Bioprocess Biosyst Eng (2017) 40:1–7
DOI 10.1007/s00449-016-1669-7

ORIGINAL PAPER

Immobilization of proline-specific endoprotease on nonporous silica nanoparticles functionalized with amino group

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Received: 17 April 2016 / Accepted: 8 August 2016 / Published online: 22 August 2016

Abstract Enzyme immobilization is believed to provide an excellent base for increasing environmental tolerance of enzyme and considerable period of time. In this work, a kind of nonporous silica nanoparticles functionalized with amino group was synthesized to immobilize proline-specific endoprotease (PSEP). PSEP is known to specifically cleave peptides (or esters) at the carboxyl side of proline, thus can prevent the formation of haze and prolong the shelf life of beer. After immobilization, the environmental tolerance (temperature and pH, respectively) was obviously improved, and the immobilized enzyme can retain above 90 % of its original activity after 6 uses. Moreover, the immobilized enzyme can effectively prevent the formation of chill-haze using fresh beer fermentation liquid.

Keywords Enzyme immobilization · Proline-specific endoprotease · Enzymatic activity · Beer · Chill-haze

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Introduction

Beer is a complex mixture of over 450 constituents, which contains macromolecules such as proteins, nucleic acids, poly-saccharides, and lipids. Due to the complicated component, the stability of beer is relatively weak, and cloudy precipitation can be easily formed during the storage. It is observed that the chill-haze in beer is mainly formed by the non-covalent bond (hydrophobic effects) between sensitive protein and polyphenol. The permanent haze forms in the same manner initially, but covalent bonds (hydrogen bonding) are soon formed and insoluble complexes are created which cannot dissolve when heated [1–3]. In the protein–polyphenol aggregate, the haze-active protein plays an important role. The special protein contains rich proline segments which can easily cause turbidity and precipitation because of the easy formation of hydrogen bond between the ketonic oxygen in pyrrole ring of proline and specific polyphenols [4–8]. In the traditional brewing industry, the major part of the protein–polyphenol complexes is precipitated out by cooling the fermentation liquid during beer maturation. In the subsequent clarification process, the haze forming reaction can be prevented by removing either the haze-active protein component with silica gel, or the remaining polyphenol component with PVPP (polyvinylpolypyrrolidone) [4, 9–11].

Although PVPP and silica gel has been commonly used in brewing industrial, their application as a treatment does have a number of disadvantages. For example, PVPP has the problems in the high capital costs for regeneration and the inherent lowering of the natural antioxidant potential of the beer; for silica gel, its protein absorbing capacity is limited and the regeneration is also a headache during the industrial use.

In recent years, researchers tried to develop alternative means to prevent chill-haze formation in beer. Among them, the application of proline-specific endoprotease (PSEP) in beer is an effective method [12]. PSEP [EC.3.4.21.26] is a kind of restriction enzyme, which exhibits narrow substrate specificity and is capable of preferentially cleaving peptides (or esters) at the carboxyl side of proline: [12]

$$Y-\mathrm{Pro}-Y',$$

where *Y* stands for a peptide or protected amino acid while *Y*' stands for an amino acid (except proline), peptide, amide or aromatic amine or alcohol [13]. It is known that haze-active proteins contain significant amounts of proline while the proteins which lack proline form little or no haze when adding polyphenols. So adding appropriate PSEP in beer can effectively prevent the formation of chill-haze [12–14]. Otherwise, because the proteins involved in foam formation are known to have only low proline contents, such an enzyme treatment would hardly affect the foam stability of beer [15–18].

Due to the low content of PSEP in organisms, to extract PSEP directly from tissue is cumbersome and inefficient, which lead to high cost thus hinder their industrial application. However, Immobilization technology of enzyme is an effective measure to solve this problem. Furthermore, fixing enzyme on an appropriate carrier can also improve the enzymatic utilization and operational stability, and overcome the drawbacks of highly environmental sensitivity and the difficulties in recovery and recycling [19–21].

In recent years, a broad variety of enzyme immobilization protocols have been developed, involving physical or chemical methods [20, 22–24]. Among them, chemical covalent method refers to the formation of chemical covalent bond between the active functional groups of amino acid residues on the enzyme molecule and the solid support surface. Compared with the non-covalent immobilization method based on adsorption, the covalent binding between the enzyme and carrier is much more firm, and has good stability and reusability, thus become a frequently used enzyme immobilization method. Although chemical methods for enzyme immobilization often result in the decreasing of enzymatic activity, the reaction conditions can be controlled as mild as possible to weaken or avoid this drawback.

Herein, we synthesized a kind of nonporous silica nanoparticles functionalized with amino group (SiNPs-NH_2) with diameter of about 250 nm. The SiNPs-NH_2 was used to immobilize PSEP through the chemical crosslinking method with glutaraldehyde (GA). Interestingly, it was found that the immobilized PSEP could exhibit better environmental tolerance and reusability, which is crucial for the industrial application of immobilized enzyme. Moreover, the immobilized enzyme could effectively improve the non-biological stability of beer.

Materials and methods

Materials

PSEP from *Aspergillus niger* was purchased from Aladdin and provided from Tsingtao Brewery Company Limited. Silica was also offered by Tsingtao Brewery Company Limited. *N*-benzyloxycarbonyl-glycyl-prolyl-p-nitroanilide (Z-Gly-Pro-pNA) was obtained from Sigma-Aldrich, Co., Ltd. Tetraethylorthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), and GA were provided from Sinopharm Chemical Reagent Co., Ltd. All other reagents were of analytical grade from Sinopharm Chemical Reagent Co., Ltd. and used without further purification.

Preparation of the nonporous SiNPs-NH_2 material

Amino-functionalized silica nanoparticles (NPs) were synthesized in a reaction mixture of deionized water (DI water), ethanol, and ammonium hydroxide. In detail, 15 mL ethanol, 1.3 mL DI water, and various ammonium hydroxide (0.4, 0.6, 1.2, 1.8 mL) were added in a 100 mL of round-bottom flask and stirred for 15 min. Then, 0.25 mL TEOS and 0.05 mL APTES were fully blended and quickly added into the mixture, leaving them gently stirring overnight at room temperature (15 °C). After reaction, the solid product was separated by centrifugation, and washed them with ethanol and water until that the centrifugal liquid was neutral. Finally, the solid was dried at 40 °C in a vacuum drying oven.

Next, the primary amino groups on the SiNPs-NH_2 carrier were modified with GA molecules to effectively combine with enzyme. In detail, 0.6 g of the amino-functionalized silica NPs was dispersed in 3 mL of pH 7 buffer solution, and then 2 mL of 25 % (v:v) GA was then added in the dispersion solution, which was stirred overnight at room temperature. Samples were then centrifuged and washed with DI water four times to remove the excess of GA. Finally, the solid was dried at 40 °C in a vacuum drying oven.

Immobilization of enzyme on silica NPs

For the immobilizing procedure, 50 mg of carriers was added into 5 mL of 10 mg/mL PSEP solution (pH 5 of acetate buffer solution, 0.1 M), stirring gently for 12 h in an ice bath, followed by being centrifuged at 7000 rpm for 10 min. The solid samples were centrifuged and

rinsed alternately three times with the same buffer solution to remove the nonspecific adsorbed enzymes. All the supernatant obtained was collected to determine the enzyme loading. The amount of the immobilized enzyme was evaluated according to Lowry method by the enzyme concentration change of the solution before and after loading [25].

Activity assay

The enzymatic activities of free or immobilized PSEP were evaluated using Z-Gly-Pro-pNA as a substrate as described by Lopez [12]. Z-Gly-Pro-pNA was dissolved in 1,4-dioxane (40 %, v/v in water) at 60 °C to give a 2 μM solution. The reaction products were monitored spectrophotometrically at 410 nm. All the activities mentioned in this paper were relative activity. When investigating the effects of temperature and pH on the activities of free and immobilized PSEP, the highest activity of each enzyme under its optimal conditions was taken as a reference, that is, 100 %. The activity comparison of the free and immobilized enzyme was carried out at the optimum conditions of themselves, and the activity of free PSEP was set to 100 %. In the reusability experiments, the first run activity of the immobilized enzyme was taken as 100 %.

Beer haze test

Fresh beer fermentation liquid was taken out from fermentation tank and filtered to remove the yeast cells. 0.2 g silica was added into 1 L fermentation liquid after filtering and stirred for 15 min at room temperature. The mixture was centrifuged for 5 min with 8000 rpm. 600 μL of 50 mg/mL immobilized PSEP dispersion was added into 1 L fermentation liquid after filtering and stored in 37 °C constant temperature bath for 1 h. Then the mixture was centrifuged for 5 min with 8000 rpm.

The turbidity of the beer fermentation liquid, the fermentation liquid absorbed by silica and the fermentation liquid handled by immobilized PSEP as well as three samples stored at 0 °C for 48 h were analyzed by a turbidity instrument (HZ013, Denmark).

Characterizations

UV/Vis spectra were recorded on a UV-250 spectrophotometer. Scanning electron microscopy (SEM) images were recorded on a Hitachi S-4800 equipment. Prior to image, the samples were sputter-coated with gold thereby making the samples conductive.

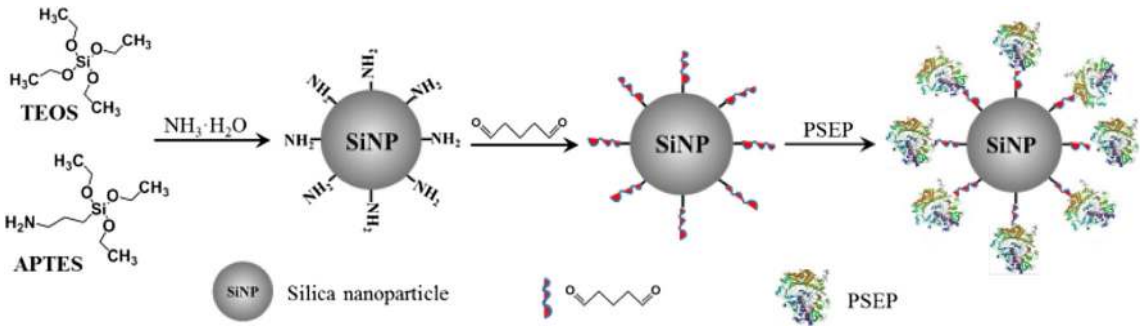
Results and discussion

Preparation of carrier and PSEP immobilization

The proposed schematic of the preparation for the nonporous SiNPs-NH_2 and the immobilization of PSEP on the NPs were shown in Scheme 1. First, SiNPs-NH_2 was synthesized in the mixture of DI water, ethanol, and ammonium hydroxide with TEOS and APTES as precursors. Then, GA as a crosslinker was connected to the amino-functionalized silica NPs to form covalent linkages which could act as an anchor for the enzyme immobilization. At last, enzyme was immobilized through the covalent bond between the active amino of amino acid residues on the PSEP molecules and aldehyde group on the solid support surface.

The impact of the various ratio of ammonia and water (v:v) during the reaction on the morphology and size of SiNPs-NH_2 was investigated and shown in Fig. 1. It was found from Fig. 1a–c shows that the size of the SiNPs-NH_2 was increasing with the increase of the ratio of ammonia and water. Moreover, the nanoparticles were uneven in morphology, and some of them were conglomerated together. When the ratio (v:v) of ammonia and water increased to 1.8:1.3, the morphology of SiNPs-NH_2 was rather uniform with a size around 250 nm and almost no conglomeration happened, which demonstrated that it was the optimal ratio of ammonia and water when synthesizing SiNPs-NH_2, and at this pH condition, all the nanoparticles were almost monodispersed. Monodispersed nanoparticle carriers were more beneficial for enzyme immobilization compared with the conglomerate ones.

We also investigated the effect of different proportion of TEOS and APTES on the amount of immobilized PSEP (Table 1). After SiNPs-NH_2 was synthesized, GA was linked onto the nanoparticles to immobilize enzymes. When GA was linked, the color of the nanoparticles turned pink from white, and with the increase of APTES proportion, the color deepened gradually, which suggested that the number of the amino group on the surface of the nanoparticles increased. As shown in Table 1, the amount of immobilized PSEP and the immobilization efficiency was rising with the increase of the APTES proportion. The reason was that with the increasing of the amino group on the surface of the SiNPs-NH_2, the amount of aldehyde group was also increasing when enough GA was linked, which can consequently combine more enzyme. Notably, when no APTES added, there was no amino group on the surface of SiNPs, however, still considerable amounts of PSEP (0.1678 mg/mg) were immobilized on the silica NPs carrier, which suggested that it is not only a simple covalent linkage between carrier and enzyme, but also physical



Scheme 1 Schematic diagram of carrier synthesis and enzyme immobilization

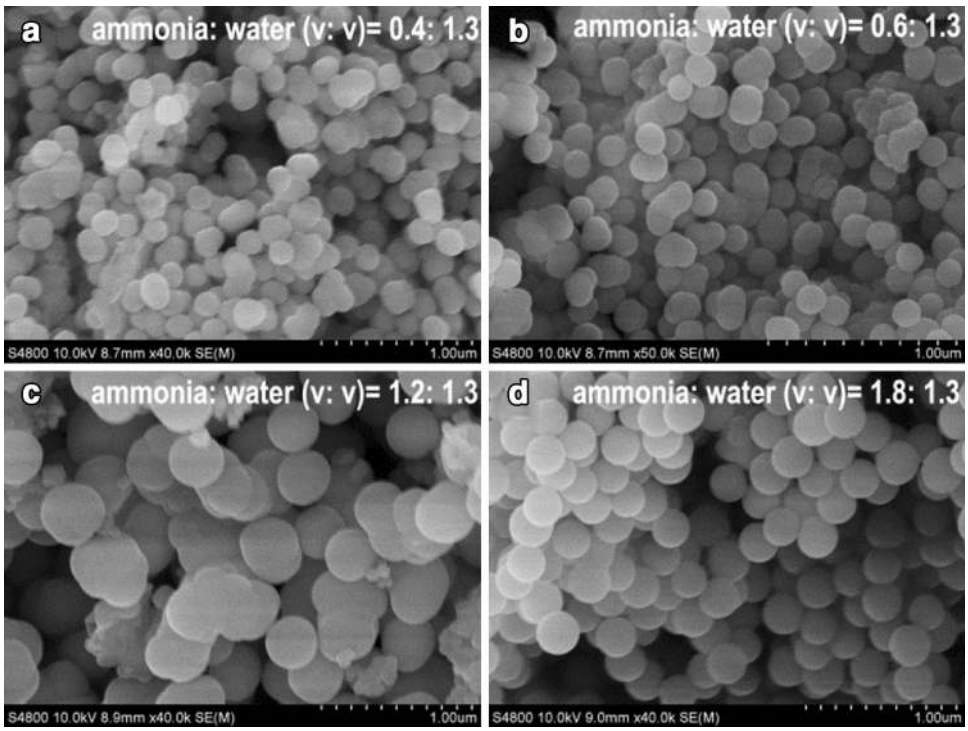


Fig. 1 SEM images of the SiNPs-NH_2 synthesized with various ratio of ammonia and water

Table 1 The effect of different proportion of TEOS and APTES on the amount of immobilized PSEP

Sample number	TEOS value (mL)	APTES value (mL)	APTES: TEOS (v:v)	Amount of immobilized PSEP (mg/mg)	Immobilization efficiency (%)
1	0.3	0	0	0.1678	16.78
2	0.25	0.05	0.2	0.3894	38.94
3	0.225	0.075	0.33	0.4006	40.06
4	0.2	0.1	0.5	0.4398	43.98
5	0.15	0.15	1	0.4576	45.76

adsorption (electrostatic effect, hydrophilic interaction, hydrophobic flocculation, etc.) exists. When the ratio of APTES: TEOS (v:v) increased from 0 to 1, the amount of immobilized PSEP increased from 0.1678 to 0.4576 mg/mg. However, APTES is more expensive than TEOS, considering the cost and enzymatic activity of immobilized PSEP, we will choose a proper ratio of APTES:TEOS (v:v = 0.5) to synthesize the nanoparticle carrier.

Because of the effects of various factors during the immobilization procedure, such as solvent, chemical bond, pH, etc., and the mass-transfer limitations in solid supports [27–29], immobilized enzyme usually exhibits lower

activity compared with free ones. Before our experiments,the activities of free as well as each immobilized PSEP were evaluated, respectively, to determine the optimal reaction conditions.

Activity assay

The activity of PSEP before and after immobilization was determined by UV/Vis spectra with Z-Gly-Pro-pNA as a substrate. PSEP is capable of preferentially cleaving peptides at the carboxyl side of proline, producing p-nitroaniline (Fig. 2, inset). As shown in Fig. 2, the substrate Z-Gly-Pro-pNA showed an absorption peak at around 313 nm while the product p-nitroaniline exhibited a main peak at about 381 nm. However, Z-Gly-Pro-pNA still has some absorbance at 381 nm, which was overlapped with the absorbance of p-nitroaniline. To completely eliminate the effect of Z-Gly-Pro-pNA, we choose 410 nm to evaluate the activity of enzyme where there was only the absorbance of p-nitroaniline but Z-Gly-Pro-pNA without.

Figure 3 shows the relative activity of the free and immobilized PSEP. The immobilized enzyme 1 to 5, respectively, corresponded to sample 1 to 5 in Table 1. As can be seen from Fig. 3, after immobilization, the enzymatic activity declined to different extent compared with free PSEP. The enzymatic activity of immobilized PSEP-1 was the lowest compared with other immobilized PSEP, which could only retain 23 % of its original enzyme activity after the immobilization. Immobilized PSEP-2 could retain 61 % enzymatic activity, and immobilized PSEP 3–5 could retain about 65 % enzymatic activity after the immobilization. Taking into account of the immobilization efficiency, the enzymatic activity of immobilized PSEP and the material costs, we chose immobilized PSEP-4 as a sample to carry out the following experiment.

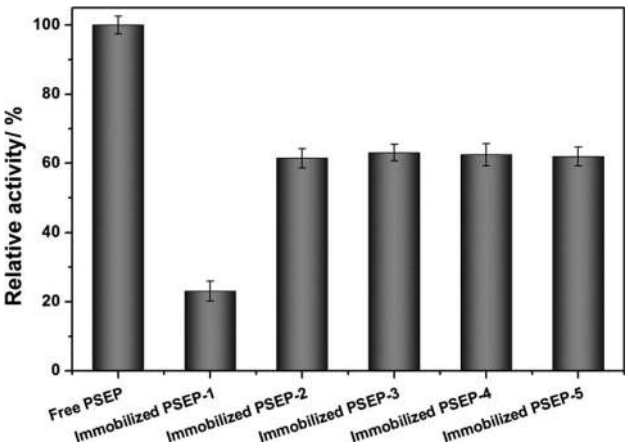


Fig. 3 Relative activities of the free and a series of immobilized PSEP. The specific activity of free PSEP was set to 100 %

Generally speaking, free enzyme is sensitive to external environment, such as temperature and pH. When using enzyme to catalyze a reaction, the reaction conditions need to be strictly controlled within a relative small range to keep its optimum activity, which is also one of the problems for the enzyme application. To immobilize enzyme on a proper carrier has the potential to change the microenvironment surrounding the enzyme molecules, thus usually can enhance the stability of enzyme to external environment. Therefore, we examined the enzymatic activities of free and immobilized PSEP under different pH and temperature conditions.

The influence of temperature on the activities of free and immobilized PSEP was shown in Fig. 4. It was found that the optimum temperature for the free PSEP was 40 °C. After immobilizing PSEP on the SiNPs-NH_2 carrier, the

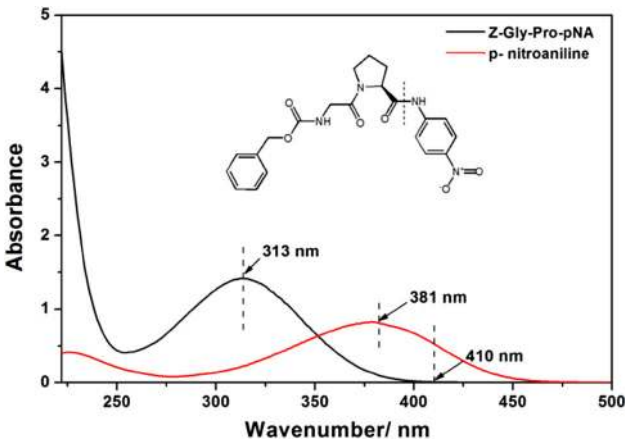


Fig. 2 UV/Vis spectra of the substrate of Z-Gly-Pro-pNA and the product of p-nitroaniline

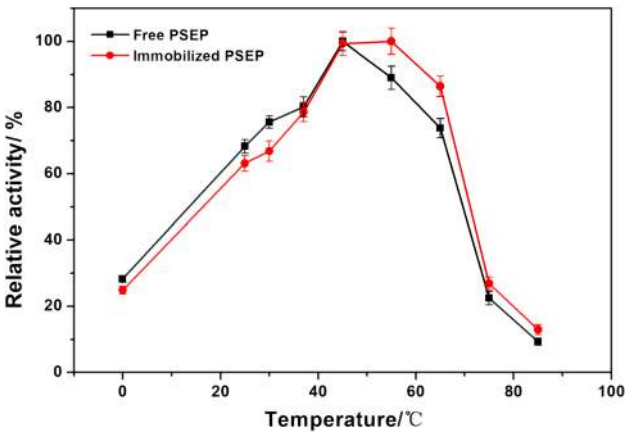


Fig. 4 Effect of temperature on the activities of free and immobilized PSEP. The highest activity of each enzyme under its optimum temperature was set to 100 %

optimum temperature was broadened to 40–50 °C. Besides, under higher temperature conditions, the immobilized PSEP could also retain relatively higher activity than the free one, which verified that the heat tolerance for the immobilized PSEP improved compared with the free enzyme. Figure 5 showed the effect of pH on the activities of free and immobilized PSEP. It is found that the optimal pH of free PSEP was pH 4.5. However, when immobilizing PSEP onto the SiNPs-NH_2 carrier, the enzyme could still retain its optimum enzymatic activity to a higher degree (more than 90 %) over a broad pH range of 3–5. This suggests that the range of pH change (pH 3–5) has less impact on the enzymatic activity of immobilized PSEP, which is preferable in the practical application. However, for the free PSEP, when the pH was below 4 or above 5.5, the enzyme activity decreased below 80 %. Moreover, at the pH range of 5.5–7, the enzyme activity of immobilized PSEP was also higher than the free one, which suggested that the ability of resistance to alkali for immobilized PSEP was stronger than the free one. In short, the optimum pH of immobilized PSEP was much broadened, and the ability of resistance to acid and alkali also got great improvement compared to its free form. In a word, after immobilization of PSEP on the SiNPs-NH_2 carrier, the enzyme possessed a good environmental tolerance (temperature and pH, respectively), which will facilitate the application of the enzyme.

Reusability

The reusability of immobilized enzyme is always taken into account for industrial application with the purpose of cost reduction. Silica nanoparticles were excellent carrier and used for a long time to immobilize enzyme. In this work, the reusability was evaluated at the optimum conditions by examining the remaining activity of immobilized PSEP after each cycle, and the immobilized enzyme could be recycled by simple centrifugal separation. Figure 6 exhibited the reusability of the immobilized PSEP, which showed that the immobilized enzyme retained above 90 % of its original activity after 6 cycle uses. The higher stability of the immobilized enzyme could be ascribed to the main covalent binding between enzymes and the carrier, which effectively prevented the enzymes detaching from the carrier. More importantly, the commendable reusability can availably reduce the cost of enzyme.

Turbidity analysis of beer samples

Subsequently, the immobilized PSEP was used to process beer fermentation liquid. Figure 7 exhibited the turbidity analysis of the beer samples treated with immobilized PSEP and silica gel,with untreated beer fermentation liquid as a comparison. As shown in Fig. 7, before refrigeration, the beer turbidity treated by immobilized PSEP and silica gel was obviously reduced, which suggested that the cold turbidity protein can be dramatically reduced in the treated beer. After refrigeration, the turbidity of the untreated beer fermentation liquid increased to about 3.1 from about 0.6 after storing the sample at 0 °C for 48 h. However, when treated with immobilized PSEP or silica gel, no obvious cold muddy phenomenon could be visually observed, and the beer turbidity only had a little rise after refrigeration compared with blank sample. The reason was that the cold turbidity protein was efficiently removed by silica gel or hydrolysed by immobilized PSEP. Therefore, the immobilized PSEP had a positive effect to improve the non-biological stability of beer, and it would have a potential application in the brewing industry.

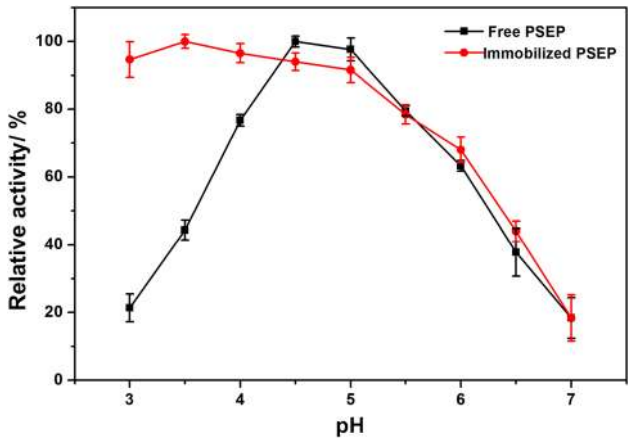


Fig. 5 Effect of pH on the activities of free and immobilized PSEP. The highest activity of each enzyme under its optimum temperature was set to 100 %

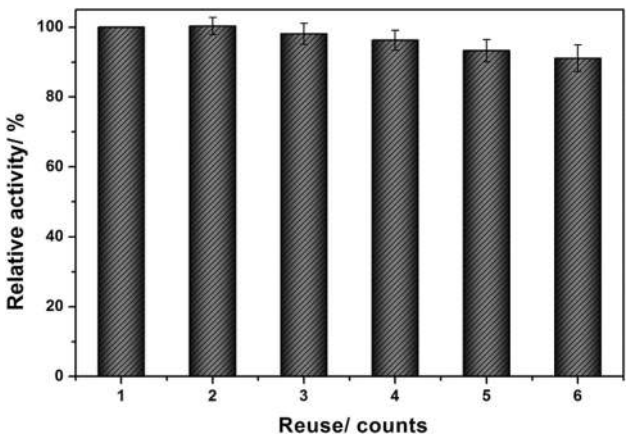


Fig. 6 Reusibility of the immobilized PSEP. The first run activity of the immobilized enzyme was taken as 100 %

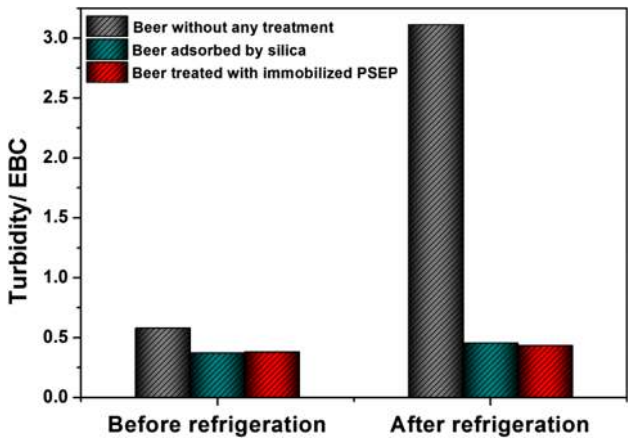


Fig. 7 Turbidity analysis of the beer samples treated with immobilized PSEP and silica gel, as well as the blank beer fermentation liquid before and after refrigeration

Conclusions

We synthesized a kind of nonporous silica nanoparticles functionalized with amino group with diameter of about 250 nm, which was first used to immobilize PSEP. The carrier had higher enzyme immobilization efficiency, and the immobilized PSEP could retain 65 % enzymatic activity compared with the free one. After immobilization of the enzyme, the environmental tolerance (temperature and pH, respectively) was obvious improved, and the immobilized enzyme could retain above 90 % of its original activity after 6 cycle uses. Moreover, the immobilized enzyme can effectively prevent the formation of chill-haze in beer sample, which rendered it a promising application value in brewing industry.

Acknowledgments This work is supported by the National Natural Science Foundation of China (Nos. 21433001, 21273260), Applied Basic Research Foundation of Qingdao City (No. 14-2-4-21-jch), and Shandong Provincial Natural Science Foundation for Distinguished Young Scholar, China (No. JQ201305).

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