



Synthesis of Antibiotic Penicillin-G Enzymatically by *Penicillium chrysogenum*

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Received: 20 October 2018;

Accepted: 2 July 2019;

Published online: 30 August 2019;

AJC-19553

Penicillin-G antibiotic was used as the basic ingredient of making antibiotic type β -lactam such as tetracycline, amoxicillin, ampicillin and other antibiotics. Penicillin-G was split into 6-amino penicillanic acid as the source of β -lactam. The biosynthetic pathway for the formation of penicillin-G in *Penicillium chrysogenum* cell through the formation of intermediates was carried out in the form of amino acids such as α -amino adipate, L-cysteine, L-valine which are formed from glucose (food ingredients). The formation of 6-amino penicillanic acid is an amino acid combination of L-cysteine and L-valine, a step part of the formation of antibiotic penicillin-G in *P. chrysogenum* cells, thus, it is obvious that there are enzymes involved in its formation. The objective of this study was to examine the use of enzymes present in *P. chrysogenum* cells to produce penicillin-G and 6-amino penicillanic acid using the intermediate compounds α -amino adipate, L-cysteine, L-valine and phenylacetic acid assisted by NAFA[®] coenzymes in *P. chrysogenum* cells which is more permeable. The research method started from producing biomass of *P. chrysogenum* cells that demonstrated penicillin-producing antibiotic capability, as the source of the enzyme, followed by addition of permeability treatment of *P. chrysogenum* cell membrane to get immobile of enzyme by its own cell therefore it can be used more than once. After that the enzyme activity was proven by adding α -amino adipate, L-cysteine, L-valine, phenylacetic acid and NAFA[®] coenzyme for the formation of penicillin-G, whereas the addition of L-cystein, L-valine and NAFA[®] coenzyme were aimed to form 6-amino penicillanic acid. The results showed that *P. chrysogenum* is able to produce antibiotics with stationary early phase on day 6. The best increased permeability of *P. chrysogenum* cell membranes was obtained using a 1:4 of toluene:ethanol ratio mixture with the highest antibiotic concentration (130.06 mg/L) after testing for the enzymatic formation of antibacterial penicillin-G.

Keywords: *Penicillium chrysogenum*, Penicillin-G, 6-Amino penicillanic Acid, Immobile enzyme.

INTRODUCTION

Penicillium chrysogenum is a fungus that can produce penicillin benzyl antibiotics (penicillin-G) [1,2]. A cell used in its fermentation intact, all intermediate biosynthetic processes for penicillin-G formation has taken place in the producing *P. chrysogenum* cells. In penicillin-G production plant, the cell or biomass of *P. chrysogenum* produced at the end of the fermentation was removed, while the liquid was taken from the fermentation containing antibiotics separated to obtain pure antibiotics.

Demain [3] has reported the process of penicillin-G biosynthesis as well as Cooney and Acevedo [4] also reported in *P. chrysogenum* producing cells. The biosynthesis pathway is a transformation of glucose that goes into intermediate

compounds *i.e.* α -amino adipate, L-cysteine and L-valine assisted coenzyme. Nawfa [5] has successfully used the intermediate compound outside *P. chrysogenum* cells whose cell membranes are made more permeable to form antibiotics without biosynthesis in the cell. This suggests that the more permeable *P. chrysogenum* cells, still contain forming enzymes. This method is called enzymatic formation of penicillin-G by using enzyme in an immobilized state, thus it can be used many times. Takeshige and Ouchi [6] have also reported a reconstruction of ethanol fermentation in permeabilized cells of the yeast *Saccharomyces cerevisiae*. Factor that affects the formation of antibiotics enzymatically by this method is the composition of ethanol toluene mixture used to increase permeability membrane *P. chrysogenum* cell. Therefore, it is needed for optimization to get better results.

EXPERIMENTAL

Microorganisms: Pure culture of *P. chrysogenum* was inoculated on slanted agar (solid media) which was sterilized at 121 °C for 15 min, then incubated at 30 °C for 7 days. The solid medium used by potato dextrose agar (PDA) by weighing 39 g was then dissolved in 1 L of distilled water. *P. chrysogenum* on this solid medium was used as the stock culture. Pure culture of *Staphylococcus aureus* was prepared in the same way, followed incubated at 30 °C for 24 h. The solid medium used was nutrient agar by weighing 20 g which was then dissolved in 1 L of distilled water. *S. aureus* in this solid medium becomes a culture stock that was used in the antibiotic test.

Antibiotic activity: The antibiotic content in fermented liquid medium was determined by diffusion test in which *S. aureus* was used as a microbial tester. *S. aureus* bacteria obtained on slant agar media were suspended using 0.5 % NaCl physiological solution. The suspension of *S. aureus* bacteria (1 mL) was filled into a sterilized petri-dish and then shaken so that bacteria soaked the base surface of petri-dish. The growth medium in the form of the melted solid at 40 °C was added and shaken well to mix homogeneously with the added medium. The mixture of bacteria and culture media was left for a while until it freezes. The circular shaped paper was used as a place for a solution containing antibiotics to be tested placed on it. The test solution was dripped as much as 25 µL on paper and incubated for 24 h at 30 °C. The test was to be positive if a clear area was obtained around the paper. The relationship between the concentration of antibiotics to the diameter of clear zone could be determined by using standard penicillin-G antibiotics at different concentrations and measuring the diameter of clear zone formed for each concentration.

Time of production of *P. chrysogenum* antibiotics: Spores obtained from *P. chrysogenum* growth on sloping agar media were inoculated on liquid media that had been sterilized, then incubated at 30 °C for 10 days. The liquid medium used was mixture of 3 g of KH₂PO₄, 35 g of glucose, 0.75 g of phenyl acetate, 3.5 g of ammonium acetate, 0.25 g of MgSO₄·7H₂O, 0.1 g of FeSO₄·7H₂O, 0.5 g of Na₂SO₄, 5 mL of metal solution A and 5 mL of metal B solution which was then dissolved in 1 L of distilled water. Metal solution A was a mixture of 4 g of MnSO₄·H₂O, 4 g of ZnSO₄·7H₂O and 1 g of CuSO₄·5H₂O which was then dissolved in 1 L distilled water, while metal solution B was 10 g of CaCl₂·2H₂O dissolved in 1 L of distilled water. The pH of liquid medium was adjusted at 6.7 either by adding HCl or NaOH. A 1 mL spore suspension was poured into 50 mL of liquid growth medium and incubated using shaking at 30 °C. After 3 days, the culture was transferred to other growth media in 250 mL Erlenmeyer flask. Sampling was carried out every 24 h of incubation and determined the penicillin-G antibiotic content by taking the fermented liquid medium which was then tested by using *S. aureus* as a microbial tester.

Increased permeability of *P. chrysogenum* cells: *P. chrysogenum* cells (1 g) obtained on the sixth day were taken and increased its cell permeability by adding 2 mM EDTA then incubated for 5 min at 30 °C, then added 0.075 % SDS and ethanol:toluene with different concentration as follows: 4:1; 3:2; 2:3, and 1:4 then incubated for 5 min at 30 °C. The

permeable *P. chrysogenum* cells were separated from the supernatant by centrifuging at 3000 rpm at 30 °C for 5 min. The filtrate which has been separated from the biomass was then absorbed by UV spectrophotometer at a wavelength of 280 nm.

Formation of penicillin-G antibiotics and 6-amino penicillanic acid (6-APA) enzymatically: The permeable *P. chrysogenum* cells were incubated with a mixture of 50 mM L-valine, 50 mM L-cysteine, 50 mM α-amino adipic acid, and 50 mM, and phenylacetate acid as precursor as well as 1 mM NAFA[®] coenzyme. The mixtures were incubated for 12 h and then the antibiotic content was tested in diffusion agar. For 6-APA, same procedure was conducted by mixing of 50 mM L-valine, 50 mM L-cysteine and 1 mM NAFA[®] coenzyme.

RESULTS AND DISCUSSION

The method used in this study was the agar diffusion method using dish paper [7]. Disinfected paper consists of penicillin-G antibiotic gave a barrier zone (clear area) around the paper. This is because there were no *S. aureus* bacteria grew. Antibacterial penicillin-G blocked the cell wall biosynthesis of *S. aureus* bacteria due to block the transpeptidation reaction involved in cross-linking of peptidoglycan biosynthesis. Cell wall synthesis did not occur because cross-woven peptide side chains were blocked by penicillin-G.

Table-1 showed the different concentrations of penicillin-G antibiotics provided the different barrier zones. The higher the concentration of penicillin-G antibiotics used, wider the zone of inhibition.

TABLE-1
DIAMETER OF BARRIER ZONE ON DIFFERENT
PENICILLIN-G CONCENTRATIONS

Diameter of barrier zone (cm)	Penicillin-G concentration (mg/L)
1.10	5000
1.45	7500
1.70	10000
2.00	12500
2.25	15000

Based on the results in Table-1, a diameter relation zone curve for the concentration of antibiotic penicillin-G was made. The standard curve regression equation with linear line equation was found to be $y = 0.000114x + 0.56$ with $r^2 = 0.996$. The correlation coefficient (r^2) of curve has fulfilled the requirements to be used as a standard antibiotic curve because it was located at an interval of $0.9 < r^2 < 1$. The value of r^2 indicates that between the diameter of barrier zone and the concentration of antibiotic has a linear correlation.

Determination of antibiotic production time by *P. chrysogenum* using diffusion method after sampling every 24 h was conducted. Based on the measurement of diameter of barrier zone, the results of diffusion method are shown in Table-2. As seen, antibiotics began to be produced on the fourth day and continued to be produced until the tenth day. From the above results, the obtained time for the treatment of cell membrane permeability of *P. chrysogenum* was on the sixth day. This was because at this time, the maximum antibiotic formation speed which shows the enzyme content for the formation of antibiotics was present in large quantities. The amount of absor-

TABLE-2
DIAMETER OF BARRIER ZONE IN
MAKING GROWTH CURVES

Time (day)	Diameter of barrier zone (cm)	Time (day)	Diameter of barrier zone (cm)
1	0.00	6	2.20
2	0.00	7	2.20
3	0.00	8	2.30
4	1.05	9	2.30
5	1.10	10	2.35

bance at a wavelength of 280 nm per solution of *P. chrysogenum* cell permeabilization results is shown in Table-3.

TABLE-3
ABSORBANCE OF SOLUTION OF PERMEABILIZATION

Ethanol (%)	Toluene (%)	Absorbance (280 nm)
4	1	0.2165
3	2	0.2317
2	3	0.2470
1	4	0.2630

Table-3 showed the greater the toluene concentration used, the greater the absorbance. This indicates that more protein was extracted from the cell membrane and results in increased permeability of the cell membrane. To prove the best condition, the four permeability treatments for the formation of antibiotic penicillin-G were enzymatically used. Naturally, L-valine, L-cysteine and α -aminoadipic acid are the intermediates produced by *P. chrysogenum* for antibiotic biosynthesis in cells. The three intermediate compounds and phenylacetic acid as a precursor was added outside the stabilized *P. chrysogenum* cells (enzymatic antibiotic formation) then incubated for 12 h. Table-4 showed the largest concentration of penicillin-G as

TABLE-4
RELATIONSHIP BETWEEN THE DIAMETER OF THE
BARRIER TO THE CONCENTRATION OF PENICILLIN-G

Ethanol (%)	Toluene (%)	Diameter of the barrier zone (cm)	Penicillin-G concentration (mg/L)
4	1	1.15	51.93
3	2	1.40	73.77
2	3	1.65	95.62
1	4	2.05	130.60

much as 130.60 mg/L with a diameter of 2.05 cm in the zone of inhibition, indicated by an increase in the cell membrane of a mixture of ethanol and toluene in the ratio of 1:4. This condition was an optimum enzymatic condition for the manufacturing of the penicillin-G.

Conclusion

The production of penicillin-G naturally by *P. chrysogenum* had occurred on the sixth day so that *P. chrysogenum* cells were taken for treatment of increased cell membrane permeability. The condition of ethanol and toluene mixture to increase the permeability of *P. chrysogenum* cell membrane was obtained at an optimum ratio of 1:4 as evidenced by the best results for the formation of enzymatic penicillin antibiotics at 130.60 mg/L.

ACKNOWLEDGEMENTS

This work was supported by Laboratory grant research 2018 from the Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia (number: 1312/PKS/ITS/2018).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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