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Enhancing stability and activity of β-galactosidase from *Kluyveromyces lactis* through immobilization on polymethacrylate monolith and optimisation using response surface methodology

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ABSTRACT

Aims: This study investigates the potential of porous polymethacrylate monoliths as enzyme support materials for large-scale enzyme commercialization.
Methodology and results: It focuses on their preparation and various immobilization techniques, such as adsorption, covalent-binding and cross-linking, specifically applied to β-galactosidase for bioprocess applications. The research assesses immobilization performance, operational stability, reusability and optimization using response surface methodology (RSM). The results reveal that covalent-binding exhibited the highest enzyme activity recovery, while cross-linking showed superior performance at lower enzyme concentrations but decreased at higher concentrations. Covalent-bound enzymes demonstrated reusability for up to four cycles, with optimal pH ranging between 7 and 8 and optimal temperature ranging between 30 °C and 40 °C. Furthermore, RSM optimization highlighted the significant influence of substrate concentration on enzyme activity, with a reliable model (R^2 = 0.9163) and adequate precision (S/N = 13.1409).
Conclusion, significance and impact of study: Overall, this study provides valuable guidelines for effectively employing porous monoliths in large-scale industrial bioprocessing, offering potential cost-saving benefits and enhanced efficiency in enzyme commercialization.

Keywords: β-galactosidase, immobilization, polymethacrylate monolith, reusability, stability

INTRODUCTION

Microorganisms that produce enzymes have garnered significant biotechnological interest due to their pivotal roles in various industrial, environmental and biotechnological applications. Enzymes, often derived from bacteria, fungi or yeast, are highly effective catalysts used in fields like sensing, separation, and bio-catalysis (Feng *et al.*, 2023). Enzymes have been known as universal catalysis which possess many advantages because of their unique characteristics and structure. However, their practical use is limited because they are sensitive to harsh conditions such as strong acids, bases, organic solvents, and high temperatures (Cai *et al.*, 2021). To make enzymes more versatile, various methods have been explored, including chemical modification, protein engineering and enzyme immobilization using solid materials. Immobilization has shown great promise in stabilizing enzymes and expanding their applications. In addition, immobilization could facilitate the recovery of enzymes and re-use in a large-scale continuous process. Moreover, compared to their free forms, immobilized enzymes are generally more stable and easier to handle (Maghraby *et al.*, 2023). It has been commonly recognized that this technology can provide protection and stability to enzymes against harsh chemical and environmental conditions (Misson *et al.*, 2015a). Fathi *et al.* (2022) reported that substrate concentration, temperature and pH could influence enzyme activity.

β-galactosidase, an enzyme of significant industrial importance, has attracted attention in the industry for its pivotal role in catalyzing the breakdown of lactose molecules, leading to the creation of enzyme-galactosyl

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complexes (Misson *et al.*, 2020). These complexes then proceed down either the hydrolysis or transgalactosylation pathways. It has gained substantial attention in the industry for its applications in food and pharmaceutical sectors. According to Pázmándi *et al.* (2018), β-galactosidase serves as the catalyst in the bioconversion of lactose into galacto-oligosaccharides (GOS). GOS, known for their prebiotic properties, selectively enhance the growth and activity of beneficial gut bacteria like *Bifidobacteria* and *Lactobacilli*. Sun *et al.* (2016) demonstrated the successful attainment of high-purity GOS through this process utilizing β-galactosidase from *Kluyveromyces lactis*. Rodriguez-Colinas *et al.* (2011) revealed in their study that β-galactosidase from *Kluyveromyces lactis*, when employed using permeabilized cells, results in higher stability and efficient GOS production. As reported by Maghraby *et al.* (2023), immobilized enzymes are generally more stable and easier to handle compared to their free forms. It has been commonly recognized that this technology can provide protection and stability to enzymes against harsh chemical and environmental conditions (Misson *et al.*, 2015a). Fathi *et al.* (2022), reported that substrate concentration, temperature and pH could influence enzyme activity.

The choice of support materials plays a crucial role in enhancing the usefulness of an immobilized enzyme. Materials with large surface area could accommodate large amounts of immobilized enzymes besides facilitating rapid mass transport of substrates and products (Ashkan *et al.*, 2021). The advancement of immobilization carriers as well as the natural/synthetic polymers or inorganic materials as enzyme supports from nanomaterials have been extensively studied recently, as reported in the review paper by Misson *et al.* (2015b). Polymer-based monolithic materials have the potential as enzyme support due to their chemical functionality and morphology that can be achieved readily during in situ preparation. In addition, monolithic materials with interconnected porous structures are beneficial for enzyme immobilization due to their large surface area and excellent biocompatibility (Feng *et al.*, 2023). Despite the promising properties of monoliths, there is a scarcity of reports on the preparation and immobilization techniques of enzymes on these materials. There is limited information available in the literature that investigates the protocols for immobilizing enzymes on monolith surfaces and optimizes the process, hence the objective of this study.

Monoliths are typically prepared from a mixture of monomers, free radical initiators and porogenic solvents which are carried out within the confines of a closed container such as a microfluidic channel (Lafleur *et al.*, 2015). In this study, polymethacrylate monolith was employed as a solid matrix for enzyme immobilization. This monolith was prepared in our previous study (Ongkudon *et al.*, 2013) in which the pore morphology of the materials was optimized for enzyme immobilization by varying the porogen contents. The monolith was prepared to possess epoxy groups that could facilitate the binding of enzymes via covalent-binding. Epoxy-activated support was known as an almost ideal way to perform very easy immobilization of proteins and enzymes at both laboratory and industrial scale as described by Mateo *et al.* (2000). Previous research on enzyme immobilization onto monoliths has predominantly focused on silica monolith while limited research has been devoted to examine polymethacrylate monolith for industrial enzymes for bioprocess application. Polymethacrylate monolith has a stronger mechanical strength and ease of handling on a large scale.

The method of enzyme immobilization is essential for maintaining the enzyme's stability and activity. Various methods and strategies have been thoroughly investigated and effectively developed for retaining enzymes either on or within nanomaterials. Misson *et al.* (2015b) have comprehensively described the strategies to immobilize enzyme, that encompass physical adsorption through electrostatic interactions, hydrophobic interactions, hydrogen bonding or van der Waals forces (Lu *et al.*, 2022), as well as covalent-binding (Sigurdardóttir *et al.*, 2018), enzyme cross-linking and physical entrapment or encapsulation. The enzyme contains functional groups such as amino (-NH2), carboxylate (-COOH), thiol (-SH) and hydroxyl (-OH) groups, which are present in lysine, arginine, glutamic, and aspartic acid residues (Zhang *et al.*, 2011). These functional groups lead to binding of enzymes by interacting with the functional groups introduced on support materials through surface modifications.

The immobilization approach must efficiently immobilize the enzymes to prevent enzyme leaching while ensuring the protection of the enzyme's active site. However, a study conducted by Buthe *et al.* (2011), which involved immobilizing enzymes using a spacer arm between the enzyme and an activated matrix through amine activation with glutaraldehyde as a coupling agent, resulted in reduced biological activity due to enzyme denaturation. Despite the effectiveness of these bridge molecules in reducing enzyme leaching, the strong binding method had unintended consequences. Furthermore, as noted by Binhayeeding *et al.* (2020), the use of potent chemical reagents in covalent-binding or crosslinking techniques may induce changes in enzyme conformation.

Therefore, in this study, three techniques for immobilizing enzymes on a polymethacrylate monolith were examined and compared including physical binding, covalent-binding, and cross-linking. β-galactosidase from *Kluyveromyces lactis*, was chosen as a model enzyme for the immobilization on monolith surface. The study systematically investigates the industrial application potential of monoliths by examining the procedures for immobilizing β-Galactosidase and assessing their immobilization performance and effects on operational stability and reusability. Subsequently the immobilization conditions were optimized using response surface methodology (RSM). The outcomes of this research

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endeavor are anticipated to offer valuable insights into the practical utilization of porous monoliths in large-scale industrial bioprocessing.

MATERIALS AND METHODS

Materials and chemicals

Glycidyl methacrylate (GMA, ≥97%), ethylene glycol dimethacrylate (EDMA, 98%), cyclohexanol (99%), azobisisobutyronitrile (AIBN, 98%), glutaraldehyde (25% in H_2O), hexamethylene diisocyanate (≥99%), bovine serum albumin (BSA), fluorescence isothiocyanate (FITC) for protein labeling, 2-nitrophenyl β-D-galactopyranoside (≥98%), o-nitrophenyl-β-D-galactopyranoside (ONPG), coomassie Brilliant Blue G-250) and phosphate buffer (pH 7) were purchased from Sigma-Aldrich, USA. β-galactosidase enzyme from *Kluyveromyces lactis* was purchased from Sigma-Aldrich, USA.

Preparation of polymethacrylate monolith

Poly(GMA-co-EDMA) monolith was prepared via free radical copolymerizations of EDMA and GMA monomers in the presence of the initiator (azobisisobutyronitrile) and cyclohexanol as the porogenic solvent following the method established by Ongkudon *et al.* (2013). The mixture of GMA: EDMA combined with porogenic solvent at 70:30 of monomer with 1% v/v of AIBN was dissolved in 150 mL of solution. All polymerizations were performed within a confined tubular glass column with a constant temperature of 60 °C in a water bath for 3 h. The solid polymer was washed with methanol overnight. After that, the polymer was washed with distilled water to remove the traces of porogen. The monolith then was crushed into a powder form for enzyme immobilization study.

Immobilization of β-galactosidase on monolith

β-galactosidase was immobilized on monoliths through physical method (adsorption technique), covalent-binding and crosslinking method. The physical immobilization method was performed according to Misson *et al.* (2016). Approximately, 10 mg of polymethacrylate monolith was immersed into 1 mL β-galactosidase in PBS solution (1 mg/mL) and gently mixed for 2 h to allow enzyme immobilization to take place. Subsequently, the unbound enzyme was removed by thoroughly washing with distilled water. Meanwhile, the covalent-binding was carried out using glutaraldehyde. The surface of the polymethacrylate monolith was initially activated prior to the binding of the enzyme. A total of 0.3% of glutaraldehyde was added to the monolith and mixed for 1 h for the activation. The method was then followed by an adsorption technique. The crosslinking technique was carried out by conducting the adsorption step prior to the addition of cross-linker following the method described by Chen *et al.* (2013). Approximately, 10 mg of polymethacrylate monolith was immersed into 1 mL of β-galactosidase in PBS solution (1 mg/mL) and gently mixed for 2 h to allow enzyme immobilization to take place. Afterward, the unbound enzyme was removed by thoroughly washing it with distilled water. Then, the immobilized enzyme was crosslinked with glutaraldehyde (0.05% v/v) for 1 h at room temperature. The particles were collected through centrifugation and washed with water to remove unbound enzymes (Misson *et al.*, 2015a).

Morphological and structural characterization of monolith and immobilized enzyme

The textural and morphological properties of the monolith and the β-galactosidase-loaded monolith via physical, covalent and crosslinking methods were observed on a scanning electron microscope (Hitachi High Technologies America Inc S-3400 N, USA) operated at 10 kV. Free and enzyme-loaded monolith samples were deposited on the sampling holder and coated with conductive gold before the morphological examination. Zeiss Axio Fluorescence microscope was used to image the presence and distribution of FITC-labeled β-galactosidase on monolith particles. The labeled sample was prepared by mixing a proportion of 100 mg of the enzyme with 10 µg of fluorescent isothiocyanate (FITC) in ethanol solution with a gentle mixing before immobilization via physical, covalent-binding and cross-linking on monolith before reacting for 2 h at 4 °C in dark condition. A thin layer of the monolith carrying FITC-labeled β-galactosidase was placed onto a glass microscope slide, observed and analyzed using a fluorescence microscope. Non-labeled enzymes on monoliths were used as a negative control. Fourier transform infrared spectroscopy (Agilents Technology Cary 630, USA) was used to characterize the functional groups of the immobilized enzyme. The changes were observed by comparing the enzyme-loaded monolith with the enzyme-free monolith and the spectra of the enzyme alone. The analysis was carried out using OMNIC software.

Effect of pH and temperature on free and immobilized β-galactosidase

The effect of pH on the activity of the free and immobilized enzyme was evaluated by assaying in 50 mM buffers of various pH ranges (pH 4.0-10.0). The buffers used were citrate buffer (pH 4.0, 5.0, and 6.0), phosphate buffer (pH 7.0 and 8.0) and glycine-NaOH (pH 9.0 and 10.0). Likewise, the effect of temperature was observed by calculating the activity of free and immobilized enzymes at various temperatures (20-70 °C).

Reusability

Reusability of the immobilized β-galactosidase on monolith support was assessed by hydrolyzing ONPG substrate using PBS buffer (pH 7) at 37 °C. Reusability of immobilized β-galactosidase was taken in triplicates for assaying the activity of the enzyme. The reactions were conducted in 10 batches. After each cycle, the immobilized enzyme was separated from the supernatant

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Table 1: The experimental range for different parameters or variables in central composite design.

Parameters	Units	-1 Level	+1 Level
Enzyme concentration	mg/mL	0.2	2.2
Reaction time	min	0.5	13
Substrate concentration	mM	5	40

Table 2: Comparison of recyclability of immobilized enzymes using various immobilization techniques and diverse support materials.

Support	Source	Immobilization method	Recyclability (cycles)	Reference
Polymethacrylate monolith	β-galactosidase	Covalent binding	3-4	This study
Polystyrene nanofibers	β-galactosidase	Covalent binding	4 -9	Misson *et al.* (2015a)
Silica nanoparticles	lipase	Covalent binding	8	Dandavate *et al.* (2009)
Chitosan biomimetic-calcium phosphate	sucrose phosphorylase	Entrapment	10	Xu and Liang (2022)
Nano-molecular cages	lipase	Entrapment	7	Cao *et al.* (2021)
Polyurethane	lipase	Entrapment	6	Facin *et al.* (2018)
Alginate-based nanofibers	lipase	Cross-linking	6	Doğaç *et al.* (2017)
Magnetic graphene oxide nanocomposite	lipase	Cross-linking	5	Badoei-Dalfard *et al.* (2019)

and washed with water three times. In the next cycle, fresh ONPG was introduced to the immobilized enzyme under the same assay conditions. The activity determined at the first cycle was considered as control and attributed to a relative activity of 100% for the determination of remaining activity after repeated uses. Each cycle was defined here as the complete hydrolysis of substrate present in a reaction mixture.

Optimisation of enzyme immobilization and RSM experimental design

The enzyme immobilization study on monolith was employed using response surface methodology (RSM) and the central composite design (CCD) using Design-Expert® software (V 6.0.8). The experimental setup included three types of parameters with low (-1) and high (+1) levels, as presented in Table 1. A total of 20 experiments (Table 2) were conducted to explore the relationship between independent variables (enzyme concentration, reaction time and substrate concentration) and the dependent variable (enzyme activity). The significance of the relationship was assessed using *p*-values from Analysis of Variance (ANOVA), the *p*-value used for assessing significance difference ($p<0.05$).

Enzymatic analysis

The enzyme concentration was analyzed using the Bradford assay (Bradford, 1976). A total of 100 µL samples were added into 5 mL of the Bradford reagent, mixed homogeneously and allowed to react for 5 min. The sample was then measured using a UV spectrophotometer (Shimadzu, Japan) at 595 nm. Protein concentration was determined from a calibration curve using BSA as a standard.

The activity assay of free and immobilized β-galactosidase was measured according to the procedure designated by Husain *et al.* (2011). The activity of the enzyme before immobilization was used as a control study. The reaction mixture containing 0.1 mL of enzyme solution (2U), 1.7 mL phosphate buffer solution (PBS) with pH 7.2 and 0.2 mL of 20 mM 2-nitrophenyl-β-D-galactopyranoside (ONPG) was incubated at 37 °C for 10 min. The reaction was terminated by adding 2 mL of 1M sodium carbonate (Na_2CO_3) and the absorbance value was measured at 405 nm. The enzyme activity was determined from the plotted o-nitrophenol standard curve. The standard curve was prepared by measuring o-nitrophenol solutions at different concentrations and plotting their absorbance at 405 nm to determine enzyme activity in the reaction samples. One unit (1U) of β-galactosidase is defined as the amount of enzyme that liberates 1 µmol of o-nitrophenol/min under the standard assay condition.

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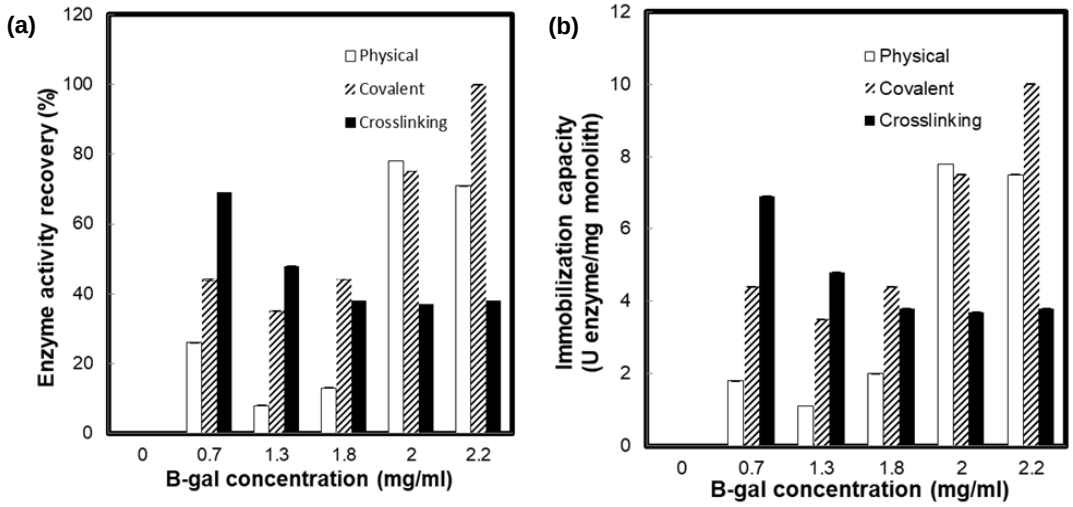

Figure 1: (a) Activity recovery and (b) immobilization capacity of β-galactosidase immobilization on polymethacrylate monolith by physical, covalent binding and cross-linking.

RESULTS AND DISCUSSION

Immobilization of β-galactosidase on polymethacrylate monolith

The successful immobilization of enzymes is evaluated based on the immobilization capacity and recovery of enzyme activity upon enzyme immobilization. Immobilization capacity signifies the amount of enzyme effectively bound to a support material while enzyme activity recovery indicates how well the enzyme retains its functionality and efficiency for catalyzing reactions after being immobilized (Zhou *et al.*, 2021). Figure 1 shows enzyme activity recovery and capacity of β-galactosidase immobilization on polymethacrylate monolith by physical, covalent-binding and cross-linking techniques. Adsorption involves physical attachment of enzymes to a solid support covalent-binding and cross-linking employ chemical methods for enzyme immobilization. According to Figure 1a, the enzyme activity recovery for each technique shows an increasing trend as the concentration of β-galactosidase increases, except for cross-linking. A notably higher enzyme activity recovery was observed in covalent-binding compared to adsorption and cross-linking methods. The highest recovery, reaching 100%, was achieved at a concentration of 2.2 mg/mL. This phenomenon could be attributed to the formation of stronger bonds between the active amino acid residues on the enzyme's surface and the active epoxy groups present on the monolith surface. According to Mateo *et al.* (2000), enzymes have the capacity to first adhere to the support material via various mechanisms and subsequently form covalent bonds by linking the epoxy group with nucleophilic groups found on the enzyme.

Figure 1b exhibits a similar trend in enzyme capacity as observed for enzyme activity recovery. A notable pattern of enzyme activity recovery was observed in the case of cross-linking at lower concentrations. However, this recovery gradually declined, from 69% to 38%, as the concentration increased. A marginal loss of 31% in enzyme activity recovery was evident when employing cross-linking with glutaraldehyde at higher concentrations. It's worth mentioning that the chemistry of glutaraldehyde in aqueous solutions and its relationship to cross-linking or enzyme immobilization remain not fully elucidated (Melo *et al.*, 2017). For the physical adsorption method, there was an initial increase in activity recovery followed by a decline from 78% to 75% as the concentration increased from 2 to 2.2 mg/mL. This decline in enzyme activity recovery about 3% loss could be attributed to the enzyme disassociating itself from the monolith due to weak interactions.

Characterization of β-galactosidase-polymethacrylate monolith

The successful binding of β-galactosidase on polymethacrylate monolith surface through various immobilization techniques was further confirmed using SEM, fluorescence microscope and Fourier Transform Infrared (FTIR) analyses. Figure 2 shows the SEM images of (a) polymethacrylate monolith and the β-galactosidase-loaded monoliths via (b) physical binding (c) covalent and (d) cross-linking techniques. The monolithic structure exhibits a porous structure as illustrated in Figure 2a**.** When β-galactosidase was loaded onto the monolith via physical binding, similar observations were made in comparison to the β-galactosidase-free monolith (Figure 2a) except for the surface displayed smaller particles evident due to the physical attachment of the enzyme. On the other hand, when covalent-binding was employed (Figure 2c), some agglomeration was observed following the activation of the monolith surface with glutaraldehyde before enzyme binding through adsorption, as observed in the study by Kaur *et al.* (2021). Comparatively, crosslinked enzyme

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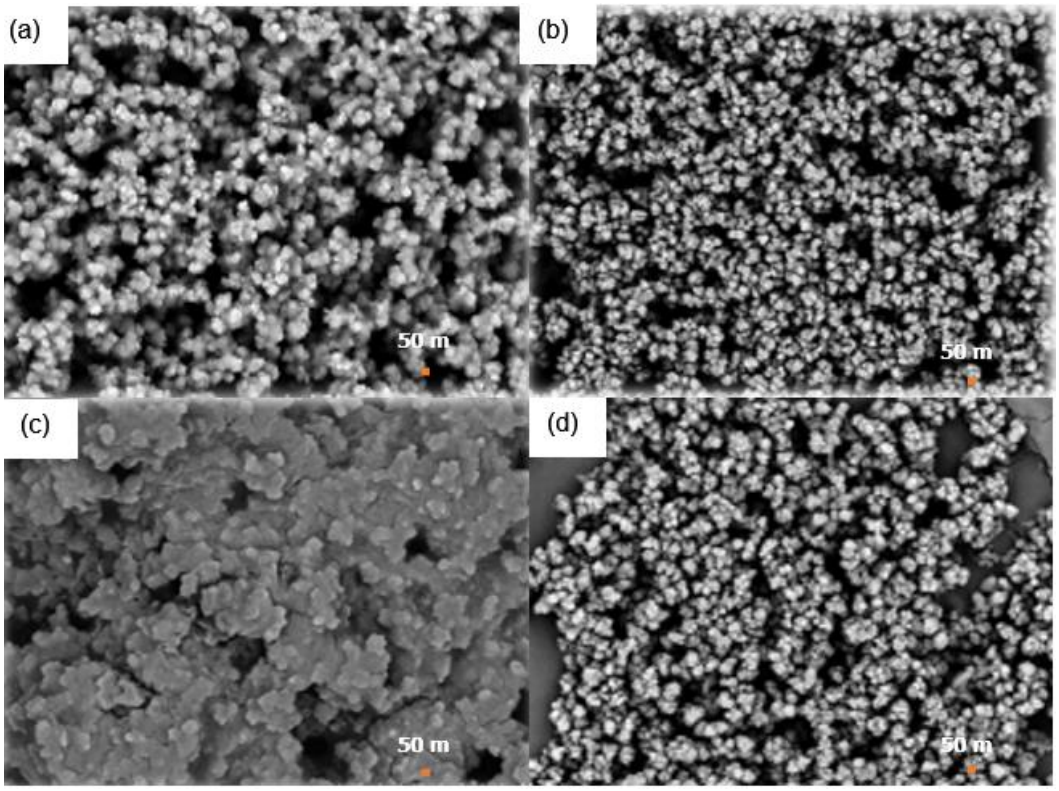

Figure 2: SEM images of (a) β-galactosidase-free monolith and β-galactosidase-loaded monolith via (b) physical binding, (c) covalent and (d) cross-linking.

immobilization bore a resemblance to physical immobilization but with slightly larger particles (Figure 2d) attributable to the crosslinking of the enzyme.

For the fluorescence microscope, β-galactosidase was tagged with fluorescein isothiocyanate (FITC) for preparing the FITC-labeled immobilized enzyme. Figure 3 shows the fluorescence background, FITC-monolith β-galactosidase fluorescence image excitation and negative control monolith-β-galactosidase without fluorescence excitation of β-galactosidase through physical (Figure 3i), covalent-binding (Figure 3ii) and cross-linking (Figure 3iii), respectively. From the visualization, it can be distinguished that the fluorescence in the form of green color was portrayed all through the monolith which demonstrated that the enzyme could permeate laterally and vertically and thus became uniformly distributed across the solid support (Valikhani *et al.*, 2017). A strong fluorescence signal was observed at the FITC-labeled enzyme representing fluorescein isothiocyanate having reacted with amino groups on the surface of enzyme-bound monolith.

FTIR results can provide insights into the interaction between the monolith matrix and β-galactosidase by identifying specific functional groups involved. Changes or shifts in peaks related to functional groups in the spectrum can indicate interactions. Figure 4 shows the FTIR spectra of β-galactosidase free monolith, free β-galactosidase and β-galactosidase loaded monolith via physical, covalent-binding and cross-linking. Spectra (a), (A), (B), (C) shows the band appeared between 600 and 1000 cm^{-1} at 652 cm^{-1} and 989 cm^{-1} was related to asymmetric stretch vibration of epoxy C-O bonds which is glycidyl methacrylate (GMA) (Sattarzadeh and Golipour, 2012). GMA was one of the monomers used for polymethacrylate monolith synthesis asides of porogenic solvent and initiator which is the epoxy-containing stationary phase can easily be modified to form a different chemical nature as mentioned by Li and Zhu (2014). There are also two bands between 1300 cm^{-1} to 1500 cm^{-1} which are 1252 cm^{-1} and 1474 cm^{-1} attributed to the -CH_2 scissoring and bending modes (δHCH) of both GMA and EDMA (Ur *et al.*, 2011). The increase in the intensity of C-O stretch mode of the alcohol group could be observed between 1000 cm^{-1} and 1200 cm^{-1} from 1034 cm^{-1} and to 1042 cm^{-1} which indicates that cross-linking reaction between enzyme and monolith surface has occurred. Moreover, the slight peaks at (C) between 2600 cm^{-1} and 3000 cm^{-1} correlate to the C-H stretch mode of the cross linked molecules also reported by Hu *et al.* (2011). Besides, another band found at (B) which is 1148 cm^{-1} related to the stretching of C-O-C bond which indicated the formation of the covalent bond between the amino groups of enzyme and epoxy groups of monolith (Morhardt *et al.*, 2014). Then, the band at 1636 cm^{-1} was ascribed to C=O stretch vibration of peptide linkage found on glutaraldehyde produced by amide I (Zhang *et al.*,

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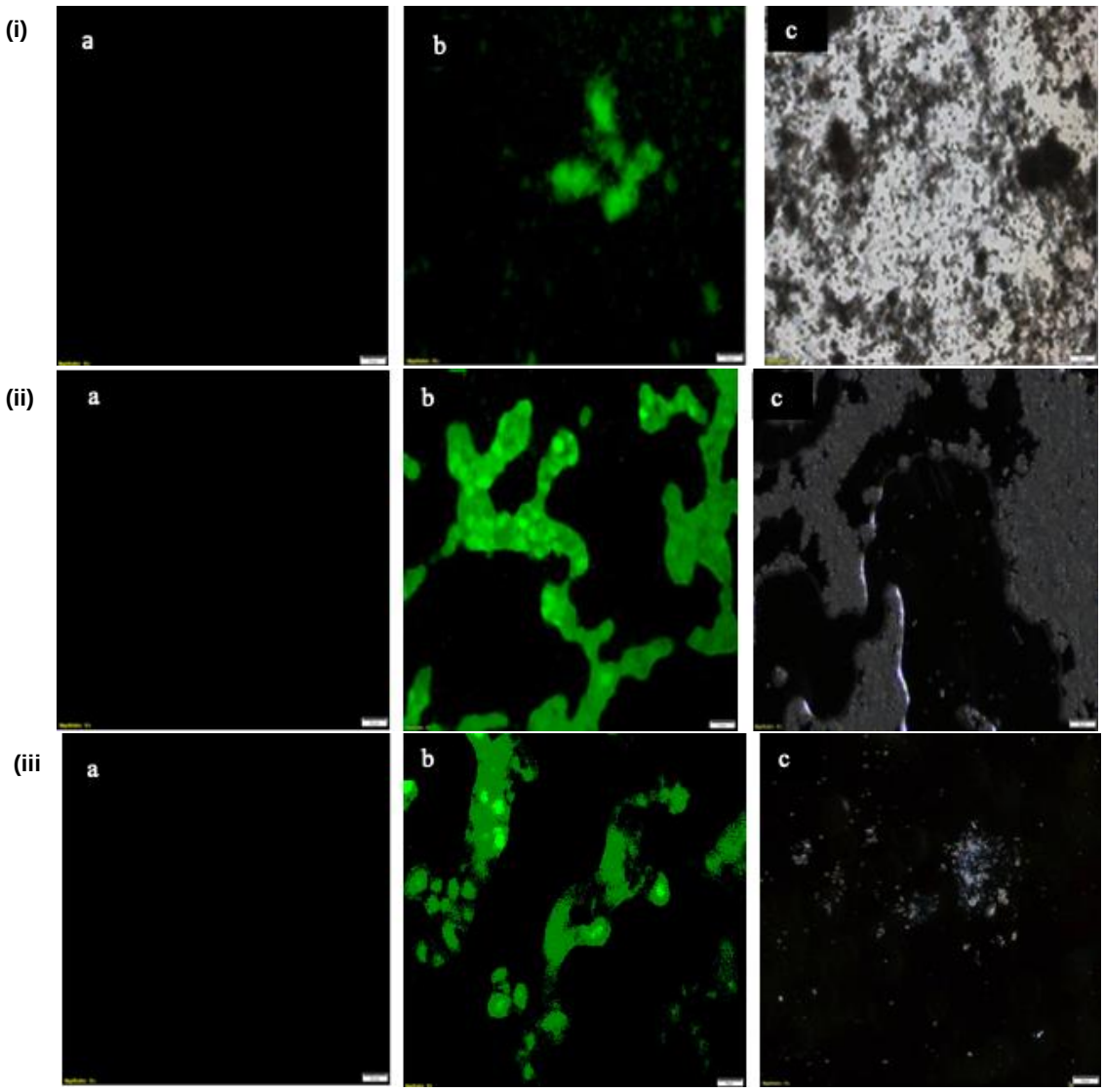

Figure 3: Visualization of FITC-tagged enzyme using fluorescence microscope. (a) Fluorescence background, (b) FITC-monolith-β-galactosidase fluorescence image excitation, (c) negative control monolith-β-galactosidase without fluorescence excitation of β-galactosidase immobilized via (i) physical binding (ii) covalent and (iii) cross-linking.

2015) and NH_2 group found in the enzyme forming C=N bond (Elnashar *et al.*, 2014). The wide and blunt absorption band at 3000 cm^{-1} and 3600 cm^{-1} attributed to the hydroxyl group which is an indication of a ring opening reaction. Based on the result, all the spectra (A), (B) and (C) show the presence of the epoxide group from the monolith which indicates that the enzyme successfully immobilized on polymethacrylate monolith.

Operational stability of β-galactosidase-polymethacrylate monolith

The influence of pH on enzyme immobilization on the polymethacrylate monolith was evaluated by testing a pH range from 4 to 10 using different buffer solutions. Figure 5a showing a steep increase in enzyme activity from pH 4, pH 5 and pH 6 for free, covalent-binding and physical, respectively. Both free enzyme and covalent-bound enzyme demonstrated steady activity up to the tested pH (pH 10). The covalent-binding of β-galactosidase to the monolith surface likely modifies the enzyme's microenvironment and structure, creating a more stable pH range favorable for its stability, comparable to its free form (Maghraby *et al.*, 2023). This is facilitated by the formation of strong and stable bonds between the enzyme and the support matrix, potentially leading to alterations in the enzyme's pH sensitivity and active site accessibility. The pH dependency curve displays unusual stability for the free enzyme, particularly noticeable at pH >5. The likely reason for this could be the utilization of a

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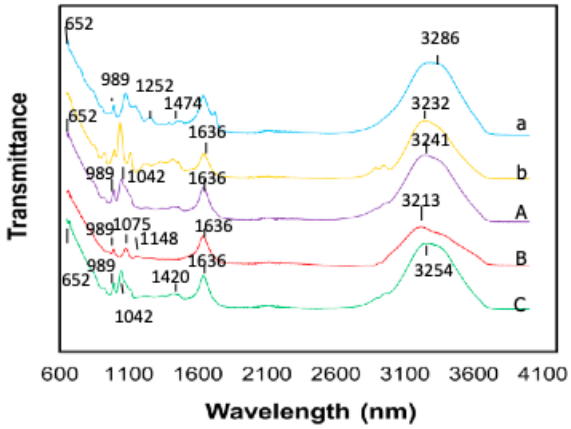

Figure 4: FTIR spectra of (a) β-galactosidase-free monolith, (b) free β-galactosidase and β-galactosidase-loaded monolith of β -galactosidase immobilized via (A) physical binding (B) covalent and (C) cross-linking.

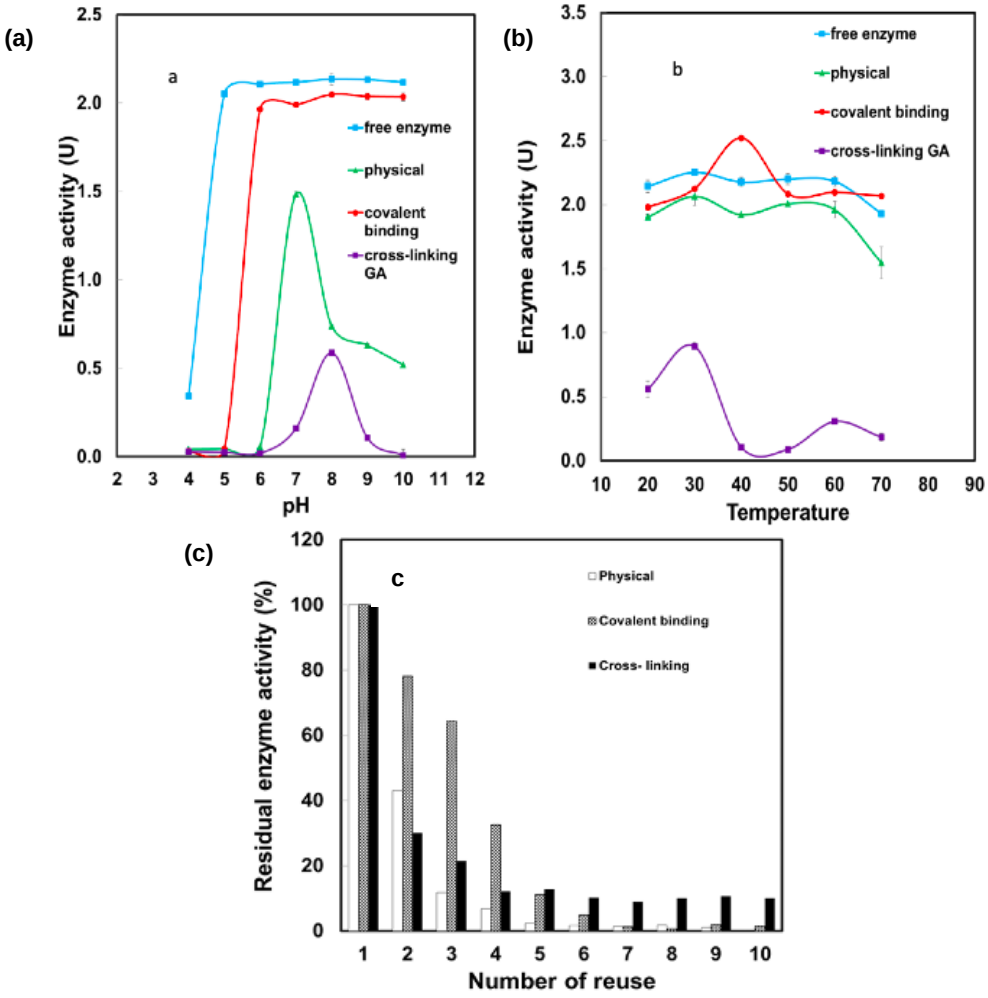

Figure 5: Enzyme activity profiles of free- β-galactosidase and immobilized β-galactosidase via physical binding, covalent and crosslinking techniques at various (a) pH and (b) temperature conditions using 1 mg/mL enzyme concentration and (c) reusability of immobilized β-galactosidase via physical binding, covalent and crosslinking techniques. The activity determined at the first cycle was considered as a control (100%).

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high enzyme concentration during immobilization, where the enzyme activity may have already saturated and reached its maximum level of 2.2 U beyond pH 5. For the physical and cross-linked enzyme, a drastic drop of enzyme activity beyond pH 8 was observed which could be attributed to the denaturation of the enzyme at extreme pH conditions. The immobilization process may have induced conformational changes in the enzyme, altering the active site of crosslink-immobilized enzymes (Kartal *et al.*, 2011). Meanwhile, physical immobilization may not effectively protect the enzyme from extreme pH conditions, resulting in a loss of enzyme activity (Zdarta *et al.*, 2018). Enzymes have an optimal pH range at which they exhibit the highest activity, and deviations from this range can lead to changes in the enzyme's conformation, affecting its catalytic activity (Chen *et al.*, 2013). Besides, the loss in enzymatic activity at highly acidic or alkaline conditions might be also contributed to the alterations in the conformation of the enzyme resulting in a decrease in immobilization efficiency for the crosslinking technique (Cabuk *et al.*, 2014). Moreover, variations in β-galactosidase characteristics, depending on the source (fungi, yeast, or bacteria), contribute to the lack of a consistent optimal pH for the enzyme (Carević *et al.*, 2015). For β-galactosidase obtained from *K. lactis*, the optimal pH falls between 6.5 and 8.5, demonstrating the lack of steadiness in the enzyme's optimal pH, regardless of the method of enzyme binding (Figure 5a).

The enzyme activity of both free and immobilized β-galactosidase was evaluated at different temperatures ranging from 20 °C to 70 °C, as shown in Figure 5b. The trends for free, physical, and cross-linked enzymes showed a gradual increase from 20 °C to 30 °C, followed by a slight decrease at 40 °C and a continuous decline from 50 °C to 70 °C. This suggests that immobilization on the polymethacrylate monolith did not cause a significant shift in the optimal temperature compared to the free enzyme. However, covalent-binding exhibited a sharp increase in activity from 20 °C to 40 °C, indicating greater stability over a wider temperature range than the free enzyme and other techniques. The shift of the optimum temperature towards higher values after covalent-binding confirms the enhanced thermal stability of the enzyme post-immobilization. As described by López-Gallego *et al.* (2004), this condition is likely due to the rigidification of its native conformation from the multipoint covalent attachment to the polymethacrylate monolith. Overall, the optimum temperature ranged from 30 °C to 40 °C, suggesting that the immobilized enzyme retains its activity better at lower temperatures but outperforms the free enzyme at higher temperatures. These results align with previous findings by Chen *et al.* (2013) which suggest that at excessively high temperatures, the aldehyde groups in glutaraldehyde and lactase amino bind too quickly, leading to increased enzyme steric hindrance and decreased activity.

Enzyme reuse is an important indicator of the effectiveness of the immobilization process and is crucial for cost-effective industrial applications (Maghraby *et al.*, 2023). Unlike immobilized enzymes, which are attached

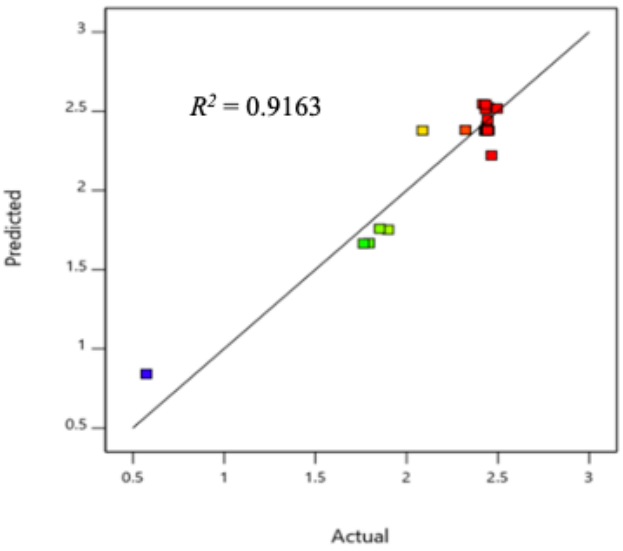

Figure 6: Predicted vs actual responses of the immobilized β-galactosidase on polymethacrylate monolith.

to a solid support and can be easily separated and reused, free enzymes are in soluble form and cannot be recovered for reuse after a single use. Figure 6 demonstrates the reusability of the immobilized enzyme for ten cycles for all immobilization techniques. Covalent-binding showed the highest residual enzyme activity, retaining 50% of its initial activity after the third cycle, while other techniques had below 20% residual activity. This marginal loss of activity can be attributed to the strong linkage of the enzyme to the support, which reduces enzyme "leaching" during catalysis (Pal and Khanum, 2011). Cross-linking displayed consistent remaining activity of approximately 10% for up to ten cycles, whereas the physical technique showed a decline after three cycles probably due to enzyme inactivation through continuous use (Hassan *et al.*, 2018). The ability of covalent-binding and cross-linking to be reused for multiple cycles with higher residual activity makes them promising candidates for cost-effective industrial enzyme applications. Since covalent-binding demonstrated superior operational stability and reusability compared to physical binding and cross-linking, this immobilization technique was selected for further optimisation study. By benchmarking the findings with previously reported studies as tabulated in Table 2, it indicates that the monolith-immobilized β-galactosidase exhibits comparable application and recyclability.

Optimisation of immobilization technique using response surface methodology

Control composite design (CCD) was employed to optimize the enzyme immobilization via covalent-binding on polymethacrylate monolith. The studied reaction variables were enzyme concentration, reaction time and

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Table 3: Experimental conditions for the various runs of central composite design of variables for the immobilization of enzymes on polymethacrylate monolith.

Run	Independent variable			Dependent variable
	Enzyme concentration (mg/mL)	Reaction time (min)	Substrate concentration (mM)	Enzyme activity (U)
1	1.1	6.5	19	2.4
2	1.1	12.4	19	2.51
3	0.5	10	8	1.9
4	1.1	6.5	0.5	0.6
5	0.5	3	8	1.8
6	1.7	3	8	1.8
7	1.1	6.5	19	2.4
8	1.7	10	8	1.9
9	0.5	10	30	2.4
10	2.1	6.5	19	2.4
11	1.1	6.5	19	2.5
12	1.1	6.5	19	2.4
13	1.1	6.5	19	2.4
14	1.1	6.5	19	2.1
15	0.5	3	30	2.4
16	0.9	6.5	19	2.3
17	1.7	3	30	2.4
18	1.1	0.6	19	2.4
19	1.1	6.5	37.4997	2.5
20	1.7	10	30	2.4

Table 4: ANOVA of parameters affecting the enzyme immobilization on polymethacrylate monolith.

Source	Sum of squares	Degree of freedom	Mean square	F-value	P value
Model	3.68	9	0.4086	12.16	0.0003
A-Enzyme concentration	0.0006	1	0.0006	0.0187	0.8939
B-Reaction time	0.0058	1	0.0058	0.1722	0.6869
C-Substrate concentration	2.30	1	2.30	68.36	<0.0001
AB	0.0000	1	0.0000	0.0010	0.9753
AC	0.0003	1	0.0003	0.0080	0.9304
BC	0.0046	1	0.0046	0.1374	0.7186
A^2	0.0004	1	0.0004	0.0115	0.9168
B^2	0.0195	1	0.0195	0.5813	0.4634
C^2	1.29	1	1.29	38.46	0.0001
Residual	0.3361	10	0.0336		
Lack of Fit	0.2329	5	0.0466	2.26	0.1964
Pure Error	0.1032	5	0.0206		
Cor total	4.01	19			

substrate concentration, aiming to achieve the highest enzyme activity. The experimental design and response to enzyme activity are presented in Table 3, demonstrating varied responses to different levels of variables. The ANOVA data in Table 4 confirm the statistical significance of the generated model, with a low *p*-value ($p<0.05$) and a high F-value, indicating the significance of the estimated model (*p*-value=0.0003) (Yaakob *et al.*, 2011).

As shown in Table 4, the enzyme activity was significantly influenced by the substrate concentration ($p<0.0001$). The enzyme concentration refers to the amount of enzyme used, and reaction time is the duration of contact with the immobilization matrix. A higher substrate concentration has the potential to increase the efficiency of the immobilization process by enabling more interactions between the enzyme and substrate as explained by Damnjanović *et al.* (2012). On the other hand, both enzyme concentration and reaction time were found to be insignificant factors in the immobilization process ($p>0.05$). These parameters showed no significant variation in the experiment.

Figure 6 demonstrates the reliability of the model in predicting the best immobilization process of β-galactosidase on polymethacrylate monolith, favoring high enzyme activity. The high coefficient of determination (R^2 = 0.9163) and comparable adjusted R^2 values (0.8409) further support the model's accuracy. According to

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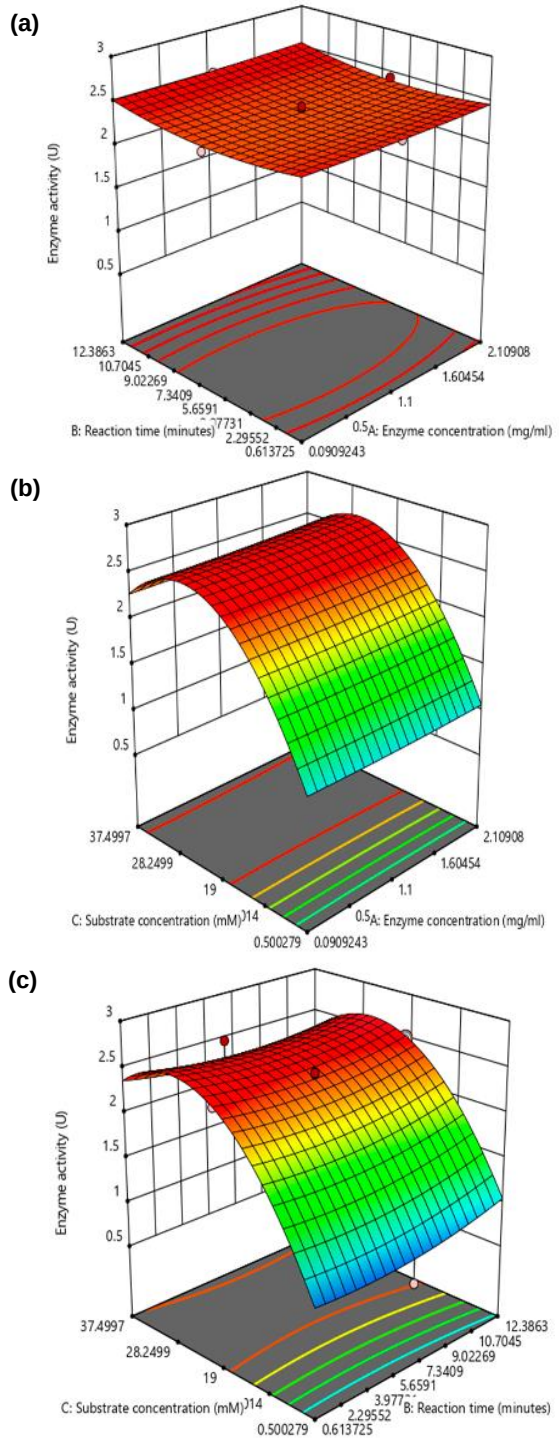

Figure 7: The response surface plot showing the interaction of the effects of (a) reaction time and enzyme concentration, (b) substrate concentration and enzyme concentration and (c) substrate concentration and reaction time on enzyme activity of the immobilized β-galactosidase via covalent-binding on polymethacrylate monolith.

Yaakob *et al.* (2011), the R^2 value above 80%, indicates an adequate model for the response variables. Additionally, the high value of adequate precision (signal-to-noise ratio) (13.1409) confirms the model's adequacy in navigating the design space (a value greater than 4 is desirable).

Figure 7 displays three-dimensional plots illustrating the interactions between reaction time and enzyme concentration (Figure 7a), substrate concentration and enzyme concentration (Figure 7b) and substrate concentration and reaction time (Figure 7c) on the enzyme activity of immobilized β-galactosidase via covalent-binding on polymethacrylate monolith. The plots show that the highest enzyme activity was achieved at 2.5 (U) for all tested reaction times (0.6-12.3 min) and enzyme concentrations (0.09-2.1 mg/mL) (Figure 7a). Similarly, an optimal substrate concentration of 19 mM resulted in the highest enzyme activity at 2.5 (U) (Figure 7b), while increasing substrate concentration facilitated more enzyme-substrate interactions, leading to enhanced enzyme activity. This finding aligns with enzyme kinetics, as higher substrate concentrations lead to increased reaction rates until reaching maximum velocity, with enzymes forming enzyme-substrate complexes in the initial step (Kou *et al.*, 2005). However, beyond the optimal concentration of 19 mM, the enzyme activity began to decrease, highlighting the importance of finding the optimal substrate concentration for maximum enzyme activity during the immobilization process.

In summary, enzyme immobilization on the polymethacrylate monolith shows significant promise for industrial applications. The study highlights the potential for optimizing the immobilization process to enhance its feasibility for more efficient and practical use across various industries. Further research can explore broader parameters and operating conditions to expand the scope of this immobilization technique and unlock new possibilities for its application in industrial processes. By benchmarking the findings with previously reported studies as tabulated in Table 5, it indicates that the monolith-immobilized β-galactosidase exhibits comparable application and recyclability.

CONCLUSION

This study successfully established a protocol for immobilizing β-galactosidase on polymethacrylate monoliths. The β-galactosidase immobilization via covalent-binding exhibited a strong and stable binding between the enzyme and the monolith surface. Microscopic and FTIR analyses provide confirmation of the successful and uniform distribution of the enzyme across the monolith. The immobilization protocol preserved the enzyme's native structure, as evidenced by the comparable pH and thermal stability of the immobilized β-galactosidase with the free enzyme. Furthermore, the immobilized enzyme showed reusability for up to four cycles. The optimisation study indicated that substrate concentration had a significant impact on enzyme activity, while enzyme concentration and reaction

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Table 5: Comparison of recyclability of immobilized enzymes using various immobilization techniques and diverse support materials.

Support	Source	Immobilization method	Recyclability (cycles)	Reference
Polymethacrylate monolith	β-galactosidase	Covalent binding	4-10	This study
Polystyrene nanofibers	β-galactosidase	Covalent binding	4-9	Misson *et al.* (2015a)
Silica nanoparticles	lipase	Covalent binding	8	Dandavate *et al.* (2009)
Chitosan biomimetic-calcium phosphate	sucrose phosphorylase	Entrapment	10	Xu and Liang (2022)
Nano-molecular cages	lipase	Entrapment	7	Cao *et al.* (2021)
Polyurethane	lipase	Entrapment	6	Facin *et al.* (2018)
Alginate-based nanofibers	lipase	Cross-linking	6	Doğaç *et al.* (2017)
Magnetic graphene oxide Nanocomposite	lipase	Cross-linking	5	Badoei-Dalfard *et al.* (2019)

time had negligible effects. Overall, the immobilization of β-galactosidase on polymethacrylate monolith showed great promise for industrial applications due to its stability, reusability, and potential for optimisation. This study offers valuable insights into enzyme immobilization techniques and their potential for industrial biocatalysis.

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