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# Immobilization of Lactase onto Various Polymer Nanofibers for Enzyme Stabilization and Recycling

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Five different polymer nanofibers, namely, polyaniline nanofiber (PANI), magnetically separable polyaniline nanofiber (PAMP), magnetically separable DEAE cellulose fiber (DEAE), magnetically separable CM cellulose fiber (CM), and polystyrene nanofiber (PSNF), have been used for the immobilization of lactase (E.C. 3.2.1.23). Except for CM and PSNF, three polymers showed great properties. The catalytic activities ($k_{cat}$) of the free, PANI, PAMP, and magnetic DEAE-cellulose were determined to be 4.0, 2.05, 0.59, and 0.042 mM/min·mg protein, respectively. The lactase immobilized on DEAE, PANI, and PAMP showed improved stability and recyclability. PANI- and PAMP-lactase showed only a 0–3% decrease in activity after 3 months of vigorous shaking conditions (200 rpm) and at room temperature (25°C). PANI-, PAMP-, and DEAE-lactase showed a high percentage of conversion (100%, 47%, and 12%) after a 1 h lactose hydrolysis reaction. The residual activities of PANI-, PAMP-, and DEAE-lactase after 10 times of recycling were 98%, 96%, and 97%, respectively.

**Keywords:** Lactase, nanomaterials, enzyme recycle, enzyme immobilization

## Introduction

Lactase (E.C. 3.2.1.23) from *Agaricus bisporus* BioChemika powder catalyzes the hydrolysis of lactose to glucose and galactose, which are more digestible and soluble than lactose. Lactase is an important enzyme in industrial and medical applications [6, 7, 11, 23]. Mammalian milk contains approximately 3–8% (w/v) lactose. Moreover, lactose comprises 70–80% of the solid components in cheese whey. A large part of the world population, particularly in Asia, has lactose intolerance, and lactose-intolerant people are discouraged from consuming lactose-containing milk.

Lactase can be used as a blood sensor because an excessive amount of lactose in the blood can indicate a gastrointestinal malignancy. The acid hydrolysis of lactose is not favored owing to color formation and fouling of the ion exchange resins used in processing. An alternative method is the use of enzymatic processing. Many studies have reported enzymatic lactose hydrolysis by lactase, which is commercially available and used in large-scale processes [1, 2, 21, 22].

Lactases have been immobilized on a variety of support materials, such as Sephadex, alginate, k-carrageenan, chitosan, porous glass, agarose, polyvin[4]yl alchohol polymer, DEAE-cellulose, Eupergit C (epoxy activated acrylic beads), nylon, polyurethane foams, and zeolite [8, 12, 18, 19, 20, 29, 30, 32, 33]. The immobilized enzymes have been used in both batch and continuous reactors [1, 2, 4, 14, 16, 17]. Extensive studies have examined the applications of lactase in dairy technology. Enzymes have a high sensitivity to a number of denaturing agents, and are unstable at room temperature and too expensive for large-scale processes. However, these limitations can be overcome using immobilized enzymes. The immobilized enzyme is more stable than the free enzyme at room temperature, can be recycled, and has activity in several denaturing agents [28].

This study examined the stability of lactase immobilized on the following five different polymer nanofibers: polyaniline nanofiber (PANI), polyaniline magnetically separable nanofiber (PAMP), magnetically separable DEAE cellulose fiber (DEAE), magnetically separable CM cellulose fiber (CM), and polystyrene nanofiber (PS). The activities of the free and immobilized lactases were compared under various conditions, including pH and temperature.

## Materials and Methods

### Materials

Lactase (E.C.3.2.1.23) from *Agaricus bisporus* BioChemika powder was purchased from Fluka AG (Switzerland). *O*-Nitrophenyl-D-galactopyranoside (ONGP), bovine serum albumin standard (BSA), polyaniline, DEAE-cellulose, CM-cellulose, and lactose were obtained from Sigma-Aldrich (St. Louis, USA) and used without further purification. All chemicals used were of analytical grade, and all solvents were of the highest quality commercially available.

### Preparation of Polyaniline and Polyaniline Magnetically Separable Nanofibers

Polyaniline nanofibers were synthesized from rapidly mixed reactions. First, 0.1% (weight fraction) of an initiator (ammonium peroxy disulfate) and 5 ml of an aniline monomer solution (with different weight fractions) in 1 M HCl were mixed rapidly. Addition of 10% (w/v) of iron oxide (nanoparticle) to the polymerization mix rendered the polyaniline magnetically separable nanofiber. Once the initiator molecules had been depleted during nanofiber formation, there was no further polymerization that could lead to overgrowth. The samples were washed four times with distilled water to remove the remaining HCl and were stored in a refrigerator for further use.

### Preparation of Magnetically Separable DEAE and CM Cellulose Fibers

Magnetically separable DEAE and CM cellulose fibers were synthesized from rapidly mixed reactions. For a typical preparation, nanoferrite (100 mg) was introduced to 10 ml of swelled CMC and DEAE. This mixture was agitated vigorously for 2 h at 500 rpm. The mixture was washed with distilled water to remove ferrite nanoparticles that were located outside of the bead. The supernatant was decanted and the sediment was washed with distilled water several times until further free ferrite was observed. Finally, the sediments were washed twice with 100 mM phosphate buffer and dried for future use.

### Preparation of Polystylene Nanofiber

Nanofibers were prepared *via* electrospinning, as previously described [19]. PS + PSMA polymer solutions (1:2) were prepared at room temperature by dissolving a mixture in THF, followed by magnetic stirring for 2 h. The polymer solution was loaded into a 3 ml capillary of glass syringe. A bias of 8–10 kV was applied to the needle using a high-voltage supply (ES30P-10W; Gamma High Voltage Research, Ormond Beach, FL, USA). The electrospun fibers were collected on clean aluminum foil (connected to the ground) placed at a suitable distance (8–10 cm) from the tip of the needle. To prepare the magnetic nanofibers, the magnetic nanoparticle in hexane (0.1 ml) was loaded into a 0.9 ml polymer solution. The magnetic nanofibers were produced by following a similar eletrospinning protocol.

### Enzymatic Activity Assays of Lactase

The activities of the free and immobilized lactases were determined at 37°C using chromogen ONPG as the substrate. The reaction mixture contained 20 mM phosphate buffer (pH 6.5), and 1.6 mM ONPG. After adding the free or immobilized lactase, a 50 µl sample was taken every minute and diluted 20-fold. The absorbance was detected at 410 nm with a spectrophotometer. One unit of enzyme activity (EU) was defined as the amount of enzyme hydrolyzing one mole of the substrate per minute under the above defined conditions.

### Immobilization of the Enzyme

A solution of 10 mg lactase in 1 ml of a 20 mM phosphate buffer (pH 6.5) was mixed with 2 mg of the polymer nanofiber and stirred gently for 30 min at room temperature. Then 1 ml of a glutaraldehyde solution (1%) as a crosslinker was added. The mixture was agitated for 2 h at room temperature, which was enough to establish a crosslink between the enzymes. The supernatant was taken to measure the protein concentration. Subsequently, 1.5 ml of 100 mM Tris-HCl (pH 7.9) was added for Tris-capping and shaken for 30 min. The immobilized lactase was washed more than 10 times with a 20 mM phosphate buffer (pH 6.5) to remove the free enzymes.

In this process, the immobilization yield (IY) and catalytic activity (CA) would be calculated. The calculation equation is given below.

$$\text{IY } (\%) = \frac{E_t - E_e}{E_t} \times 100\%$$

$E_t$: Amount of total free lactase used
$E_e$: Amount of lactase eluted in washing buffer

$$\text{CA } (\%) = \frac{E_i}{E_f} \times 100\%$$

$E_f$: Activity of free lactase
$E_i$: Activity of immobilized lactase

In this equation, $E_f$ was the amount of enzyme used for immobilization.

### Protein Assay

The protein concentration was determined by the Bradford

**Table 1.** Immobilization yield (IY) and catalytic activity (CA) of lactases immobilized on various polymer nanofibers.

| Polymer nanofiber | IY (%) | CA (% of free enzyme) | Activity retention after 96$^{th}$ day (%) |
|---|---|---|---|
| PANI | 73.5 ± 0.7 | 31.2 ± 0.56 | 95.1 |
| PAMP | 64.4 ± 0.5 | 14.5 ± 0.41 | 90.6 |
| DEAE-Cellulose | 34.3 ± 8.0 | 1.8 ± 0.007 | 19.8 |
| CM-Cellulose | 28.0 ± 2.9 | 0.032 ± 0.003 | 0.02 |
| PS/PSMA | 59.4 ± 6.4 | 0.016 ± 0.002 | 17.6 |

The activity of commercial free enzyme was 11.5 U/mg protein.

method using a Bio-Rad protein assay kit (Hercules, CA, USA) with BSA as the standard. The absorbance was measured at 595 nm using a spectrophotometer.

### Enzyme Characterization

The kinetics of the immobilized enzymes was determined using the standard experiments to measure the kinetic parameters using ONPG as the substrate. The rate of enzymatic hydrolysis of ONPG was expressed using the Michaelis-Menten equation. The kinetic constants ($V_{max}$ and $K_m$) were calculated using linear regression analysis based on the least-square method.

### Lactose Analysis

The properties of lactose were tested in a batch reactor that was incubated at 37°C under shaking conditions. The immobilized lactase was used in lactose analysis, where the lactose solution was changed at every 1 h reaction, and then the residual lactose concentration was calculated.

## Results and Discussion

### Lactase Immobilization

At present, many nanoparticles, nanofibers, and nanotubes are used as enzyme immobilization materials. Because of the great properties of the immobilized enzyme, they have been used in many fields such as drug delivery, bioconversion, biosensors, and so on [24–26, 29, 32]. In this study, we designed five immobilization materials for used on lactase immobilization. The immobilization efficiency was determined by measuring the activity of the immobilized enzyme. The immobilization yield was calculated from the difference in protein concentration before and after the glutaraldehyde treatment. The catalytic activity (CA) was measured as the ratio of immobilized enzyme to free enzyme activity. The results are shown in Table 1. Among the five immobilized lactases, lactase immobilized on PANI and PAMP showed great activity and stability. As shown in Figs. 1A–1F, PANI and PAMP have a large surface area and they can be easily recovered by centrifugation and magnets. The highest immobilization yield (IY, %) obtained with PAMP was 71.8%, and the catalytic activity was 14.5%. The stability of PANI- and PAMP-lactase was quite high, and 95.1% and 90.6% of activity remained after 96 days of exposure to shaking conditions at room temperature. Both PS-lactase and CMC-lactase had a low immobilization yield and low activities. The stability of CM-, and PS-lactase was as low as 0 and 17.6, respectively. Since the lactases were immobilized inside the CM pores, there was a problem with mass transfer of the substrate from the bulk solution to the internal pores. Because of their low activities, the subsequent research did not study PS- and CMC-lactase. Moreover, DEAE-lactase was better than PS and CMC-lactase, but also showed low activity. In Figs. 1G–1J, DEAE cellulose is seen as a microsize polymer with fiber form; lactase was immobilized on the surface of the fiber, so its surface area and mobility will be less than PANI and PAMP. This results in the low activities of the immobilized enzymes.

### Effect of Lactase Concentration on Enzyme Loading

The optimal lactase concentration for immobilization was determined by examining the effects of the lactase concentration in the range of 1–20 mg/ml (20 mM phosphate buffer, pH 6.5) on the immobilization yield and recovery activity. The catalytic activity of the immobilized enzyme was the highest when 5 mg/ml of free lactase was immobilized. The enzyme activity recovery and the catalytic activity per enzyme concentration increased with decreasing enzyme concentration. At high lactase concentrations, the lactase can aggregate, which can decrease the catalytic activity. Since the optimal lactase concentration showing the highest catalytic activity was found to be 5 mg/ml, it was used for all subsequent lactase immobilization experiments.

### Estimation of Kinetic Parameters of Immobilized Enzymes PANI and PAMP

As shown in Table 2 [13], we could predict that if we use the GA as a crosslinker, material with -$NH_2$ as a functional group could be a great material for lactase immobilization.

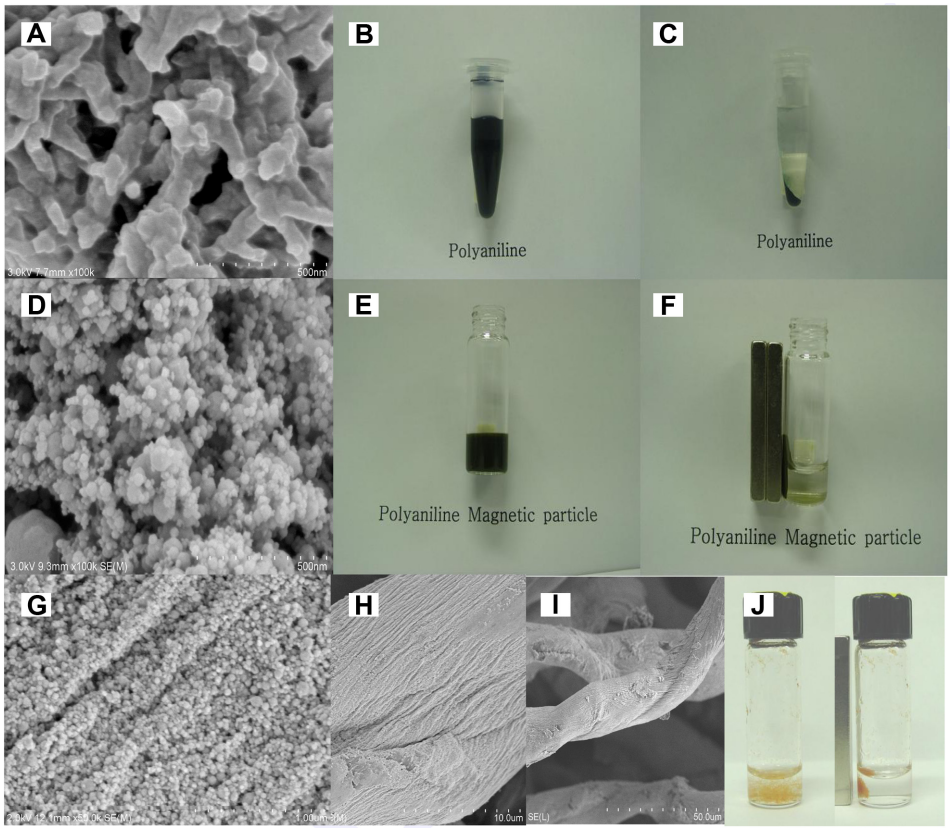



**Fig. 1.** SEM images of PANI (**A**), PAMP (**D**), and DEAE (**G**).
PANI was separated with centrifugation (**C**) and PAMP and DEAE were separated with magnets (**F**, **J**).

CM-cellulose, whose functional group is –COOH, showed a low immobilization yield. In this study, ONPG was used as a substrate to measure the initial enzyme reaction rate. The maximum reaction rate ($V_{max}$) and Michaelis-Menten constant ($K_m$) for the free and immobilized lactases were estimated. The catalytic activity ($k_{cat}$) represents the maximum enzyme reaction rate for the same amount of free and immobilized enzymes. Table 3 shows the estimated kinetic parameters of the free and immobilized lactases. The $K_m$ value of PAMP-lactase was 2.34 times lower than that of the free enzyme (see Table 3), but that of PANI-lactase was 4.39 higher. The decrease in $K_m$ in PAMP-lactase might be

**Table 2.** Immobilization materials and their functional groups, protein functional groups, and reactions.

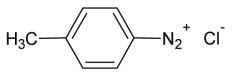
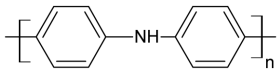
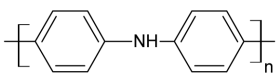
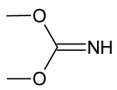
| Immobilization materials | Reactive group of materials | Reactive group of enzyme | Coupling reaction |
|---|---|---|---|
| PS |  | -$NH_2$/-SH | Glutaraldehyde (GA) as a crosslinker to make amine linkages |
| PANI |  | -$NH_2$ | |
| PAMP |  | -$NH_2$ | |
| DEAE-cellulose |  | -$NH_2$ | |
| CM-cellulose | -$COO^-$ | -$NH_2$ | |

**Table 3.** Catalytic activity of free lactase and immobilized lactases with various supporting nanofibers.

| Polymer nanofiber | Amount of lactase used (mg/ml) | $K_m$ (mM) | $V_{max}$ (mM/min) | $k_{cat}$ (mM/min $mg^{-1}$ protein) |
|---|---|---|---|---|
| Free | 0.5 | 2.7 | 2.01 | 4.02 |
| PANI | 1 | 4.39 | 2.05 | 2.05 |
| PAMP | 1 | 2.34 | 0.59 | 0.59 |
| DEAE | 10 | 6.53 | 0.42 | 0.042 |

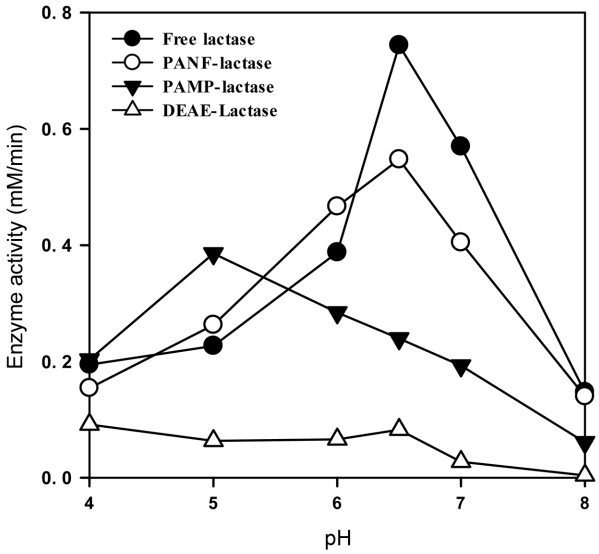



**Fig. 2.** Effect of pH on the activity of free lactase, PANI-lactase, PAMP-lactase, and DEAE-lactase.

due to structure changes in the enzyme after immobilization. The substrate can easily access the active sites after immobilization. The $k_{cat}$ values for free, PANI, PAMP, and magnetic DEAE-cellulose were 4.02, 2.05, 0.59, and 0.042 mM/min $mg^{-1}$ protein, respectively.

### Effect of pH on Enzyme Activity

The effect of pH after lactase immobilization was determined by measuring the activity from pH 4 to 8 and is presented in Fig. 2. Like the previous research results, the optimal pH for free lactase and PANI-lactase was pH 6.5 [3, 9, 15]. PAMP-lactase showed the maximum activity at pH 5, whereas PANI-lactase showed maximum activity at pH 6.5. Some books and paper announced that after immobilization, the enzyme maximum activation pH was changed [5]. The reason predicted was the oxidation of the polymer synthesis; the most possible reason was that the surface of the PAMP had ferric ions. The PANI-lactase showed high durability under a wide range of pH. The activity of immobilized lactase after 4 days of incubation

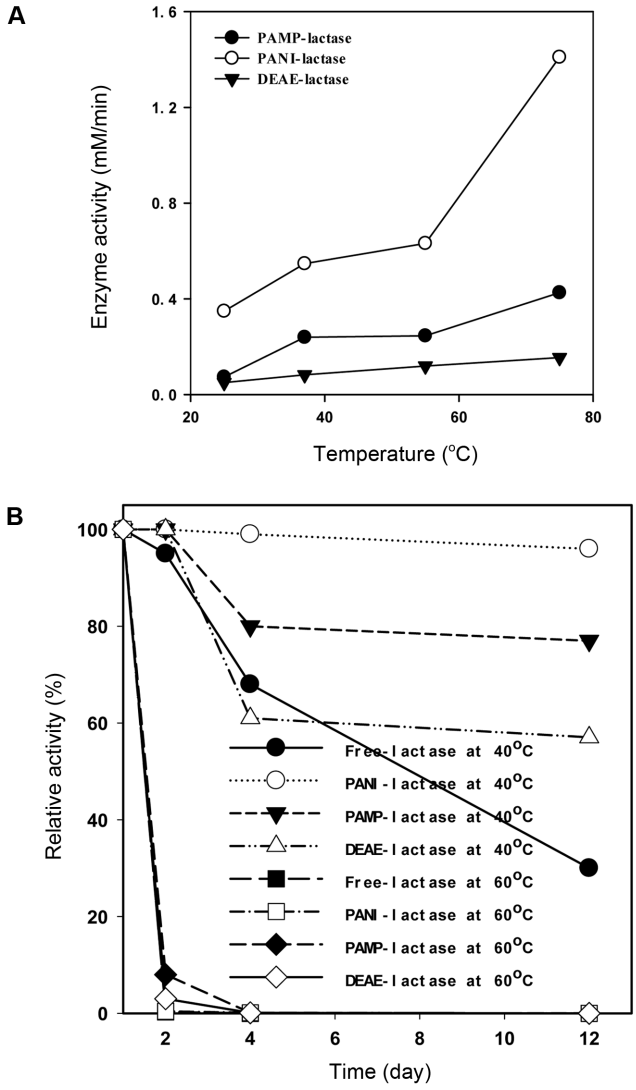



**Fig. 3.** Effects of temperature on the (**A**) enzyme activities and (**B**) stability of immobilized lactases.
The activity of immobilized LP rapidly increased with temperature increase, but the stability of immobilized lactase rapidly decreased with temperature increase.

under room temperature and shaking conditions was determined in order to check the effect of pH on stability.

PANI-lactase was quite stable and there was no loss of activity at pH 6.5.

**Effect of Temperature on Immobilized Enzyme Stability**

The effect of temperature on the initial activity of immobilized lactase was examined at temperatures between 25°C and 75°C. As shown in Fig. 3A, the activity of the immobilized lactase increased with temperature increasing. Fig. 3B shows the thermostability of the immobilized lipase. At 60°C, the complete loss of lactase activity was observed after 2 days. The thermostability increased with decreasing temperature. After 12 days incubation at 40°C, the retention activity of the lactase immobilized on PANI, PAMP, and DEAE was 96%, 77%, and 57%, respectively.

**Long-Term Operational Stability of Immobilized Lactases**

The free and the immobilized lactases were incubated at room temperature under rigorous stirring conditions at 200 rpm. PANI- and PAMP-lactase showed no decrease in activity after 3 months of incubation (Fig. 4). On the other hand, PSNF- and DEAE-lactase showed low stability with residual activities of 17.6% and 19.8%, respectively, after 2 months under the same conditions. Many studies have reported that immobilized lactase has excellent stability at 4°C [10]. Table 4 gives a summary of the experimental results published in other papers [4, 9, 10, 15, 23, 27, 31, 34]. The lactases immobilized onto PANI and PAMP showed above 90% residual activity under room temperature and vigorous shaking conditions.

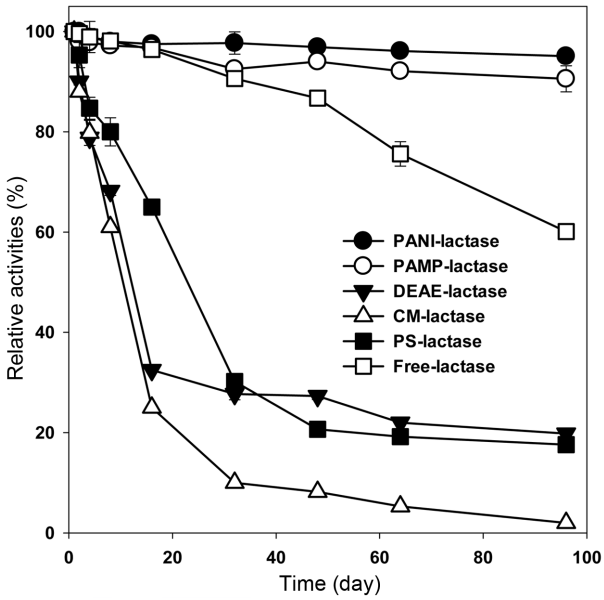



**Fig. 4.** Long-term operational stability of the free and immobilized lactases under room temperature (25°C) and rigorous shaking conditions (200 rpm).

**Lactose Hydrolysis with Immobilized Lactase**

The immobilized enzymes were used for the hydrolysis of lactose. A vial reactor with immobilized enzyme was incubated at 37°C under shaking conditions. For a 1 h reaction, the lactose conversion with PANI-, PAMP-, and DEAE-lactase was 100%, 47%, and 12%, respectively.

The immobilized lactase was recycled 10 times after lactose hydrolysis. After 30 min hydrolysis, the glucose concentration was measured using the glucose oxidase-peroxidase method (Fig. 5A). The concentration of glucose produced using PAMP-lactase after recycling 10 times was

**Table 4.** Stability of immobilized lactase using various supporting materials.

| Support materials | Retention activities (%) | Storage condition | Storage period | Reference |
|---|---|---|---|---|
| Crosslinked concanavalin A-β-galactosidase complex | 93 | 4°C | 2months | [28] |
| Magnetic poly (GMA-MMA) beads | 94 | 4°C, dry sample | 2 months | [13] |
| | 59 | 4°C, wet sample | 2 months | |
| CPC-silica and agarose | 100 | 4°C | 2 months | [27] |
| Langmuir-Blodgett films of poly(3-hexyl thiophene) stearic acid) | 80–82 | 4°C | 3 months | [4] |
| Graphite surface | 86 | 4°C | 1 month | [32] |
| Cellulose-gelatine carrier system | 80 | 4°C | 1 month | [26] |
| Fiber composed of alginate and gelatin | 56 | 4°C | 1 month | [31] |
| Functionalized silicon dioxide nanoparticles | 47 | 35°C | 10 h | [29] |
| Magnetic DEAE | 19.8 | 25°C | 3 months | This study |
| PANI | 95.07 | 25°C | 3 months | This study |
| PAMP | 90.56 | 25°C | 3 months | This study |

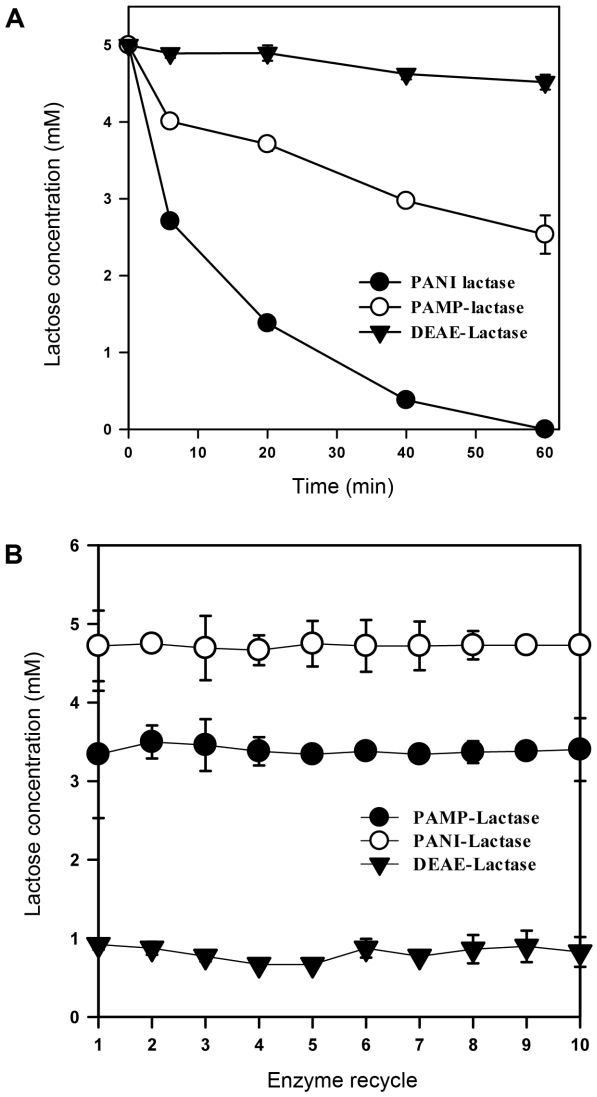



**Fig. 5.** Batch lactose hydrolysis using recycled immobilized lactase.

(**A**) Lactose degradation with the immobilized lactase, and lactose concentration (**B**) after immobilized lactase recycling. The lactose concentration was measured after 30 min of reaction. After 10 recycling steps, lactase residual activity was 98%, 96%, and 97%, respectively.

similar to that observed with the first-time use. PANI-, PAMP-, and DEAE-lactases showed 98%, 96%, and 97%, respectively, of their original activity after 10 recycles (Fig. 5B).

In conclusion, the lactases immobilized on PSNF, DEAE, PANI, and PAMP showed improved stability and recyclability. PANI- and PAMP-lactase were quite stable with less than 10% decrease in activity being observed after 3 months of rigorous shaking conditions. PANI-, PAMP-, and DEAE-lactase showed a high level of lactose conversion (100%, 47%, and 12%) after a 1 h batch reaction. The immobilized lactases were easily recovered and recycled after the reaction, and their residual activities after recycling 10 times were 98%, 96%, and 97%, respectively. These immobilized enzymes could be used in lactose analysis and biosensors to detect the lactose concentration. In future research in the lactase immobilization field, we have the expectation to make biosensors with PANI-lactase.

## Acknowledgments

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