Mutant Isolation, Optimization and Comparison On Production of Penicillin Acylase

[1]Dr. Anila Rani. P, [2]Ms. Saikalyani Kasarabada,[3] Dr. M. Kishore Babu

[1]Associate Professor,[2]Assistant Professor,[3]Assistant Professor

[1]Biotechnology,

[1]Maris Stella College, Vijayawada, India

Abstract - Penicillin acylase (EC 3.5.1.11) plays a crucial function in the deacylation of the penicillin into the 6- amino penicillanic acid (6-APA) and the side-chain organic acids. The ATCC cultures used were compared with high yield and therefore selecting. E. coli ATCC 194 for the mutation by UV inducing at specific distance and time, the mutated strain and other standard cultures were possessed to different temperature, pH, and substrates for analyzing the highest penicillin acylase activity. However, the mutant of this new isolate was found to possess even higher penicillin acylase activity under the same conditions and genetic manipulations could be developed for industrial use.

Index Terms - Penicillin acylase (EC 3.5.1.11), E. coli ATCC, Para dimethyl amino benzaldehyde (PDAB),

I. INTRODUCTION

Penicillin acylases (E.C. 3.5.1.11) catalyze the hydrolysis of the amide bond in penicillin molecules fabricating the b-lactam nucleus, 6-aminopenicillanic acid (6-APA) and the corresponding side chain. Enzymatic method for large scale production of 6-aminopenicillanic acid employs penicillin acylase or penicillin amido hydrolase thus is one of the most important enzymes applied in the pharmaceutical industry [1]. 6-aminopenicillanic acid is the starting material for synthesis of semi synthetic penicillin [2].

Penicillin is a broad range antibiotic and used to treat many bacterial infections *viz.* diphtheria (*Corynebacterium diphtheriae*), Pneumonia (*Streptococcus pnemoniae*), Wound infection (*Staphylococcus aureus*), Syphilis (*Treponemapallidum*), gonorrhea (*Neisseria gonorrhoeae*) and various general ailments. Penicillin is the most important antibiotic in terms of annual production and prescription volume, despite the availability of an ever-growing number of alternative antimicrobials [3]. Many genera of molds, yeast and bacteria produce penicillin acylases. Among them, enzyme produced by E. coli is the most well-characterized and common one for industrial application. E. coli is known to produce an intracellular penicillin acylase that can be induced by phenylacetic acid [4]. Due to the high industrial importance of penicillin acylase, numerous efforts have been made towards screening for strains overproducing this enzyme [5]. Penicillin acylase is, in general, produced in fermentative process and is obtained from either mutated or natural variant strains [6]. The E. coli penicillin acylase being intracellular is quite difficult to purify. Even if whole cell is used for catalysis, it would pose substrate/product diffusion problem thereby putting break on speed of reaction.

The present work describes obtaining isolates of *E. Coli* that would be suitable for production of penicillin acylase, working with isolate to obtain mutant 194-1, estimation and comparison of the standard and mutated culture kinetics.

II. Materials and methods:

Organisms

The organisms used in the current work were *E. coli* ATCC 9637, *E. coli* ATCC 194, *E. coli* ATCC 458 and *E. coli* ATCC 111105 a known penicillin acylase producer, the cultures were maintained on nutrient agar slants and subcultures every two weeks and the stock cultures were maintained at4^{o}C.

Isolation of penicillin resistant bacteria

The cultures were grown in 500 ml conical flasks containing 125 ml of the medium. The flasks were placed in orbital shaker at 250 rpm for 20-24 hr, at 28^{o}C as specified. The conical flask contains Peptone of 0.5%, Beef extract of 0.3% and phenylacetic acid of 0.064%. The pH of the medium was adjusted to 7.0 before sterilization.

Mutation of *E. coli* ATCC 194-1

Cells were grown in the nutrient broth at 27^{o}C for 24 hours were later centrifuged, washed and re suspended in the phosphate buffer of 0.05M with pH 7.5. The bacterial suspension was subjected to UV radiation of different time and distance. Then 0.1 ml of each sample was spread on each of the two agar plates with peptone 0.5%, beef extract 0.3% and agar 1.2% adjusted to pH of 7.0 before sterilization which were incubated overnight at 37^{o}C. The next day they were over layered with 5ml of nutrient agar containing 25mg of penicillin-G and 0.01 ml of overnight culture broth with Peptone of 1% and NaCl 0.5% for *S. marcescens.* The over layered plates

incubated at 27ºC for 24 hours then examined for clear zone of inhibition around *E.Coli* colonies, these zones results from the failure of the growth of *S. marcescens* to grow and it indicates the capacity of mutant organism produce penicillin acylase.

Penicillin acylase assay

Penicillin acylase assay was done by Para dimethyl amino benzaldehyde (PDAB) method [7]. The PDAB reagent is composed of PDAB 0.75g, Acetic acid 50mL, Methanol 30mL, and water 20mL. Reaction mixture composed of Cell suspension 1mL and 0.5% Penicillin in 0.1M Phosphate buffer 5mL. After incubation at 40ºC for 50 min 1.5 mL PDAB reagent was added. The 6-APA produced was measured spectrophotometrically at 415 nm. One unit of penicillin acylase was taken as amount of enzyme required to liberate 1 µMol of 6-amino penicillanic acid per minute under assay conditions.

Optimization of Growth Conditions for Penicillin Acylase Production

For time course and temperature optimization the production media used composed of g/L, Beef extract of 3.0%, peptone of 5.0%, NaCl of 5.0% and Benzyl penicillin of 10%, pH 7. For time course optimization the 5% inoculums of test organism were inoculated in production medium. Penicillin acylase activity was determined after every 24hrs for 4 days. For temperature optimization inoculated production medium was incubated at different temperatures (20, 30, 40, 50 and 60ºC). Effect of glucose, lactose, and sucrose on penicillin acylase production was studied. For this the production media was supplemented with 0.2% test sugar.

Characterization of Penicillin Acylase

The effect of temperature on penicillin acylase activity in the range 20 - 80ºC was determined. To study the effect of pH on penicillin activity the substrate solution was prepared in buffers with different pH 4 - 10. To check specificity penicillin acylase was reacted with different beta lactum antibiotics – ampicillin, piperacillin, benzathine penicillin, Benzyl penicillin.

In this study strains used were selected for penicillin acylase producing bacteria by groups. Strain 194 was selected for higher penicillin acylase activity than other cultures. Percent conversions to 6 APA in 194-1 show the highest and least represented by 11105 cultures [Table 1]. *E. coli* 194-1, a mutant of *E. coli* 194 was selected on the basis of high enzyme activity.

Strains	%6 APA converted
E. coli ATCC 194-1	92.2
E. coli ATCC 9637	64.3
E. coli ATCC 194	79.0
E. coli ATCC 458	66.5
E. coli ATCC 11105	58.7

Table 1: Effect of growth conditions on penicillin acylase production by E. Coli

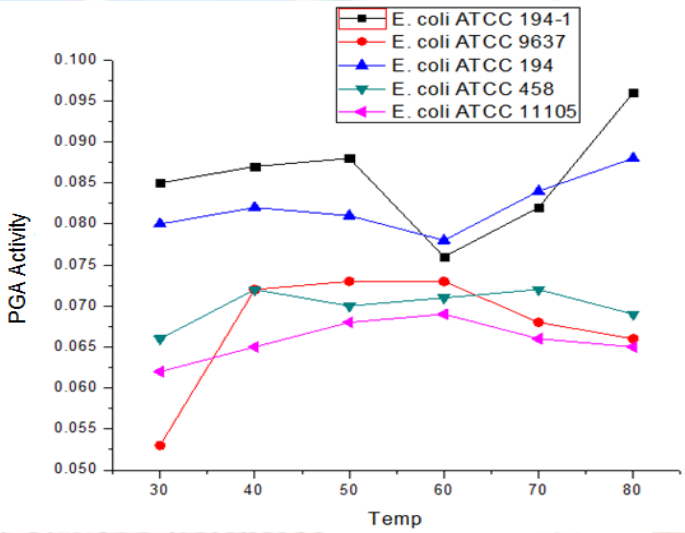
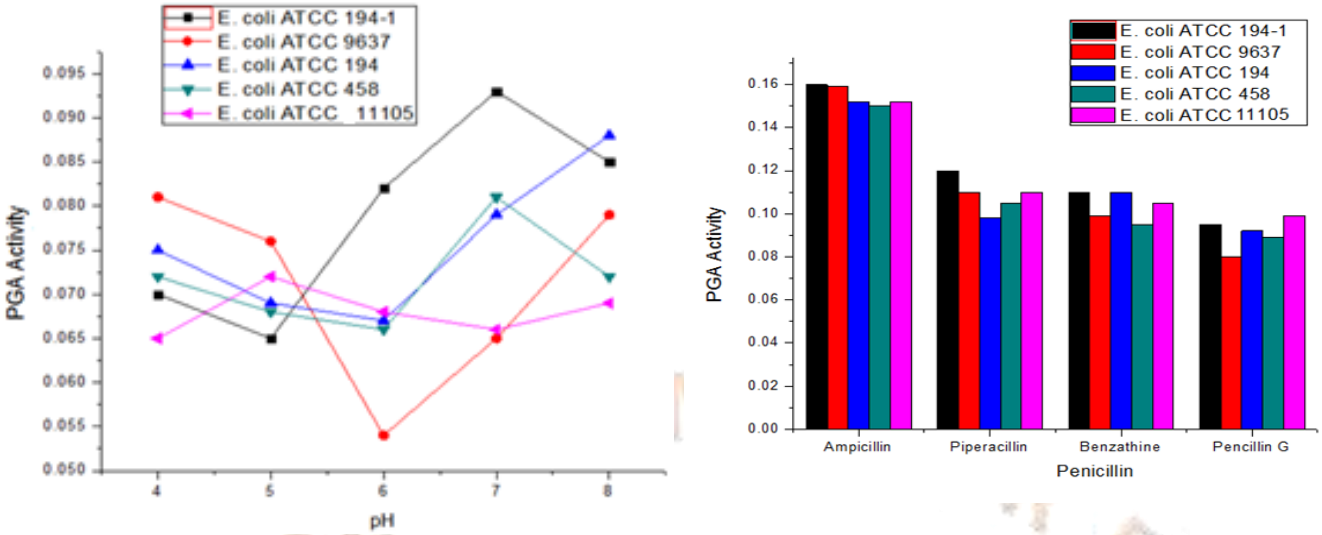


Figure 1: Effect of temperature on penicillin acylase activity.

The effect of temperature on penicillin acylase activity shows in the figure 1, mutated strain 194-1 shows best activity at 80ºC but optimized for 50ºC, and most culture shows the best yield between 50 – 60ºC, strain 9637 shows least activity at 30ºC about 0.0527. The effect of pH on penicillin acylase activity show in the figure 2, where strain 11105 show the least initial activity at pH 4 and strain 9637 shows the high activity of 0.081 at pH 4, the mutated strain 194-1 shows the high activity compared to all other stains at pH 7.

Figure 2: Effect of pH on penicillin acylase activity. **Figure 3: Substrate specificity of penicillin acylase.**



The Substrate specificity of penicillin acylase against few other beta lactum antibiotics was checked. The results are presented in histogram Figure 3. All three penicillin acylase worked with almost similar efficiency against Penicillin G, Piperacillin and Benzathine penicillin. Whereas all three much more rapidly deacylated ampicillin. The inhibitory effect of carbohydrates on penicillin acylase production when added to medium at higher concentration was stated [8].

The mutant strain 194-1 show the highest 92.2 percent conversions to 6 APA and the high PGA activity at temperature 50 and 80°C with pH range of 7 was suitable, the ampicillin was substrate specificity for penicillin acylase, similarly for *E. coli* ATCC 9637 shows the 64.3percent conversions to 6 APA and the high PGA activity at temperature 40 and 60°C with pH range of 4 and 8 was suitable, the ampicillin was substrate specificity for penicillin acylase, and for *E. coli* ATCC 194 shows the 79.0 percent conversions to 6 APA and the high PGA activity at temperature 70 and 80°C with pH range of 6 to 8 was suitable, the ampicillin was substrate specificity for penicillin acylase, and for the *E. coli* ATCC 458 shows the 66.5 percent conversions to 6 APA and the high PGA activity at temperature 40 to 70°C with pH range of 7 was suitable, the ampicillin was substrate specificity for penicillin acylase, and for *E. coli* ATCC 11105 shows the 58.7 percent conversions to 6 APA and the high PGA activity at temperature 40 to 70°C with pH range of 7 was suitable, the ampicillin was substrate specificity for penicillin acylase.

Conclusions

The strains isolated and were used here to produce extracellular penicillin acylase. Production of enzyme is improved at eminent temperature. The enzyme is of commercial importance since 6-aminopenicillanic acid is a key intermediate for the synthesis of numerous semi synthetic penicillin. This enzymatic conversion of penicillin has received considerable interest during recent years [9-11]. Enzyme of both tolerated higher temperature but pH should be near impartiality. The presence of glucose and sucrose at small concentration stimulated enzyme production by mutant strain. Penicillin acylase from these strains has broad substrate specificity even Penicillin G was added to production medium as inducer for enzyme production. And astonishingly the enzyme was additionally active. Finally, it was done with isolation of the mutant that produces penicillin acylase resistant to high pH and temperature and provides the better yield comparatively, continuing with mutant 194-1 to obtain further mutant with more yield and optimization.

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