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# Screening, isolation and identification of bacterial isolates having potential for bioconversion of cephalosporin C

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## ABSTRACT

The total worldwide market value for cephalosporin antibiotics ranks fifth among the leading therapeutic agents. Cephalosporin C is one of most important antibiotic produced by filamentous fungi such as *Cephalopsorium acremonium*. Cephalosporin C shows antibiotic activity against Gram-negative bacteria by hindering cell wall synthesis, but it is not the clinically active form. Thereby all clinically important semi synthetic derivatives of cephalosporin are manufactured from 7-aminocephalosporanic acid (7-ACA) which is an intermediate compound produced from cephalosporin C. The present investigation deals with the bioconversion of cephalosporin C using bacterial isolates obtained from various natural sources such as soil, waste water and hospital wastes. The bioconversion of cephalosporin was detected by thin layer chromatography and spectrophotometrically. Two bacterial isolates exhibiting bioconversion potential were isolated and identified as *Pseudomonas* and *Achromobacter* species.



## KEYWORDS

7-aminocephalosporanic acid;
Cephalosporin C;
Biotransformation;
Bioconversion.

## INTRODUCTION

Cephalosporin and its derivatives are the best sold antibiotics worldwide, with global sales of $ 8.3 billion of $ 466.3 billion of the total pharmaceutical products in 2003[1]. Due to lower clinical value of cephalosporin C, broad-spectrum semisynthetic cephalosporins are prepared from important intermediate 7-aminocephalosporanic acid. The chemical conversion of cephalosporin C to 7- aminocephalosporanic acid was accomplished by using chemicals which creates serious disadvantages such as requirement of multistep and complex process, and low quantity & quality of product[2,5,9]. Therefore, the chemical method has been replaced by an enzymatic method for preparing 7-ACA which is regarded as an environmentally acceptable method. Cephalosporin C antibiotic can be transformed either by single step transformation of cephalosporin C to 7-amino-cephalosporanic acid[3] using the enzyme cephalosporin acylase or by two step conversion using the enzyme D-amino acid oxidase. This enzyme converts cephalosporin C to glutaryl-7-

aminocephalosporanic acid and then glutaryl-7-aminocephalosporanic acid is transformed to 7-aminocephalosporanic acid by glutaryl-7-aminocephalosporanic acid acylase[4]. Many microorganisms such as *Pseudomonas* species, *Pseudomonas diminuta*, *Bacillus megaterium*, *Aeromonas sp*., *Arthrobacter viscous* etc. are found to convert cephalosporin C into 7-aminocephalosporanic acid in single step[4,5,9] while some transform cephalosporin C into 7-aminocephalosporanic acid via GL- 7 – aminocephalosporanic acid. Attempts were accomplished to isolate microorganisms with higher cephalosporin biotransformation activity from natural environments like wastewater, chemical industry wastes and hospital wastes. The present investigation deals with the bioconversion of cephalosporin C which was detected by thin layer chromatography and spectrophotometrically. Two bacterial strains were isolated which exhibited the bioconversion of cephalosporin C into its derivative.

## MATERIALS AND METHODS

### Chemicals and media

7- aminocephalosporanic acid and G l-7-aminocephalosporanic acid were obtained from Ira Pharmaceuticals, Mumbai. p-dimethylaminobenzaldehyde was procured from Merck Millipore, India. Cephalosporin C was supplied by Sigma Pharmaceutical Industry, India. Yeast extract, minimal broth ingredients and all other chemical reagents were purchased from Hi Media Laboratories, India.

### Screening of cephalosporin biotransforming organisms

Organisms obtained from natural sources such as waste water, industrial effluents and hospital wastes were used for inoculation of enrichment medium. Minimal broth was used for the purpose which contained grams per litre of Dextrose, 1.0; Dipotassium phosphate, 7.0, Monopotassium phosphate, 2.0; Sodium citrate, 0.5; Magnesium sulfate, 0.1; Ammonium sulfate, 1.0; final pH 7.0 +/- 0.2 at 25°C with 0.1 g % CPC and 0.5 g % dextrose. After incubation visible growth was observed in each flask in the form of turbidity. Small aliquots of medium were transferred from each the final liquid enrichment culture flasks to solidified minimal agar medium, containing 0.1 gram percent cephalosporin C as a stimulant for biotransforming activity of the organism and decreased concentration of dextrose (0.1 g %). Plates were incubated at room temperature for 48 hrs. After incubation pure culture was obtained on the plates in the form of bacterial & fungal colonies.

### Growth in presence of high concentration of cephalosporin C

Trials were carried out to check the ability of individual isolates to grow in presence of cephalosporin C by exposing them to increased concentration of cephalosporin C (1 %). Minimal broth was prepared with 1 gram percent cephalosporin C. Broth was distributed in 50 small tubes and sterilized. Individual colonies showing different growth characters (50 numbers) obtained by solid enrichment culture technique were transferred into tubes containing 1 gram percent CPC. The tubes were incubated at room temperature for two weeks. The tubes were routinely observed for visible growth in the form of turbidity.

### Analysis by Thin Layer Chromatography (TLC)

Individual colonies of isolates were selected for testing biotransforming potential by thin layer chromatography[6]. In all thirty nine isolates were screened using cephalosporin C containing medium. Each isolate was further tested for biotransformation of Cephalosporin C and GL-7- amino cephalosporanic acid into 7-aminocephalosporanic acid by thin layer chromatography. Washed cells were used for biotransformation in medium containing cephalosporin in phosphate buffer and incubated for four hours at $37^0$C with intermittent shaking. The supernatant obtained after centrifugation was used for TLC. The solvent system used was acetone:water:acetic acid:: 85:12:3. The plates were then sprayed with fluorescamine and observed under ultraviolet light at 365 nm. Two of the isolates which exhibited satisfactory biotransformation were further identified by 16S rRNA sequencing.

### Quantitative determination of 7-amino cephalosporanic acid

The detection of 7-aminocepalosporanic acid is possible through imines' formation of its amino functionality with p-dimethylaminobenzaldehyde (p-DAB).

The assay of quantitative detection of 7-aminocephalosporanic acid was performed as per the standard method[7]. p-Diaminobenzaldehyde (p-DAB) reacts with 7-aminocephalosporanic acid (7 amino group) resulting in the formation of imine of cephalosporin C. Quantitative estimation of 7-aminocephalosporanic acid from by isolates was done by performing the assay as per standard protocol in a total volume of 500 µl. In reaction mixture 40 µl of sample and different concentrations of standard 7-aminocephalosporanic acid (1mM - 10 mM) were combined with 280 µl of p-Diaminobenzaldehyde reagent. The mixture was incubated at room temperature for 3 minutes. After incubation content of tubes were immediately diluted with 180 µl of phosphate buffer at pH 7 to facilitate measurement of absorbance in routine 1 ml microcuvette. The absorbance was measured at room temperature at 414 nm using buffer solution of respective sample as reference. The reaction of p-DAB with 7-ACA completes within the incubation time. The reaction of Cephalosporin C proceeds after 30 minutes of incubation. Thus, working in time frame of 3 minutes the interfering absorbance value of Cephalosporin C imines can be neglected[7].

After performing p-DAB assay absorbance was measured for each known concentration of 7-ACA and standard curve was plotted. The test samples obtained from the isolates in the form of supernatant after growth and biotransformation were also assayed in parallel. The absorbance obtained was extrapolated on a standard curve made by plotting varying concentrations of the 7-ACA (mM per milliliters) against absorbance at 414 nm. The values for concentration of 7-ACA thus obtained were recorded.

## RESULTS AND DISCUSSION

### Screening and identification of the isolates

Thirty nine isolates were obtained by screening protocols used. All the isolates were subjected to growth in liquid medium followed by testing the supernatant for biotransformation of cephalosporin C to 7-amino cephalosporanic acid. Two isolates were found to exhibit considerably good biotransformation potential and were further subjected to identification on the basis of biochemical characters and 16S rRNA sequencing. The isolates were identified as *Pseudomonas* species and *Achromobacter* species.

### TLC

The thin layer chromatographic studies of *Pseudomonas* species and *Achromobacter* species revealed identical Rf value (As shown in Plate 1) as that exhibited by 7- aminocephalosporanic acid obtained where both of them were grown in broth in presence of cephalosporin C and GL 7-aminocephalosporanic acid respectively.

### Quantitative estimation of 7-ACA

The p-Dimethylaminobenzaldehyde (p-DAB) assay carried out for both the isolates exhibited considerably significant absorbance value. The values of 7-ACA were calculated from standard curve. The isolated *Pseudomonas* species was found to form 4.5 mM of 7-ACA per milliliter of inoculated broth and *Achromobacter* species formed 4.2 mM of 7-ACA per milliliter of inoculated broth. The *Pseudomonas* species converted cephalosporin C into 0.00122526 gm of 7-aminocephalosporanic acid per milliliter of incubated broth while *Achromobacter* species converted cephalosporin C into 0.001143576 gm of 7-aminocephalosporanic acid per milliliter of incubated broth. The bioconversion was quite satisfactory at preliminary scale which can further be enhanced by optimization studies which are underway. The bioconversion is parallel good as compared to the conversions obtained with known standard strains of *Pseudomonas*. Several research reports have revealed that *Pseudomonas* species and *Pseudomonas diminuta* produced cephalosporin C acylase enzyme which converted cephalosporin C into 7- aminocephalosporanic acid in single step[3,5,8,9]. On the contrary some organisms produced enzyme D-amino acid oxidase which converted cephalosporin C to glutaryl-7-aminocephalosporanic acid and then glutaryl-7-aminocephalosporanic acid was transformed to 7-aminocephalosporanic acid by glutaryl-7-aminocephalosporanic acid acylase[5]. It was observed during the present investigation that *Pseudomonas* species and *Achromobacter* species utilized glutaryl 7-ACA acylase for the bioconversion as investigated form the enzyme assays performed.

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