Original Article

Penicillin Production by Penicillium Chrysogenum PCL 501: Effect of UV Induced Mutation

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Keywords

agro-wastes, mutant strains, penicillin, penicillium chrysogenum, uv irradiation

Citation

B M Onyegeme-Okerenta, V I Okochi, S N Chinedu. *Penicillin Production by Penicillium Chrysogenum PCL 501: Effect of UV Induced Mutation* . The Internet Journal of Microbiology. 2013 Volume 12 Number 1.

Abstract

Penicillin production by Penicillium chrysogenum (PCL501) fermented on glucose, lactose and four agro-wastes (cassava shavings, corncob, sawdust, and sugarcane pulp) was monitored with HPLC. The highest amount of penicillin was obtained with sugarcane pulp. Penicillin yield from a 7-day culture of the fungus was 8.65 ± 0.05, 7.68 ± 0.03, 6.85 ± 0.05, 5.54 ± 0.01, 5.32 ± 0.05 and 2.23 ± 0.02µg/ml respectively on sugarcane pulp, glucose, cassava shavings, corncob, lactose, and sawdust. Two mutant strains, UVP1 and UVP2, were obtained by exposing the wild strain (PCL501) to ultraviolet irradiation for 20 and 25 minutes respectively. The 7-day culture of UVP1 and UVP2 on sugarcane pulp yielded 14.83 ± 0.05 and 14.97 ± 0.05 µg/ml penicillin respectively. This represented over 70% increase in penicillin production over the parent strain. There is a good prospect of producing cheaper and effective penicillin using the mutant strains of P. chrysogenum and

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INTRODUCTION

Penicillium chrysogenum, since its discovery in 1943, has remained the species of choice for industrial penicillin production; it yields much more penicillin than P. notatum, the first antibiotics-producing fungus isolated by Alexander Fleming, or any of its derivatives1,2,3. Efforts at producing cheaper and more effective penicillins had centered on isolation of new strains of the fungus, optimization of fermentation technique and improvement of available strains by classical mutagenesis procedures1,4.

Mutation of P. chrysogenum with X-Rays, UV rays or other mutagens has been engaged since 1950s as a tool for enhancing penicillin production3. Mutant strains with 1000-fold increase on the amount of penicillin produced in Fleming's original culture have been developed from such improvement procedures5,6. Stauffer and Backus7 reported of high penicillin yielding P. chrysogenum Q-176 mutant obtained by UV irradiation. Plasma mutation method was recently used to develop a mutant strain of P. chrysogenum with high productivity of penicillin8.

A strain of P. chrysogenum (PCL501) isolated from wood-wastes in Lagos, Nigeria9, is currently being investigated for penicillin production. Previous studies showed that the fungus produces β-lactam antibiotics with significant antibacterial effects against clinical isolates of E. coli and B. subtilis10. The fungus thrives on media supplemented with agro-wastes and utilizes such waste plant materials as sawdust, sugarcane pulp, cassava shavings, and corncob as carbon and energy sources for growth and antibiotics production10,11,12. In the present study, agro-wastes (cassava shavings, corncob, sawdust, and sugarcane pulp) and refined sugars (glucose and lactose) were compared as fermentation substrates for penicillin production by P. chrysogenum (PCL501). Ultraviolet (UV) irradiation was also used to produce mutant strains which yielded higher amounts of the antibiotics than the parent fungus.

MATERIALS AND METHODS

Preparations of Agro waste materials:

Mature sugarcane stem (Saccharum offinarum), fresh maize (Zea mays) and cassava shavings (Manihot esculenta) were purchased from a local market in Mushin, Lagos, Nigeria. Sawdust of Abora wood (Mitragyna ciliata) was collected from sawmills at Ikorodu, Lagos, Nigeria.

Peeled sugarcane stem was crushed and soaked overnight in distilled water. The pulp was washed in distilled water repeatedly until no trace of sugar was detected. Corncob, obtained by removing the maize grains, was cut into small sizes. The agro-wastes were dried to constant weight in the oven at 80oC and milled using Marlex Exceller grinder (Mumbai, India). Fine powder obtained after passing each agro-waste through 0.5 mm pore sized sieve was used as substrate in the fermentation media.

Penicillium chrysogenum strain (PL 501)

The wild strain of P. chrysogenum (PCL 501) was obtained from wood-wastes in Lagos, Nigeria as described previously9 and maintained on PDA slant at 4oC. Subcultures of the fungus on PDA plates were incubated at 30oC for 3-5 days to obtain spores for the study.

Media preparation:

The fermentation media contained: 6.0g Ammonium acetate, 0.5g NaSO3, 0.02g ZnSO4.7H2O, 0.25g MgSO4.7H2O, 6.0g KH2PO4, 0.01g FeSO4.7H2O and 10.0g substrate (cassava shavings, corncob, glucose, lactose, sawdust or sugarcane pulp) per litre of distilled water. Fifty millilitres (50 ml) of each media was dispensed into 250 ml Erlenmeyer flask and used for the growth and penicillin production. Fermentation media were prepared in duplicates and autoclaved at 121oC for 15 minutes after the pH was adjusted to 6.5.

Fungal growth

Spores were washed from agar plates into a sterile beaker with 0.1% Tween 80 in 0.1M potassium phosphate buffer (pH 7.0). The spore suspension was standardized such that 1 in 10 dilutions gave an optical density of 0.48 at 530nm. Five millimeters (5 ml) of the spore suspension was used to inoculate 50 ml of the respective sterile liquid medium in the flask. The flask was covered with sterile cotton wool and incubated at 30℃ with continuous agitation at 100 Osci/min using Griffin flask shaker. Growth was monitored by measuring mycelia weight and protein content of the cell-free culture filtrate. Cultures were harvested by filtration in triplicates at 3-day intervals for 21 days. Filtrate from the 7-day culture was used for HPLC analysis of penicillin concentration.

Mycelia weight measurement:

Mycelia was collected on a weighed filter paper and washed with distilled water. The filter paper (with the mycelia) was dried to a constant weight at 70oC and weighed, and the dry weight of the mycelia was deducted13,14,15. Change in mycelia weight was calculated as difference of the mycelia weight from the previous value.

Protein assay:

The protein content of cell-free culture filtrates of P. chrysogenum (PL 501) was assayed by Folin-Lowry method16. Change in protein yield was calculated as difference of the protein content from the previous value.

UV mutation

Agar slant of P. chrysogenum (PL 501) was incubated for 5 days to fully sporulate. The spores were scraped into 5 ml of sterile water and serially diluted to 1 in 105. The spore suspension (0.1 ml) was aseptically poured on a PDA plates and uniformly distributed using a sterile spreader.

The spore suspension was exposed to UV irradiation from a UV Illuminator fitted with TUP 40w Germicidal lamp with about 90% of its radiation at 2540-2550 A°. The exposure was carried out in the dark at a distance of 16 cm away from the centre of the Germicidal lamp (UV light source) with occasional shaking. The exposure times were 5, 10, 15, 20, 25, and 30 minutes. Each UV exposed spore suspension was sealed with a foil paper and stored overnight in the dark to avoid photo reactivation. The plates were incubated for 3 days at 28℃. Selection was based on a 1% survival rate and changes in morphology, size and shape17.

The best two mutant strains with 1% survival rate were isolated, streaked on PDA slants and incubated for 3 days at 28℃. The two UV mutant strains were inoculated into a fermentation media containing sugarcane pulp and penicillin production was monitored and quantified after 7 days using HPLC analytical method18.

HPLC analysis

HPLC analysis of penicillin from 7-day culture of P. chrysogenum (PCL 501) and mutant strains (UV1 and UV2) was carried out with a µBondapak C18 column after a careful validation of the method of Laich et al.18. The extract was filtered through Whatman No.1 filter paper and left at 4oC for 1 hour. This was further filtered through a Whatman No. 1, 0.45 micron and 0.22 micron filters. This filtrate was divided into 50 ml aliquots and stored in the dark at 4oC until use. Benzyl Penicillin G at a concentration of 0.1 mg/ml was used as the control sample. A C18 gravity column was wetted with 100 ml of HPLC grade methanol and then washed with 50 ml of Milli-Q water. A 50 ml aliquot of crude extract was added to the column and allowed to adsorb. The flow rate was 1.0 ml/min, and the running conditions were as follows: 1 – 3 min, buffer A (50 mM sodium acetate); 3 – 15 min, gradient buffer B (0 to 60% acetonitrile); 15 – 18 min, gradient buffer B (60 to 80% acetonitrile); 18 – 20 min, buffer B (80% acetonitrile); 20 – 22 min, gradient buffer B (80% to 0% acetonitrile); and 22 – 25 min, buffer A (50 mM sodium acetate)18,19,20. Penicillin eluted at approximately 2.35 minutes.

Data analysis

All assays were done in triplicates and results were expressed as Mean ± standard error of mean (SEM). Significant difference between values was determined by Fisher's protected least significant different t-test with two-tail probabilities of less than 0.05 considered significant. Statistical significant difference in the rate of microbial growth between media types was assessed by a one-way analysis of variance.

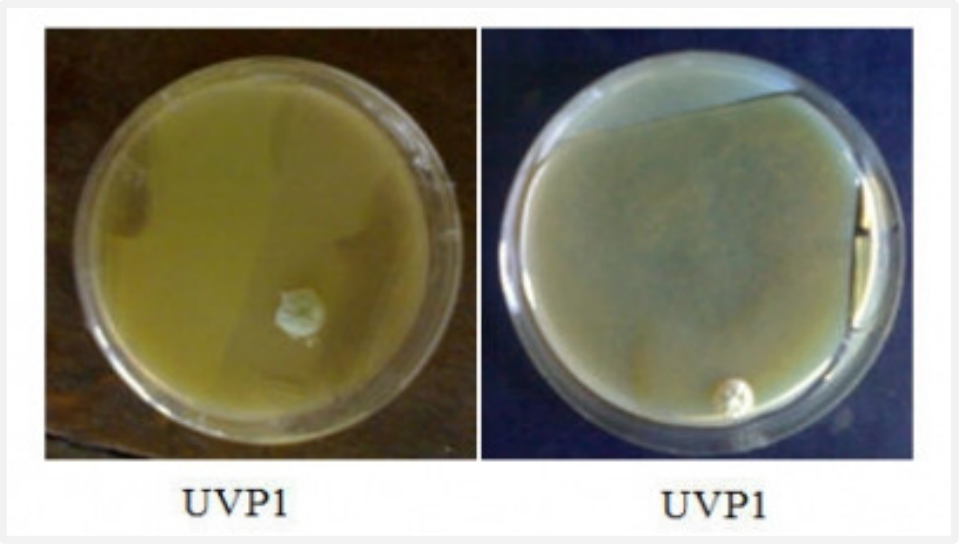
RESULTS

Mutant strains of P. chrysogenum (UVP1 and UVP2)

The UV modified strains of P. Chrysogenum, UVP1 and UVP2, were obtained after exposing the culture plate with cell suspension to UV light for 20 and 25 minutes respectively. The survival rate was 1%. Figure 1 shows the culture of UV modified strains of P. chrysogenum (UVP1 and UVP2) on basal medium containing sugarcane pulp as sole carbon source.

Figure 1

Plate 1: Mutant strains of P. chrysogenum, UVP1 and UVP2, obtained by exposure of the wild strain of P. chrysogenum (PCL501) to UV irradiation for 20 and 25 minutes respectively.

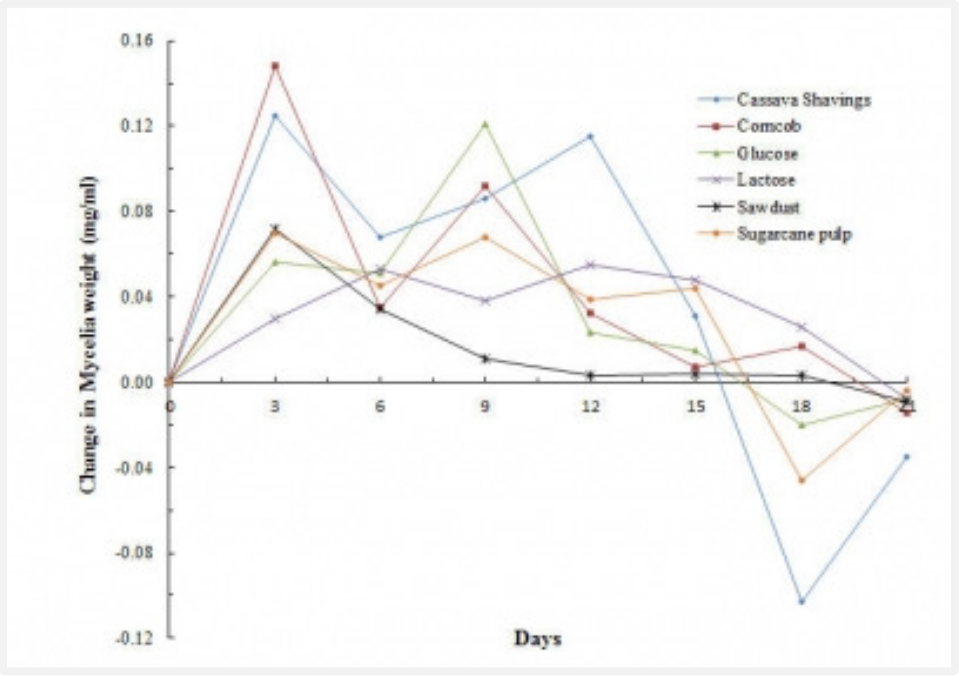


Mycelia Weight

Figure 2 shows the changes in mycelia weight of P. chrysogenum (PCL 501) fermented on the different substrates for 21 days. There were wide fluctuations in the mycelia weight of the fungus within the period of study. The mycelia weight increased significantly on day 3 of fermentation in all the media, and continued to appreciate at different rates until day 15. Highest increase in mycelia weight was obtained with corncob; it gave two peaks, a major peak of 0.148 mg/ml (at day 3) and a minor peak of 0.092 mg/ml (at day 9). Cassava shavings also had two peaks of mycelia weight increases, 0.125 mg/ml (at day 3) and 0.115 mg/ml (at day 12). The peak values of mycelia weight gain was 0.121, 0.073, 0.070, and 0.055 mg/ml respectively in media containing glucose, sawdust, sugarcane and lactose. There was significant loss of mycelia weight in media containing glucose, sugarcane and cassava shavings in the 18th day of fermentation and by the 21st day, the mycelia weight had declined in all the media indicating lack of growth.

Figure 2

Changes in mycelia weight of Penicillium chrysogenum (PCL 501) fermented on cassava shavings, corncob, glucose, lactose, sawdust, and sugarcane pulp measured at 3-day intervals for 21 days.

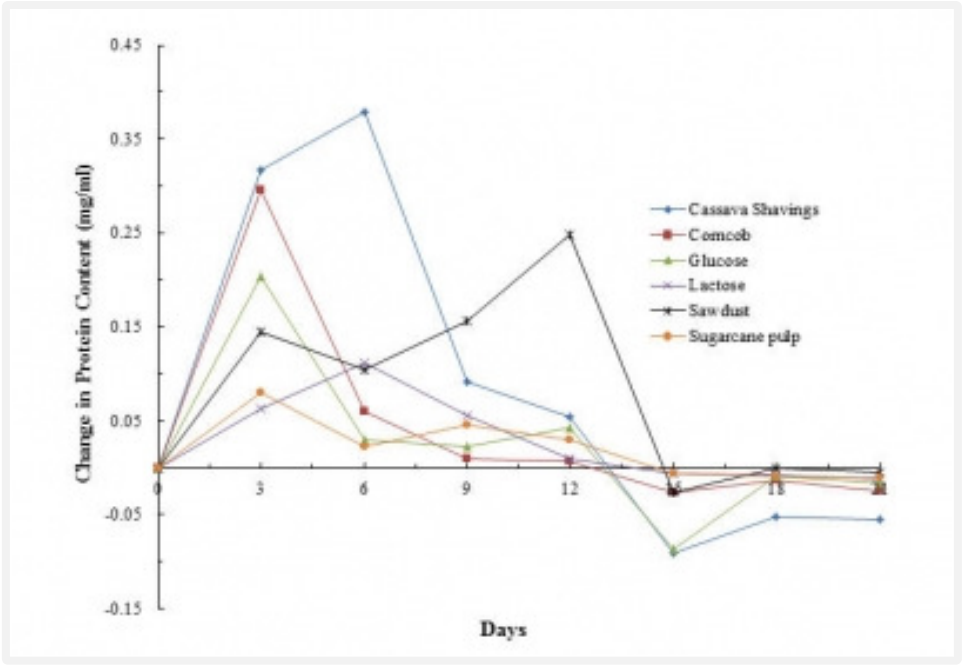


Protein yield

Changes in the amount of protein secreted by P. chrysogenum (PCL 501) fermented on the different substrates for 21 days is shown in Figure 3. Culture filtrate from media supplemented with cassava shavings gave the highest increase in protein yield (0.38 mg/ml) on day 6 while the least peak value was obtained with sugarcane pulp. Protein yield in all the media appreciated until day 12. Peak protein yield of 0.30, 0.20 and 0.08 mg/ml respectively was obtained for corncob, glucose and sugarcane pulp at day 3. Sawdust gave two protein peaks, 0.15 mg/ml (at day 3) and 0.25 mg/ml (at day 12). Protein secretion in all the media reduced drastically from day 15. The negative value of protein yield indicates an end in the growth of the organism.

Figure 3

Changes in the protein content of culture filtrate of Penicillium chrysogenum (PCL 501) fermented on cassava shavings, corncob, glucose, lactose, sawdust, and sugarcane pulp measured at 3-day intervals for 15 days.



Penicillin yield:

The yield of penicillin from media containing refined sugars (glucose and lactose) and agro-wastes (cassava shavings, corncob, sawdust, and sugarcane pulp) is shown in Figure 4. The highest yield was obtained with sugarcane pulp followed by glucose. The yield from cassava shavings and corncob was higher than that from lactose while the least yield came from sawdust. Figure 5 shows the rate of production of penicillin monitored by the wild strain of P. chrysogenum (PL 501) and the UV mutant strains. There was 70% increase in penicillin yield by the two mutant strains of P. chrysogenum over the yield of the parent (wild) strain.

Figure 4

Penicillin yield of 7-day culture of Penicillium chrysogenum (PCL501) fermented on agro-wastes (cassava shavings, corncob, glucose, lactose, sawdust, and sugarcane pulp).

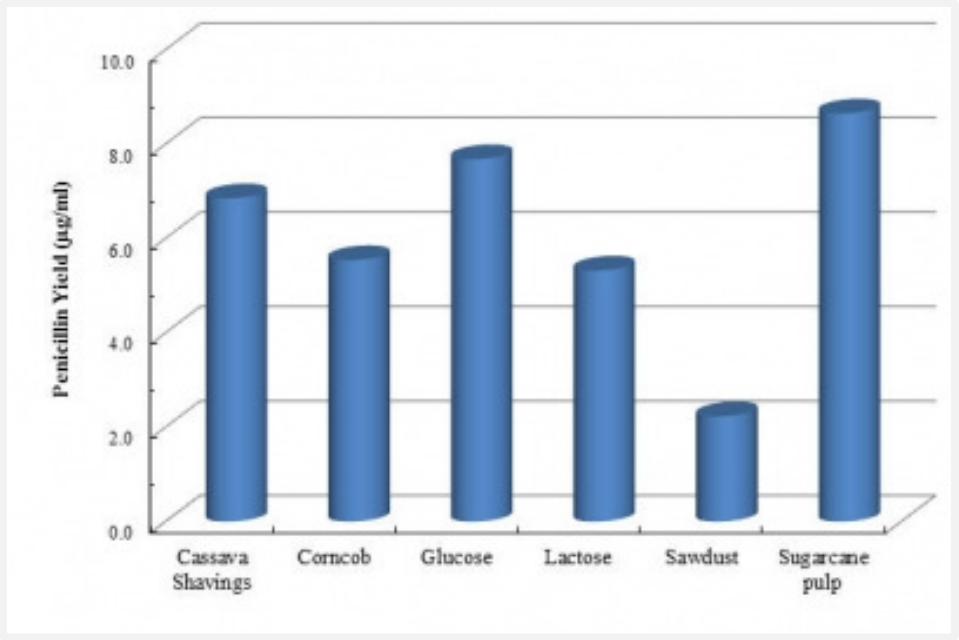
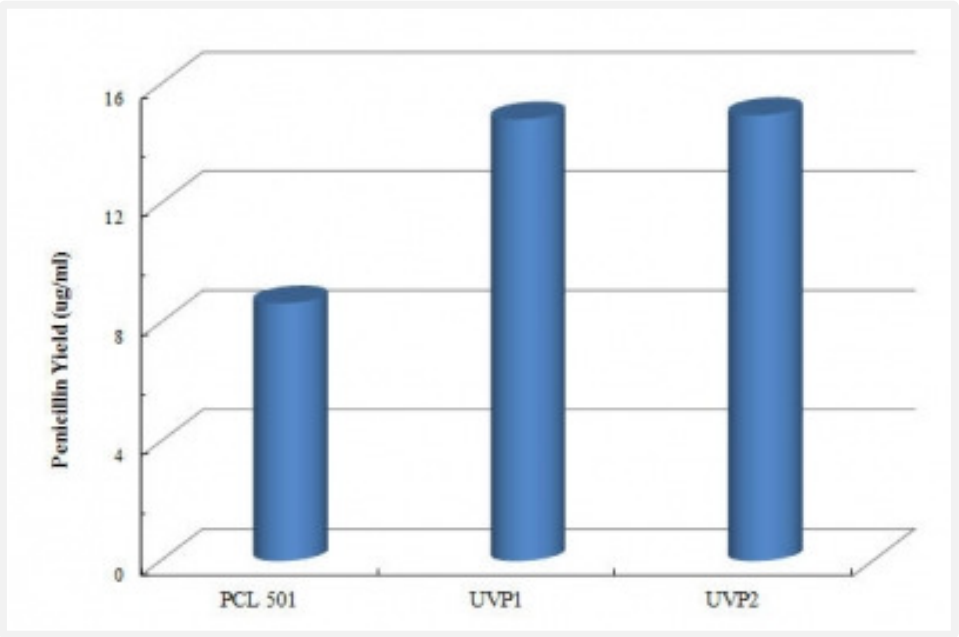


Figure 5

Penicillin yield of 7-day culture of wild strain (PCL501) and mutant strains (UVP1 and UVP2) of Penicillium chrysogenum fermented on sugarcane pulp.



DISCUSSION

The strain of P. chrysogenum (PCL 501) thrives on refined sugars as well as the agro-wastes and utilizes them as fermentation substrates for the production of penicillin antibiotics. The results showed that the fungal growth was profoundly affected by the fermentation substrates. Cassava shavings and corncob were the best substrates for the growth of the fungus. Growth was significantly ($P<0.001$) higher on the two agro-wastes than on the refined sugars (glucose and lactose). The fungal growth on sugarcane pulp, however, did not statistically differ from that on the refined sugars. These are positive indicators that the agro-wastes are suitable substrates for the growth of P. chrysogenum.

The peak periods of mycelia weight and protein yield also varied with the fermentation substrates. In all the substrates, best growth period was between 3 and 9 days; there was rapid decline in growth from 12th day of fermentation. It is interesting to note that the period of peak mycelia growth did not correspond to the time of peak protein yield. This suggests that growth rate is not necessarily a function of protein secretion. The protein yield may be a reflection of the complexity of the fermentation substrate. Protein yield from more complex agro-wastes, cassava shavings, corncob and sawdust was statistically ($p<0.05$) higher than that from sugarcane pulp and the refined sugars. These substrates contain secondary cell wall polymers such as cellulose, hemicelluloses and lignin which need to be hydrolyzed into simpler sugars by the organism21. The enzymes (proteins) required for the hydrolysis of the macromolecules are synthesized by the fungi and secreted into the culture medium; this may account for the higher protein yield10,11,12.
Penicillin production by the fungus varied with the fermentation substrates; it was not however a function of mycelia growth. It is interesting to note that penicillin production was highest in media supplemented with sugarcane pulp, followed by glucose, cassava shavings, corncob, lactose and sawdust, in that order. Penicillin yield from media containing sugarcane pulp was significantly ($p<0.05$) higher than that obtained from glucose- and lactose- containing media. Cassava shavings and corncob were also found to be better substrates for penicillin production than lactose. In our earlier work, culture extracts of P. chrysogenum from media containing sugarcane pulp and cassava shavings exerted better antibacterial activity against clinical isolates of E. coli and B. subtilis than culture extracts from glucose- and lactose- containing media (10).
UV irradiation is a classical technique used for genetic modification17. It had been used in the 1950s to produce high penicillin yielding P. chrysogenum Q-176 mutant (7). The strains, UVP1 and UVP2, were obtained after UV irradiation of P. chrysogenum (PCL 501) for 20 and 25 minutes respectively and had a survival rate of 1%; they were therefore considered mutant strains of the wild type. The mutant strains yielded higher levels of penicillin, 70% more than the parent strain. Thus, the UV induced modification was considered effective. UV irradiation has been shown to have mutational effect on P. chrysogenum with significant impact on antibiotic production3. The method of UV irradiation was also used to enhance lipase production by mutation induced Aspergillus japonicas17.
In conclusion, two hyper penicillin producing mutant strains of P. chrysogenum, UVP1 and UVP2, were obtained by mutating wild strain of P. chrysogenum (PCL 501) with UV irradiation. Residual plant waste materials in urban refuse can serve as cheap carbon and energy sources for growth and penicillin production by P. chrysogenum. Sugarcane pulp was found to be a better alternative substrate for penicillin production than refined sugars (glucose and lactose) by the strain of P. chrysogenum (PCL 501). Cheaper and more effective penicillin antibiotics could be obtained by fermenting the UV induced mutant strains of P. chrysogenum on the agro-wastes. The use of agro-wastes as fermentation substrates for the production of penicillin by the UV induced mutant strains of P. chrysogenum is not only cost effective and sustainable but also an environmentally friendly approach to natural penicillin production.

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