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Cephalosporin C acylase from *Pseudomonas* species: Production and enhancement of its activity by optimization of process parameters

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ABSTRACT

The bacterium, *Pseudomonas* species was isolated from rhizosphere soil and screened out for its ability to produce cephalosporin C acylase (CCA). Cephalosporin C acylase has the potential to transform cephalosporin C to 7- aminocephalosporanic acid which is an important intermediate for production of broad-spectrum semi-synthetic cephalosporins. This article describes the selection of medium components and parameters like temperature, pH, carbon sources, nitrogen sources and inoculum level for the optimal production of cephalosporin C acylase by *Pseudomonas* species. The biotransformation was conducted utilizing all optimized parameters for the production of cephalosporin C acylase at the end of study. The amount of product 7- aminocephalosporanic acid formed was measured by glutaryl p-nitroanilide (GpNA), p-Dimethyl aminobenzadehyde (p-DAB) and HPLC assays. The CCA activity in *Pseudomonas* species was measured 33.1 and 35.5 U/mL using substrate Cephalosporin C (CPC) and glutaryl p-nitroanilide (GpNA) respectively at optimized parameters. The maximum CCA acylase activity was detected at 72 h of incubation. The highest CCA activity for *Pseudomonas* species 42.9 U/mL was detected at 72 h of incubation by HPLC.

1. Introduction

Cephalosporin C (CPC[1]) is an antibiotic that was first isolated from cultures of *Cephalosporium acremonium* from sewer in 1945 by Giuseppe Brotzu (Pollgioni et al., 2013). The semi-synthetic cephalosporins became the popular antibiotics due to their excellent characteristics such as broad-spectrum, low toxicity, and resistance to the β-lactamase and it made tremendous contribution to fight with bacterial infection (Ren, 2013). The chemical conversion of cephalosporin C to 7-aminocephalosporanic acid (7-ACA[2]) is usually accomplished by using chemicals which creates serious disadvantages such as requirement of multistep and complex process, low quantity and quality of product (Zhang et al., 2005; Kim et al., 2000; Lein, 1989; Nigam et al., 2005). Thereby the chemical method has been replaced by an enzymatic method for preparing 7-ACA which can be regarded as an environmentally safe method. From last few years the production of 7-ACA is shifted from traditional chemical method to enzymatic method. Although the two step production of 7-ACA has potential rate of bioconversion but still single stage production of 7-ACA from CPC may possible for desirable outputs. Several Research reports are available on one step biotransformation of CPC to 7-ACA. Still today attempts are continue to find out cost effective and product effective one step bioconversion of CPC.

Cephalosporin C antibiotic can be transformed either by single step transformation of cephalosporin C to 7-amino-cephalosporanic acid using the enzyme cephalosporin acylase (Saito and Shimomura, 1996) or by using the enzyme D-amino acid oxidase (Pillone et al., 1995) and glutaryl-7 ACA acylase produced by bacteria (Shibuya et al., 1981). The two-step enzymatic method has become commercially popular because it is considered environmentally safe method. But it is still need to be an economical to satisfy the industrial production (Ren, 2013). Many bacteria, such as *Pseudomonas* species strains N176, V222 and SE83 acyII16 and SE 83 (Deshpande et al., 1996; Hirve et al., 2008) *Pseudomonas diminuta, Bacillus megaterium, Aeromonas* species, *Arthrobacter viscous*, *Bacillus* species etc. have been reported to convert cephalosporin C into 7-aminocephalosporanic acid (Lein, 1989;

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[1] CPC: Cephalosporin C.
[2] 7-Aminocephalosporanic acid.

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Sonawane et al., 1996; Acai et al., 1995). The bioconversion of cephalosporin C into of 7-ACA in a single step pathway is of great pharmaceutical importance. Thereby the process of screening new isolates with improved cephalosporin C acylase activity is underway by researchers. In this study the organisms were isolated which exhibited the property to convert CPC to its derivatives and found that few isolates had potential to transform CPC to 7-ACA. Cephalosporin C acylases (CCA[3]) were isolated from few bacteria which were active on both substrates CPC and glutaryl 7-ACA. The isolated organism *Pseudomonas* species was detected for bioconversion of cephalosporin C into 7-aminocephalosporanic acid and further optimization process was conducted. Limited reports were available on optimization studies for production of cephalosporin C acylase which was encouraging to conduct this study.

2. Materials and methods

2.1. Analysis of biotransformation by thin layer chromatography and plate assay

The biotransformation of Cephalosporin C into 7-aminocephalosporanic acid was detected by thin layer chromatography (Sonawane et al., 1996). The isolates which exhibited comparable biotransformation were further tested by using model substrate glutaryl p-nitroanilide. Fresh growth of the isolates was spread over nutrient agar medium containing 1 g/L of glutaryl p-nitroanilide. The colonies were checked for formation of yellow coloured zone due to 4-nitroaniline (Khang and Yoo, 1997).

2.2. Analysis of bio-transformed derivatives by HPLC

After extensive review of the literature, it was found that most of the bacterial transformers of cephalosporin C produced intracellular Cephalosporin C acylase and glutaryl-7-aminocephalosporanic acid acylase. Thereby *Pseudomonas* species was grown in Casein Hydrolysate Broth (Himedia M200 A) at 25 °C for 24 h. The cells were harvested by centrifugation at 4 °C (8000 rpm for 8 min) and cell pellet was washed thrice with sterile phosphate buffer pH 7. Cells were suspended in 2 mL of sterile phosphate buffer pH 7 and subjected to disruption by sonication (Hirve et al., 2008). The supernatant was collected after centrifugation at 4 °C (10,000 rpm for 10 min) and used as enzyme preparation for biotransformation of CPC. Enzyme assay was performed according to Deshpande et al. (1996) with slight change. An aliquot of 0.1 mL of enzyme preparation in the form of cell free extract was incubated with 2 mg/mL of cephalosporin C in 50 mM Tris buffer pH 8.0 at 40 °C for 60 min in triplicates. The reaction was terminated by the addition of equal volume of 1 M acetate buffer pH 4.0 and the amount of 7-ACA formed in reaction mixture was detected by HPLC. Reverse phase Enable H C-18 column (250 mm × 4.6 mm × 5 μm particle size) was employed for the assay of CPC and 7-ACA. The flow rate of the mobile phase 25 mM sodium phosphate (pH 3.5) and acetonitrile in ratio of 92:8% v/v (Qiang et al., 2006) respectively was 1 mL and the peaks were monitored at 254 nm.

2.3. Determination of CPC acylase activity

Glutaryl p-nitroanilide (GpNA[4]) was used as substrate for estimation of cephalosporin C acylase (CCA) enzyme (Hirve et al., 2008). With slight modifications (Hirve et al., 2008), the assay for measuring enzyme activity was conducted as 0.1 mL of enzyme preparation (cell free extract) incubated with 2 mg/mL of glutaryl p-nitroanilide in 50 mM Tris buffer pH 8.0 at 40 °C for 30 min. The reaction was terminated by the addition of equal volume of 1 M acetate buffer pH 4.0 and the absorbance was measured at 410 nm (Hirve et al., 2008). Similarly, the assay for quantitative detection of 7-aminocephalosporanic acid was also performed under optimized conditions using Cephalosporin C as a substrate (Patett and Fischer, 2006). The assay was performed in total volume of 320 μl. In reaction mixture 40 μl of sample was combined with 280 μl of p-Dimethyl aminobenzaldehyde (pDAB) reagent. The mixture was incubated at room temperature for 3 min. The absorbance was measured at room temperature at a 414 nm. Enzyme activity was calculated as the amount of enzyme required to form 1 μmole of product per minute under standard assay conditions.

2.4. Synthesis of model substrate

Glutaryl p-nitroanilide an artificial chromogenic substrate for cephalosporin C acylase (CCA) enzyme (Hirve et al., 2008) was used for detection of CCA activity. CCA degraded this substrate into was yellow coloured product which could be measured spectrophotometrically at 410 nm. The glutaryl-p-nitroanilide was synthesized by employing method used according to Franzosi et al. (1995). The formation of glutaryl-p-nitroanilide was confirmed by TLC and ^{1}H NMR.

2.5. Optimization of parameters for CPC acylase production

Optimization of process parameters were performed for *Pseudomonas* species by detecting higher CCA enzyme activity. The best medium for the organism was screened amongst five media used. Best carbon and nitrogen sources were screened out and optimum concentration of both carbon and nitrogen source was detected. Parameters like temperature, pH, and inoculum level were optimized for maximum CCA activity. Optimization of parameters was done by classical approach-one factor at a time by referring methods of Simplex and Box (Stanbury et al., 1998). At each step, the selected factor was included in the control medium for getting a set of conditions that enabled maximum production of CPC acylase from *Pseudomonas* species. In all cases, batch fermentation was carried out by inoculation of 150 mL volume of medium in 500 mL Erlenmeyer flasks. Small aliquots of samples were withdrawn at every 12 h interval and analysed for CPC acylase activity.

2.6. Optimization of media

Primarily nutrient broth (Himedia), minimal broth (Himedia M-390), Casein Hydrolysate broth (Himedia M200 A), Soybean Casein Digest broth (Himedia M011), and Luria Bertani broth (Himedia) were used for production of CCA enzyme. Batch fermentation was carried out in 500 mL Erlenmeyer flasks. After inoculation of *Pseudomonas* species all the media were incubated at ambient temperature for 120 h at 120 rpm. Intermittently after every 24 h small volume of samples were withdrawn from each flask and processed for measurement of CCA activity. After calculating enzyme activity best medium was selected for each organism for further optimization studies.

2.7. Influence of temperature

Soybean Casein Digest Broth (Himedia) supported maximum production of CCA enzyme for *Pseudomonas* species. Optimization of temperature was done by incubating Soybean Casein Digest medium inoculated with respective organisms at varying

[3] CCA: Cephalosporin C acylase.
[4] Glutaryl p-nitroanilide.