A hydantoin hydrolysing bacteria: Isolation, characterization and bioconversion

D-phenyl glycine and D-P-hydroxy phenyl glycine (D-P-HPG) are valuable synthons for the production of semisynthetic penicillin and cephalosporins such as ampicillin and amoxicillin. DL-5-substituted hydantoins are the starting materials for the chemical synthesis of DL-amino acids. However, this chemical process is cumbersome as the chemical hydrolysis to obtain optically pure amino acids requires recemate resolution. Dihydropyrimidinase, an enzyme widely distributed in nature, catalyses the hydrolytic ring cleavage of DL-5-substituted hydantoin to *N*-carbamyl amino acids[1]. Several optically active amino acids[2–4] including D-P-HPG are suggested for possible production using the enzymatic route, starting from the corresponding racemic 5-substituted hydantoins.

We report here the isolation of a novel *Pseudomonas* species with the ability to stereospecifically transform DL-5-P-hydroxy phenyl hydantoin to C-N-P hydroxy phenyl glycine (C-N-P-HPG). This strain was able to cleave hydantoin and was adapted to cleave P-hydroxy phenyl hydantoin (P-HPH) in the presence of uracil as an inducer to produce selectively C-N-P-HPG which can be chemically or enzymatically converted to produce D-P-HPG. Improvement of D-amino acid production by resting cells was investigated through enhancement of the hydantoinase during fermentation. The optimal conditions for the bioconversion were determined.

Hydantoin degrading bacterial isolates were isolated using soil samples enriched with hydantoin and P-HPH for several weeks. The soil samples were diluted with sterile water and processed for isolation of the microflora employing a basal salt medium containing (g/l) NH_4NO_3, 1.0; K_2HPO_4, 0.25; $MgSO_4.7H_2O$, 0.25; NaCl, 0.005; Hydantoin, 1.0; P-HPH, 1.0; pH 7.2, 0.5 g of the sample was taken to inoculate 50 ml of the medium in a 250 ml Erlenmeyer flask and then incubated on a shaker at 30°C for 6 days. The broth of mixed culture was reinoculated with a volume equivalent to 5% into a fresh medium. This procedure was repeated two more times at an interval of 6 days. To isolate the pure culture, the mixed bacterial culture was plated on N-agar plates. Representative colonies with similar colony characteristics and abundance throughout the plates were picked and further purified through single colony isolation. The ability of the isolates to hydrolyse hydantoin was examined individually in liquid medium. The selected hydantoin hydrolysing strains were identified on the basis of morphological and biochemical characteristics. From the enriched soil samples 23 organisms were isolated and only 3 organisms, viz. *Pseudomonas* sp. H1, *Pseudomonas* sp. H2 and *Bacillus* sp. H3 were found to be accumulating the hydrolysed product. Among these, the most efficient strain on the basis of hydrolysis of P-HPH was a *Pseudomonas* sp. This species was further investigated for determination of P-HPH hydrolysis along with a culture known for having hydantoinase enzyme *Agrobacter radiobacter* NCIM-2986 obtained from National Chemical Laboratory, Pune.

The *Pseudomonas* sp. was grown in a medium containing (g/l) glucose, 5; Soybean flour, 5; $(NH_4)_2SO_4$, 3; KH_2PO_4, 2; $CaCO_3$, 2; pH 7.2 at 30°C for 48 h. The *Agrobacter* sp. was grown in a medium containing (g/l) sucrose, 10; peptone, 5; yeast extract, 5; KH_2PO_4, 1; NaCl, 0.5; $MgSO_4$ 0.1; $MnSO_4$, 0.1; pH 7 at 30°C for 48 h. Two litres of respective standardized growth medium was taken in the fermenter and inoculated with 10% inoculum and allowed to grow. The cells were harvested by centrifugation at 3000 rpm and washed twice with N-saline. Free cells were then used for the bioconversion study. The bioconversion was carried out in the bioreactor with continuous pH and temperature monitoring and control system. The cells were suspended in the phosphate buffer with the substrates at different proportions to find the optimum concentration required. Similarly different pH and temperatures were maintained to optimize the physical parameters. The pH was maintained by addition of 1 N NH_4OH or 1 N HCl. The reaction medium was continuously stirred and kept under nitrogen atmosphere. The progress of the reaction was continuously monitored by taking out samples and analysing them by TLC and HPLC for the substrate P-HPH or the converted product C-N-HPG and D-P-HPG. TLC was done by spotting the sample in silica gel 60 F_{250}. TLC plate (Merck) and was developed on 1-butanol/acetic acid/water (65:13:22). P-HPH could be detected directly under UV light, C-N-HPG gives a yellow spot by spraying Ehrlich's reagent on the plate and P-HPG gives a violet spot on spraying ninhydrin. HPLC was conducted by injecting the diluted samples into HPLC column – ODS/Hypersil (C_{18}) and the mobile phase used was H_2O/CH_3CN/H_3PO_4 85% (95:5:0.01) by volume with a flow rate of 1 ml per minute. The detection was done using a UV detector at 210 nm.

The reaction products were identified after purification by IR and NMR studies. The isolated *Pseudomonas* sp. H1 showed maximum hydrolysing capacity and the product was analysed and identified by IR, NMR studies and measuring the optical rotation which was –158.3° (authentic D-HPG = –159.1°). Studies were conducted using free resting cells of this strain at 2% cell mass concentration and substrate concentration of 0.5% to investigate the effect of different reaction temperatures (range: 20, 30, 40, 50, 60°C) at pH 8. The optimum temperature for D-hydantoinase activity of the isolated organism was found to be 40°C as the concentration of N-C-HPG measured in the reaction medium was 0.3, 1.1, 1.3, 0.1, 0.0 g/l, respectively. Similarly different reaction pH (range: 6.0, 7.5, 8.0, 8.5, 9.0) on the conversion activity of the enzyme system under the same reaction conditions at 40°C was investigated and optimal pH was found to be 8.0 as the concentration of N-C-HPG measured in the reaction medium was 0.0, 0.4, 1.2, 0.9, 0.8 g/l, respectively.

The hydantoinase enzyme is known to be both inducible[5] and constitutive[6]. This study was conducted to establish the effect of induction of various

Table 1. Comparison of the two organisms for their productivity

Enzyme source	P-HPH added in the reaction (g/l)	C-N-HPG produced (g/l)	Total HPG produced after decarbamylation (g/l)
Agrobacterium	5.0	2.3	2.3
Pseudomonas sp. H1	5.0	4.0	3.2
	50.0	22.0	–

Cell mass concentration of 2–5%, pH 8 and temperature 40°C.

hydantoin derivatives like hydantoin, hydroxy phenyl hydantoin and structural analogues like uracil on the formation of hydantoinase in the isolated micro-organism in comparison to uninduced cell mass. Under standard reaction conditions the concentration of N-C-HPG measured in the reaction mixture with the uninduced cell mass was 1.25 g/l whereas cell mass induced with hydantoin, hydroxy phenyl hydantoin and uracil showed concentration of 1.5, 1.4, 2.0 g/l, respectively. There was improvement in the enzyme activity due to induction with all the above compounds tested, with uracil showing the highest. From the study on the course of progress of reaction with time, conversion reaction proceeded till around 20–25 h, and further incubation did not result in any improvement in yield at 5 g/l of substrate concentration with 2% cell mass concentration. There are a few reports on the direct conversion of DL-P-HPH to D-P-HPG. Olivery *et al.*[7] have shown that the production of D-P-HPG may be achieved in one step using resting cells of *A. radiobacter*. A comparative study along with *A. radiobacter* NCIM 2986 was conducted to examine the efficiency of the enzyme system. *Pseudomonas* sp. H1 was seen to have 1.7-fold more yield in comparison to *Agrobacterium* (Table 1). The enzyme seems to be different from that reported by Dong-Man Kim and Hak-Sung Kim[6] who used hydantoin, dihydrouracil, DL-P-phenyl hydantoin and DL-P-HPH to induce the enzyme system in the isolated micro-organism, but found the enzyme to be a constitutive one, as the activity was similar in spite of addition of all the inducers. The optimum temperature and pH for the activity of the enzyme was more or less similar to those in case of substrate concentration at 10 g/l. Investigation of the activity with higher substrate concentration showed the enzyme to be economically promising for higher hydrolysis of the substrate. About 50% conversion is achieved at 50 g/l substrate concentration.

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Behavioural and chemical aspects of scent marking in the Asiatic lion

Scent marking by the big cats was first described by Locke[1] who studied the tiger in Malay but it was Schaller who first clearly mentioned that tigers spray a mixture of urine and secretion from the anal gland upwards and backwards which presumably leaves odourous signals on salient environmental objects. This spraying was subsequently observed in detail by Smith *et al.*[2] in the wild and by Brahmachary *et al.*[3] in captivity. Schaller's statement that the odourous secretion from the anal gland is mixed with urine in both tiger[4] and the African lion[5] runs into a difficulty because the anal gland opens towards the anus and has no connection with the urinary tract[6]. Both male and female tigers spray with a high frequency[3,7] but in the African lion it is predominantly a male activity[5].

The present note is intended to document the behavioural and chemical aspects of scent marking in the Asiatic lion. This investigation was made in two enclosures at the Interpretation Zone, Sasan-Gir, Gir National Park, India. Each enclosure has an area of 81 m^2 but occasionally the lions were released in the 420 ha fenced forest area where they could freely roam and even hunt prey. Thus, they were exposed to a more natural environment than that of most zoos. Observations were carried out during 2 h in the morning and again for 2 h in the afternoon when they were generally most active. The relative frequencies of urination and marking fluid (MF) and their location within the enclosures