Improving β-galactosidase catalyzed transglycosylation yields by cross-linked layer-by-layer enzyme immobilization

Masih Karimi Alavijeh [a, b], Anne S. Meyer [c], Sally L. Gras [a, b], Sandra E. Kentish [a, *]

[a] Department of Chemical Engineering, The University of Melbourne, Parkville, VIC 3010, Australia

[b] The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Vic 3010, Australia

[c] Protein Chemistry and Enzyme Technology Division, Department of Biotechnology and Biomedicine, Technical University of Denmark, DTU, DK-2800, Kgs Lyngby, Denmark

*Corresponding author:

E-mail: sandraek@unimelb.edu.au (Sandra Kentish).

Abstract

The biotransformation of lactose into gut-bioactive glycans catalyzed by β-galactosidase can give economic value to lactose-rich side streams generated in the food or dairy industry. Herein, we study the immobilization of the commercially used β-galactosidase from *Bacillus circulans* onto silica particles using an enzyme immobilization technology involving a cross-linked layer-by-layer encapsulation method. The immobilized β-galactosidase was used for the synthesis of N-acetyllactosamine (LacNAc) as an important precursor for numerous bioactive compounds and a prebiotic in itself. Techniques including molecular analysis, enzyme activity determination, secondary structure analysis, thermodynamic characterization as well as the determination of thermal and operational stability were conducted to characterize the immobilized enzyme. Changes in the activity of the enzyme after immobilization were attributed to possible changes in electrostatic, covalent and protein-protein interactions. Immobilization significantly improved enzymatic LacNAc yield compared to the free enzyme. In turn, this improved the economics and the sustainability of the process. The immobilized enzyme encapsulated in multilayer films was significantly more stable in the presence of divalent cations and its thermostability also substantially increased, with the thermal denaturation activation energy increasing from 53 kJ mol^{-1} to 294 kJ mol^{-1}. The immobilized enzyme was successfully reused in eight consecutive reaction cycles with no significant reduction in the LacNAc yield. The improved transgalactosylation yield and productivity, higher stability and

reusability obtained with this immobilization method provide new opportunities for industrial applications.

Keywords: Layer-by-layer; Galacto-oligosaccharide; Enzyme; Immobilized β-galactosidase; Stability; Silica.

INTRODUCTION

The global dairy industry produces large amounts of lactose-rich by-product streams, particularly whey generated from manufacturing of cheese, casein and yoghurt.[1-2] Lactose valorization—the use of lactose in the synthesis of functional food and pharmaceutical products—can enhance the economic and environmental sustainability of the industry.[1, 3] Although separation of proteins as a value-added product from whey streams has become an established process over the past 30 years, lactose valorization is still under development. Large amounts of the lactose generated are not used and so regarded as low-value streams, imposing adverse environmental impacts.[1, 4-5] One solution to exploit this abundant resource is to synthesize lactose-derived prebiotics, including galacto-oligosaccharides or human milk oligosaccharides, via β-galactosidase-catalyzed transgalactosylation reactions. In a transgalactosylation reaction, lactose typically acts as a galactosyl donor, which is cheaper and less toxic than nitrophenyl glycosides.[6-7]

Glycosyltransferases can catalyze the formation of such prebiotics with very high selectivity and high yields.[8-9] Glycosyltransferases are not readily available, however, making this process expensive. Further, most glycosyltransferases are also incapable of undergoing variations in donor or acceptor. They act on an activated nucleotide donor, which is unstable and expensive; the released nucleotide can also strongly inhibit the action of these enzymes.[9-10] In view of these limitations, the favored enzymatic method for catalyzing transgalactosylation is based on the use of β-

galactosidases, which are able to act on lactose as the starting substrate. In this method, however, hydrolysis of lactose can simultaneously be catalyzed in competition with transgalactosylation, leading to lower yields and selectivity.

Different methods, including protein engineering,[11-12] water activity reduction [13-14] and optimization of operational parameters,[15-16] have been developed by researchers to increase the yield of transgalactosylation products in preference to hydrolysis. In an alternative approach, enzyme immobilization has been shown to improve the transgalactosylation efficiency in several studies.[17-20] Moreover, immobilization increases the options for controlling the reaction (via reaction time control) and eases enzyme separation and reuse after reaction, whilst also improving the storage and operational enzyme stability.[21] The thermal stability of β-galactosidases often increases upon immobilization. This is a significant advantage for transgalactosylation reactions, as at elevated temperatures higher concentrations of starting substrates can be dissolved into solution, which favors transgalactosylation relative to hydrolysis.[7, 22] The solubility of lactose is relatively low in aqueous solutions at ambient temperatures, about 19 g per 100 g of water at 20 °C, whereas it is 60 g and 100 g per 100 g of water at 60 °C and 80 °C, respectively [23] illustrating the large potential gains that may be achieved at higher temperature.

Silica is widely used as a support material for enzyme immobilization, due to its thermal and mechanical stability, microbial resistance and non-toxicity. Such silica supports are commercially available and are easy to handle and synthesize.[24-25]

89 Electrostatic rather than Van der Waals forces are reported to be the main forces
90 governing adsorption of enzymes onto silica supports.[26-27] Given the theoretical
91 isoelectric points of 3-4 and 4.5 for SiO_2[28-29] and β-galactosidase,[30] respectively,
92 attractive electrostatic interactions between this enzyme and silica supports are not
93 likely to be very strong, especially at the optimum pH of β-galactosidase from *Bacillus*
94 *circulans* (pH=6), where both are negatively charged. This makes the enzyme more
95 susceptible to desorption from the support, which is problematic in terms of enzyme
96 reusability in successive cycles. This potential problem can be overcome by modifying
97 the silica surface using a chemical agent to activate the silanol groups on their surface.
98 Silanized SiO_2 can then be further treated by a cross-linker to prepare it for covalent
99 immobilization of an enzyme,[31-33] such as β-galactosidase.

100 Layer-by-layer assembly is another established method to encapsulate an enzyme and
101 to prevent its leakage from the support. In this technique, the adsorbed protein is
102 coated with oppositely charged polymers or polyelectrolytes. It is a simple and
103 extremely versatile technique for immobilization of various enzymes onto different
104 supports based on electrostatic interactions.[34] This method can be performed in
105 aqueous solutions under mild conditions, which is environmentally favorable and
106 reduces the likelihood of enzyme degradation. Furthermore, this method allows the co-
107 immobilization of multiple enzymes for cascade reactions.[35-36] Enzyme covalent
108 immobilization on silicon substrates, on the other hand, involves in the use of
109 hazardous and toxic non-aqueous solutions such as organosilane reagents and toluene
110 with longer preparation times and a greater risk of enzyme denaturation.[31, 37-38] Due to

111 the creation of very robust pairs with a high chemical and physical stability, polystyrene
112 sulfonate (PSS) and polyallylamine hydrochloride (PAH) have been widely employed in
113 layer-by-layer encapsulation methods, including the immobilization of enzymes.[39-41]

114 While a number of workers have studied the covalent immobilization of β-
115 galactosidases for galactooligosaccharide production, no study has been performed to
116 analyze the effect of layer-by-layer immobilization on transglycosylation reactions.
117 Microenvironments and molecular interactions generated in multilayer films may
118 present some desirable impacts on the β-galactosidase enzyme stability and
119 hydrolysis/transgalactosylation activities. Hence, in the present study, we investigate
120 the suitability of a cross-linked layer-by-layer technique for the immobilization of β-
121 galactosidase derived from *Bacillus circulans*. Negatively charged silica particles are
122 first coated by the positively charged PAH followed by the electrostatic adsorption of
123 the negatively charged β-galactosidase, cross-linking with glutaraldehyde and
124 deposition of further PAH and PSS layers. In addition, for the first time, an analysis is
125 performed to analyze the economics and sustainability of such an immobilization
126 method.

127 **EXPERIMENTAL SECTION**

128 Sources of chemicals are provided in the Supporting Information as are detailed
129 descriptions of experimental protocols such as the hydrolytic assay.
130 transgalactosylation activity, determination of immobilization efficiency, activity

retention and thermal stability. Characterization methods and computational molecular analysis are also described in the Supporting Information.

Layer-by-layer adsorption of β-galactosidase onto SiO_2 particles

Polyelectrolyte solutions of PSS or PAH (2 mg·ml^{-1}) were prepared in Tris buffer (pH=7.2; 50 mM). The SiO_2 particles (SIPERNAT®)(50 mg) were dispersed in 1 ml of the PAH solution. The suspension was mixed and sonicated using a water bath sonicator (Ultrasonic Bath FXP4, 50 Hz, Ultrasonics, Australia) for 1 min. After 10 min shaking on a tube rocker, the particles were collected by centrifugation at 7,000g for 1 min, followed by re-suspension and vortex mixing in 1 ml Tris buffer (pH=7.2) to remove free PAH and any impurities. This step was repeated three times, with the supernatant discarded by centrifugation at 7,000g for 1 min after each rinsing. After the deposition of this first layer of PAH, a total of 1 ml of the enzyme solution (0.1 mg·ml^{-1}) in phosphate-citrate buffer (pH=6; 50 mM) was added to the tube containing the particles. The adsorption of the enzyme took place for 20 min under mild shaking on a tube rocker. The same procedure as already described for the free PAH removal was used to collect and wash the particles and the supernatant as well as washings were collected for measuring the un-adsorbed free enzyme. The immobilized enzyme was further stabilized by cross-linking with 1 ml of 0.1% w/w glutaraldehyde solution at pH=7.2. The solution containing 0.1% w/w glutaraldehyde in Tris buffer was made from a 25% w/w glutaraldehyde stock solution. The excess glutaraldehyde was removed by centrifugation and the particles were then washed three times.

In addition to the electrostatic interaction between the negatively charged β-galactosidase with positively charged PAH, glutaraldehyde reacts with the amine groups of PAH (and to a minor extent with exposed amine groups in the enzyme). Thus, a subsequent layer of PAH was deposited on the particles as described earlier. The particles were then exposed to 1 ml of PSS (2 $mg \cdot ml^{-1}$) for 10 min followed by three washings. The strong polyanionic PSS was used as the terminal layer as this polyelectrolyte is more resistant to re-solubilization in comparison with a PAH layer.[42-43] A schematic representation of this cross-linked layer-by-layer assembly is shown in Figure 1.

For comparison, the enzyme was also pretreated by contact with lactose, as there is evidence that this might protect the active site during the immobilization process.[44] To this end, the enzyme solution (0.1 $mg \cdot ml^{-1}$) was prepared in a phosphate-citrate buffer (pH=6; 50 mM) containing 30 mM lactose. This solution was incubated at 37 °C for 1 h at 150 rpm before immobilization.[44]

(Figure 1)

In all experiments, statistical significance was determined by the two-tail Student's t-test ($p<0.05$) and all experimental data were indicated as mean value ± one standard deviation. Linear regression analysis (Excel Data Analysis ToolPak) was used to determine the error associated with the regression line slope where needed. The error propagation analysis was taken into account to calculate the overall error of a parameter from the individual errors [45].

RESULTS AND DISCUSSION

Characterization of the Immobilized Enzyme

The TGA thermograms (see Figure S1(B) in the Supporting Information) showed a significantly greater mass loss upon heating from the silica particles with immobilized enzyme compared to the bare silica supports. The mass loss below 200 °C is because of dehydration.[46] The decomposition of the PAH side chains starts at around 230 °C followed by the sulfonate groups in PSS.[47-48] The loss at higher temperatures is attributed to the degradation of the main polymeric backbones.[47-49]

Figure 2(A) shows the ATR-FTIR spectra of the native and immobilized enzyme. The silica particles display a broad peak at 1,070 cm^{-1} resulting from asymmetric vibrations of Si–O.[50] The enzyme immobilization results in new peaks, particularly the Amide I band (1,600 cm^{-1} to 1,700 cm^{-1}) related to C-O stretching vibrations and the Amide II band (1,510 cm^{-1} to 1,580 cm^{-1}) related to N-H bending.[51-52] New peaks between 3,000 cm^{-1} and 3,700 cm^{-1} are indicative of N-H stretching and side chain stretching.[53-54] In addition, zeta potential measurements reveal surface charge inversion, confirming the successful deposition of layers (Figure 2(B)).

The characteristic Amide I band is highly sensitive to small changes in molecular geometry and hydrogen bond patterns and can be used to obtain more detailed information on the secondary structure components of a protein.[55-57] As shown in Figure 2(C), the β-galactosidase enzyme immobilized using the cross-linked layer-by-layer technique had an altered conformation. Changes in bands after immobilization

have been reported previously.[58-59] Schwinté et al.[60] showed that encapsulating the protein into the oppositely charged polyelectrolyte layers (PAH or PSS) can increase the intermolecular β-sheet content. The enhanced protein-protein interactions are associated with increased formation of intermolecular β-sheets, which can lead to partial unfolding and denaturation.[61-62] Such changes in the secondary structure of the β-galactosidase may contribute to the modification of its catalytic activity.

(Figure 2)

Evaluation of β-galactosidase hydrolytic activity after immobilization

Layer-by-layer encapsulation of the β-galactosidase onto the SIPERNAT 50S particles with an enzyme dosage of 2 mg per g silica resulted in a protein immobilization efficiency of 100%. However, the hydrolytic enzyme activity after immobilization was reduced to 22 ± 2%. Such a reduction in activity is common during immobilization due to interactions between the surface and enzyme, which can vary depending on the immobilization method employed.[63] As shown in Figure 3, electrostatic, covalent and protein-protein interactions might occur in the present case. The positively charged PAH can interact with the enzyme at different locations, as the enzyme is mainly negatively charged at pH=6, which is indicated by the red colour, with some limited regions of positive charge shown in blue colour in Figure 3(A), leading to the formation of polyelectrolyte-protein complexes.[64] The aldehyde groups in glutaraldehyde can also form covalent bonds with free amine groups within the enzyme, shown by a green colour in Figure 3(B). Covalent binding typically alters

enzyme activity.[32] It has been reported that clusters or multilayers of the enzyme on the support surface can be formed as a result of protein-protein interactions.[63, 65] Figure 3(C) shows the protein-protein interaction sites (yellow regions) of the β-galactosidase predicted by the PSIVER server. Protein-protein interactions dominate as greater initial enzyme dosages are contacted with the support, resulting in a reduction in enzyme activity due to steric hindrance and limited diffusion.[32, 63, 65]

Increasing the enzyme dosage to 10 mg per g silica decreased the β-galactosidase activity after immobilization to 17 ± 2%, while all the enzyme protein was still adsorbed by the support (see Table 1). A further increase in the enzyme dosage to 1000 $mg \cdot g^{-1}$ silica reduced the activity so that only 0.6 ± 0.4% of the free enzyme activity was retained after immobilization. Further, the immobilization efficiency reduced to 22 ± 7%. In this case, the large increase in the amount of the enzyme loaded on the support (220±70 $mg \cdot g^{-1}$) led to a greater number of protein multilayers and interactions. Figure 3(D) shows the surface representation of the β-galactosidase with its surface catalytic pocket region (cyan colour). According to this figure, the interactions described in Figure 3(A-C) may block this region and hinder efficient access of the substrate to the enzyme.

Table 1. Hydrolytic activity retention, immobilization efficiency and amount of immobilized β-galactosidase adsorbed on the SIPERNAT 50S particles by the layer-by-layer encapsulation method used in this study at different enzyme dosages.

Enzyme dosage ($mg \cdot g^{-1}$ silica)	Activity retention (%)	Immobilization efficiency (%)	Amount of immobilized enzyme ($mg \cdot g^{-1}$ silica)
2	22± 2	100	2
10	17 ± 2	100	10
30	13.5 ± 1.5	98.4 ± 0.6	29.5 ± 0.2
1000	0.6±0.4	22± 7	220 ± 70

We also examined the effect of lactose pre-treatment on the loss of enzyme activity during the immobilization. It was reported that lactose binding to the active site of β-galactosidase can act as a shield against the formation of covalent bonds near the active site.[44] This protection method, however, did not result in any significant change in the enzyme activity compared to the immobilized enzyme without lactose pre-treatment. This result demonstrates that interactions other than covalent bonding play a part in the change in enzyme conformation in this cross-linked layer-by-layer assembly.

(Figure 3)

Transgalactosylation reaction

The immobilized biocatalyst with a load of 2 mg enzyme per g silica was then used in the transgalactosylation reaction. Starting with the same initial hydrolytic activities of 0.14 $U \cdot ml^{-1}$ (corresponding to 40 $\mu g \cdot ml^{-1}$ of the immobilized enzyme and 8 $\mu g \cdot ml^{-1}$ of the free enzyme), the immobilized enzyme produced approximately two-fold to three-fold more LacNAc compared to the free enzyme at each time point (Figure 4(A)). The ratio of initial transgalactosylation rate (r_{trans}) to hydrolysis rate (r_{hydro}) for the immobilized enzyme is also about seven times greater than that for the free enzyme (Figure 4(B)). Even when the free enzyme concentration is increased to 0.63 $U \cdot ml^{-1}$ (corresponding to 36 $\mu g \cdot ml^{-1}$ of the free enzyme), which is equivalent to the initial transgalactosylation activity of the immobilized enzyme, the LacNAc production over time did not reach to the same level as observed for the reaction with the immobilized enzyme (Figure 4(A)), due to competition from the hydrolysis reaction. This is an important result, which shows that the immobilization technique employed in this study not only makes it possible to recover the enzyme after the reaction but also that immobilization increases yield, which can significantly influence the economy of large-scale LacNAc production.[2]

For further elucidation, as shown in Figure 4(C), the reaction specific productivity (also known as biocatalytic productivity rate) was calculated at two time points when the highest LacNAc concentrations were obtained in the reactions catalyzed by the free enzyme at 0.63 $U \cdot ml^{-1}$ (90 min) and the immobilized enzyme at 0.14 $U \cdot ml^{-1}$ (150 min), respectively. When the free enzyme is used, a greater amount of

271 LacNAc can be achieved using 0.63 U·ml^{-1} rather than 0.14 U·ml^{-1} but at the expense of
272 a reduction in the specific productivity. In contrast, the specific productivity of the
273 reaction catalyzed by 0.14 U·ml^{-1} of the immobilized enzyme is greater than that of the
274 reaction run by 0.63 U·ml^{-1} of the free enzyme for either time point. The higher specific
275 productivity of 0.14 U·ml^{-1} of the free enzyme is due to the lower enzyme mass
276 concentration (8 μg·ml^{-1}) of the immobilized enzyme (40 μg·ml^{-1}) at the same
277 hydrolytic activity. Nonetheless, the ability to re-use the immobilized enzyme may
278 compensate for the loss of activity. Changes in the transgalactosylation yield and
279 reaction productivity upon immobilization of β-galactosidases have been reported by
280 several researchers.[17, 66-68]

281 As previously shown,[69] high concentrations of divalent cations, i.e. 100 mM Ca^{2+}
282 or 100 mM Mg^{2+} can lead to the formation of salt bridges between the negatively
283 charged β-galactosidases and subsequent protein aggregation. Thus, in the present
284 study, we also examined the effect of these cations on the product formation when the
285 immobilized enzyme is used. As can be seen in Figure 4(A), no significant change in the
286 concentration of LacNAc in the presence of 100 mM Ca^{2+} or Mg^{2+} is observed. This result
287 indicates that the enzyme is well encapsulated into the support and is unable to form
288 aggregates with neighbouring molecules. It also confirms our previous conclusion that
289 these cations have no role as co-factors of this enzyme.

290 (Figure 4)

In our previous work,[69] we developed a kinetic model to describe the multi-pathway reactions including transgalactosylation and hydrolysis involved in the conversion of lactose to LacNAc. Based on this model (see the Supporting Information), the reaction rate constants were estimated for the free enzyme and the immobilized enzyme (Table S1). The altered kinetic parameters are indicative of changes in the catalytic active site and/or the restriction on substrate diffusion into the layer-by-layer coating, which decreases the reaction rate constants after immobilization.[70-71] Importantly, the ratio of the LacNAc synthesis rate constant (k_3) to galactose release rate constant (k_2) is 14 M^{-1} for the free enzyme while that for the immobilized enzyme is about 20 M^{-1}, which demonstrates improved transgalactosylation.

Thermal stability of the immobilized enzyme versus free enzyme

The immobilized enzyme could retain about 92% and 83% of its initial activity after 90 min and 180 min at 55 °C, respectively (Figure 5(A)), while only 8% of the initial activity of the free enzyme was maintained after 30 min (Figure 5(B)). This means that with an immobilized enzyme it is possible to run reactions at elevated temperature and thus at a higher reaction rate. At even higher temperatures, the immobilized enzyme begins to denature more rapidly (Figure 5(C)), so that at 70°C, the residual activity is more comparable (7% for the free enzyme and 15% for the immobilized after 15 minutes). This observation is consistent with the inactivation rate constant (k_D) data shown in Table 2. However even at 70°C, the immobilized enzyme remains more thermally robust.

Table 2. Inactivation rate constant (k_D), half-time values ($t_{1/2}$) and activation energy of thermal denaturation reaction (E_a) for the immobilized and free enzyme.

T (°C)	$k_D \times 10^3$ (min^{-1})			$t_{1/2}$ (min)		
	Free enzyme	Immobilized enzyme	Two-tailed p-value	Free enzyme	Immobilized enzyme	Two-tailed p-value
55	77 ± 9	1.2 ± 0.3	5×10^{-8}	9 ± 1	580 ± 140	3×10^{-10}
60	96 ± 7	6 ± 0.5	3×10^{-9}	7 ± 0.4	117 ± 9	2×10^{-22}
65	142 ± 13	33 ± 2	4×10^{-6}	5 ± 0.4	21 ± 1	5×10^{-17}
70	174 ± 10	127 ± 18	2×10^{-5}	4 ± 0.2	5 ± 1	2×10^{-2}

E_a (kJ mol^{-1})	
Free enzyme	Immobilized enzyme
53 ± 1	294 ± 2

(Figure 5)

The activation energy (E_a) for the thermal denaturation reaction, or the minimum energy needed for triggering enzyme inactivation, is 53 ± 1 kJ mol^{-1} for the free enzyme and 294 ± 2 kJ mol^{-1} for the immobilized enzyme (Table 2). The higher E_a of the immobilized enzyme, which is more than five-fold that of the free enzyme, corroborates the suitability of the cross-linked layer-by-layer immobilization technique as a method for improving the thermal stability of β-galactosidase.

323 The positive ΔS observed for the immobilized enzyme (Table 3) is directly
324 indicative of protein unfolding (greater disorder) during inactivation.[72-73] By contrast,
325 the corresponding negative ΔS values for the free enzyme can be related to the
326 formation of protein aggregates during inactivation as a result of denaturation. The
327 aggregates are less disordered compared to the soluble proteins moving freely in the
328 solution, hence the negative ΔS values.[74-75]

329 The positive values of ΔH indicate that thermal inactivation of the β-
330 galactosidase is an endothermic reaction, and the differences in the ΔH values of the
331 free vs the immobilized enzyme implies that more energy required to destabilize the
332 immobilized form (Table 3). The formation of an electrostatic cage as a result of the
333 interaction between the enzyme and polyelectrolytes can restrict structural
334 fluctuations, leading to improved structural stability within the protein.[60] It has been
335 demonstrated that a greater β-sheet content in a protein also leads to a more rigid
336 protein structure.[76] As described above, the FTIR spectra of the immobilized enzyme is
337 consistent with such changes in secondary structure confirmation and the formation of
338 a more rigid structure following immobilization.[59]

339

340 **Table 3.** Thermodynamic parameters of thermal inactivation of the immobilized and
341 free enzyme.

T (°C)	ΔH (kJ mol^{-1})		ΔS (J mol^{-1} K^{-1})	
	Free enzyme	Immobilized enzyme	Free enzyme	Immobilized enzyme
55	50 ±1	291 ±2	-148 ± 4	552 ± 6
60	50 ±1	291 ±2	-149 ± 4	551 ± 0.7
65	50 ±1	291 ±2	-148 ± 4	552±0.5
70	50 ±1	291 ±2	-149 ± 4	551 ± 1

342

343

344 The immobilized enzyme and free enzyme were also incubated at these elevated
345 temperatures for 30 min and the remaining transgalactosylation activity then
346 measured at 50 °C. The transgalactosylation activity of the immobilized enzyme
347 incubated at 55 °C was unchanged, while around 86% of this activity was retained after
348 incubating the immobilized enzyme at 60 °C (Figure 6). A much more significant
349 decrease in activity was observed for the free enzyme after incubation at temperatures
350 higher than the optimum temperature of this β-galactosidase (50 °C).

351 (Figure 6)

352

353 **Operational stability of the immobilized enzyme**

354 A control sample containing the immobilized enzyme (0.14 $U \cdot ml^{-1}$) but without
355 substrate was incubated at 50 °C for 150 min (under the same conditions of the
356 transgalactosylation reaction) and then the supernatant was separated by
357 centrifugation. No enzyme was found to be released from the support during this
358 incubation when the protein content of the supernatant was measured. This is an
359 important advantage of the cross-linked layer-by-layer encapsulation. The reusability
360 of the immobilized β-galactosidase in successive reactions was also examined, as this is
361 one of the key factors in evaluating the usefulness of immobilization for industrial
362 applications. After eight reuse cycles no significant reduction in the molar yield of
363 LacNAc was observed (Figure 7(A)), indicating a good operational stability for β-
364 galactosidase immobilized by this method.

365 (Figure 7)

366 **Preliminary economic analysis results**

367 We have previously developed a simulation of a LacNAc production process using
368 this β-galactosidase.[2] This simulation was now used to compare the amount of LacNAc
369 that could be obtained from 20 tons of lactose derived from whey, as well as the annual
370 cost of LacNAc production, for both the free and the immobilized enzyme. The results
371 are shown in Table S2 in the Supporting Information, indicating the annual production
372 of 8.5 tons LacNAc with the use of immobilized enzyme and 6.3 tons LacNAc with the
373 use of free enzyme. The two simulations used enzyme concentrations that provided the

same initial transgalactosylation activities; 0.14 $U \cdot ml^{-1}$ (40 $\mu g \cdot ml^{-1}$) for the immobilized case and 0.63 $U \cdot ml^{-1}$ (36 $\mu g \cdot ml^{-1}$) for the free enzyme. The cost of immobilizing the enzyme at a production scale was estimated as $104 per kg of the immobilized support, based on the calculations and process flowsheet of unit operations shown in the Supporting Information (Figure S3). Other assumptions and economic parameters used in the simulation are shown in Table S2.

The annual cost of raw materials per kg of product is 149 US dollars for the process based on the immobilized enzyme with eight reuse cycles, while it is 178 US dollars for the process based on the free enzyme (Table 4). This difference reflects the greater yield obtained when using the immobilized enzyme. As shown in Figure S4 in the Supporting Information, with no reuse of the immobilized biocatalyst, 72% of the total raw material costs arises from the immobilization of the enzyme, whereas the share of the free enzyme cost in the total raw material costs is about 19%. This finding is in agreement with previously reported results.[77] As the number of reuse cycles increases, however, this share falls, so that these costs are comparable after 11 reuse cycles.

The Net Present Value (NPV) was then calculated as an indicator of the project profitability (see Supporting Information). The NPV for the plant processing 20 tons of lactose per year based on the immobilized enzyme (463 million US dollars) is greater than that for the process based on the free enzyme (333 million US dollars). This increase in the NPV is mainly due to the increase in the yield after immobilization. As shown in Figure 4(A), starting with the same initial transgalactosylation activities and

395 a lactose concentration of 50 mM, the molar yield of LacNAc is about 50% when the
396 immobilized enzyme is used, versus 35% for the free enzyme. For both enzyme types,
397 the process costs are close to each other, as no change in the equipment sizes occurs
398 (Table S2 in the Supporting Information), although there is an additional cost for
399 immobilization that is included for this case. Nonetheless, in each batch more LacNAc
400 is produced with the same amount of lactose when the immobilized enzyme is used,
401 indicating a higher annual revenue. Further analysis of the effect of the immobilized
402 enzyme reuse on the NPV is also shown in Figure 7(B). According to this figure,
403 variations in the NPV values are significant as the number of reuses increases up to
404 about 10. Further increases in the NPV are not very significant for higher reuse
405 numbers assuming the yield of product does not change.

406 The sustainability of the process based on the immobilized enzyme with eight reuse
407 cycles and free enzyme is further outlined in Table 4. The water used in the wash cycles
408 of the enzyme immobilization process can be recycled for the production of fresh
409 polyelectrolyte and enzyme solutions, so a 90% water recovery is assumed. As the
410 immobilized enzyme can be recycled at least eight times, the net water usage becomes
411 small, only 14 kg per kg LacNAc. The net electricity usage from the centrifugation in this
412 immobilization process is also low (0.004 kWh per kg LacNAc). Recycling of the
413 immobilized enzyme within the LacNAc reactor requires an additional filtration and
414 washing process and this also increases the total waste generated and water consumed
415 per kg of product. Nonetheless, it can be assumed that most of the water from this wash

step can be recycled to the reactor and so a water recovery of 90% is again used for this step.

The use of the immobilized enzyme results in a lower chemical oxygen demand (COD) per kg of product. In turn, this reduces the CO_2 equivalent emissions per kg of LacNAc in the subsequent wastewater treatment processes, when emission factors for either an aerobic or anaerobic treatment are applied.[78] The electricity, steam and chilled water consumption also fall, which will further reduce CO_2 emissions if these utilities are supplied using a fossil fuel energy source. Further, the amount of chemical agents used in Clean-In-Place (CIP) for cleaning equipment is 354 kg per kg LacNAc when the immobilized enzyme is applied, while 440 kg of these agents per kg LacNAc are needed when the free enzyme is utilized. The reduction in these chemicals will further reduce the environmental impact.

Table 4. Evaluation of the process sustainability based on either the free enzyme; or the immobilized enzyme with eight reuse cycles.

Sustainability Parameter	Units	Quantity per kg product for:	
		Free enzyme	Immobilized enzyme
Waste	kg	582	486
Total chemical oxygen demand (COD)	kg	10	8
Emission from waste[1]	kg CO_2 equivalents		
- Anaerobic treatment or:		10	8
- Aerobic treatment		23	18
Raw materials	kg	210	188
Cleaning-In-Place (CIP) solutions [2]	kg	440	354
Water consumption	kg	201	181
Electricity	kWh	3	2
Steam	kg	338	265
Chilled water	tonne	23	18
Raw material cost	USD	178	149
Waste treatment cost	USD	0.32	0.28
Utility cost	USD	11	9
Labor-dependent cost	USD	499	408
CIP cost	USD	21	17

[1] Emission factors of 1 kg CO_2eq.kg^{-1} COD for anaerobic treatment and 2.4 kg CO_2eq.kg^{-1} COD for aerobic treatment were assumed.[78]

[2] Water rinse (WFI: water for injection), caustic rinse (0.5 M NaOH) and acid rinse (5% w/w H_3PO_4).

Conclusions

In this work, we have demonstrated that cross-linked layer-by-layer encapsulation of β-galactosidase enzyme derived from *Bacillus circulans* can effectively improve the transgalactosylation activity, whilst also improving stability and enabling reuse of the enzyme. An enzyme dosage of 2 mg per g support resulted in an immobilization efficiency of 100% with 21.5% of hydrolytic activity retained. Molecular analysis indicates a range of different interactions during immobilization that may be associated with the loss of enzyme activity. Further increase in the initial enzyme dosage resulted in greater protein-protein interactions, leading to a more severe loss of activity. The immobilized enzyme produced more LacNAc in comparison with the free enzyme at the same hydrolytic activity. This can be attributed to conformational changes observed by spectroscopy. The free enzyme was thermally unstable at 55 °C, losing 92% of its initial activity after 30 min, while the immobilized counterpart was significantly more thermostable and for instance retained 92% of its initial activity after 90 min at 55 °C. The enhanced thermal stability and strong retention of the enzyme on the silica support facilitated the reuse of the enzyme for at least eight successive cycles with no significant reduction in the LacNAc yield. The enhanced yield at the same initial transgalactosylation activity led to a significantly greater economic value for LacNAc production, based on simulation results. The simulations also indicate that the use of an immobilized enzyme would lead to reduced greenhouse gas emissions and reduced need for wastewater treatment facilities. These enhancements in conjunction with the

use of a commercially available silica support meet the essential requirements for large-scale application.

Acknowledgements

M. Karimi Alavijeh acknowledges The University of Melbourne for a Melbourne Research Scholarship.

Supporting Information

The Supporting Information is available online free of charge.

Materials and methods; estimated rate constants for the free and immobilized enzymes; assumptions and economic parameters used for economic evaluations; raw materials and utility costs; TGA of silica before and after enzyme immobilization; calculated time-course variations in sugar concentrations based on the kinetic model; a schematic representation of the unit operations for immobilizing the enzyme; changes in the share of the total raw material cost that arises from enzyme immobilization versus the number of reuse cycles.

References

(1) Andler, S. M.; Goddard, J. M., Transforming food waste: how immobilized enzymes can valorize waste streams into revenue streams. *NPJ Sci. Food* **2018,** *2*, 19-19, DOI: 10.1038/s41538-018-0028-2.

(2) Karimi Alavijeh, M.; Meyer, A. S.; Gras, S. L.; Kentish, S. E., Simulation and economic assessment of large-scale enzymatic N-acetyllactosamine manufacture. *Biochem. Eng. J.* **2020,** *154*, 107459, DOI: 10.1016/j.bej.2019.107459.

(3) Gänzle, M. G., Chapter 4 - Lactose—a conditional prebiotic? In *Lactose*, Paques, M.; Lindner, C., Eds. Academic Press: 2019; pp 155-173.

(4) Tavares, T.; Malcata, F. X., Whey and Whey Powders: Fermentation of Whey. In *Encyclopedia of Food and Health*, Caballero, B.; Finglas, P. M.; Toldrá, F., Eds. Academic Press: Oxford, 2016; pp 486-492.

(5) Boland, M., 3 - Whey proteins. In *Handbook of Food Proteins*, Phillips, G. O.; Williams, P. A., Eds. Woodhead Publishing: 2011; pp 30-55.

(6) Zeuner, B.; Jers, C.; Mikkelsen, J. D.; Meyer, A. S., Methods for Improving Enzymatic Trans-glycosylation for Synthesis of Human Milk Oligosaccharide Biomimetics. *J. Agric. Food Chem.* **2014,** *62* (40), 9615-9631, DOI: 10.1021/jf502619p.

(7) Gosling, A.; Stevens, G. W.; Barber, A. R.; Kentish, S. E.; Gras, S. L., Recent advances refining galactooligosaccharide production from lactose. *Food Chem.* **2010,** *121* (2), 307-318, DOI: 10.1016/j.foodchem.2009.12.063.

(8) Downey, A. M.; Hocek, M., Strategies toward protecting group-free glycosylation through selective activation of the anomeric center. *Beilstein J. Org. Chem.* **2017,** *13*, 1239-1279, DOI: 10.3762/bjoc.13.123.

(9) Seeberger, P.; Finney, N.; Rabuka, D., Chemical and Enzymatic Synthesis of Glycans and Glycoconjugates. In *Essentials of Glycobiology. 2nd edition*, Varki, A.; Cummings, R.; Esko, J., Eds. Cold Spring Harbor Laboratory Press: NY, 2009.

(10) Wang, Z. A.; van der Wel, H.; Vohra, Y.; Buskas, T.; Boons, G. J.; West, C. M., Role of a cytoplasmic dual-function glycosyltransferase in O2 regulation of development in Dictyostelium. *J Biol Chem* **2009,** *284* (42), 28896-904, DOI: 10.1074/jbc.M109.022574.

(11) Qin, Z.; Li, S.; Huang, X.; Kong, W.; Yang, X.; Zhang, S.; Cao, L.; Liu, Y., Improving Galactooligosaccharide Synthesis Efficiency of β-Galactosidase Bgal1-3 by Reshaping the Active Site with an Intelligent Hydrophobic Amino Acid Scanning. *J. Agric. Food Chem.* **2019,** *67* (40), 11158-11166, DOI: 10.1021/acs.jafc.9b04774.

(12) Lundemo, P.; Adlercreutz, P.; Karlsson, E. N., Improved Transferase/Hydrolase Ratio through Rational Design of a Family 1 β-Glucosidase from Thermotoga

neapolitana. *Appl. Environ. Microbiol.* **2013,** *79* (11), 3400, DOI: 10.1128/AEM.00359-13.

(13) Gosling, A.; Stevens, G. W.; Barber, A. R.; Kentish, S. E.; Gras, S. L., Effect of the Substrate Concentration and Water Activity on the Yield and Rate of the Transfer Reaction of β-Galactosidase from Bacillus circulans. *J. Agric. Food Chem.* **2011,** *59* (7), 3366-3372, DOI: 10.1021/jf104397w.

(14) Kwon, S. J.; Jung, H.-C.; Pan, J.-G., Transgalactosylation in a Water-Solvent Biphasic Reaction System with β-Galactosidase Displayed on the Surfaces of Spores. *Appl. Environ. Microbiol.* **2007,** *73* (7), 2251, DOI: 10.1128/AEM.01489-06.

(15) González-Delgado, I.; López-Muñoz, M.-J.; Morales, G.; Segura, Y., Optimisation of the synthesis of high galacto-oligosaccharides (GOS) from lactose with β-galactosidase from Kluyveromyces lactis. *Int. Dairy J.* **2016,** *61*, 211-219, DOI: 10.1016/j.idairyj.2016.06.007.

(16) Carević, M.; Veličković, D.; Stojanović, M.; Milosavić, N.; Rogniaux, H.; Ropartz, D.; Bezbradica, D., Insight in the regioselective enzymatic transgalactosylation of salicin catalyzed by β-galactosidase from Aspergillus oryzae. *Process Biochem.* **2015,** *50* (5), 782-788, DOI: 10.1016/j.procbio.2015.01.028.

(17) Guerrero, C.; Aburto, C.; Suárez, S.; Vera, C.; Illanes, A., Effect of the type of immobilization of β-galactosidase on the yield and selectivity of synthesis of transgalactosylated oligosaccharides. *Biocatal. Agric. Biotechnol.* **2018,** *16*, 353-363, DOI: 10.1016/j.bcab.2018.08.021.

(18) Sen, D.; Sarkar, A.; Gosling, A.; Gras, S. L.; Stevens, G. W.; Kentish, S. E.; Bhattacharya, P. K.; Barber, A. R.; Bhattacharjee, C., Feasibility study of enzyme immobilization on polymeric membrane: A case study with enzymatically galacto-oligosaccharides production from lactose. *J. Membr. Sci.* **2011,** *378* (1), 471-478, DOI: 10.1016/j.memsci.2011.05.032.

(19) Mozaffar, Z.; Nakanishi, K.; Matsuno, R., Effect of glutaraldehyde on oligosaccharide production by β-galactosidase from Bacillus circulans. *Appl. Microbiol. Biotechnol.* **1987,** *25* (5), 426-429, DOI: 10.1007/BF00253312.

(20) Misson, M.; Du, X.; Jin, B.; Zhang, H., Dendrimer-like nanoparticles based β-galactosidase assembly for enhancing its selectivity toward transgalactosylation. *Enzyme Microb. Technol.* **2016,** *84*, 68-77, DOI: 10.1016/j.enzmictec.2015.12.008.

(21) Panesar, P. S.; Kumari, S.; Panesar, R., Potential Applications of Immobilized β-Galactosidase in Food Processing Industries. *Enzyme Res.* **2010,** *2010*, 473137-473137, DOI: 10.4061/2010/473137.

(22) Zeuner, B.; Nyffenegger, C.; Mikkelsen, J. D.; Meyer, A. S., Thermostable β-galactosidases for the synthesis of human milk oligosaccharides. *New Biotechnol.* **2016,** *33* (3), 355-360, DOI: 10.1016/j.nbt.2016.01.003.

(23) Fox, P. F., Lactose: Chemistry and Properties. In *Advanced Dairy Chemistry: Volume 3: Lactose, Water, Salts and Minor Constituents*, McSweeney, P.; Fox, P. F., Eds. Springer New York: New York, NY, 2009; pp 1-15.

(24) Bhatia, R. B.; Brinker, C. J.; Gupta, A. K.; Singh, A. K., Aqueous Sol−Gel Process for Protein Encapsulation. *Chem. Mater.* **2000,** *12* (8), 2434-2441, DOI: 10.1021/cm000260f.

(25) Bernal, C.; Sierra, L.; Mesa, M., Design of β-galactosidase/silica biocatalysts: Impact of the enzyme properties and immobilization pathways on their catalytic performance. *Eng. Life Sci.* **2014,** *14* (1), 85-94, DOI: 10.1002/elsc.201300001.

(26) Hüttner, S.; Zezzi Do Valle Gomes, M.; Iancu, L.; Palmqvist, A.; Olsson, L., Immobilisation on mesoporous silica and solvent rinsing improve the transesterification abilities of feruloyl esterases from Myceliophthora thermophila. *Bioresour. Technol.* **2017,** *239*, 57-65, DOI: 10.1016/j.biortech.2017.04.106.

(27) Deere, J.; Magner, E.; Wall, J. G.; Hodnett, B. K., Mechanistic and Structural Features of Protein Adsorption onto Mesoporous Silicates. *J. Phys. Chem. B* **2002,** *106* (29), 7340-7347, DOI: 10.1021/jp0139484.

(28) Essa, H.; Magner, E.; Cooney, J.; Hodnett, B. K., Influence of pH and ionic strength on the adsorption, leaching and activity of myoglobin immobilized onto ordered mesoporous silicates. *J. Mol. Catal. B Enzym.* **2007,** *49* (1), 61-68, DOI: 10.1016/j.molcatb.2007.07.005.

(29) Givens, B. E.; Diklich, N. D.; Fiegel, J.; Grassian, V. H., Adsorption of bovine serum albumin on silicon dioxide nanoparticles: Impact of pH on nanoparticle-protein interactions. *Biointerphases* **2017,** *12* (2), 02D404-02D404, DOI: 10.1116/1.4982598.

(30) Food Standards Australia & New Zealand *β-galactosidase derived from Bacillus circulans as a processing aid*; Food Standards Australia & New Zealand: 2012.

(31) Eskandarloo, H.; Abbaspourrad, A., Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. *Food Chem.* **2018,** *251*, 115-124, DOI: 10.1016/j.foodchem.2018.01.068.

(32) Zeuner, B.; González-Delgado, I.; Holck, J.; Morales, G.; López-Muñoz, M.-J.; Segura, Y.; Meyer, A. S.; Mikkelsen, J. D., Characterization and immobilization of engineered sialidases from Trypanosoma rangeli for transsialylation. *AIMS Mol. Sci.* **2017,** *4* (2), 140-163, DOI: http://dx.doi.org/10.3934/molsci.2017.2.140.

(33) Song, Y.-S.; Lee, H.-U.; Park, C.; Kim, S.-W., Optimization of lactulose synthesis from whey lactose by immobilized β-galactosidase and glucose isomerase. *Carbohydr. Res.* **2013,** *369*, 1-5, DOI: 10.1016/j.carres.2013.01.002.

(34) Zhao, S.; Caruso, F.; Dähne, L.; Decher, G.; De Geest, B. G.; Fan, J.; Feliu, N.; Gogotsi, Y.; Hammond, P. T.; Hersam, M. C.; Khademhosseini, A.; Kotov, N.; Leporatti, S.; Li, Y.; Lisdat, F.; Liz-Marzán, L. M.; Moya, S.; Mulvaney, P.; Rogach, A. L.; Roy, S.; Shchukin, D. G.; Skirtach, A. G.; Stevens, M. M.; Sukhorukov, G. B.; Weiss, P. S.; Yue, Z.; Zhu, D.; Parak, W. J., The Future of Layer-by-Layer Assembly: A Tribute to ACS Nano Associate Editor Helmuth Möhwald. *ACS Nano* **2019,** *13* (6), 6151-6169, DOI: 10.1021/acsnano.9b03326.

(35) Sakr, O. S.; Borchard, G., Encapsulation of Enzymes in Layer-by-Layer (LbL) Structures: Latest Advances and Applications. *Biomacromolecules* **2013,** *14* (7), 2117-2135, DOI: 10.1021/bm400198p.

(36) Feifel, S. C.; Kapp, A.; Lisdat, F., Protein Multilayer Architectures on Electrodes for Analyte Detection. In *Biosensors Based on Aptamers and Enzymes*, Gu, M. B.; Kim, H.-S., Eds. Springer Berlin Heidelberg: Berlin, Heidelberg, 2014; pp 253-298.

(37) Gunda, N. S. K.; Singh, M.; Norman, L.; Kaur, K.; Mitra, S. K., Optimization and characterization of biomolecule immobilization on silicon substrates using (3-

aminopropyl)triethoxysilane (APTES) and glutaraldehyde linker. *Appl. Surf. Sci.* **2014,** *305*, 522-530, DOI: 10.1016/j.apsusc.2014.03.130.

(38) Bernal, C.; Urrutia, P.; Illanes, A.; Wilson, L., Hierarchical meso-macroporous silica grafted with glyoxyl groups: opportunities for covalent immobilization of enzymes. *New Biotechnol.* **2013,** *30* (5), 500-506, DOI: 10.1016/j.nbt.2013.01.011.

(39) Reurink, D. M.; te Brinke, E.; Achterhuis, I.; Roesink, H. D. W.; de Vos, W. M., Nafion-Based Low-Hydration Polyelectrolyte Multilayer Membranes for Enhanced Water Purification. *ACS Appl. Polym. Mater.* **2019,** *1* (9), 2543-2551, DOI: 10.1021/acsapm.9b00689.

(40) Godoy-Gallardo, M.; Labay, C.; Hosta-Rigau, L., Tyrosinase-Loaded Multicompartment Microreactor toward Melanoma Depletion. *ACS Appl. Mater. Interfaces* **2019,** *11* (6), 5862-5876, DOI: 10.1021/acsami.8b20275.

(41) Kozlovskaya, V.; Kharlampieva, E.; Sukhishvili, S. A., 1.25 Polymer Films Using LbL Self-Assembly. In *Comprehensive Biomaterials II*, Ducheyne, P., Ed. Elsevier: Oxford, 2017; pp 554-569.

(42) Adamczyk, Z.; Zembala, M.; Warszyński, P.; Jachimska, B., Characterization of Polyelectrolyte Multilayers by the Streaming Potential Method. *Langmuir* **2004,** *20* (24), 10517-10525, DOI: 10.1021/la040064d.

(43) Jurin, F. E.; Buron, C. C.; Magnenet, C.; Quinart, M.; Lakard, S.; Filiâtre, C.; Lakard, B., Predictive tools for selection of appropriate polyelectrolyte multilayer film for the functionalization of organic membranes. *Colloid. Surf. A Physicochem. Eng. Asp.* **2015,** *486*, 153-160, DOI: 10.1016/j.colsurfa.2015.09.043.

(44) Song, Y. S.; Lee, J. H.; Kang, S. W.; Kim, S. W., Performance of β-galactosidase pretreated with lactose to prevent activity loss during the enzyme immobilisation process. *Food Chem.* **2010,** *123* (1), 1-5, DOI: 10.1016/j.foodchem.2010.04.043.

(45) Taylor, J. R., *Introduction To Error Analysis: The Study of Uncertainties in Physical Measurements*. University Science Books: 1997.

(46) Zhuravlev, L. T., The surface chemistry of amorphous silica. Zhuravlev model. *Colloid. Surf. A Physicochem. Eng. Asp.* **2000,** *173* (1), 1-38, DOI: 10.1016/S0927-7757(00)00556-2.

(47) Meléndez-Ortiz, H. I.; Puente-Urbina, B.; Castruita-de León, G.; Mercado-Silva, J. A.; Saucedo-Salazar, E.; García-Cerda, L. A., Covalent attachment of poly(allylamine hydrochloride) onto ordered silica foams. *J. Porous Mater.* **2020,** *27* (3), 929-937, DOI: 10.1007/s10934-020-00870-8.

(48) Sui, C.; Preece, J. A.; Zhang, Z., Novel polystyrene sulfonate–silica microspheres as a carrier of a water soluble inorganic salt (KCl) for its sustained release, via a dual-release mechanism. *RSC Adv.* **2017,** *7* (1), 478-481, DOI: 10.1039/C6RA25488H.

(49) Zhao, H. C.; Wu, X. T.; Tian, W. W.; Ren, S. T., Synthesis and Thermal Property of Poly(Allylamine Hydrochloride). *Adv. Mater. Res.* **2011,** *150-151*, 1480-1483, DOI: 10.4028/www.scientific.net/AMR.150-151.1480.

(50) Jadhav, S. A.; Nisticò, R.; Magnacca, G.; Scalarone, D., Packed hybrid silica nanoparticles as sorbents with thermo-switchable surface chemistry and pore size for fast extraction of environmental pollutants. *RSC Adv.* **2018,** *8* (3), 1246-1254, DOI: 10.1039/C7RA11869D.

(51) Chan, W. Y.; King, E. J.; Olsen, B. D., Hydrophobic and Bulk Polymerizable Protein-Based Elastomers Compatibilized with Surfactants. *ACS Sustainable Chem. Eng.* **2019,** *7* (10), 9103-9111, DOI: 10.1021/acssuschemeng.8b03557.

(52) Pirozzi, D.; Abagnale, M.; Minieri, L.; Pernice, P.; Aronne, A., In-situ sol-gel modification strategies to develop a monolith continuous microreactor for enzymatic green reactions. *Chem. Eng. J.* **2016,** *306*, 1010-1016, DOI: 10.1016/j.cej.2016.08.035.

(53) Yasar Mahlicli, F.; Şen, Y.; Mutlu, M.; Alsoy Altinkaya, S., Immobilization of superoxide dismutase/catalase onto polysulfone membranes to suppress hemodialysis-induced oxidative stress: A comparison of two immobilization methods. *J. Membr. Sci.* **2015,** *479*, 175-189, DOI: 10.1016/j.memsci.2014.12.025.

(54) Nyanhongo, G. S.; Rodríguez, R. D.; Prasetyo, E. N.; Caparrós, C.; Ribeiro, C.; Sencadas, V.; Lanceros-Mendez, S.; Acero, E. H.; Guebitz, G. M., Bioactive albumin

functionalized polylactic acid membranes for improved biocompatibility. *React. Funct. Polym.* **2013,** *73* (10), 1399-1404, DOI: 10.1016/j.reactfunctpolym.2012.12.007.

(55) Yang, H.; Yang, S.; Kong, J.; Dong, A.; Yu, S., Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy. *Nat. Protoc.* **2015,** *10* (3), 382-396, DOI: 10.1038/nprot.2015.024.

(56) Ciolacu, D.; Chiriac, A. I.; Pastor, F. I. J.; Kokol, V., The influence of supramolecular structure of cellulose allomorphs on the interactions with cellulose-binding domain, CBD3b from Paenibacillus barcinonensis. *Bioresour. Technol.* **2014,** *157*, 14-21, DOI: 10.1016/j.biortech.2014.01.027.

(57) Barth, A., Infrared spectroscopy of proteins. *BBA-Bioenergetics* **2007,** *1767* (9), 1073-1101, DOI: 10.1016/j.bbabio.2007.06.004.

(58) Kumar, A.; Park, G. D.; Patel, S. K. S.; Kondaveeti, S.; Otari, S.; Anwar, M. Z.; Kalia, V. C.; Singh, Y.; Kim, S. C.; Cho, B.-K.; Sohn, J.-H.; Kim, D. R.; Kang, Y. C.; Lee, J.-K., SiO2 microparticles with carbon nanotube-derived mesopores as an efficient support for enzyme immobilization. *Chem. Eng. J.* **2019,** *359*, 1252-1264, DOI: 10.1016/j.cej.2018.11.052.

(59) Bedade, D. K.; Muley, A. B.; Singhal, R. S., Magnetic cross-linked enzyme aggregates of acrylamidase from Cupriavidus oxalaticus ICTDB921 for biodegradation of acrylamide from industrial waste water. *Bioresour. Technol.* **2019,** *272*, 137-145, DOI: 10.1016/j.biortech.2018.10.015.

(60) Schwinté, P.; Ball, V.; Szalontai, B.; Haikel, Y.; Voegel, J. C.; Schaaf, P., Secondary Structure of Proteins Adsorbed onto or Embedded in Polyelectrolyte Multilayers. *Biomacromolecules* **2002,** *3* (6), 1135-1143, DOI: 10.1021/bm025547f.

(61) Puente-Santiago, A. R.; Rodríguez-Padrón, D.; Quan, X.; Muñoz Batista, M. J.; Martins, L. O.; Verma, S.; Varma, R. S.; Zhou, J.; Luque, R., Unprecedented Wiring Efficiency of Sulfonated Graphitic Carbon Nitride Materials: Toward High-Performance Amperometric Recombinant CotA Laccase Biosensors. *ACS Sustainable Chem. Eng.* **2019,** *7* (1), 1474-1484, DOI: 10.1021/acssuschemeng.8b05107.

(62) Khakshoor, O.; Nowick, J. S., Artificial beta-sheets: chemical models of beta-sheets. *Curr. Opin. Chem. Biol.* **2008,** *12* (6), 722-729, DOI: 10.1016/j.cbpa.2008.08.009.

(63) Secundo, F., Conformational changes of enzymes upon immobilisation. *Chem. Soc. Rev.* **2013,** *42* (15), 6250-6261, DOI: 10.1039/C3CS35495D.

(64) Cooper, C. L.; Dubin, P. L.; Kayitmazer, A. B.; Turksen, S., Polyelectrolyte–protein complexes. *Curr. Opin. Colloid Interface Sci.* **2005,** *10* (1), 52-78, DOI: 10.1016/j.cocis.2005.05.007.

(65) Zhang, D. H.; Yuwen, L. X.; Peng, L. J., Parameters Affecting the Performance of Immobilized Enzyme. *J. Chem.* **2013**, 1-7, DOI: 10.1155/2013/946248.

(66) Song, Y. S.; Suh, Y. J.; Park, C.; Kim, S. W., Improvement of lactulose synthesis through optimization of reaction conditions with immobilized β-galactosidase. *Korean J. Chem. Eng.* **2013,** *30* (1), 160-165, DOI: 10.1007/s11814-012-0105-1.

(67) Urrutia, P.; Bernal, C.; Wilson, L.; Illanes, A., Improvement of Chitosan Derivatization for the Immobilization of Bacillus circulans β-Galactosidase and Its Further Application in Galacto-oligosaccharide Synthesis. *J. Agric. Food Chem.* **2014,** *62* (41), 10126-10135, DOI: 10.1021/jf500351j.

(68) Osman, A.; Symeou, S.; Trisse, V.; Watson, K. A.; Tzortzis, G.; Charalampopoulos, D., Synthesis of prebiotic galactooligosaccharides from lactose using bifidobacterial β-galactosidase (BbgIV) immobilised on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose. *Biochem. Eng. J.* **2014,** *82*, 188-199, DOI: 10.1016/j.bej.2013.11.020.

(69) Karimi Alavijeh, M.; Meyer, A. S.; Gras, S.; Kentish, S. E., The role of cations in regulating reaction pathways driven by Bacillus circulans β-galactosidase. *Chem. Eng. J.* **2020,** *395*, 125067, DOI: 10.1016/j.cej.2020.125067.

(70) Cai, Z.; Wei, Y.; Wu, M.; Guo, Y.; Xie, Y.; Tao, R.; Li, R.; Wang, P.; Ma, A.; Zhang, H., Lipase Immobilized on Layer-by-Layer Polysaccharide-Coated Fe3O4@SiO2 Microspheres as a Reusable Biocatalyst for the Production of Structured Lipids. *ACS Sustainable Chem. Eng.* **2019,** *7* (7), 6685-6695, DOI: 10.1021/acssuschemeng.8b05786.

(71) Sarma, R.; Islam, M. S.; Miller, A.-F.; Bhattacharyya, D., Layer-by-Layer-Assembled Laccase Enzyme on Stimuli-Responsive Membranes for Chloro-Organics Degradation. *ACS Appl. Mater. Interfaces* **2017,** *9* (17), 14858-14867, DOI: 10.1021/acsami.7b01999.

(72) Agrawal, D. C.; Yadav, A.; Kesarwani, R.; Srivastava, O. N.; Kayastha, A. M., Immobilization of fenugreek β-amylase onto functionalized graphene quantum dots (GQDs) using Box-Behnken design: Its biochemical, thermodynamic and kinetic studies. *Int. J. Biol. Macromol.* **2020,** *144*, 170-182, DOI: 10.1016/j.ijbiomac.2019.12.033.

(73) Konermann, L., Protein Unfolding and Denaturants. *eLS. John Wiley & Sons, Ltd: Chichester* **2012**, DOI: 10.1002/9780470015902.a0003004.pub2.

(74) Zaboli, M.; Zaboli, M.; Torkzadeh-Mahani, M., From in vitro to in silico: Modeling and recombinant production of DT-Diaphorase enzyme. *Int. J. Biol. Macromol.* **2020,** *143*, 213-223, DOI: 10.1016/j.ijbiomac.2019.12.029.

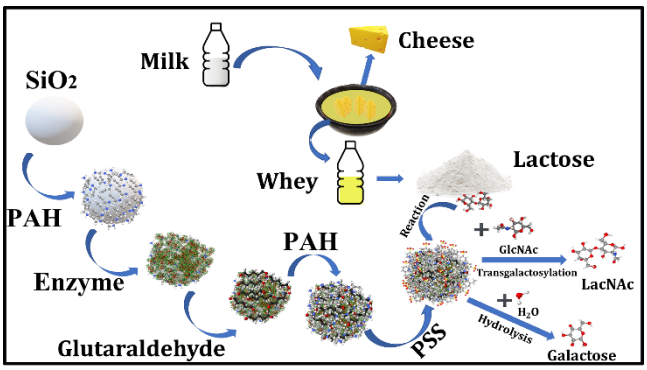
(75) Dhiman, S. S.; Kalyani, D.; Jagtap, S. S.; Haw, J.-R.; Kang, Y. C.; Lee, J.-K., Characterization of a novel xylanase from Armillaria gemina and its immobilization onto SiO2 nanoparticles. *Appl. Microbiol. Biotechnol.* **2013,** *97* (3), 1081-1091, DOI: 10.1007/s00253-012-4381-9.

(76) Perticaroli, S.; Nickels, Jonathan D.; Ehlers, G.; Sokolov, Alexei P., Rigidity, Secondary Structure, and the Universality of the Boson Peak in Proteins. *Biophys. J.* **2014,** *106* (12), 2667-2674, DOI: 10.1016/j.bpj.2014.05.009.

(77) DiCosimo, R.; McAuliffe, J.; Poulose, A. J.; Bohlmann, G., Industrial use of immobilized enzymes. *Chem. Soc. Rev.* **2013,** *42* (15), 6437-6474, DOI: 10.1039/C3CS35506C.

(78) Keller, J.; Hartley, K., Greenhouse gas production in wastewater treatment: process selection is the major factor. *Water Sci. Technol.* **2003,** *47* (12), 43-48, DOI: 10.2166/wst.2003.0626.

For Table of Contents Use Only



Synopsis:

Whey-derived lactose was transformed to a bioactive compound (N-acetyllactosamine) via enzyme encapsulation into polyelectrolyte layers resulting in improved yield and stability.

745 **Figure Captions**

746 **Figure 1.** A schematic representation of the cross-linked layer-by-layer encapsulation
747 of the enzyme onto silica particles in this study. PAH: Poly(allylamine hydrochloride),
748 PSS: Poly(styrene sulfonate). The shapes do not represent the exact particle
749 morphology, molecule sizes or interaction between different layers but provide a visual
750 guide.

751 **Figure 2.** (A) ATR-FTIR spectra of SiO_2, native enzyme and immobilized enzyme. (B)
752 Zeta potential measurements for the layer-by-layer depositions in Tris buffer at pH=7.2.
753 (C) ATR-FTIR spectrum of Amide I band for the native and immobilized enzymes.

754 **Figure 3.** (A) Poisson-Boltzmann electrostatic surface potential of the enzyme
755 estimated at pH 6 showing negatively charged regions (red), uncharged regions (white)
756 and positively charged regions (blue). (B) Free amine groups of the enzyme coloured
757 green, which can potentially react with glutaraldehyde. (C) Surface regions (yellow)
758 involved in enzyme-enzyme interactions were predicted by PSIVER (protein-protein
759 interaction sites prediction server). (D) The 3D surface structure of the enzyme
760 showing the region of its catalytic pocket (cyan). The visualization was generated using
761 Chimera software and the 3D structure was obtained from the Protein Data Bank (PDB)
762 with a code of 4ypj. Four different views of the enzyme are represented in A to C.

763 **Figure 4**. (A) time-course of the synthesis of LacNAc with the use of immobilized and
764 free enzyme at 50 °C with initial lactose and GlcNAc concentrations of 50 mM and 500
765 mM, respectively. (B) The ratio of transgalactosylation to hydrolysis rates for

766 immobilized and free enzyme. (C) The specific productivity of LacNAc after 90 min and
767 150 min of the reaction with the use of immobilized or free enzyme.

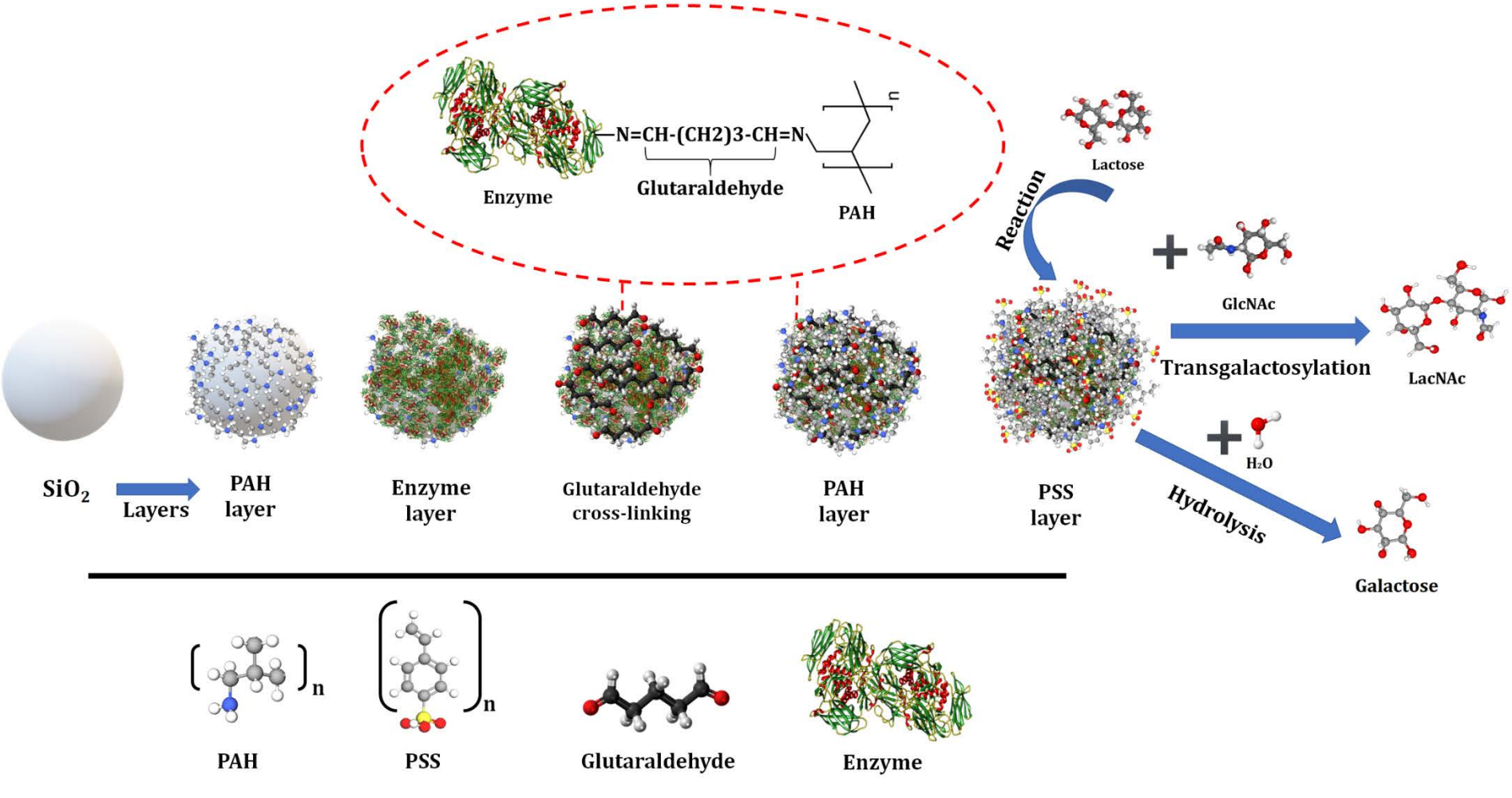
768 **Figure 5**. Natural logarithm of the residual activity of (A) the immobilized enzyme and
769 (B) the free enzyme versus incubation time at elevated temperatures. (C) Natural
770 logarithm of the thermal inactivation rate constant versus the reciprocal of the absolute
771 temperature.

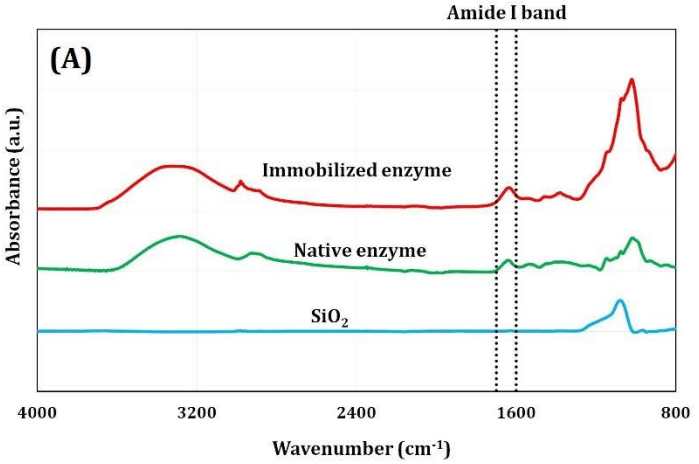
772 **Figure 6**. The transgalactosylation activity of the immobilized and free enzymes after
773 incubation for 30 min at elevated temperatures, relative to the case of the same enzyme
774 with no incubation. All activity tests were conducted at 50 °C for 10 min.

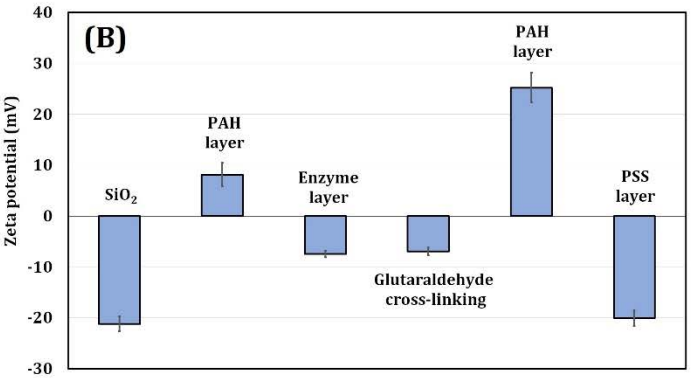
775 **Figure 7**. (A) Variations in the molar yield of LacNAc after eight reuse cycles of the
776 immobilized enzyme. (B) Changes in the profitability parameter (Net Present Value;
777 NPV) as a function of number of reuse cycles of the immobilized enzyme for a plant with
778 a downstream process based on selective crystallization. The cost parameters and
779 assumptions for the NPV calculation are described in Tables 1S and 2S in the
780 Supplementary Information.

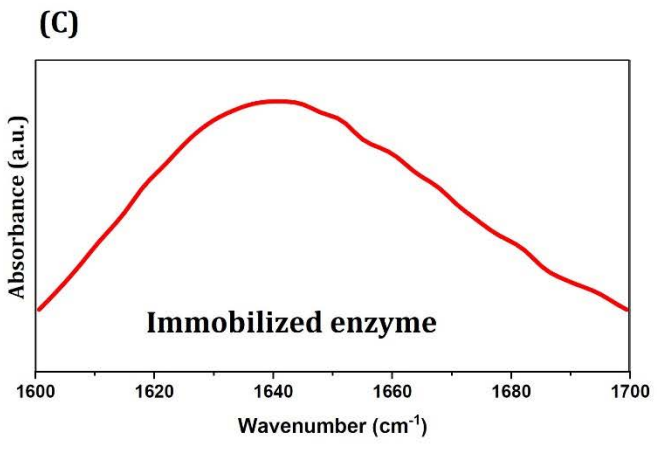
781

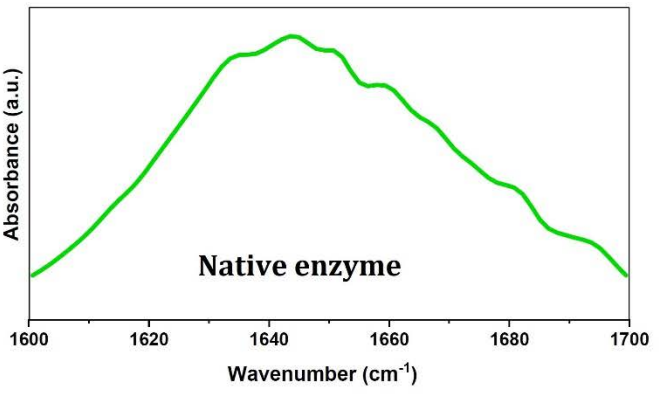
782

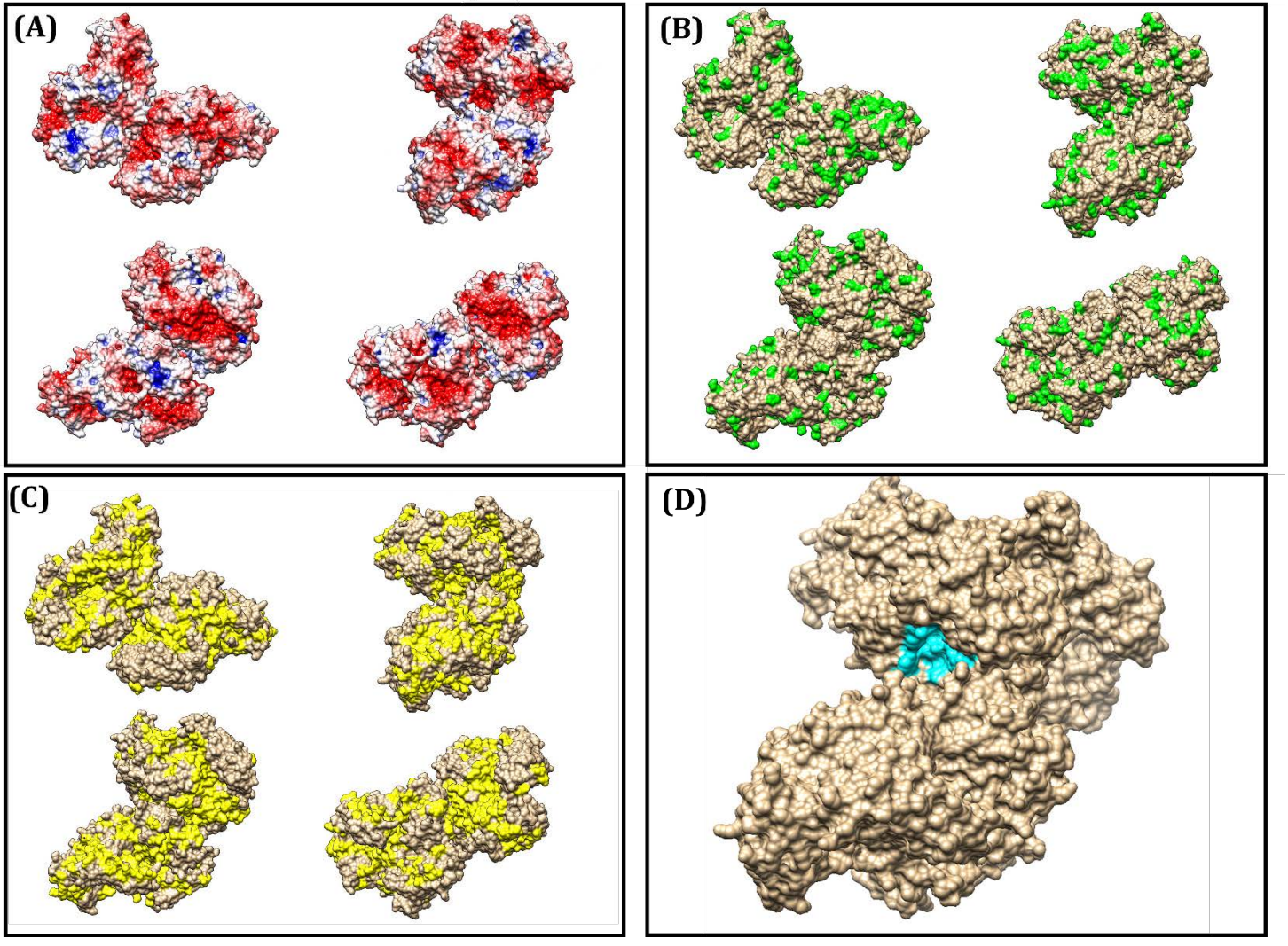

Enzyme
N=CH-(CH2)3-CH=N
Glutaraldehyde
n
PAH
Lactose
Reaction
GlcNAc
Transgalactosylation
LacNAc
H2O
Hydrolysis
Galactose
SiO2
Layers
PAH layer
Enzyme layer
Glutaraldehyde cross-linking
PAH layer
PSS layer
PAH
PSS
Glutaraldehyde
Enzyme

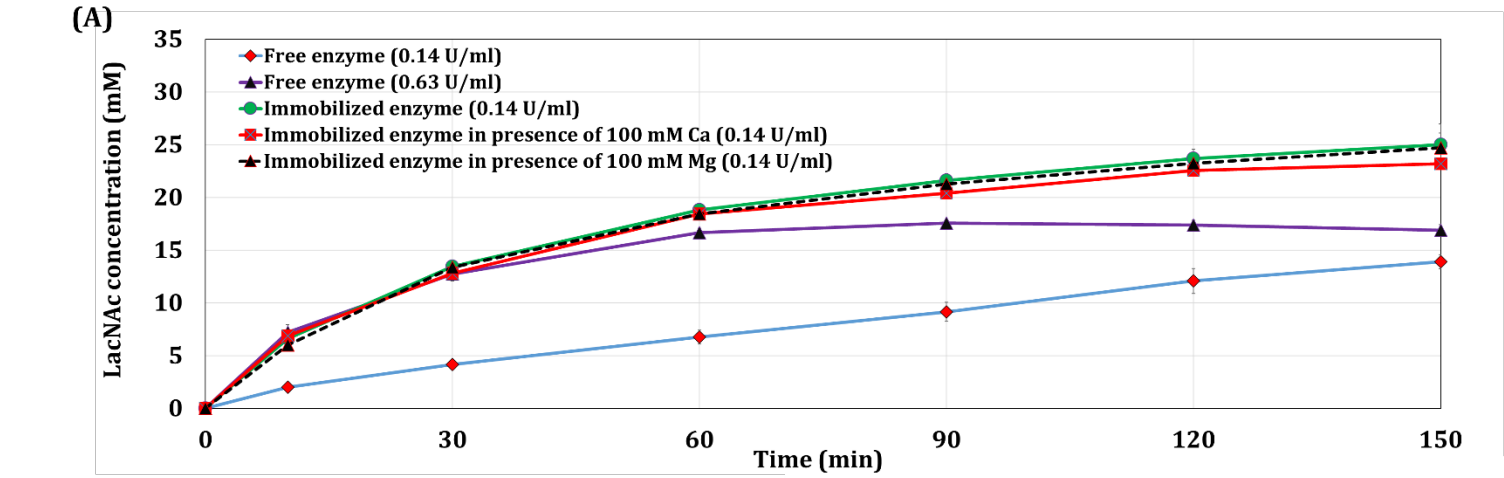

(A)
Amide I band
Absorbance (a.u.)
Immobilized enzyme
Native enzyme
SiO2
4000
3200
2400
1600
800
Wavenumber (cm-1)

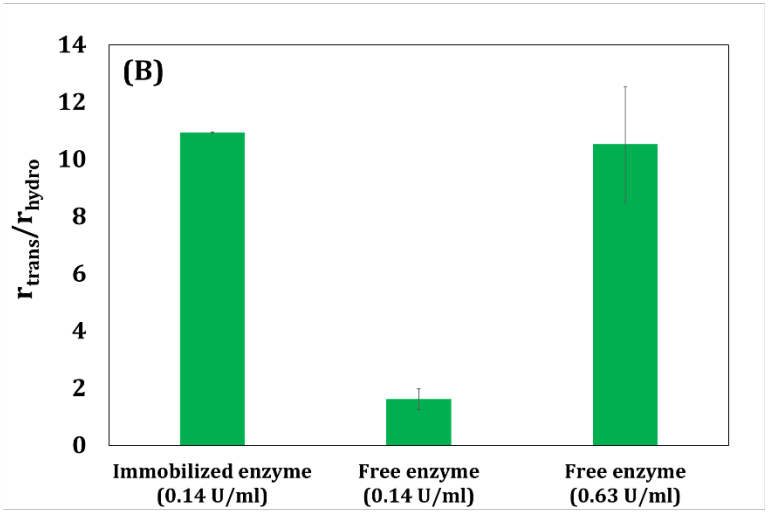

(B)
Zeta potential (mV)
40
30
20
10
0
-10
-20
-30
SiO2
PAH layer
Enzyme layer
Glutaraldehyde cross-linking
PAH layer
PSS layer

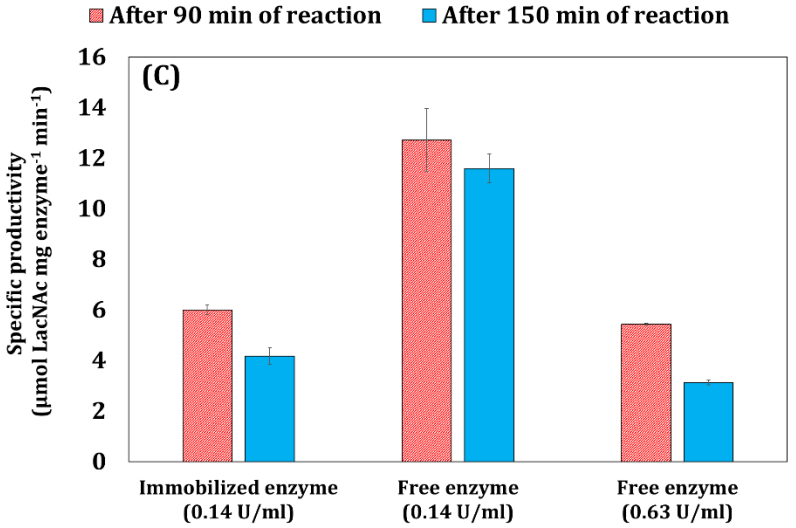

(C)
Absorbance (a.u.)
Immobilized enzyme
1600
1620
1640
1660
1680
1700
Wavenumber (cm-1)

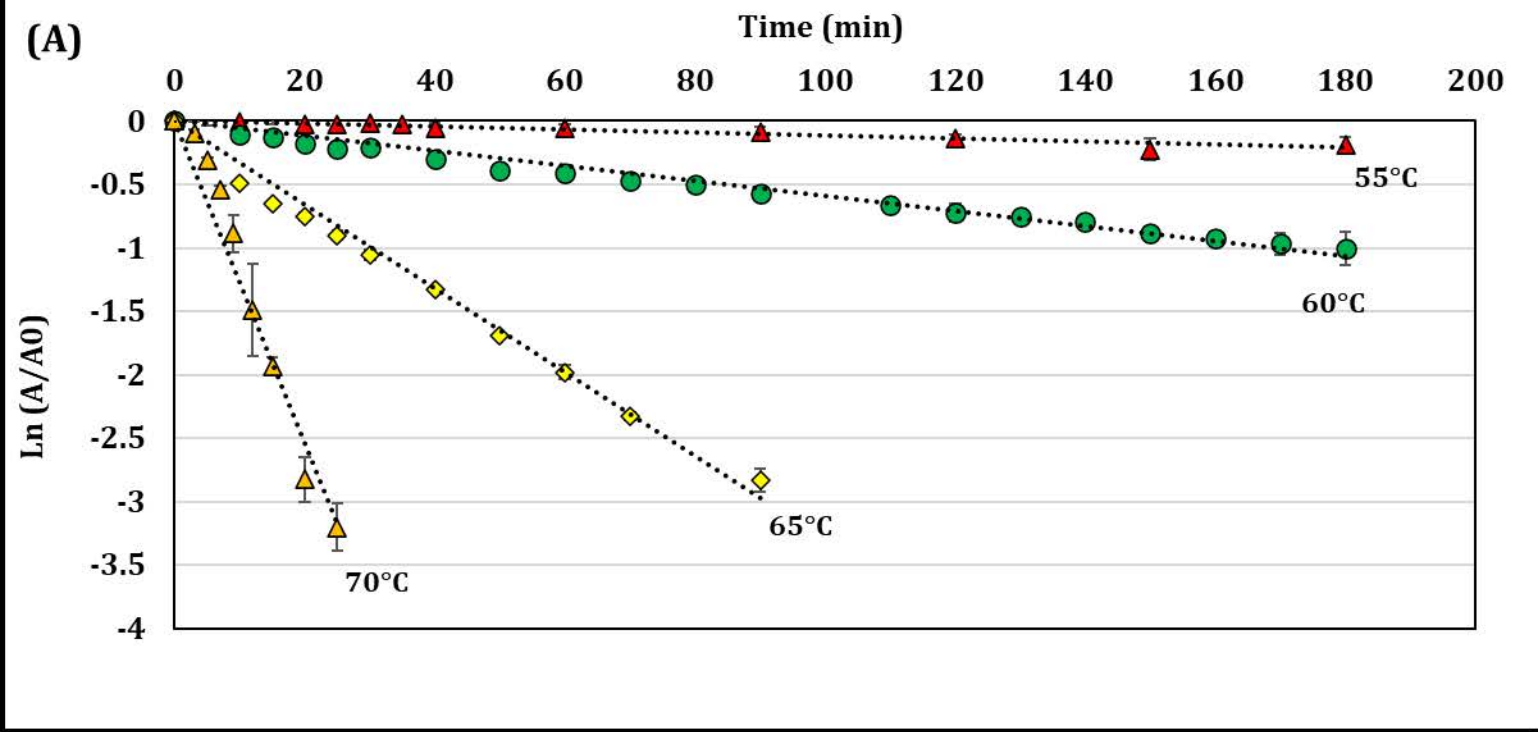

Absorbance (a.u.)
Native enzyme
1600
1620
1640
1660
1680
1700
Wavenumber (cm-1)

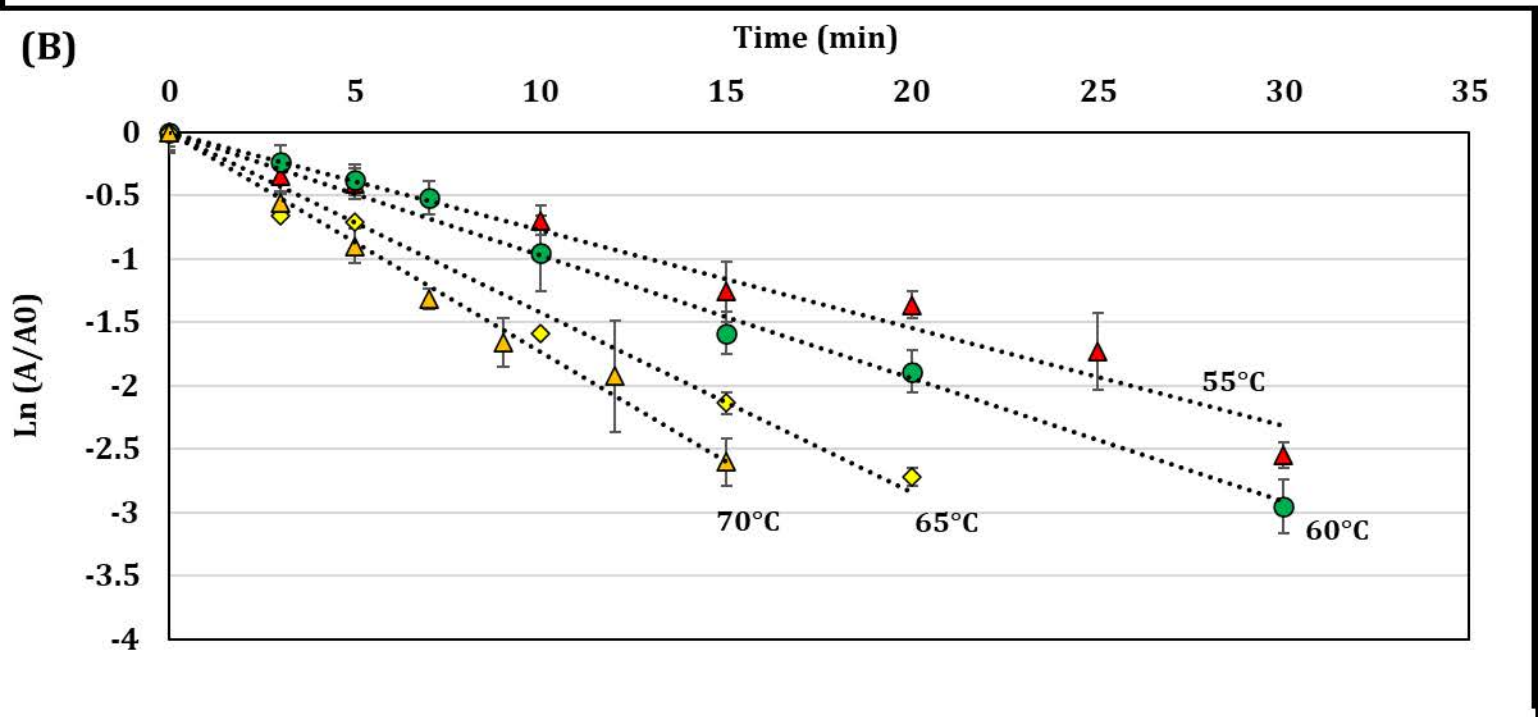

(A)
(B)
(C)
(D)

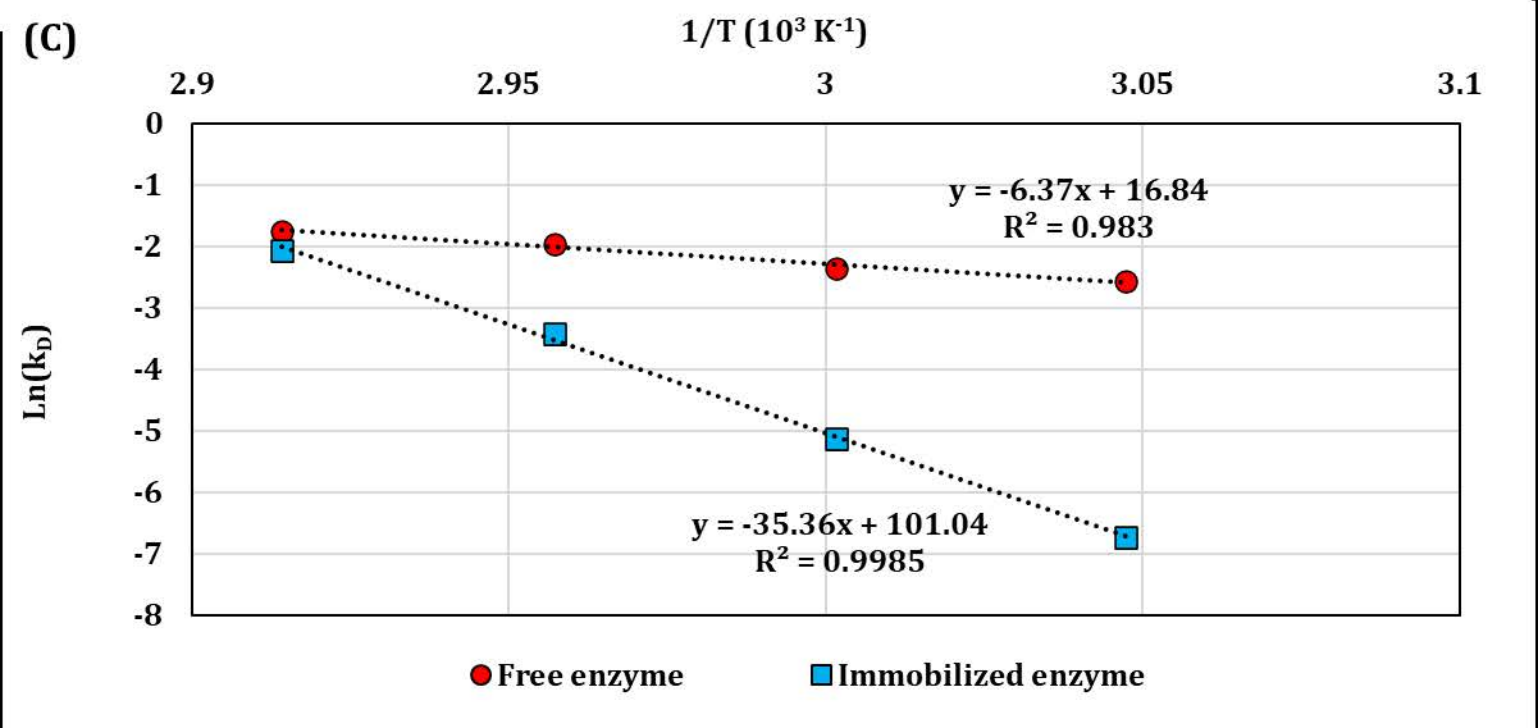

(A)
LacNAc concentration (mM)
Time (min)
Free enzyme (0.14 U/ml)
Free enzyme (0.63 U/ml)
Immobilized enzyme (0.14 U/ml)
Immobilized enzyme in presence of 100 mM Ca (0.14 U/ml)
Immobilized enzyme in presence of 100 mM Mg (0.14 U/ml)

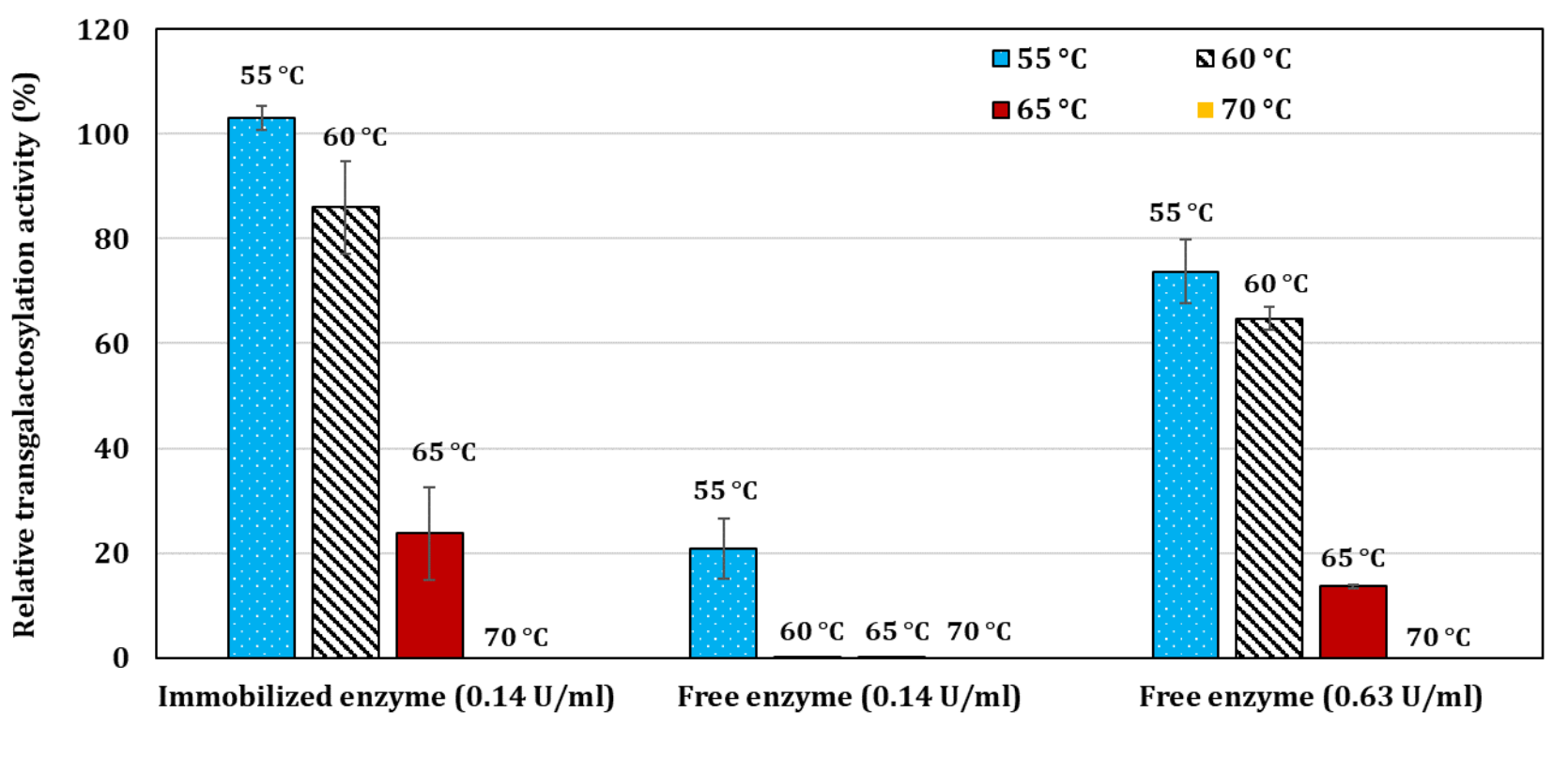

(B)
r_{trans}/r_{hydro}
Immobilized enzyme (0.14 U/ml)
Free enzyme (0.14 U/ml)
Free enzyme (0.63 U/ml)


After 90 min of reaction
After 150 min of reaction
(C)
Specific productivity (µmol LacNAc mg enzyme^{-1} min^{-1})
Immobilized enzyme (0.14 U/ml)
Free enzyme (0.14 U/ml)
Free enzyme (0.63 U/ml)

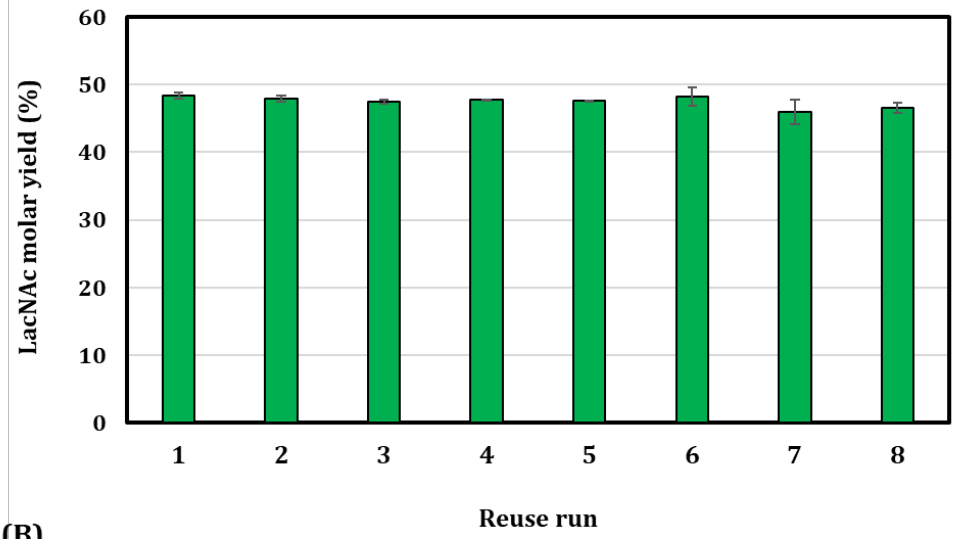

(A)
Time (min)
0
20
40
60
80
100
120
140
160
180
200
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
Ln (A/A0)
55°C
60°C
65°C
70°C


(B)
Time (min)
0
5
10
15
20
25
30
35
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
Ln (A/A0)
55°C
60°C
65°C
70°C

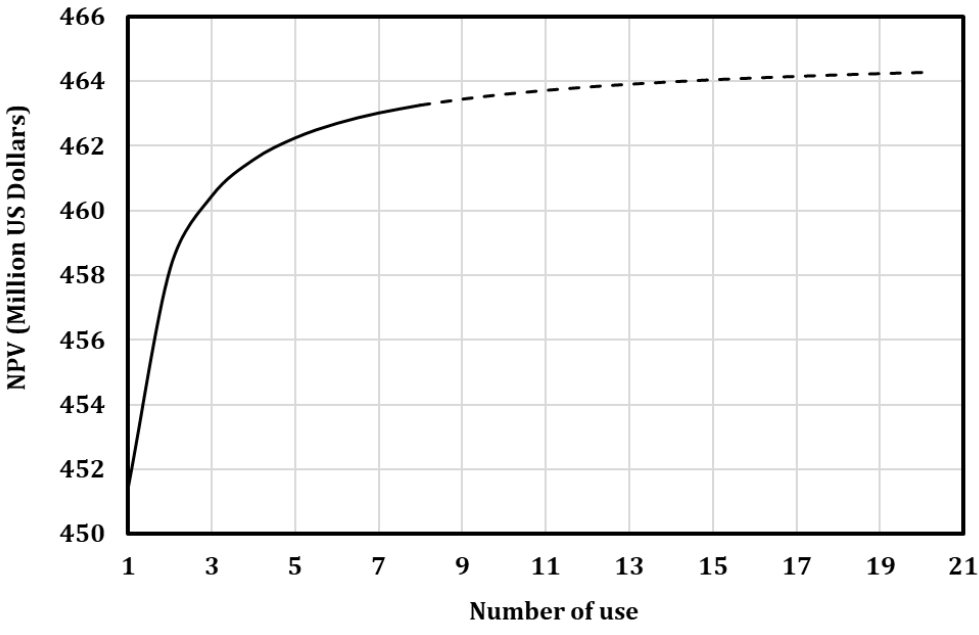

(C)
1/T (10³ K⁻¹)
2.9
2.95
3
3.05
3.1
0
-1
-2
-3
-4
-5
-6
-7
-8
Ln(k_D)
y = -6.37x + 16.84
R² = 0.983
y = -35.36x + 101.04
R² = 0.9985
Free enzyme
Immobilized enzyme


Relative transgalactosylation activity (%)
120
100
80
60
40
20
0
55 °C
60 °C
65 °C
70 °C
55 °C
60 °C
65 °C
70 °C
55 °C
60 °C
65 °C
70 °C
55 °C
60 °C
65 °C
70 °C
Immobilized enzyme (0.14 U/ml)
Free enzyme (0.14 U/ml)
Free enzyme (0.63 U/ml)

(A)


LacNAc molar yield (%)
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
Reuse run

(B)


NPV (Million US Dollars)
466
464
462
460
458
456
454
452
450
1
3
5
7
9
11
13
15
17
19
21
Number of use