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RESEARCH PAPER

Improving the biocatalytic performance of co-immobilized cells harboring nitrilase via addition of silica and calcium carbonate

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Abstract

To improve nicotinic acid (NA) yield and meet industrial application requirements of sodium alginate-polyvinyl alcohol (SA-PVA) immobilized cells of *Pseudomonas putida* mut-D3 harboring nitrilase, inorganic materials were added to the SA-PVA immobilized cells to improve mechanical strength and mass transfer performance. The concentrations of inorganic materials were optimized to be 2.0% silica and 0.6% $CaCO_3$. The optimal pH and temperature for SA-PVA immobilized cells and composite immobilized cells were both 8.0 and 45 °C, respectively. The half-lives of composite immobilized cells were 271.48, 150.92, 92.92 and 33.12 h, which were 1.40-, 1.35-, 1.22- and 1.63-fold compared to SA-PVA immobilized cells, respectively. The storage stability of the composite immobilized cells was slightly increased. The composite immobilized cells could convert 14 batches of 3-cyanopyridine with feeding concentration of 250 mM and accumulate 418 g $\cdot L^{-1}$ nicotinic acid, while the SA-PVA immobilized cells accumulated 346 g L^{-1} nicotinic acid.

Keywords Biocatalysis · Co-immobilization · Nitrilase · Inorganic materials

Introduction

Nicotinic acid (NA) belongs to the vitamin B family and is a member of the 13 vitamins necessary for the human body. NA plays an extremely important role in the production of additives for foods and feeds as well as chemical and pharmaceutical intermediates. The nitrilase can catalyze 3-cyanopyridine (3-CP) to biosynthesize NA in one step [1, 2]. To a certain extent, it alleviates problems in conventional chemical hydrolysis such as the use of strong acids, relatively low production yield and serious environmental pollution. This is in line with the development trend of green chemistry and has a wide range of prospects for industrial applications [3].

The NA supply has been unable to meet the growing demand in recent years due to the limited existing NA production capacity at present. Improving the NA production technology to expand production capacity and reduce production costs will surely attract attention from scholars and entrepreneurs in the future [4–6].

The use of cell immobilization is most suitable in biocatalysis for those cells producing intracellular enzyme and also the corresponding reaction catalyzing small-molecule substrates as the purified intracellular enzymes might lead to more activity loss than extracellular ones [7, 8]. The nitrilase is such an intracellular enzyme, and the surface of the enzyme contains thiol groups. After being purified, the thiol groups on the surface are easily reduced. And both the substrate 3-CP and the product NA are small molecules, which could easily transfer in and out of the cells. Therefore, using whole-cells as the catalyst is common to catalyze 3-CP into NA. Through further immobilization, the product separation and purification process can be simplified and the cell utilization efficiency can be improved [9, 10]. For

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improving the NA production, optimizing the production process, screening for new enzymes, improving the enzyme properties by protein engineering or enzyme immobilization were all available strategies [11–14].

Selecting an ideal immobilized cell carrier is the key to the immobilization technology. Suitable carriers should exhibit the following characteristics: the carrier should have a certain capacity to couple enough biological molecules; the role of the carrier is only to immobilize the biomolecules; the carrier should have good biocompatibility, moderate particle size and pore size structure; the carrier should be inexpensive and readily available [8]. In fact, there are few cases where only one carrier material satisfies all the above conditions. Besides, a bottleneck in practical application is that the mechanical strength of the immobilized particles is not strong enough. At present, researchers have put their eyes on composite carriers, which combines the organic carrier and inorganic carrier for immobilization [15]. This way reorganizes the performance advantages each other and make up for deficiencies in each other.

In our previous work, the sodium alginate-polyvinyl alcohol (SA-PVA) cell immobilization methods of *P. putida* mut-D3 have been established [16]. However, the SA-PVA immobilized cells still showed insufficient mechanical strength and relatively poor mass transfer performance. In this study, inorganic materials were employed to enhance the catalytic performance of SA-PVA immobilized cells. Three typical inorganic materials SiO_2, $CaCO_3$ and bentonite were used as additives for co-immobilization, mechanical mildness, mass transfer capacity and residual catalytic activity were evaluated as indices to select suitable inorganic materials.

Materials and methods

Materials

The strain *P. putida* mut-D3 used in this research was screened and constructed in our previous work [16]. The culture medium (g L^{-1}) contained: tryptone 15, yeast extract 7.5, glycerol 15, urea 1.5, NaCl 1, KH_2PO_4 2, K_2HPO_4 2, pH 7.0. The medium was sterilized at 121 °C for 20 min.

Preparation of free cell suspension

The activated strain mut-D3 was cultured at 30 °C, 120 rpm for 24 h as a seed liquid, and transferred to a fresh fermentation medium with 1% inoculum, and cultured again for 40 h in the same manner. The cells were collected at 50 mL centrifuge tubes by centrifugation (8000 rpm, 4 °C, 10 min). Then the cells were washed and then prepared into a suspension of 20 mg mL^{-1} cells with PBS buffer (100 mM, pH 7.2).

Preparation of SA-PVA immobilized cells

SA (4%) and PVA (10%) were mixed at a ratio of 9:1 as immobilization material and continuously mixed with the bacterial suspension at the ratio of 1:1. The mixture was aspirated with a syringe and slowly added dropwise into the solidification solution (0.6% $CaCl_2$ saturated boric acid solution, 4 °C) for 5 h. The prepared immobilized cells were also washed with PBS and resuspended for use.

Addition of inorganic materials

In the SA-PVA immobilized materials, SiO_2 (1.0, 1.5, 2.0, 2.5, and 3.0%), $CaCO_3$ (0.2, 0.4, 0.6, 0.8, and 1.0%) and bentonite (2, 3, 4, 5, and 6%) were added separately to investigate the effects of single inorganic material on SA-PVA immobilized cells. Then 1.5% SiO_2 + 0.6% $CaCO_3$, 1.5% SiO_2 + 0.8% $CaCO_3$, 1.5% SiO_2 + 1.0% $CaCO_3$, 2.0% SiO_2 + 0.6% $CaCO_3$, 2.0% SiO_2 + 0.8% $CaCO_3$, 2.0% SiO_2 + 1.0% $CaCO_3$, 2.5% SiO_2 + 0.6% $CaCO_3$, 2.5% SiO_2 + 0.8% $CaCO_3$, 2.5% SiO_2 + 1.0% $CaCO_3$ were added separately to investigate the effects of composite inorganic materials on the immobilized beads.

Transmission electron microscopy (TEM)

TEM was used to directly investigate the cell morphology. The copper grid was immersed in the cell suspension for 10 min and blotted dry with fresh filter paper, then 1% (w/v) uranyl acetate was used for negative staining.

Effect of pH on immobilized cells

At the reaction temperature of 30 °C, the immobilized cells were placed into different pH buffers (pH 6.0–7.0, sodium citrate buffer; pH 7.0–8.0, PBS; pH 8.0–9.0, Tris–HCl buffer) and incubated for 5 min. The reaction was then initiated and examined for their catalytic ability after the substrate was added into the reaction system.

Effect of temperature on immobilized cells

To examine the optimal reaction temperature, the immobilized cells were incubated in a metal bath at 30 °C for 5 min, then the substrate solution was added. The reaction was carried out at the water bath of 25, 30, 35, 40, 45, 50 and 55 °C, respectively. Finally, adding 2.0 M HCl to terminate the catalytic reaction and measure the enzyme activity.

Thermal stability of the immobilized cells

Composite immobilized cells were incubated in PBS (100 mM, pH 7.2) at water bath of 30, 35, 40 and 45 °C, respectively, and the residual enzyme activities were measured by the samples which were taken at regular intervals. The natural logarithm of residual catalytic activity, Ln(RA), was plotted versus time to calculate the half-life ($t_{1/2}$).

Storage stability of the immobilized cells

The appropriate amount of composite immobilized cells was stored at the refrigerator of 4 °C, and the residual catalytic activities were detected by the samples every 5 days. The same weight of the immobilized cells was taken for enzyme activity assay.

Effect of initial 3-CP concentration on immobilized cells

The 3-CP feed concentration was set at 50, 100, 150, 200, 250, and 300 mM, respectively, and added into the reaction system after incubated in a metal bath of 30 °C for 5 min. Then investigated the effect of different substrate concentrations on transformation ability according to the enzyme activity.

Enzyme assay

The nitrilase can catalyze the production of the corresponding carboxylic acids from nitrile compounds and generate by-product ammonia in an equal molar ratio. Therefore, the ammonia in the reaction system can be determined and the catalytic activity of nitrilase can be calculated. In this study, phenol-sodium hypochlorite method was employed, and blue soluble compounds were finally generated, which can be determined by an ultraviolet spectrophotometer at 630 nm [17].

Under standard reaction conditions, the catalytic activity unit (1 U) is defined as the amount of enzyme to product 1 μmol L^{-1} of ammonia per minute. The specific activity unit (U g^{-1}) is defined as the catalytic activity possessed by 1 g of cells.

The reaction was performed in a 50 mL conical flask containing 150 mM 3-CP and 100 mM PBS in a total volume of 10 mL. A suitable amount of composite immobilized cells was placed in a flask and pre-incubated at 30 °C, and the reaction was performed on an oscillating shaker at 30 °C. The free cells were used as control. Finally, 2.0 M HCl was added to the reaction system to terminate the reaction. After the reaction was completed, an appropriate amount of the conversion solution was taken for enzyme activity assay. All assays were performed in triplicate.

HPLC method

In batch conversion experiments, HPLC was used to accurately measure the NA and 3-CP concentration. HPLC (Dionex Ultimate 3000 series) conditions were as follows: column: Atlantis dC18 column (5 μm, 4.6 × 150 mm), mobile phase: acetonitrile/water (3/2, containing 0.02% trifluoroacetic acid), flow rate: 0.5 mL min^{-1}, detection wavelength: 268 nm, column temperature: 30 °C, injection volume: 10 μL.

Results and discussion

Effect of single inorganic materials

The organic polymer carrier is non-toxic, but it also exhibits several disadvantages, such as low strength and low mass-transfer performance. These disadvantages can be improved by adding inorganic materials. In terms of improving the strength of cell-immobilized beads, studies have shown that adding appropriate amounts of activated carbon, $CaCO_3$, $Ca(OH)_2$ powders and SiO_2 can not only directionally adjust the specific gravity of the immobilized beads, but also improve the compression strength [18]. Chotani et al. added inorganic materials (such as silica sand) during the PVA immobilization process to increase the mechanical strength, sediment ability, and permeability of immobilized beads [19, 20]. The investigations have also proven that the addition of 2–30% calcium silicate to PVA-immobilized materials can greatly enhance the stability of immobilized beads and the activity of cells [15]. In terms of improving the transfer characteristics of cell-immobilized beads, studies have shown that the addition of $CaCO_3$ can improve the transfer characteristics of immobilized particles. $CaCO_3$ is hard to be utilized by microorganisms on the one hand, and on the other hand, it can slowly decompose and produce CO_2 during the process of immobilization under neutral acidic conditions. The internal voids of the pellets increase, which is conducive to the proliferation of the cells, the ventilation and the water permeability inside the immobilized pellets, and the diffusion and transfer of the substrate. Microporous structures inside the immobilized spheres are crucial for cell immobilization performance and the subsequent mass transfer reactions. Wang et al. added $CaCO_3$ to provide the mass transfer so as to support the growth of immobilized cells of *Pseudomonas* sp. DG17 [21]. Liu et al. added bentonite, $CaCO_3$ and SiO_2 to improve the mechanical strength and mass transfer capacity of immobilized beads [22].

In this study, the inexpensive inorganic materials SiO_2, $CaCO_3$ and bentonite were used as additives (Fig. 1). The mechanical strength and residual catalytic activity were used as indices to examine the properties of these three materials.

From the experimental results (Fig. 2), it can be seen that as the SiO_2 concentration increased, the mechanical strength of the SA-PVA immobilized beads first increased and then decreased, while the residual activity was in a downward trend. With the increase of $CaCO_3$ concentration, both the residual activity and mechanical strength were in the trend of increasing first and then decreasing. With the increasing concentration of bentonite, the residual catalytic activity and mechanical strength were in a downward trend. Considering the results of the comprehensive experiment, this study would employ SiO_2 and $CaCO_3$ for composite immobilization.

Effect of composite inorganic materials

As can be seen from Fig. 3, with the increase of SiO_2 concentration, the mechanical strength generally increased, which indicated that SiO_2 can increase the specific gravity and

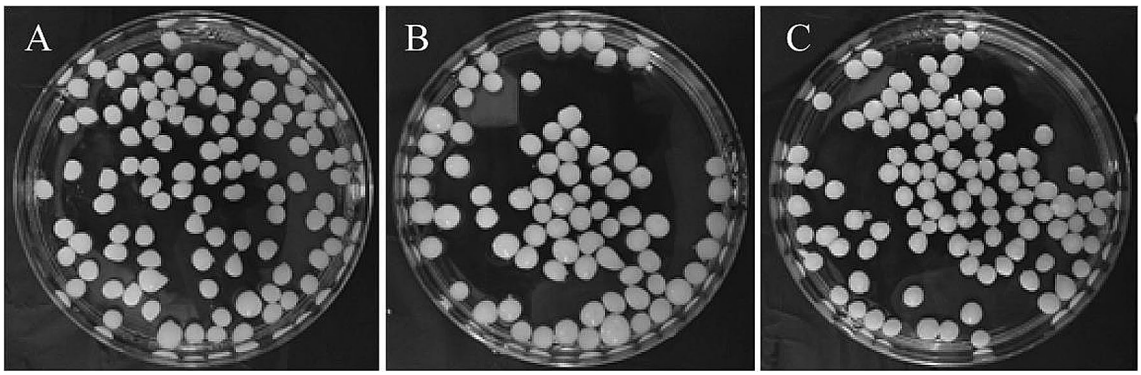

Fig. 1 Immobilized bead shape. **a** SiO_2, **b** $CaCO_3$, **c** bentonite

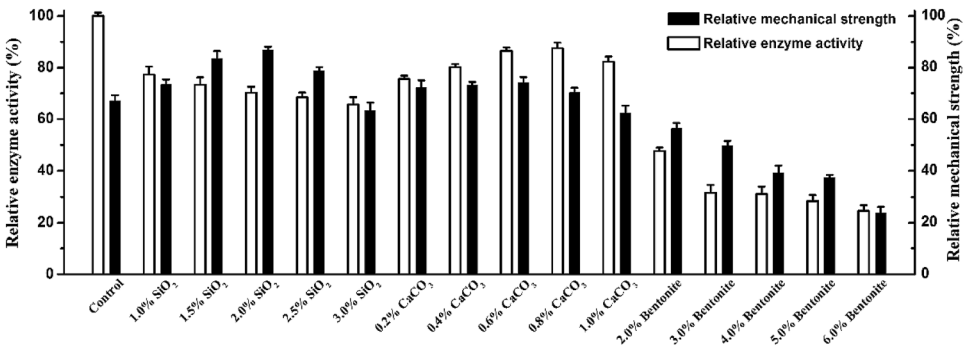

Fig. 2 Effect of single inorganic materials on the performance of SA-PVA immobilized cells

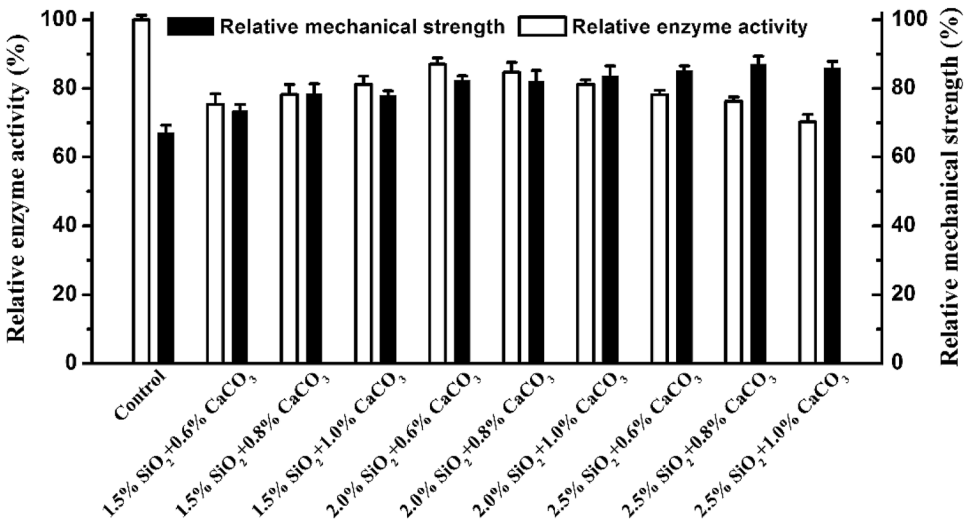

Fig. 3 Effect of composite inorganic materials on the performance of SA-PVA immobilized cells

strength of immobilized beads. However, with the increase of SiO_2 concentration, the catalytic activity first rose and then decreased. Therefore, with the mechanical strength and relative catalytic activity as indicators, 2.0% SiO_2 and 0.6% $CaCO_3$ were finally added to the SA-PVA immobilized materials.

Transmission electron microscopy (TEM)

The scanning electron microscopy image showed that the SA-PVA immobilized beads which added with 2.0% SiO_2 and 0.6% $CaCO_3$ had significantly larger and looser internal micro-pore structure (Fig. 4). It was more conducive to the attachment of microorganisms to increase the number of cells in each hole, ventilation, water permeability, and reduce the mass transfer resistance of the reaction.

Effect of pH on SA-PVA composite immobilized cells

As shown in Fig. 5, the optimal pH for both SA-PVA immobilized cells and composite immobilized cells was 8.0. Between pH 7 and pH 8, SA-PVA immobilized cells and composite immobilized cells showed little difference in catalytic ability, and they both exhibited the maximum activity at pH 8. It was consistent with the immobilized nitrilase as Zhang et al. [23] reported. However, the composite immobilized cells had better adaptability and catalytic ability under acidic and alkaline conditions.

Effect of temperature on SA-PVA composite immobilized cells

Both of the optimal reaction temperatures for SA-PVA immobilized beads and composite immobilized beads was 45 °C (Fig. 5), it was similar with the immobilized cells as Jin et al. [24] reported, which optimal temperature was 50 °C. While in the temperature range of less than 30 °C and higher than 45 °C, the composite immobilized cells exhibited superior stability to temperature stimulation than SA-PVA immobilized cells. Especially at 25 °C, the relative enzyme activity of the composite immobilized beads still remained 61%, which was 9% higher than the SA-PVA immobilized beads.

Thermal stability of the SA-PVA composite immobilized cells

The half-lives ($t_{1/2}$) at 30, 35, 40, 45 °C of the composite immobilized cells were 271.48, 150.92, 92.92, 33.12 h, respectively (Fig. 5). The half-lives of SA-PVA immobilized cells were 192.54, 111.79, 76.17, 20.32 h. The thermal stability was further improved by 1.40, 1.35, 1.22 and 1.63 times, respectively. To extend the working life and obtain the highest yield of NA, the reaction temperature in the batch conversion was still set to 30 °C.

Storage stability of the SA-PVA composite immobilized cells

The storage stability of the mut-D3 composite immobilized beads was slightly increased compared to SA-PVA immobilized cells at 10–50 days. When stored at 4 °C for 40 days, the composite immobilized cells retained 65% of the initial enzyme activity, whereas SA-PVA immobilized cells were only about 60% (Fig. 5). The storage stability was similar to that of the nitrilase immobilized with acryloyl crosslinked cellulose dialdehyde (ACCD) [25], which remained 58% (ACCD-N_{II}) of the residual enzyme activity after 30 days.

Batch conversion of 3-CP for the synthesis of NA

As shown in Fig. 6, when the initial 3-CP concentration was in the range of 150–550 mM, the ability of SA-PVA immobilized cells which added with inorganic materials to transform 3-CP was greater than that of SA-PVA immobilized cells, especially when the 3-CP concentration reaches 550 mM. At

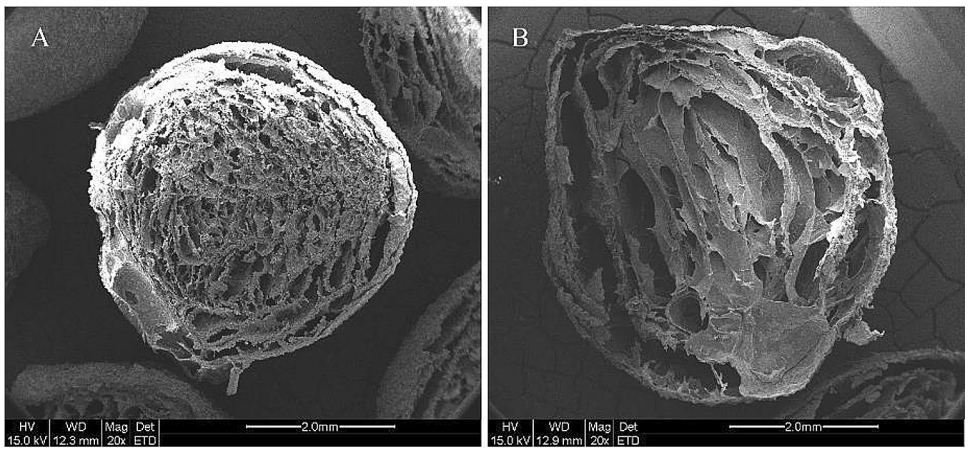

Fig. 4 Scanning electron microscopy images. **a** SA-PVA immobilized beads, **b** SA-PVA immobilized cells added with 2.0% SiO_2 and 0.6% $CaCO_3$

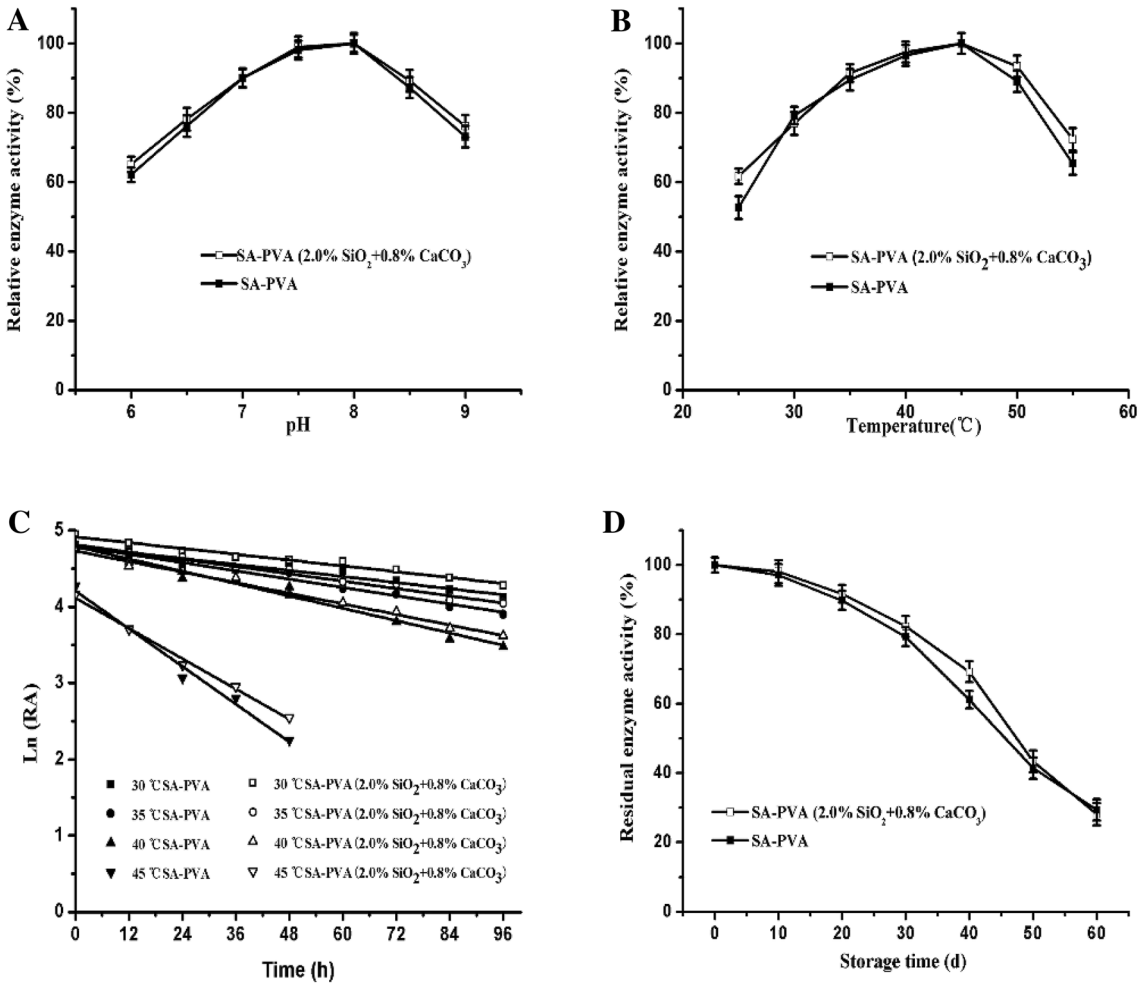

Fig. 5 Effect of pH (**a**), temperature (**b**) on the activity of SA-PVA immobilized cells added with inorganic materials and their thermal stability (**c**) as well as storage stability (**d**)

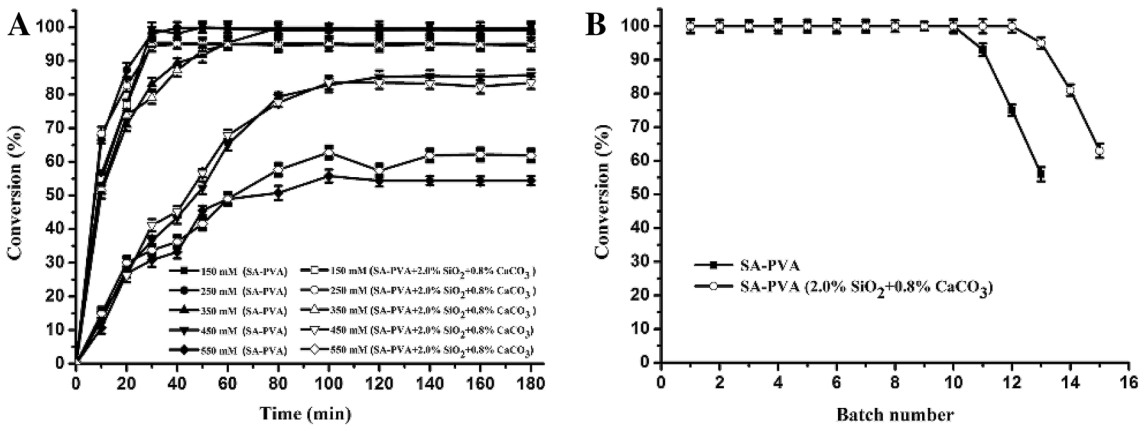

Fig. 6 Effect of initial 3-CP concentration on conversion (**a**) and batch conversion (**b**) for NA synthesis

the optimum substrate concentration of 250 mM, 14 batches of the substrate can be fully converted and 418 g L^{-1} NA can be accumulated, while the SA-PVA immobilized cells could accumulate 346 g L^{-1} NA.

Conclusion

To improve the mechanical strength, mass transfer performance and meet industrial application requirements, inorganic material was added to *P. putida* mut-D3 SA-PVA immobilized cells, and their catalytic capabilities and batch conversion were performed. The optimum concentrations of added inorganic materials were determined: 2.0% SiO_2 and 0.6% $CaCO_3$. The half-life ($t_{1/2}$) was significantly enhanced compared to SA-PVA composite immobilized cells of mut-D3. The storage stability was slightly increased composed of SA-PVA immobilized cells at 10–50 days. At the optimum substrate feeding concentration of 250 mM, 14 batches of the substrate could be converted, which contributed to the accumulation of 418 g L^{-1} NA.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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