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Antibacterial and toxicological evaluation of ß-lactams synthesized by immobilized ß-lactamase-free penicillin amidase produced by *Alcaligenes* sp.

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Search for anti-ß-lactamase and synthesis of newer penicillin were suggested to overcome resistance to penicillin in chemotherapy. It was found that clavulanic acid, an ant-ß-lactamase was ineffective due to its structural modification by bacteria. Thus, there is a need for the synthesis of newer pencillins. Retro-synthesis was inspired by the success of forward reaction *i.e.*conversion of penicillin G to 6-aminopenicillanic acid (6-APA) by biological process. In the present study a better enzymatic method of synthesis of newer pencillin by a ß-lactamase-free penicillin amidase produced by *Alcaligenes* sp. is attempted. Antibacterial and toxicological evaluation of the enzymatically synthesized ß-lactams are reported. Condensation of 6-APA with acyl donor was found to be effective when the reaction is run in dimethyl formamide (DMF 50% v/v) in acetate buffer (25 m*M p*H 5.0) at 37°C. Periplasm entrapped in calcium alginate exihibited the highest yield (~34%) in synthesis. The minimum inhibitory concentration of the synthetic products against *Staphylococcus aureus* and *Salmonella typhi* varied between 20–80 µg/ml. Some of the products exhibited antibacterial activity against enteric pathogens. It was interesting to note that product A was potent like penicillin G. LD_{50} value of three products (product A, B and C) was more than 12mg/kg. Furthermore, these synthetic ß-lactams did not exihibit any adverse effect on house keeping enzymes viz., serum glutamate oxalacetate-*trans*-aminase, serum glutamate pyruvate -*trans*-aminase, acid phosphatase, alkaline phosphatase of the test animals. The hematological profile (RBC and WBC) of the test animals also remained unaffected.

Keywords: *Alcaligenes sp.*, Antibacterial, Penicillin amidase, Synthetic ß-Lactams, Toxicological evaluation

Indiscriminate use of penicillins for the treatment of various diseases has resulted in the development of penicillin resistance by pathogenic microorganisms. Of three unrelated enzymes present in penicillin amidase producing microorganisms, ß-lactamase is the key component, which hydrolyses the ß-lactam moiety of penicillin and makes it ineffective. Two lines of approach have been adopted to overcome the problem of penicillin resistance. Clavulanic acid, a good anti-ß-lactamase in combination with penicillin, is recommended to combat penicillin resistant strains[1]. However, bacteria got the machinery to modify this compound and as a result, the combination becomes ineffective. The newer penicillins provide a better alternative. Biological synthesis of new penicillins is preferred over to chemical synthesis because of low cost of production and eco-friendly nature[2]. Successful transformation of pen G to 6-APA by biological method attracted attention for retro-synthesis.

Penicillin amidase (PA) (EC 3.5.1.11) transforms pen G to 6-APA and furnishes pen G from 6-APA and phenyl acetic acid by condensation. Isolation, characterization and purification of a ß-lactamase-free penicillin amidase produced by *Alcaligenes* sp. (A-13) have been reported[3,4]. The test strain (A-13) was capable of both hydrolysis and synthesis of pen G *in vivo* and *in vitro* conditions[4]. PA produced by *Alcaligenes* sp. is a thermostable enzyme like other PAs reported so far from gram negative bacteria[5,6]. Because of the promise, and high demand for semi-synthetic penicillins, attempts were made to develop enzymatic synthesis of newer ß-lactams by immobilized penicillin amidase produced by

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Alcaligenes sp. In the present study, the anti-bacterial and toxicological properties of enzymatically synthesized ß-lactams are reported.

Materials and Methods

Source of test organism—The test organism *Alcaligenes* sp. (A-13) was isolated from local soil by dilution plate method[3] and was maintained on nutrient agar slant (composition% w/v: peptone1, beef extract 1, sodium chloride 0.1, agar 2, *p*H7.0).

Preparation of periplasmic fraction—For the production of enzyme, the organism was grown in 500 ml of sterile PBP medium (peptone 1%, beef extract 0.5%, phenyl acetic acid 0.1%) in an Erlenmeyer flask (2 L).The culture was allowed to grow at 28°C on a rotary shaker (180 rpm) for 16 hr. After incubation, the culture broth was centrifuged at 5000 g for 15 min. The pellet of cells obtained was washed with Tris- HCl buffer (10 mM, *p*H 8.0). The washed cells were suspended in the same buffer (30 mM, *p*H 8.0) supplemented with sucrose (20% w/v). The suspended cells were then treated with lysozyme (500 μg) (Sigma USA) at 10°C for 30 min. It was then centrifuged (10000 g for 20 min).The supernatant was kept aside as periplasmic fraction[4].

Immobilization of periplasmic fraction—Periplasmic fraction was mixed with sodium alginate (3.3% w/v) in the ratio of 2:3. The mixture was then dropped into a stirring solution of $CaCl_2$ (0.1 M). Immobilized beads were washed and used for synthesis.

Preparation of acyl donors—Methyl or ethyl esters of aromatic acids, used as acyl donors, were prepared by refluxing the acid with excess of methanol or ethanol saturated with HCl vapour[7]. The ester was then extracted with solvent, washed with water, dried over anhydrous sodium sulphate and finally evaporated to dryness under reduced pressure. The homogeneity of the ester was tested by thin layer chromatography (TLC) on silica gel G plate using ethanol-chloroform-ammonium hydroxide (53:30:17) as solvent system. The spots were detected under UV light or in iodine vapour.

Reaction conditions—6-APA and acyl donor (in the ratio of 1:2) were allowed to react in a mixture of solvents i.e. dimethyl formamide (DMF 50% v/v) and acetate buffer (25 mM, *p*H 5.5) in presence of immobilized beads (equivalent to 50 mg cell) for 3 hr at 37°C.

Isolation of the product—The enzyme beads were separated by filtration, the filtrate was extracted with diethyl ether, the ether extract was then washed with sodium bicarbonate solution (5% w/v). The bicarbonate fraction was in turn extracted with ether, the extract processed and finally evaporated to dryness. The homogeneity of the product was tested on silica gel G plate (TLC) using butyl acetate- butanol-acetic acid-water (80:15:40:24) as solvent system. The spots were detected in iodine vapor. The product was also resolved on Waters Nova pack C-18 reverse phase column by high pressure liquid chromatography (HPLC) using acetic acid (50 mM) and methanol (1:1) as mobile phase at a flow rate of 0.5 ml/min.

Assay of antibacterial activity—Antibacterial activity of synthetic products was assayed by agar cup diffusion method[8,9] using freshly grown (24 hr old) test organisms. *Staphylococcus aureus, Salmonella typhi (BC924), Escherichia coli (H25034), Shigella sonnei (NK2752), Vibrio cholerae O1 (32430), Vibrio cholerae O139 (*H18016*), Shigella flexneri* type 2a (33220), *Shigella flexneri* type 6 (E03489) and *Salmonella infantis* (15739) were maintained on nutrient agar (NA) slant and used for determination of antibacterial activity. The test organism was seeded in sterile nutrient agar taken in a petri dish. After solidification of the medium, agar cups were cut and they were filled with individual product (100 μg). Penicillin G (100 μg) dissolved in minimum volume of aqueous methanol (50% v/v) was used as reference. A solvent control was maintained. All plates were incubated at 37°C for 24-48 hr. The zone of inhibition was measured and compared with control.

MICs—MICs of the synthetic products were determined against *Staphylococcus aureus* and *Salmonella typhi* by test tube dilution method[10].

Toxicity study—Swiss albino mice (18–22 g) supplied by Bengal Chemical Pharmaceutical Works Ltd., Kolkata were used for the toxicity study. The mice were kept over husk bed in cages in an animal room having a relative humidity of 45-55% at 25°C. The animals were fed on a stock diet (pellet composed of wheat flower 65%, casein 15%, sucrose 10%, ground nut oil 5%, shark oil 2%, AOAC vitamin mixture 2% and USP XVII salt mixture 1%) in addition to boiled drinking water. The mice were fed at *ad libitum* for 18 hr before each experiment.

LD_{50}—For LD_{50} study[11], two groups containing 6 animals each were used. The control group received

normal saline and the experimental group was given increasing doses (0.5, 1, 2, 4, 8, and 12 mg/kg) of synthetic product by intra-peritoneal route. Then the test animals were kept under observation for 48 hr after which they were sacrificed.

The effect of the synthetic product on house keeping enzymes viz., serum glutamic-oxalaacetic-*trans*-aminase (SGOT), serum glutamic-pyruvic-*trans*-aminase (SGPT), serum alkaline phosphatase (ALP), serum acid phosphatase (ACP) and blood count (red blood cells, RBC; white blood cells, WBC) of the test animals were examined. Blood samples were taken directly from heart of the animals. One portion of blood was used for RBC/WBC count and the rest was kept in test tubes without anti-coagulant. After coagulation, the clotted blood was centrifuged at 3000 g for 10 min in a refrigerated centrifuge. The serum was analyzed for the enzymes. Statistical analysis was done by students'*t*-test [12].

Counting of RBC—Blood samples were taken in red cell counting pipette up to 0.5 mark and diluted (1 to 200) with RBC diluting fluid. One drop of diluted blood was put in the counting chamber (0.1 mm sample depth) of hemocytometer and covered by a cover slip. Volume of each small square is 1/4000 cu.mm Red blood cells were counted in five groups of 16 small suares[13].

$$\text{Number of RBC per cu.mm blood} = \frac{\text{number of cells counted} \times \text{dilution} \times 4000}{\text{number of 1 square mm counted}}$$

Counting of WBC—Blood samples were taken in white cell counting pipette and diluted (1 to 20) with WBC diluting fluid. One drop of diluted blood was put in the counting chamber (0.1 mm sample depth) of hemocytometer and covered by a cover slip. WBC was counted at four corners of 1 square and in the central ruled area on both sides of the counting chamber[13].

$$\text{Number of WBC per cu.mm blood} = \frac{\text{number of cells counted} \times \text{dilution} \times 10}{\text{number of 1 square mm counted}}$$

Serum ALP and ACP determination—Acid and alkaline phosphatase activity of serum were measured using p-nitro phenyl phosphate (PNPP) as substrate. Buffer (0.5 ml) mixed with 0.5 ml of PNPP received 0.1 ml of serum and it was incubated at 37°C for 30 min. After the incubation, the reaction mixture received 10 ml of NaOH (0.1N), mixed and color developed was read at 420 nm. The amount of p-nitro phenol (PNP) formed was calculated from the standard curve and the activity was expressed as μg of PNP/min/ml serum[14.] Glycin-NaOH buffer (0.1M, *p*H 9.3) and citric acid-NaOH (0.09M, *p*H 5.0) were used for determination of ALP and ACP activity respectively.

SGOT and SGPT activity—SGO*T* and SGPT activity was determined using alpha-keto glutaric acid, DL-aspartic acid, DL-alanine as substrates. Non-hemolysed serum (0.1 ml) was mixed with SGOT/SGPT reagent separately and incubated at 37°C for 1 hr and 30 min. Dinitro phenyl hydrazine (DPNH, 0.5 ml) was added to the reaction mixture and kept at room temperature for 20 min followed by the addition of NaOH (0.4 N, 5 ml), mixed and allowed to stand at room temperature for 30 min. The color developed was read at 505 nm. The amount of oxalacetic acid formed was calculated from the standard curve. The activity was expressed as μg of oxalacetic acid or pyruvic acid/min/ml serum[15] .

Results

Percentage conversion of 6-APA to newer penicillins was 34%. Among the solvents used, dimethyl formamide (DMF 50% v/v) in acetate buffer (25 mM, *p*H5.0) proved to be best for synthesis. The stochiometry of reactants i.e. 6-APA and acyl donor in ratio of 1:2 furnished maximum yield of the product. The optimum *p*H for synthesis was 5.5. The synthetic products were found to be homogeneous because they had retention time (HPLC) and R_f value (TLC) different from that of the reactants. MIC values of the synthetic products against *S. aureus* and. *S .typhi* are given in Table 1. It varied from 20–60

Table 1—MIC (μg/ml) of the synthetic products by serial dilution method

Product	*S. aureius*	*S. typhi*
6-APA + CAME (Product A)	20	50
6-APA + MCSA (Product B)	30	50
6-APA + E M (Product C)	20	50
6- APA + PAME (Product D)	60	80
6- APA + HPMA (Product E)	50	70

6-APA = 6-Amino penicillanic acid, CAME = cinnamic acid methyl ester, CSAME = chloro salicylic acid methyl ester, PAME = phenyl alanine methyl ester, HPMA = hydroxyl phenyl methyl acetate, EM = ethyl mandelate

and 50-80 μg/ml against *S. aureus* and *S. typhi* respectively. Among them, the products A, B and C had low MIC values and were chosen for further study. Antibacterial profile of the selected products A, B and C are given in Table 2. They were found to be active against gram positive and gram negative bacteria including some enteropathogens. *S.aureus* is most sensitive while *S. flexinery* type 6 appeared to be most resistant to these compounds. Product A was found to be active against *S.aureus* at par with pen G. Moreover, products A, B and C were least toxic. LD_{50} value of these compounds was more than 12 mg/kg body weight of mice. There was no death of experimental animal upto the dose of 12 mg/kg body weight. Further, the products did not exhibit any adverse effect on the house keeping enzymes *viz*., SGOT, SGPT, ALP and ACP of the test animals and their RBC, WBC count remained unaltered (Table 3).

Discussion

For synthesis of newer ß-lactams, esters of aromatic acid were preferred for condensation with 6-APA because most of the ß-lactams used in chemotherapy have got aromatic acid residue at 6 position of penicillin nucleus. Conversion of pen G to 6-APA by microbial enzymes is well established[16]. In fact the success of forward reaction has inspired the retro-synthesis. In the present study, the synthesis of newer ß-lactams by immobilized penicillin amidase was found to be best in mixed solvents. Reports are available about improvement in yield of ß-lactams by penicillin amidase when the reaction is run in frozen media[17]. The role of *p*H on synthesis proved to be critical; the optimum *p*H was 5.5 in the present study. Excellent yield in conversion of 6-APA to ampicillin (96%) had been reported under *p*H gradient[18]. Like natural penicillins, the newer penicillins are active against both gram positive and gram negative bacteria (Table 1). It was interesting to note that the condensation product of 6-APA and methyl cinamate (Product A) was almost at par with pen G in its antibacterial activity. The LD_{50} of synthetic penicillins was more than 12 mg/kg body weight of mice. They were found to be least toxic like natural penicillins. This was further supported by house keeping enzyme and hematogical profile of the test animals which remained almost unaffected.

It may be suggested that PA produced by *Alcaligenes* sp. seems to be a unique enzyme and may be used for building up of amide bond irrespective of nature of substrate other than 6-APA. It also shows promise of an acylating and deacylating agent for preparing 6-APA and newer penicillins by biological means.

Table 2—Antibacterial activity of product A, B and C
[Values are zone of inhibition in mm]

Product	*S. aureus*	*S. typhi*	*E. coli*	*S. sonnei*	*V. cholerae* 01	*V. cholerae 0139*	*S. flexneri 2a*	*S. flexneri* 6	*S. infantis*
A	11.5	5.5	0	8	6	5	0	0	4
B	7	6	0	2	3	3	6	0	0
C	10	6	0	0	5	4	1	0	0
Solvent	0	0	0	0	0	0	0	0	0
Pen G (authentic)	12	2	0	5	4	5	3	0	5

Table 3—Effect of selected products (A, B and C) on blood count and house keeping enzymes of test animals
[Values are mean ± SD of 6 animals]

Product	RBC (lacs cells/cu. mm blood)	WBC (cells/cu.mm blood)	ACP (μg of PNP/min/ml serum)	ALP (μg of PNP/min/ml serum)	SGOT (μg of oxalacetic acid/min/ml serum)	SGPT (μg of pyruvic acid min/ml serum
Control	21.15 ± 6.30	140 ± 7	23.69 ± 2.43	11.85 ± 3.39	14.77 ± 3.1	142.60 ±18.9
A	21.10 ± 6.21	120 ± 10	24.04 ± 5.35	11.53± 4.61	13.83 ± 1.9	145.31 ± 21.5
B	21.90 ± 5.25	130 ± 6	29.79 ± 4.21	12.29 ± 3.25	14.75 ± 2.7	154.38 ± 28.8
C	20.55 ± 8.19	120 ± 12	23.47 ± 3.52	09.47 ± 2.56	14.47 ± 2.2	132.41 ± 26.5

ACP = acid phosphatase, ALP = alkaline phosphatase, SGOT = serum glutamic–oxalaacetic-*trans*-aminase, SGPT = serum glutamic-pyruvic-*trans*-aminase, n = 6; *P*> 0.1 Compared to control

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