous silica carriers, a higher agitation speed of 200 rpm was employed [4,6].

The specific activities of both free and immobilized glucoamylase were evaluated using soluble starch in 0.30 L of 0.05 M sodium acetate buffer at a pH of 4.5. The analysis revealed that the immobilized enzyme exhibited a slightly lower specific activity compared to its counterpart (Figure 6). Specifically, the specific activities for free and immobilized glucoamylase were recorded at 23,848 U.g⁻¹ and 20,578 U.g⁻¹, respectively. These reductions could be caused by conformational changes on the enzyme structures during the immobilization process as some enzyme active sites are difficult to be accessed by the substrate [47,48]. Milosevic *et al.* [49,50] explained the diffusional limitations had occurred caused by the support that reduces the access of the substrate to the active sites of the bound enzyme. Further, Milosevic *et al.* [51] concluded the immobilization process interferes with the enzyme catalytic site and/or by diffusional problems generally produced when immobilized

enzymes are acting on macromolecular substrates. The obtained specific activity surpassed the specific activity of glucoamylase immobilized on TEOS-based MCF silica, which was measured at 1,856.78 U.g⁻¹ [2]. Additionally, the specific activities from the immobilization of glucoamylase on functionalized magnetic nanoparticles, mesoporous carbon, and poly(CMA-co-EGDMA) were lower, yielding 1,408.6 U.g⁻¹ [52], 84-540 U.g⁻¹ [50,51], and 700-1,100 U.g⁻¹ [49,51], respectively. It is common for the activity of free enzymes to diminish when glucoamylase is immobilized on inorganic carriers; for instance, glucoamylase immobilized on Sibunit retained less than 20% of the activity of free glucoamylase [53,54]. Moreover, immobilization on polymeric compounds often resulted in even lower specific activities, typically below 10% [49-51]. However, a notable increase in specific activity, reaching up to 55%, was observed when free glucoamylase was immobilized in magnetic clays [55].

Hydrolysis of starches, including tapioca, wheat, potato, and corn, were subjected to operational variations through the OFAT experiments over a duration of eight hours in 30 mL of 0.1 M sodium acetate buffer. The processes yielded low to moderate Dextrose Equivalent (DE) values; however, the free glucoamylase exhibited higher DE values compared to the immobilized enzyme, as shown in Table 3. Previous studies demonstrated that the free Sigma-Aldrich *Aspergillus niger* glucoamylase activity was enhanced through its adsorption and covalent bonds onto TEOS-based mesoporous silicas [4]. In contrast, the Gist-Broekades glucoamylase (Amigase, batch 8187/SPE 0551) immobilized on the TEOS-based MCF silicas via adsorption and covalent bonds gave products with greater activity than the free glucoamylase, with the unique pore structure of the MCF playing a pivotal role in the enzyme immobilization [7]. Additionally, the hydrolysis of tapioca starch using glucoamylase immobilized on TEOS-based MCF silica achieved a maximum DE value of 76.3% at a temperature of 70 °C, a buffer pH of 4.1, and an agitation speed of 140 rpm. In starch hydrolysis processes, the immobilization of Sigma-Aldrich *Aspergillus niger* glucoamylase on mesoporous silica through adsorption or covalent bonding enhances the enzyme's stability and results in products with higher catalytic activity compared to free glucoamylase [6].

Activity of the immobilized glucoamylase was found to be lower than that of the free enzyme during variation of the sodium acetate buffer pH, which exhibited peak activity at pH of 4.6, whereas the immobilized enzyme demonstrated optimal performance at pH of 4.1. The DE values for the immobilized glucoamylase throughout the experiments ranged from 8.1% to 45.6% (uncertainty analysis: average value = 29.69

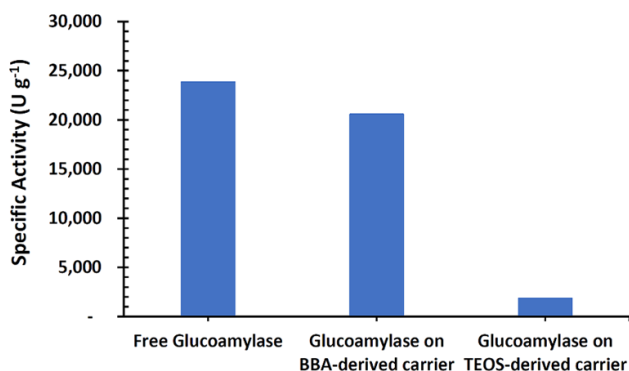


Figure 6. The comparison of enzyme specific activity.

(tapioca), 11.13 (potato), 15.55 (corn), 10.76 (wheat), confidence interval at 95% confidence level = 5.58 (tapioca), 15.96 (corn), 6.39 (wheat), 3.56 (potato)), with hydrolysis of tapioca and corn starch yielding superior DE results. In contrast, the free enzyme achieved DE values as high as 65% during the hydrolysis of corn (uncertainty analysis: average value = 39.96 (tapioca), 14.79 (potato), 58.51 (corn), 15.59 (wheat), confidence interval at 95% confidence level = 4.24 (tapioca), 48.49 (corn), 6.69 (wheat), 5.43 (potato)). A significant disparity in enzyme activities was noted in the hydrolysis of corn; however, both enzymes displayed minimal DE differences when hydrolyzing tapioca, wheat, and potato starch. The small variation between the maximum and minimum DE values suggests a limited impact of this factor on the performance of the immobilized enzyme during starch hydrolysis. A similar optimal pH was observed in the hydrolysis of tapioca using glucoamylase adsorbed on the TEOS-based MCF silica [2]. In contrast, covalently bonded glucoamylase on certain mesoporous silicas demonstrated optimal hydrolysis of a 3% (w/v) starch solution at a higher pH range of 5.0 to 6.0 [6].

The operational temperature for the hydrolysis process was adjusted within the range of 40-80 °C. Similar to the buffer pH, activity of the immobilized glucoamylase remained lower than that of the free enzyme. Both enzyme types exhibited the same optimal temperature. The derived DE values reached as high as 35.4% for the immobilized enzyme (uncertainty analysis: average value = 27.50 (tapioca), 11.20 (potato), 19.52 (corn), 13.20 (wheat), confidence interval at 95% confidence level = 8.23 (tapioca), 8.80 (corn),

5.66 (wheat), 2.99 (potato)) and up to 79.1% for the free glucoamylase (uncertainty analysis: average value = 33.09 (tapioca), 13.45 (potato), 56.97 (corn), 21.82 (wheat), confidence interval at 95% confidence level = 12.46 (tapioca), 16.86 (corn), 5.38 (wheat), 2.68 (potato)), with both potato and tapioca starch hydrolysis yielding comparable DE values for each enzyme. When varying this factor, the hydrolysis of tapioca and corn starch resulted in superior DE values compared to other starches. The factor significantly influenced the hydrolysis process, as evidenced by the minimal difference between the highest and lowest DE values produced by the immobilized glucoamylase. During the hydrolysis, the optimal temperature varied considerably among the starches. The hydrolysis of tapioca and corn starch using the immobilized glucoamylase was optimal at 50 °C, whereas the hydrolysis of wheat and potato starch required higher optimal temperatures of 60 °C and 70 °C, respectively. Previous studies indicated that the effect of process temperature on the activity of immobilized *Aspergillus niger* glucoamylase in starch hydrolysis within the same buffer was optimal at temperatures ranging from 30-40 °C [6]. However, Zhao *et al.* [56] demonstrated that the immobilized *Aspergillus niger* glucoamylase exhibited higher optimal temperatures, specifically between 55-65 °C, compared to the free glucoamylase in the soluble starch hydrolysis process.

Additional operational parameters, including the initial concentration of enzyme, starch concentration, and agitation speed, were also varied to assess their impacts. Performance of the immobilized glucoamylase remained inferior to that of the free enzyme, consistent with the

Table 3. DE values of various starch hydrolysis.

Factor	Wheat		Corn		Potato		Tapioca	
	Free	Adsorbed	Free	Adsorbed	Free	Adsorbed	Free	Adsorbed
Buffer pH	10.2-21.9	8.1-17.2	40.4-65.0	37.9-45.6	9.5-18.8	8.9-14.7	36.1-43.6	26.7-35.4
Temperature	13.2-40.7	5.1-25.0	42.9-79.1	10.7-31.8	9.6-16.0	7.8-14.7	26.8-40.2	23.1-35.4
Enzyme Concentration	20.8-43.0	17.3-34.5	7.4-79.1	5.6-31.8	16.9-24.4	12.2-19.5	23.3-42.5	21.6-35.4
Starch Concentration	20.9-45.4	25.9-32.2	35.6-87.8	12.1-49.1	24.4-38.3	14.3-52.9	17.6-38.3	19.1-45.6
Agitation Speed	27.1-46.4	14.8-29.9	13.6-87.8	7.1-49.1	24.6-46.8	43.4-63.9	40.4-65.0	37.9-45.6
Optimum pH	4.6		4.1		4.1		4.1	
Optimum Temperature	60 °C		50 °C		70 °C		50 °C	
Optimum [Enzyme]	7500 U		5000 U		7500 U		5000 U	
Optimum [Starch]	5 mg.mL ⁻¹		2 mg.mL ⁻¹		3 mg.mL ⁻¹		5 mg.mL ⁻¹	
Optimum Speed	140 rpm		140 rpm		120 rpm	160 rpm	120 rpm	140 rpm

OFAT Design: 1) Effect of buffer pH (tapioca: 50 °C, 140 rpm; 5% (w/v) starch, 5,000 U enzyme; potato: 70 °C, 140 rpm; 5% (w/v) starch, 5,000 U enzyme; corn: 50 °C, 140 rpm; 4% (w/v) starch, 5,000 U enzyme); wheat: 60 °C, 140 rpm; 5% (w/v) starch, 5,000 U enzyme); 2) Effect of temperature (tapioca, wheat & potato: pH 4.6, 140 rpm, 5% (w/v) starch, 5,000 U enzyme; corn: pH 4.6, 140 rpm, 4% (w/v) starch, 5,000 U enzyme); 3) Effect of free enzyme concentration (tapioca: 50 °C, pH 4.6, 140 rpm, 5% (w/v) starch; wheat: 60 °C, pH 4.6, 140 rpm, 5% (w/v) starch; potato: 70 °C, pH 4.6, 140 rpm, 5% (w/v) starch; corn: 50 °C, pH 4.6, 140 rpm, 4% (w/v) starch); 4) Effect of starch concentration (tapioca, corn: 50 °C, pH 4.6, 140 rpm, 5,000 U enzyme; wheat: 60 °C, pH 4.6, 140 rpm, 7,500 U enzyme; potato: 70 °C, pH 4.6, 140 rpm, 7,500 U enzyme); 5) Effect of agitation speed (tapioca: 50 °C, pH 4.6, 3% (w/v) starch, 5,000 U enzyme; wheat: 60 °C, pH 4.6, 5% (w/v) starch, 7,500 U enzyme; potato: 70 °C, pH 4.6, 3% (w/v) starch, 7,500 U enzyme; corn: 50 °C, pH 4.6, 2% (w/v) starch, 5,000 U enzyme)

findings of the previous two factors, where the produced DE ranged from 7.1% to 63.9% for the immobilized enzyme (uncertainty analysis: average value = 28.62-41.06 (tapioca), 16.13-54.00 (potato), 18.08-36.44 (corn), 22-55-28.29 (wheat), confidence interval at 95% confidence level = 3.74-15.08 (tapioca), 13.47-23.89 (corn), 3.88-8.56 (wheat), 4.12-9.92 (potato)) and from 7.4% to 87.8% for the free enzyme (uncertainty analysis: average value = 29.22-53.39 (tapioca), 20.83-36.58 (potato), 35.19-65.50 (corn), 29.62-34.32 (wheat), confidence interval at 95% confidence level = 8.78-12.60 (tapioca), 31.66-43.11 (corn), 3.88-15.51 (wheat), 3.88-12.81 (potato)). The optimal initial enzyme concentrations were determined to be 5,000 U for corn and tapioca, and 7,500 U for wheat and potato. Starch sources such as potato, corn, and tapioca yielded superior results compared to wheat starch. The influence of starch concentration on the hydrolysis process was found to be optimal within the range of 2-5 mg.mL⁻¹. The ideal agitation speed for hydrolysis processes utilizing immobilized glucoamylase was identified as 140 rpm, with the exception of potato starch hydrolysis, which exhibited performance nearly equivalent to that of free glucoamylase. The agitation factor was deemed to have a minimal effect on the hydrolysis process. Notably, variations in agitation speed led to increased DE values in potato starch hydrolysis when employing immobilized glucoamylase compared to the free enzyme.

Reusability on an immobilized enzyme is considered a must for large-scale operations [57]. Hence, the repeated uses of the immobilized glucoamylase were studied to observe its capability after many hydrolysis cycles of tapioca and potato starch (Figure 7). In general, the immobilized enzyme still showed activity of more than 50% after 4 (four) cycles. The results did not differ greatly with the TEOS-based MCF silica that reached > 50% after 5 (five) cycles [1]. Reductions of the immobilized glucoamylase activity could occur as it must be washed several

times with the buffer solutions that caused the enzyme leaching from the carrier [58,59].

4. Conclusion

The use of bagasse boiler bottom ash (BBA) has proven to be a sustainable and efficient alternative for synthesis of salt-assisted mesoporous MCF silica. The carrier demonstrated favorable characteristics for glucoamylase immobilization, including a good surface area and adequate pores, confirming the potential of BBA as a raw material source for these processes. Enzyme immobilization occurred effectively, with significant yield, especially under optimized conditions of enzyme concentration, pH, temperature, and agitation speed. The results highlighted that, although the specific activity of the immobilized enzyme was slightly lower than that of the free enzyme, the immobilization process provided greater stability and consistent performance throughout the hydrolysis of different starches. This reinforces the viability of using supports derived from agro-industrial waste for biotechnological applications. Therefore, this study paves the way for the development of more sustainable industrial processes by converting natural waste into functional materials for biocatalysis. BBA-derived MCF silica, with its favorable properties and promising performance in enzyme immobilization, can be applied in various industrial contexts, especially in starch hydrolysis and potentially in other enzymatic biotransformations.

Declaration of AI Technology

We declare that no Artificial Intelligence (AI) technologies or AI-assisted tools were utilized in any capacity during the writing and preparation of this article.

Conflict of Interest

No conflict of interest was declared by the authors.

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Credit Author Statement

Author Contributions: J. Agustian: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources;

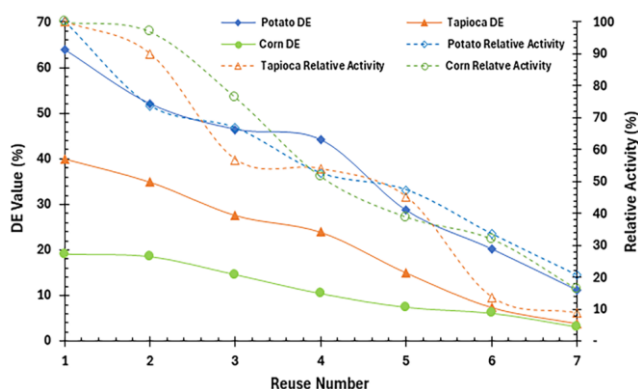


Figure 7. Reusability of the immobilized glucoamylase.

Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing; L. Hermida: Conceptualization; Data curation; Investigation; Methodology; Supervision; Validation; Roles/Writing - original draft; Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

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