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# SCREENING OF BACTERIAL ISOLATES PRODUCING PENICILLIN G ACYLASES

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The stream water, soil and plant organs, leaves and petals were screened to obtain bacterial isolates that may produce good to excellent amount of penicillin G acylase. Stream water and soil proved promising sources in providing *Escherichia coli* strains that produced fair to good amount of penicillin G acylase, however; each of these strains also produced betalactamase together with penicillin G acylase. The bacterial isolates from plant habitats, leaves and petals; were found poor in producing penicillin G acylase but were quite good in producing betalactamase activity. Penicillin G acylase producing isolates were not so wide spread as betalactamase bioactivity.

***Key words:*** Penicillin G acylase, Bacterial isolates.

## Introduction

Penicillin G or V is used to cure infections due to Gram positive bacteria, however, these are ineffective against infections caused by Gram negative bacteria that harbour penicillinases (betalactamases) which inactivate these antibiotics [1]. As penicillins specifically interfere with biosynthesis of bacterial cell wall, that is absent in animal cells, therefore there are little side effects and these are considered much safer as compared to other antibiotic. aminoglycosides, macrolides, tetracyclines etc. [2]. Excessive use of penicillin G or V has resulted in the emergence of resistant strains of Gram positive bacteria that are know to acquire the ability to produce betalactamase [1]. For this point in view semisynthetic penicillins insensitive to betalactamases have been synthesized and are in clinical use [1-3].Semisynthetic penicillins are synthesized from 6-aminopenicillanic acid (6-APA) which is obtained by deacylation of either penicillin G or V, using chemical or enzymic methods [3-5]. The enzymes that catalyze penicillin deacylation and 6-APA acylation, a reversible reaction, are called penicillin acylases, and these were called penicillin amidases in early literature (1,10). 6-APA can also be converted to cephalosporins [6].

In Pakistan penicillin G and V are produced locally, however; no technology exists in the country to convert penicillins to 6-APA. A research programme has been started to convert penicillin G to 6-APA. We report herein the isolation of penicillin G acylase producing bacteria from some local habitats, stream water, soil, plant leaves and flower petals.

## Materials and Methods

Stream water samples, soil samples and plant organs were collected from around NIAB Campus. From stream water and soil samples only *Escherichia coli* isolates were studied. All types of bacterial isolates were studied for plant organs and no attempt was made to identify them.

*Isolation of* Escherichia coli *from stream water or soil samples.* Stream water, freshly collected sample was diluted, 2ml of it to 5ml, by addition of sterile distilled water. Freshly collected soil sample 2g, was hand shaken with 10ml of sterile water for ten minutes in a sterile screw capped tube. The stream water or soil suspension was passed through a millipore prefiltration pad to remove the heavy particles. The prefiltrate, 2 to 3ml, was passed through sterile millipore filter, 0.45um, using syringe filter holder. The filter which retained the bacteria was placed on MacConkey agar plates, incubated overnight at 37° and large red colonies were collected. These were streaked on eosin methylene blue (EMB) agar plates and incubated at 37° overnight. The colonies with blue black centres and green reflected metallic sheen were saved that are supposed to be *E.coli* and subcultured on nutrient agar slants. Further biochemical reactions Methyl Red (+), Voges-Proskauer (-) and Citrate utilization (-) were made to confirm them being *E.coli* [7] which were maintained on nutrient agar.

*Bacterial isolates from plant organs.* Plant organs, leaves and petals, were used for bacterial isolation. Leaves and petals were taken from plants listed in Tables 2 and 3 respectively. Those were washed with sterile distilles water separately and cut into small pieces, that were placed on nutrient agar plates and allowed to incubate overnight at 37°. Next day bacterial colonies were picked up and maintained on nutrient agar slants. Gram reaction was studied [8] and these bacterial strains were used for fermentation and production of penicillin G acylases.

*Culture conditions of bacterial isolates for penicillin G acylases production.* Composition of the media used for the cultivation of bacteria was:- yeast extract, 1.0%; peptone,

1.0%; meat extract, 0.5%; NaCl, 0.25%, pH 7.3. The medium was sterilized at 15 lb for 15 minutes. Fermentation was carried out in Erlenmeyer flasks (250 ml) in a gyrotory shaker at 28° and 100 rpm for 2 days. Cells were harvested at 2500 g and suspended in deionized water at 6 mg dry cell weight per ml. Relationship between dry weight and packed cell mass determined separately.

*Penicillin G acylases bioassay.* The penicillin G acylase reactions were carried out essentially according to Nara *et. al.*, [9] and Cole *et,al.* [10]. Reaction mixture used for penicillin G acylases bioassay was composed of 2ml of 1% penicillin G in water 2ml of 0.1M phosphate buffer (pH 7.4 to 7.5) + 0.05 toluene + 2ml of cell suspension in water. Reaction mixture was incubated at 35° for 4 hr. Then it was filtered through millipore filters (0.45 μM) to remove cells. Reactions products were separated by thin layer chromatography using silica gel plates 0.25 mm thick with upflow development for 1.5 hr. with solvent system:- *n*-butyl acetate, *n*-butylalcohol, acetic acid and water (80:15:40:24). After development the plates were exposed to ammonia vapours for 20 minutes, then sprayed with 2% starch solution in saturted NaCl. After that the plates were exposed to iodine vapours, the white spots appeared for penicillin G (Rf:0.88), penicilloic acid (Rf:0.63) and 6-APA (Rf: 0.28).

*Estimation of 6-APA and penicillin G acylase.* The quantity of 6-APA produced in the reaction mixture was evaluated from the area of its chromatographic spot appeared on TLC plates. The areas of spots were considered directly related to penicillin G acylase quantities present in the cells. The spot areas of 6-APA were graded as:- (negative,- <20mm$^2$ as slight, ± ; = 20 mm$^2$ as fair, + ;> 20mm as moderate, ++;> 50mm$^2$ as good, +++).

*Estimation of betalactamase.* Betalactamase activity found to occur invariably together with penicillin G acylase activity in one and the same bacterial isolate. Betalactamase transforms penicillin G into its corresponding penicilloic acid which is completely devoid of antibacterial activity. The penicilloic acid appeared as a distinct spot on the TLC plate and its quantity produced in the reaction mixture was evaluated from the areas of its chromatographic spot. The spot areas for penicillic acid were graded as:- (negtive, -;< 20mm$^2$ as slight, ± ; = 20mm$^2$ as fair, +;> 20 mm$^2$ as moderate, ++;> 50 mm$^2$ as good, +++ ;> 100 mm$^2$ as excellent ++++).

*Sources of microbiological media and chemicals.* All the microbiological media were from Difco Laboratories, Detroit Michigan, USA. Penicillin G was a gift from Antibiotics (P) Limited, Iskanderabad (Daudkhel), Mianwali, 6-aminopenicillonic acid was purchased from Sigma Chemical Co., St. Louis, Missouri, USA. The silica gel TLC plates and all the other chemicals were purchased from E. Merck, Frankfurter Strasse 250, D-6100 Darmstadt, Federal Republic of Germany.

## Results and Discussion

One hundred *Escherichia coli* isolates obtained through selective procedure, fifty from stream waters and fifty from soil samples, were studied for their production of penicillin G acylase and betalactamase bioactivities. Betalactamase bioactivity is undesirable as it inactivates penicillin G by converting it into penicillioic acid. The aim is to obtain bacterial isolates producing penicillin G acylase alone and lack any ability to produce betalactamase bioactivity. The chromatographic monitoring system used in these studies had the advantage to bioassay both the enzymes simultaneously and any bacterial isolate lacking the betalactamase production ability and producing penicillin G acylase alone could be easily detected.

The bacterial isolates from stream waters and soils can be placed in different groups, depending upon the amount of penicillin G acylase alongwith betalactamase produced. Diagram showing the placement of 100 isolates of *E. coli*, from stream waters and soils, producing the penicillin G acylase and betalactamase in different combinations is shown in Table 1. The group of isolates that produce no penicillin G acylase but slight amount of betalactamase is depicted in column 1 and row of 2 of the Table 1. There were 15 such isolates obtained from stream waters and 4 were from soil samples. It can be seen that there was only one isolate that produced good amount of penicillin G acylase with slight amount of betalactamase, column 5 row 2 of the Table 1.

Among the 50 isolates studied from stream waters samples, 35 did not produce any penicillin G acylase, 5 produced slight, other 5 produced fair and still other 5 produced good penicillin G acylase bioactivity. However, all

TABLE 1. DIAGRAM SHOWING *ESCHERICHIA COLI* ISOLATES, FROM STREAM WATER OR SOIL, IN VARIOUS GROUPS PRODUCING PENICILLIN G ACYLASE AND BETALACTAMASE IN DIFFERENT COMBINATIONS.

| Acylase group | - | ± | + | ++ | +++ | Total |
|---|---|---|---|---|---|---|
| Betalactamase groups | | | | | | |
| - | WO/SO | WO/SO | WO/SO | WO/SO | WO/SO | WO/SO |
| ± | W15/S4* | W1/SO | WO/SO | W3/S1 | W1/SO | W20/55 |
| + | W3/S3 | WO/SO | WO/SO | W2/S3 | WO/SO | W5/56 |
| ++ | W16/S26 | W3/S5 | WO/SO | WO/S4 | W3/S4 | W22/539 |
| +++ | W1/SO | W1/SO | WO/SO | WO/SO | W1/SO | W3/SO |
| Total | W35/S33 | W5/S5 | WO/SO | W5/S8 | W5/S4 | W50/S50 |

* W15/S4 is showing a group of bacterial isolates that produced no penicillin G acylase (-) but produced slight betalactamase (±) and there were 15 such isolates from stream waters and 4 isolates from soils.

the isolates produced betalactamase activity and it varied from slight to good in amount. No bacterial isolate was obtained that lacked the ability to produce betalactamase, Table 1.

Among the fifty isolates studied from soil samples, 33 did not produce any penicillin G acylase, 5 produced slight, and other 8 produced fair and still other 4 produced good penicillin G acylase bioactivity. All the isolates, like those obtained from stream waters, produced betalactamase that varied from slight to fair in amount. No bacterial isolate, as it was the case with stream waters, was obtained that lacked the ability to produce betalactamase, Table 1.

It is expected that further screening may be able to show up some isolates with good production of penicillin G acylase but lacking the ability to produce betalactamase. The other alternative is to improve upon the isolates that produced good amount of penicillin G acylase and slight amount of betalactamase by inactivating or deleting the genes of betalactamase using mutagenic or genetic engineering procedures.

Bacterial isolates, 50 in number, obtained from leaf habitats of seven different plants, were studied for their Gram reaction, penicillin G acylase production and betalactamase bioactivity. Among the 6 bacterial isolates obtained from the leaf habitat of *Celosia cristata*, 2 were Gram positive and 4 were Gram negative; all the six produced slight penicillin G acylase bioactivity, however 2 of these were fair, three were good and one was excellent producer of betalactamase, Table 2. Both Gram negative and Gram positive isolates were found inhabiting the plant leaves, and all the 50 isolates had only slight ability to produce penicillin G acylase, but quite a large number of those could produce fair to good amount of betalactamase. From Table 2, it appears that bacterial isolates from leaf habitats of seven plants proved poor acylase producers and good to excellent producers of betalactamase.

Similarly the flower petal habitats, from seven different plants, were poor to good producers of penicillin G acylase but at the same time these were good to excellent producers of betalactamase, Table 3.

Studies of other workers have also shown penicillin acylase activity to occur together with betalactamase activity in one and the same microbial strain and this co-existence was demonstrated in *Escherichia coli* strains by Cole and Sutherland [11] and Pruess and Johnson [12]. Similarly the screening studies have demonstrated that penicillin G acylase bioactivity is not so wide spread as betalactamase bioactivity [9-16].

It is concluded from this study that plant habitats are poor sources of bacterial isolates producing penicillin G acylase, whereas stream waters and soils are good sources for obtaining bacterial isolates that may yield penicillin G acylase in good amount. A penicillin G acylase producing strain lacking betalactamase bioactivity is difficult to obtain and the screening is effected by trial and error method.

TABLE 2. BACTERIAL ISOLATES FROM PLANTS LEAVES PRODUCING PENICILLIN G ACYLASE AND BETALACTAMASE

| Plant | Bacterial isolates studies | Gram reaction | Acylase activity | Betalactamase activity |
|---|---|---|---|---|
| *Celosia cristata* | 6 | +(2)<br>- (4) | ±(6) | +(2)<br>++(3)<br>+++(1) |
| *Citrus reticulata* | 6 | +(0) | ±(0) | ++(6 |
| *Clerodendron inerme* | 6 | +(3) | ±(6) | ++(6) |
| *Chrysanthemum morifolium* | 12 | +(9)<br>-(3) | ±(12) | +(3)<br>+++(9) |
| *Populus hybrida* | 6 | +(2)<br>-(4) | ±(6) | +(2)<br>++(4) |
| *Rosa indica* | 6 | +(2)<br>-(4) | ±(6) | +(1)<br>++(5) |
| *Tagetes erecta* | 8 | +(4)<br>-(4) | ±(8) | ++(8) |

TABLE 3. BACTERIAL ISOLATES FROM PETALS PRODUCING PENICILLIN G ACYLASE AND BETALCTAMASE.

| Plant | Bacterial isolates studied | Gram reaction | Acylase activity | Betalac tamase activity |
|---|---|---|---|---|
| *Antirrhinum majus* | 6 | +(6)<br>-(0) | +(6) | ++(5) |
| *Chrysanthemum morifolium* | 12 | +(4)<br>-(8) | +(1)<br>++(11) | ++(1)<br>++++(11) |
| *Lathyrus odoratus* | 8 | +(5)<br>-(3) | ±(8) | +++(7)<br>++++(1) |
| *Nasturtium officinale* | 6 | +(3)<br>-(3) | ±(5)<br>-(1) | ++(1)<br>+++(5) |
| *Rosa indica* | 6 | +(3)<br>-(3) | ±(6) | ++++(6) |
| *Tagetes erecta* | 6 | +(3)<br>-(3) | ±(6) | +(2)<br>++(4) |
| *Partulaca grandiflora* | 6 | +(4)<br>-(2) | ±(6) | ++++(6) |

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