Effects of Additives on Macroporous Adsorption Resin-Immobilized Lipase (Postprint)

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Date: 2018-12-16T00:00:00+00:00

Abstract

Marine lipase was immobilized using macroporous adsorption resin DA-201 as the carrier, and the effects of additives on the immobilization process were investigated. Using NH4Cl, mannose, and glycine as additives respectively, the conditions were optimized through a combination of single-factor and orthogonal experiments. The results showed that the optimal conditions with NH4Cl as the additive were: citric acid-sodium citrate buffer pH 6.0, immobilization temperature 30°C, carrier amount 0.5 g, NH4Cl concentration 25 mmol/L, immobilization time 3.0 h, with enzyme activity reaching 115.27 U/g; this represented a 47.42% improvement in immobilization efficiency compared to the additive-free immobilized enzyme. The optimal conditions with mannose as the additive were: potassium dihydrogen phosphate-sodium hydroxide buffer pH 7.0, immobilization temperature 35°C, carrier amount 0.5 g, mannose concentration 10 mmol/L, immobilization time 4.5 h; enzyme activity reached 122.75 U/g, representing a 6.50% improvement in immobilization efficiency compared to the additive-free immobilized enzyme. The optimal conditions with glycine as the additive were: potassium dihydrogen phosphate-sodium hydroxide buffer pH 7.0, immobilization temperature 20°C, carrier amount 0.5 g, glycine concentration 25 mmol/L, immobilization time 7.5 h; enzyme activity reached 141.69 U/g, representing a 26.12% improvement in immobilization efficiency compared to the additive-free immobilized enzyme. The use of different additives exhibited significant effects on the adsorption immobilization process of macroporous adsorption resin DA-201, substantially improving adsorption efficiency; simultaneously, it was found that buffer type, pH, temperature, additive concentration, and immobilization time markedly influenced the adsorption of lipase by DA-201 resin, providing valuable reference for subsequent research on the adsorption immobilization of industrial enzymes.

Full Text

Effects of Additives on the Immobilization of Lipase by Macroporous Adsorption Resin

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Abstract

Macroporous adsorption resin DA-201 was employed as a carrier to immobilize marine-derived lipase, and the influence of additives on the immobilization process was investigated. NH Cl, mannose, and glycine were used as additives, and the immobilization conditions were optimized through a combination of single-factor and orthogonal experiments. The results demonstrated that the optimal conditions with NH Cl additive were: citric acid-sodium citrate buffer at pH 6.0, immobilization temperature of 30°C, carrier quantity of 0.5 g, NH Cl concentration of 25 mmol/L, and immobilization time of 3.0 h, achieving an enzyme activity of 115.27 U/g—47.42% higher than that of the immobilized enzyme without additives. The optimal conditions with mannose additive were: potassium dihydrogen phosphate-sodium hydroxide buffer at pH 7.0, immobilization temperature of 35°C, carrier quantity of 0.5 g, mannose concentration of 10 mmol/L, and immobilization time of 4.5 h, yielding an enzyme activity of 122.75 U/g, representing a 6.50% improvement over the additive-free control. The optimal conditions with glycine additive were: potassium dihydrogen phosphate-sodium hydroxide buffer at pH 7.0, immobilization temperature of 20°C, carrier quantity of 0.5 g, glycine concentration of 25 mmol/L, and immobilization time of 7.5 h, resulting in an enzyme activity of 141.69 U/g, which was 26.12% higher than the control. These findings indicate that different additives substantially influence the adsorption immobilization process on macroporous adsorption resin DA-201 and can significantly enhance adsorption efficiency. Furthermore, buffer type, pH, temperature, additive concentration, and immobilization time were identified as critical factors affecting lipase adsorption onto DA-201 resin, providing valuable insights for future research on industrial enzyme immobilization.

Keywords: macroporous adsorption resin; marine lipase; immobilization; additives

Introduction

Lipase (EC 3.1.1.3) belongs to the carboxyl ester hydrolase family and catalyzes the stepwise hydrolysis of triglycerides into glycerol and fatty acids, as well as

various chemical reactions including alcoholysis, esterification, and transesterification. Due to its broad application potential in food, agriculture, and pharmaceutical industries, lipase has attracted significant research interest. However, free lipases are highly sensitive to environmental conditions, exhibiting poor stability and substantial variability in practical applications. Moreover, the high cost of enzyme catalysts and the difficulty of separating and recycling free enzymes from reaction systems increase production costs, creating a technological bottleneck for industrial enzyme applications. To address these challenges, enzyme immobilization technology has been developed. Immobilized lipases maintain high catalytic activity and recovery rates while demonstrating improved stability and tolerance—critical properties for industrial applications—making them more economically viable than their free counterparts.

Common immobilization techniques include adsorption, cross-linking, entrapment, and covalent binding, each with distinct advantages and limitations. Among these, adsorption offers the benefits of preserving enzyme conformation, providing a wide selection of carriers, low cost, and operational simplicity. Macroporous adsorption resins are organic polymer adsorbents with large pore structures (average pore diameter of 10–13 nm) and no exchange groups. They adsorb molecules through van der Waals forces or hydrogen bonds and possess screening capabilities due to their macroporous network structure and large specific surface area. These resins are insoluble in acids, bases, and organic solvents, and exhibit excellent stability. In recent years, macroporous adsorption resins have been increasingly used for enzyme immobilization. For instance, Xie et al. immobilized catalase using macroporous adsorption resin AB-8, Zhao et al. immobilized lipase on resin HZ-841 for catalytic resolution of racemic naproxen, and Wang et al. immobilized porcine pancreatic lipase using macroporous adsorption resin. Therefore, this study selected macroporous adsorption resin as the carrier for adsorption immobilization to avoid the functional damage to industrial enzymes often associated with chemical immobilization methods.

Previous studies have shown that certain additives can enhance or inhibit enzyme activity. For example, isopropanol treatment of immobilized lipase significantly enhanced enantioselectivity and reaction rate in the synthesis of (S)-ketoprofen hydroxyalkyl esters in organic solvents. Peng et al. investigated the effects of metal ions on -glucanase and xylanase activities, finding that Na , K , Mg^2 , Mn^2 , Fe^3 , and Ca^2 activated -glucanase while Cu^2 inhibited it, and that Na , K , and Ca^2 activated xylanase while Mg^2 , Mn^2 , Fe^3 , and Ca^2 were inhibitory. However, research on the effects of other additives such as inorganic salts, carbohydrates, and amino acids on the immobilization process, particularly using macroporous resins, remains limited. This study focuses on the immobilization of marine-derived lipase, first screening the optimal macroporous adsorption resin DA-201 and additive types, then systematically investigating the effects of three selected additives on lipase immobilization. Furthermore, critical factors influencing adsorption immobilization—including temperature, pH, carrier quantity, and immobilization time—were optimized to determine the best process conditions.

1. Materials and Methods

1.1 Materials and Instruments

Materials: Marine lipase (from *Candida* sp.) was purchased from Shanghai Guchen Biological Co., Ltd. Macroporous adsorption resins were obtained from Zhengzhou Hecheng New Materials Technology Co., Ltd. Polyvinyl alcohol (PVA), copper acetate anhydrous, olive oil, anhydrous ethanol, isooctane, carbohydrates, amino acids, and inorganic salts were acquired from various suppliers (Tianjin Damao Chemical Reagent Factory, Aladdin, Shanghai Macklin Biochemical Technology Co., Ltd., Tianjin Jindong Tianzheng Fine Chemical Reagent Factory, Tianjin Fuyu Fine Chemical Co., Ltd., and Guangzhou Dongju Co., Ltd.).

Instruments: PB-10 pH meter (Sartorius, Germany), Allegra X-30R centrifuge (Beckman Coulter), DJS-2012R constant temperature shaker (Shanghai Shiwei Experimental Instrument Technology Co., Ltd.), SCIENTZ-IID ultrasonic cell disruptor (Ningbo Xinzhi Biological Co.), Infinite M200 Pro microplate reader (Tecan, Switzerland), Tomos vortex mixer, DK-8D water bath (Shanghai Yiheng Scientific Instrument Co., Ltd.), and electric thermostatic drying oven (Shanghai Yiheng Scientific Instrument Co., Ltd.).

1.2 Enzyme Activity Assay

1.2.1 Measurement of Enzyme Activity Enzyme activity was determined using an improved copper soap spectrophotometric method. In a 25 mL test tube, 1 mL of olive oil substrate emulsion and 1.25 mL of 0.05 mol/L sodium phosphate buffer (pH 7.0) were mixed and preheated at 40°C for 5 min. Lipase supernatant was then added, and the mixture was incubated at 40°C with shaking at 200 rpm for 15 min. The reaction was terminated by rapid addition of 0.5 mL of 6 mol/L HCl and 3 mL of 95% ethanol. After mixing, 1.5 mL of isooctane was added, and the mixture was vortexed for 2 min, then placed in a 60°C water bath for phase separation. Following cooling at room temperature for 5 min, 0.5 mL of the upper isooctane layer was transferred to a new tube, mixed with 2 mL of isooctane and 0.5 mL of copper salt chromogenic agent, and vortexed for 2 min. After standing for at least 1 min, 0.1 mL of the upper layer was transferred to a microplate for OD measurement.

1.2.2 Definition of Enzyme Activity Unit One unit of lipase activity (U) was defined as the amount of enzyme required to catalyze the conversion of 1 mol of substrate to fatty acid per minute under the assay conditions (40°C, pH 7.0).

1.2.3 Preparation of Olive Oil Substrate Emulsion Polyvinyl alcohol (PVA, polymerization degree 1750, 8.0 g) was dissolved in 200 mL of deionized water by heating in a boiling water bath with stirring. After cooling, 30 mL of

4% PVA solution was mixed with 10 mL of olive oil and homogenized using an ultrasonic cell disruptor to form a white emulsion, which was stored at 4°C.

1.2.4 Lipase Immobilization Procedure A predetermined amount of carrier was placed in a plastic tube, followed by addition of 10 mL of lipase supernatant. The mixture was incubated in a shaking incubator at 200 rpm for 3 h. After immobilization, the carrier was washed with 0.05 mol/L PBS buffer to remove unadsorbed enzyme molecules, then filtered and dried for enzyme activity measurement.

1.3 Screening of Macroporous Adsorption Resins

Nine macroporous adsorption resins were evaluated as carriers: D4020, DA-201, HPD100, NKA-9, NKA-II, H103, HPD700, X-5, and GDX-104. Immobilization was performed at pH 6.5, 30°C for 3 h using 1.0 g of carrier. The resin yielding the highest enzyme activity was selected for subsequent experiments.

1.4 Effects of Additives on Lipase Immobilization

Lipase powder was dissolved in 0.05 mol/L PBS buffer to prepare a 2.0 mg/mL enzyme solution. Ten milliliters of this supernatant was added to a 50 mL plastic tube containing 1.0 g of DA-201 resin, along with additives at three concentration levels (2, 5, and 10 mmol/L). The additive categories included: (1) carbohydrates: xylitol, mannose, trehalose, D-xylose, sorbitol, sucrose, galactose, maltose, glucose, and fructose; (2) inorganic salts: KCl, NH Cl, MgCl , CaCl , Na CO , NaCl, (NH) SO , Na SO , and MgSO ; and (3) amino acids: alanine, threonine, glycine, serine, and valine. Control groups without additives were prepared in parallel. All samples were incubated at 30°C with shaking at 200 rpm for 3 h, then washed with 0.05 mol/L PBS buffer, filtered, and dried for activity measurement.

1.5 Optimization of Immobilization Conditions

1.5.1 Selection of Buffer Type Four buffer systems at pH 6.5 were prepared: sodium phosphate buffer, citric acid-sodium citrate buffer, citric acid-disodium hydrogen phosphate buffer, and potassium dihydrogen phosphate-sodium hydroxide buffer. Lipase powder was dissolved in each buffer to prepare 2.0 mg/mL enzyme solutions. Ten milliliters of each solution was added to 1.0 g of DA-201 resin and incubated at 30°C with shaking at 200 rpm for 3 h.

1.5.2 Effect of Buffer pH on Adsorption Immobilization Using the selected buffer type, pH gradients were prepared. Citric acid-sodium citrate buffer was adjusted to pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5. Potassium dihydrogen phosphate-sodium hydroxide buffer was adjusted to pH 6.0, 6.5, 7.0, 7.5, and 8.0. Lipase was dissolved in these buffers to prepare 2.0 mg/mL solutions. Citric acid-sodium citrate buffer was used for NH Cl additive experiments, while

potassium dihydrogen phosphate-sodium hydroxide buffer was used for mannose and glycine additives. All samples were incubated at 30°C with shaking at 200 rpm for 3 h.

1.5.3 Effect of Temperature on Adsorption Immobilization One gram of DA-201 resin was placed in a 50 mL plastic tube. Ten milliliters of lipase supernatant (prepared in the appropriate buffer based on additive type) was added, and the mixture was incubated at 20, 30, 40, 50, or 60°C with shaking at 200 rpm for 3 h.

1.5.4 Effect of Carrier Quantity on Adsorption Immobilization Different quantities of DA-201 resin (0.5, 1.0, 1.5, 2.0, and 2.5 g) were placed in 50 mL plastic tubes. Ten milliliters of lipase supernatant (2.0 mg/mL, prepared in the appropriate buffer) was added to each tube. Immobilization was performed at the temperature determined in section 1.5.3 with shaking at 200 rpm for 3 h.

1.5.5 Effect of Additive Concentration on Adsorption Immobilization Stock solutions of additives (500 mmol/L) were prepared and diluted in 10 mL of enzyme supernatant to final concentrations of 2, 5, 10, 20, and 30 mmol/L. The mixtures were added to 50 mL plastic tubes containing DA-201 resin. Immobilization was conducted using the buffer type, temperature, and carrier quantity determined previously, with shaking at 200 rpm for 3 h.

1.5.6 Effect of Immobilization Time on Adsorption Immobilization Using the optimized conditions from previous sections, immobilization time was varied: carbohydrate additives at 2 mmol/L were tested at 1.5, 3.0, 6.0, 9.0, and 12.0 h; inorganic salt additives at 20 mmol/L were tested at the same time points; and amino acid additives at 20 mmol/L were also evaluated across this time gradient.

1.6 Orthogonal Experimental Design for Lipase Immobilization

Based on single-factor experiments, a 4-level, 5-factor orthogonal experimental design was employed to optimize immobilization conditions, with enzyme activity recovery as the response variable. The experimental factors and levels are presented in , , and for NH Cl, mannose, and glycine additives, respectively.

2. Results and Discussion

2.1 Screening of Macroporous Adsorption Resins

Nine macroporous adsorption resins (D4020, DA-201, HPD100, NKA-9, NKA-II, H103, HPD700, X-5, and GDX-104) were evaluated for lipase immobilization at pH 6.5, 30°C for 3 h using 1.0 g of carrier. As shown in Figure 1: see original paper, the adsorption capacity varied dramatically among different resins. DA-201, a styrene-type polar resin with stable physicochemical properties and

excellent selectivity for organic compounds, demonstrated superior adsorption performance compared to other carriers.

2.2 Effects of Additives on Lipase Immobilization

The influence of various additives on lipase adsorption immobilization was investigated. Nine inorganic salts (KCl, NH Cl, MgCl , CaCl , Na CO , NaCl, (NH) SO , Na SO , and MgSO) were tested at three concentrations (2, 5, and 10 mmol/L). As depicted in Figure 1: see original paper, NH Cl and (NH) SO promoted lipase immobilization, while Na SO and MgSO exhibited inhibitory effects. KCl, MgCl , CaCl , Na CO , and NaCl showed minimal impact. NH Cl demonstrated the most significant enhancement, likely by altering the surface charge of the resin or the ionic state of the lipase. Therefore, NH Cl was selected for further optimization.

Ten carbohydrates (xylitol, mannose, trehalose, D-xylose, sorbitol, sucrose, galactose, maltose, glucose, and fructose) were evaluated at 2, 5, and 10 mmol/L. Figure 1: see original paper shows that all carbohydrates provided some enhancement, with mannose exhibiting the most pronounced effect, leading to its selection for subsequent experiments.

Five amino acids (alanine, threonine, glycine, serine, and valine) were tested at the same concentration levels. Figure 1: see original paper reveals that alanine, threonine, glycine, and serine promoted adsorption, while valine had negligible effect. Glycine showed the best performance and was chosen for further study.

2.3 Optimization of Immobilization with NH Cl Additive

2.3.1 Effect of Buffer Type and pH Four buffer systems at pH 6.5 were compared: sodium phosphate, citric acid-sodium citrate, citric acid-disodium hydrogen phosphate, and potassium dihydrogen phosphate-sodium hydroxide. Citric acid-sodium citrate buffer yielded the highest immobilization efficiency Figure 2: see original paper and was selected for subsequent experiments.

The effect of pH was examined using citric acid-sodium citrate buffer across a pH range of 3.0–6.5. Maximum enzyme activity was observed at pH 6.0, with activity decreasing at higher pH values Figure 2: see original paper. The pH effect was more pronounced under acidic conditions than in alkaline environments.

2.3.2 Effect of Temperature Enzyme activity decreased with increasing immobilization temperature Figure 2: see original paper, likely due to temperature-induced alterations in protein structure and active site conformation. The optimal immobilization temperature was determined to be 20°C.

2.3.3 Effect of Carrier Quantity Varying carrier quantities (0.5–2.5 g) were added to 10 mL of 2.0 mg/mL lipase solution. Enzyme activity per gram of

carrier decreased as carrier quantity increased Figure 2: see original paper, possibly because at lower carrier amounts, more enzyme molecules were adsorbed per unit mass of resin, while higher carrier quantities resulted in fewer enzyme molecules per unit mass.

2.3.4 Effect of NH Cl Concentration NH Cl concentrations ranging from 2 to 30 mmol/L were evaluated. Maximum activity was achieved at 20 mmol/L Figure 2: see original paper.

2.3.5 Effect of Immobilization Time Time courses from 1.5 to 12.0 h were examined. Enzyme activity increased with time, peaking at 6.0 h before declining Figure 2: see original paper. This pattern suggests that initial extension of immobilization time facilitates greater enzyme loading in the resin pores, but prolonged exposure leads to pore blockage and reduced catalytic efficiency due to constrained enzyme conformation.

2.3.6 Orthogonal Experimental Results for NH Cl Additive A 5-factor, 4-level orthogonal array was employed. Analysis of variance indicated optimal conditions of pH 6.0, temperature 30°C, carrier quantity 0.5 g, NH Cl concentration 25 mmol/L, and immobilization time 3.0 h. The order of factor importance was: carrier quantity > temperature > time > pH > concentration. Under these optimized conditions, immobilized enzyme activity reached 115.27 U/g, compared to 78.19 U/g in the additive-free control .

2.4 Optimization of Immobilization with Mannose Additive

2.4.1 Effect of Buffer Type and pH Among the four buffer systems tested at pH 6.5, potassium dihydrogen phosphate-sodium hydroxide buffer provided the best immobilization performance Figure 3: see original paper. Using this buffer, pH optimization across 6.0–8.0 revealed maximum activity at pH 6.5 Figure 3: see original paper.

2.4.2 Effect of Temperature Immobilized enzyme activity increased with temperature up to 30°C, then declined at higher temperatures Figure 3: see original paper. The temperature optimum of 30°C reflects the balance between enhanced molecular motion facilitating enzyme-carrier contact and thermal denaturation of the enzyme.

2.4.3 Effect of Carrier Quantity As observed with NH Cl, increasing carrier quantity from 0.5 to 2.5 g resulted in decreased specific activity Figure 3: see original paper, with 0.5 g identified as optimal.

2.4.4 Effect of Mannose Concentration Mannose concentrations of 2–30 mmol/L were tested, with 10 mmol/L providing maximum activity Figure 3: see original paper.

2.4.5 Effect of Immobilization Time Enzyme activity generally decreased with prolonged immobilization time from 1.5 to 12.0 h Figure 3: see original paper, suggesting rapid saturation of resin pores followed by blockage.

2.4.6 Orthogonal Experimental Results for Mannose Additive Optimal conditions were determined to be pH 7.0, temperature 35°C, carrier quantity 0.5 g, mannose concentration 10 mmol/L, and immobilization time 4.5 h. Factor importance ranked as: carrier quantity > time > pH > concentration > temperature. Under these conditions, immobilized enzyme activity reached 122.75 U/g, compared to 115.26 U/g in the control .

2.5 Optimization of Immobilization with Glycine Additive

2.5.1 Effect of Buffer Type and pH Potassium dihydrogen phosphate-sodium hydroxide buffer proved optimal Figure 4: see original paper, similar to mannose but differing from NH Cl, possibly due to differential effects of amino acids and carbohydrates on resin charge, buffer properties, and enzyme ionization states. Using this buffer, maximum activity was observed at pH 7.0 Figure 4: see original paper.

2.5.2 Effect of Temperature The optimal immobilization temperature was 30°C, with activity declining above this temperature Figure 4: see original paper.

2.5.3 Effect of Carrier Quantity As with other additives, 0.5 g of carrier provided the highest specific activity Figure 4: see original paper.

2.5.4 Effect of Glycine Concentration Maximum activity was achieved at 25 mmol/L glycine Figure 4: see original paper.

2.5.5 Effect of Immobilization Time Activity increased with time up to 6.0 h, then decreased Figure 4: see original paper, consistent with pore saturation and blockage mechanisms.

2.5.6 Orthogonal Experimental Results for Glycine Additive Optimal conditions were pH 7.0, temperature 20°C, carrier quantity 0.5 g, glycine concentration 25 mmol/L, and immobilization time 7.5 h. Factor importance ranked as: carrier quantity > pH > temperature > time > concentration. Under these conditions, immobilized enzyme activity reached 141.69 U/g, compared to 112.34 U/g in the control .

3. Conclusion

Adsorption using macroporous resins represents an important method for enzyme immobilization. This study screened nine macroporous adsorption resins

and identified DA-201 as the most effective carrier for lipase immobilization. The effects of three additives (NH Cl, mannose, and glycine) on the immobilization process were systematically investigated, and optimal conditions were established for each additive system.

The optimal conditions for NH Cl-mediated immobilization were: citric acid-sodium citrate buffer at pH 6.0, 30°C, 0.5 g carrier, 25 mmol/L NH Cl, and 3.0 h immobilization time, yielding 115.27 U/g enzyme activity with 11.06% activity recovery—47.42% higher efficiency than the additive-free control. For mannose: potassium dihydrogen phosphate-sodium hydroxide buffer at pH 7.0, 35°C, 0.5 g carrier, 10 mmol/L mannose, and 4.5 h immobilization time, achieving 122.75 U/g with 12.24% recovery (6.50% efficiency improvement). For glycine: potassium dihydrogen phosphate-sodium hydroxide buffer at pH 7.0, 20°C, 0.5 g carrier, 25 mmol/L glycine, and 7.5 h immobilization time, producing 141.69 U/g with 10.11% recovery (26.12% efficiency improvement). These results demonstrate that NH Cl and glycine additives can substantially enhance immobilization efficiency after systematic process optimization.

DA-201 resin, a styrene-type polar copolymer with strong adsorption capacity for polar compounds, proved highly effective for lipase immobilization. The physical adsorption process preserves enzyme active sites and higher-order structures, minimizing detrimental effects on catalytic activity and selectivity compared to chemical immobilization methods.

The study revealed that buffer type, pH, temperature, additive concentration, and immobilization time significantly influence the adsorption process, though the optimal conditions varied among additives. These differences likely stem from variations in additive molecular structure and charge, which affect resin-enzyme interactions under different conditions. The findings demonstrate that appropriate additive selection and process optimization can effectively promote enzyme immobilization on organic adsorption carriers, providing a valuable reference for future industrial enzyme immobilization research.

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Note: Figure translations are in progress. See original paper for figures.