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Invertase Activity of *Saccharomyces cerevisiae* Cells Immobilized in Gelatin Hydrogels: Kinetics, Thermostability, and Reusability

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In this work, a novel biocatalyst possessing medium feeding capability was prepared by immobilizing Saccharomyces cerevisiae cells into gelatin by crosslinking with chromium salts. Optimum chromium salt concentrations were found as 0.016 mol/dm^3 chromium III acetate and 0.008 mol/dm^3 chromium III sulfate. The developed biocatalyst was characterized with respect to its pH, temperature tolerances, kinetic properties and reusability. It was observed that immobilization shifted the pH for maximum activity from 4.6 to 7.2. Thermal stabilities below 60°C were positively affected by immobilization. V_{max} values obtained for immobilized samples were 1.85 and 1.87 μmol sucrose/mg.min, whereas the value was 0.0262 μmol sucrose/mg.min for free cells. Thus, a 70-fold increase of V_{max} was obtained by immobilization and reuse experiments showed no activity decline for 10 reuses in 28 days which were attributed to continuous cell growth of immobilized whole cells using glucose (from sucrose hydrolysis) and gelatin as nutrients.

Keywords *Saccharomyces cerevisiae*, cell immobilization, sucrose hydrolysis, invertase, gelatin, chromium salts, yeast, thermal stability

Introduction

Recently, immobilization of whole microbial cells has been recognized as practically desirable for its advantages, such as the reuse of expensive catalyst, simplification of downstream process, and the easy adaptation for continuous operation. Bioprocesses carried out with immobilized viable cells are very complex because of simultaneously occurring growth, mass transfer, and conversion of substrate processes. *Saccharomyces cerevisiae* cells that are rich in both intracellular and extracellular enzymes are good models for whole cell immobilization. The surface-immobilized proteins in the *S. cerevisiae* cells are covalently linked to glucan in the cell wall, making them resistant to extraction. Thus, in order to repeatedly use its invertase activity, either whole cell immobilization or difficult and expensive enzyme purification plus enzyme immobilization is required (1).

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Saccharomyces cerevisiae cell enzyme invertase (β-D-fructofuranosidase, E.C.3.2.1.26) is one of the enzymes attracting attention for many industrial applications. It splits sucrose into glucose and fructose (invert, syrup) and can be applied for any inversion of sucrose especially liquefied cherry centers, creams, mints, truffles, marshmallow, and invert syrup. Invertase is also used to improve the shelf life of confections. In addition, enzymatic hydrolysis using invertase avoids the production of colored by-products generated by processes involving acidic hydrolysis (2).

In order to utilize these benefits in an economic way, many attempts have been made to immobilize *Saccharomyces cerevisiae* cells. Covalent binding of yeast cells with polyethyleneimine and glutaraldehyde (3), entrapment of yeast cells with films of poly (2-hydroxyethyl methacrylate) (4), immobilization by chelation/metal-link process onto porous inorganic carriers (5), immobilization in liquid-core alginate capsules (6), immobilization on celite, and on polyacrylamide by absorption (7) are some remarkable examples for these efforts.

Selection of support material is more critical in food technology than other applications of immobilization. Gelatin has suitable chemical properties to be used in food technology. It is inexpensive and physiologically inert. Gelatin is a water soluble protein obtained from partial hydrolysis of collagen. It has carboxyl, amino, and hydroxyl functional groups. The most characteristic property of gelatin is its ability to form reversible elastic gels when chilled in water and its capability to absorb water up to 600–700% of its weight when allowed to swell in cold water.

In aqueous solutions, the molecules of amino acids behave like dipolar ions. At the isoelectric point, where pH is equal to 5, they are electrically neutral. Equilibrium between the ionized acidic and basic groups at the isoelectric point results in strong intramolecular attractions which make the gelatin molecules curl up, but at other pH values they remain stretched. This clustering or stretching out influences the physical and mechanical properties of gelatin and therefore, leads to considerable variations of the viscosity of gelatin solutions, which reaches the minimum at the isoelectric point. Gelatin hardens when reacted with some aldehydes and metal salts (8, 9). In this work, immobilization of *Saccharomyces cerevisiae* whole cells into gelatin with chromium (III) acetate (CA) and chromium (III) sulfate (CS) and the effect of pH, temperature, and incubation time on free and immobilized whole cell enzyme were investigated. We also studied the kinetics of free and immobilized *S. cerevisiae* cell invertase activity and reusability of developed biocatalyst.

Materials and Methods

Chemicals

Chromium (III) acetate, chromium (III) sulfate and sucrose (pure grade), were purchased from Merck, Darmstadt (Germany). Granular photographic gelatin was obtained from Croda Gelatin Co. (U.K.) and glucose was obtained from Sigma Chemical Company (St Louis, MO). Yeast extract, trypton, and agar were supplied by Oxoid (U.K.). All other chemicals were of analytical grade.

Strain and Growth Conditions

All the experiments were carried out with *Saccharomyces cerevisiae* ATCC 26786 strain obtained from American Type Culture Collection (USA) and were used as invertase

sources. Free cells were cultivated aerobically at 30°C in 500 cm^3 shake flasks (300 rpm), containing 200 cm^3 of medium having the following composition (w/v) 1% yeast extract, 2% trypton, and 2% glucose. The pH value was adjusted to 4.5 with a citrate buffer before sterilization was carried out for 25 min at 120°C. We traced the cells growth by measuring the optical density (OD) increase at 650 nm. The strain was subcultured at 4-month intervals on agar slant and maintained at 4°C.

Immobilization Procedure

Saccharomyces cerevisiae cells collected at mid-exponential phase ($OD_{650} = 0.500$) were immobilized by crosslinking and entrapment within gelatin. The standard cell density was 2.71 mg (dry weight) cm^{-3} of gel. For immobilization, 0.75 g gelatin was swelled in the appropriate amount of ethylenediaminetetraacetic acid (EDTA) buffer (0.1%, pH 7.2) at room temperature to obtain a final volume of 10 cm^3, and then dissolved at 50°C. *Saccharomyces cerevisiae* cells (27.1 mg dry weight) and (appropriate amount of cross-linkers to obtain the specified final concentrations) 0.004–0.04 mol/dm^3 chromium (III) acetate and 0.002–0.02 mol/dm^3 chromium (III) sulfate were added to the solution at 32°C. The mixture was stirred for 1 min and 0.1 cm^3 aliquots containing 0.271 mg dry cell were taken. The whole cell gelatin spots were left to dry for 24 h to establish cross-linking at 25°C. Free cells and excess crosslinkers were removed by washing 3 times for 12 min at 25°C with (3×0.9 cm^3) EDTA buffer. 0.016 mol/dm^3 chromium (III) acetate or 0.008 mol/dm^3 chromium (III) sulfate were found as optimum chromium salt concentrations. Developed biocatalysts were stored in dry test tubes at 25°C for future use.

Measurement of Invertase Activity

Free and immobilized invertase activities were determined according to the Nelson's method from the amounts of reducing sugars produced (10). In the determination of invertase activity of free and immobilized whole cells, a fallowing procedure was applied. We added 0.1 cm^3 of sucrose solution (0.3 mol/dm^3 sucrose solution in 0.05 mol/dm^3 acetate buffer (pH 4.6)) and 0.7 cm^3 0.1% EDTA buffer (pH 7.2) to test tubes containing immobilized or free *Saccharomyces cerevisiae* cells and then incubated the tubes for 10 min at 25°C. At the end of a 10 min incubation period, we added 1 cm^3 Nelson reagent and plunged the tube into boiling water to stop the reaction and rested it there for 20 min. Then, we cooled to tubes to 25°C and added 1 cm^3 arseno-molybdate reagent to tubes. Finally, the reaction volume was diluted to 10 cm^3 with double-distilled water and we measured optical densities at 540 nm against a blank for both free and immobilized whole cells. Solutions with equimolar amounts of glucose and fructose were used as standards.

Invertase activities were calculated in units (U) where one unit corresponds to one micromole of sucrose hydrolyzed to D-glucose and D-fructose per minute at 25°C. Both free and immobilized whole cell invertase activities were calculated per 1 mg dry *Saccharomyces cerevisiae* cells.

Percentage maximum activities were calculated according to the following formula:

$$\text{ra} = [\text{activity of free or immobilized cells at any operating condition} / (\text{total activity of free cell} - \text{activity loss by enzyme leakage})]$$

ma = maximum value of ra in series of experiments with variation of an operating variable

$$\% \text{ Maximum activity} = \text{ra} \times 100/\text{ma}$$

Results and Discussion

Effect of pH on Activity

The effect of pH on the activities of both free and immobilized *Saccharomyces cerevisiae* cells invertase were studied in the pH range of 1.5–10.7. Optimum pH values were found as 4.6 for free cells and 7.2 for both crosslinkers. Results are presented in Figure 1.

As seen from the figure, general trends of immobilized samples were almost the same, but they differ from free cell graph with respect to pH at maximum values. We obtained maximum invertase activity at pH 4.6 for free *Saccharomyces cerevisiae* cells and at pH 7.2 for both immobilized whole cell samples. This behavior was attributed to the curling up of gelatin molecules at pH 5 (isolectric point) thus increasing resistance to the diffusion in gel matrix. Our results for free cells were consistent with Hasal et al. (3) (pH 4.6), Cantarella et al. (4) (pH 4.75), and Mansour et al. (7) (pH 4.6) but different for immobilized samples. Our immobilization procedure shifted the optimum pH from a acidic to a neutral zone. Whereas their procedures did not effect optimum pH. In all probability, curling up of gelatin molecules at pH 5 and stretching out at higher and lower pH values caused such a result. Further experiments were performed at pH 7.2 because this is the physiological pH and invertase is a food technology related enzyme.

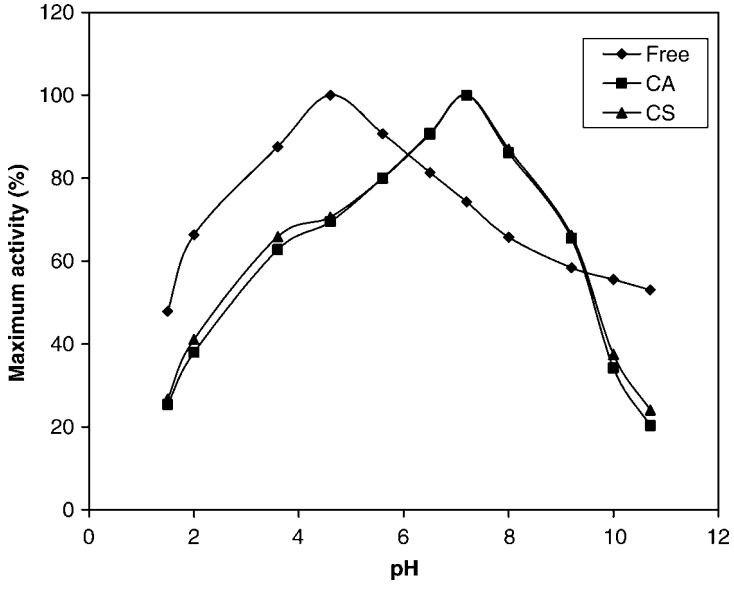

Figure 1. Effect of pH on β-D-fructofuranosidase activity of free and immobilized *Saccharomyces cerevisiae* cells. At 25°C, 10 min reaction time, 0.016 mol/dm^3 CA, 0.008 mol/dm^3 CS.

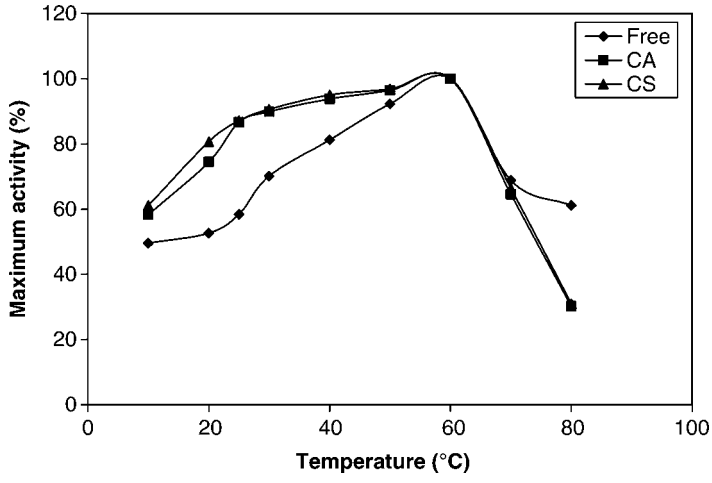

Figure 2. Effect of temperature on β-D-fructofuranosidase activity of free and immobilized *Saccharomyces cerevisiae* cells. At pH 7.2, 10 min reaction time, 0.016 mol/dm^3 CA, 0.008 mol/dm^3 CS.

Effect of Reaction Temperature on Activity

To determine the thermal stability of free and immobilized whole cell invertase, experiments were performed for a series of temperatures from 10 to 80°C. Results are given in Figure 2.

As seen in the figure, peak points for maximum activities were obtained at 60°C for all samples. The main difference between immobilized cells and free cells was the higher stability of immobilized samples for the temperatures up to 60°C. Similar results with reference to maximum activities were obtained by Chang et al. (6) (65°C) and Mansour et al. (7) (60°C).

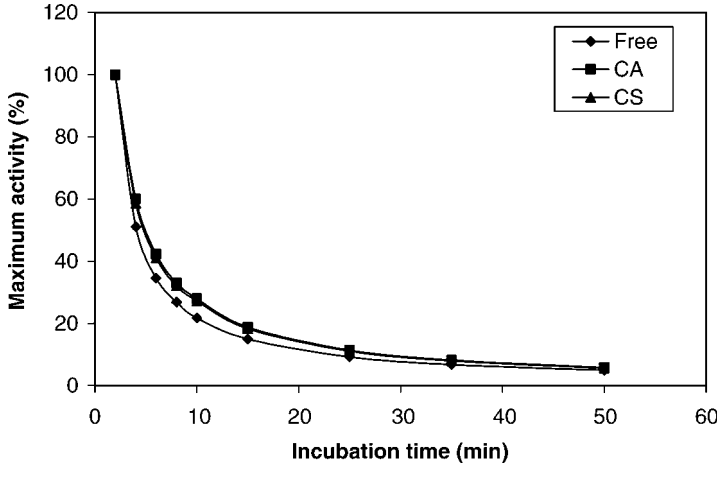

Figure 3. Effect of incubation time on β-D-fructofuranosidase activity of free and immobilized *Saccharomyces cerevisiae* cells. At pH 7.2, 25°C, 0.016 mol/dm^3 CA, 0.008 mol/dm^3 CS.

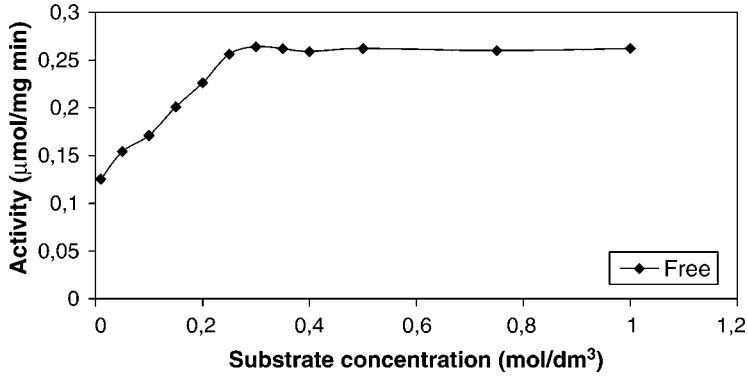

Figure 4. Michaelis-Menten curve for free *Saccharomyces cerevisiae* cell invertase. At pH 7.2, 25°C.

Effect of Incubation Time on Activity

Incubation time was varied from 2 to 50 min in order to analyze the effect of increasing product concentration on the reaction rate and thus to find the most suitable reaction period, maximum activities obtained for free and immobilized samples are given in Fig. 3.

As presented in the figure, as incubation time increased, maximum activity decreased for both free and immobilized whole cell samples as expected. This progress was attributed to increased product (or decreased substrate) concentration around the enzyme's active sites due to diffusion limitations. The suitable reaction time was considered as 10 min since more than 60% of the product was obtained in first 10 min. This period was used in the rest of the work as incubation time.

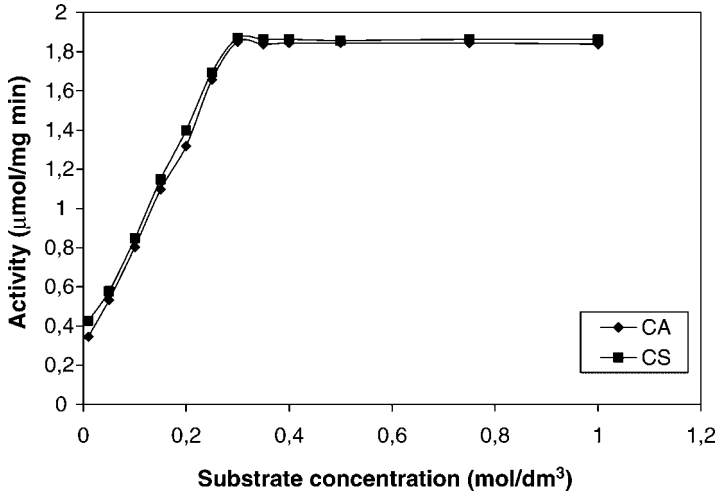

Figure 5. Michaelis-Menten curve for *Saccharomyces cerevisiae* cell invertase immobilized with chromium III acetate (CA) and chromium III sulfate (CS). At pH 7.2, 25°C, 0.016 mol/dm^3 CA, 0.008 mol/dm^3 CS.

Kinetic Properties of Saccharomyces cerevisiae Cell Invertase

Effect of substrate concentration on the invertase activities of free and immobilized *Saccharomyces cerevisiae* cell enzymes were studied with different concentrations of sucrose ($0.01-1.00\,mol/dm^3$). The results are given in Figures 4 and 5 for free and immobilized whole cell samples.

In the case of free *Saccharomyces cerevisiae*, cell invertase activity changes linearly with increasing sucrose concentration up to $0.30\,mol/dm^3$ and stayed constant after this concentration (Figure 4). The maximum enzymatic activities for immobilized whole cell invertase were also obtained at $0.30\,mol/dm^3$ sucrose concentration for both crosslinkers CA and CS (Figure 5). In this work, the common sucrose concentration of 0.30 mol/dm3 was employed. Using Figures 4 and 5 as Michaelis-Menten curves, K_m (substrate concentration at half V_{max} in graph) and V_{max} (highest activity in graph) were determined from the graphs.

For free *Saccharomyces cerevisiae* cell invertase;

$$K_m = 0.01\ mol/dm^3$$

$$V_{max} = 0.0262\ \mu mol\ sucrose\ mg^{-1}\ min^{-1}$$

For *Saccharomyces cerevisiae* cell invertase immobilized with chromium III acetate;

$$K_m = 0.12\ mol/dm^3$$

$$V_{max} = 1.85\ \mu mol\ sucrose\ mg^{-1}\ min^{-1}$$

For *Saccharomyces cerevisiae* cell invertase immobilized with chromium III sulfate;

$$K_m = 0.11\ mol/dm^3$$

$$V_{max} = 1.87\ \mu mol\ sucrose\ mg^{-1}\ min^{-1}$$

We obtained about a 70-fold increase in the V_{max} values for immobilized cell samples and this was attributed to continuing cell growth in immobilization media (medium feeding capability) due to mixing with glucose (produced in hydrolysis reaction) and gelatin (immobilization media).

Our immobilized cell invertase activities ($1850-1870\,Ug^{-1}$) were higher than the activities obtained by Cantarella et al. (4) ($300-1500\,Ug^{-1}$) and Cabral et al. (5) ($161-517\,Ug^{-1}$), but lower than Chang et al. (6) ($2980\,Ug^{-1}$). On the other hand, since our initial cell activities ($26.2\,Ug^{-1}$) are very low compared to Chang et al. (6) ($3010\,Ug^{-1}$), we can state that our % immobilization performance is better than their work.

Reusability

Immobilized *Saccharomyces cerevisiae* cells prepared by using $0.004-0.040\,mol/dm^3$ CA and $0.002-0.020\,mol/dm^3$ CS were used with 3 day intervals to investigate the effect of shelf life and reusing on β-D-fructofuranosidase activity. This process was continued for 30 days and 10 uses. The results are presented in Figures 6 and 7.

Activity improvements obtained with increasing salt concentration (for low concentration values) in both figures were attributed to increased amount of cell immobilized due to increased cross-linker concentration. For higher concentrations of salt, this

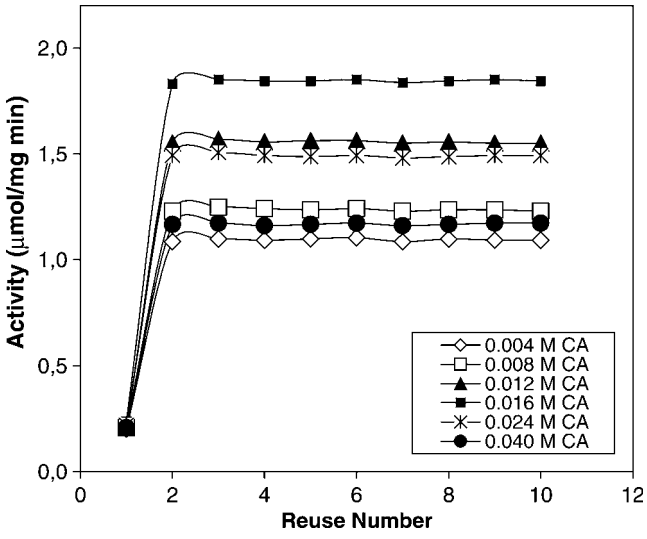

Figure 6. Effect of reuse on *Saccharomyces cerevisiae* cell invertase activity immobilized with chromium III acetate (CA). At pH 7.2, 25°C.

activity increase was first balanced and then reversed due to decreasing diffusion rate caused by the tightening of matrix.

As seen from the figures, immobilized cells could be reused 10 times within 30 days with negligible activity loss. Best results were obtained for CA concentration 0.016 mol/dm^3 and CS concentration 0.008 mol/dm^3. We observed a sudden increase of activity after first use and credited this increase to cell growth in shelf due to usage of nutrient (reaction

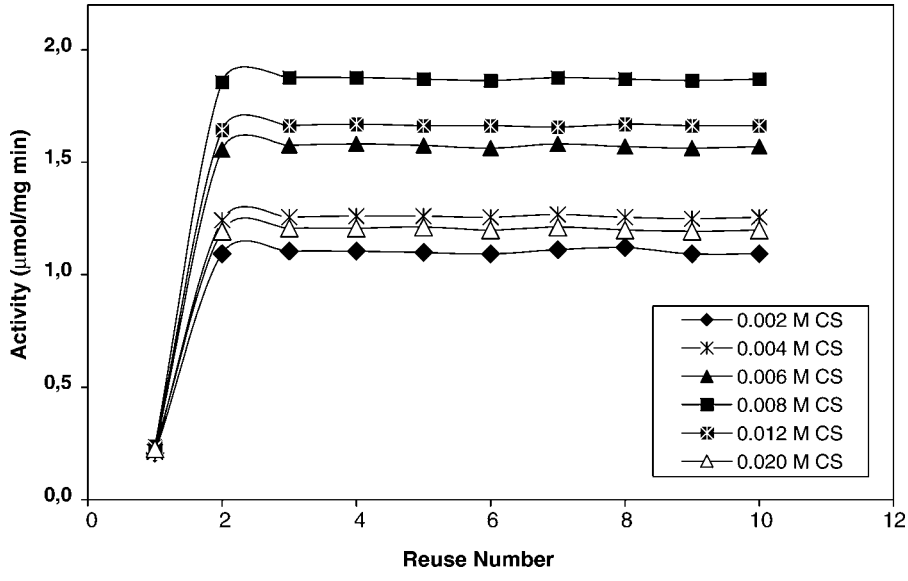

Figure 7. Effect of reuse on *Saccharomyces cerevisiae* cell invertase activity immobilized with chromium III sulfate (CS). At pH 7.2, 25°C.

product left in the pores of biocatalyst) sucrose. Excluding the first use, average activities for these concentrations were 1.84 μmol mg^{-1} min^{-1} for CA and 1.87 μmol mg^{-1} min^{-1} for CS with standard deviations 0.0637 and 0.0676, respectively. These results confirmed the success of immobilization method developed in this research.

Conclusions

In this work, we developed and characterize a new biocatalyst with invertase activity. We managed to obtain an activity approximately 70 times greater than the activity of the same amount of free cells due to continuing cell growth, thus getting the benefit of medium feeding. Our biocatalyst could be reused 10 times within a period of 30 days with insignificant activity loss and possessed better thermal stability than free cells.

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