Study on co-immobilized cellulase by carbon nanotubes and sodium alginate

Lijuan Li[1,*], Guiping Ma[1], Wenjing Xia[2], Yueyu Ma[1], Yuelin Chen[1]

[1]Department of Medical Examination, Wulanchabu Medical College, Wulanchabu, Inner Mongolia 012000, China. [2]Department of Biological Engineering, Nanjing Normal University Taizhou College, Taizhou, Jiangsu 225300, China.

Received: May 16, 2019; **accepted:** July 2, 2019.

Cellulase has been potentially applied in ethanol production, extraction of medicinal ingredients, food, brewing, oil exploration, environmental protection, and other industries. However, the widespread use of cellulase is limited by its relatively high production costs and low biological activity. In this study, multiwalled carbon nanotubes and sodium alginate were used as carriers to co-immobilize enzyme for the first time. The objective of this study was to provide a method for immobilizing enzyme that allowed the enzyme to be reused in multiple cycles, reducing production costs, and overcoming technical bottlenecks. The conditions for co-immobilization of enzyme with multiwalled carbon nanotubes and sodium alginate were studied. An orthogonal experimental design was applied to optimize critical conditions for the immobilization process. The results showed that the optimum process conditions for immobilized enzyme were as follows: enzyme concentration of 3 mg/mL, temperature of 40°C, and pH of 3.0. The research provides a new method for immobilizing cellulase and a basis for industrial manufacturing processes.

Keywords: carbon nanotubes; sodium alginate; enzyme activity; immobilization yield; co-immobilized enzyme activity.

***Corresponding author:** Lijuan Li, Department of Medical Examination, Wulanchabu Medical College, Wulanchabu, Inner Mongolia 012000, China. Phone: +86 13624741848. E-mail: lilij2002@126.com.

Introduction

The immobilized enzyme technology has been utilized in binding or limiting an enzyme to a particular surface, yet still allowing it to catalyze a reaction and reuse [1]. In most cases, the storage stability and reusability of an immobilized enzyme is higher than that of a free enzyme, which can solve the problems of low biological activity and high production costs. Researchers typically immobilize enzymes via covalent and non-covalent crosslinking. Covalent crosslinking can maintain the activity of the enzyme, and moreover, this kind of bond is durable, yet the structure of the enzyme can be vulnerable to damage [2]. Non-covalently cross-linked immobilization is typically conducted by physical adsorption, which can retain characteristics of the enzyme and carrier by π-π hydrophobic and electrostatic bonds [3]. These types of interactions normally help retain the characteristics of the enzyme and carrier. However, due to the weak forces of non-covalent crosslinking, the immobilized enzyme gradually unbinds from the carriers after repeated use [4]. In contrast to covalent crosslinking, the carriers can be reused, which can further reduce the cost of immobilized enzyme systems. Most of the reported immobilized enzymes show higher thermal stability and a wider pH range than that of free enzymes [5].

Current carrier materials used for immobilizing enzyme include nanomaterials [6], natural polymers [7], mesoporous materials [8], and magnetic materials [9], in which nanomaterials and natural polymers have both attracted much attention in the scientific community as they are easy to prepare and the lower costs.

Nanoscale materials used for immobilized enzyme include carbon nanotubes (CNTs) [10], nanoporous gold [11], copper [12], and graphene oxide [13]. Natural polymers are easy to obtain and show good biocompatibility. They can be used to improve the stability and efficiency of enzyme as well as the carrier materials including alginate, chitosan, chitin, and starch [14, 15] for immobilized enzyme.

CNTs have shown excellent chemical stability, biocompatibility, and dispersibility [16, 17], as well as larger specific surface area [18], higher concentrations of enzyme loading [19], easier preparation, and low cost [16]. Therefore, the most promising prospect for immobilized enzyme has been showed. There are two types of CNTs used for immobilized enzyme, multiwalled carbon nanotubes (MWCNTs) and singlewalled carbon nanotubes (SWCNTs) [20]. MWCNTs are formed by several graphite sheets curled around a central axis. Comparing to SWCNTs, MWCNTs can load more enzyme and have better physical and chemical stability, lower price, easier to obtain, and lower toxicity, which are more widely used than SWCNTs formed with only one sheet of graphite flake curled around a central axis. Due to specific gel properties, sodium alginate can be added to calcium chloride solution to form microspheres, thereby improving mechanical strength and flexibility, which is also widely used to immobilize enzyme [21, 22]. However, MWCNTs or sodium alginate used as carriers to immobilize enzyme directly has high leakage rates of enzyme. Although the leakage rates can be reduced through the introduction of functional groups, the processes are complex and tend to increase the cost of biocatalysis [10]. In this research, MWCNTs and sodium alginate co-immobilized cellulase were studied. The objective of this investigation was to provide a simple, reusable, and inexpensive method to immobilize enzyme. The conditions of immobilization were optimized in terms of enzyme concentration, immobilization time, temperature, and pH to obtain the optimal immobilization effect.

Materials and Methods

Co-immobilized enzyme by MWCNTs and sodium alginate

75 mg of Trichoderma cellulase (10 U/mg) (Shanghai Ryon Biological Technology, Shanghai, China) was added to 25 mL citrate-Na_2HPO_4 buffer (50 mmol/L, pH 5.0) followed by 20 mg of MWCNTs with an outer diameter of 15-30 nm and a length of 1.5 μm (Shenzhen Nanotech Port, Shenzhen, China). The solution was shaken in a 40°C water bath at 200 rpm for 3 h [23]. 100 mL of 3.5% sodium alginate (Sinopharm Chemical Reagent, Beijing, China) was added with continuous mixing. The mixture was drawn with a 5-mL syringe to 50 mL, and 2% $CaCl_2$ (stock solution concentration) was injected to form smooth globules immediately. Subsequently, the globules were filtered by a stainless-steel filter spoon, and the $CaCl_2$ solution was replaced with static hardening in a 4°C refrigerator for 2 h. The globules were filtered out again, and the water surface was blotted with a filter paper. The globules were placed on the filter paper, and the filter paper were replaced 3-4 times until the water was blotted from the surface of the globules [24].

Determination of enzyme activity

Carboxymethyl cellulose (CMC) (Sinopharm Chemical Reagent, Beijing, China) was used as a substrate. The 3,5-dinitrosalicylic acid (DNS) method [25] was employed to determine the enzyme activity. First, 1.0 mL of citrate-Na_2HPO_4 buffer (50 mmol/L, pH 4.8) and 0.5 mL of 1% CMC were added into a 25 mL graduated test tube. Then, 0.5 mL (U/mL) of enzyme solution was added and the tube was put in a 50°C water

bath for 30 min. 3 mL of DNS was added afterward and the tube was put in boiling water bath for 5 min. Subsequent to cooling, distilled water was added to maintain a constant volume of 25 mL. The absorbance of the 3-amino-5-nitrosalicylic acid was detected via a colorimetric method at 540 nm (0.5 ml distilled water was used to replace the enzyme solution as the blank control). The reducing sugar produced by the hydrolysis of cellulose can reduce DNS to 3-amino-5-nitrosalicylic acid, thus the product was red under alkaline conditions and has a maximum absorbance at 540 nm. The optical density was proportional to the reducing sugar content within the linear dynamic range. The reducing sugar content was calculated via a standard glucose curve equation, the enzyme activity calculated by the following formula. The co-immobilized enzyme activity was determined by replacing 0.5 mL of enzyme solution with 0.5 mL distilled water and immobilized enzyme, the other steps were consistent with the determination of free enzyme activity.

$$\text{Free enzyme activity (U/mL)} = \frac{\text{reducing suger content (mg)} \times N}{0.18 \times t \times V}$$

$$\text{Immobilized enzyme activity (U/mg)} = \frac{\text{reducing suger content (mg)}}{0.18 \times t \times g}$$

Where t is the reaction time of enzyme and substrate; V is the volume of added enzyme solution during the determination of enzyme activity; N is the dilution ratio; g is the amount of added immobilized enzyme; and the factor 0.18 represents that 1 μmol glucose is equivalent to 0.18 mg glucose.

Determination of enzyme immobilization rate

The total activity of the free enzyme added during the immobilization process (M_0) and the activity of enzyme in the supernatant after immobilization (M_1) were detected. The immobilization rate was calculated as:

$$\frac{M_0 - M_1}{M_0} \times 100\%$$

Effect of enzyme concentration on co-immobilized enzyme activity and immobilization yield

At 30°C and pH 5.0, the co-immobilized enzyme activity and immobilization yield were measured by altering enzyme concentrations (mg/mL) of 1, 2, 3, 4, 5, and 6.

Effect of pH on co-immobilized enzyme activity

The activity of co-immobilized enzyme was measured at 50 mmol/L with citrate-Na_2HPO_4 buffer with different pH values as 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 in a 50°C water bath.

Effect of temperature on co-immobilized enzyme activity

The co-immobilized enzyme activity was measured by altering water bath temperatures at 30°C, 40°C, 50°C, 60°C, and 70°C, respectively.

Results and discussion

Effect of enzyme concentration on co-immobilized enzyme activity and immobilization yield

Figure 1 showed that the activity of co-immobilized enzyme gradually increased with the increasing enzyme concentration. The increased activity of the co-immobilized enzyme is due to the large number of pores on the surface of the MWCNTs, which can absorb cellulase. The sodium alginate can perform the similar function. Meanwhile, Figure 1 also showed that the immobilization yield of enzyme gradually decreased with the increasing enzyme concentration, which indicated that the enzyme loss rate increased. These results determined that an enzyme concentration of 3 mg/mL was optimal for further studies.

Effect of pH on co-immobilized enzyme activity

pH also has a significant effect on enzyme activity as it can affect the dissociation and charge of proteins. At 30°C and an enzyme concentration of 3 mg/mL, the effect of pH was studied on the co-immobilized enzyme activity. Figure 2 showed that enzyme loading was the

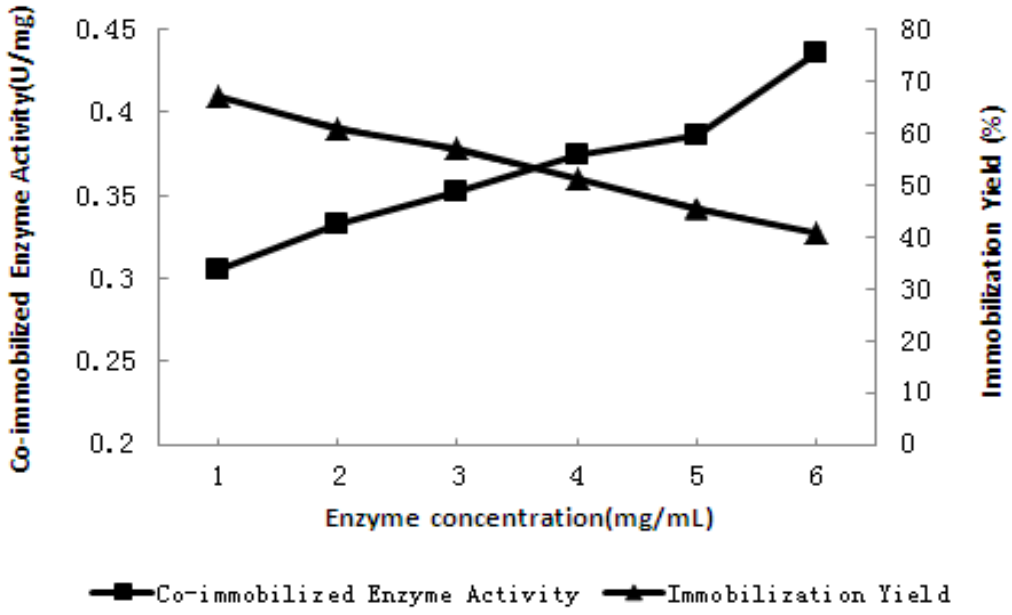

Figure1. Effect of enzyme concentration on co-immobilized enzyme activity and immobilization yield.

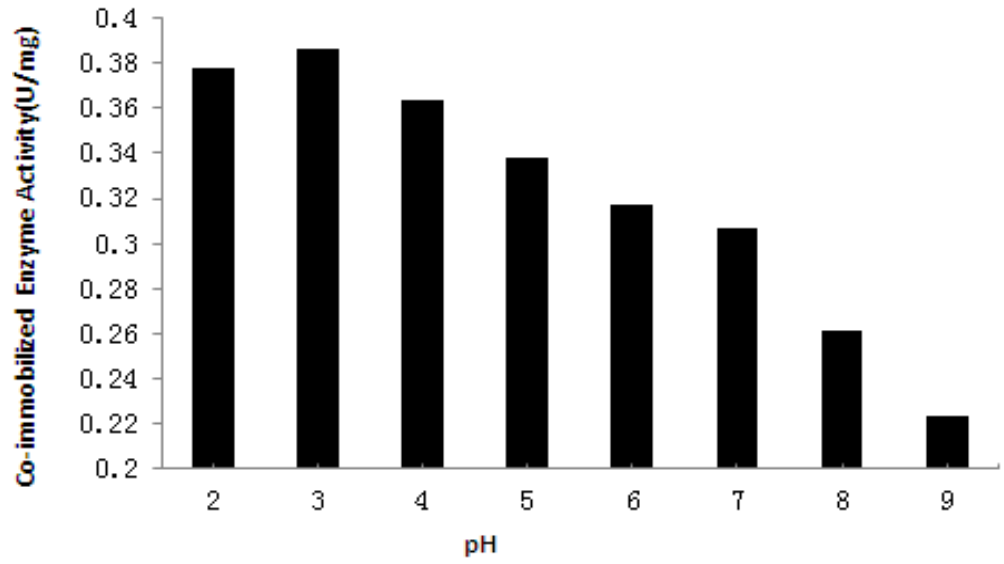

Figure 2. Effect of pH on co-immobilized enzyme activity.

highest at an optimal pH of 3 with an enzyme activity of 0.386 U/mg. When the pH is greater than 7.0, the ionic groups on the surface of the enzyme have strong electrostatic repulsions, which leads to changes in the enzyme activity center and will reduce the overall enzyme activity. The results were similar to previous studies [26, 27].

Effect of temperature on co-immobilized enzyme activity

The temperature has a great influence on enzyme activity because enzyme activity is inhibited at low temperature and the enzyme is denatured at high temperature. Figure 3 showed that the co-immobilized enzyme activity was high at the temperature of 40°C, and gradually

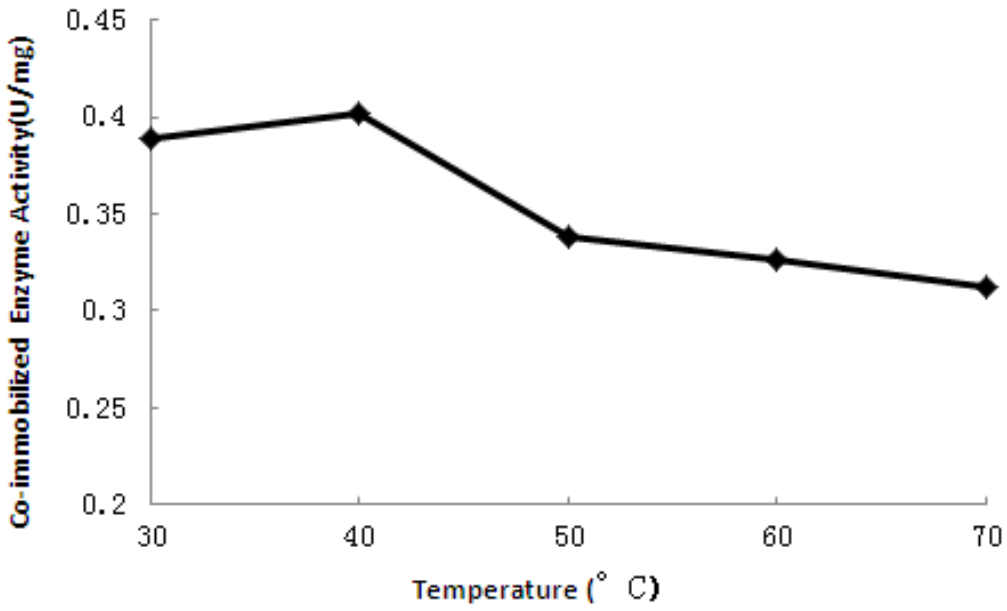

Figure 3. Effect of temperature on co-immobilized enzyme activity.

Table 1. Orthogonal experimental factors table.

Serial number	Temperature (℃)	pH	enzyme concentration (mg/mL)	co-immobilized enzyme activity (U/mg)
1	30	2	2	0.365
2	30	3	3	0.391
3	30	4	4	0.387
4	40	2	4	0.383
5	40	3	3	0.401
6	40	4	2	0.377
7	50	2	4	0.323
8	50	3	2	0.284
9	50	4	3	0.316
Mean value 1	0.381	0.506	0.342	
Mean value 2	0.536	0.359	0.512	
Mean value 3	0.308	0.360	0.370	
range	0.228	0.147	0.170	

declined as the temperature was increased. At a high temperature, the enzyme structure changed, and the enzyme active center was affected, and thereby reduced enzyme activity. Therefore, the optimum temperature of immobilized enzyme is 40°C.

Optimization of preparation conditions for co-immobilized enzyme

The activity of co-immobilized enzyme was studied in the context of enzyme concentration, temperature, and pH. The experiment was designed based on the orthogonal table of L9 (3^4). Table 1 shows that co-immobilized enzyme activity is the highest, 0.401 U/mg, at condition 5. Therefore, the optimal process conditions for co-immobilized enzyme are enzyme concentration of 3 mg/mL, temperature of 40°C, and pH of 3. According to the orthogonal factor

test, the orders of factors were temperature > enzyme concentration > pH.

Conclusion

With MWCNTs and sodium alginate as carriers, the optimal process conditions for co-immobilization of enzyme by physical adsorption are enzyme concentration of 3 mg/mL, temperature of 40°C, and pH of 3.0. The optimal process conditions showed that co-immobilized enzyme activity was 0.401 U/mg. The carrier materials, MWCNTs and sodium alginate, have changed the immobilization method of the enzyme, which reduces the number of steps in the process and can subsequently reduce production cost. Co-immobilized enzyme by multi-walled carbon nanotubes and sodium alginate was studied for the first time. The results of this study provide a new method for immobilizing enzyme and a basis for industrial manufacturing processes.

Acknowledgement

The authors are grateful to the Education Department of Inner Mongolia Autonomous Region for the financial support provided for this work. This work was financially supported by the scientific research project of the Inner Mongolia Autonomous Region Education Department Higher Education Institutions of China (No. NJZY17574).

References

1. Liu XJ, Li T, Huo ZQ, et al. 2011. Research status and application of cellulose. Anhui J Agr Sci. 39(4):1920-1924.
2. Mahsan K, Isthier C, Cheng JJ, et al. 2014. Immobilization of endo-inulinase on non-porous amino functionalized silica nanoparticles. J Mol Catal B-Enzym. 104:48–55.
3. Koji M, Takeshi S, Toshiya O, et al. 2006. Selectivity of watersoluble proteins in single-walled carbon nanotube dispersions. Chem Phys Lett. 429(4–6):497-502.
4. Feng W, Ji PJ. 2011. Enzymes immobilized on carbon nanotubes. Biotechnol Adv. 29:889–895.
5. Wan XM, Zhang C, Yu DH. 2015. Enzyme immobilized on carbon nanotubes. Prog Chem. 27(9):1251-1259.
6. Raghvendra AB, Nanasaheb DT, Shivaji HP, Shivaji HP. 2016. Immobilization of cellulase on functionalized cobalt ferrite nanoparticles. Korean J Chem Eng. 33(1):216–222.
7. Andriani D, Sunwoo C, Ryu HW, et al. 2015. Immobilization of cellulase from newly isolated strain Bacillus subtilis TD6 using calcium alginate as a support material. Bioproc Biosyst Eng. 1(1-2):29-33.
8. Zhang DZ, Hisham EH, Yuri L, et al. 2016. Immobilization of cellulose on a silica gel substrate modified using a 3-APTES self-assembled monolayer and James Palmer. Springer Plus. 5(1):48-68.
9. Han J, Rong JH, Wang Y, et al. 2018. Immobilization of cellulase on thermo-sensitive magnetic microspheres: improved stability and reproducibility. Bioproc Biosyst Eng. 41(7):1051–1060.
10. Tais BG, Caroline TW, Rodrigo K, et al. 2014. Carbon nanotubes as supports for inulinase immobilization. Molecules. 19:14615-14624.
11. Yan XL, Wang X, Zhao P, et al. 2012. Xylanase immobilized nanoporous gold as a highly active and stable biocatalyst. Micropor Mesopor Mat. 161:1-6.
12. Qiu HJ, Lu L, Huang XR, et al. 2010. Immobilization of horseradish peroxidase on nanoporous copper and its potential applications. Bioresource Technol.101:9415 –9420.
13. Seyed Z, Aldin SS, Fatemeh B, Mohammad RM, et al. 2018. Immobilization of lactoperoxidase on graphene oxide nanosheets with improved activity and stability. Biotechnol Lett. 10:1-11.
14. Urszula G, Katarzyna HK, Ariel M, et al. 2014. Enhancement of biodegradation potential of catechol 1,2-dioxygenase through its immobilization in calcium alginate gel. Electron J Biotechn. 17:83–88.
15. Abd El-Ghaffar, Hashem. 2013. Calcium alginate beads encapsulated PMMA-g-CS nano-particles for chymotrypsin immobilization. Carbohyd Polym. 92:2095-2102.
16. Zahra R, Abbas AK, Farzaneh V, et al. 2014. Facile surface functionalization of multiwalled carbon nanotubes bysoft dielectric barrier discharge plasma: Generate compatible interface for lipase immobilization. Biochem Eng J. 90:16-26.
17. Lee YM, Kwon OY, Yoon YJ, et al. 2006. Immobilization of horseradish peroxidase on multi-wall carbon nanotubes and its electrochemical properties. Biotechnol Lett. 28:39-43.
18. Irina MA, Constantin A. 2015. The biocomposite screen-printed biosensor based on immobilization of tyrosinase onto the carboxyl functionalised carbon nanotube for assaying tyramine in fish products. J Food Eng. 149:1–8.
19. Pang R, Li MZ, Zhang CD. 2015. Degradation of phenolic compounds by laccase immobilized on carbon nanomaterials: Diffusional limitation investigation. Talanta. 113:38-45.
20. Wang L, Xu R, Chen Y, et al. 2011. Activity and stability comparison of immobilized NADH oxidase on multi-walled carbon nanotubes, carbon nanospheres and single-walled carbon nanotubes. J Mol Catal B-Enzym. 69:120-126.

21. Gul O, Esra G. 2010. Production of aroma esters by immobilized Candida rugosa and porcine pancreatic lipase into calcium alginate gel. J Mol Catal B-Enzym. 64:140-145.
22. Benjamas C, Prawit J, Aran HK. 2009. Optimizing an alginate immobilized lipase for monoacyl glycerol production by the glycerolysis reaction. J Mol Catal B-Enzym. 59:206- 211.
23. Zhou H, Qu YY, Kong CL, et al. 2014. Catalytic performance and molecular dynamic simulation of immobilized C-C bondhydrolase based on carbon nanotube matrix. Colloids Surf B Biointerfaces. 116:365-371.
24. Zhao LG, Li LJ, Wang P. 2007. Immobilization of β- Glucosidase by Sodium Alginate. Chinese J Bioproc Eng. 5(4):25-31.
25. Zhou Z. 2008. Enhancement in hydrolysis of corncob by cellulase with the addition xylanases. Nanjing Forest Univ.
26. Mubarak, Wong, Tana. 2014. Immobilization of cellulase enzyme on functionalized multiwall carbon nanotubes. J Mol Catal B-Enzym. 107:124–131.
27. Sławomir B, Aurelia Z, Katarzyna SS. 2013. Alkaline lipase from Pseudomonas fluorescens non-covalently immobilised on pristine versus oxidised multi-wall carbon nanotubes as efficient and recyclable catalytic systems in the synthesis of solketal esters. Enzyme Microb Tech. 53:263-270.