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p-Phenylene Diisothiocyanate-Based Covalent Immobilization of β-D-Galactosidase and Determination of Enzyme Activity by Cleavage of X-Gal and ONPG on Solid Support

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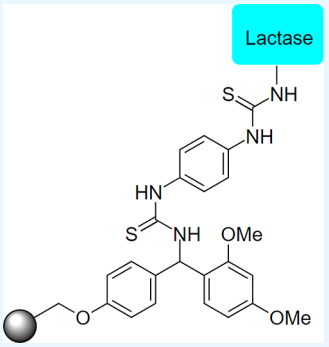
ABSTRACT: Herein, we present the immobilization of a technical grade β-D-galactosidase on amino-functionalized microtiter plates. Afterward, we transferred the results to a resin-based approach. For the covalent binding of the enzyme, an amino-functionalized microtiter plate was prefunctionalized with 1,4-phenylendiisothiocyanate. The cleavage of the substrate 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) produces a deep blue dye, which was quantified in a microtiter plate reader at 595 nm. The maximum reaction rates and the Michaelis−Menten constant were calculated. In addition, the unwanted blue precipitate formed during the experiments could be minimized by optimizing the experiments. When transferring the immobilization method to Rink amide resin, *o*-nitrophenyl-β-D-galactopyranoside was used as the substrate and the measurement was carried out in a photometer at 420 nm.



1. INTRODUCTION

The immobilization of enzymes has become a common method for various purposes, especially in biotechnological applications such as biomedical techniques,[1] biosensor development,[2,3] or the food industry.[4] Therefore, the intention to improve enzyme characteristics such as stability, activity, and usability/reusability is the central idea of most of these immobilization efforts.[5,6] In particular, reusability is, in addition to its scientific aspects, an important economic reason for the immobilization of enzymes. The use of enzymes in solution is often connected to the loss of these enzymes, and although the prices for different enzymes can vary enormously, not only economic reasons but also sustainability plays an important role.[7]

Therefore, several methods for the immobilization of enzymes have been examined by different techniques, such as adsorption on silanized or mesoporous molecular sieves[8,9] or on polypropylene-based hydrophobic granules.[10] While adsorption-based techniques rely on nonspecific hydrophobic interactions, affinity-based immobilization has also been investigated, including one-step purification and immobilization of the enzyme.[11,12] A method that often results in significantly increased stability and activity is covalent immobilization.[5] Normally, the functionalities of amino acid side chains of enzymes react with organic functionalities on a solid support, often using crosslinking reagents such as glutaraldehyde or *p*-phenylene diisothiocyanate (PDC).[13−15]

β-D-Galactosidases (β-Gal) are a group of exoglycosidases, a type of enzyme that hydrolyse β-glycosidic bonds between a terminal galactose and its organic aglycon. They are widespread in all organisms and play a key role in the degradation of polysaccharidic structures for energetic and carbon supply reasons.[16] Industrially and technically, β-Gal is an important enzyme that allows the production of lactose-free milk.[17] A significant number of adults cannot produce β-Gal (Lactase), resulting in gastrointestinal problems if lactose-containing food, especially milk, is consumed.[18] Technically used β-Gal is produced from yeasts or fungal sources,[19] but methods using *lactobacilli*[20] have also been developed.

Several methods have been developed for the production of lactose-free milk, such as the use of soluble β-Gal under various conditions, such as before or after packaging of raw or pasteurized milk products.[21,22] Although these methods have made lactose-free milk available to a broad number of customers, these products are still more expensive than nonprocessed products, resulting in reduced consumer acceptance.[23] As a result, some studies have immobilized β-Gal on solid supports such as chitosan[15] using tris(hydroxymethyl)phosphine (THP) and glutaraldehyde as crosslinking reagents or on the surface of epoxy-activated magnetic beads.[24] Although both methods showed good results in thermal stability and activity, the reusability of glutaraldehyde-crosslinked β-Gal seemed to be limited, whereas the THP-crosslinked β-Gal could be reused several times without losing much of its activity. However, THP

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itself is a very toxic substance that should at least be considered problematic in potential food industrial applications.

In this work, we present the investigation of PDC-based crosslinking of β-Gal on amino-functionalized 96 microwell plates and on rink amide resin, as well as the determination of enzymatic activity.

2. MATERIALS AND METHODS

Chemicals and reagents were purchased from Carl Roth and were used without further purification. Rink amide resin (4-

Table 1. Following Pipetting Scheme Shows the X-Gal Concentrations 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, and 0.6 mM, Which were Used for the Enzyme Dilution 1:50

X-Gal 0.6	X-Gal 0.55	X-Gal 0.5	X-Gal 0.45	X-Gal 0.4	X-Gal 0.35	X-Gal 0.3	X-Gal 0.25	X-Gal 0.2	X-Gal 0.15	X-Gal 0.1	Zero value

Table 2. Following Pipetting Scheme Shows the X-Gal Concentrations 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, and 0.6 mM, Which were Used for the Enzyme Dilution 1:500

Zero value	X-Gal 0.1	X-Gal 0.15	X-Gal 0.2	X-Gal 0.25	X-Gal 0.3	X-Gal 0.35	X-Gal 0.4	X-Gal 0.45	X-Gal 0.5	X-Gal 0.55	X-Gal 0.6

(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, Peptipure, 100−200 mesh, 1% DVB, loading: 0.3-0-8 mmol/g) was purchased from carl roth. The experiments for immobilization on amino-functionalized microtiter plates are based on the immobilization of amino-PEG-glycosides described by Tong et al.[25] For the enzymatic activity, the experiments of Manera et al.[26] were adapted. Lactase L extracted from *Kluyveromyces* lactis was purchased from ASA Spezialenzyme GmbH. As per manufacturer information, the activity of the lactase used is approximately 45.000 U/mL and was determined by hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside and observation of the increase in the extinction to a value of one over 10 min. Verification of the activity by the method of Manera et al. (Manera, A. P., da Costa Ores, J., Amaral Ribeiro, V., Veiga Burkert, C. A. & Kalil, S.J., 2008) results in an activity of approximately 300 U/mL due to the different procedure.

Table 3. Following Pipetting Scheme Shows the X-Gal Concentrations 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 and the BSA Dilutions 1:10, 1:5, 1:2, and 1:1, Which were Used for the First Attempts[a]

	X-Gal 0.6		X-Gal 0.5		X-Gal 0.4		X-Gal 0.3		X-Gal 0.2		X-Gal 0.1	
BSA 1:10												
BSA 1:5												
BSA 1:2												
BSA 1:1												

[a]Only half of the microtiter plate is shown in this scheme. When the entire plate was used, the BSA dilutions were repeated in the same order for subsequent wells.

Table 4. Following Pipetting Scheme Shows X-Gal Concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 and BSA Dilutions of 1:5, 1:2, and 1:1[a]

		Zero value	X-Gal 0.1	X-Gal 0.2	X-Gal 0.3	X-Gal 0.4	X-Gal 0.5	X-Gal 0.6
BSA 0	Enzyme dilution 1:50							
BSA 1:5								
BSA 1:2								
BSA 1:1								
BSA 0	Enzyme dilution 1:500							
BSA 1:5								
BSA 1:2								
BSA 1:1								

[a]The previously used BSA dilution of 1:10 was replaced with BSA 0, where no BSA was added to these wells. This scheme was used for the latter experiments examining the influence of BSA on the enzyme activity of the immobilized lactase. In this scheme, the entire microplate is shown, with the top half of the microplate working with an enzyme dilution of 1:50 and the bottom half working with an enzyme dilution of 1:500 for the immobilization.

Plates with covalently binding surfaces from Thermo Scientific CovaLink and Immobilizer Amino were used for the experiments.

2.1. Solutions for Immobilization of β-D-Galactosidase on Amino-Functionalized Microtiter Plates. *2.1.1. Preparation of Phosphate Buffer Saline (PBS Buffer).* 10 mM, pH 7.3 sodium dihydrogen phosphate dihydrate (0.362 g, $NaH_2PO_4 \times 2H_2O$), disodium hydrogen phosphate dodecahydrate (2.75 g, $Na_2HPO_4 \times 12H_2O$), sodium chloride (8.75 g, 150 mM, NaCl), magnesium chloride hexahydrate (0.0203 g, $MgCl_2 \times 6H_2O$), calcium chloride (0.0111 g, $CaCl_2$), and manganese(II) chloride tetrahydrate (0.0198 g, $MnCl_2 \times 4H_2O$) were dissolved in deionized water. The pH was adjusted to pH 7.3 with 0.1 M

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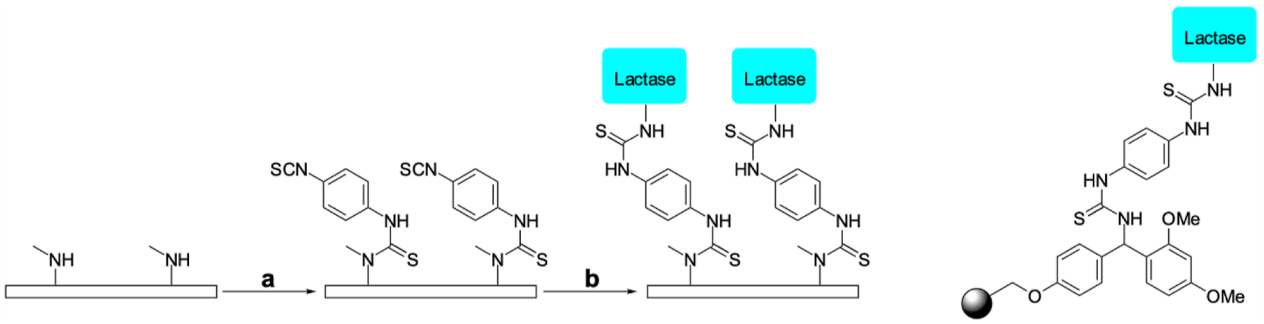

Figure 1. This figure illustrates the covalent immobilization of β-galactosidase using PDC as a spacer molecule on an amino-functionalized microtiter plate (left side) and an amino-functionalized (rink) resin (right side). The immobilization process involves prefunctionalization with PDC followed by the immobilization of the enzyme itself by thiourea linkage.

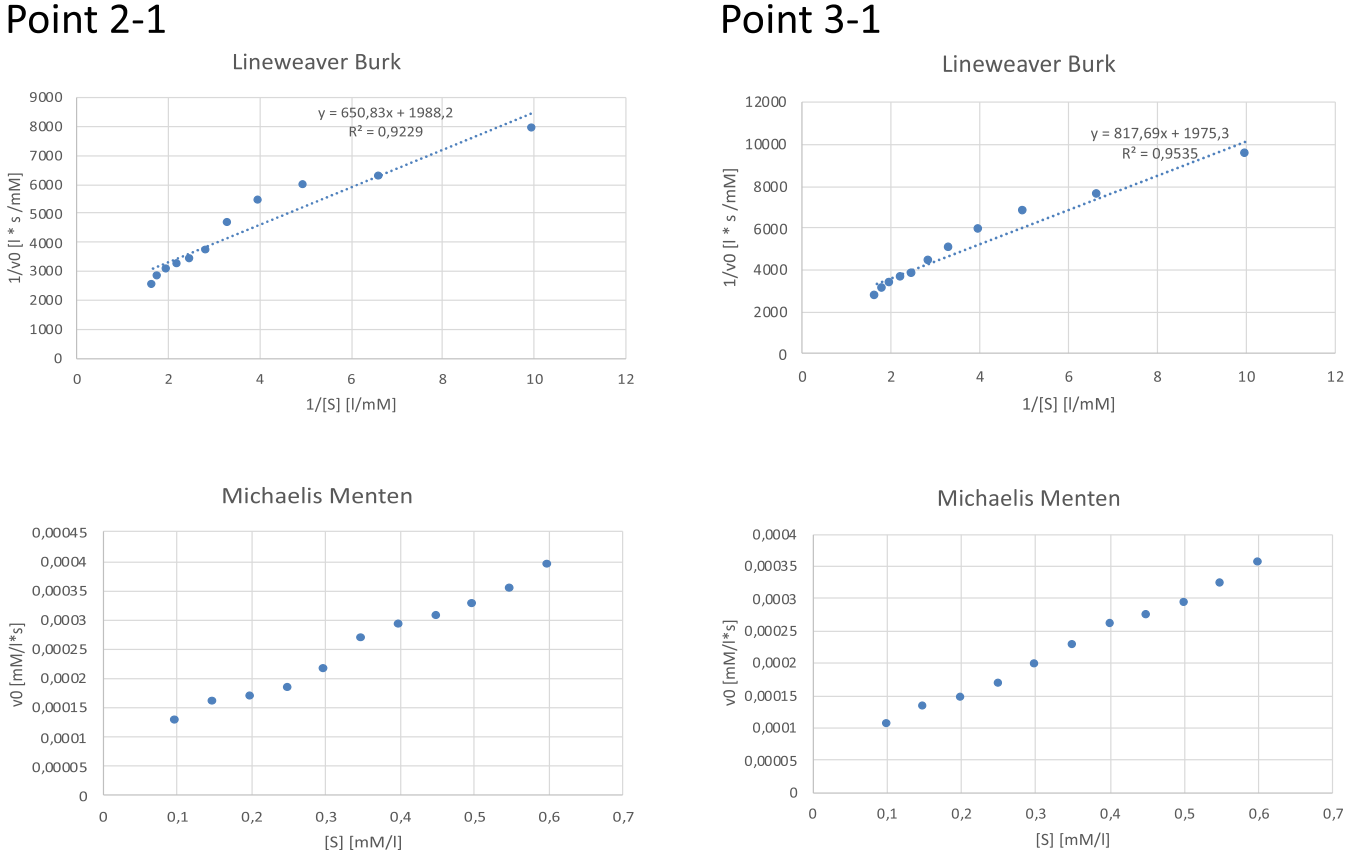

Figure 2. Michaelis–Menten and Lineweaver Burk plots of the first two measuring points (left) and the first three measuring points (right).

hydrogen chloride (HCl) or 0.1 M sodium hydroxide (NaOH) and then filled up to 1 L.

2.1.2. Preparation of PBST Buffer (PBS + 0.05% Tween 20). 10 mM Tween 20 (0.5 mL) was dissolved in 1 L of PBS buffer.

2.1.3. Preparation of the X-Gal Solution. 0.6 mM X-Gal (0.245 g/L) was dissolved in a PBS/DMSO ratio of 9:1.

2.1.4. Preparation of the Enzyme Dilutions. 1:500; 1:1000; 1:100; 1:50 the enzyme stock solution of β-D-galactosidase from ASA Spezialenzyme GmbH was diluted with PBS buffer.

2.1.5. Preparation of the Carbonate/Bicarbonate Buffer. 100 mM, pH 10.1 Sodium carbonate (1.33 g, Na_2CO_3) and sodium bicarbonate (1.05 g, $NaHCO_3$) were dissolved in deionized water. The pH was adjusted to pH 10.1 with 0.1 M HCl or 0.1 M NaOH and then filled up to 250 mL.

Table 5. Calculated V_{max} and K_m Values for the Different Measuring Points

measuring points	V_{max} [mmol/L·s]	K_m [mmol/L]
first two measurement points	5.030 × 10−4	0.327
first three measurement points	5.063 × 10−4	0.414

2.1.6. Preparation of the BSA Stock Solution. 10 g/L BSA (250 mg) was dissolved in PBS buffer and brought up to 25 mL.

2.1.7. Preparation of the BSA Dilutions. 1:10; 1:5; 1:2 the BSA stock solution was diluted with PBS buffer.

2.1.8. Preparation of the PDC Solution. 5 g/L a total of 125 mg PDC was dissolved in DMSO, and 100 μL *N,N*-diisopropylethylamine (DIPEA) was added and brought to 25 mL with DMSO.

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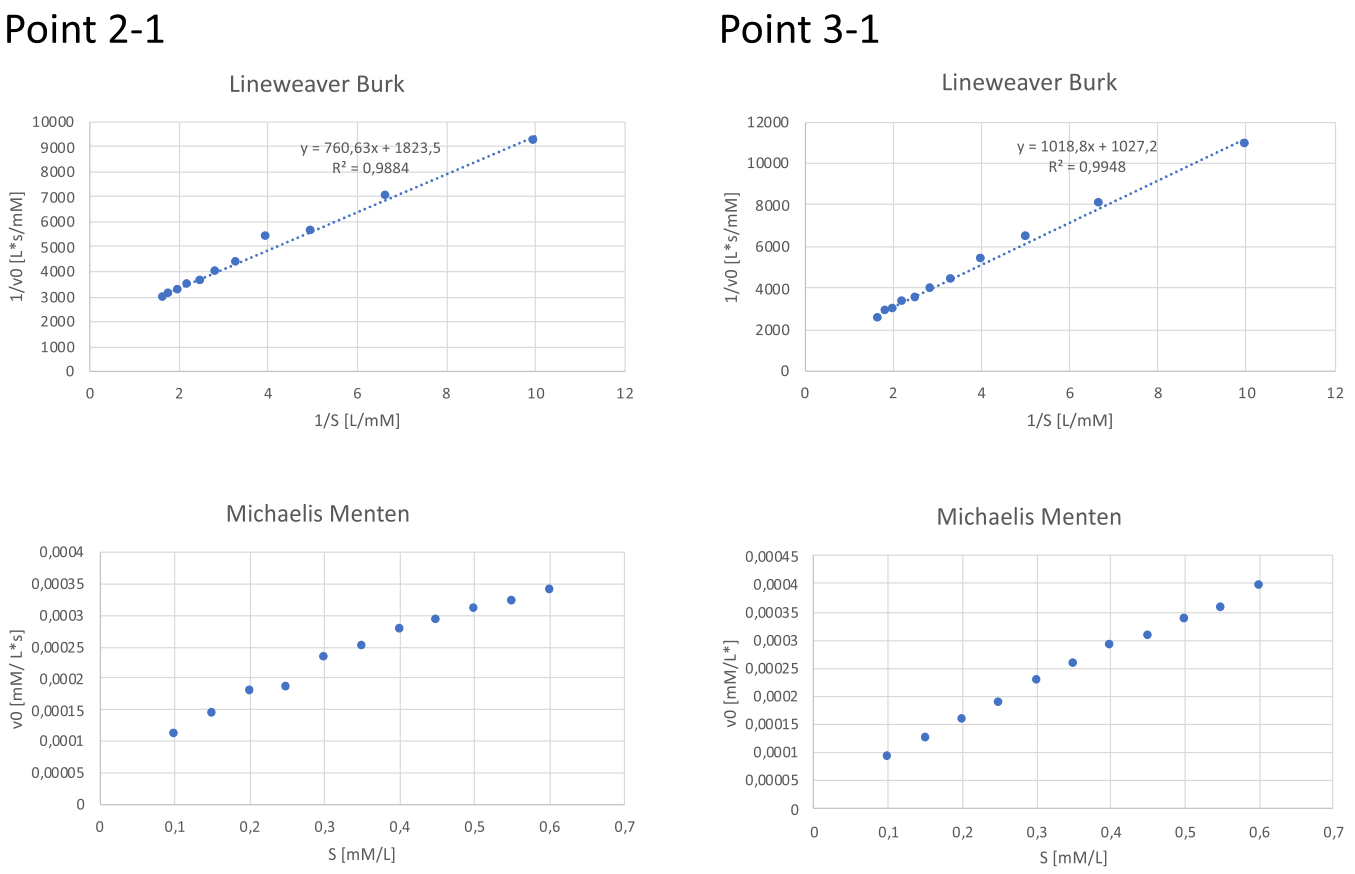

Figure 3. Michaelis–Menten and Lineweaver Burk plots of the first two measuring points (left) and the first three measuring points (right).

Table 6. Calculated V_{max} and K_m Values for the Different Measuring Points

measuring points	V_{max} [mmol/L·s]	K_m [mmol/L]
first two measurement points	0005.4846×10^{-4}	0.417
first three measurement points	9.74×10^{-4}	0.992

2.2. Solutions for the Immobilization of β-D-Galactosidase on Rink Amide Resin. *2.2.1. Preparation of the PEM Buffer.* pH 4, pH 6.5 then, 27.2 g potassium dihydrogen phosphate (KH_2PO_4), 37 g ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2 × 2 H_2O), and 20.3 g $MgCl_2$ × 7 H_2O were dissolved in deionized water. The pH was adjusted.

2.2.2. Preparation of the Sodium Carbonate Solution. 0.94 mol/L Na_2CO_3 (99.6 g) was dissolved in 1 L of deionized water.

2.2.3. Preparation of the ONP Solution for the Calibration Lines. 5 mM ONP (0.007 g) was dissolved in 10 mL PEM buffer and 5 mL sodium carbonate solution.

2.2.4. Preparation of the ONPG Solution. 5 mM For the 5 mM ONPG solution, 0.009 g ONPG was dissolved in 6 mL PEM buffer.

2.2.5. Preparation of the 20% Piperidine Solution. Piperidine (1.4 mL) and DMF (5.6 mL) were mixed.

2.2.6. Preparation of the PDC Solution. 5 g/L a total of 125 mg PDC was dissolved in DMF, and 100 μL DIPEA was added and brought to 25 mL with DMF.

2.3. General Procedure for the Determination of Enzyme Kinetics in Solution. To use the highest possible substrate concentration for the experiments, the solubility of X-Gal in PBS–DMSO buffer at a mixing ratio of 9:1 (PBS/DMSO) was examined, resulting in a substrate concentration of 0.6 mM, which was used as the highest substrate concentration for all subsequent experiments.

For the experiments, 100 μL of X-Gal solution starting with a concentration of 0.6 mM was pipetted into the well of the microtiter plate using a multichannel pipette according to the pipetting scheme below. The experiment was started by adding 100 μL of a 1:50 or 1:500 enzyme dilution of the purchased stock solution to the well of the microtiter plate, and the plate was immediately measured in the plate reader with the parameters shown in Tables 1 and 2. To decide on the appropriate enzyme dilution, a preliminary test was carried out in which different enzyme dilutions were measured with the same substrate concentration in the plate reader. The parameters of the measurement method used on the microplate reader were absorbance as the measurement mode, a 595 nm wavelength, three flashes, 40 kinetic cycles with an interval of 60 s, a temperature range between 40 and 42 °C and a shake duration of 10 s.

2.4. General Procedure for Enzyme Immobilization. The freshly prepared PDC solution was immediately transferred to the amino-functionalized plate, and the plate was covered with Parafilm and left for 3 h at room temperature.

Then, the wells were emptied with a microtiter plate washer and washed twice with 300 μL of isopropanol, deionized water, and carbonate/bicarbonate buffer. After the last washing step, the wells were washed again with deionized water. Then, 100 μL of the enzyme dilution per well was pipetted with a multichannel pipette. The plate was covered with parafilm and left at room temperature for 24 h. After 24 h, the plate was emptied with a microtiter plate washer and washed once with 300 μL of

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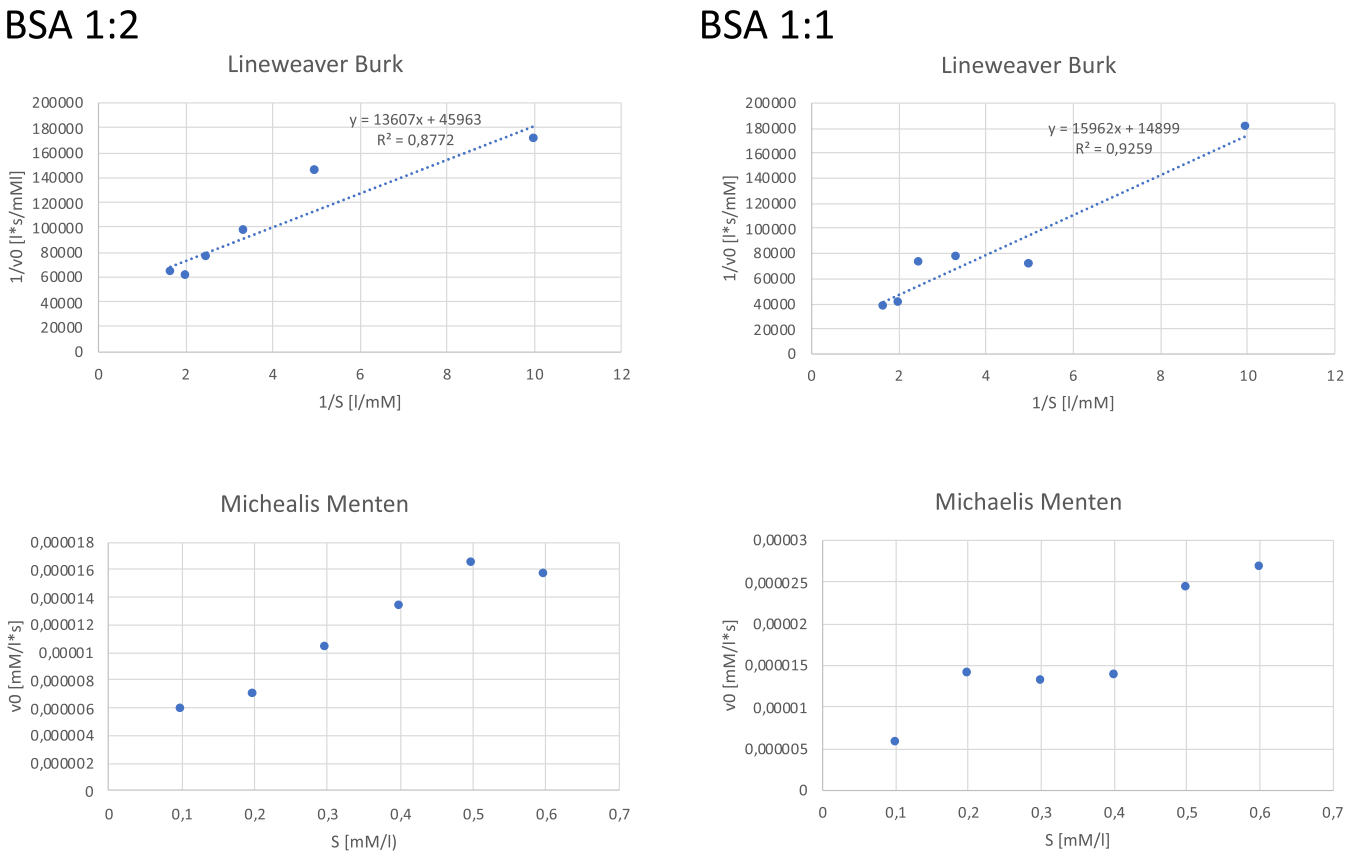

Figure 4. Michaelis−Menten and Lineweaver Burk plots of BSA dilutions of 1:2 and 1:1.

Table 7. Calculated V_{max} and K_m Values for the BSA Dilutions

BSA dilution	V_{max} [mmol/L·s]	K_m [mmol/L]
1:2	2.176×10^{-5}	0.296
1:1	6.712×10^{-5}	1.071

isopropanol and four times with 300 μL of PBST buffer per well. Next, 300 μL of BSA dilution was pipetted into each well according to the pipetting scheme in Tables 3 and 4 and left in the incubator at 37 °C for 30 min. In the first experiments, BSA dilutions of 1:10, 1:5, 1:2, and 1:1 were examined, and for the latter experiments, the BSA dilution of 1:10 was replaced with no BSA.

After 30 min, the wells were emptied and washed three times with 300 μL of PBST buffer for each well. One hundred microliters of the X-Gal concentrations shown in the pipetting scheme above were pipetted into the well, and the plate was immediately measured in the plate reader with the same parameters as before, except for the long-term measurements of the amino-functionalized microtiter plates, where the kinetic interval was set to 360 s. After the measurement, the data were saved, and the wells were washed with a new cleaning method due to problems removing the blue dye. For cleaning, 300 μL of a PBST−DMSO (50%) buffer mixture was pipetted into the well. The plate was then placed in the incubator at 37 °C for 48 h; afterward, the wells were emptied and rinsed with 300 μL of deionized water. The plate was then measured in the plate reader for five kinetic cycles. The principle of the covalent immobilization of the enzyme onto a carrier material is shown in Figure 1.

2.5. Determination of a Calibration Line for the Evaluation of the ONPG Concentration during the Immobilization Tests on Rink Amide Resin. PEM buffer and a 0.94 mM sodium carbonate solution were mixed in a 2:1 ratio, in which a 5 mM stock solution of ONP was prepared. The concentrations of 0.05, 0.1, 0.15, 0.175, 0.5, 0.75, 1, 2, 3, and 4 mM were prepared by diluting the 5 mM stock solution with the PEM buffer sodium carbonate mixture. The prepared dilutions, including the 5 mM stock solution, were measured photometrically twice at 420 nm. After 24 h, the samples were measured again.

2.6. Preparation and Implementation of the Immobilization of β-D-Galactosidase on Rink Amide Resin. First, 0.5 g of the rink amide resin was transferred to a peptide synthesis reactor and swelled in 5 mL of DMF at 4 °C for 24 h. After 24 h, the DMF was removed, and the resin was shaken twice with 5 mL of 20% piperidine solution for 5 min the first and 10 min the second time. After removal of the 20% piperidine solution, the resin was washed four times with dichloromethane (DCM). The deprotection was monitored by Kaiser test kit from Sigma-Aldrich.

The peptide synthesis reactor was filled with 2 mL of PDC solution and left at room temperature for 3 h. After 3 h, the reactor was washed twice with 5 mL each of isopropanol, deionized water, and carbonate/bicarbonate buffer. After the second washing step, a final wash was performed with deionized water. Then, a 1:50 enzyme dilution was drawn into the reactor and left at room temperature for 24 h.

At the end of 24 h, the content of the reactor was washed once with 5 mL isopropanol and four times with 5 mL PBST buffer. Then, 6 mL of a 2 mM ONPG solution was added, and the reactor was placed in a REAX 2 overhead mixer, which was placed in an incubator. The experiment ran for 120 min at 37 °C.

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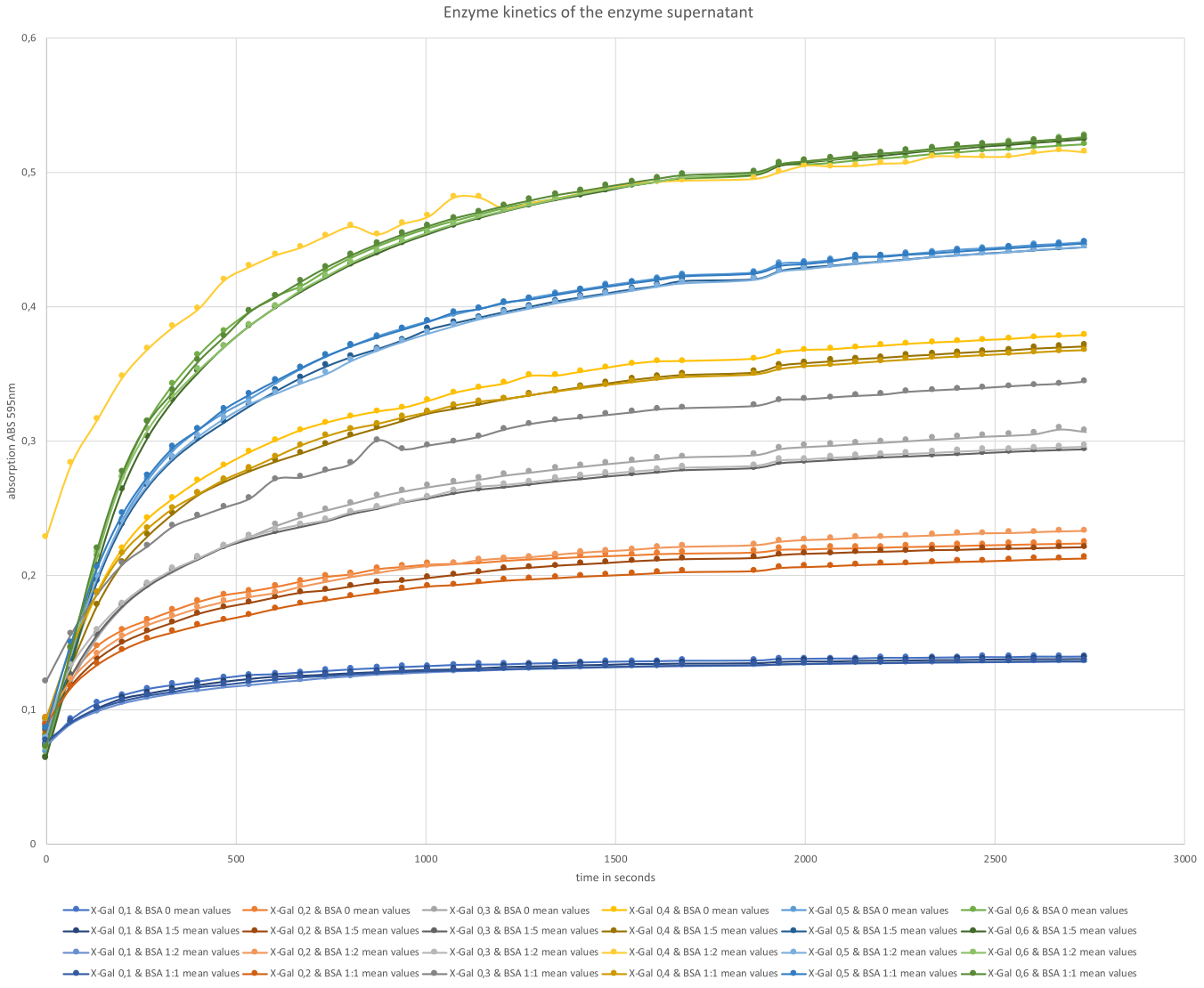

Figure 5. Enzyme kinetics of the enzyme supernatant separated by BSA dilutions of the amino-functionalized plate. The plate reader took a measurement break between 1500 and 2000 s.

Every 15 min, 0.5 mL of the solution was transferred to a cuvette, and 0.5 mL of sodium carbonate solution was added. The samples were then measured in a photometer at 420 nm. Afterward, the reactor was washed three times with 5 mL of a PBST-(20%) DMSO mixture. Another 6 mL of the 2 mM ONPG solution was added, and the experiment was repeated.

3. RESULTS AND DISCUSSION

3.1. Enzyme Kinetics of Lactase in Solution. For all the following experiments time zero is the start of the measurement in the plate reader and not the actual start of the experiment. A certain amount of time elapses before all the solutions have been pipetted into the well and the measurement can be started in the plate reader, which is kept as short as possible by working quickly. In these experiments, no enzyme was immobilized; instead, we worked in solution in corresponding microtiter plates to adjust the concentrations for perfect enzyme activity without producing a blue precipitate.

In all experiments on microtiter plates, it was possible to visually recognize that the test was successful. In addition, no blue precipitate could be observed. In all experiments, the highest substrate concentration had the highest absorption values, and the lowest substrate concentration had the lowest absorption values, as expected.

For the first enzyme kinetics experiment with an enzyme dilution of 1:50, we obtained poor results. The subsequent attempt has widely distributed absorbance values between 0.05 and 0.2. Positive K_m and V_{max} values, calculated using the Michaelis−Menten and Lineweaver Burk plot from Figure 2, were found to be very similar and extremely small for the first two points and the first three points, as shown in Table 5. Low K_m values indicate a high affinity between the enzyme and substrate.[27] Overall, this experiment demonstrates that the enzyme kinetics have been successfully reproduced.

In the enzyme kinetics experiment with an enzyme dilution of 1:500, V_{max} and K_m values calculated using the plots in Figure 3 differ from the experiments with an enzyme dilution of 1:50. However, they are still very low, as it can be seen in Table 6. This indicates that good enzyme kinetics can also be obtained with a higher enzyme dilution.

3.2. Enzyme Kinetics of on Amino-Functionalized Microtiter Plates Immobilized Lactase. First experiments did not result in feasible kinetics since the absorption values for the different substrate concentrations showed extreme fluctua-

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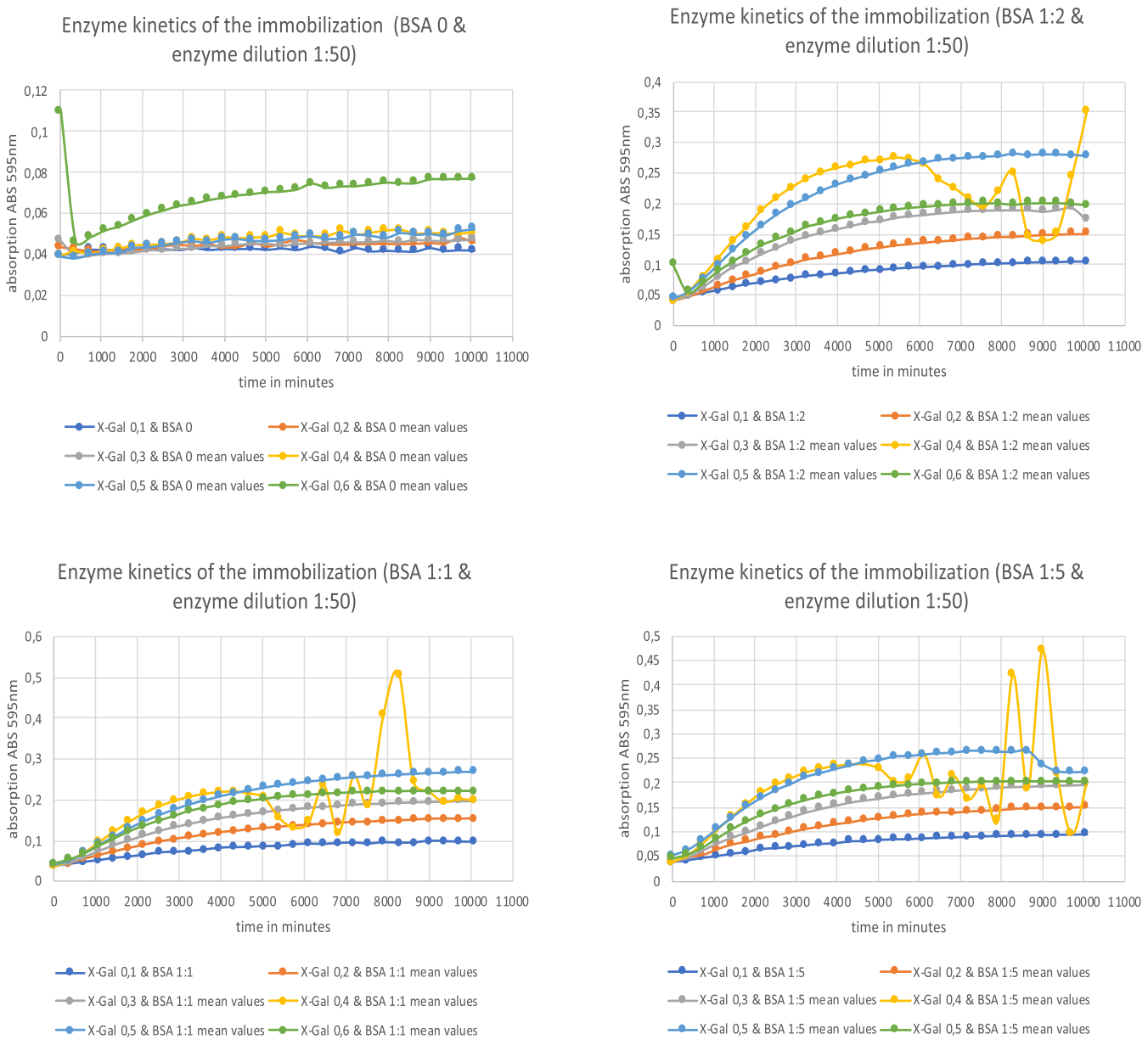

Figure 6. Enzyme kinetics of the immobilized plate for the 1:50 enzyme dilution. For a better comparison of the absorbances with the experimental procedure, the graphs were separated after the four BSA dilutions with all X-Gal concentrations. The *y*-axes of the individual graphs were chosen in such a way that the absorbances of the individual X-Gal concentrations are recognizable and the axes start at zero.

tions. These were most likely caused by the strong precipitation of the blue dye, which occurred most noticeably in the experiments using a BSA dilution of 1:10. It is known, that cleavage of X-Gal results in formation of a deep blue indigo derivative and therefore is of limited suitability for the measurement of enzyme kinetics. To optimize our experiments, we applied washing steps after the cleavage reaction with PBST buffer and DMSO in a 1:1 ratio. On the other side, we tried to avoid formation of the precipitate by varying the surface properties by examination of the influence of BSA on the functionalized surface. Therefore, we used various dilutions of the BSA stock solution.

The following graphs show the results of the optimized experiment, which showed nice kinetics and had fewer fluctuations in the absorption because less blue precipitate was formed. This also indicates that the enzyme was immobilized in all wells that included BSA, even though much of the enzyme remained in the supernatant solution, which changed the order of the substrate concentrations. The substrate concentration of 0.4 mM is above the 0.5 mM concentration, and this in turn is above the 0.6 mM concentration. Subsequently, BSA dilutions of 1:2 and 1:1 were used to determine the V_{max} and K_m values since their process of the substrate concentrations came closest to what was expected.

However, the well without BSA (BSA 0) showed practically no process, and the absorbances measured remained in a narrow range. BSA was used to prevent X-Gal from binding nonspecifically to the plastic surface.[28] The influence of BSA concentration on the enzyme kinetics is clearly evident from the noticeable difference observed in the two V_{max} and K_m values obtained through the plots in Figure 4. The higher BSA concentration has somewhat higher V_{max} and K_m values, as shown in Table 7, confirming the influence of the BSA solution on the enzyme kinetics. The higher V_{max} value also indicates that a greater

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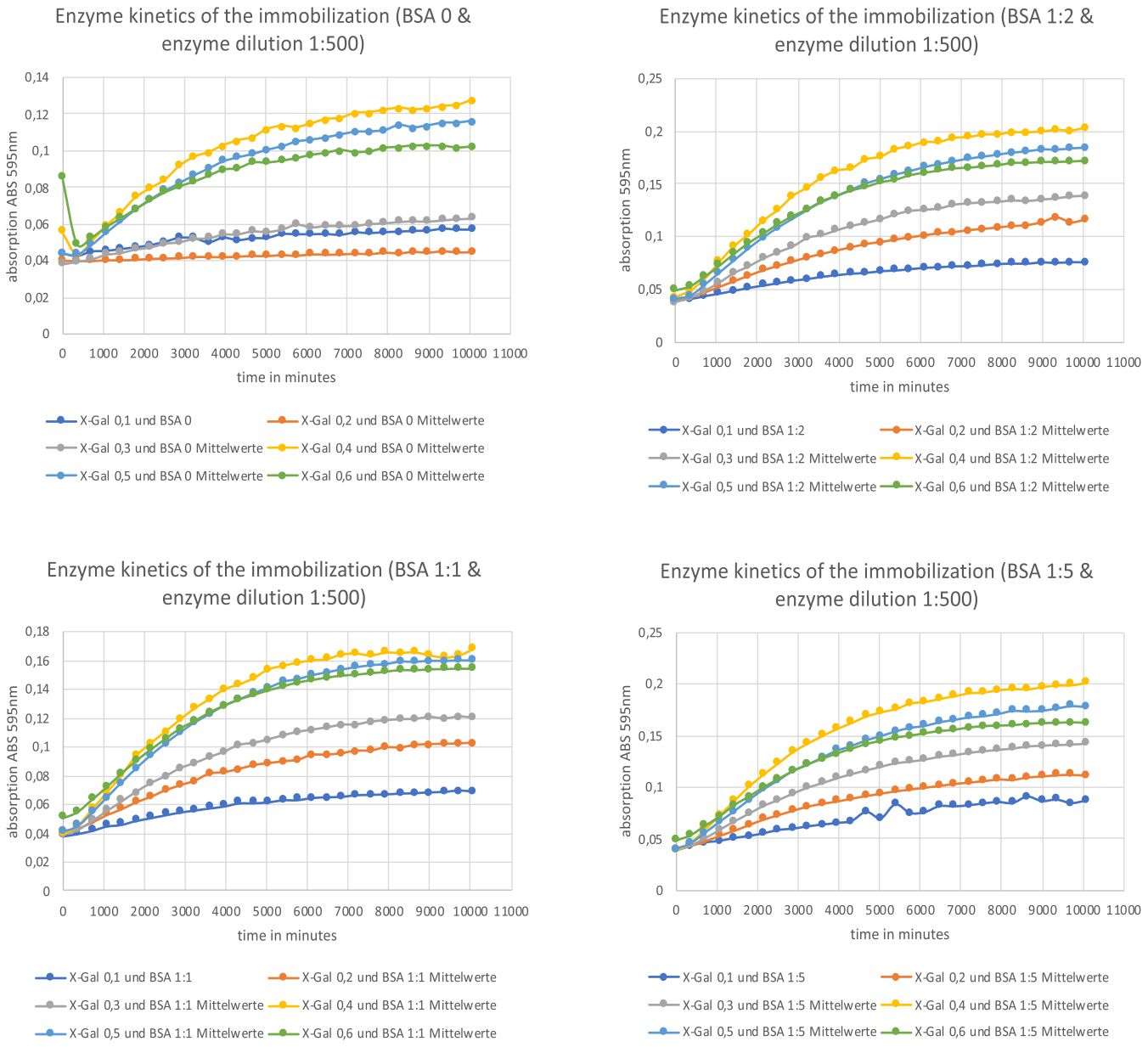

Figure 7. Enzyme kinetics of the immobilized plate for the 1:500 enzyme dilution. For a better comparison of the absorbances with the experimental procedure, the graphs were separated after the four BSA dilutions with all X-Gal concentrations. The *y*-axis of the individual graphs were chosen in such a way that the absorbances of the individual X-Gal concentrations are recognizable and the axes start at zero.

Table 8. Calculated V_{max} and K_m Values for the Respective BSA Dilutions of the 1:500 Enzyme Dilution

BSA dilution	V_{max} [mmol/L s]	K_m [mmol/L]
0	$3.190 \times 10-6$	0.687
1:5	-5.789×10^{-5}	−2.849
1:2	1.262×10^{-4}	4.869
1:1	1.364×10^{-4}	6.049

amount of enzyme is accessible at higher BSA concentrations, and therefore, more enzyme−substrate complexes are formed.

From the examination of the enzyme supernatant from the immobilization experiment, complete enzyme kinetics could be established on a nonamino-functionalized plate with the remaining enzyme in solution, proving the presence of excess enzymes. The enzyme kinetics plot, illustrating the observed enzymatic activity, is shown in Figure 5.

3.3. Long-Term Measurement. In the long-term measurement, the plate was functionalized with 1:50 and 1:500 enzyme dilutions. The 1:50 enzyme dilution, as shown in Figure 6, caused large fluctuations in absorbance values over the course of the measurement of the cleavage of X-Gal, while the 1:500 enzyme dilution, depicted in Figure 7, resulted in smaller fluctuations. The well in which these fluctuations were observed, a blue precipitate formed after the measurement. Conversely, the well without BSA showed neither a visually recognizable color gradient nor clear enzyme kinetics. Shah et al.[29] discovered that in the presence of BSA, 100% of the enzyme activity of the starting enzyme preparation was retained, while without BSA, only 0.4% of the activity was retained. Thus, in our immobilization experiment, BSA should also retain a large part of the enzyme activity. This observation would also explain the absence or very low absorbance values measured in the wells without BSA.

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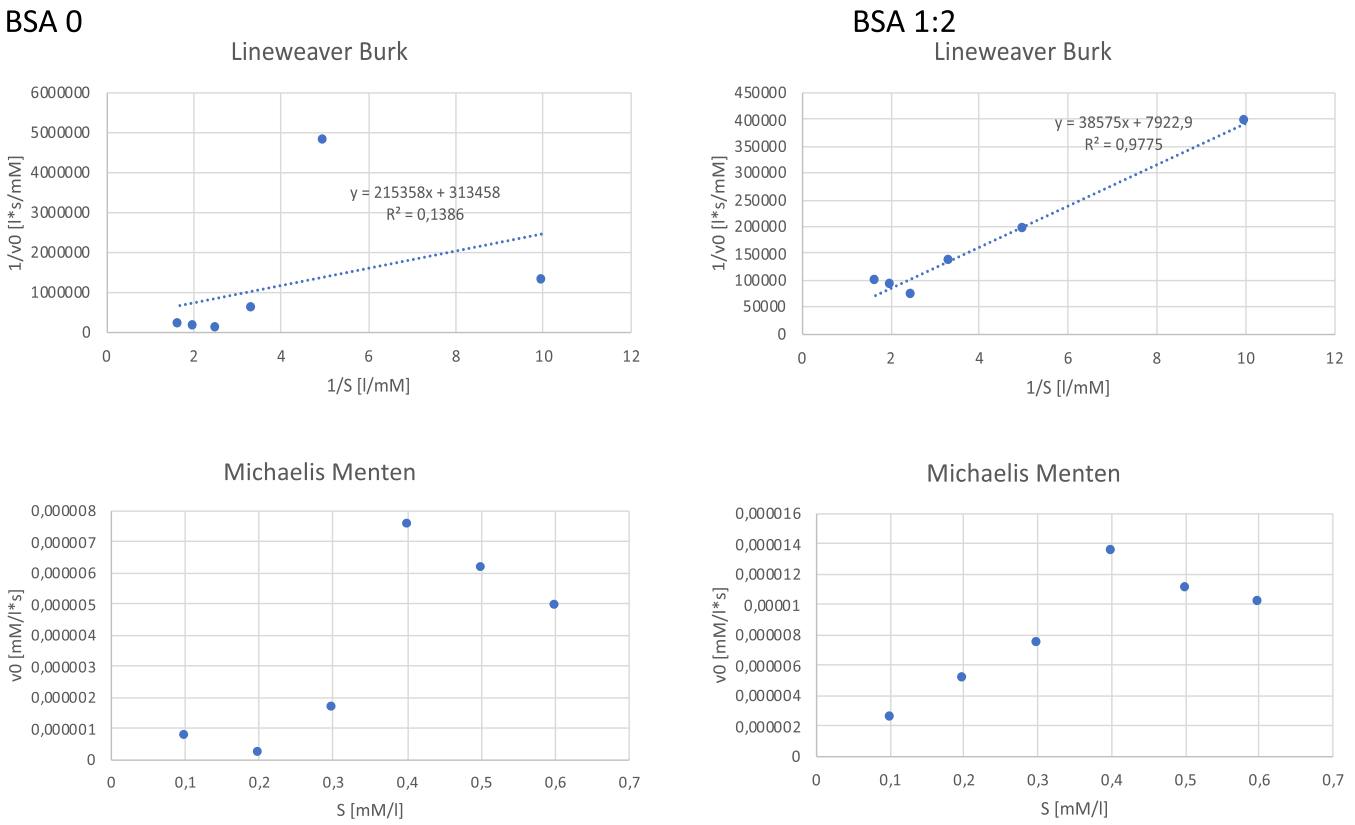

Figure 8. Michaelis–Menten and Lineweaver Burk plots of BSA dilutions 0 and 1:2 for the 1:500 enzyme dilution.

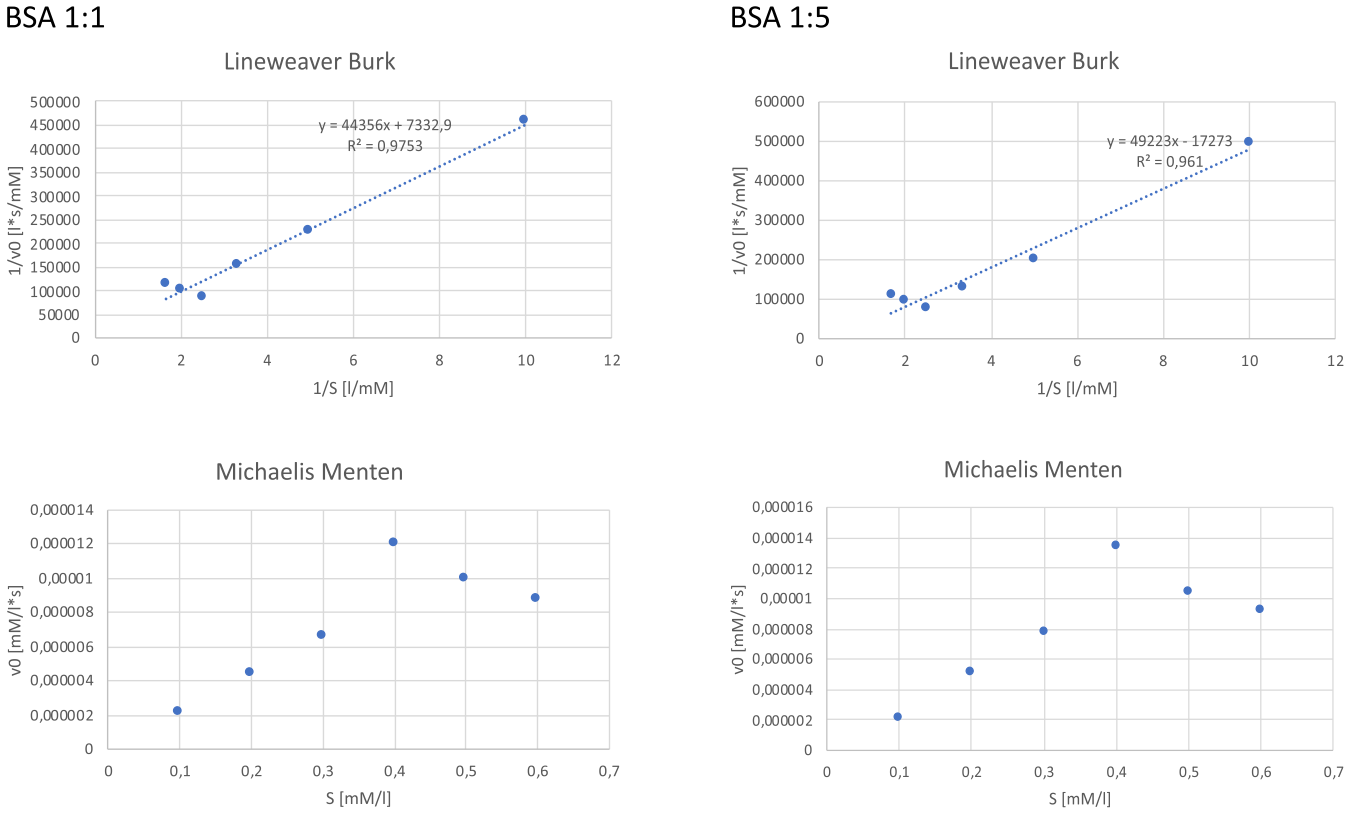

Figure 9. Michaelis–Menten and Lineweaver Burk plots of BSA dilutions of 1:1 and 1:5 for the 1:500 enzyme dilution.

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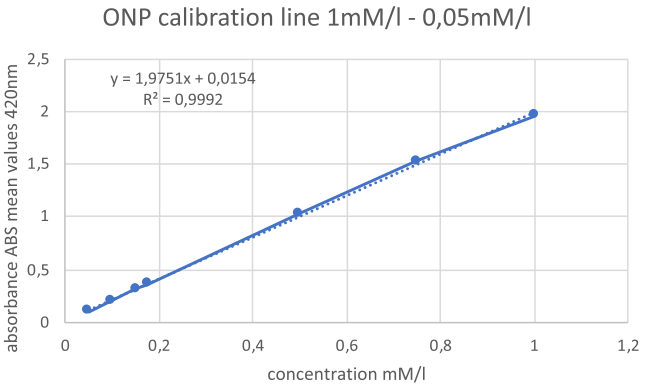

Figure 10. ONP calibration line.

Comparing the enzyme kinetics of the covalently bound enzyme with the kinetics of the enzyme in solution, it is noticeable that the kinetics of the covalently bound enzyme show a lag phase at the beginning of the measurement. It is assumed that the reason for this lag phase is the formation of a laminar boundary layer through which the substrate can only reach the enzyme by diffusion.[30]

Using a more dilute enzyme solution results in a noticeable reduction in precipitation and lower absorbance values, making the calculated dynamic data more reliable. For these reasons, V_{max} and K_m values were calculated from the 1:500 enzyme dilution only. The negative K_m value, shown in Table 8, at the BSA 1:5 dilution may be attribute to an experimental error inherent in double reciprocal plots. This error is most often caused by the lowest substrate concentration, which has a higher probability of being defective. In addition, the lowest substrate concentration had a very high influence on the slope of the plot. The Michaelis−Menten plot in Figures 8 and 9 illustrates that the reaction rate increases up to a substrate concentration of 0.4 mM and then decreases again. This behavior indicates uncompetitive inhibition by substrate, with the substrate acting as an inhibitor. The binding site for the inhibitor is created by the interaction between the substrate and the enzyme, causing it to bind to the enzyme−substrate complex, thereby forming an

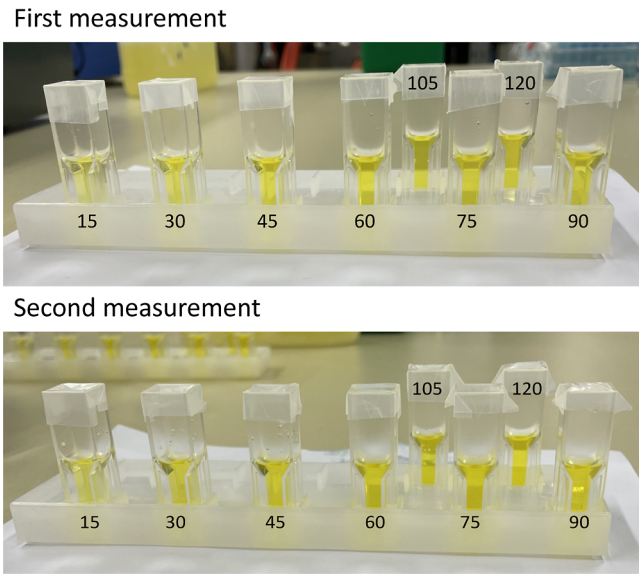

Figure 12. Results of immobilization on the rink amide resin. The upper half of the figure shows the cuvettes of the first measurement, and the lower half of the figure shows the cuvettes of the second measurement with the content of the peptide synthesis reactor mixed with the sodium carbonate solution after the measurements in the photometer. An increase in the yellow coloration with increasing reaction time can be clearly seen up to minute 75. After the 75th minute, the intensity of the yellow color decreases. In addition, there was a difference in color between the first and second experiments.

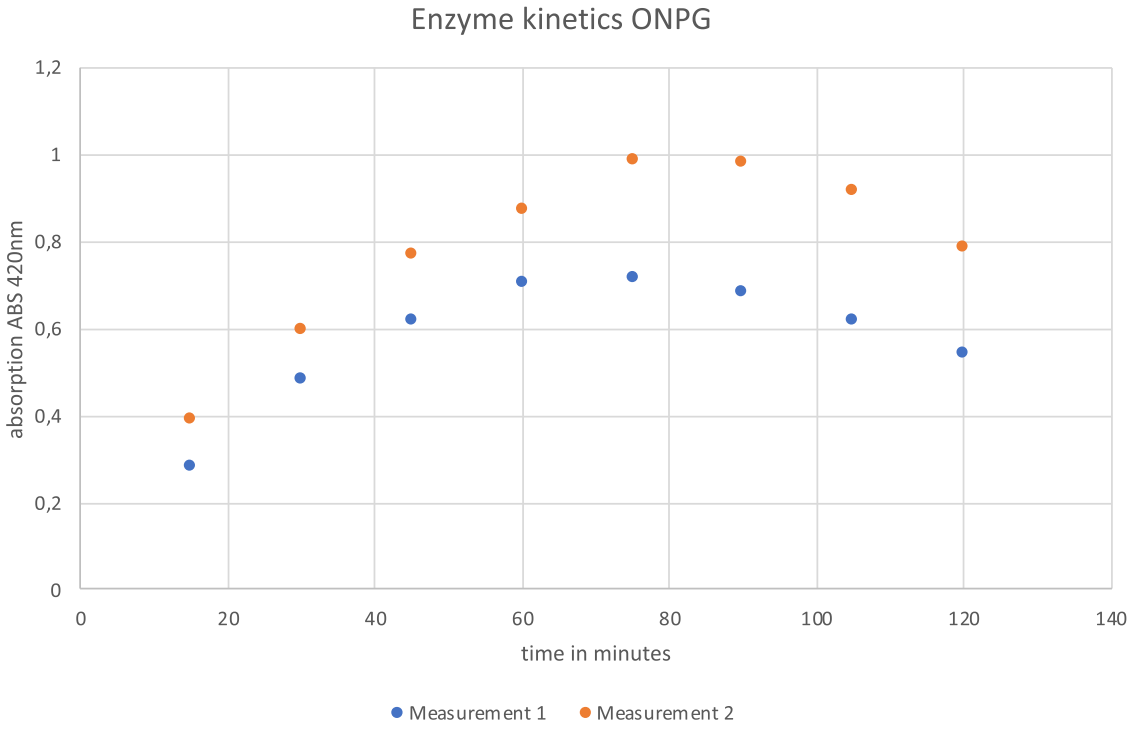

Figure 11. Absorption values of the first and second series of measurements of the first immobilization test in a peptide synthesis reactor.

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additional complex. This causes the V_{max} value to drop, the substrate not to be converted and the enzyme−substrate balance to be shifted. On the Lineweaver Burk plot of this inhibition, the points initially increase, so that the double-reciprocal reaction rate values rise steeply as the double-reciprocal concentrations approach zero.[27,28] Despite the inhibition, the highest BSA solution again has the highest K_m and V_{max} values. A higher K_m value indicates that the affinity between the enzyme and substrate is low.[27] Comparing the experiments with enzyme dilutions of 1:50 and 1:500, it is noticeable that the V_{max} values of the 1:2 and 1:1 BSA dilutions of the lower enzyme concentration are higher. One explanation for this could be that more enzyme was immobilized in the experiment with a lower enzyme dilution (see Figure 10).

3.4. Calibration Line of ONP. When measuring the absorption, the photometer oscillated because the absorption values at concentrations above 0.175 mM were too high.

The first calibration line shows a flattening of the curve after the concentration of 2 mM, which causes a coefficient of determination of $R^2 = 0.7667$.

In a second approach, the calibration line was generated between 0.05 and 1 mM, resulting in a coefficient of determination of $R^2 = 0.992$. Since the absorbance values remained the same at concentrations above 2 mM, the maximum ONPG concentration for all subsequent experiments was set at 2 mM.

3.5. Immobilization of β-D-Galactosidase on Rink Amide Resin. From the results of the absorption values, it is noticeable that the absorption decreases after 75 min, as can be seen in Figure 11. A reason for this could be that ONP is unstable, similar to 2,4,6-trinitrophenol, and the nitro group is removed in the alkaline environment. For example, picric acid (2,4,6-trinitrophenol) is very sensitive to heat, friction, and impact.[31] Furthermore, in Figure 12, this decrease in absorption can be visually observed by the loss of yellow color intensity in the cuvettes containing the probes.

In addition, the reaction of the enzyme in solution was significantly faster than in the resin experiments. Most likely, this is because only a fraction of the enzymes were immobilized compared to the experiments in the microtiter plates with immobilized enzyme. From the loading density of the rink amide resin, we can deduce that in our experiment max. 0.15−0.4 mmol can be immobilized on 0.5 g resin. The amount of linker we used was approximately 0.057 mmol. Even assuming quantitative reactions during the immobilization process means that not the full loading capacity has been used. Therefore, a part of the enzyme activity may have remained in the enzyme solution. However, it must be noted that this is an assumption that cannot be verified. Nonetheless, these first results are promising and demand further experiments to evaluate the immobilization on the resin further.

4. CONCLUSION

The methods described have proven to be reliable for the successful covalent immobilization of β-D-galactosidase on amino-functionalized microtiter plates and on rink amide resin. In addition, the successful transfer of the immobilization method from the microtiter plate to the rink amide resin shows that the described method can also be used on different surfaces. Since covalent immobilization of enzymes can potentially decrease or even prohibit enzyme activity due to negative effects on the tertiary structure, these results show that PDC immobilization retains enzymatic activity and therefore is a sufficient method. With the help of this immobilization method, a more sustainable process, for example, for the continuous production of lactose-free milk, can be created and expanded to a larger scale. Nonetheless further experiments and optimizations for the immobilization on resins need to be performed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03279.

Measured values of the analysis of the free enzyme and the substrate X-Gal, measured values of the nonamino-functionalized microtiter plates, the calculated values of the enzyme kinetics of the nonamino-functionalized microtiter plates, measured values of the amino-functionalized microtiter plates, the calculated values of the enzyme kinetics of the amino-functionalized microtiter plates, and measurements of β-D-galactosidase immobilization experiments on the Rink amide resin inside a peptide synthesis reactor (PDF)

Raw data from the plate reader (XLSX)

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Conzeptualization: D. D., H. S., M.S.S. Methodology: D. D., U. E., H. S. Formal analysis: D. D., U. E. Investigation: D. D. Resources: H. S., M.S.S. Writing— Original Draft: D.D., M.S.S. Writing—Review & Editing: H. S., U. E., M.S.S. Visualization: D. D. Supervision: M.S.S. **Credit**: Photograph courtesy of Defne I. Dayi. Copyright 2022 Defne I. Dayi. Images are free domain.

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Notes

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■ ABBREVIATIONS

PDC, 1,4-phenylendiisothiocyanate;; X-Gal, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PDC, *p*-phenylene diisothiocyanate; β-Gal, β-D-galactosidases; THP, tris(hydroxymethyl)phosphine; PBS buffer, phosphate buffer saline; $NaH_2PO_4 \times 2H_2O$, sodium dihydrogen phosphate dihydrate; $Na_2HPO_4 \times 12H_2O$, disodium hydrogen phosphate dodecahydrate; NaCL, sodium chloride; $MgCl_2 \times 6H_2O$, magnesium chloride hexahydrate; $CaCl_2$, calcium chloride; $MnCl_2 \times 4H_2O$, manganese(II) chloride tetrahydrate; HCl, hydrogen chloride; NaOH, sodium hydroxide; Na_2CO_3, sodium carbonate; $NaHCO_3$, sodium bicarbonate; DIPEA, *N*,*N*-diisopropylethylamine; KH_2PO_4, potassium dihydrogen phosphate; EDTA-$Na_2 \times 2H_2O$, ethylenediaminetetraacetic acid disodium salt dihydrate; DCM, dichloromethane; KCN, potassium cyanide

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