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Screening of Beta-lactam Acylase Producers from Soil and Characterization of Isolates for Substrate Specificity for Cephalosporins

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Abstract

Beta-lactam acylase enzyme producers were isolated from soil samples. Soil samples were induced with phenyl amino acid derivatives for selelctive isolation. Out of the two potential isolates obtained, one of the isolates was studied for presence of beta-lactam acylase enzyme with cephalexin as model substrate. Enzyme activity of isolate grown in media at varying pH showed it to be tolerant to alkaline pH 8.5. Also the substrate specificity for various cephalosporins showed it most selective to cephalexin.

Keywords: Betalactam Acylase Enzyme, Cephalexin, Soil Isolate

1. Introduction

Beta lactam antibiotics have been in clinical use for more than 60 years and are currently the most widely used group of antibiotics utilized to treat bacterial infections by virtue of beta-lactam nuclei and different acyl side chain in semisynthetic antibiotics responsible spectrum of antibacterial activity and increased chemical stability and lesser known toxicity to mammalian cells[1].

Beta-lactam antibiotics particularly derivatives of Penicillins and cephalosporins represent world's major biotechnology products and comprise ~65% of the total world market for antibiotics. Beta-lactam antibiotics have been in clinical use for treatment of bacterial infections since the discovery of Penicllin in 1960 and produced fermentatively from Penicillium and Cephalosporium sp. However wide spread use of these during first world war led to resistance by various bacteria producing betalactamase enzyme which cleaves the beta-lactam nucleus. The susceptibility was also found to be influenced by acyl side chain which on alteration resulted in changed pharmacological properties. These observations led to introduction of semisynthetic beta-lactams containing synthetic side chain attached to beta-lactam nucleus. Amoxicillin, Ampicillin, Cephalexin, Cefadroxil were amongst initial ones.

Manufacturing of semisynthetic beta-lactam antibiotics involved 2 steps - i) Enzymatic hydrolysis of antibiotic to generate nucleus ii) Coupling of synthetic side chain to beta-lactam nucleus. Biocatalysis has paved its way for enzymatic hydrolysis of Penicillin and Cephalosporin to generate key intermediates like 6 Amino Penicillanic Acid (6APA) and 7 Amino Desacetoxycephalosporanic Acid (7ADCA) using Penicillin G Acylase (PGA E.C.3.5.1.11) enzyme widely used for this purpose on commercial scale since 1985[2].

Betalactam acylase enzymes belonging to Ntn hydrolase family include Penicillin amidase or amino acid

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ester hydrolase which were till 1960 were explored for their ability to hydrolyse amide bond in phenyacetyl ring producing useful antibiotic intermediate nucleus like 6APA from Penicillin and like wise 7ADCA from cephalosporin[3] by hydrolysis of CO-NH amide bond which are specifically acted upon by betalactam acylase enzymes. These nucleus are then used for production of semisynthetic antibiotics like amoxicillin, ampicillin and cephalexin and next generation cephalosporins by chemical route.

With growing demand for semisynthetic betalactams especially cephalosporin, the enzymes are looked upon for their potential to synthesize the semisynthetic antibiotic by enzymatic acylation of nucleus with different side chain in cephalosporin nucleus, generates new antibiotic with altered antibacterial spectrum. Further use of nucleus with specific group like - Cl at C3 position forms next generation semisynthetic antibiotic like cefaclor which shows different antibacterial spectrum and pharmacokinetic property form cephalexin. By virtue of use of this property of enzyme, it is possible to synthesize semisynthetic cephalopsorins in aqueous reaction thus replace the chemical coupling step used otherwise and eliminate use of extreme temperature and pressure conditions and use of solvents generating hazardous waste in industry and taking a step towards green revolution[4].

The present research is aimed to screen for bacterial culture which synthesize betalactam acylase enzyme which shows enzyme activity towards cephalosporin derivatives. Since enzymes are reported in various bacteria, actinomycetes and fungi, these vary in their substrate specificity. PGA from *Escherichia coli, Alcaligenes faecalis, Kluyvera citrophila* and *Proteus (Providencia) rettgeri* are localized in the periplasmic space. *Arthrobacter viscosus* and *Bacillus megaterium* produce the acylase extracellularly[5]. In addition to penicillin acylases, enzymes from *Acetobacterturbidans* and *Xanthomonas citri* capable of hydrolysis and synthesis of ampicillin and cephalexin have been described. Since only α -amino acid derivatives could act as substrates, these enzymes were named alpha amino acid ester hydrolases[6]. Ampicillin acylases from *Pseudomonas melanogenum* with a rather narrow substrate specificity[7] relative to AEH, can both hydrolyze and synthesize ampicillin, amoxicillin and cephalexin. DNA sequence analysis showed that AEH are members of a new class of beta-lactam antibiotic acylases[8,9].

Molecular biology advancement further enhanced the prospects of screening and recombinant organisms carrying the gene of enzyme of interest can be explored by high throughput screening techniques[10].

There lies an extensive scope to look for more potential bacterial strains in various environment like industrial soil, effluents from antibiotic industry and explore the synthetic capacity of these enzymes. Preliminary screening from various different soils in vicinity of industry was used as source for isolation by conventional media with addition of inducer such as phenyl acetic acid or phenylalanine which trigger enzymes in these organisms[11–14].

Isolates from soil were studied for their morphology, biochemical characteristics and activity for cephalexin[15,16]. The enzyme tolerance to alkaline pH and its substrate specificity was studied which served as important preliminary finding which was different from the presently known organisms[15,16].

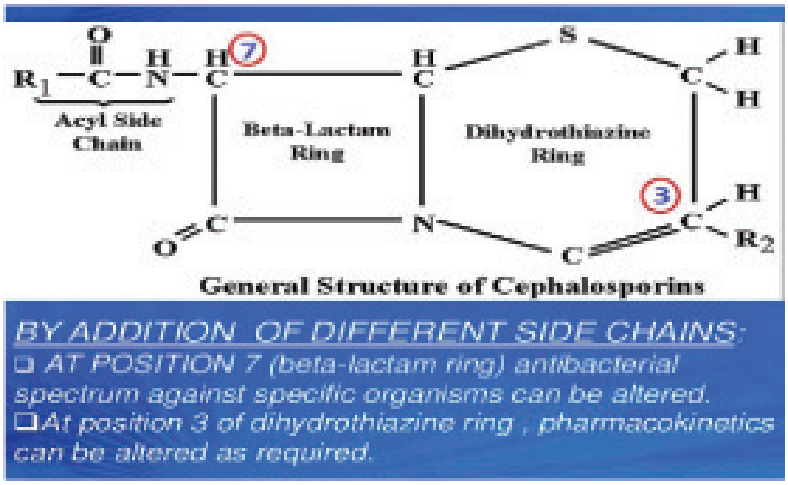

Figure 1. Site of action of betalactam acylase on Cephalopsorin G nucleus at position 7 and generation of 7ADCA nucleus.

2. Materials and Method

E1: Ground soils near ETP area from DIL complex, Thane, India.

G2: Ground soil from factory site of Fermenta Biotech Ltd, Mandi, Himachal Pradesh, India

D3: Ground soil near R and D from DIL complex, Thane, India.

Phenylglycine Methyl ester Hydrchloride (PGMe) and Phenylglycine (PGM) and NIPAB were purchased from Sigma Aldrich.

Cephalosporin G(Ceph G), Penicillin G(Pen G), Cephalexin(CPX), Cefaclor(CCL), Cefadroxil(CDL), Cefprozil(CZL) and their derivatives (7ADCA, 7APCA, 7ACCA) were kind gift from Fermenta Biotech Ltd, DIL complex, India.

All other chemicals used were of analytical grade from local suppliers.

2.1 Screening for Potential Bacteria

100 mL of Sterile Nutrient Broth (NB) was inoculated with 10 g soil E1, G2 and D3 and incubated for 14 days at 28 degrees. Loopful from each flask was inoculated in each 10 mL NB containing 50 mg PGMe in one flask and 50 mg PGM in other flask. PGM was insoluble in medium. The flask were incubated for 48 h at 30 degrees at 150 rpm. From each flask, loopful was isolated on to Nutient agar plates. Plates were incubated at 28 degrees for varying time period as mentioned in results in Table 1 below.

Colonies from each plates were labelled a-j in sequence mentioned below and studied for their morphological characters, gram characteristics and colour development with chromogenic substrate NIPAB (Table 2).

Selected colonies showing positive reaction with NIPAB were grown in 10 mL nutrient broth for 48h at 30 degrees, 150 rpm and pure cultures stored on NA slants for further study.

Table 1. Screening for potential bacteria

Soil	E1			G2			D3		
Medium	NB			NB			NB		
Additive	PGMe	PGM		PGMe	PGM		PGMe	PGM	
Colonies	A	B	C	D	E	F	G	H	I	J

Table 2. Colony morphology

Colonies	Growth conditions	Colony characteristics	Gram stain	Colour Test with NIPAB
A	48h	0.5 mm, round, off white to yellow, transclucent, regular margins	Gram negative thin rods	Positive
B	24h	1mm, round, off white, opaque	Gram positive thick rods	Negative
C	48h	Tiny, irregular, white, opaque	Gram positive rods	Negative
D :	48h	0. 5 mm, cream coloured transcluscent round regular	Gram negative thin rods	Positive
E	72h	0.3 mm, yellow, trascluscent, round regular margins	Gram negative short rods	Positive
F	24h	0.1 mm, irregular margins, thick, white coloured	Gram positive cocci	Negative
G	72h	About 1mm, off white, irregular filamentous	Gram positive rods with spores	Negative
H	48h	1mm, cream coloured, regular round margins, transcluscent	Gram positive rods	Negative
I	48h	Tiny, off white, transcluscent, round margins	Gram negative thin rods	Positive
J	72h	Tiny round, off white, transparent, regular margins	Gram positive cocci	Negative

2.2 Screening for Betalactam Acylase Enzyme

Each of the positive culture was grown in 10 mL of Basic Media (Media B) consisting of 2% w/v Yeast extract, 1% w/v Peptone and 1% w/v glucose pH 7.2 for 24 h. Each of Cell suspension (1mL) was added to 1mL of 0.1M sodium phosphate buffer pH 7.0 containing 0.5%w/v of cephalexin substrate and allowed to incubate at 37 deg for 30 mins. 7ADCA formed in the reaction mixture due to hydrolysis of cephalexin by enzyme was assayed by addition of chromogenic substrate p-dimethylaminobenzaldehyde and extent of yellow colouration was measured spectrophotometrically at 415 nm. (Table 3) Two of the cultures (d and e) showing cephalexin activity were further studied for biochemical characteristics and one of the colonies e renamed as FRCC 71 found to be potential isolate was selected for further studies of substrate specificity and growth in different media.

2.3 Biochemical Characteristics and Media

Colonies d and e tested for various biochemicals according to Bergeys manual of systematic bacteriology (Table 4). FRCC 71 isolate were inoculated in Media B and Media N at different pH. Media B was basic media mentioned above. Media N included salts ammonium sulphate (0.4%w/v), potassium dihydrogen orthophosphate (1.2%w/v), sodium hydroxide (0.3%) and trace elements like magnesium sulphate (200pm), calcium chloride (100ppm) and ferrous sulphate (10ppm). Cultures were allowed to grow at 30 deg for 48h. Cells were separated by centrifugation at 7000 rpm for 30 mins and washed twice with distilled water.

The supernatant media and cells grown in above media and varying pH was checked for cephalexin synthesis activity by method described below.

2.4 Cephalexin Synthesis Activity Assay

Activity was determined by cephalexin synthesis rate in reaction volume of 10 mL containing 100 mM sodium phosphate buffer pH 6.3, 30 mM 7ADCA and 36 mM of PGMe at 28 degrees for 30mins. The cephalexin concentration in reaction mixture was determined by HPLC Inertsil C8 Column 250mmX4.6mm (5micron), Column

Table 3. Biochemical characteristics

Colonies	D	E (FRCC71)
Citrate	-	+
Lysine decarboxylase	+	+
Lysine iron agar	Purple slant and butt	Purple slant and butt
TSI	Red slant and yellow butt	Red slant and yellow butt
Dextrose/Sucrose	+	+
Facaultative/aerobe	Facaultative	Facaultative
H2S	+	+
Cetrimide agar	-	-
Oxidase	-	+
Gelatinase	-	-
Methyl red	+	-

Table 4. Media and pH influence

Culture type	Cells	Supernatent	Cells	Supernatent
	Media B		Media N	
6.5	1.058	0.606	0.769	0.496
7	1.143	0.587	0.791	0.418
7.5	0.719	0.700	1.218	0.440
8.0	0.838	0.427	0.393	0.506
8.5	2.098	0.474	1.303	0.039
7.2	1.272	0.710	0.722	0.349

Table 5. Substrate specificity

Substrate	% specificity
Pen G	131.9
Ceph G	71.31
CPX	100
CDL	83.48
CCL	69.32
CZL	82.95

oven temperature 35 deg C, Detection Wavelength 225 mm. HPLC was performed in isocratic mode with mobile phase containing potassium phosphate Buffer 100mM : Methanol : Acetonitrile in ratio of 700 : 230 : 70 and flow rate 1.2 mL/min. 20µL of sample injected was diluted 1:31 with mobile phase before injection. Unit activity of cephalexin synthesis defined as amount of enzyme catalyzing formation of 1 micromole of cephalexin product per mL per min under the above mentioned conditions.

2.5 Substrate Specificity

Substrate specificity of FRCC71 with various betalactam antibiotics substrates as listed in Table 5. Each of 0.5%w/v substrate hydrolysed with cell suspension in 50 mm sodium phosphate buffer for 15 mins resulted in intermediate betalactam product which was assayed by reaction with chromogenic substrate p-dimethylaminobenzaldehyde and extent of yellow colouration was measured spectrophotometrically at 415 nm. Absorbance value of each corresponds to degree of respective substrate hydrolysed.

3. Result and Discussion

3.1 Screening for Potential Bacteria

Soil samples used were selected from sites underlying antibiotic containment since such places can serve as promising site to screen for isolates with betalactam acylase. Phenylamino acids and derivatives serve as triggers to isolate such organisms, so suitable traces were added in enrichment media (Table 1). The isolates were studied for colony morphology abd gram characteristics and potential isolates were identified by primary test based on hydrolysis of NIPAB which is chromogenic phenyl amino derivative used by enzyme to give yellow coloured product (Table 2). The positive colonies A, D, E and I were Gram negative stored as pure culture and promising strains were screened further for beta lactam acylase activity.

3.2 Screening for Betalactam Acylase Enzyme

To study betalactam acylase enzyme specifically for cephalosporin substrate selected, cephalexin was selected as model substrate as it is most common first generation semisynthetic antibiotic. The cell suspension of each isolate grown basic media subjected to cephalexin hydrolysis showed varied response. Table 3 show that Cultures A and I did not show cephalexin activity. Culture E showed higher activity than D. Both cultures could be considered as potential enzyme producers. However initially culture E was taken forward for study.

3.3 Biochemical Characterisitics and Media

Both the strains were positive Lysine decarboxylase and H_2S. Culture E however showed positive for oxidase and citrate while Culture D showed positive methyl red (Table 4). Culture E was renamed as FRCC 71 listed in series of cultures in Fermenta Research Culture Collection (FRCC). To observe the influence of pH and trace elements on betalalctam acylase enzyme, FRCC 71 was grown in basic media (Media B) and Media N with trace elements at varying pH. Cephalexin synthesis activity was determined for cells and broth separated from cells.

Table 3 and Figure 2 show Media B to be suitable probably the concentration of trace elements in Media N needs to be optimized further. Activity of cells grown at pH 8.5 was highest but range between 7.5 and 8 showed relatively lower activity. As depicted in Figure 2 cells show higher activity then supernatant indicating intracellular location of enzyme.

The cells and enzyme tolerance to alkaline pH is significant and interesting characterisitics for enzyme development.

3.4 Substrate Specificity

Betalactam acylase enzyme belonging to Ntn Hydrolase family comprise of Penicillin acylase and Alpha amino ester hydrolase enzymes which show specificity for betalactam moiety with phenyl acetate derivative side chain. Since the work was aimed to screen enzyme producers specific for cephalosporin derivative, a range of cephalosporin substrates were included to be tested under hydrolysis conditions. Penicillin was only penam antibiotic included for comparison.

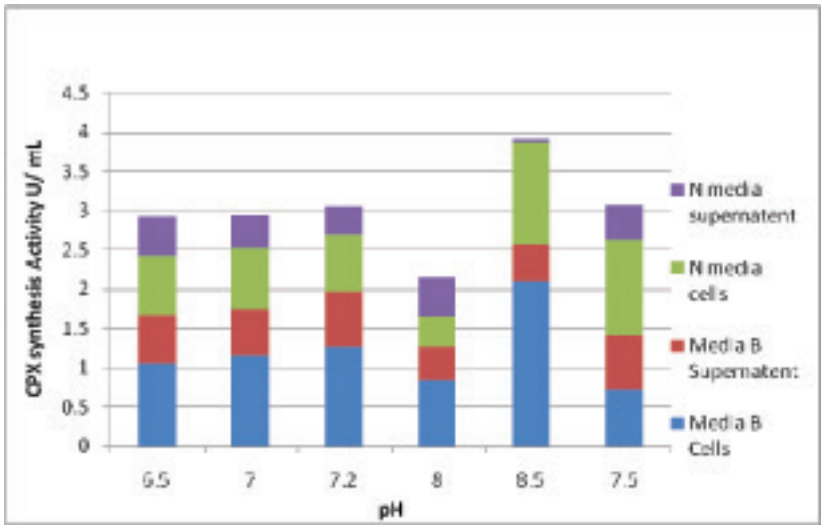

Figure 2. Media and pH influence.

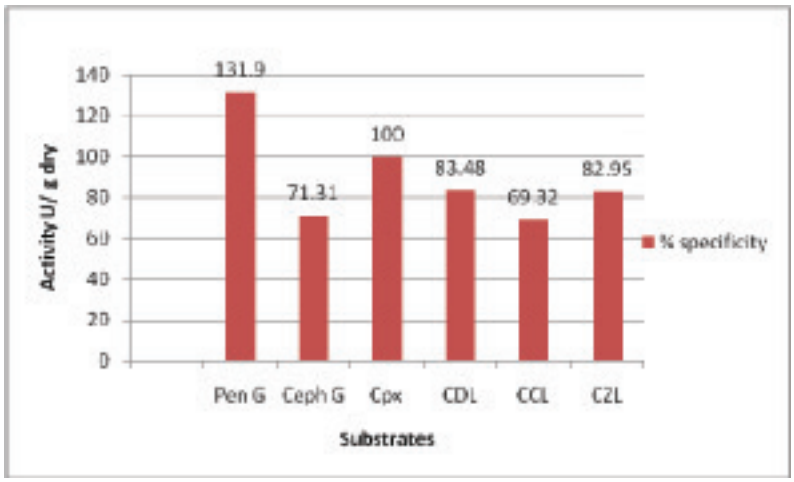

Figure 3. Substrate specificity of FRCC 71 cells.

Results in Table 5 and Figure 3 show that amongst cephalosporins, FRCC 71 show specificity in order with highest for cephalexin > Cefadoxil > Cefprozil > Cephalosporin G > Cefaclor.

However the specificity may further change under influence of pH and temperature. A separate study of kinetics of the enzyme and effect of inhibitors will be conducted to determine these parameters.

4. Conclusion

The screening for betalactam acylase producers resulted in isolates from soil which could serve as viable cultures for enzyme characterization. FRCC 71 isolate was primarily selected based on activity for cephalexin and found to be stable under alkaline conditions. This isolate FRCC 71 could serve as viable option for industrial production. Hence enzyme will be isolated, characterized and studied further.

5. Acknowledgement

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6. References

1. Andersson I, Scheltinga T, Valegard K. Towards New Beta-lactam antibiotics. Mol Life Sci. 2001; 58:1897–906.

2. Wegman M, Janssen M, Van Rantwijk F, Sheldon R. Towards biocatalytic synthesis of β-Lactam antibiotics. Adv Synth Catal. 2001; 343(6-7):559–76.
3. Arroyom et al. Biotechnological applications of penicillin acylases: state-of-the-art. Applied Microbiology and Biotechnology. 2003; 60(5):507–14.
4. Bruggink A. Synthesis of β-Lactam antibiotics. 2001. Chapter 3, Biocatalyts and Biocatalysis in the synthesis of β-Lactam antibiotics; p. 102–49.
5. Plhackova K, Becka S, Skrob F, Kyslik P Isolation and characterization of a new strain of Achromobacter with β-lactam antibiotic acylase activity. Appl Microbiol Biotechnol. 2003; 62:507–16.
6. Takahashi T, Yamazaki Y, Kato K. Substrate specificity of an a-amino ester hydrolase produced by Acetobacter turbidans A.T.C.C 9325. Biochem J. 1974; 137:497–503.
7. Okachi R, Kato F, Miyamura Y, Nara T. Selection of *Pseudomonas melanogenum* KY 3987 as a new ampicillin-producing bacteria. Agric Biol Chem. 1973; 37:1953–57.
8. Polderman-Tijmes J, Jekel P, de Vries E, van Merode A, Floris R, van der Laan J, Sonke T, Janssen D. Cloning, sequence analysis, and expression in *Escherichia coli* of the gene encoding an α-amino acid ester hydrolase from *Acetobacter turbidans*. Appl Environ Microbiol. 2002; 68:211–8.
9. Kurochkina V, Sklyarenko A, Berzina O, Yarotskii S. Alpha–Amino Ester Hydrolases: Properties and Applications. Applied Microbiology and Biochemistry. 2013; 49(8): 672–94.
10. Oh B, Kim K, Park J, Yoon J, Han D, Kim Y. Modifying the Substrate Specificity of Penicillin G Acylase to Cephalosporin Acylase by Mutating active-site residues. Biochemical and Biophysical Research Communications. 2004; 319:486–92.
11. Arshad R, Farooq S, Ali S. Characterization and Documentation of Bacterial Diversity collected from Various Local Habitats-1 Diversity in *Esherichia coli*. Pak J Bot. 2006; 38(3):791–7.
12. Javadpour S, Norouzian D, Akbarzadeh A, Mirdamadi S, Farahmand B. Isolation of Penicillin acylase producing *E. coli* and Kinetic Characterization of Whole cell Enzyme Activity. Iranian Biomedical Journal. 2002; 6(2 and 3):93–6.
13. Shiau C, Pai S, Lin W, Ji D, Liu Y. Purification and Characterization of Inducible Cephalexin Synthesizing Enzyme in Gluconobacter oxydans. Biosci Biotechnol Biochem. 2005; 69(3):463–9.
14. Babu P, Panda T. Role of Phenyl acetic acid in biosynthesis of Penicillin amidase in *E. coli.* Bioprocess Engineering. 1991; 6:71–4.
15. Sklyarenko A, Berezina O, Satarova D, Fedorchuk V, Fedorchuk E, Savin S, Yarotsky S, Tishkov V. Recombinant alpha_amino ester acid hydrolase from *Xanthomonas rubrilineans* vkpm b_9915 is a highly efficient biocatalyst of cephalexin synthesis. Moscow university chemistry bulletin. 2014; 69(2):86–92.
16. Tembhurkar V, Patil A, Chaudhari C, Kulkarni M, Harke S. Penicillin Acylase Production by *Micrococus luteus* and *Staphylococcus* spp. Isolated from Soda Lake. IOSR Journal of Pharmacy. 2012; 2(2):296–301.