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Mathematically optimized production, purification and characterization of penicillin G acylase from soil bacterial isolates AA17A and AA17B

Abhishek Ajamani[1] Rajesh Kumar[1], Prachi Bhargava[2] and Siddharth Vats[2]*
[1]University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra 136 119;
[2]Faculty of Biotechnology, IBST, Shri Ramswaroop Memorial University, Lucknow 225 003

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This research article deals with production of industrial enzyme penicillin G acylase from soil bacterial isolates namely AA17A and AA17B, which are selected from 80 soil samples. The strains were selected based on qualitative (turbidity) and quantitative (HPLC) test for 6-aminopenicillanic acid (6APA) production. The enzyme was assayed for its activity and optimized for production of enzyme using design of experiments software (DOE) “Design Expert 8.0.7.1”. Optimization of enzyme production of four carbon sources (glucose, glycerol, sucrose and starch), four nitrogen sources (beef extract, tryptone, peptone and yeast extract), for temperature (25°C, 30°C, 35°C and 40°C), four pH (6, 7, 8, 9), four inoculum volumes (2.5 ml, 5.0 ml, 7.5 ml, 10.0 ml) and the phenyl acetic acid (PAA) level (0.15%, 0.17%, 0.185%, 0.2%). The penicillin acylase activity was enhanced to 1.2 fold under following optimized culture conditions: carbon source - glucose (8%), nitrogen source - beef extract (2%), pH 9.0, temperature 30ºC, phenyl acetic acid 0.185%, inoculum volume 5 ml. Approximately 1.22-fold purification from the initial culture broth was achieved during ammonium sulphate precipitation (70-80%) with a yield of 4.6% enzyme. The specific activity of the final partially purified enzyme was 13.73 IU/mg protein.

Keywords: Penicillin acylase, mathematical modeling, design of experiments software

Introduction

Enzymes extracted from microorganisms are applicable in baking, brewing, alcohol production, cheese making, pharmaceuticals and bioethanol based energy sectors. Pharmacy sector is also not untouched by their applications for example enzyme like penicillin acylase amidase (penicillin amidohydrolase). Penicillin acylase was first reported from *Penicillium chrysogenum* Wisc. Q 176[1]. This enzyme splits benzylpenicillin into phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA) which is used to produce sem-synthetic β-lactam antibiotics. Penicillin acylase is produced by yeast, bacteria and mold. This is a heterodimeric periplasmic protein consisting of a small ‘a’ subunit and a large ‘b’ subunit, which are formed by processing of a precursor protein. The catalytic nucleophile, a serine, is located at the N-terminus, which is a hallmark of the family of N-terminal nucleophile (Ntn) hydrolases, a class of enzymes which share a common fold around the active site and contain a catalytic serine, cysteine or threonine at the N-terminal position[2]. About 7,500 tons of 6-APA is produced annually and 10-30 tons of immobilized penicillin acylase is consumed. Based on their substrate specificity, penicillin acylases were originally divided into three groups: penicillin G acylases (PGAs), penicillin V acylases (PVAs) and ampicillin acylases. At present, however, ampicillin acylases are classified as α-amino acid ester hydrolases. PGAs preferentially hydrolyze penicillin G and have wide substrate specificity. They were found produced by many bacteria, i.e. *Alcaligenes faecalis*, *Arthrobacter viscosus*, *Bacillus megaterium*, *Proteus rettgeri*, *Pseudomonas melanogenum*, *Bovista plumbea*, *Escherichia coli*, *Kluyvera citrophila*, and *Providencia rettgeri*. Penicillin acylases are of great importance to pharmaceutical industry for their application in the production of semi-synthetic β-lactam antibiotics via the key intermediates, 6-aminopenicillanic acid (6-APA) and 7-amino-3-deacetoxycephalosporanic acid (7-ADCA). In this work, we have isolated penicillin G acylase producing bacteria from soil. Further, the enzyme was for its activity and the production was optimized for enzyme using design of experiments software. Partial purification of enzyme was done followed by enzyme

*Author for correspondence:
vatssidd@gmail.com
Tel: 9140951132

characterization for various parameters. The isolated bacteria were sent to Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh for phenotypic characterization.

Materials and Methods

The chemicals, reagents, dehydrated media, their components all were of analytical grade and were obtained from CDH, Rankem, Sigma, and Himedia. Penicillin G was bought from veterinary chemist shop located near Old Bus Stand, Kurukshetra, Medical grade 6-APA was kindly provided by Surya Pharmaceuticals Limited, Panchkula, Haryana. Glasswares were procured from Perfit, Labco and borosilicate.

Soil Sample Collection

Eighty soil samples of 10 g each were collected from Kurukshetra University campus and Karnal District. Samples were prepared by inoculating 5-8 g soil into tube containing 50 ml of minimal medium and 30 µg/ml pencillin G and incubated at 37°C for 24 h in BOD incubator (Popular Traders, India) for enrichment with occasional shaking. These tubes were incubated until growth was observed in the form of turbidity.

Primary Screening

Tubes were selected on the basis of higher growth in the presence of penicillin G, with increased turbidity. The broth from these tubes was spread plated onto minimal medium containing 30 µg/ml penicillin G.

Secondary Screening

Secondary screening involved the analysis of tubes showing high turbidity for the presence of 6-APA produced as a result of conversion of penicillin G by enzyme PGA through the high performance liquid chromatography (HPLC) (Waters Corporation, USA). The system was operated at a flow rate of 1 ml/min, analysis was done at 210 nm and the run time was 15 min. Hypercil C18 (250 X 4.6) 5 µm BDS column was used. Mobile phase consisted of 6.8 g KH_2PO_4 in 1 L double distilled water, pH maintained at 6.0 by potassium hydroxide (KOH). Buffer consists of 80% mobile phase + 20% acetonitrile.

Isolation

Sample from test tube showing the highest 6-APA level was used for the isolation of penicillin acylase producing strains. For isolation, samples were spread on nutrient agar containing 30 µg/ml penicillin G and colonies were streaked for pure culture. Out of them, one strain was randomly chosen for further studies.

Morphological and Biochemical Characterization

Preliminary morphological and biochemical tests were performed using microscope and standard kit from Himedia Laboratories Pvt Ltd (Mumbai, India). For further identification, bacterial strains were sent to MTCC, IMTECH (Chandigarh, India) for analysis.

Inoculum Preparation

Inoculum was prepared by adding a loop full of pure culture into 50 ml of sterile nutrient broth (NB) medium. The cultured broth was diluted with the appropriate amount of fresh broth to obtain OD 600 nm = 0.5.

Enzyme Extraction

Intra-cellular enzymes were isolated for enzyme assay by using a 0.5 ml lysozyme-ethylenediaminetetraacetic acid (EDTA) solution (including 100 mM of EDTA in 1.0 mg/ml of lysozyme, respectively in 100 mM Tris-HCl buffer, pH 7.5) was added to 4.5 ml of cell suspension and left at 37°C for 20 min[3]. The cultures were centrifuged at 10,000 rpm for 15 min at 4°C in refrigerated centrifuge (REMI, India). The supernatant was kept and the pellet was discarded. The procedure followed for extraction was same throughout the study.

Assay of Penicillin Acylase Activity

Penicillin acylase activity was assayed by modification of method[4]. The culture was centrifuged, and the sediment was suspended and readjusted to 30 mg (cell dry weight) per mililitre (ml) with 0.05 M phosphate buffer (pH 7.5). The reaction mixture contained 5 mg of benzylpenicillin per ml, phenoxymethylpenicillin or ampicillin, and 1 ml of bacterial suspension (30 mg, cell dry weight) in 4 ml of phosphate buffer (0.05 M, pH 7.5). The mixture was incubated at 40°C for up to 30 min and then 3 ml of inhibitor (1:1 mixture of 0.05M NaOH and 20% acetic acid) and centrifuged. Production of 6-APA was estimated by incubation of 2 ml of supernatant with 0.5 ml of 0.5% para-dimethylaminobenzaldehyde (PDAB) in methanol. Absorbance was taken at 415 nm using computer based double beam spectrophotometer 2202 (Systronics, India). One international unit (IU) is the amount of enzyme that catalyses the

formation of 1 μmol of 6-APA from penicillin G in one minute. The enzyme activity was calculated using the standard curve prepared by the different concentration of 6-APA. The procedure followed for all the enzyme assays throughout the study.

Optimization of Enzyme Production

For the purpose of optimizing the media and culture conditions for the chosen isolate, "Design Expert 8.0.7.1" software was used. Optimization was done for four carbon sources viz. glucose, glycerol, sucrose and starch; four nitrogen sources viz. beef extract, tryptone, peptone, yeast extract; four temperature levels viz.- 25°C, 30°C, 35°C, 40°C; four pH levels viz.- 6, 7, 8, 9; four PAA levels viz. 015%, 0.17%, 0.185%, 0.2% and four inoculum volumes viz. 2.5 ml, 5.0 ml, 7.5 ml, 10.0 ml. The response of the experiment to be fed was set as enzyme activity in terms of IU. Random combinations of levels of different factors (carbon source, nitrogen source, temperature and pH) were generated by the software. All of these were executed experimentally in 500 ml flasks containing 300 ml of the production medium for the optimum time period of enzyme production found. The results or enzyme activity for each run/combination was calculated and fed as response in the program. These were used to evaluate and analyze the results to find out the optimum combination of levels of different factors.

Enzyme Production

Penicillin acylase production was carried out for the chosen isolate, under submerged fermentation conditions using Luria Bertani (LB) broth. PAA was added after 8 hrs, of incubation at a concentration of 0.15%. The total volume was 1.25 L. Production experiments were carried out in 500 ml Erlenmeyer flasks containing 250 ml of the production medium. Flasks were inoculated with 1% inoculum and incubated at 30°C for 48 h under shaking conditions of 150 rpm in an incubator shaker (Sunline Instruments, India). Enzyme activity and total protein content was measured and specific activity was determined after completion of incubation period.

Partial Purification of Penicillin Acylase

Penicillin acylase was produced by cultivating the bacterial isolate under optimized conditions. All the purification steps were carried out at 4°C. The supernatant was collected after centrifugation of culture broth at 10,000 rpm for 15 min and the crude enzyme broth was used for purification. Initially, ammonium sulphate precipitation was carried out in narrow range with 10% increments to attain different saturation levels (20% - 80%) using varying salt concentrations. Each precipitated fraction was dissolved in minimal volume of phosphate buffer. Specific activity was found to be highest in 70 - 80% fractions. Therefore, for large scale purification crude broth was precipitated at 0 - 70% to remove unwanted proteins and the supernatant was further precipitated at 70-80% and dialyzed against 0.1 M phosphate buffer pH 7.5 to obtain partially purified enzyme. This partially purified enzyme was used for further studies.

Protein Estimation

Total protein contents of the enzyme solution were measured according to the method described by Bradford *et al* (1976)[5], using bovine serum albumin (BSA) as a standard. The equation for the graph was found to be $y = 0.005x + 0.006$. The values of O.D. for the samples were substituted for "y" and the corresponding values for "x" were found, which gave concentration of the protein in the samples.

Characterization of Partially Purified Penicillin Acylase

Effect of pH on Enzyme Activity

The effect of pH on the penicillin acylase activity was studied over a pH range of 6.0 to 10.0, at 40°C using the following buffer: 0.1 M sodium phosphate (pH 6.0 - 7.0); 0.1 M Tris-HCl (pH 8.0); 0.1 M glycine-NaOH (pH 9.0-12.0).

Effect of Temperature on Enzyme Activity

The effect of temperature on penicillin acylase activity was determined by estimating the activity at pH 7.5 and temperatures ranging from 30ºC-70ºC.

Production and Separation of 6-APA

For the production, isolate AA17B was inoculated in 1 L ml flask containing 600 ml minimal medium with 1% casein. Penicillin G was added in the beginning and at subsequent intervals for its conversion into 6-APA. For the separation of 6-APA, dimethyl chloride 2X broth volume was taken at 0-5°C. The pH was adjusted to 0.8-1.0 by addition of hydrochloric acid. Solution was stirred for 10 min while maintaining the temperature. The volume was allowed to settle for 10 min. Layer was allowed to form and aqueous layer was collected. Next, pH was adjusted to 4.15-4.2 using NaOH. 6-APA was allowed to crystallize at this pH and methanol was added for washing. 6-APA crystals were separated using Buchner funnel with suction pump arrangement. The

obtained crystals were washed with methanol, dried and weighed.

Antibiotic Pattern of the Two Isolates

The antibiotic pattern of the two isolates viz. AA17A and AA17B were obtained using antibiotic discs (ICOSA).

Results

Primary Screening

The samples collected were inoculated in 50 ml test tubes containing 30 ml broth and they were numbered 1 - 80. Out of 80 tubes inoculated, growth was observed in 32 tubes.

Secondary Screening

Out of 32 test tubes showing growth, 4 test tubes showing comparatively high growth viz. test tube #7, #17, #33, #47 were subjected to HPLC analysis. 6-APA presence was detected in all of the 4 samples, with the highest 6-APA level of 87.86% in test tube #17.

Isolation

The sample from test tube #17 was spread plated onto nutrient agar in the presence of penicillin G, two isolates were obtained and coded as AA17A and AA17B. AA17B was chosen for penicillin acylase production. Isolates AA17A, AA17B and other isolates as well streaked onto nutrient agar plates and preserved for further use.

Morphological and Biochemical Characterization

The results of the morphological and biochemical tests of two isolates AA17A and AA17B can be seen in Table 1 and Table 2, respectively.

Optimization of Enzyme Production

Experiments were carried out according to the different combinations of the levels of factors generated by the Design Expert- 8.0.7.1 software. The absorbance at 415 nm and their corresponding enzyme activities obtained as a result of practical execution of runs are shown in Table 3. Optimum enzyme activity of 0.604 IU was obtained with the experimentation of third run having following factors: carbon source - sucrose, nitrogen source - beef extract, pH - 9, temperature - 25ºC, PAA - 0.185%, inoculum volume - 10 ml. The enzyme activities fed in the software as the responses of the practical execution of the runs shown in Table 3, was used for the generation of different graphs for various factors. These graphs demonstrated the scenario displayed by the factors during the experiment.

After analyzing the results and the graphs generated from the data, it was found that optimum activity of 0.80 IU could be obtained under following setting: carbon source - glucose, nitrogen source - beef extract, pH - 9, temperature - 30ºC, PAA - 0.185%, inoculum volume - 5 ml. Production was carried out in 300 ml medium in 500 ml flask under these conditions and resulted in an enzyme activity of 0.73 IU.

Production was carried out by inoculating isolate AA17B in 300 ml LB broth along with 0.185% PAA as an inducer taken in 500 ml flask. Enzyme activity was found to be 0.63 IU from the centrifuged extract. Protein content and specific activity were found to be 0.091 mg/ml and 6.92 IU/mg, respectively.

Partial Purification

Ammonium sulphate precipitation cut-off for the enzyme was found to be from 70-80%. The above

Table 1 — Morphological characteristics of isolate AA17A

Morphological tests	Results
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	Flat
Surface	Smooth
Colour	Yellow
Opacity	Opaque
Gram's reaction	-
Cell shape	Cocci
Size (µm)	1-1.5 x 2-5
Motility	-

Table 2 — Biochemical characteristics of two isolates viz. AA17A and AA17B

S. No.	Test	AA17A	AA17B
1	Indole production	-	+
2	MR test	+	-
3	VP test	-	+
4	Citrate utilization	+	+
5	Lysine decarboxylase	+	+
6	Ornithine decarboxylase	+	+
7	Nitrate reduction	-	-
8	Urease test	-	-
9	Phenylalanine deamination	-	-
10	H_2S production	-	-
11	Glucose utilization	+	+
12	Sorbitol utilization	-	-
13	Sucrose utilization	+	+
14	Adonitol utilization	-	-
15	Lactose utilization	-	-
16	Arabinose utilization	-	-

Table 3 — Run sheet generated by the software and the fed response data (EA - IU) after experimentation of the runs

Std	Run	Factor 1 A:CS	Factor 2 B:NS	Factor 3 C:pH	Factor 4 D:Temp	Factor 5 E:PAA	Factor 6 F:Inoc	Response 1 EA IU
1	1	Glycerol	Beef extract	6	30	0.150	2.5	0.285
21	2	Glycerol	Peptone	8	25	0.185	10	0.169
22	3	Sucrose	Beef extract	9	25	0.185	10	0.604
7	4	Sucrose	Yeast extract	9	35	0.5	5	0.443
12	5	Starch	Peptone	7	25	0.15	7.5	0.462
6	6	Sucrose	Peptone	7	35	0.200	2.5	0.041
13	7	Sucrose	Tryptone	7	30	0.150	7.5	0.278
17	8	Starch	Beef extract	9	40	0.200	7.5	0.574
10	9	Glycerol	Beef extract	7	35	0.185	5	0.514
23	10	Starch	Yeast extract	8	30	0.200	10	0.514
16	11	Sucrose	Yeast extract	6	40	0.185	7.5	0.503
2	12	Glucose	Beef extract	8	40	0.150	2.5	0.356
3	13	Starch	Tryptone	9	25	0.170	2.5	0.409
20	14	Starch	Peptone	6	35	0.170	10	0.029
15	15	Glucose	Tryptone	8	35	0.185	7.5	0.157
24	16	Glycerol	Tryptone	7	40	0.200	10	0.338
5	17	Starch	Yeast extract	7	40	0.185	2.5	0.285
9	18	Starch	Tryptone	6	30	0.185	5	0.330
8	19	Sucrose	Peptone	8	40	0.170	5	0.191
11	20	Glucose	Yeast extract	6	25	0.200	5	0.420
18	21	Glucose	Peptone	9	40	0.150	10	0.409
19	22	Glucose	Beef extract	7	30	0.170	10	0.552
4	23	Glucose	Peptone	9	30	0.185	2.5	0.601
14	24	Glycerol	Yeast extract	9	30	0.170	7.5	0.334

Table 4 — Absorbance values (OD) for calculation of enzyme activity (IU) and the protein concentration (mg/ml) during purification steps

S. No.	Steps	OD (Enzyme activity)	Enzyme activity (IU/ml)	OD (Protein)	Protein (mg/ml)
1	Centrifugation	0.176	0.63	0.50	0.091
2	$(NH_4)_2SO_4$ precipitation	0.198	0.71	0.42	0.083
3	Dialysis	0.254	0.92	0.34	0.067

Table 5 — Results of enzyme purification

S.No.	Step	Total recovery		Specific activity (IU/mg)	Fold purification	% Yield
		Protein (mg)	Enzyme (IU)			
1	Centrifugation	27.300	189	6.92	-	-
2	$(NH_4)_2SO_4$ precipitation	1.250	10.65	8.52	1.23	4.6
3	Dialysis	1.005	13.8	13.73	1.98	3.7

filtrate was used to perform ammonium sulphate precipitation. The enzyme activity of the 15 ml buffer dissolved precipitate solution came out to be 0.71 IU with a protein concentration of 0.083 mg/ml. Increase in enzyme activity to 0.92 IU was shown by the 15 ml dialyzed enzyme with a protein concentration of 0.067 mg/ml (Table 4 & 5).

Characterization of Enzyme

Effect of pH on Enzyme Activity

Penicillin acylase obtained from isolate AA17B showed optimum enzyme activity at pH 8.0 and was found to be active over pH range of 6.0-10.0, exhibiting more than 70% relative activity within this range.

Effect of Temperature on Enzyme Activity

Penicillin acylase obtain from isolate AA17B activity increased with an increase in the temperature from 25°C to 40°C and declined thereafter. The temperature optima was 40°C, though it showed more than 80% relative activity in the temperature range of 25-45°C. The procedure for the separation of 6-APA was followed and the obtained 6-APA was 100 mg in quantity.

Table 6 — Antibiotic patterns of two isolates obtained from test tube #17.

S.	Antibiotic		AA17A		AA17B	
No	Name	Conc. (µg)	Zone	Size (mm)	Zone	Size (mm)
1	Norfloxacin	10	+	27	+	34
2	Gentamicin	10	+	14	+	10
3	Chlorampheniol	30	+	26	+	17
4	Cefuroxime	30	-	-	-	-
5	Ciprofloxacin	5	+	35	+	35
6	Cefoperazone	15	+	19	-	-
7	Ceftazidime	30	+	10	-	-
8	Roxithromycin	30	+	14	+	10
9	Clarithromycin	15	+	17	+	15
10	Cotrimoxazole	25	-	-	-	-
11	Netillin	30	+	13	+	17
12	Cefaclor	30	-	-	-	-
13	Cephotaxime	30	+	18	-	-
14	Cefadroxil	30	-	-	-	-
15	Azithromycin	15	+	33	+	29
16	Ampicillin	10	-	-	-	-
17	Penicillin	10	-	-	-	-
18	Amikacin	30	+	13	+	14
19	Sparfloxacin	5	+	33	+	40
20	Sulbactam	10	-	-	+	13

Antibiotic Pattern of Isolates

The result of the antibiotic pattern of the two isolates viz. AA17A and AA17B is shown in Table 6. Figure 1 and 2 shows the effect of antibiotic sparfloxacin on AA17A and AA17B, respectively, which is highly sensitive to both the strains. The penicillin acylase activity was enhanced to 1.2 fold under following optimized culture conditions: carbon source - glucose (8%), nitrogen source - beef extract (2%), pH - 9.0, temperature - 30ºC, PAA - 0.185%, inoculum volume - 5 ml. Approximately 1.22 fold purification from the initial culture broth was achieved during ammonium sulphate precipitation (70-80%) with a yield of 4.6% enzyme. The specific activity of the final partially purified enzyme was 13.73 IU/mg protein. The penicillin acylase exhibited its optimal activity at 40°C and at pH 8.0. The enzyme was active in the pH range of 6.0-10.0. The enzyme showed high activity in the temperature range 30-45°C, while the enzyme activity was lower at 25°C. The penicillin acylase was good in conversion of penicillin G to 6-APA, an important intermediate in the production of all semi-synthetic penicillins like ampicillin etc. with a production of approximately 100 mg 6-APA from 1 g penicillin G carried out in 1 L broth.

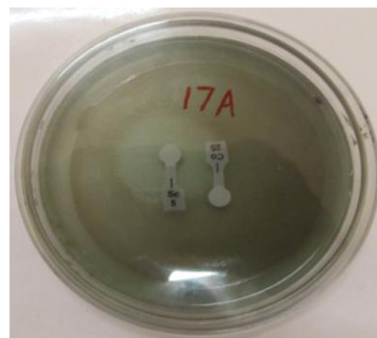

Fig. 1 — Antibiotic (sparfloxacin) showing largest size zone formation against AA17A.

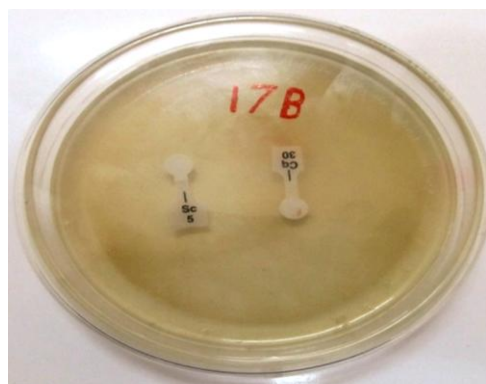

Fig. 2 — Antibiotic (sparfloxacin) showing largest size zone formation against AA17B.

Discussion

With increasing emphasis on the environmental protection, the use of biocatalysts gained considerable attention in this biotechnological era. Extremozymes are now-a-days replacing chemical catalysts in manufacturing of chemicals, textiles, pharmaceuticals, paper, food, agricultural chemicals. Penicillin acylases are of particular interest due to their wide applications in pharmaceutical industry like hydrolysis of natural β-lactams, production of new β-lactam antibiotics[6] and other applications like peptide synthesis such as stereoisomers of 3, 6-diphenylpiperazine-2, 5-dione[7], separation of racemic mixtures (2-amino-1-butanol and amines)[8]. Application potential of these enzymes depend on the nature of catalytic activity with respect to different natural penicillins like penicillin G, penicillin V which led to the classification of penicillin acylases as penicillin G acylase and penicillin V acylase. Hence, these penicillin acylases and their producing organisms attracted more attention of the research activists to understand the protein chemistry and protein

engineering to enhance their use to protect the environment as well as carrying out the same activity economically.

Penicillin G acylase has been found in almost all microbes types whether its bacteria, yeast, or fungi[9]. At the present time, penicillin acylase (PAs) from more than 40 different microorganisms have been described. However, the great economic value of PAs still gives an impetus to search for new penicillin acylases with novel properties. The important PAs producing bacteria are *Alcaligenes faecalis*, *Arthrobacter viscosus*, *Bacillus megaterium*, *Proteus rettgeri, Pseudomonas melanogenum, Bovista plumbea, Escherichia coli*, *Kluyvera citrophila*, and *Providencia rettgeri*[10-11]. Penicillin acylase of species *Escherichia* and *Bacillus* are of great importance due to their intracellular enzyme with good properties and extracellular enzyme with less cost for enzyme purification, respectively. In the present work, a penicillin acylase producing bacterial strain was isolated from soil; the culture conditions for the penicillin acylase production by isolated strain were optimized, the penicillin acylase expressed was partially purified, characterized and studied for its capability to produce 6-APA[12-13].

Soil is considered to be the hub of all the microorganisms including bacteria, fungi, yeast etc. So, in this study, in order to isolate a new bacteria producing penicillin acylase, soil samples were collected from Kurukshetra University Campus as well as Karnal. Soil samples were incubated with 30 µg/ml penicillin G for enrichment. The test tubes were screened for showing growth in the presence of penicillin G, used as a sole source of carbon. The growth of microorganisms in the presence of penicillin G shows penicillin G hydrolysis for their growth. But the presence of growth is not a good indicator of the presence of penicillin acylase activity. So, the concentrate from these tubes were screened for the presence of 6-APA using HPLC technique as previously done by Kim and Lee (1996) and Serra *et al* (2009)[14-15]. Silva *et al* (2006), performed HPLC technique for the estimation of phenyl acetic acid concentration[16]. In this study, the HPLC was carried out for the test tubes #1, #7, #17, #47 for the estimation of penicillin G, PAA and 6-APA. The test tube #17 showed the highest conversion of penicillin G to 6-APA with a conversion of 87.6%. On the basis of these results, isolates from test tube #17 were used for further study. The commonly accepted procedure for quantitatively estimating the activity of penicillin acylase is to determine the 6-APA concentration. The 6-APA concentration is determined by its reaction with p-dimethylaminobenzaldehyde (PDAB) to form Schiff's base. Several authors have used the PDAB method[17] as a standard method for the estimation of penicillin acylase activity[18]. Choi *et al* (1992), used the method described by Bomstein and Evans for the estimation of enzymatic activity[19]. In this study, penicillin acylase activity was assayed by modification of the methods.

The optimization experiments showed wide variation in penicillin acylase activity owing to the different conditions of incubation. The microbial growth and enzyme production is known to be strain specific and depends on composition of culture media and fermentation conditions. Since, the production level under un-optimized conditions was low; optimization of culture conditions was desirable for enhancing the penicillin acylase production. Penicillin acylase production is greatly influenced by the nutritional factors (carbon and nitrogen sources) and physical factors (pH, temperature, inoculation volume and inducer)[20-25]. These factors play important role in the growth of bacteria, enzyme synthesis. No defined medium has been established for the best production of penicillin acylase from different microbial sources or processes. Each organism or strain has its own special requirements for maximum enzyme production. In this study, further we used "Design Expert 8.0.7.1" software for the optimization of various factors that affect the growth of culture as well as expression of enzyme. This software takes into consideration the separate effects of various factors while they are used combined and the output is in the form of separate graphs for various factors studied.

The penicillin acylase production is dependent on the availability of both carbon and nitrogen sources in the medium. Thus, to optimize the conditions for production of PGA, it was necessary to optimize the carbon and nitrogen sources. Various sources of carbon such as sucrose, glucose, starch, and glycerol were used. Results obtained showed that glucose instigated highest penicillin acylase production compared to other carbon sources. The most widely used bacterial strains of *E. coli* are grown in a standard medium contained glucose as carbon source (Schoomer *et al*, 1984)[26]. Our result accedes with these reports. Nitrogen is an essential nutrient which is needed as a precursor for protein synthesis; besides it also plays an important role in regulating pH of the

medium and is crucial for maintenance of the activity and stability of the enzyme. In this study, four sources of nitrogen were used. They were beef extract, yeast extract, peptone and tryptone. The optimization experiment reveals the beef extract to be the optimum nitrogen source giving the highest enzymatic activity. Phenyl acetic acid (PAA) acts as a strong inducer in the production of penicillin acylase in the culture medium. The production of penicillin acylase in *E. coli* is induced by PAA. Szentirmai (1964) reported that addition of 0.2% phenylacetic acid increased the enzyme level eight to tenfold. Panbangred *et al* (1990), used 0.15% PAA as an inducer in penicillin acylase production. The most widely used bacterial strains of *E. coli* are grown in a standard medium contained 0.1-0.2% PAA as an inducer (Schoomer *et al*, 1984) and microbial enzymes were purified by neutral salt based purification[27]. In this study, PAA was used at a conc. of 0.150%, 0.170%, 0.185% and 0.200%. The optimum PAA conc. came out to be 0.185%. The penicillin acylase activity was enhanced to 0.73 IU from 0.604 IU during stationary phase of growth maximum at 48 h under following optimized culture conditions: glucose 8%; beef extract 2%; pH 9.0; temperature 30°C; 150 rpm, and inoculums 2% (v/v). This study has enabled the optimum formulation of media composition for maximum penicillin acylase production by this organism. In this study, we used LB broth with 0.185% PAA as an inducer for the production of penicillin G acylase separately in addition to the optimization experiment. Enzyme purification and characterization of enzyme have been carried out from this medium only.

Purification or partial purification of enzyme is required for further studies like enzyme characterization and for obtaining the enzyme in purified form too. In this study, we partially purified the enzyme produced using the ammonium sulphate precipitation followed by dialyses against the phosphate buffer (0.1 M, pH 7.5) and approximately 1.22-fold purification from the initial culture broth was achieved during ammonium sulphate precipitation (70-80%) with a yield of 4.6% enzyme. The penicillin acylase produced by us exhibited its optimal activity at 40°C and at pH 8.0. The enzyme was active in the pH range of 6.0-10.0. The enzyme showed high activity in the temperature range 30-45°C, while the enzyme activity was lower at 25°C. Without a doubt this study supports the fact that mathematical modeling based bioprocess optimization has helped in better production and in research in the field of pharma industries[28].

Conclusion

This study concluded that mathematical modeling for production of penicillin G acylase enzyme, an industrial biocatalyst for the production of penicillin is a much required industrial process. Since there are various disadvantages associated with one factor at a time method for production, a mathematical model based optimization of carbon and nitrogen sources, different pH, different temperature ranges and concentration of PAA was done. Maximum yield was production of 6-APA with a high 87.6% conversion of penicillin into 6-APA. Various medium constituents and fermentation conditions were standardized for maximum penicillin acylase production. This study concludes that by employing the mathematical model based production of penicillin acylase, we can enhance the yields.

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