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PENICILLIN PRODUCTION BY WILD ISOLATES OF *PENICILLIUM CHRYSOGENUM* IN PAKISTAN

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ABSTRACT

The present study was aimed at exploring the native wild isolates of *Penicillium chrysogenum* series in terms of their penicillin production potential. Apart from the standard medium, the efforts were made to utilize suitable agro-industrial wastes for the maximum yield of penicillin. Two series of *P. chrysogenum* were isolated from local sources and named as *P. chrysogenum* series UAF R1 and *P. chrysogenum* series UAF R2. The native series were found to possess better penicillin production potential than the already reported series of *P. chrysogenum*. However, *P. chrysogenum* series UAF R1 was found to be the best candidate for high yield of penicillin starting at 100 hour as compared to *P. chrysogenum* series UAF R2 which produced the highest yield of penicillin at 150 hours for a shorter period of time. Addition of Corn Steep Liquor (CSL) to the fermentation medium resulted in the production of 1.20g/L penicillin by *P. chrysogenum* series UAF R1 and *P. chrysogenum* series UAF R2. The fermentation medium in which Sugar Cane Bagasse (SCB) was replaced with CSL resulted in the highest yield of penicillin (1.92g/L) by both native series of *P. chrysogenum*. The penicillin production was increased by 62.5% in medium with SCB as compared to that with CSL. The penicillin yield of medium containing lactose and phenyl acetate was higher than that of control medium. Overall results revealed that *P. chrysogenum* series UAF R1 and *P. chrysogenum* series UAF R2 may be recommended for better yield of natural penicillin and this efficiency may be further enhanced by utilizing SCB as substrate in the growth medium.

Key words: Penicillin; *Penicillium chrysogenum*; Corn Steep Liquor; Sugar Cane Bagasse

INTRODUCTION

Production of penicillin has been the subject of many studies because of its academic and industrial importance. It is one of the oldest discoveries among the naturally occurring antibiotics. More than thirty different derivatives are being prepared from 6-aminopencillanic acid and playing a vital role in the treatment of mixed infections (1). Penicillin is most actively produced by representatives of the species *P. chrysogenum*. Several strains of *P. chrysogenum* are being used for penicillin production at laboratory and commercial levels in various countries of the world (7, 11). Research is still continuing in an effort to economize its production by fermentation. Special attention is being paid to develop strains

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for maximum penicillin productivity, to use cheap raw materials as substrate and to employ simple methods of cultivation for better penicillin production (13).

Agro-industrial wastes are generated annually and their use as raw materials in technological processes for obtaining products of high added value is gaining interest. Different kinds of agro-industrial wastes have been reported for the production of various enzymes and other products using biotechnological processes (10). Among such wastes are sugar cane bagasses (SCB), a plentiful biomass with high contents of sugar and corn steep liquor (CSL), a waste product of starch (maize) industry. These wastes can be used for penicillin production by maintaining different series of *P. chrysogenum.* This type of fermentation is quite feasible in an agricultural country like Pakistan where these agro-industrial wastes are available in large amounts for their commercial utilization. (8, 14).

Keeping in view the importance of the subject, the present work was carried out to isolate *P. chrysogenum* from local sources and to further evaluate it on the basis of penicillin production potential by shake-flask cultivations using simple agro-industrial wastes like sugarcane bagasse and corn steep liquor as substrates for mold growth.

MATERIALS AND METHODS

Primary isolation

Spoiled fruits (mangoes, apple, and citrus), vegetables, bread and grains (wheat, maize) were collected as samples for primary isolation of *P. chrysogenum* using Sabourauds' glucose agar medium as described by Malik (12). The medium composed of glucose 40.0g, peptone 10.0g, agar 15.0g dissolved in 1000ml distilled water with pH adjusted to 5.4 (6). The fruiting bodies of mold from the samples were grasped and rubbed with a pair of forceps on Sabourauds' glucose agar medium plates to spread the spores as described by Cappuccino and Sherman (3). The inoculated plates were incubated in inverted position at 25^{O}C for seven days in moist incubator. The fungal isolates were identified by slide culture method as recommended by Awan and Rahman (2). Each slide was examined under low and high power objective of microscope for the arrangement of hyphae, conidiophores, sterigmata and conidia.

Purification of seed culture

Czapek Yeast Autolysate (CYA) agar medium was used for the purification of seed culture as described by Singh *et al* (15). The CYA agar medium composed of (g/L): $NaNO_3$, 3.0; K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; yeast extract 5.0; sucrose, 30.0; agar, 15.0 and trace metal solution, 1.0ml. Trace element solution composed of $ZnSO_4.7H_2O$, 1.0g and $CuSO_4.5H_2O$, 0.5g in 100ml distilled water with pH adjusted to 5.4. Different colonies of *P. chrysogenum* growing on Sabourauds' agar plates were transferred to CYA agar medium plates and slants under sterile conditions (15). The inoculated plates and slants were incubated at 25^{O}C for seven days in darkness. Colony characters were recorded and slide culture method was used for re-identification of seed culture. Slants with pure growth of *P. chrysogenum* were kept in refrigerator for further use.

Inoculum preparation

After seven days spores from the surface of CYA agar slants were rubbed off in sterile water. The numbers of spores/ml were determined through Breed smear method as described by Tortora *et al.* (16). Briefly, 0.01ml of spore suspension was spreaded over a marked area of 1 cm^2 on glass slide. A drop of lactophenol cotton blue stain was added and a cover slip was placed on the slide. The slide was viewed under an oil immersion objective lens. Numbers of spores were calculated in different microscopic fields at random to calculate the average spores/microscopic field. The average count was multiplied by microscopic factor and 100 to get the final spore count/ml.

Shake flask cultivations

Shake flask cultivations were performed in 500ml Erlenmeyer flasks containing 100ml growth medium as recommended by Chen *et al*. (4). The growth medium composed of (g/L): glucose, 20.0; yeast extract, 10.0; Corn Steep Liquor (CSL), 5.0; beef extract, 0.075; peptone, 0.125; $(NH_4)_2SO_4$, 4.0; KH_2PO_4, 3.0; $ZnSO_4.7H_2O$, 0.01; $MgSO_4.7H_2O$, 2.3. The medium was modified by using polyethylene glycol (PEG), corn steep liquor (CSL), sugarcane bagasse (SCB), phenyl acetate (PA) and lactose each @10%. In each flask, 1.3×105 spores/100ml were inoculated and resulting mixtures were incubated at 25^{O}C at 125 rpm on rotary shaker (Eyela, Japan) for 300 hours.

Sampling

Culture sample of 10ml volume was collected separately from each system at 50 hours interval and studied for morphology, pH change and glucose concentration. Since the inoculum was comprised of spores therefore morphological transitions from spores to mycelia and from mycelia to pellets were observed. Glucose was assayed as reducing sugar by Benedicts' test. When glucose was depleted after 2 hours, 0.4% (w/v) lactose and 0.1% (w/v) phenyl acetate were added to the medium to induce the penicillin production.

Penicillin production analysis

The qualitative analysis was done through β-lactamase test using penicillin resistant *Staphylococcus aureus* (5). Briefly, filter paper was soaked in 0.2% bromophenol blue and 2% culture sample from different shake flask culture medium. The filter paper was dried and loopful culture of penicillin resistant *S. aureus* was placed on it. The change in color was noted to see the presence of β-lactamase enzyme and to confirm the penicillin production. Quantitative analysis was performed by measuring the diameter of zones of inhibition of all the culture samples and comparing them with the standard curve drawn by measuring the diameter of zones of inhibition of standard dilutions of commercially available penicillin G.

Extraction of penicillin

A four step solvent transfer method was used for the extraction of penicillin from different shake flask cultivation systems as described by Rafi (14). Briefly, first penicillin produced in shake flask culture was extracted in to amyl acetate and then transferred from amyl acetate into phosphate buffer. In third step extraction was made from buffer solution in to chloroform and finally transferred from chloroform in to water. The extracted material was soaked in to sterilized filter paper discs for quantitative analysis.

RESULTS AND DISCUSSION

Different samples comprising of fruits, vegetables, bread and grains were processed on Sabourauds' glucose agar medium for primary isolation of Penicillium species. The same medium has also been attempted by other scientists for primary isolation of mold (3, 12). After seven days of incubation all the samples exhibited different colony characteristics. On identification most of the isolates belonged to Penicillium, Aspergillus, Mucor and other un-identified species of fungi. As it was difficult to process a complete range of mold isolates, only those isolates which were having morphological and cultural characteristics similar to Penicillium species were selected. A total of four isolates which showed dark green to grayish blue green colored colonies were further processed. The findings are in line with the work of Malik (12) who also reported that Penicillium species effect food and fruit quality.

The colonial and microscopic morphology of two isolates, one from the spoiled mango and other from maize were similar to *P. chrysogenum.* Being the local isolates, these were provisionally named as *P. chrysogenum* series UAF R1 and *P. chrysogenum* series UAF R2. The comparative colonial and microscopic morphology of these two series on Czapek Yeast Autolysate (CYA) agar medium is given in Table 1. The

successful isolation of Penicillium species on CYA medium from different colonies growing on Sabourauds' glucose agar medium has also been reported by Singh *et. al.* (15). Slide culture method used in the present study has also been recommended by Malik (12) for the proper identification of Penicillium species. Most of the morphological and cultural characteristics of *P. chrysogenum* series UAF R1 and *P. chrysogenum* series UAF R2 were similar to those described in the literature (15).

Table 1. Comparison of colonial and microscopic morphology of *P. chrysogenum* series on Czapek Yeast Autolysate (CYA) agar medium

Colonial morphology	***P. chrysogenum* series UAF R1**	***P. chrysogenum* series UAF R2**
Colony diameter	4.5-5.0 cm	5.5-6.0 cm
Texture	Sulcate, velutinous, and white borders 2-3 cm wide	Sulcate, velutinous, and white borders 2-3 cm wide
Obverse	Bluish green to dark green	Greyish green to dark green
Reverse	Pale yellow	Creamish yellow
Odor	No	No
Diameter at 37 ^{O}C	1.0-1.5 cm	1.5-2.0 cm
Microscopic morphology	***P. chrysogenum* series UAF R1**	***P. chrysogenum* series UAF R2**
Stipes	Short, smooth	Short, smooth
Penicilli	Treverticillate	Biverticillate
Phialides	Ampulliform	Ampulliform
Collula	Short	Medium sized
Conidia	Spherical to ellipsoidal smooth, greenish	Ellipsoidal to spherical, smooth, dark green

A total of four fermentation for each of *P. chrysogenum* series UAF R1 and *P. chrysogenum* series UAF R2 were carried out with different combinations of growth medium to see their comparative penicillin production potential. The penicillin titers calculated from diameter of zone of inhibitions before and after extraction in each group for *P. chrysogenum* series UAF R1 and *P. chrysogenum* series UAF R2 are given in Table 2. Addition of polyethylene glycol (PEG) to the fermentation medium resulted in more and smaller pellets. In first group (CSL), the maximum penicillin titer was 1.20g/L at 150-200 h followed by 1.08g/L at 250 h and 0.72g/L at 300 h in UAF R1, whereas it was 1.20g/L at 200-250 h followed by 0.72g/L at 300 h in UAF R2. The similar results have been reported by Chen *et. al.* (4). One of the favorable effects of PEG in penicillin fermentation was its ability to suppress foaming (4).

Table 2. Comparative Penicillin Production Potential of *P. chrysogenum* series at different time intervals

Time (hours)	**Penicillin titers before extraction* (g/L)**				**Penicillin titers after extraction* (g/L)**			
UAF R1	**CSL**	**SCB**	**PA**	**C**	**CSL**	**SCB**	**PA**	**C**
50	-	-	-	-	-	-	-	-
100	0.48±0.01	1.20±0.03	-	-	0.72±0.02	1.92±0.06	-	-
150	1.08±0.03	1.68±0.05	0.48±0.03	-	1.20±0.05	1.92±0.04	0.72±0.03	-
200	1.08±0.02	1.08±0.02	0.72±0.04	0.48±0.01	1.20±0.03	1.20±0.02	1.08±0.02	0.72±0.01
250	0.72±0.04	1.08±0.03	0.72±0.01	0.48±0.01	1.08±0.02	1.20±0.02	1.08±0.04	0.72±0.03
300	0.48±0.01	0.48±0.02	0.48±0.01	0.48±0.03	0.72±0.02	0.72±0.01	0.72±0.02	0.72±0.02
UAF R2	CSL	SCB	PA	C	CSL	SCB	PA	C
50	-	-	-	-	-	-	-	-
100	0.48±0.02	0.72±0.02	-	-	0.72±0.02	1.08±0.03	0.48±0.01	-
150	0.72±0.03	1.68±0.06	0.72±0.03	-	1.08±0.03	1.92±0.06	1.08±0.02	-
200	1.08±0.04	1.20±0.04	0.72±0.02	0.48±0.01	1.20±0.04	1.44±0.04	1.08±0.04	0.72±0.01
250	1.08±0.04	1.08±0.03	0.72±0.02	0.48±0.02	1.20±0.03	1.20±0.03	1.08±0.03	0.72±0.02
300	0.48±0.01	0.48±0.01	0.48±0.01	0.48±0.02	0.72±0.02	0.72±0.02	0.72±0.02	0.72±0.03

CSL = Corn Steep Liquor
SCB = Sugar Cane Bagasse
PA = Phenyl Acetate
C = Control
- = Zero titers
* Extraction of penicillin was carried out by 4 step solvent transfer method

Chen *et. al.* (4) and Weng *et. al.* (17) also experienced similar effects of CSL on penicillin production in shake flask cultivations. Addition of sugar cane bagasse (SCB) to fermentation medium resulted in the highest yield of penicillin. The maximum titer was 1.92g/L at 100-150 h followed by 1.20g/L at 200-250 h and 0.72g/L at 300 h in case of UAF R1, whereas it was 1.92g/L at 150 h followed by 1.44g/L at 200 h and 0.72g/L at 300 h in UAF R2. The penicillin titer was increased by 62.5% in fermentation medium with SCB as compared to that with CSL. These findings were in line with those of Gonzalez *et. al.* (9) who reported that the use of a large particle size supported the increased penicillin production by 37%, however this effect was due to a higher sugar concentration in bagasse fraction.

The effect of lactose and phenyl acetate (PA) on penicillin production was also observed in third group (PA). The maximum titer was 1.08g/L at 200-250 h followed by 0.72g/L at 300 h in UAF R1 whereas it was 1.08g/L at 150-250 h followed by 0.72g/L at 300 h in case if UAF R2. The mold grew in a non-uniform morphology with a large portion existing as compact smooth pellets. As the fermentation proceeded, the pellet became bigger and eventually broke in to clumps. Similar observations have been made by Chen *et. al.* (4) and Weng *et. al.* (17). The penicillin production was the lowest in control group (C) with simple growth medium. The maximum titer was 0.72g/L at 200-300 h in both the series of *P. chrysogenum*.

The highest penicillin production of *P. chrysogenum* series UAF R2 was 1.20g/L at 200-250 h in fermentation medium with CSL and 1.92g/L at 150 h in fermentation medium with SCB. Similar observations have been made by Chen *et. al*. (4) who reported that maximum penicillin titer of a strain of *P. chrysogenum* ATCC 48271 was 1.25g/L at 250-300 h in fermentation medium with CSL. On the basis of these results, it was concluded that penicillin production potential of *P. chrysogenum* series UAF R1 was better as compared to *P. chrysogenum* series UAF R2 in terms of early recovery of penicillin. However both *P. chrysogenum* series UAF R1 and *P. chrysogenum* series UAF R2 locally isolated proved to be the best species in terms of their penicillin production potential. Moreover, the addition of 5% SCB in growth medium resulted in the highest yield of penicillin followed by CSL. These agro-industrial wastes can be efficiently used in future to further enhance the penicillin production potential of native strains of *P. chrysogenum*.

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