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Optimization of penicillin G production by *Penicillium chrysogenum*

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ABSTRACT

Microorganisms are a source of many biotechnological products in modern world. Antibiotics are one of the secondary metabolites produced by some fungi and bacteria. The aim of this study was to determine the optimal conditions for penicillin production and extraction from the Penicillium chrysogenum culture filtrates. Two isolates of the fungus were subjected to experiments. Metabolites were detected using TLC and HPLC compared with the penicillin G standard . It was found that, maximum production of penicillin in broth culture media was the interval of 8-6 days after inoculation. Optimal culture conditions was the presence of 3 g yeast extract, 21 g of sucrose per liter ,the optimal temperature conditions between 28-25 ° C with 120 rpm of the Shaker movement. No meaningful differences were observed between two tested isolates.

Key words: Antibiotic, *Penicillium*, Culture media, Optimization, HPLC, TLC

INTRODUCTION

Nowadays, the use of microorganisms has created a huge revolution in all aspects of human's life with numerous studies which have been conducted by scientists in different fields. Since the identification of microorganisms, human are able to control their undesirable activities [1]. In fact, man can use them against their own or take advantage of them in various fields. Currently, we observe the use of microorganisms in production of pharmaceutical products specially antibiotics [2]. Antibiotics are valuable substances. Man has realized the necessity of their use from years and is able to extract them from different source microorganisms [3]. Today, antibiotics have an important role in health of living organisms. Penicillin is a type of antibiotics that achieved from *P.chrysogenum* or *P.notatum* cultures [4]. In normal conditions, the rate of penicillin production is low. But in terms of controlled substances in the culture media and good physical condition, fungus can grow more and the rate of penicillin production will increase, consequently [5]. Penicillin is effective on gram-positive bacteria, but some gram-negative are also affected. Therefore, penicillin has a great value in treating a broad category of infections [6]. Penicillin is the first known antibiotic. Its mode of action is to prevent the production of peptide glycan polymerase enzyme. This causes the bacterial cell to absorb too much water and bursting of the cell will prevent it from working and growth [7].

Discovery of the first antibiotic substance that was obtained accidentally from Fungus *Penicillium notatum* was conducted by Fleming, in 1928. Howard Florey purified this material and succeeded to cure infections by a systemic approach [8]. Over time, antibiotics have been needed to produce more due to the emergence and spread of various diseases. As a result, scientists are trying to produce and discover more antibiotics [9].

[10] did a research, in which the presence of residual antibiotics in animal tissues were examined. They separate and analyzed different beta_ lactam antibiotics, using TLC method. Different antibiotics were identified, using different

systems of various solvents and their isolation rates in the thin layer chromatography and making different colors in a variety of RF, in this method [11].

In another study that was done by P.Z. Wang et al. in 2007, production of penicillin G (benzyl penicillin) was evaluated in fungal culture media. In this study it was shown that, extraction and quality of antibiotic production increases, by using ultrafiltration.
[9] evaluated accumulation effect of Cytrinin on fungal growth and assessed antibiotic activity, using of *P.chrysogenum* 5108 MTCC cultured in PDB.

In this study the antimicrobial activity on different bacteria has been investigated by the disk diffusion method. A combination of chromatographic techniques, ie, thin layer chromatography and column chromatography has been used, in this method. The researchers also took advantage of TLC, UV_Visspectrophotometer, HNMR and ESI_ MS/MS methods.

In a research which were conducted by [12], antimicrobial activity was observed from 200 *Penicillium* isolated from Soils of different regions of Brazil.

In a survey that was performed by [13], survival of *Penicillium chrysogenum* spores in front of UV rays and their production of antibiotics, was investigated. Obtained results showed that, changes in *P. chrysogenum* which was due to mutation, leading to more production of penicillin.

In a survey, antibacterial and antifungal compounds obtained from fungal species which were isolated from agricultural lands in northern Iran was studied [14]. Type of these anti-vital materials was identified by TLC method [15] and was found that these fungi produce Penicilic acid Cytrinin.

Considering the efforts taken for the extraction of antibiotics in the years after identification of this valuable substance; we decided to optimize Penicillin G extraction produced by *Penicillim chrysogenum* in PDB medium. For this purpose, *Penicillium chrysogenum* isolates which are able to produce high efficiency of penicillin G were selected, until different environmental conditions and the amount of material in the growth medium, are investigated.

MATERIALS AND METHODS

Two 5031 and 5037 isolates of *Penicillium chrysogenum* were prepared for this study. They were sub cultured and proliferated in PDB medium. Fungal growth in liquid medium is needed for antibiotics extraction. In order to extract the penicillin produced in this environment, solvents such as chloroform and butyl acetate were used. After the necessary studies to obtain an optimal culture medium for *Penicillium chrysogenum* growth, a medium was prepared with 3 g yeast extract and 21 grams sucrose in one liter of pure distilled water. *Penicillium* grew well in this environment at room temperature. After 5 to 8 days of fungal growth, the medium was contaminated using various dilutions of bacteria to evaluate anti-microbial activity of the fungi. Ability of antibiotic production and anti-microbial activity of fungi were assessed, after culturing them on solid medium in the presence of different bacteria. Fungi with production of secondary metabolites prevent bacterial growth and build inhibition zone around itself. The inhibition zone is a distinct amount for each bacterium.

The amount of antibiotics produced by the fungus was realized by calculating the diameter of the halo [16].

Extraction of antibiotics from culture media

Chloroform and butyl acetate solvents were used, for isolation of antibiotics from fungal medium. For this purpose, environment temperature was reduced to 0-4 ° C, after separation of impurities and mycelia and spores from the medium. PH of medium was brought to about 2. So, in these conditions antibiotics will mix better with the desired solvent in the environment [17, 18]. Then, solvent was added to the medium. When solvent completely combined with the medium, the antibiotic was transferred to the solvent. At this stage solvent and the medium are in different phases and need to be separated [19]. Using a separating funnel, the solvent that was included penicillin, isolated from the medium. For complete separation of the phases, centrifugation was also used [20].

Using TLC for detection of Penicillin G in Fungal medium

Thin layer chromatography (TLC) was used to detect the presence of penicillin in medium.

Silica gel 60HF was used to build TLC plates. After covering a thin layer of silica on the plates and drying them in the oven, they were spotted by samples. Spots were placed on TLC plates using Hamilton syringe. Then, different

solvents were used for loading samples on TLC plates. Solvent was poured in TLC tank for 60 to 90 minutes and they were given time until move [21, 22]. Then, using different wavelengths of UV, colors and RF produced by each of them was compared with standard of penicillin G.

Using HPLC to determine the amount of penicillin produced in fungi medium

Samples of both isolates were spotted with standard of penicillin G on TLC plates after filtration. Then, they were put in solvent. Samples started to move on TLC plates. The spots were scrape using a sterile blade, when the same color with the standard penicillin G and RF standards under UV light, were observed. Then, they were dissolved in methanol, separately. Methanol containing penicillin G was isolated, using centrifugation. Then, the solvent was filtered for injection into the HPLC system, in order to determine the amount of Penicillin G production by two *Penicillium* isolates. In next stage, Penicillin production rate in different environmental conditions and with changes in medium components, were measured, using HPLC. The results of these shots were used to determine the optimum conditions for penicillin production.

RESULTS AND DISCUSSION

Fungal growth in liquid base used in this study showed that two strains used are able to grow well in this medium. After fifth day of cultivation, the fungus is able to produce substances inhibiting growth of bacteria. This growth inhibitory effect was mostly on gram-positive bacteria. Anti-critical composition in this medium was determined penicillin G type, using TLC method and compared with standard penicillin G. Comparison of chromatogram obtained from injection of extracted sample of fungus medium in eighth day, to HPLC showed that retention time for injected standard sample and Penicillin extracted from *Penicillium* medium growing on the eighth day, is the same, in equal conditions for HPLC. This result proved presence of Penicillin G in obtained extractions. As seen in the following figure, chromatograms obtained from both injections are consistent with each other, completely.

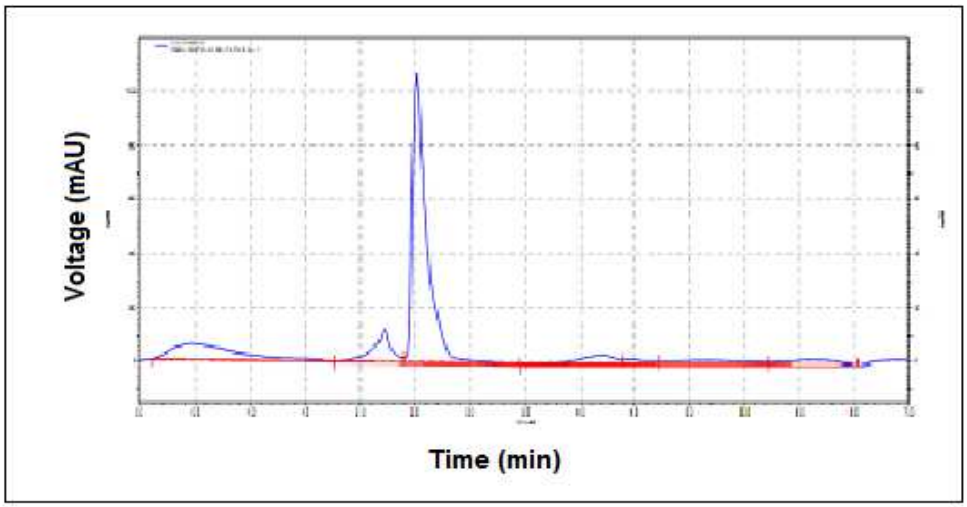

Figure 1) Chromatogram resulting from injection of penicillin G standard to HPLC system

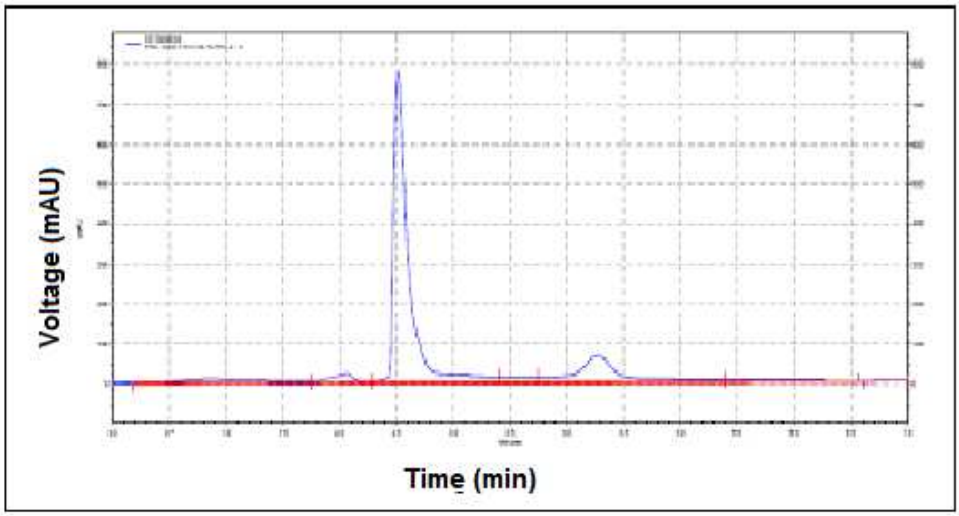

Figure 2) Chromatogram obtained from extraction of fungal medium of *P.chrysogenum*, isolate 5031

In comparison of different methods of extraction using different solvents, the highest rate of penicillin extraction was by using butyl acetate solvent and a three-step method.

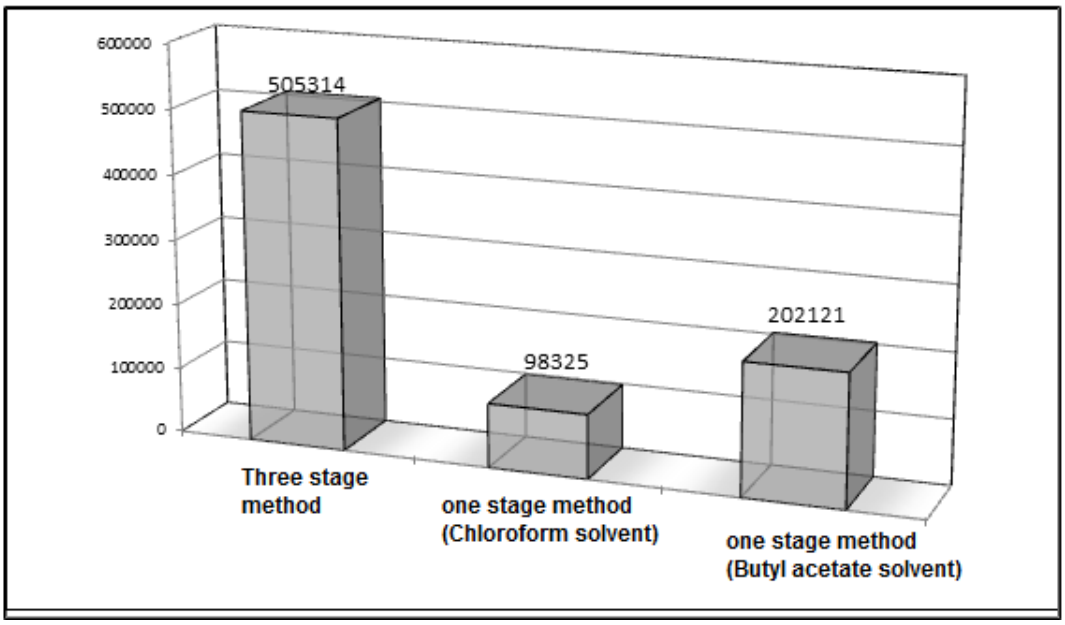

Figure 3) Comparison of obtained results from different extraction methods

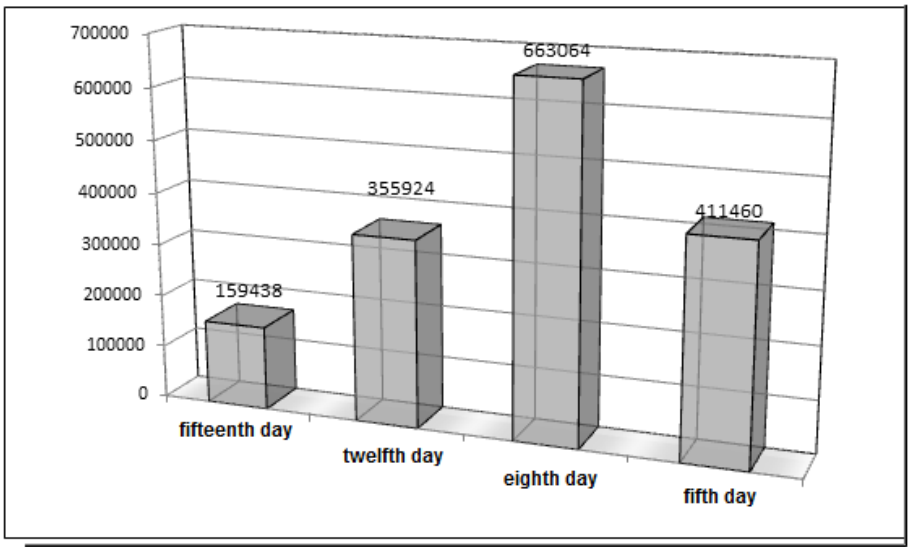

Figure 4) Comparison of obtained results from injection of extractions with different duration of cultures

Highest rate of antibiotics produced by *P. chrysogenum* was from 6-8 days after its cultivation. Before the fifth day, the fungus can't produce growth inhibitor substances. So, duration of fungal cultivation is effective on production of penicillin. According to the results of Chromatograms obtained from injection of samples which were extracted in different temperature conditions, temperature variations cause changes in the rate of penicillin G production in *P. chrysogenum* medium. The highest rate of production was in 28˚C. The production rate of penicillin was reduced significantly at temperatures above 30 ° C.

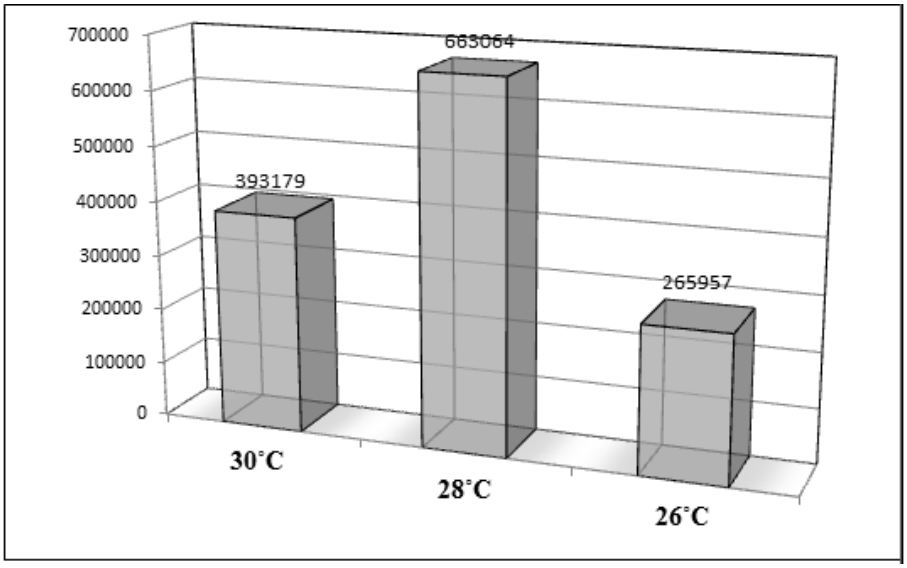

Figure 5) Comparison of obtained results from injection of extractions in different temperature conditions

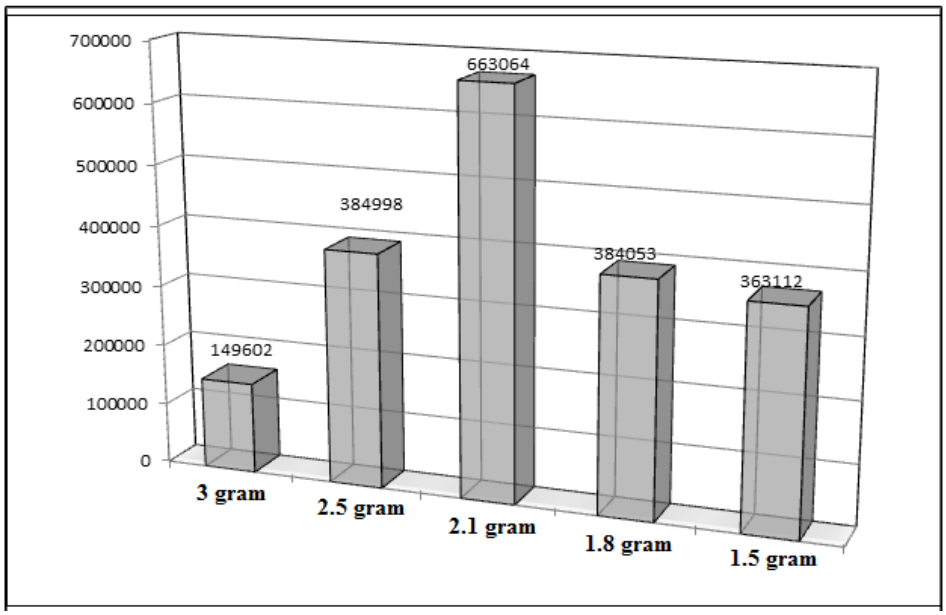

Figure 6) Comparison of obtained results from injection of extractions from 5031 isolate media with different levels of sucrose

After comparing the results of chromatograms which were obtained from injections in different conditions, optimum values of medium components was determined as: 3 g yeast extract and 2 g of sucrose in one liter of pure distilled water and the optimal amount of Shaker movement was set at 120 rpm.

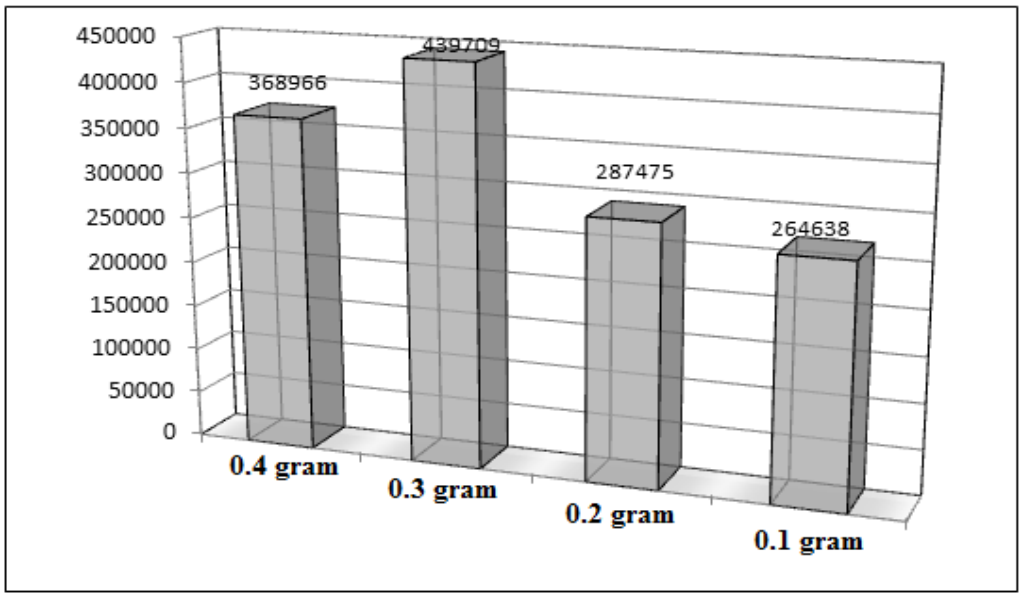

Figure 7) Comparison of obtained results from injection of extractions from isolate 5031 media with different levels of yeast extract

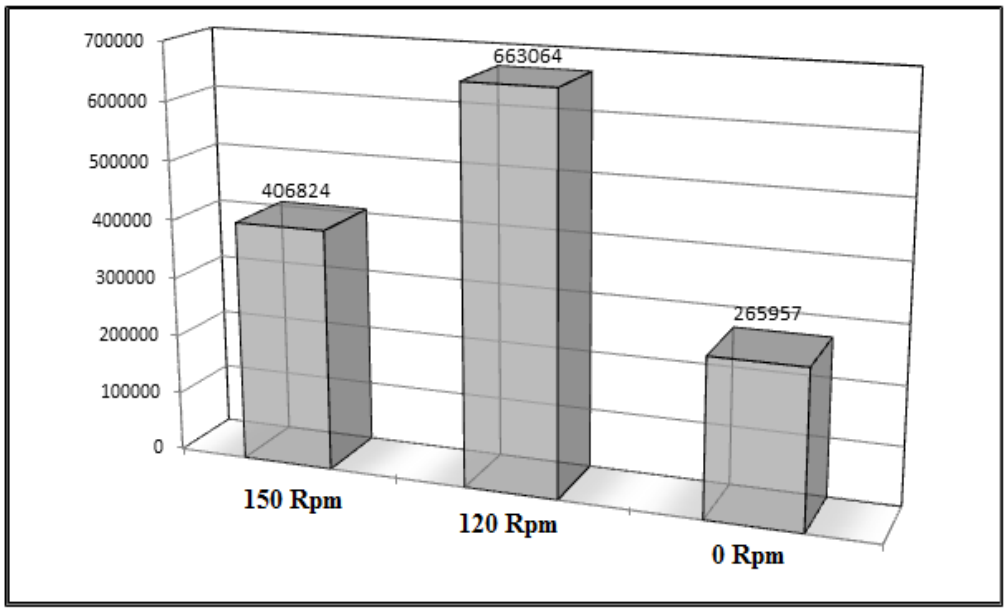

Figure 8) Comparison of obtained results from injection of extractions from isolate 5031 media Located on different rotates of Shaker

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