Penicillin Production from Transformed Protoplast of Penicillium chrysogenum by Fermentation

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Abstract
The Protoplast was prepared from the Penicillium chrysogenum and confirmed by microscopic observation and strains from the Penicillium chrysogenum were intralinfused and protoplast was regenerated. The Penicillin was produced by fermentation method. The DNA was isolated from the Sclerotium rolfsii and it was characterized by electrophoresis and Protoplast of Penicillium chrysogenum was transformed with DNA of Sclerotium rolfsii. Penicillin was produced from transformed protoplast of Penicillium chrysogenum and at last HPLC was used to quantify the penicillin. Penicilllin was isolated and purified and injected into experimental animals, where it was found not only to cure infections but also to possess incredibly low toxicity for the animals. This fact ushered into being the age of antibiotic chemotherapy, and an intense search for similar antimicrobial agents of low toxicity to animals that might prove useful in the treatment of infectious disease. Penicillium and Cephalosporium molds produce beta-lactam antibiotics such as penicillin and cephalosporin and their relatives. They also produce the base molecule for development of semisynthetic beta-lactam antibiotics, such as amoxicillin and ampicillin. Beta-lactams are used to treat about one-third of outpatients with bacterial infections.

Keywords: Protoplast; Penicillium chrysogenum; Sclerotium rolfsii; Penicillin and Chemotherapeutic agents

Introduction
Most microbiologists distinguish two groups of antimicrobial agents used in the treatment of infectious disease: antibiotics, which are natural substances produced by certain groups of microorganisms, and chemotherapeutic agents, which are chemically synthesized. A hybrid substance is a semisynthetic antibiotic, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties. Furthermore, some antimicrobial compounds, originally discovered as products of microorganisms, can be synthesized entirely by chemical means. In the medical and pharmaceutical worlds, all of these antimicrobial agents used in the treatment of disease are referred to as antibiotics, interpreting the word literally. Chemotherapeutic agents are chemical substances used for the treatment of infectious diseases or diseases caused by the proliferation of malignant cells. Some antibiotics are prepared synthetically, but most of them are prepared commercially by microbial biosynthesis.

Penicillin is a class of β-lactam antibiotics of related structure with slightly different properties and activities [1]. All penicillin has a common basic nucleus, a fused β-lactam-thiazolidine ring with different side chains which give each its unique properties. Natural penicillin can be prepared as salts of sodium, potassium, procaine, and other bases. Some of the new semisynthetic penicillin may be much more stable than those produced by biosynthesis, the ‘natural penicillins’

Penicillium is known as the blue green mold on bread, fruits, and nuts. It is mostly found on rotted tangerines. It is also used for the production of green and blue mould cheese. Penicillium grows at its peak during the spring and winter, but the mold has no seasonal variation [2]. It is a well – known group of brushed – shaped microorganisms belonging to the fungi mycin family, and has no known sexual state. Penicillium is heterotrophic. This means that it gets nutrients through food digestion. The colonies of Penicillium are rapid growing, flat, filamentous, and velvety, woolly, or cottony in texture. The colonies are initially white and become blue green, gray green, olive gray, yellow or pinkish in time. The plate reverse is usually pale to yellowish.

The name “Penicillin” can be used in reference to a specific member of the Penicillium group. All Penicillin’s possess the basic Penam Skeleton, which has the molecular formula R-C9H11N2O4S, where R is a variable side chain [3]. The chemical structure of Penicillin was determined by Dorothy Crowfoot Hodgkin in the early 1940’s, enabling synthetic production. Penicillin inhibits cell wall synthesis of gram- Positive bacteria. Penicillin causes no ill effects in humans and other animals, except for allergies in about 10% of humans. (Penicillin allergies are usually caused by its binding to serum proteins, causing an IgE-mediated inflammation).

Penicillin acts by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms [4]. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin binding proteins (PBPs). The β – lactam nucleus of the molecule irreversibly binds to (acylates) the Ser403 residue of the PBP active site. This irreversible inhibition of the PBPs prevents the final cross linking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis. Inhibition of PBPs may also lead to the activation of autolytic enzymes in the bacterial cell wall (Scott Williams 2004).

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Protoplast preparation

1 ml of 24-hour fungal culture was taken in an eppendorf tube. Culture was centrifuged at 4000 rpm. The cells were washed once with saline solution. Then equal amount of E buffer containing 20 g of Novozyme 234 was added. To form protoplast, the cells were incubated at room temperature in a shaker at low speed. The sample was checked periodically at every half an hour using microscope for protoplast formation [13]. After 2 hour the incubated cells were separated by centrifugation at 2000rpm for 20 min. The protoplasts were resuspended in the 0.7M NaCl solution. It was stored at 20°C.

Protoplast conformation

Protoplasts were taken in a slide and covered by a cover slip. It was seen under microscope for round protoplasts. 1 drop of water was taken and added with the sample in the slide, seen under microscope for the deformed protoplasts.

Intra fusion of Penicillium chrysogenum

1 ml of the 0.7 M NaCl stored protoplasts of two different strains was taken in an eppendorf tube. It was then added to equal amount of the fusion buffer. The mixture was placed in a shaker for incubation [14].

The sample was periodically checked for the aggregation and fusion of the protoplast using microscope. After 3 hours the fused protoplasts were allowed for regeneration.

Regeneration of the fused protoplast

The fused protoplasts of Penicillium chrysogenum were plated on the regeneration medium. The regeneration medium composed of YPD agar and 0.7 M NaCl. It was then incubated at 30°C for 24 hours.

Screening of fused cells

The regenerated colonies were scratched and dissolved in 1ml of distilled water in separate tubes. Each sample was spread plated in the individual plates. After the colonies are started to grow the plates were spreaded with the Gram-positive bacterial culture. After few days the zone of inhibition were seen around the colonies [14]. By measuring the diameter of the colonies and the zone of inhibition the good colonies were selected for shake flask production.

Fermentation of penicillin

Screened cells from fusion are tested for penicillin production by cultivating them first in seed medium to get a good growth and then with fermentation medium to check the yield. The colonies in the screening medium are scraped using the loop. It was then suspended to the sterilized distilled water [15]. The suspended cells were inoculated in to the seed medium. For 45 hours the culture was incubated in a shaker at 30°C. 225µl of seed culture/50ml of fermentation medium was inoculated. For 60 hours the culture was incubated in a shake at 30°C. The microscopic view of Penicillium was shown in Figure 1.

DNA isolation from Sclerotium rolfsii (Using CTAB method)

10 ml of the culture was taken in a tube. It was then centrifuged at 5000 rpm for 10 min. The pellet was resuspended with 2 ml of the Enzyme buffer containing Novoyme 234. It was then incubated for 2 hours in room temperature [2]. Using a syringe the cells were sucked and pierced back into the tube for several times to disrupt the cells. Equal amount of extraction buffer was added the tube and incubated at 65°C for 30 min in water bath. It was then cooled at room temperature. Equal volume of chloroform: Isoamyl
To prepare samples for μμμμμμμμμμ: 10ml of the fermented broth incubated for 24 hours for regeneration of the protoplast. Liquid culture medium was added progressively. The sample was allowed to resuspend over night at 4°C.

Electrophoresis of DNA (Julie Wolf)

Gel preparation and electrophoresis: 100 ml of 0.7% Agarose solution was prepared in a glass beaker. Micro waved on a hot plate until Agarose is dissolved and solution is clear. Solution was allowed to cool to approximately 50°C before pouring into a gel casting. Gel tray was prepared by sealing ends with tape [16]. Comb was placed in gel tray approximately 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray.

Gel solution was poured into tray to a depth of approximately 5 mm. Gel was allowed to solidify (approximately 20 min at room temperature). To run the gel, the comb was gently removed and placed the tray in electrophoresis chamber, and covered with electrophoresis buffer.

Sample preparation and loading: To prepare samples for electrophoresis, every 5μl of DNA solution 1μl of 6X gel loading Dye was added and mixed well. 5 to 12μl of DNA per well was loaded. Electrophoresed at 50-150V until the dye markers had migrated an appropriate distance.

Staining of agarose gel: The gel was removed from the casting. In a 0.5μg/ml Ethidium Bromide solution the gel was completely submerged. The gel was incubated in Ethidium Bromide for an appropriate amount of time to properly stain the DNA. The gel was extensively washed in distilled water.

Elution of DNA from agarose gel (Steve Hahn)

Glass wool spin column preparation. Using a clean needle, a hole was made into the end of a 650μl micro centrifuge tube from the inside out and then the tube cap was cut off. The glass wool plug was placed in the bottom of the 650μl tube. The tube with the glass wool was placed in a 1.7ml micro centrifuge tube [17]. Elution of the DNA from Agarose gel fragments. The fragment of interest was excised with a clean razor blade. After removing the excess liquid with a tissue the Agarose fragment was placed in the spin column. The tube was centrifuged at 3000rpm for no more than 45 sec to elute the DNA. Using the transilluminator, elute in the bottom of the outer tube was checked for the presence of DNA [18]. The DNA fragments were further purified by Ethanol precipitation.

PEG-mediated transformation of protoplast

The protoplast was isolated from the penicillium chrysogenum and shown in Figure 2. The protoplast was resuspended in 1 ml of MM solution. 30μl of the sclerotium rolfsii DNA was taken in a 14 ml centrifuge tube. 300μl of the protoplast was added with DNA in the tube and mixed well. 300μl of PEG solution was added at last to the tube.

The tube was heat shocked for 5 min at 45°C. Then it was cooled to room temperature with gentle mixing. 10 ml of the protoplast liquid culture medium was added progressively. The sample was incubated for 24 hours for regeneration of the protoplast.

Screening of transformed cells

Centrifuge the regenerated sample at 4000rpm for 10 min. The pellet was resuspended in 1 ml of the distilled water. The resuspended sample was plated in the plate containing screening medium. Plates were incubated for few days until the colonies were appeared. Then the colonies were picked and used for shake flask production. The fused protoplast was shown in Figure 3.

Fermentation of penicillin

Screened transformed cells were inoculated to the seed medium containing cellulose as carbon source. For 45 hours the culture was incubated in a shaker at 30°C. 225μl of seed culture/50ml of fermentation medium containing cellulose as carbon source were inoculated. For 60 hours the culture was incubated in a shake at 30°C.

Quantification of penicillin using HPLC

Standard preparation and calibration: About 200mg of penicillin v was dissolved in 100ml of distilled water. 100 mg of phenoxy acetic acid was dissolved in 500ml of distilled water. 5ml of the penicillin v and 5ml of phenoxy acetic acid were mixed well. From the mixture 20μl of the sample was injected to the column. The chromatogram reading was noted and taken as standard.

Sample preparation and analysis: 10ml of the fermented broth was taken in a centrifuge tube. Sample was centrifuged at 4000rpm. The supernatant was taken in a separate tube leaving the pellet. The whole broth was weighed and transferred to a STD volumetric flask.

It shacked and filtered through wattman 1 filter paper. 20μl of the filtrate was injected to the system containing Hypresil ODS(Octa Decyl Silone) 25cmx 4.6mm column with CH3CN:0.1M KH2PO4 (20:80) as mobile phase at a flow rate at 1.4ml/min. The sample chromatogram was analyzed at 225 nm and the concentration was noted.

alcohol (24:1) was added. Mixed well and centrifuged for 10 min at 10000rpm. The aqueous supernatant was transferred to new tube. Equal volume of isopropanol was added. High molecular weight DNA was spooled out upon mixing with glass rod [2]. The supernatant was discarded and rinsed the spooled DNA with 70% Ethanol. 1 ml of TE buffer containing 20μg/ml R nase was added. The sample was allowed to resuspend over night at 4°C.
and affects the enzyme action during the protoplast preparation. Various chemicals at various concentrations were tried and the NaCl at 0.7M gave a large number of protoplasts. Penicillin type antibiotic used to treat staphylococcal infections that are resistant to penicillin. Administered by mouth or injection, it should not be taken with acidic fruits or juices or aged cheese and if taken with alcohol, this drug could cause stomach irritation. It should be used with birth control pills that may impair the efficacy of the contraceptive. Penicillin is the treatment of choice for treating syphilis. The long half-life of azithromycin and its clinical efficacy in vitro against syphilis support its use in treating early syphilis; however, clinical data are currently insufficient to recommend its use. No good evidence indicates that the non-β-lactam antibiotics, which are used as alternatives to penicillin, are clinically effective in syphilis.

References