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# Biotransformation of iminodiacetonitrile to iminodiacetic acid by *Alcaligenes faecalis* cells immobilized in ACA-membrane liquid-core capsules

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Biotransformation of iminodiacetonitrile (IDAN) to iminodiacetic acid (IDA) was investigated with a newly isolated *Alcaligenes faecalis* ZJUTBX11 strain showing nitrilase activity in the immobilized form. To reduce the mass transfer resistance and to increase the toleration ability of the microorganisms to the toxic substrate as well as to enhance their ability to be reused, encapsulation of the whole cells in alginate–chitosan–alginate (ACA) membrane liquid-core capsules was attempted in the present study. The optimal pH and temperature for nitrilase activity of encapsulated *A. faecalis* ZJUTBX11 cells were 7.5 °C and 35 °C, respectively, which is consistent with free cells. Based on the Michaelis–Menten model, kinetic parameters of the conversion reaction with IDAN as the substrate were: $K_m$ = (17.6 ± 0.3) mmol $L^{-1}$ and $V_{max}$ = (97.6 ± 1.2) μmol $min^{-1}$ $g^{-1}$ of dry cell mass for encapsulated cells and (16.8 ± 0.4) mmol $L^{-1}$ and (108.0 ± 2.7) μmol $min^{-1}$ $g^{-1}$ of dry cell mass for free cells, respectively. After being recycled ten times, the whole cells encapsulated in ACA capsules still retained 90 % of the initial nitrilase activity while only 35 % were retained by free cells. Lab scale production of IDA using encapsulated cells in a bubble column reactor and a packed bed reactor were performed respectively.


**Keywords:** biotransformation, iminodiacetic acid, microencapsulation, *Alcaligenes faecalis*, nitrilase, bubble column reactor

## Introduction

Nitrilases have recently attracted increasing attention and interest in the biochemistry fields owing to their ability of conducting nitrile hydrolysis at ambient conditions with high chemo-, regio- and enantioselectivity, which also meet the demand of green chemistry and environmental protection (Banerjee et al., 2002; O'Reilly & Turner, 2003; Huang & Xu, 2006; Martínková & Křen, 2010). A lot of microorganisms possessing high nitrile-converting enzyme activity were obtained from soil environment by employing the high-throughput screening methods (Sosedov et al., 2009; He et al., 2011; Xue et al., 2011; Zhang et al., 2011; Jin et al., 2013).

Iminodiacetic acid (IDA) is an important compound widely utilized as a key intermediate in fine chemical industry, especially in the production of glyphosate which has been a dominant herbicide all over the world for the past decade (Woodburn, 2000). Biotransformation of IDA from iminodiacetonitrile (IDAN) by nitrilase led to a promising technique with the potential to substitute the conventional process (He et al., 2011; Liu et al., 2011a, 2011b, 2012; Zhang et al., 2012, 2013). Regarding the future industrial applications, it is necessary to achieve a biocatalyst with high enzyme activity and to improve the re-

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peated use capacity as well as the operational stability (Kabaivanova et al., 2005; Banerjee et al., 2006; Kaul et al., 2006). Great efforts have been made to achieve these goals: formation of gene recombination microorganisms by using nitrilase separated and purified from different kinds of sources (Xue et al., 2010; Liu et al., 2011a, 2011b) and design of a bioreactor that can be operated in continuous regime even at high dilution rates (Shukla et al., 2001; Nigam et al., 2009).

Encapsulation technology has been one of the most used to immobilize microbial cells since 1993; it has some obvious advantages over conventional gel beads entrapment including lower mass transfer resistance and larger growing space for viable microbial cells within the capsules (Park & Chang, 2000; Dembczynski & Jankowski, 2000; Kuan et al., 2010). Microencapsulation of whole cells consists in the enclosing of the active material within a polymeric matrix surrounded by a semi-permeable membrane. A lot of materials such as alginate, chitosan, polyvinyl alcohol and other polymers are used to form microcapsules. An alginate–polylysine–alginate (APA) microcapsule is one of the most widely studied capsules for its excellent biocompatibility and characteristics, but the high cost of polylysine may limit its use in cell encapsulation (Chang et al., 1994; Gugerli et al., 2002). Subsequently, chitosan has been studied to substitute polylysine for its good properties and lower cost of the ACA microcapsule preparation; here, alginate and chitosan, natural anionic or cationic polysaccharides widely used for immobilization, were combined to form an alginate–chitosan membrane of the capsules (Lin et al., 2008).

In the present study, *Alcaligenes faecalis* ZJUTBX11 strain, isolated previously from soil in our laboratory and demonstrated to possess nitrilase enzyme activity, was used in the biotransformation of IDA from IDAN. Several immobilization methods based on the alginate matrix were attempted and various properties of the immobilized cells such as optimal pH and temperature, pH and temperature stability, Michaelis-Menten kinetic parameters with IDAN as the substrate, and operational stability of the encapsulated cell enzyme were also investigated.

## Experimental

High viscosity sodium alginate (SA) and chitosan (CTS, degree of deacetylation: 95 %) were purchased from Sinopharm Chemical Reagent (Shanghai, China). Iminodiacetonitrile (IDAN, > 95 mass %) was obtained from Zhejiang Wynca Chemical Industry Group (Hangzhou, China). Iminodiacetic acid (IDA, > 98 mass %) was purchased from J&K Chemical (Shanghai, China). Solvents used for HPLC were purchased from Merck & Co. (Shanghai, China). All other reagents and chemicals purchased from commercial source were of analytical grade.

*A. faecalis* ZJUTBX11 strain from culture collections screened by our laboratory was used in this work and stored at 4 ℃ on a BPD (beef and peptone deposit) agar slant. The agar slant medium consisted of beef extract (5 g), peptone (10 g), NaCl (5 g), and agar (15 g) per liter (pH 7.0). The strain was grown aerobically at 30 ℃ for 48–72 h in 50 mL of a sterile medium consisting of glucose (10 g), peptone (12 g), yeast powder (5 g), ammonium acetate (7.5 g), NaCl (5 g), $MgSO_4$ (0.2 g), $KH_2PO_4$ (2 g), and $K_2HPO_4$ (3 g) per liter, and the pH was adjusted to 7.5 with 1 M NaOH before the autoclaving, and then supplemented with butanenitrile (butyronitrile) at the final concentration of 0.1 vol. % after the sterilization for the induction of nitrilase. The microbial cultures were carried out at 30 ℃ and 150 $min^{-1}$ on a shaking incubator. Cells were harvested by centrifugation at 10000 × *g* for 20 min under 4 ℃ and then thoroughly washed three times with distilled water to remove residuals from the culture medium.

### *Methods of whole cells immobilization*

For entrapment of cells, 2 g (wet mass) of cells were suspended in 10 mL of a sodium chloride solution (8.5 g $L^{-1}$) and then mixed well with 10 mL of a sodium alginate solution (40 g $L^{-1}$) under thorough stirring at room temperature. The mixture was extruded drop wise by means of a hypodermic needle with the diameter of 0.5 mm into a calcium chloride aqueous solution (20 g $L^{-1}$) to yield calcium alginate gel beads with a 2 mm diameter. The resulting gel beads were hardened in the same aqueous solution for 2 h under appropriate agitation, and stored in a calcium chloride solution (50 g $L^{-1}$) before use.

Multilayer hydrogel microcapsules were prepared by a ionic gelation method at room temperature. 2 g (wet mass) of microorganism cells suspended in 10 mL of a normal saline solution was mixed well with same volume of alginate solutions (40 g $L^{-1}$) prepared by dissolving sodium alginate in an aqueous solution under heating. The cell-alginate suspension was then extruded through a 0.5 mm needle into a 20 g $L^{-1}$ calcium chloride solution to form calcium alginate gel beads. After a 30 min gelation, micro gel beads containing microbial cells were obtained; they were washed with sterile deionized water. The beads were incubated in a 50 g $L^{-1}$ chitosan solution dissolved in a 1 vol. % acetic acid solution at the volume ratio of 1 : 10 (beads : solution) to form polyelectrolyte membranes for a 30 min incubation. After being rinsed three times with sterilized physiological saline, a 20 g $L^{-1}$ alginate solution was added to counteract the excessive positive charge on the membrane; the membranes were rinsed three times. Then, microcapsules were incubated in a chitosan and alginate solutions again to form dense membranes. As a result, ACA microcapsules containing cells with a

solid core and the diameter of 2 mm were prepared and stored in physiological saline at 4 ℃ prior to use. Furthermore, the ACA microcapsules containing microorganism cells with a liquid core were also prepared using the treated microcapsules mentioned above in a 55 mM sodium citrate solution for 20 min under constant stirring to give capsules with a 1.5 mm diameter.

For the preparation of ACA capsules with liquid cores by a one-step method, 10 mL of the microorganism suspension containing 2 g (wet mass) of cells and 3 % of calcium chloride were mixed well with 10 mL of a chitosan solution (50 g $L^{-1}$) prepared by dissolving a certain amount of chitosan in a 10 g $L^{-1}$ acetic acid solution under the conditions of heating. The mixture was then extruded drop by drop into 300 mL of a 50 g $L^{-1}$ sodium alginate solution using a 0.5 mm hypodermic needle under constant stirring at room temperature. After 30 min of the membrane formation process, capsules with the diameter of approximately 2 mm were washed three times with physiological saline and stored at 4 ℃ prior to use.

The surface and the layer structure of the capsules membrane were observed by a scanning electric microscope (SEM). Both capsule samples, with and without microbial whole cells, were dehydrated on a lyophilizer and mounted on specimen stubs with silver paint, gold-coated and examined by SEM at the acceleration of 20 kV.

Kinetic parameters including the maximum reaction rate ($V_{max}$) and the apparent Michaelis–Menten constant ($K_m$) were determined based on different substrate concentrations, from 20 mM to 240 mM, and the yield of the product in the biotransformation of IDAN to IDA by free and encapsulated cells at 35 ℃ and pH 7.5. Apparent $K_m$ sand $V_{max}$ values were calculated by function fitting. Catalytic efficiency ($K_{cat}$) was expressed as the ratio $V_{max}/K_m$. All experiments were carried out in triplicates.

To investigate the performance of encapsulated cells in the long-term use, repeated batch conversion of IDAN to IDA was carried out using the resuspended whole cells and encapsulated cells of *A. faecalis* ZJUTBX11. The reaction mixture (10 mL), containing a Tris-HCl buffer (50 mM, pH 7.5), 0.2 g of wet mass of resting cells (equivalent of 0.03 g dry cell mass, DCW) or the equivalent cells-immobilizing capsules and 100 mM IDAN, was immobilized on an orbital shaker at 35 ℃ and 150 $min^{-1}$. After each cycle, the cells or the capsules were recovered by filtration and then washed with distilled water prior to their transfer into another fresh reaction mixture for the next conversion run under the same conditions.

The batch biotransformation of IDA from IDAN was conducted in a 500 mL bubble column reactor made of glass (height: 35 cm, inner diameter: 5 cm) with the working volume of 400 mL. The conversion system consisted of: 100 mM Tris-HCl buffer of pH 7.5, different concentrations of IDAN from 50 mM to 200 mM and a certain amount of the ACA membrane capsules containing 10 g of wet cells (corresponding to 1.49 g of dry cells). The reactor was incubated at 35 ℃ in a controlled temperature chamber. Air was supplied from the bottom of the reactor with an air pump at the rates of 0.6 L $min^{-1}$, 0.9 L $min^{-1}$, and 1.2 L $min^{-1}$. Every batch, the conversion mixture was withdrawn at intervals from the reactor and the concentration of IDA was analyzed using HPLC. All experiments were carried out in triplicates.

Continuous biotransformation of IDAN to IDA was also carried out in a packed-bed reactor made of glass with the ACA-membrane cells-encapsulated capsules as the catalyst. Parameters of the reactor were: height of 35 cm and diameter of 4.5 cm, the reactor was packed with a certain amount of capsules containing 10 g of wet cells (approximately 1.49 g of DCM). Temperature of the reaction system was controlled at 35 ℃ by water circulation in the outer jacket of the glass column. The substrate solution (various amounts of IDAN dissolved in the 50 mM Tris-HCl buffer of pH 7.5 to yield a certain concentration of the influent) was fed into the bioreactor at the bottom of the column using a peristaltic pump to regulate the flow rate. The biotransformation was conducted at different residence times and the effluent samples were collected at defined intervals from the top of the column and subjected to HPLC. All experiments were carried out in triplicates.

### *Analytical methods*

The nitrile-hydrolyzing enzyme activities were assayed using IDAN as the substrate according to Liu et al. (2011b). One unit of the enzyme activity was defined as the amount of enzyme which produced 1 μmol of IDA per min at 35 ℃ and pH 7.5. All presented data were the mean values of triplicate assays in which the standard deviations were always lower than 5 %.

The amount of IDA was also determined by high performance liquid chromatography (HPLC, LC-10AS, Shimadazu, Japan) using a 4.6 mm × 250 mm × 5 μm Hypersil SAX column (Elite, Dalian, China). Here, 1 mL of the reaction mixture was centrifuged by 10000 × *g* for 10 min under 4 ℃ and then microfiltrated by a 0.45 μm membrane (Millipore, USA). The resultant 20 μL of the supernatant were subjected to HPLC to determine the amount of IDA formed. A UV detector set at the wavelength of 210 nm and a mobile phase of 20 mM $(NH_4)_2HPO_4$ buffer (pH 4.0) at the flow rate of 1.0 mL $min^{-1}$ were used for the detection. The mobile phase was filtered through a 0.45 μm filter membrane. The analysis time was 10 min per sample.

## Results and discussion

### *Biotransformation by free A. faecalis ZJUTBX11 cells*

*A. faecalis* ZJUTBX11, possessing nitrile-converting activities, was isolated from soil samples in our laboratory using IDAN as the sole nitrogen source of the biotransformation of IDAN to IDA. IDAN is a dicyano compound mainly used in the production of glyphosate and it is a poor substrate for the nitrile hydrolysis to the nitrilase reported previously. It has been reported that *A. faecalis* JM3 arylacetonitrilase is completely inactive against IDAN (Nagasawa et al., 1990). However, Kobayashi et al. (1990) firstly demonstrated the nitrilase from *Rhodococcus rhodochrous* K22 to be active on aliphatic dinitriles, such as pentanedinitrile (glutaronitrile), 2,2′-iminodiacetonitrile, 3,3′-oxydipropionitrile, and 3,3′-iminodipropionitrile, with nitrilase activity of 1.924 μmol $min^{-1}$ $mg^{-1}$ of the protein to iminodiacetonitrile. Nitrilase from *Alcaligenes* sp. ECU0401 also showed low activity over IDAN, only 0.49 μmol $min^{-1}$ $mg^{-1}$ protein (Zhang et al., 2011). Except these enzymes, no more information about the hydrolysis of IDAN was reported. In recent years, our research group focused our attention on the biotransformation of IDAN to IDA for the improvement of green production of glyphosate. It has been indicated that *A. faecalis* ZJUTBX11 exhibited excellent specific nitrilase activity and thus can hydrolyze IDAN effectively employing nitrilase. Specific enzyme activity of the *A. faecalis* ZJUTBX11 is 109.4 μmol $min^{-1}$ $g^{-1}$ of dry cells considering the increase of the nitrilase-producing capacity and optimization of the fermentation conditions.

In the previous study, the fact that the *A. faecalis* ZJUTBX11 strain produces nitrilase rather than nitrile hydratase and amidase systems was confirmed for no amide by-product detected throughout the hydrolysis reaction, also no activity was observed when some amide compounds were used as the substrates. As it is known, most nitrilases are inducible by certain compounds, such as nitriles or azepan-2-one (ε-caprolactam), except for *Bacillus subtilis* ZJB-063 (Chen et al., 2008) and *Rhodococcus* R 312 (CBS 717.73) (Osprian et al., 1999). A comparative study of the biotransformation of IDAN to IDA was also carried out using non-induced and butyronitrile-introduced *A. faecalis* ZJUTBX11 cells. Non-induced cells yielded 8 % of IDA form IDAN after 3 h of the conversion reaction, whereas 35 % of IDA for induced cells were achieved, which revealed that the *A. faecalis* ZJUTBX11 nitrilase is primarily inducible.

The effect of IDAN concentration ranging from 50 mM to 250 mM on the conversion to IDA was investigated at 35 ℃ in a 50 mM Tris-HCl buffer (pH 7.5) (data not shown). Increasing the IDAN concentration from 50 mM to 250 mM led to an increased IDA concentration and an extended reaction time. In the batch reaction, 150 mM of IDAN were completely converted in 8 h of the incubation with free cells as the biocatalyst. Furthermore, no substrate and product inhibition was observed even though the IDAN concentration was increased up to 200 mM . In view of the results, this strain can be considered as the most efficient microorganism for the production of IDA in future studies.

### *Selection of immobilization method based on alginate matrix*

As it is well known, the cost of complex purification and immobilization of enzymes is too high for it to be used in industrial scale production; however, immobilization of whole cells is simpler and provides higher enzyme activity recovery than the purified enzymes used (Léonard et al., 2011). In this regard, for a large-scale biotransformation process, immobilized whole cells of *A. faecalis* ZJUTBX11 were used as the biocatalyst in the production of IDA from IDAN.

Microbial cells immobilized in a hydrogel matrix can be protected from outer harsh environmental conditions such as pH, temperature, organic solvent, and toxicity of substrate or product. Various methods

**Table 1.** Comparison of different methods of whole cells immobilization ($n = 3$)

| Method | Carrier | Cells loading rate | Enzyme activity recovery | Total intact beads rate after 10 runs |
|---|---|---|---|---|
| | | % | | |
| Entrapment | Ca-alginate beads | > 99 | 79 ± 1.5 | 45 ± 3 |
| Microencapsulation | ACA capsules with liquid core (two-step method) | 65 ± 2 | 55 ± 1 | 67 ± 1 |
| | ACA capsules with solid core (two-step method) | > 99 | 67 ± 3 | 65 ± 2 |
| | ACA capsules with liquid core (one-step method) | > 95 | 88 ± 2.5 | 87 ± 4 |

based on alginate carrier were utilized for the immobilization of whole cells of *A. faecalis* ZJUTBX11. Results of the immobilization methods used are listed in Table 1. Conventional entrapment of microbial cells in Ca-alginate beads is the most widely used immobilization technique for whole cells as well as for purified enzymes. However, the problems of Ca-alginate beads entrapment are mainly related to the membrane weakening with time. The presence of chelating reagents including phosphate, lactate and citrate and the growth of microorganisms inside the beads can result in a burst of the Ca-alginate bead (Kabaivanova et al., 2005).

In the present study, only 45 % of total intact Ca-alginate beads remained after ten conversion runs, whereas 67 %, 65 %, and 87 % of ACA capsules with liquid and solid core prepared via the two-step method and ACA capsules prepared via the one-step method remained respectively, though the cells loading rate of the entrapment in Ca-alginate beads was 100 %. Meanwhile a large loss of whole cells was observed in the liquefaction procedure of the microencapsulation via the two-step method, which led to a 65 % of the cells loading rate compared to approximately 100 % of the other methods. The achieved 67 % of enzyme activity recovery of ACA capsules with solid core via the two-step method is similar to that of the Ca-alginate beads entrapment but much lower than that of the ACA capsules with liquid core, which is primarily caused by the diffusion limitations from the surface into the interior of the beads. Among the four cell immobilization methods, ACA capsules with liquid core prepared via the one-step method showed the highest enzyme activity recovery and total intact cell rate, which means the probability of long-term use, although the diameter of the capsules (2 mm) prepared using this method was slightly larger than that of the other ones. Furthermore, encapsulation of viable whole cells in one step can significantly simplify the preparation procedure and subsequently improve the recovery of enzyme activity.

Bioencapsulation has been widely used as one of the most important immobilization techniques in a number of applications, including biotechnological (Park & Chang, 2000), environmental (Stormo & Crawford, 1992), etc. In particular, microbial whole cells and enzymes were encapsulated as industrial biocatalysts for the production of fine chemicals and pharmaceuticals (Liese et al., 2006; Schmid et al., 2001; Straathof et al., 2002). Liquid core capsules have many advantages over other methods in microbial cell immobilization, such as adsorption, entrapment, etc. (Förster et al., 1996). Capsules made of high viscosity sodium alginate and chitosan have shown a high potential in biotransformation. Their liquid cores provide a larger space for the encapsulated cells and reduces the mass transfer resistance. The cells can be confined inside the capsule and the substrates and the products can easily cross through the semi-permeable membrane (Bučko et al., 2005). The capsule membrane also protects the cells from contact with the toxic organic solvent and provides a mild, comfortable environment for the cells. The encapsulation method enables high cell loading, low mass transfer resistance, and better ability of repeated use in industrial applications than any other immobilization method used until now (Park & Chang, 2000).

In view of the results mentioned above, whole cells of *A. faecalis* ZJUTBX11 were encapsulated into the ACA capsules with liquid core using the one-step method for the production of IDA from IDAN in the present study.

### *Characterization of ACA membrane liquid-core capsules*

Morphologies of both capsules with cells and empty samples were investigated. The capsules with the diameter of 1.5–2.0 mm were smooth, spherical, and covered with a thin layer of sodium alginate which diminished during the conversion process due to the shear force or was redissolved in the reaction system. The size of the liquid-core capsules is controlled by the gravity force, drag force, and surface tension (Park & Chang, 2000), while in the present experiment, the size of a liquid drop falling from a needle was due to the gravity and the drag force larger than 1 mm. It is very important for the capsules to have good mechanical stability to support their proliferation and long-term use in bioreactors (Kurillova et al., 2000). Whole cells of *A. faecalis* ZJUTBX11 within the capsules were uniformly distributed in the interior of the capsules containing chitosan because of its incomplete combination with sodium alginate. However, cells encapsulated in the capsules with liquid core tended to form large aggregates in standing state, especially in a packed bed reactor. The surface and the layer structure of the ACA membrane of the capsules were also observed by a scanning electric microscope (SEM), Fig. 1. The thin gelatin film formed around the droplet spontaneously when it trickled into the alginate solution because of the electrostatic interaction of the oppositely charged polymers. The layer thickness of the ACA membrane of the capsules was approximately 15 μm which is negligible compared to their diameter of 2 mm. Encapsulated whole cells can be formed for every type of enzyme using genetically engineered microbial cells (Park et al., 1999).

### *Temperature and pH profiles*

The effect of basic enzymatic properties such as pH and temperature on the nitrile-converting enzyme activity of free and encapsulated cells was investigated. In all cases, the highest enzyme activity of the free and encapsulated cells was taken as 100 % and the relative

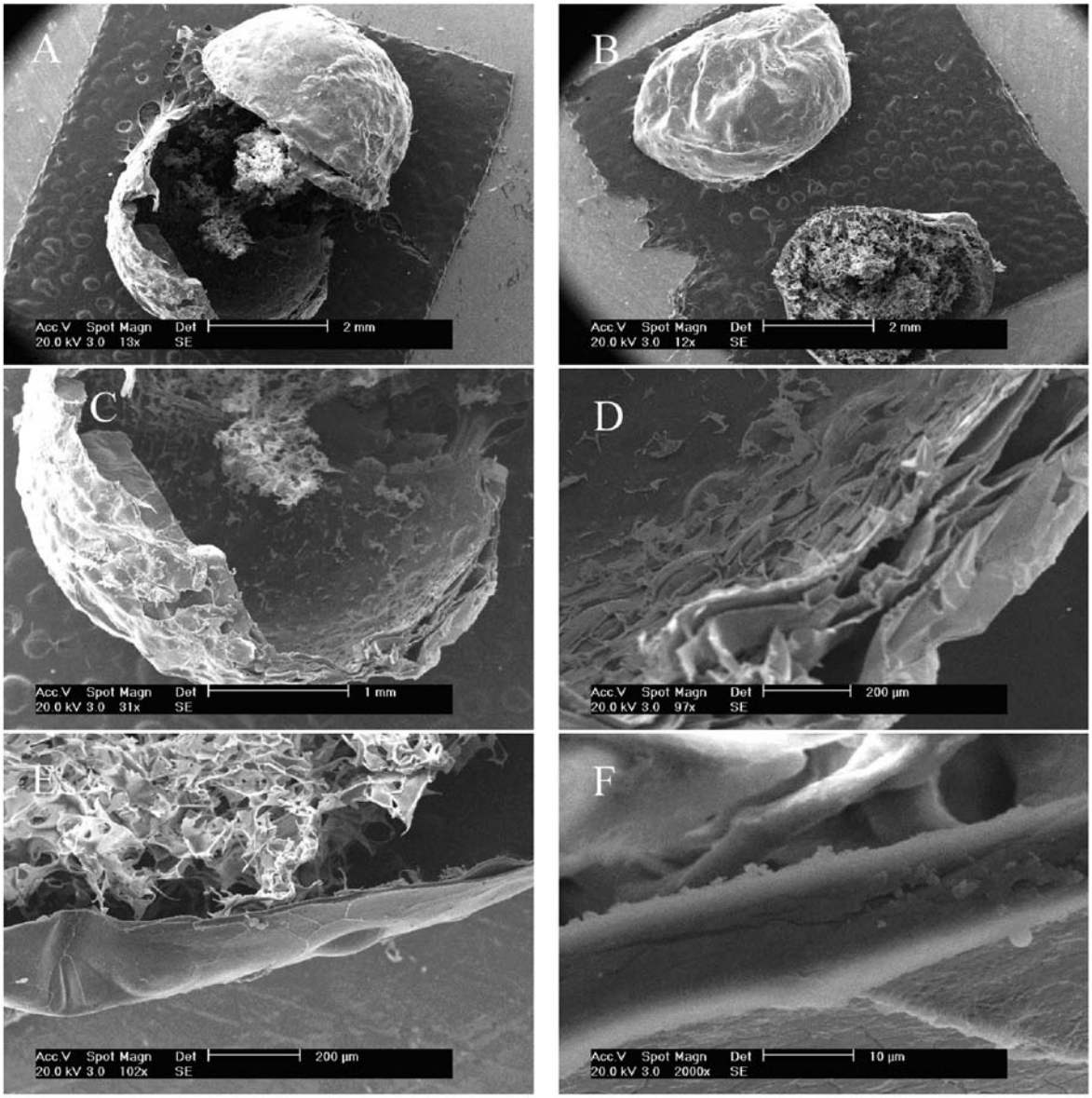


**Fig. 1.** SEM photographs of ACA-membrane capsules: empty capsules (A, C, E) and cell-encapsulated capsules (B, D, F).

activity was expressed in percentage of the maximum activity.

The effect of pH on the enzyme activity of free and encapsulated cells was assessed using IDAN as the substrate in the pH range of 4.0–10.0 at 35 ℃, as well as an acetate buffer (pH 4–6), Tris-HCl buffer (pH 7–9), and glycine–NaOH buffer (pH 8.5–10). As can be seen from Fig. 2A, the optimal pH value of encapsulated cells in the Tris-HCl buffer system was 7.5, which is consistent with that of the free cells. This result is in agreement with the fact that the optimal pH of nitrilase from *A. faecalis* is always between pH 7–8, e.g. pH 7.5 for nitrilase from *A. faecalis* JM3 (Nagasawa et al., 1990), or pH 7.0 for nitrilase from *A. faecalis* ECU0401 (He et al., 2010). Fig. 2B depicts the pH stability of the free and encapsulated cells. As it can be seen, encapsulated cells are more stable than the free ones in the pH range of 5–10, and 65 % of the initial enzyme activity were recovered for encapsulated cells when stored at pH 6.0 for 3 h compared to 44 % for free cells; and 57 % for encapsulated cells compared to 45 % for free cells when incubated at pH 10.0.

The effect of temperature on the enzyme activity of free and encapsulated cells was studied in the range of 20–50 ℃ in the Tris-HCl buffer system (50 mM, pH 7.5) and it is presented in Fig. 2C. The maximum activity was observed at 35 ℃ for both free and encapsulated cells. These results show that the encapsulation procedure has no obvious effect on the physical and chemical properties of the enzyme. Generally, thermal stability of the biocatalyst has to be improved by its immobilization before its utilization in the industrial scale. In the current study, the temperature stability of the enzyme in free and encapsulated cells was evalu-

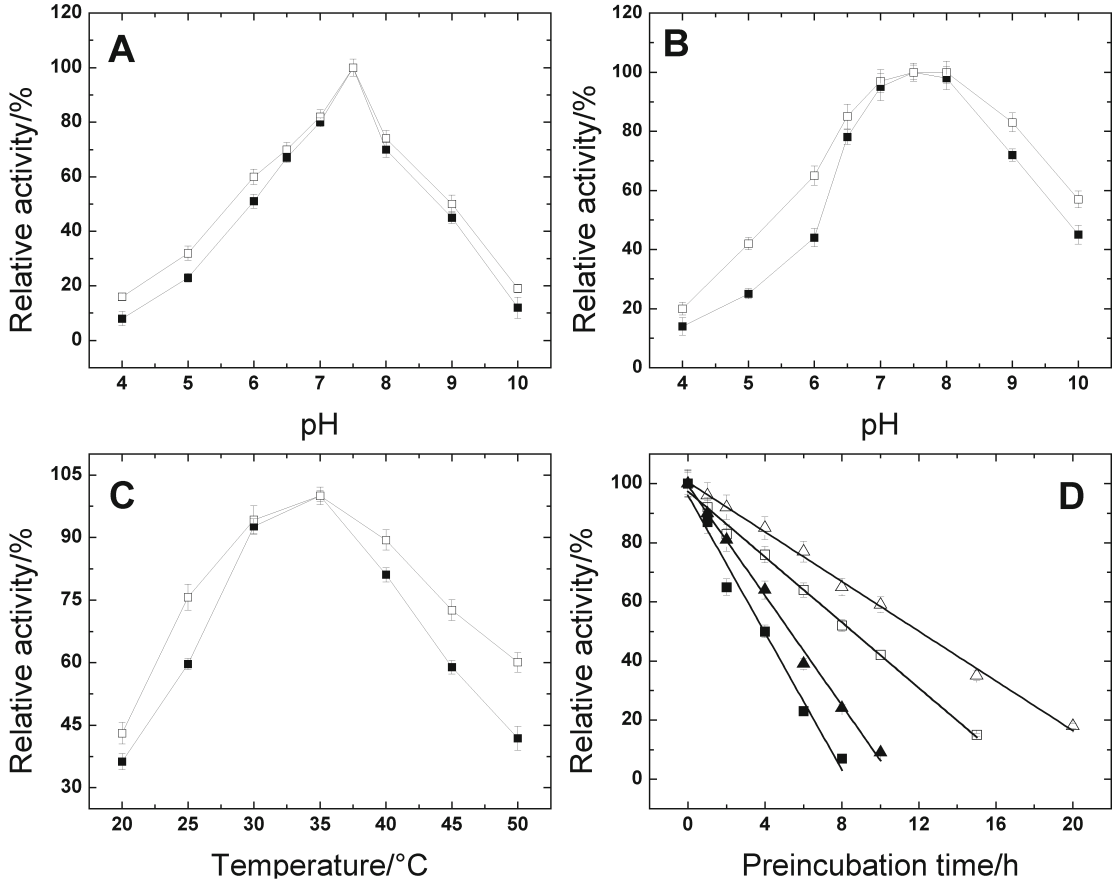


**Fig. 2.** Effect of optimal pH (A) and pH stability (B), optimal temperature (C) and thermal stability (D) on the enzyme activity. Highest enzyme activities of free and encapsulated cells obtained were assigned to be 100 %. Free cells (■), encapsulated cells (□), free cells at 45 °C (○), at 55 °C (●), encapsulated cells at 45 °C (△), at 55 °C (▲).

ated by measuring the residual enzyme activity at different times of the incubation at 45 °C and 55 °C. The activity of both types of cells at 0 h of the incubation was taken to be 100 %. The results in Fig. 2D show an increase in the thermal stability both at 45 °C and 55 °C after the encapsulation which is probably due to the better protection provided by the membrane of the capsules. Thermal stability of the encapsulated cells was enhanced by immobilization which is a great advantage in large scale applications.

### *Kinetic parameters of free and encapsulated cells nitrilase*

The biotransformation reaction of IDAN to IDA followed the Michaelis–Menten kinetics and the corresponding kinetic parameters of the free and immobilized cells were determined at pH 7.5 and 35 °C using the Lineweaver–Burk plot to calculate the $V_{max}$ and the apparent $K_m$ values (Fig. 3). Both free and encapsulated cells showed a typical hyperbolic response with the increasing substrate concentration from 20 mM to 240 mM. The $V_{max}$ and apparent $K_m$ values were found to be (108.0 ± 2.7) μmol min$^{-1}$ g$^{-1}$ of DCM and (16.8 ± 0.4) mmol L$^{-1}$ for free cells,

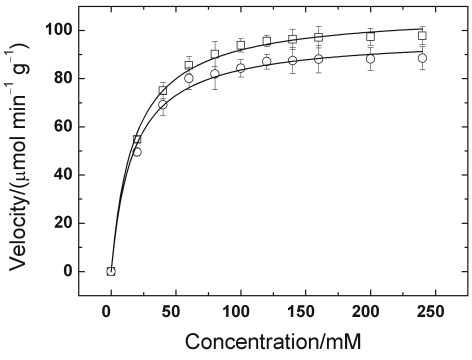


**Fig. 3.** Initial reaction rate at different concentrations of IDAN in its bioconversion to IDA catalyzed by free cells and the encapsulated counterparts (○) with fitting curves (—).

and (97.6 ± 1.2) μmol min$^{-1}$ g$^{-1}$ of DCM and (17.6 ± 0.3) mmol L$^{-1}$ for encapsulated cells, respectively (Table 2). The $K_m$ value obtained for encapsulated cells was slightly higher than that obtained for their free counterparts showing a decrease in the affinity of the enzyme of encapsulated cells to the substrate compared to that of free cells. Generally,

**Table 2.** Kinetic parameters of free and encapsulate cells at pH 7.5 and 35 ℃ ($n = 3$)

| Parameter | Free cells | Encapsulated cells |
|---|---|---|
| Apparent $K_m$/(mmol $L^{-1}$) | 16.8 ± 0.4 | 17.6 ± 0.3 |
| $V_{max}$/(μmol $min^{-1}$ $g^{-1}$) | 108.0 ± 2.7 | 97.6 ± 1.2 |
| Catalytic efficiency, $V_{max}/K_m$/$min^{-1}$ | 0.018 | 0.017 |

the differences observed in the enzyme affinity can be due to the conformational changes of the enzyme caused by its immobilization or the lower accessibility of active sites of the immobilized enzyme. The $V_{max}$ value for encapsulated cells was lower than that for free cells which probably attributed to the restricted diffusion of the substrate into the capsules. Bučko et al. (2005) prepared polyelectrolyte complex capsules immobilizing a whole-cell epoxide-hydrolyzing enzyme with sodium alginate and cellulose sulfate as polyanions and poly(methylene-*co*-guanidine) as a polycation. Encapsulation of the biocatalyst led to a two-fold increase in the CES hydrolase activity and a shorter time required for total biotransformation.

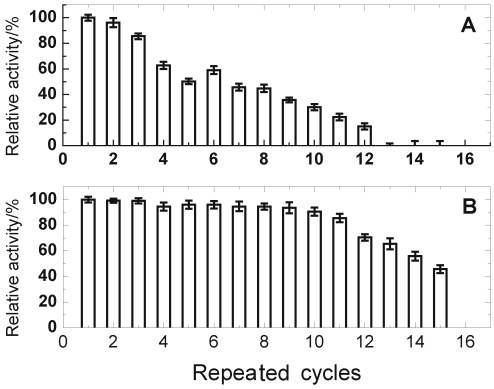


**Fig. 4.** Operational stability of free (A) and encapsulated (B) cells. The enzyme activity of both types of cells at 0 h was considered to be 100 %.

### *Operational stability of encapsulated cells*

A significant advantage of the immobilized enzyme is its excellent recycling stability compared to its free form. Therefore, enzyme activity of the ACA membrane encapsulated biocatalyst is very stable during the sequencing batch or continuous reaction processes. To investigate the long-term operational stability, several repetitive applications of encapsulated cells in the conversion of IDAN to IDA in a batch reaction process were performed under optimal conditions. Initial activity of the encapsulated cells in the first run of the reaction was considered as 100 %, the relative enzyme activities of encapsulated cells in each run were calculated by the ratio of nitrilase activity to the total enzyme activity in the first run. After ten repeated batches, the encapsulated cells still retained 80 % of their original activity, whereas only 40 % were observed for free cells (Fig. 4). The results revealed that cells encapsulated in the ACA membrane capsules have better operational stability compared to their suspended counterparts, which indicates that the encapsulation of whole cells is a promising alternative technology in the long-term production field. Furthermore, only a small amount of cell loss was observed in the conversion reaction system within six cycles, indicating excellent mechanical strength of the ACA capsules; another controlled experiment with alginate gel beads used in the conversion system was carried out, and a large amount of cells observed in the reaction mixture due to their leakage from the hydrogel revealed weak mechanical strength of the membrane. Kabaivanova et al. (2005) demonstrated the immobilization of whole cells of moderately thermophilic *Bacillus* sp. UG-5B harboring nitrilase on polysulfone membranes using chemical binding, the retention of 100 % activity was obtained after eight reaction cycles.

### *Batch production of IDA in a bubble column reactor (BCR)*

In order to reuse the biocatalyst improving thus the productivity and reducing the production costs, consecutive batch reactions with capsule immobilized whole cells of *A. faecalis* ZJUTBX11 were performed in a bubble column bioreactor containing 100 mM IDAN. Conversion of the substrate and productivity of the reactor were determined at various incubation times under different conditions possibly affecting the bioconversion process in the BCR system.

The reason why a BCR reactor was used in the present study is the mix function provided by the upflow air bubble, since the shear force in a BCR is rather lower than that caused by the impeller in a stirred tank reactor (STR), which significantly reduced the breakage of the cell immobilized capsules in the conversion process (Xu et al., 2005). Factors significantly influencing the production of IDA in a BCR are mainly pH, temperature, air flow rate, and concentration of the substrate. In the present experiments, pH and temperature of the reaction system were fixed at 7.5 and 35 ℃ by the Tris-HCl buffer and a thermostatic water cycle was set up to maintain optimal conditions of nitrilase-mediated hydrolysis of IDAN catalyzed by encapsulated *A. faecalis* ZJUTBX11 cells. The air flow rates of 0.6 L $min^{-1}$, 0.9 L $min^{-1}$, and 1.2 L $min^{-1}$

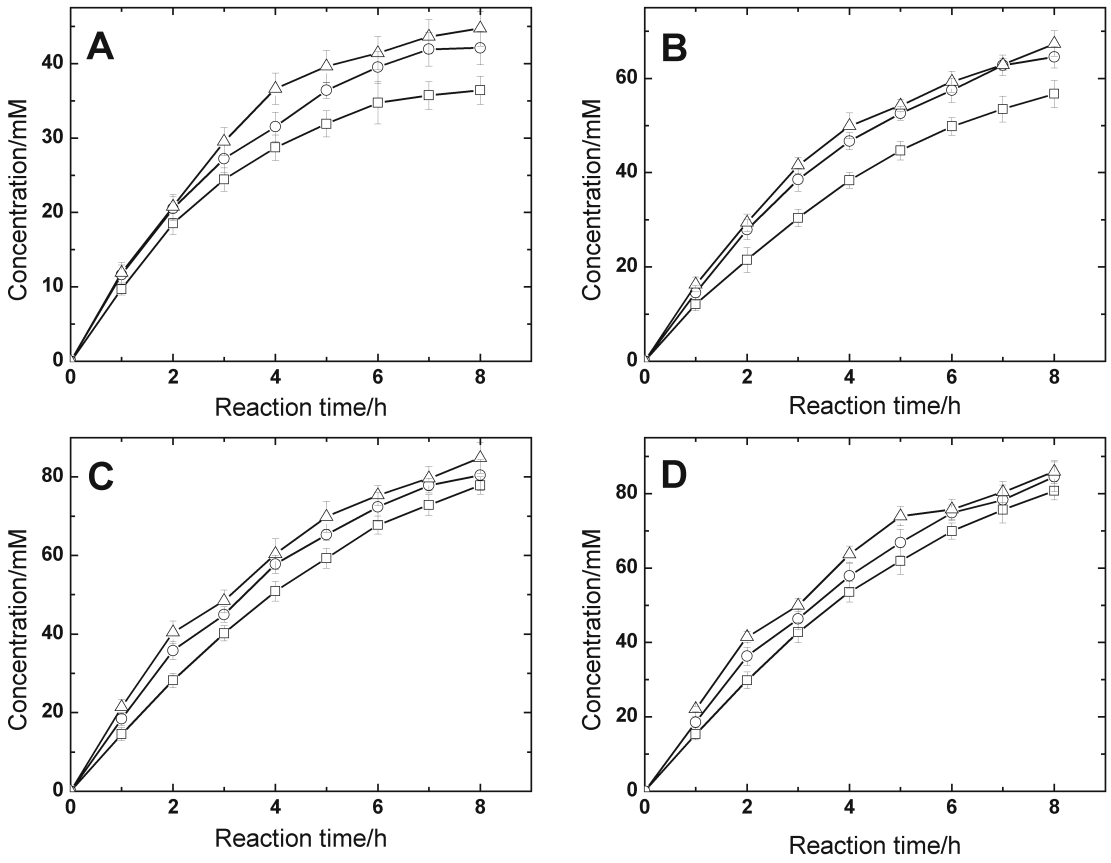


**Fig. 5.** Time course of the batch production of IDA with BCR at different air flow rates: 0.6 L $min^{-1}$ (□), 0.9 L $min^{-1}$ (○), 1.2 L $min^{-1}$ (△) and different concentrations of IDAN: 50 mM (A), 1.00 mM (B), 150 mM (C), 200 mM (D).

were applied to evaluate the effect of the bubble rate on the production of IDA because flow rates lower than 0.6 L $min^{-1}$ cannot provide efficient lifting force to achieve good mixing results and flow rates higher than 1.2 L $min^{-1}$ result in a large amount of air bubbles possibly causing overflow of the reaction mixture from the reactor; this could be resolved by an addition of antifoaming agents but at higher production costs. All batch operations were terminated after 8 h of the conversion and at predetermined interferes; 1 mL of the reaction mixture was withdrawn to determine the concentration of IDA by HPLC.

The effect of the air flow rate on the production of IDA at different concentrations of the substrate are shown in Fig. 5, where four different concentrations of the substrate resulted in an increase of the product content with the increase of the air flow rate in the BCR system. The faster the air flow rates the better the mix of the substrate and the biocatalyst in the system which subsequently decreased mass transfer resistance and resulted in an increase of the reaction rate. The results show the conversion of the reaction and the productivity as a function of the substrate concentration and air flow rate (Figs. 6A and B). As it can be seen from the figure, the conversion ratio decreased with the increase of the substrate concentration from

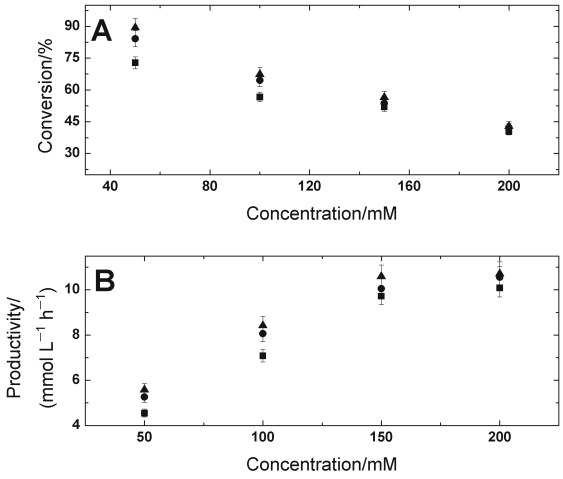


**Fig. 6.** Conversion (A) and productivity (B) of IDAN to IDA by encapsulated cells in a bubble column reactor at different concentrations of the substrate under air flow rate of: 0.6 L $min^{-1}$ (■), 0.9 L $min^{-1}$ (●), and 1.2 L $min^{-1}$(▲), respectively.

50 mM to 200 mM whereas the difference in the conversion ratio under various air flow rates diminished as the substrate concentration increased. As the sub-

**Table 3.** Results of the batch production of IDA in a bubble column reactor at different concentrations of IDAN ($n = 5$)

| Concentration of IDAN | Conversion | Initial specific activity | Biocatalyst productivity (DCW) |
|---|---|---|---|
| mM | % | μmol $min^{-1}$ $g^{-1}$ | g $g^{-1}$ |
| 50 | 89.4 ± 2.5 | 47.5 ± 3.6 | 1.4 ± 0.05 |
| 100 | 67.4 ± 3.2 | 65.1 ± 1.4 | 2.1 ± 0.02 |
| 150 | 56.5 ± 1.4 | 85.4 ± 2.1 | 2.7 ± 0.03 |
| 200 | 42.9 ± 2.3 | 88.2 ± 2.8 | 2.7 ± 0.01 |

strate content is too high and will inhibit the biocatalyst, the conversion ratio cannot be improved by enhancing the stirring rate. On the other hand, productivity of the conversion system increased significantly, from 4.5 mmol $L^{-1}$ $h^{-1}$ to 10.6 mmol $L^{-1}$ $h^{-1}$, as the substrate concentration increased up to 150 mM, and then it did not increase though the continuous increase of the substrate concentration and the air flow rate. As it can be seen from Table 3, the initial specific activity reached the highest value of 88.2 μmol $min^{-1}$ $g^{-1}$ of DCM when applying the substrate concentration of 150 mM and the air flow rate of 1.2 L $min^{-1}$, achieving thus the highest biocatalyst productivity of 2.7 g $g^{-1}$ of DCM.

During the conversion process, no breakage of the capsules and no cell accumulation in the reactor was observed, which shows that the mechanical strength of the capsule membrane was strong enough to bear the shear force caused by air bubble flowing up and indicates that the BCR system is a good alternative for the biotransformation applying the encapsulation technique.

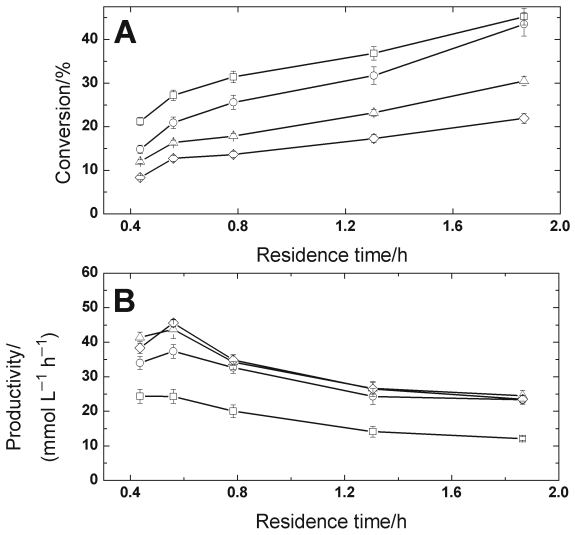


**Fig. 7.** Conversion (A) and productivity (B) of IDAN to IDA by encapsulated cells in a packed bed reactor at different residence times at the substrate concentration of: 50mM (□), 100 mM (○), 150 mM (△), and 200 mM (♦), respectively.

### *Continuous biotransformation of IDAN in a packed bed reactor (PBR)*

To enhance the IDA productivity, continuous biotransformation of IDAN to IDA in a 400 mL PBR reactor was performed at 35 ℃ with continuous feeding of IDAN from the bottom of the reactor at the concentrations of 50 mM, 100 mM, 150 mM, and 200 mM, pH 7.5 and different flow rates. The substrate residence time was set to 0.4 h, 0.6 h, 0.8 h, 1.3 h, and 1.9 h, respectively, by changing the flow rate controlled by a peristaltic pump. In each case when achieving a steady state of the reactor, when the pH and concentration of the product remained constant, relevant results were determined calculating the conversion ratio and productivity of the reaction in the PBR. As it can be seen in Fig. 7A, the conversion ratio increased rapidly as the residence time was prolonged from 0.4 h to 1.9 h and it was obviously reduced with the increase of the substrate concentration. When applying the residence time of 1.9 h and the substrate concentration of 50 mM, the conversion ratio reached the maximum value of 45.2 % which is lower than that obtained in the BCR system; however, in a PBR, only approximately 1 h is required to achieve a steady state and can maintain in the best conditions for a longer time than that in a BCR. Obviously higher conversion ratio can be obtained by prolonging the residence time whereas the productivity of the reactor decreases significantly (Fig. 7B). The maximum productivity of 45.6 mmol $L^{-1}$ $h^{-1}$ was achieved at the residence time of 0.6 h and the substrate concentration of 200 mM ; the conversion ratio was rather low compared to the results obtained in a BCR.

Based on the above results, continuous biotransformation of IDAN to IDA in a PBR using encapsulated *A. faecalis* ZJUTBX11 cells was performed (Fig. 8). The results showed that the concentration of the product changes a little even after ten hours of the process, which indicates perfect performance of the cell immobilized capsules in a PCR; low concentration of cells leaking from the capsules indicate that the ACA membrane liquid core capsules are a promising immobilization technique for the biocatalysis industry.

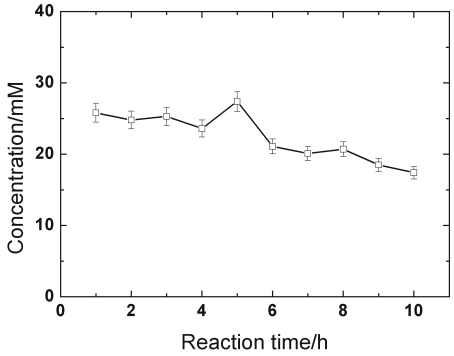


**Fig. 8.** Continuous production of IDA from IDAN by encapsulated cells in a packed bed reactor at the residence time of 0.6 h and the substrate concentration of 150 mM.

## Conclusions

Biotransformation of IDAN to IDA by the immobilization of whole cell nitrilase of *A. faecalis* ZJUTBX11 in an ACA capsule with liquid core was attempted. In addition, the excellent mechanical performance of the capsules enabled the utilization of the encapsulated cells in a larger scale biotransformation under long-term continuous operational conditions. No breakage of capsules and no cell accumulation were observed in either of the reactors indicating lower shear force in the reactors and higher mechanical strength of the capsules. However, the low enzyme activity is a drawback of the presented method. Increasing the activity of the enzyme in its genetically engineered strain is attempted in our lab.

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