Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 15(4), 1411-1422, 2025
Journal of the Institute of Science and Technology, 15(4), 1411-1422, 2025

ISSN: 2146-0574, eISSN: 2536-4618

Chemistry **DOI: 10.21597/jist.1683104**

Research Article

Received: 24.04.2025 **Accepted: 29.05.2025**

Atıf İçin: Varan, N.E., ve Tükel, S. (2025). D-Laktat Dehidrogenazın Kovalent İmmobilizasyonu Ve Karakterizasyonu. *Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, 15(4), 1411-1422.

To Cite: Varan, N.E., & Tükel, S. (2025). Immobilization and Characterization of D-Lactate Dehydrogenase onto 3-aminopropyl Silica Gel Support. *Journal of the Institute of Science and Technology*, 15(4), 1411-1422.

Immobilization and Characterization of D-Lactate Dehydrogenase onto 3-aminopropyl Silica Gel Support

Nazlı Ece VARAN[1*], Seyhan TÜKEL[1]

Highlights:

- D-lactic acid
- Covalent immobilization
- Reuse stability

Keywords:

- D-lactate dehydrogenase
- 3-aminopropyl silica gel
- Glutaraldehyde

ABSTRACT:

In this study, D-lactate dehydrogenase (D-LDH) from *Leuconostoc mesenteroides* was covalently immobilized onto 3-aminopropyl-functionalized silica gel using glutaraldehyde as a bifunctional crosslinker, with the aim of developing a catalytically active and thermally stable biocatalyst for D-lactic acid production. The immobilization protocol achieved an efficiency of 86% using 1 mg/mL of enzyme and 0.250 g of support material. Comparative biochemical characterization of both free and immobilized D-LDH was performed, assessing optimal pH and temperature, thermal stability and kinetic parameters. The immobilized enzyme preparation exhibited an optimal pH of 6.5 and a temperature optimum of 45 °C. These values corresponded to 7.0 and 37 °C for the free enzyme. Kinetic analysis revealed a Michaelis constant (K_m) of 0.37 mM and maximum velocity (V_{max}) of 86.9 U/mg protein for the free enzyme, whereas the immobilized enzyme displayed a significantly reduced K_m of 0.08 mM and a lower V_{max} of 19.2 U/mg protein, indicating increased substrate affinity but reduced catalytic turnover. Thermal stability assays demonstrated enhanced resistance of the immobilized D-LDH to elevated temperatures. Furthermore, reuse studies in a batch reactor showed that the immobilized enzyme preserved 38% of its original activity after 10 successive uses, underscoring its potential for repeated use in biotechnological applications.

[1]Nazlı Ece VARAN, (**Orcid ID:** 0000- 0003-1570-9509), Seyhan TÜKEL **(Orcid ID:** 0000- 0003-2503-2781) Çukurova Üniversitesi, Çukurova Üniversitesi, Fen-Edebiyat Fakültesi, Kimya Bölümü, Adana, Türkiye

***Corresponding Author:** Nazlı Ece VARAN, e-mail: evaran@cu.edu.tr

Bu çalışma Nazlı Ece VARAN'ın Yüksek Lisans tezinden üretilmiştir.
Makale 4-7 Kasım 2021 tarihlerinde Antalyada'da düzenlenen "3. Eurasia Biochemical Approaches & Technologies Congress" de poster olarak sunulmuştur.

INTRODUCTION

Enzymes are biocatalysts that facilitate complex chemical transformations under relatively mild physiological and environmental conditions, thanks to their functional properties such as activity, selectivity, and specificity (Alagöz et al., 2021; Tan et al., 2022). Although enzymes have widespread industrial applications, including food, chemical analysis, pharmaceuticals, cleaning, cosmetics, textiles, and paper (Chapman et al., 2018; Meghwanshi et al., 2020), their uses are often limited by various issues, such as their short lifespan and lack of reusability (Tülek et al., 2023; Cavalcante et al., 2021; Vidhya et al., 2024). The most common and effective way to overcome these limitations is the use of enzyme immobilization techniques (Umari et al., 2021; Lubek-Nguyen et al., 2022; Kim et al., 2024). The goal of enzyme immobilization is to make the immobilized enzyme more resistant to environmental conditions such as pH and temperature, easily separable from the reaction medium at the end of the process, reusable, improving the enzyme's kinetic properties, controlling the product formation, and making it applicable to multi-step reactions and continuous processes (Ashkan et al., 2021; Bié et al., 2022; Zhou et al., 2021).

Lactic acid fermentation plays a significant role in the food industry, as well as in medical and chemical applications (Zaboli et al., 2021). It is an optically active molecule and produced through microbial fermentation, chemical synthesis, and enzymatic reactions (Inoue et al., 2024; Cheng et al., 2022). Lactate dehydrogenase (LDH) is an enzyme that plays a crucial role in cellular metabolism. It catalyzes the reversible conversion between pyruvate and lactate, with the simultaneous interconversion of NADH and NAD^+. L-LDH reduces pyruvate to L-lactate, while D-LDH (EC 1.1.1.28) catalyzes D-lactate formation (Jin et al., 2023). D-LDH activity is observed in conditions such as acute appendicitis, bacterial infections, and kidney damage. Elevated D-LDH concentrations in urine or serum are used as biomarkers to diagnose this illness. The optically pure D-lactic acid was used in the bioplastics industry to produce polylactic acid, which is a thermoplastic and biodegradable polyester used in applications ranging from medicine and agriculture to packaging. Hence, D-LDHs were immobilized on various supports to determine the amount of D-lactate present, to enhance their thermal stability or to synthesize enantiopure D-lactic acid derivatives. For example, the D-lactate sensing electrode was fabricated by immobilizing thermostable dye-D-LDH and multi-walled carbon nanotubes (MWCNTs) within a Nafion membrane. A clear electrocatalytic response was observed upon exposure to D-lactate (Satomura et al., 2018). D-LDH exhibited enhanced thermal stability when immobilized on carbon nanotubes (CNTs) and functionalized carbon nanotubes (fCNTs). Compared to the free enzyme, the immobilized form showed significantly improved stability at elevated temperatures (Zaboli et al., 2019). D-LDH was encapsulated within the metal–organic framework ZIF-90 via a one-pot embedding approach, yielding a biocatalyst with high catalytic activity for the efficient synthesis of D-phenyllactic acid (Wang et al., 2022).

In recent years, 3-aminopropyl silica gel has become one of the preferred derivatized support materials for enzyme immobilization due to its presence of propylamino groups, which are easily modified by glutaraldehyde before covalent immobilization of enzymes (Chikurova et al., 2023; Milek et al., 2019). Enzymes immobilized on this support material show higher thermal stability and reusability compared to those immobilized on other supports (Sosa et al., 2020; Donga et al., 2022; Putz et al., 2021).

In this study, the covalent immobilization of *Leuconostoc mesenteroides* D-lactate dehydrogenase onto silica support containing 3-aminopropyl functional groups through a

glutaraldehyde spacer arm was investigated for the first time. The optimum pH, temperature, kinetic parameters, thermal stability, and reusability of the immobilized enzyme were determined.

MATERIALS AND METHODS

Materials

D-Lactate dehydrogenase enzyme (recombinant *Leuconostoc mesenteroides*; Mr: 36.5 kDa, specific activity: 1500 U/mg) was purchased from Megazyme (USA). Sodium pyruvate, nicotinamide adenine dinucleotide (reduced form), 3-aminopropyl functionalized silica gel, glutaraldehyde (50 %, w/w), ammonium sulfate, sodium hydroxide, Folin-Ciocalteu reagent, copper(II) sulfate, sodium carbonate, bovine serum albumin, glacial acetic acid, phosphoric acid (85%, v/v), and hydrochloric acid (37%, v/v) were purchased from Sigma (USA). All other chemicals were used without further purification.

Covalent Immobilization of D-LDH

The covalent immobilization of D-LDH was performed using 3-aminopropyl-functionalized mesoporous silica activated with glutaraldehyde, as described by Tülek et al. (2023). Initially, surface functionalization of mesoporous silica particles was carried out by silanization with 3-aminopropyltriethoxysilane (APTES) to introduce primary amine groups. One gram of mesoporous silica was treated with 25 mL of a 4% (v/v) APTES solution in acetone and incubated overnight at 45°C. Following silanization, the support was activated by incubation with 25 mL of 2.5% (v/v) glutaraldehyde solution, prepared in 0.1 M sodium phosphate buffer (pH 7.0), for 2 hours at room temperature under continuous stirring. The glutaraldehyde-activated support was then thoroughly washed with distilled water until the absence of free glutaraldehyde was confirmed in the filtrate, and subsequently dried at 60°C for 2 hours. For enzyme immobilization, 1 mL of D-LDH solution (1 mg/mL protein in 0.1 M sodium phosphate buffer, pH 7.0) was added to 0.25 g of the activated support. The immobilization reaction was conducted at 4°C for 2 hours under gentle stirring. After immobilization, the support was washed repeatedly with distilled water until no protein was detected in the filtrate, and the immobilized enzyme preparation was dried overnight at 4 °C. Protein determination in the filtrate was performed according to Lowry et al. (1951).

Activity of D-LDH

The activity of the free enzyme was determined at 37°C by measuring the decrease in NADH absorbance at 340 nm using a spectrophotometer (UV-1800, Shimadzu, UV spectrometer) in the presence of sodium pyruvate and NADH, after a 2.5 minutes reaction. In the activity determination method, 100 µL of a 0.33 mM pyruvate solution prepared in 2.8 mL of 0.1 M pH 7.0 sodium phosphate buffer, 100 µL of a 0.498 mM NADH solution, and 10 µL of the free enzyme solution with a protein concentration of 0.551 mg/mL were added to the reaction mixture. One unit of activity is defined as the amount of enzyme capable of catalyzing the oxidation of 1 µmol of NADH per minute under standard conditions (37°C, pH 7.0).

To determine the activity of the immobilized D-LDH, 5 mg of the immobilized enzyme preparation was incubated in a reaction mixture containing 2.8 mL of 0.1 M sodium phosphate buffer (pH 6.5), 100 µL of 0.33 mM pyruvate solution, and 100 µL of 0.831 mM NADH solution. The reaction was carried out at room temperature, and after 15 minutes, 1 mL of the reaction medium was withdrawn for analysis. Enzyme activity was quantified by tracking the reduction in NADH absorbance at 340 nm, corresponding to the enzymatic reduction of pyruvate. The activity of the immobilized enzyme was expressed in units per milligram of immobilized preparation (U/mg), while

specific activity was reported as units per milligram of enzyme protein (U/mg protein). One unit (U) of D-LDH activity was defined as the amount of enzyme required to catalyze the conversion of 1 µmol of pyruvate per minute under the assay conditions.

Determination of the Optimum Enzyme Concentration for D-LDH Immobilization

D-LDH solutions were prepared with final concentrations of 0.5, 1.0, and 2.0 mg/mL of enzyme protein and added to 100 mg of support. The amount of enzyme protein bound to the 100 mg support was determined by a protein assay performed in the filtrate. Then, the specific activity (U/mg protein) was calculated, and the enzyme protein concentration that yielded the highest specific activity was determined.

Effect of Nicotinamide Adenine Dinucleotide Concentration on Activity

After determining the activity of the free and immobilized enzymes, the NADH concentration that yielded the highest activity was determined. To do this, the pyruvate concentration in the reaction mixture was kept constant at 0.33 mM, and NADH solutions with final concentrations ranging from 0.016 to 0.667 mM were used to measure the activity. The NADH concentration that resulted in the highest activity was then identified.

Determination of the Optimum Immobilization Time for D-LDH

To determine the optimum immobilization time, D-LDH was incubated in the immobilization medium for 0.5, 1, 2, and 4 hours, after which unbound protein was removed by washing. The amount of D-LDH bound to the support was expressed as a percentage of the total amount of D-LDH added to the immobilization medium. The time at which the maximum amount of protein bound to the support was evaluated.

Determination of the Optimum pH and Temperature

To determine the effect of pH on the activity of free and immobilized D-LDH, 0.1 M buffers at different pH values were prepared for the reaction mixture. Activity measurements were made using sodium phosphate buffers (pH 5.0-8.0) and carbonate buffers (pH 9.0-10.0), and the pH values at which the highest activity was determined. The catalytic activities were evaluated across a temperature range of 25–60 °C, and the temperature corresponding to the maximum activity was identified for each preparation.

Determination of the Kinetic Parameters

After determining the optimum pH and temperature for free and immobilized D-LDH, activities were measured at these conditions using different substrate concentrations. In these measurements, the NADH concentration in the reaction mixture was kept constant, and 100 µL of pyruvate solutions with concentrations ranging from 1 to 20 mM was added to the mixture. At the end of the reaction, K_m and V_{max} values were calculated using the Lineweaver-Burk plot.

Determination of the Thermal Stability

To determine the thermal stability of free and immobilized enzymes, the enzymes were incubated at 45°C and 60°C for 24 hours, and the remaining activities were measured at specific time intervals (1, 2, 4, 8, 16, 20, and 24 hours) and calculated as relative activities. The previously determined optimum pH values and NADH concentrations were used for the activity measurements.

Determination of the Reusability Stability of Immobilized D-LDH

For the reusability studies of immobilized D-LDH in a batch reactor, a reactor with a diameter of 1.5 cm and a length of 8 cm was used. Five milligrams of immobilized D-LDH were placed inside the

column, and 2.8 mL of pH 6.5 sodium phosphate buffer was added. After adding 100 µL of pyruvate and NADH solutions with a concentration of 10 mM to the reaction mixture at 25°C, the solution was rapidly removed from the column after 15 minutes. The decrease in NADH absorbance at 340 nm was measured to determine the activity. The procedure was repeated 10 times to calculate the activity change associated with reuse.

RESULTS AND DISCUSSION

Optimum Enzyme Concentration and Immobilization Time

To identify the optimal enzyme concentration for immobilization, D-LDH solutions with protein concentrations of 0.5, 1.0, and 2.0 mg/mL were tested. The amount of D-LDH bound to the support was calculated as the percentage ratio (%) of the enzyme protein bound to the support relative to the total amount of enzyme protein initially added to the medium. As shown in Table 1, the highest relative specific activity was obtained when the enzyme solution with a total protein amount of 1 mg in the immobilization medium was used.

Table 1. Determination of the optimum enzyme concentration of immobilization on silica gel support

Initial Protein Concentration (mg)	Bound protein (mg)	Relative Activity (%)
0.25	0.21	61
0.5	0.15	78
1.0	0.19	100
2.0	0.66	35

In the selection of the optimum enzyme concentration for D-LDH immobilization, the enzyme's activity value and the amount of bound protein were considered. When 0.5 mg of protein was added to the immobilization medium, the relative activity was 78%. However, when 1 mg of protein was added, the activity reached its highest level. Further increase in initial protein amount resulted in a decrease in the activity of immobilized D-LDH. To date, there have been no studies in the literature on the immobilization of D-LDH on activated silica gel support. Alagöz et al. (2021) reported the immobilization of *Rhizomucor miehe*i lipase (RML) on silica gel support by adding different amounts of RML protein (4, 8, and 16 mg) to the immobilization medium. They calculated that the highest activity was achieved when the RML protein amount in the immobilization medium was 4 mg. They also reported that the calculated specific activity decreased when the protein amount increased, this situation was explained by an increase in substrate diffusion limitation (Alagöz et al., 2021). After determining the optimum enzyme concentration for immobilization, the free enzyme was incubated in the immobilization medium for 0.5, 1, 2, and 4 hours. As seen in Fig. 1, the relative activity was calculated as 49% at 30 minutes, 87% at 1 hour, 100% at 2 hours, and 90% at 4 hours. Based on these results, the optimum immobilization time was determined to be 2 hours.

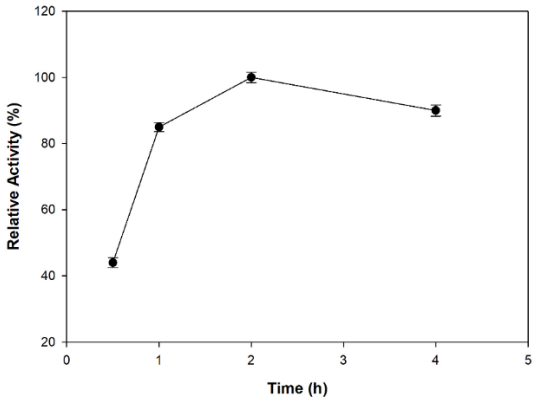

Figure 1. The change of the relative activity depends on the immobilization time

Determination of the Optimum pH and Temperature

To examine the effect of pH, a series of 0.1 M buffer solutions were prepared, including phosphate buffers (pH 5.0-8.0 and carbonate buffers (pH 9.0 and 10.0), and their activities were measured at 25°C. The optimal pH was determined to be 7.0 for free D-LDH, while the optimal pH was found to be 6.5 for immobilized D-LDH (Fig. 2). This can be explained by the effect of changes in the microenvironment of D-LDH covalently bound via glutaraldehyde (Carlsson et al., 2014). It was observed that free D-LDH had high activity from pH 5.0 to 9.0, and its activity decreased to 60% at pH 10, while the immobilized D-LDH maintained 80% of its activity under the same conditions. Zaboli et al. (2021) obtained the D-LDH gene from *Leuconostoc mesenteroides* through recombinant *E. coli* BL21 strain and calculated its optimum pH to be 8.0. In another study, Zaboli et al. (2019) reported that after immobilizing D-LDH on carbon nanotubes (CNT), the optimum pH remained at 8.0 for immobilized D-LDH. Torkzadeh-Mahani et al. (2020) reported the optimum pH for free D-LDH was 8.0, whereas the optimum pH was determined to be 7.0 when it was adsorbed onto a metal-organic framework (Fe_3O_4NPs@Ni-MOF). In a different research study, Jin et al. (2023) characterized mouse D-LDH (mLDHD) and they examined the dependence of the activity on the pH of the reaction solution. The results showed that mLDHD had maximum specific activity at pH 7.4.

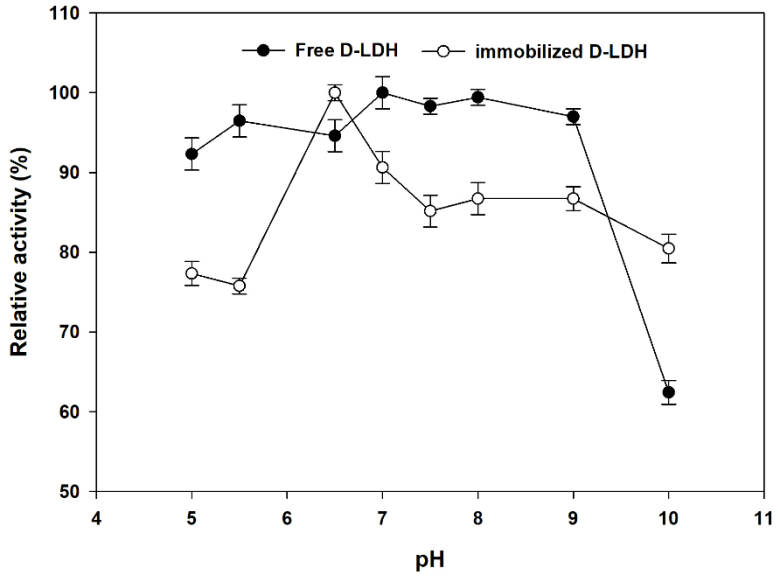

Figure 2. Changes in the activities of free and immobilized D-LDH depending on pH

As depicted in Fig. 3, free D-LDH exhibited maximum activity at 37 °C, whereas the immobilized form showed a peak activity at 45 °C. Although no significant difference in activity was observed for the free enzyme between 20°C and 50°C, its activity decreased to 53% at 60°C. In the literature, Torkzadeh-Mahani et al. (2020) reported that the optimum temperature for recombinant D-LDH was 30°C. After adsorbing the enzyme onto a metal-organic framework (Fe_3O_4NPs@Ni-MOF), they found that immobilized D-LDH maintained its enzymatic activity between 30-50°C; its optimum activity did not decrease within this temperature range. In the literature, Zaboli et al. (2021) observed that the optimum temperature for recombinant D-LDH from *Leuconostoc mesenteroides* at 30°C. In another study published in 2019, Zaboli et al. reported that the both free D-LDH and immobilized D-LDH on carbon nanotubes (CNT) exhibited optimum activity at 30°C. In the study of Yuping et al. (2024), the optimum pH and optimum temperature of D-LDH in Leuconostoc citreum KM20 enzyme were shown as 8 and 40 °C respectively.

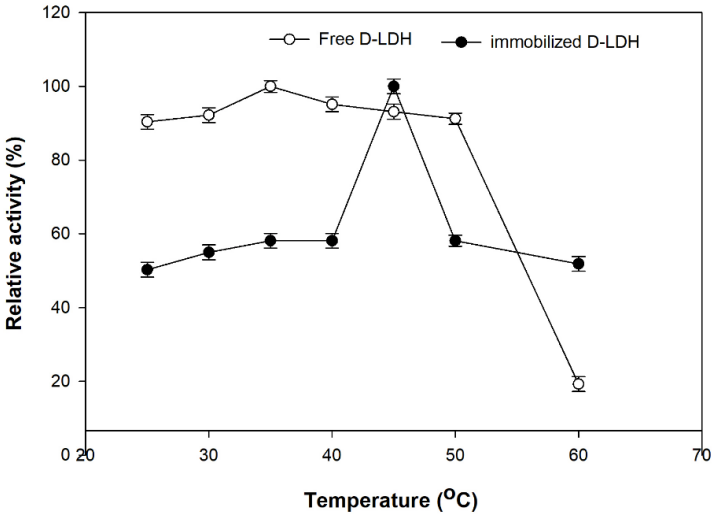

Figure 3. Changes in the activities of free and immobilized D-LDH depending on temperature

NADH Concentration for Free D-LDH Activity

Under optimal pH and temperature, the reaction rates were measured by varying NADH concentrations while maintaining a constant pyruvate concentration (0.33 mM) in the reaction medium. As demonstrated in Fig. 4, the maximum relative activity was observed at an NADH concentration of 0.332 mM. In contrast, the immobilized D-LDH exhibited optimal activity at pH 6.0 and 45 °C. To determine the optimal NADH concentration for the immobilized enzyme, a series of NADH solutions (ranging from 0.25 to 30 mM) were tested, with the pyruvate concentration kept constant at 0.33 mM. As illustrated in Fig. 4, the immobilized D-LDH reached its maximum activity at a final NADH concentration of 0.831 mM.

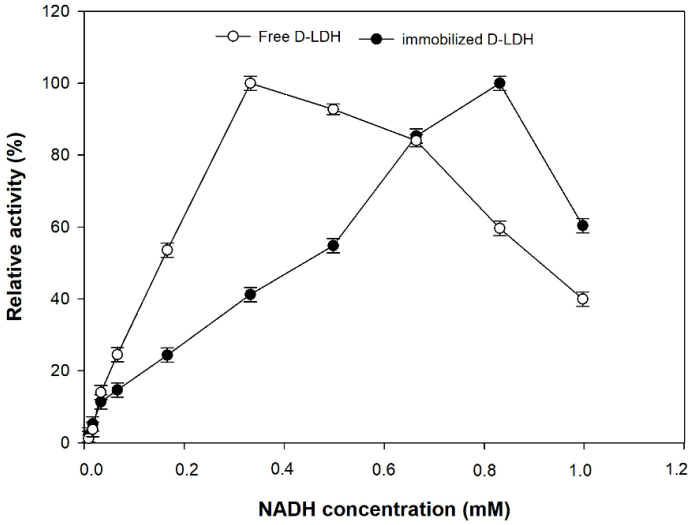

Figure 4. The activity of free and immobilized D-LDH depending on NADH concentration

Kinetic parameters

After determining the optimum NADH concentrations of 0.498 mM for free D-LDH and 0.831 mM for immobilized D-LDH, pyruvate solutions at different concentrations were prepared, and kinetic parameters were established. As given in Table 2, the K_m values were 0.37 mM for free D-LDH and 0.08 mM for immobilized D-LDH, while the V_{max} values were calculated as 86.9 U/mg protein and 19.2 U/mg protein, respectively. After immobilization, V_{max} decreased by approximately 4.5 times. The k_{cat} and k_{cat}/K_m values were calculated and they were 3173 1/dak and 8576 1/dak mM for free D-LDH, while after immobilization, they were 699 1/dak and 8738 1/dak mM. The results obtained in

this study were compared with those from the literature. Zaboli et al. (2019) determined the kinetic parameters for both free D-LDH and D-LDH immobilized on carbon nanotubes (CNT) using NADH and pyruvate as substrates. They reported that under optimum conditions, the K_m value for free D-LDH was 0.390 mM. With immobilization of D-LDH, the K_m value for pyruvate increased approximately five times to 1.92 mM. Gu et al. (2014) calculated the K_m value for free D-LDH as 0.05 mM. They calculated the k_{cat} and k_{cat}/K_m values as 370.8 1/s and 7.3 × 10^3 1/mM s, respectively. Torkzadeh-Mahani et al. (2020) determined the K_m and k_{cat} values as 0.44 mM and 0.733 × 10^4 1/s, respectively, using different pyruvate concentrations as the substrate solution for recombinant (from *E. coli* BL21 strain) D-LDH. They reported that the affinity of immobilized D-LDH for pyruvate decreased by approximately 1.75 times when D-LDH was immobilized onto a metal-organic framework (Fe_3O_4NPs@Ni-MOF).

Table 2. Kinetic parameters of free and immobilized D-LDH

Enzyme	K_m (mM)	V_{max} (U/mgprotein)	k_{cat} (1/dak)	k_{cat}/K_m (dak.mM)
D-LDH	0.37	86.9	3173	8576
Immobilized D-LDH	0.08	19.2	699	8738

Thermal Stability

In industrial settings, enzyme thermal stability is crucial. To evaluate this, the relative activities of both free and immobilized D-LDH were measured after incubation at 45°C and 60°C for various durations. The results indicated that immobilized D-LDH exhibited greater thermal stability at 60°C than its free counterpart (Fig. 5). The free and immobilized D-LDH retained 90% and 89% of their initial activities, respectively, after 4 hours of incubation at 45°C. After 24 hours of incubation, the corresponding retained activities were 52% and 20%. The free D-LDH retained 12% of its initial activity, while immobilized D-LDH retained 32% after 24 hours of incubation at 60 °C. Zaboli et al. (2019) investigated the thermal stability of free and CNT-immobilized D-LDH across a temperature range of 30-70°C and calculated the relative activities after incubating for 10 minutes at each temperature. After 10 minutes of incubation at 70 °C, free D-LDH preserved just 6% of its original activity whereas CNT-immobilized D-LDH retained 24%. Torkzadeh-Mahani et al. (2020) incubated recombinant D-LDH at 40°C and found that the free D-LDH preserved just 7% of its original activity, while D-LDH adsorbed onto (Fe_3O_4NPs@Ni-MOF) retained more than 50% of its activity after 60 minutes.

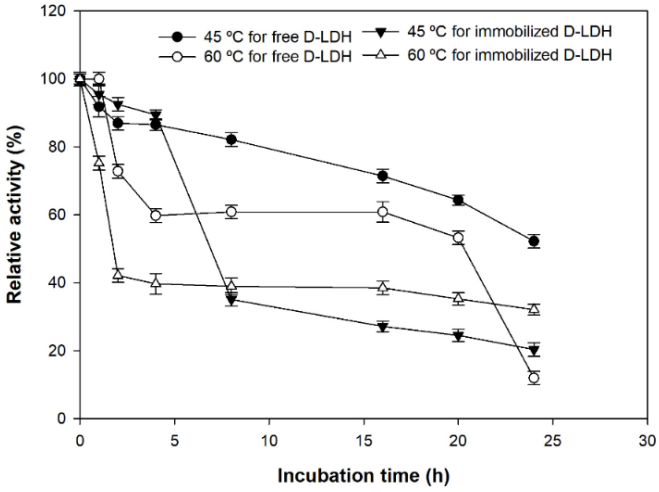

Figure 5. Change of activity of free and immobilized D-LDH depending on incubation time at 45 °C and 60 °C

Reusability Stability of Immobilized D-LDH

Figure 6 illustrates the reuse stability of D-LDH in a batch-type reactor. The immobilized enzyme retained 90% of its initial activity after four cycles of use and maintained 38% of its original activity after ten cycles. The decrease in initial activity may be due to the conformational change of D-LDH depending on the washing step after each cycle. D-LDH immobilized on $Cu_3(PO_4)_2$-based inorganic hybrid nanoflowers remained 78% of its initial activity after 8 cycles (Liu et al., 2024).

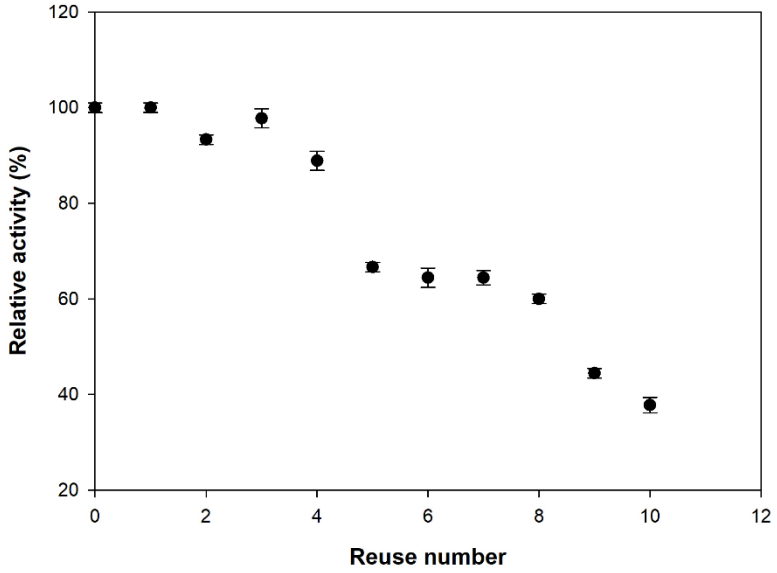

Figure 6. Change in the activity of immobilized D-LDH due to reuse

CONCLUSION

The covalent immobilization of D-LDH from *Leuconostoc mesenteroides* onto 3-aminopropyl-functionalized silica gel using glutaraldehyde as a cross-linker was optimized to enhance enzyme stability and reusability. The immobilization process achieved an efficiency of 86%, and the resulting biocatalyst exhibited shifts in optimal pH and temperature, indicative of enhanced thermal stability and a slight preference for more acidic conditions. Although immobilization resulted in a decrease in V_{max}, a notable reduction in Km was observed, suggesting an increased affinity for the substrate. Additionally, the immobilized D-LDH demonstrated improved operational stability, retaining 38% of its initial activity after 10 consecutive reuse cycles in a batch reactor. These findings underscore the potential of this immobilized enzyme system for sustainable and efficient D-lactic acid production in industrial bioprocesses.

ACKNOWLEDGEMENTS

This study, with project number FYL-2019-12462, was supported by the "Ç.Ü. Research Projects Unit".

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

Study conception and design: Seyhan Tükel, Data collection: Nazlı Ece Varan; Analysis and interpretation of results: Nazlı Ece Varan.; Manuscript draft preparation: Seyhan Tükel. All authors reviewed the results and approved the final version of the manuscript.

REFERENCES

Alagöz, D., Toprak, A., Yildirim, D., Tükel, S. S., & Fernandez-Lafuente, R. (2021). Modified silicates and carbon nanotubes for immobilization of lipase from *Rhizomucor miehei*: Effect of support and immobilization technique on the catalytic performance of the immobilized biocatalysts. *Enzyme and Microbial Technology*, 144, 109739. https://doi.org/10.1016/j.enzmictec.2020.109739

Ashkan, Z., Hemmati, R., Homaei, A., Dinari, A., Jamlidoost, M., & Tashakor, A. (2021). Immobilization of enzymes on nanoinorganic support materials: An update. *International Journal of Biological Macromolecules*, 168, 708-721. https://doi.org/10.1016/j.ijbiomac.2020.11.127

Bié, J., Sepodes, B., Fernandes, P. C., & Ribeiro, M. H. (2022). Enzyme immobilization and co-immobilization: main framework, advances and some applications. *Processes*, 10, 494. https://doi.org/10.3390/pr10030494

Carlsson, N., Gustafsson, H., Thörn, C., Olsson, L., Holmberg, K., Åkerman, B. (2014). Enzymes immobilized in mesoporous silica: a physical-chemical perspective. *Advances in Colloid and Interface Science*, 205, 339-360. https://doi.org/10.1016/j.cis.2013.08.010

Cavalcante, F. T., Cavalcante, A. L., de Sousa, I. G., Neto, F. S., & dos Santos, J. C. (2021). Current status and future perspectives of supports and protocols for enzyme immobilization. *Catalysts*, 11, 1222. https://doi.org/10.3390/catal11101222

Chapman, J., Ismail, A. E., & Dinu, C. Z. (2018). Industrial applications of enzymes: Recent advances, techniques, and outlooks. *Catalysts*, 8, 238. https://doi.org/10.3390/catal8060238

Cheng, Y. Y., Park, T. H., Seong, H., Kim, T. J., & Han, N. S. (2022). Biological characterization of D-lactate dehydrogenase responsible for high-yield production of D-phenyllactic acid in *Sporolactobacillus inulinus*. *Microbial Biotechnology*, 15, 2717-2729. https://doi.org/10.1111/1751-7915.14125

Chikurova, N. Y., Shemiakina, A. O., Kryzhanovskaya, D. S., Shpigun, O. A., & Chernobrovkina, A. V. (2023). Comparison of the properties of 3-aminopropyl silica with different nitrogen content in HILIC mode. *Moscow University Chemistry Bulletin*, 78, 118-125. https://doi.org/10.3103/S0027131423030057

Donga, C., Mishra, S. B., Abd-El-Aziz, A. S., Ndlovu, L. N., Mishra, A. K., & Kuvarega, A. T. (2022). (3-Aminopropyl) Triethoxysilane (APTES) functionalized magnetic nanosilica graphene oxide (MGO) nanocomposite for the comparative adsorption of the heavy metal [Pb (II), Cd (II) and Ni (II)] ions from aqueous solution. *Journal of Inorganic and Organometallic Polymers and Materials*, 32, 2235-2248. https://doi.org/10.21203/rs.3.rs-1184162/v1

Inoue, Y., Yamada, R., Matsumoto, T., & Ogino, H. (2024). Enhancing D-lactic acid production by optimizing the expression of D-LDH gene in methylotrophic yeast *Komagataella phaffii*. *Biotechnology for Biofuels and Bioproducts*, 17, 149. https://doi.org/10.1186/s13068-024-02596-0

Jin, S., Chen, X., Yang, J., & Ding, J. (2023). Lactate dehydrogenase D is a general dehydrogenase for D-2-hydroxyacids and is associated with D-lactic acidosis. *Nature Communications*, 14, 6638. https://doi.org/10.1038/s41467-023-42456-3

Kim, S., Ga, S., Bae, H., Sluyter, R., Konstantinov, K., Shrestha, L. K., ... & Ariga, K. (2024). Multidisciplinary approaches for enzyme biocatalysis in pharmaceuticals: protein engineering,

computational biology, and nanoarchitectonics. *EES Catalysis*, 2, 14-48. https://doi.org/10.1039/D3EY00239J

Liu, J., Jiang, X., Zheng, Y., Li, K., Zhang, R., Xu, J., Wang, Z., Zhang, Y., Yin, H., & Li, J. (2024). Expression, characterization, and immobilization of a novel D-lactate dehydrogenase from *Salinispirillum sp*. LH 10-3-1. *Processes*, 12, 1349. https://doi.org/10.3390/pr12071349

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275. https://doi.org/10.1016/S0021-9258(19)52451-6

Meghwanshi, G. K., Kaur, N., Verma, S., Dabi, N. K., Vashishtha, A., Charan, P. D., ... & Kumar, R. (2020). Enzymes for pharmaceutical and therapeutic applications. *Biotechnology and Applied Biochemistry*, 67, 586-601. https://doi.org/10.1002/bab.1919

Putz, A. M., Ciopec, M., Negrea, A., Grad, O., Ianăşi, C., Ivankov, O. I., ... & Almásy, L. (2021). Comparison of structure and adsorption properties of mesoporous silica functionalized with aminopropyl groups by the co-condensation and the post grafting methods. *Materials*, 14, 628. https://doi.org/10.3390/ma14030628

Satomura, T., Hayashi, J., Sakamoto, H., Nunoura, T., Takaki, Y., Takai, K., Takami, H., Ohshima, T., Sakuraba, H., &Suye, S. (2018). d-Lactate electrochemical biosensor prepared by immobilization of thermostable dye-linked d-lactate dehydrogenase from Candidatus *Caldiarchaeum subterraneum*. *Journal of Bioscience and Bioengineering*, 126, 425-430. https://doi.org/10.1016/j.jbiosc.2018.04.002

Sosa, N., Chanlek, N., & Wittayakun, J. (2020). Facile ultrasound-assisted grafting of silica gel by aminopropyltriethoxysilane for aldol condensation of furfural and acetone. *Ultrasonics Sonochemistry*, 62, 104857. https://doi.org/10.1016/j.ultsonch.2019.104857

Tan, Z., Bilal, M., Li, X., Ju, F., Teng, Y., & Iqbal, H. M. (2022). Nanomaterial-immobilized lipases for sustainable recovery of biodiesel–A review. *Fuel*, 316, 123429. http://dx.doi.org/10.1016/j.fuel.2022.123429

Torkzadeh-Mahani, M., Zaboli, M., Barani, M., Torkzadeh-Mahani, M. (2020). A combined theoretical and experimental study to improve the thermal stability of recombinant D-lactate dehydrogenase immobilized on a novel superparamagnetic Fe_3O_4NPs@ metal–organic framework. *Applied Organometallic Chemistry*, 34. https://doi.org/10.1002/aoc.5581

Tülek, A., Günay, E., Servili, B., Essiz, S., Binay, B., & Yildirim, D. (2023). Sustainable production of formic acid from CO_2 by a novel immobilized mutant formate dehydrogenase. *Separation and Purification Technology*, 309, 123090. https://doi.org/10.1016/j.seppur.2022.123090

Umaru, I. J., Adam, B. R., Habibu, B., Umaru, K. I., & Chizaram, B. C. (2021). Biochemical impact of microorganism and enzymatic activities in food and pharmaceutical industries. *International Journal of Advanced Biochemistry Research*, 5, 42-57. https://doi.org/10.33545/26174693.2021.v5.i1a.147

Vidhya, K., Parveen, S., Rajkumar, P., Arulmari, R., Nisha, K., & Pandiselvam, R. (2024). A comprehensive review on minimizing acrylamide in foods: rethinking ingredients, process tweaks, culinary techniques, and advanced analysis. *Journal of Food Measurement and Characterization*, 1-21. http://dx.doi.org/10.1007/s11694-024-03020-9

Wang, Y., Luo, X., Sun, X., Hu, J., Guo, Q., Shen, B., & Fu, Y. (2022). Lactate dehydrogenase encapsulated in a metal-organic framework: A novel stable and reusable biocatalyst for the synthesis of D-phenyllactic acid. *Colloids and Surfaces B: Biointerfaces*, 216, 112604. https://doi.org/10.1016/j.colsurfb.2022.112604

Yan, Y., Li, X., Yang, Q., Zhang, H., Hettinga, K., Li, H., & Chen, W. (2022). Dietary D-lactate intake facilitates inflammatory resolution by modulating M1 macrophage polarization. *Molecular Nutrition & Food Research*, 66, 2200196. https://doi.org/10.1002/mnfr.202200196

Yuping, W. E. N., Jinxi, L. I. U., Qing, J. I. N., & Hushan, C. U. I. (2024). Characterization of D-lactate dehydrogenase in Leuconostoc citreum KM20. Food and Machinery, 40(2), 36-42. https://doi.org/10.13652/j.spjx.1003.5788.2023.80549

Zaboli, M., Raissi, H., Zaboli, M., Farzad, F., Torkzadeh-Mahani, M. (2019). Stabilization of D-lactate dehydrogenase diagnostic enzyme via immobilization on pristine and carboxyl-functionalized carbon nanotubes, a combined experimental and molecular dynamics simulation study. *Archives of Biochemistry and Biophysics*, 661, 178-186. https://doi.org/10.1016/j.abb.2018.11.019

Zaboli, M., Saeidnia, F., Zaboli, M., Torkzadeh-Mahani, M. (2021). Stabilization of recombinant D-lactate dehydrogenase enzyme with trehalose: Response surface methodology and molecular dynamics simulation study. *Process Biochemistry*, 101, 26-35. http://dx.doi.org/10.1016/j.procbio.2020.11.001

Zhou, W., Zhang, W., & Cai, Y. (2021). Laccase immobilization for water purification: A comprehensive review. *Chemical Engineering Journal*, 403, 126272. http://dx.doi.org/10.1016/j.cej.2020.126272