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Maximum production of penicillin G culture media

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Abstract
Penicillin G is a natural antibiotic with secondary metabolites properties. Since the penicillin is the most widely used antibiotic in the world, efforts in the pharmaceutical industries is focused on using the ingredients which are inexpensive and easy to access but have higher yeild of production. In this study Penicillin G was produced by Penicillium chrysogenom PTCC No. 5033. The total process was formed of three stages of sporulation, preparation of inoculum -pre-fermentation- and fermentation. To determine the optimal amount of carbon, nitrogen and phenyl acetic acid we used experimental design method, Taguchi, by using Qualitek software. The carbon source was wheat starch and rice bran was our nitrogen source.

Keywords: fermentation, penicillin G, secondary metabolite, experimental design method of Taguchi.

Introduction

Penicillin G $S_4O_2N_{18}H_1$ is from B-lactam family with secondary metabolites properties. Penicillin G is a natural penicillin which extracellularly secret from penicillium to the media. Penicillin can be obtained directly from penicillium culture media by extraction. The main structure of penicillin G is 6-amino-penicillianic acid(6APA) which is containing of tiazolidin cycle and B-lactam cycle in which the 6-amino location can be replaced by any kind of acyls (Nasution U. 2007).

Since all bacterias have cell wall which contain peptidoglycan compounds, penicillin can affect on their cell walls and by destroying them, it cause bacterias death. Penicillin has tremendous impact on gram positive coccies such as streptococcus and pneumococcus, but it is sensitive to penicillinase enzyme so it is not effective for staphylococcus species. Penicillin is a secondary metabolite which will be formed near or at the end of exponential phase of growth. Forming of penicillin G depends on medium components, and by choosing the appropriate specie and changing the composition of the culture media can increase production efficiency. Penicillin G was produced for the first time in 1944 by using aerobic fermentation in pharmaceutical industry, and so the antibiotic era began (Ahmad S, Hom D.I. 2004, Harrison, K. 2006). In this study we focused on optimization of amounts of carbon and nitrogen sources and phenyl acetic acid for increasing the production of penicillin G. We used Taguchi method and Qualitek-4 software for designation and implementation of experiments.

Materials and Methods

(a) Materials and micro organism

All materials and culture medias were obtained from Merck company of Germany. Penicillin G, potassium salt which was used as standard sample was prepared from Jaber-ebn-Hayan pharmaceutical company (Tehran, Iran).

Lyophilized vials of penicillium chrysogenum PTCC No. 5033 was bought from scientific and industrial research organization of Iran (Tehran, Iran).

Sporulation medium: To grow and produce spores strains were cultured on plates containing malt extract agar medium which ingredients were as follow: 20 gr/l agar- Art NO 101613, 20 gr/l glucose- Art NO108342, 20 gr/l malt extract agar - Art NO105398 and 1 gr/l peptone - Art NO107224, for 72 hours. The formulation of this culture media was obtained from the web page of scientific and industrial research organization of Iran[http://www.ptcc.irost.org]

Inoculum: First of all we prepared 40 gr/l S.D.B culture media in a flask Meyer and sterilized it, then we transferred the grown strains into this medium. At last we let it be on stirrer with 250 rpm in 25·C for 24 hours.

Main culture media: The ingredients of main culture media in 1000ml were as follow: 15 gr/l Lactose Art NO 107657, 5 gr/l sucrose Art NO 107687, 23 gr/l sodium acetate Art NO 106268, 2 gr/l phenyl acetic acid, 3 gr/l ammonium acetate Art NO 101115, 10 gr/l sodium citrate Art NO 106431, 5 gr/l $(NH_4)_2SO_4$ Art NO 101217, 1gr/l KH_2PO_4 Art NO 104871, 1 gr/l Na_2HPO_4 Art NO 106559, 0.05 gr/l $FeSO_4.7H_2O$ Art NO 103965, 0.5 gr/l $MgSO_4.7H_2O$ Art NO 102786, 0.01 gr/l $ZnSO_4.7H_2O$ Art NO 108883, 0.01 gr/l $CuSO_4.5H_2O$ Art NO 102790, 0.01 gr/l $MnCl_2$. $4H_2O$ Art NO 105927, 0.005 gr/l $.7H_2O$ $CoSO_4$ Art NO 102546 and 0.001 gr/l NaCl Art NO 116224 (Martinez-Blanco, H., Reglero, A., *et al.* 1992).

We used wheat starch and rice bran in respect as our carbon source and as our nitrogen source. Each of carbon source, nitrogen source and phenyl acetic acid(PAA) were used in two levels that for wheat starch were 20 gr/l and 10 gr/l, for rice bran were 1.66gr/l and 1 gr/l and for PAA were 2.5 gr/l and 1 gr/l. After preparing the main culture media and sterilizing it, inoculum was added to this medium and were stored in 25 ·C, 250 rpm, pH=6 and for 120 hours. After that mycelium were separated from the fermentation medium and the remaining broth was containing penicillin G which should be extracted.

(b) Extraction of penicillin G

To prevent damage to the Penicillin by Penicillium enzyme the medium is cooled to the temperature of 2-4 and pH was

stabilized to 2.5-2. We used butyl acetate (BA) as non-organic solvent. The broth was mixed with 2 to 1 ratio to BA, and the resulting emulsion was centrifuged for 15 minutes at 1000 rpm so that penicillin transferred from aqueous phase to the organic phase.

(c) Identifying penicillin G

Identification of penicillin G and measurement of its production is done by HPLC method according to instructions of USP31. This method containing of three solutions an extracted broth, mobile phase and the standard solution. The mobile phase solution is made of phosphate buffer and acetonitrile with a ratio of 4 to1 and the standard solution is working standard Pen G 1587in/ml from Jaber-ebn-hayan pharmaceutical company. The HPLC conditions were: column type= L1, wave length = 225 nm, mobile phase flow=1ml/min, injection rate=10 m/lit, pump pressure=16-17 psi, type of detector=UV. We also used HPLC to confirm the reliability of the results of bioassay and lethal effects of penicillin G. For this purpose, we used Mueller Hinton agar medium, staphylococcus aureus was isolated and cultured on this medium. Then we fill the wholes we have made in the center of those plates with extracted samples and standard sample. We let the plates be in room temperature for 2-3 hours so extracted samples and standard sample would be absorbed into culture media. In this level we left the plates in incubator in 35·C for 48 hours. The appeared inhibition zone indicated production of Pen G and inhibition zone diameter indicates lethal effects of penicillin G. These results were consistent with the results of HPLC and confirm them·

Spectrophotometry: To measure Penicillin G production in the combination work we have done, we used spectrophotometry. We used three standard solution with concentrations of 0.05, 0.06 and 0.07 mg/4ml from penicillin 600000 iu. Then the operations of spectroscopy were done on these standard samples, and the best wave length (253nm) for spectroscopy of penicillin from scanning chart was obtain so that wave length was used for spectroscopy of the rest of the samples. After calibration of spectroscopy instrument with BA by which we dilute our samples, we did the spectroscopy operation for each of diluted samples (M. Herriott, Roger. 1946, Yves R, Chalus P, Maurer L, Nadine J. 2007)

(d) Test designing by Taguchi method

Since optimization of carbon source, nitrogen source and phenyl acetic acid was the most important issue in this work, Taguchi test designing method was utilized here for optimization. This design was done by Quali-Tek software and three factors in two levels were examined that resulted in orthogonal array L8 (table 1)**.** Eight experiments were designed by these data and software. Then each of experiments were done three times and by means of spectroscopy method the results were processed to give us the amount of penicillin production. The results (amount of penicillin production) were put in Quali-Tek and by comparing the results the best permutation which had higher amount of penicillin G production was chosen.

Results and Discussion

(a) HPLC results

For each experiments of protein source and phenyl acetic acid which were repeated for three times the HPLC operation were done and the results were as follow:

According to HPLC results for protein source between concentrations of 1.66 g/l, 3 g/l and 5 g/l penicillin G production was higher in concentration of 3 g/l rice bran with area under the peak of 274322 (fig 1)

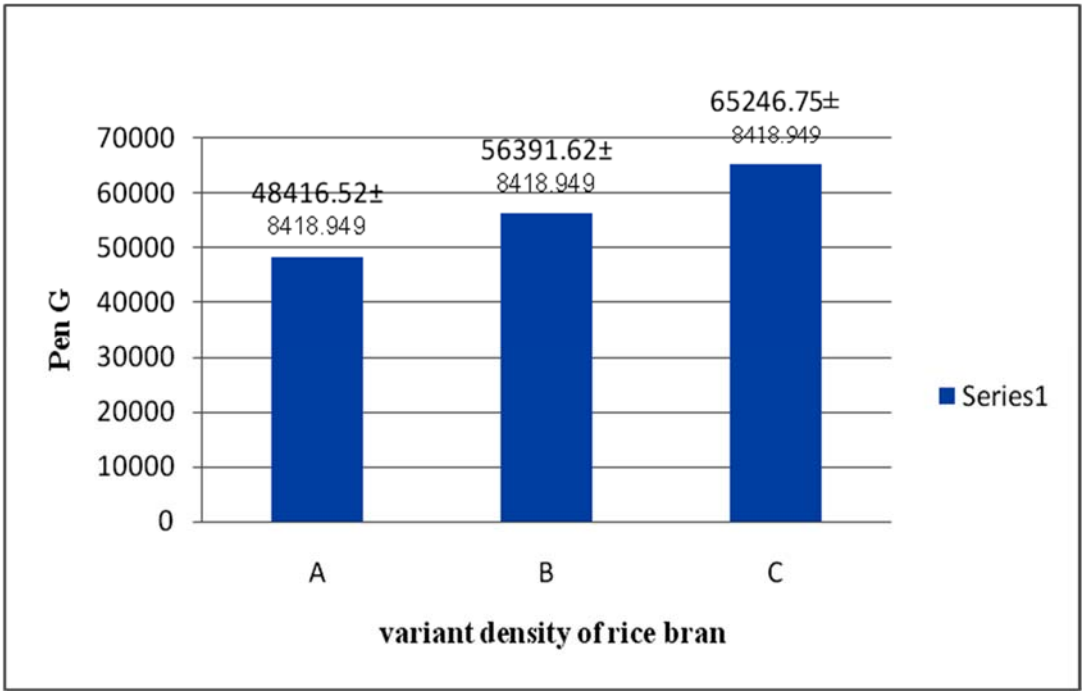

Fig (1): the bar graph of penicillin G production in different concentrations of protein source: (A) media with 1.66g/l rice bran. (B) media with 3g/l rice bran. (C) media with 5g/l rice bran

Table 1: Comparison between standard peak and media with 3g/l rice bran peak

sample	(Ret. Time) peak time
standard	12.50
3g/l rice bran	12.57

About phenyl acetic acid, the addition of this material needs an accurate diet because not only it is very expensive but also it has toxic effects on microorganism so an optimal amount should be defined for it. According to above description and our observations, 1g/l PAA was not enough for media and microorganism and the penicillin G production decreased. Also with 2.5g/l PAA penicillin G production decreased, so we tested two levels between 1g/l-2.5g/l for PAA which were 1.5g/l and 1.85 g/l PAA. According to HPLC results penicillin G production in the media with 1.5g/l PAA was higher than penicillin G production in the media with 1.85g/l PAA and this difference was remarkable and was about 1700 iu (fig 3)

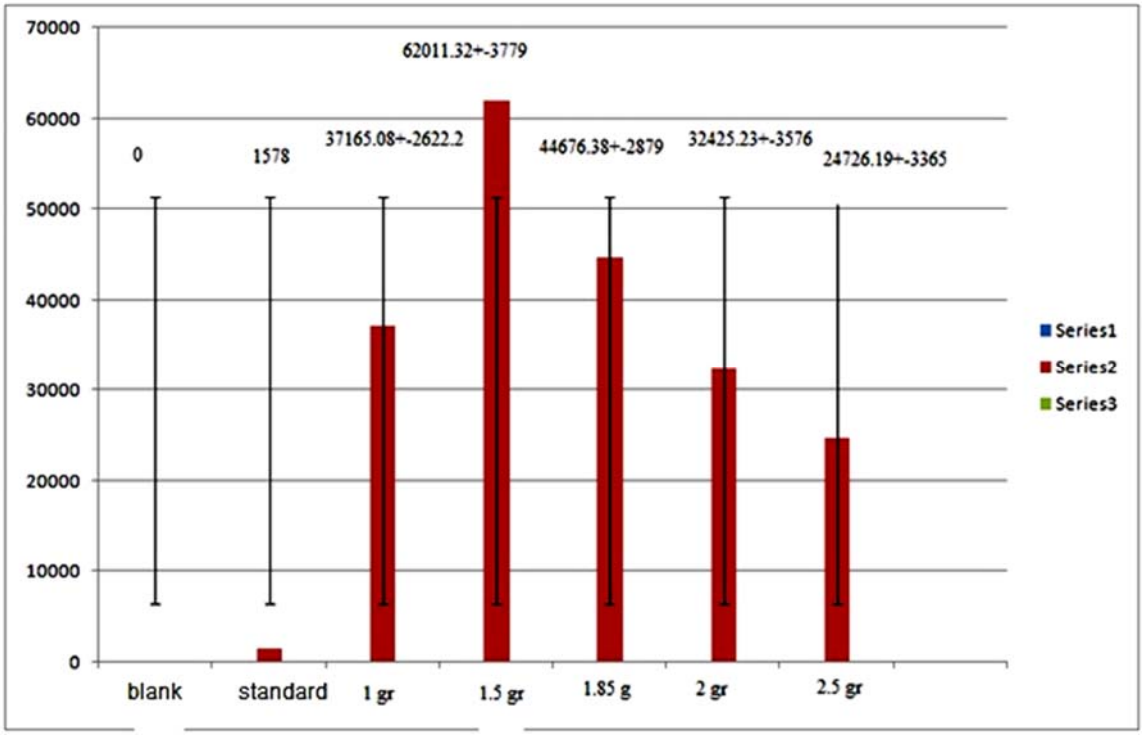

Fig (2): penicillin production in different concentrations of PAA

Table 2: comparison between standard peak and media with 1.5g/l PAA and media with 1.85g/l PAA

sample	(Ret. Time) peak time
standard	12.50
1.5 g/l	12.616
1.85 g/l	12.081

(b) Bioassay results

The bioassay test was done to determine the antibiotic power of produced penicillin G in different culture medias. the inhibitory zone for penicillin G produced in media with 3g/l rice bran had bigger diameter than inhibitory zone of penicillin G produced in medias with 1.66g/l and 5g/l rice bran, and between these two the inhibitory zone of penicillin G produced in media with 5g/l has bigger diameter than penicillin G produced in media containing 1.66 g/l rice bran.

Also according to observation for PAA the diameter of inhibitory zone for penicillin G produced in media containing 1.5g/l PAA was the biggest diameter after that the diameter of inhibitory zone of penicillin G produced in media with 1.85g/l PAA is bigger than inhibitory zone diameter of penicillin G produced in culture medias containing 2g/l, 1g/l and 2.5g/l PAA.

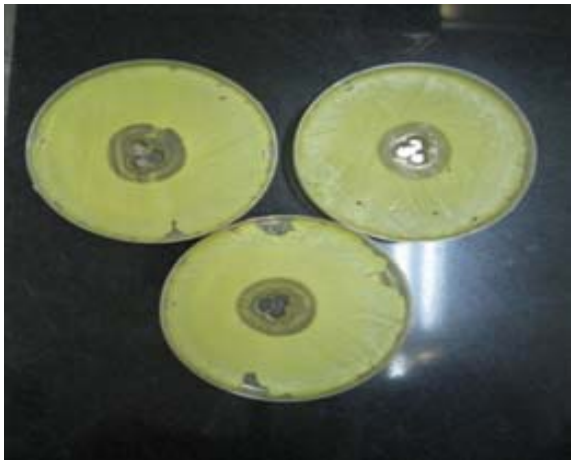

Fig (3): inhibitory zone of penicillin G

(c) Quali-Tek results

By giving the results to the Q-Tek software, it gave us the best permutation of these experiments. To verify the result of this method we can compare it with the results of spectroscopy and experimental tests, in case of matching accuracy of the results can be accepted with confidence.

Table 3: Taguchi test designing by Quali-Tek. The amount of penicillin G in results columns were obtained from spectroscopy method.

Test no	Wheat starch	Rice bran	PAA	Pen G. round1	Pen G. round2	mean
1	10	5	1	66166.66	65166.66	65666.66
2	20	5	2.5	63000	64166.66	63583.33
3	20	1.66	2.5	64333.33	63583.33	63958.33
4	20	1.66	1	63916.66	65283.33	64599.99
5	10	5	2.5	67666.66	66666.66	67166.66
6	20	5	1	68333.33	68833.33	68583.33
7	10	1.66	1	65900	67050	66457
8	10	1.66	2.5	65316.66	65883.33	65599.99

Table 4: Results that were put in Quali-Tek

	Sample# 1	Sample# 2	Sample# 3	Sample# 4	Sample# 5	Sample# 6
Trial# 1	66166.66	65166.66				
Trial# 2	63000	64166.66				
Trial# 3	64333.33	63583.33				
Trial# 4	63916.66	65283.33				
Trial# 5	67666.66	66666.66				
Trial# 6	68333.33	68833.33				
Trial# 7	65900	67050				
Trial# 8	65316.66	65883.33				

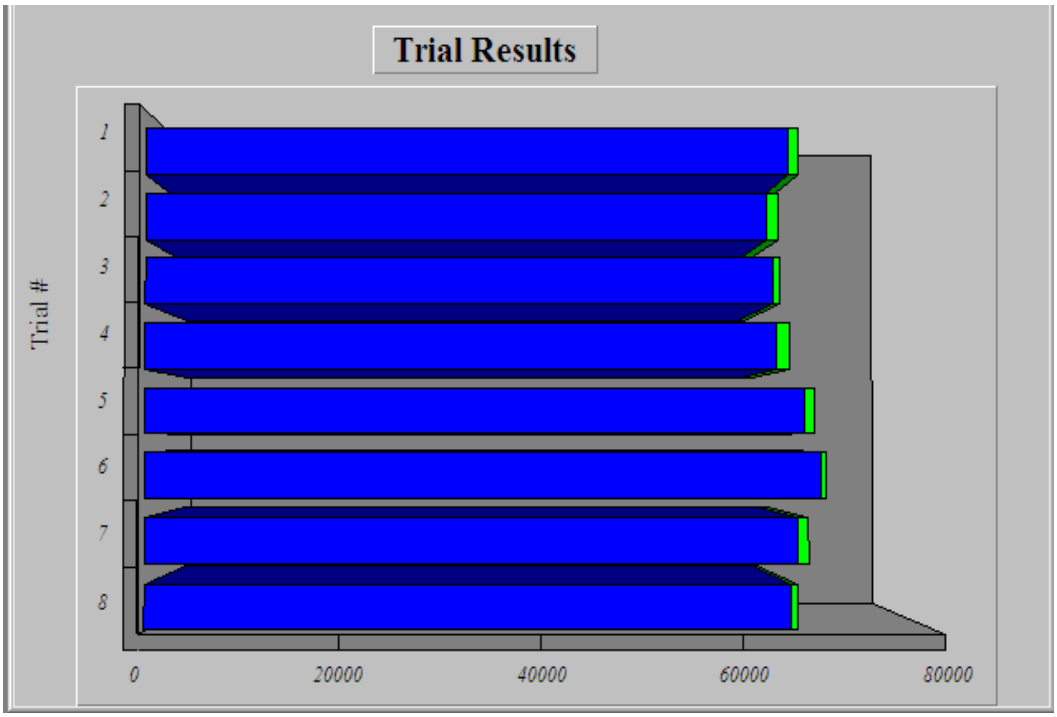

Fig (4): Bar graph of penicillin production per trial.

Quali-Tek chose permutation of 2-2-1 (level number) which was in trial 6 and was similar to the results of spectroscopy. It means that 20 gr/l wheat starch, 5 gr/l rice bran and 1 gr/l phenyl acetic acid is the best composition for producing penicillin G. According to results of spectroscopy the maximum production of penicillin G was occurred in trial 6 and the average of production between two replicate of this test is 68583.33 iu/ml.

Table 4: Efficiency and optimal conditions

	Factors	***Level Desc.***	***Level***	***Contribution***
1	Starch	20 g/lit	2	1252/082
2	Rice bran	5 g/lit	2	112/496
3	PAA	1 g/lit	1	627/082

Conclusions

According to the obtained results the optimum amount of protein source and phenyl acetic acid for high production of penicillin G are in respect 3 gr/l and 1.5 gr/l,1.85 g/l. Although when the amount of the precursor (PAA) is 1.5 gr/l the production of Pen G is higher than when it is 1.85 gr/l.

The following reasons can be cited to justify such a conclusion:

1- Compared to Soya and ammonium acetate which were used in index medium, rice bran is cheaper. Also the amount of phenyl acetic acid became lower than its amount in index medium, so because of its high price its more desirable to lowering its amount without lowering the production of Pen G.
2- Rice bran has higher productivity in comparison with ammonium acetate and wheat bran. Although its penicillin production is a little lower than culture media containing soya, we chose rice bran because it is a native nitrogen source and also because of its lower price.
3- Rice bran concentration is also effect the penicillin production. On one hand lower concentrations cause the lack of nitrogen source and as a result penicillin production would become lower, on the other hand higher concentrations (5gr/l) also lead to lower production. Because in this situation the microorganism does not need to fight for survival. Indeed higher concentrations of nitrogen source can cause inhibitory.
4- The toxicity of phenyl acetic acid in 1.5 gr/l is not dangerous for microorganism and also its amount is enough and appropriate for producing penicillin G. at the same time, if we increase the amount of PAA not only we cannot increase the production of Pen G, but also by increasing the toxicity effect of precursor on microorganism and fermentation culture media the production of penicillin G will decrease.

Generally, since the price of ingredients of culture media in fermentation processes is 20-30% of the total cost, and according to our main goal that was optimization of production of penicillin G by using native and cheaper nitrogen source and optimum amount of PAA, 3 gr/l rice bran is the optimum nitrogen source and 1.5 gr/l phenyl acetic acid is the best amount for achieving this goal. Using this optimized medium the amount of production increased by 53% or 30510.494 iu/ml in comparison with index medium ,and by decreasing the cost of culture media the total cost decreased by 10-15%. Also compared to similar work that was done by soybean in 2009 and produced 66823 iu/ml Pen G, yield have increased to 67399.98 iu/ml.

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