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Process parameter optimization for hydantoinase-mediated synthesis of optically pure carbamoyl amino acids of industrial value using *Pseudomonas aeruginosa* resting cells

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Abstract Hydantoinase-mediated enzymatic synthesis of optically pure carbamoyl amino acids was investigated as an environmentally friendly, energy-efficient alternative to the otherwise energy-intensive, polluting chemical synthesis. Hydantoinase-producing bacterial strain was identified as *Pseudomonas aeruginosa* by 16S rRNA gene sequencing and biochemical profiling using the BIOLOG Microbial Identification System. Hydantoinase activity was assessed using hydantoin analogs and 5-monosubstituted hydantoins as substrates in a colorimetric assay. The hydantoinase gene was PCR amplified using gene-specific primers and sequenced on an automated gene analyzer. Hydantoinase gene sequence of *P. aeruginosa* MCM B-887 revealed maximum homology of only 87 % with proven hydantoinase gene sequences in GenBank. MCM B-887 resting cells converted >99 % of substrate into *N*-carbamoyl amino acids under optimized condition at 42 °C, pH 8.0, and 100 mM substrate concentration in <120 min. Hydantoin hydrolyzing activity was D-selective and included broad substrate profile of 5-methyl hydantoin, 5-phenyl hydantoin, 5-hydroxyphenyl hydantoin, *o*-chlorophenyl hydantoin, as well as hydantoin analogs such as allantoin, dihydrouracil, etc. MCM B-887 resting cells may thus be suitable for bio-transformations leading to the synthesis of optically pure, unnatural carbamoyl amino acids of industrial importance.

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Introduction

Optically pure amino acids are essential for the production of semi-synthetic antibiotics, peptides, hormones, pyrethroids, pesticides, as well as pharmaceutics and developmental drugs. Conventional chemical synthesis of amino acids is an expensive, energy-intensive, and laborious process. Also, the resultant amino acids are of low optical purity [6]. Enzymatic synthesis of optically pure amino acids has become increasingly popular over the past couple of decades as enzymes can catalyze synthesis of amino acids and intermediates with almost 100 % optical purity in a mild environment [7]. Hydantoinase is one such enzyme that can catalyze the reversible stereospecific hydrolysis of chemically synthesized racemic cyclic hydantoins yielding optically pure carbamoyl amino acids [19], which can be efficiently converted into corresponding amino acids by chemical process using nitrous acid [9]. Synthesis of optically pure amino acids using resting cells as a source of hydantoinase is an environmentally friendly, energy-efficient alternative to the otherwise energy-intensive, polluting chemical synthesis [21]. Further, the process has the added advantage of easy product separation and recovery.

Optically pure carbamoyl amino acids can be used as substrates for the synthesis of industrially important amino acids via chemical/enzymatic routes. Hydantoin derivatives such as 5-methyl hydantoin, 5-phenyl hydantoin, 5-hydroxyphenyl hydantoin, and *o*-chlorophenyl hydantoin act as a source for synthesis of some of the valuable amino acids that are used as side chains for the synthesis of

semisynthetic penicillins and cephalosporins such as ampicillin, amoxicillin, etc. D-Phenylglycine and D-*p*-hydroxyphenylglycine are extensively used in the synthesis of cefbuperazone and aspoxicillin. Chlorophenylglycine is an important intermediate in the synthesis of antiplatelet agent clopidogrel [12]. There is a paucity of published literature describing hydantoinase with such broad-spectrum substrate profile in spite of several reports describing Gram-positive and Gram-negative bacteria capable of hydrolyzing hydantoins [1]. Most of these reports described enzymes from members of *Agrobacterium* sp. [8], *Bacillus* sp. [15], and *Pseudomonas* sp. [10]. However, only a few of them have been commercially explored and brought to use for industrial production of either natural or unnatural amino acids. This can be attributed to lower productivity and narrow substrate specificity of these hydantoinases. For example, *P. putida* yielded only aliphatic L-amino acids from corresponding DL-5-substituted hydantoins [3], *Arthrobacter* sp. DSM 3747 [11], and *Flavobacterium* sp. [16] converted DL-5-indolyl methyl hydantoin to L-tryptophan, etc. Also, there are non-stereoselective hydantoinases that have been reported [13]. Some of the factors for the limited exploitation of hydantoinase process in industry could be listed as (1) narrow range of substrate catalysis offered by known hydantoinase enzymes, (2) limited stability at high temperature or in organic solvents of these enzymes, and (3) slow racemization rates of certain hydantoin derivatives resulting in partial conversion of the substrate to product. Thus, there is a need for hydantoinase(s) with improved catalytic efficiency, enantioselectivity, stability at high temperature and in organic solvents, and broader/varied substrate profile. Such hydantoinase can be obtained by screening a large number of isolates from varied environmental sources. In our laboratory, hundreds of bacterial isolates obtained from a wide range of environmental sources were screened for hydantoinase activity. One of the high hydantoinase-producing strains designated as MCM B-887 was selected for further investigation. Following is the description of process parameter optimization for the enzymatic synthesis of optically pure and industrially valuable carbamoyl amino acids using resting cells of *Pseudomonas aeruginosa* MCM B-887.

Materials and methods

Materials

Hydantoin and 5-mono-substituted hydantoin derivatives were procured from Sigma Aldrich India Ltd. (Bangalore, India). Nutrient media and ingredients were procured from Hi Media (Mumbai, India). Unless otherwise stated, all reagents were of analytical grade. The solutions of salts and acids were prepared in ultrapure water (Sartorius, Germany). Oligonucleotides used in the study were procured from Imperial Life Sciences, India. All other reagents for PCR were procured from Sigma, St. Louis, MO, USA.

Growth and preservation of microorganisms

The strain MCM B-887, isolated from a soil sample, was used as a source of hydantoinase. MCM B-887 was identified as *P. aeruginosa* on the basis of 16S rRNA gene sequence homology with the reference sequences available in the GenBank database. *P. aeruginosa* MCM B-887 was maintained as glycerol stocks at −70 °C. Working stocks of the culture were maintained at 4–8 °C on Luria agar slants (1 % w/v peptone, 1 % w/v beef extract and 0.5 % w/v NaCl, 2 % w/v Agar agar, pH 7.2).

Assay of hydantoinase activity using resting cells

An actively growing MCM B-887 culture was inoculated in 100 ml of production medium (Hydantoin, 1.0 g; Yeast extract, 1.0 g; NaCl, 0.3 g; pH 8.5) at 1.0 % v/v density. The flasks were incubated at 37 °C on a rotary shaker (150 rpm) for 22 h. Cells were harvested by centrifugation at 10,000×*g* for 2 min, washed with Tris buffer (0.1 M, pH 8.0), and then used for biotransformation. Approximately 7.5 mg of biomass was re-suspended into 200 µl of substrate buffer. The hydantoinase assay was performed in 0.1 M Tris buffer (pH 8.0) containing 200 mM hydantoin and incubated at 42 °C, without agitation for 15 min unless specified otherwise. The enzyme assay was stopped by adding trichloroacetic acid to a final concentration of 0.5 N. The reaction mixture was then centrifuged at 10,000 × *g* for 2 min and the supernatant was analyzed for the presence of *N*-carbamoyl amino acids by Ehrlich's colorimetric assay [22]. Glycine was estimated by the Ninhydrin assay [18]. Hydantoinase activity was calculated as micromoles of *N*-carbamoyl amino acid produced per minute. All reactions were carried out in triplicate and results were shown as means of the data.

For studying the enantioselectivity of the enzyme, the *N*-carbamoyl amino acid formed was analyzed using HPLC (Perkin Elmer, Waltham, MA, USA) with a chiral column (ChiroBiotic™, 25 cm in length and 8 mm in diameter, manufactured by Supelco, Bellefonte, Pennsylvania, USA) with the mobile phase of 80 % (v/v) methanol at a flow rate of 0.5 ml min^{-1}. The absorbance of the injected sample was measured with an UV detector at 210 nm.

Primer designing and sequencing of hydantoinase gene from *P. aeruginosa* MCM B-887

Gene-specific PCR primers for hydantoinase and related enzymes were designed. A total of 15 complete genes sequences encoding hydantoinase or dihydropyrimidinase or dihydroorotase or phenyl hydantoinase were selected from the GenBank database. All these sequences were aligned together with MEGA (version 5.03) in order to identify the conserved nucleotide sequences including the ORF and their adjacent regions. Such conserved loci were used to design gene-specific primers that flanked either the complete gene or the gene fragments. The specificity of each primer was confirmed using in silico PCR tool as described by Bikandi et al. [2]. PCR amplification was carried out in 20 µl volume containing 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates each, 0.2 µM primers each, 6 % v/v DMSO, 1× buffer and 1 U of *Taq* DNA polymerase. The PCR conditions were 5 min at 95 °C followed by 35 cycles of 95 °C for 1 min, 58.6 °C for 30 s, and 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were purified and used as a template in cycle sequencing experiments. Big Dye terminator chemistry was used for performing cycle sequencing as per the manufacturer's instructions (Applied Biosystems Inc., Carlsbad, CA, USA). Sequencing was performed on ABI 3100 Avant automated sequencer (Applied Biosystems).

Submission of culture and gene sequence

Pseudomonas aeruginosa MCM B-887 was deposited in MACS Collection of Microorganisms (Accession Number: MCM B-887). The MCM culture collection is recognized by World Data Center for Microorganisms (Registration Number: WDCM 561). The 16S rRNA gene sequence of MCM B-887 was deposited in the GenBank database (Accession Number: JX008025). The sequence of the gene encoding hydantoinase in *P. aeruginosa* MCM B-887 was also deposited in GenBank database under the Accession Number: JX069971.

Results and discussion

In the present investigation, a total of 150 environmental isolates were screened for hydantoinase activity (data not shown). It was observed that MCM B-887 resting cells cleaved hydantoin and 5-mono-substituted derivatives of hydantoin and thus harbored hydantoinase enzymes. MCM B-887 was found to exhibit significantly higher hydantoinase activity, and hence was selected for further studies. MCM B-887 was identified as *P. aeruginosa* on the basis of 16S rRNA gene sequence homology (>99 % homology with the reference sequence available in the GenBank database) and biochemical profiling using BIOLOG Microbial Identification System.

Entire hydantoinase gene sequence was PCR amplified using FWDa1 (5′-ATGTCCCTGTTGATCCGTGGC-3′) and REVa1 (5′-TCAGCGCTCGACGGCGAC-3′) primers. Nucleotide sequence of the open reading frame revealed the highest homology of only 87 % with proven hydantoinase gene sequence, highlighting the novelty of the enzyme.

The ability of MCM B-887 resting cells to catalyze the cleavage of cyclic hydantoin and derivatives was investigated. Hydantoin was the most preferred substrate for MCM B-887 resting cells. Methyl hydantoin was also cleaved with comparable efficiency, whereas phenyl hydantoin, *o*-chloro-hydroxyphenyl hydantoin and *p*-hydroxyphenyl hydantoin were cleaved at lower but significant efficiency as evident from the biotransformation of these substrates into carbamoyl amino acids by the resting cells (Table 1). Biotransformation of hydantoin and derivatives was also investigated using the cleared cell lysate. It was observed that the activity profile of the equivalent cleared cell lysate of MCM B-887 was comparable with that of the resting cells (Table 1). This observation underscored the suitability of the resting cells as an efficient source of hydantoinase enzyme. The use of resting cells for the biotransformation also facilitated the recovery of the product from the reaction mixture. MCM B-887 hydantoinase-mediated biotransformation of methyl hydantoin and phenyl hydantoin was of interest since alanine (product of methyl hydantoin biotransformation) and phenylglycine (product of phenyl hydantoin biotransformation) are excessively required in feed and pharmaceutical industries, respectively. Substrate profile of the hydantoinase reported in the present investigation was much broader than the earlier reports in *Agrobacterium* [5, 17], *Pseudomonas* [3], *Arthrobacter* [24], and *Blastobacter* [20]. Previous studies describing saturated mutagenesis of hydantoinase genes have revealed that the substrate profile of D-hydantoinases is determined by hydrophobic nature

Table 1 Biotransformation of 5′-mono substituted hydantoin derivatives by MCM B-887 resting cells and cleared cell lysate

Substrates (1.0 % w/v)	Relative activity (%)	
	Resting cells	Cleared cell lysate
Hydantoin	100.0	100.0
5-Methyl hydantoin	82.0	89.3
5-Phenyl-hydantoin	48.3	41.3
5-*p*-hydroxy-phenyl hydantoin	25.5	28.2
5-*o*-chloro-phenyl hydantoin	18.5	28.0

originating from the hydrophobic and bulky residues Leu 65, Tyr 155, and Phe 159, which are closely placed on the chiral exocyclic substituent of the substrate [4]. Translated gene sequence of MCM B-887 hydantoinase revealed the presence of proline at 65 and isoleucine at 159 positions. Uncommon substrate profile of the MCM B-887 hydantoinase could have been due to the altered composition of amino acids at these two positions.

There is increasing interest in optically pure carbamoyl amino acids in industry. Hence, enantioselective hydantoinase is an industrially desired enzyme. Enantioselectivity of the MCM B-887-mediated biotransformation was determined by incubating resting cells with 5-DL-phenyl hydantoin under optimized conditions. The enantiomeric excess of the product formed was assessed in the supernatant by using chiral HPLC (Fig. 1). It was observed that D-phenyl hydantoin was preferentially hydrolyzed over *L*-isomer by MCM B-887 resting cells. D-carbamoyl phenyl glycine was formed at an enantiomeric excess of more than 98 %. These observations revealed that MCM B-887-mediated biotransformation as D-selective. It may be noted here that un-reacted L-hydantoin derivatives spontaneously racemize into D-hydantoin isomers due to keto-enol-tautomerism at alkaline pH [23].

Biotransformation of hydantoins into D(–)*N*-carbamoyl amino acids was enhanced through optimization of various physico-chemical parameters. The biotransformation of

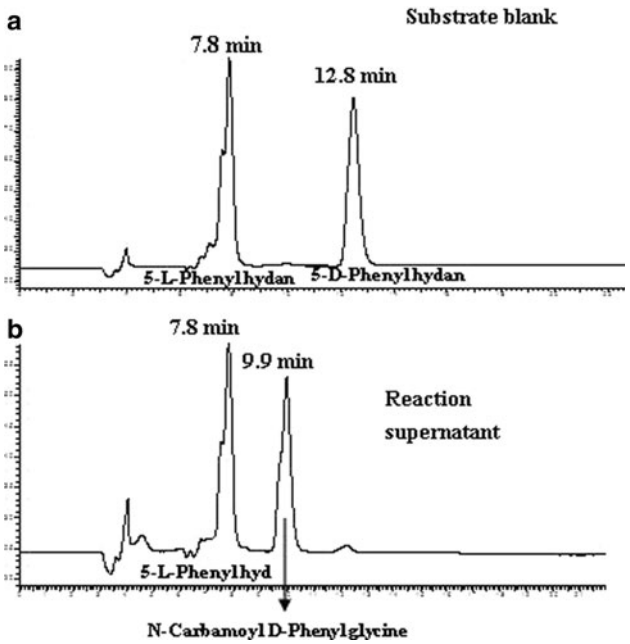

Fig. 1 Chiral HPLC chromatography of racemic DL-phenyl hydantoin (**a**) and of cell-free reaction supernatant at 15 min (**b**). MCM B-887 resting cells were incubated in Tris buffer (pH 8.0) containing 5-DL-phenyl hydantoin (200 mM) at 42 °C for 15 min. Cell-free supernatant was analyzed by HPLC using a chiral column to determine the optical purity of the product formed

hydantoin using resting cells was investigated at different pH. Data revealed that the substrate biotransformation increased with an increase in pH of the reaction with the highest activity at pH 8.0 and insignificant activity at pH 5.0 (Fig. 2a). Hydantoin derivatives have a higher solubility at alkaline pH. Also, the rate of chemical racemization is elevated under alkaline conditions, which makes the possibility of near 100 % racemic substrate conversion to a single enantiomeric form of the product [9]. Hence, optimum biotransformation observed at pH 8 was considered suitable for industrial applications. Optimum temperature for the biotransformation of hydantoin was determined by incubating the resting cells of MCM B-887 in the presence of hydantoin for 2 h at different temperatures. The optimum temperature for the MCM B-887-mediated biotransformation was found to be 42 °C (Fig. 2b). At this temperature, almost 80 % conversion efficiency was observed. Significant activity was observed even at 50 °C where more than 40 % of the conversion efficiency was seen. Thermostability of the resting cells as catalyst is desirable as many hydantoin derivatives are sparingly soluble in aqueous media at ambient temperature with significantly higher solubility at elevated temperature. Higher substrate solubility as well as thermal stability of catalyst (resting cells) ensures higher product yields at elevated temperature in industrial biotransformation of hydantoin derivatives.

Increased biotransformation efficiency at high concentration of substrate is desired in the development of an industrial process. Hence, the effect of initial concentration of hydantoin on MCM B-887-mediated biotransformation was investigated. The highest catalytic activity associated with resting cells of MCM B-887 was observed at 200 mM substrate concentration (Fig. 2c). The concentration of hydantoin when further increased resulted in a decline in catalytic efficiency of the resting cells. This can be due to either a drop in the pH, substrate-dependent inhibition, or increased end-product accumulation in the reaction mixture. In the present study as well, a significance drop in pH (<6.5) of the reaction mixture was observed as a consequence of an accumulation of carbamoyl amino acids. The effect of metal ions on the activity of hydantoinase was reported earlier in the literature. Such an effect ranged from total inhibition to significant activation of the enzyme. May et al. [13] showed that the hydantoinase of *Arthrobacter aurescens* DSM 3745 was a metalloenzyme containing a catalytically important zinc ion. Mukohara et al. [14] showed similar results with *Bacillus stearothermophilus* NS1122A, where Mn^{2+} and Co^{2+} were important for hydantoinase activity in purified extracts. The requirement of the metal ions for the hydantoinase activity of MCM B-887 resting cells was investigated in the present study. It was evident from the data illustrated in the Fig. 2d that

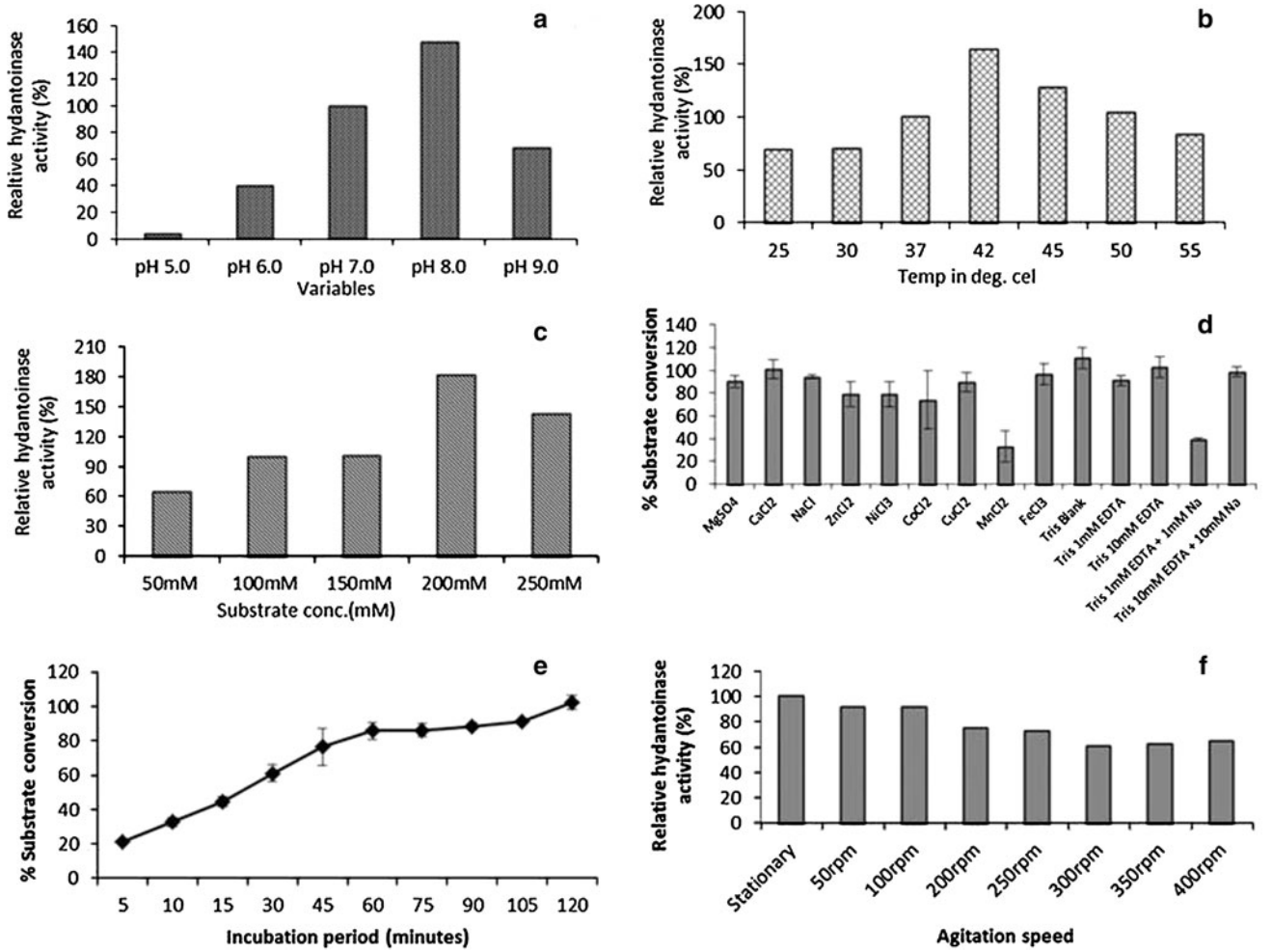

Fig. 2 Optimization of physico-chemical parameters for hydantoinase activity: Effect of pH (**a**), temperature (**b**), substrate concentration (**c**), metal ions (**d**), length of incubation (**e**), and agitation (**f**) on MCM B-887 resting cell-mediated hydantoin biotransformation is illustrated. MCM B-887 resting cells were incubated in Tris buffer (pH 8.0) containing hydantoin (200 mM) at 42 °C for 15 min unless specified otherwise. Substrate conversion was estimated colorimetrically by Ehrlich's assay as described in the text

catalytic activity of MCM B-887 was independent of most of the metal ion supplements. Addition of EDTA to the reaction mixture, which caused chelation and subsequent unavailability of metal ions, also did not adversely affect the MCM B-887-mediated hydantoin biotransformation. Unlike previous reports, MCM B-887-mediated biotransformation of hydantoins did not require the presence of divalent cations. Interestingly, Mn^{2+} supplement caused a steep decline in the biotransformation activity of resting cells.

Substrate conversion kinetics for MCM B-887 hydantoinase was evaluated by incubating resting cells of MCM B-887 in presence of hydantoin over varying lengths of incubation. The amount of substrate converted into product is illustrated in Fig. 2e. It was observed that the substrate conversion increased linearly with increasing time up to 45 min. However, substrate conversion increased only marginally on further incubation. This observation was attributed to carbamoyl amino acid accumulation and a decrease in pH, both of which are unfavorable for hydantoinase activity. Efficient mixing of the reaction mixture ensures better contact between resting cells and substrate (especially if the substrate is insoluble) and thus improves the biotransformation efficiency. Therefore, the effect of agitation on the substrate conversion efficiency was evaluated as resting cells of MCM B-887 were used as a source of hydantoinase enzyme (Fig. 2f). No significant enhancement in the hydantoin biotransformation was observed even when the agitation was gradually increased from stationary to 400 rpm. This could be due to complete solubility of hydantoins at the concentration tested in this study.

Conclusions

The present investigation has reported *P. aeruginosa* MCM B-887-mediated biotransformation of hydantoin analogues and derivatives. Low gene sequence homology with proven hydantoinase sequences, substrate profile, altered composition of critical amino acids residues (as compared to published reports), and non-dependence of catalytic activity on divalent cations underscores the novelty of MCM B-887 hydantoinase. Further, the broad substrate profile, enantioselectivity, and optimum activity at alkaline pH and elevated temperature make MCM B-887 resting cells a suitable candidate for industrial bio-transformations, leading to the production of optically pure carbamoyl amino acids of industrial importance.

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