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The Combination of Carbon Source and the Addition of Phenylacetic Acid (PAA) to Growth Medium *Penicillium chrysogenum* to Enhance of Penicillin (Pen G) Production

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Abstract

The nutrition factor governs the growth and production of Penicillin G (Pen G) by *Penicillium chrysogenum* in a broth medium. Proper nutrition can improve Pen G antibiotic production. In this research, the optimal condition for Pen G production from *P. chrysogenum* in a standard culture medium and various carbon sources medium (glucose, lactose, maltose, and sucrose) were done for ten days. Phenylacetic Acid (PAA) precursor at 0.0 – 0.6 gL^{-1} (increment 0.1) was used to improve Pen G production. The Pen G was detected by HPLC, compared with the standard (Penicillin G Sodium Salt). The results showed that the PDB standard medium and lactose medium (150 rpm, at 30°C) produced 0.425 gL^{-1} and 0.107 gL^{-1} Pen G. Addition of 0.6 gL^{-1} PAA improved the Pen G production up to 0.045 gL^{-1} in the PDB medium, become the final concentration of 0.470 gL^{-1} and 2.460 gL^{-1} in the lactose medium, become the final concentration of 2.565 gL^{-1}. The antibiotic's activity against the pathogenic bacteria, i.e., *B. subtilis*, *S. aureus*, and *S. typhi* employing the disk diffusion method, has been done. The TLC method's detection of the potential Pen G spots was conducted with ethyl acetate: distilled water: acetic acid (60:20:20) as the mobile phase. The Pen G extracts could inhibit the growth of all tested bacteria in Rf 0.65. This study informs the proper combination of carbon source and precursor effects and increases the bioproduction of Pen G from *P. chrysogenum*.

1. Introduction

Microorganism has a flexible metabolic system, especially their adaptation to chemical compounds' synthesis. The growth of microorganisms in an unfavorable (stressful) condition is frequently used to induce the production of metabolites that are more potential such as enzymes, growth factors, hormones, or antibiotics [1]. The relationship between microbes in a stressful environment contributes to selecting biotechnology products, one of which is antibiotics [2, 3]. Biotechnology products' application plays an essential role in humans and other animals and agricultural products [4]. Biotechnology products from fungi or bacteria can produce effective antibacterial compounds, e.g., penicillin [5, 6]. Antibiotics produced and used widely in the world are the β-lactam group, some of which are penicillin, amoxicillin, and cephalosporin, which account for 40% of the antibiotic market [7, 8]. To date, the local industry is still troubled to meet the demand for β-lactam antibiotics, one of which is penicillin [9]. Antibiotics derived from penicillin have a high commercial value and acts as the basis for semi-synthetic antibiotics production, e.g., Amoxicillin and Ampicillin [10]. Amoxicillin is an antibiotic derived from penicillin that is most widely used in Indonesia [11]. Penicillin's activities are effective against both Gram-positive and Gram-negative bacteria [12]. The essential ingredient of a penicillin antibiotic is Penicillin G (Figure 1). In the fungi cells, penicillin G biosynthesis involves three primary genes, namely *d*(L-a-Aminoadipyl)-L-Cysteinyl-D-Valine Synthetase (acvA), Isopenicillin N Synthase (ipnA), and Acyl Coenzyme A: Isopenicillin N

Acyltransferase (aatA) [13]. The biosynthesis of penicillin G from *P. chrysogenum* is controlled by various growth factors, including carbon source, pH, agitation speed, duration of incubation, and types of precursors [7, 14, 15]. An essential factor in increasing the metabolism and growth of fungi in a culture is the nutrition supply. The dominant portion of the nutrition source is carbon. A study from Asnaashari *et al.* [14] proved the optimum Pen G production from *P. chrysogenum* could be done for eight days of incubation with 0.3 gL^{-1} yeast extract and 21 gL^{-1} glucose substrates at 28°C and 120 rpm of agitation.

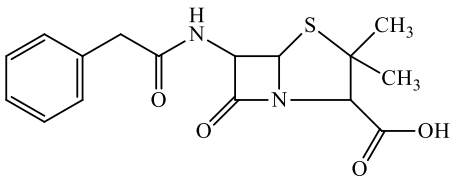

Figure 1. Structure molecule of Pen G [16]

In contrast, based on a study done by Onyegeme-Okerenta *et al.* [15], 5.32 ± 0.05 gL^{-1} of Pen G from *P. chrysogenum* is obtained at room temperature, pH 6.5 with an addition of 10 g bagasse and 50 mL distilled water for six days. Another study done by Rachman *et al.* [7] stated that the optimum penicillin production is done at 28°C and pH 7 for 192 hours with 50% glucose substrate and phenylacetic acid precursor (1 mg L^{-1}). The products showed antibacterial activities with 25 mm clear zones on average. This study aims to produce the active compound Pen G from *P. chrysogenum* by carbon source variation and PAA precursor to optimize the culture media. The second objective is to confirm the activity and the presence of Pen G compound as a spot, compared with the standard Pen G. This research is expected to play a role in developing *P. chrysogenum* strains that produce high Pen G so that it is expected to help in the supply of β-lactam antibiotic raw materials in the country.

2. Methodology

2.1. Materials

The materials used were Potato Dextro Agar (PDA) (Himedia), Nutrient Agar (NA) (Himedia), Potato Dextro Broth (PDB) (Himedia), Nutrient Broth (NB) (Himedia), (lactose, glucose, maltose, sucrose, Merck), ($Na_2SO_4.7H_2O$, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, Merck), yeast extract (Merck), and Phenylacetic Acid (PAA), (Sigma Aldrich). The instruments used in this study were shaker (Thermolyne), laminar airflow (Esco), autoclave (Tomy), microwave, HPLC (Shimadzu Prominence), and commonly used glass tools.

2.2. Culture of *P. chrysogenum* Isolate

The *P. chrysogenum* from IPBCC (IPB culture collection) isolate's hyphae were taken and inoculated in the PDA medium (3.9% b/v) and incubated at room temperature for 48 hours [7].

2.3. Culture of Pathogenic Bacteria Isolates

The pathogenic bacteria *E. coli*, *S. typhi*, *S. aureus*, and *B. subtilis* from the culture stock in the Laboratory of Applied Genetic Engineering and Protein Design, LIPI. The isolates' colonies (one pick of inoculation loop) inoculated in the NA medium (2.8% b/v) and incubated at room temperature for 24 hours [17].

2.4. Pen-G Production with Carbon Source Variation

The suspense of *P. chrysogenum* spores grown in the standard PDB medium was transferred into the activation medium, containing 0.85% NaCl and 1-2 drops of Tween 80. 2% (v/v) of the activation medium was added into the Pen G production medium (Table 1). The production medium was incubated for ten days with agitation at room temperature. The addition of the Phenylacetic Acid (PAA) precursor was done three days after the bioproduction from fungi took place [7, 18].

Table 1. Pen G production medium composition with varied carbon sources in 100 mL culture medium

No.	Production medium	Weight (g)
1	KH_2PO_4	0.4
2	$(NH_4)_2SO_4$	0.45
3	$MgSO_4.7H_2O$	0.05
4	$Na_2SO_4.7H_2O$	0.15
5	$ZnSO_4.7H_2O$	0.2
6	Yeast Extract	0.5
7	Phenylacetic Acid (PAA)	0-0.6
8	Carbon Source Variation (Lac, Glu, Suc, Mal) and PDB	1.5

Notes: Lac= Lactose, Glu= Glucose, Suc= Sucrose, Mal= Maltose and PDB as standard medium. PAA concentration variation (0, A, B, C, D, E, F). 0= 0 gL^{-1}; A= 0.1 gL^{-1}; B= 0.2 gL^{-1}; C= 0.3 gL^{-1}; D= 0.4 gL^{-1}; E= 0.5 gL^{-1}; F= 0.6 gL^{-1}

2.5. Pen G Production with Various PAA Concentration

The production media from carbon source optimization were varied with the addition of precursor (PAA). The composition of the production medium and variation of PAA concentration was employed from the previous method (Table 1). Observation of Pen G products was done by extracting 10 mL of supernatant after ten days of fermentation, in which after, Pen G concentration and pH of the medium were analyzed [19].

2.6. Analysis of Pen G from Bioproduction Process

The culture sample was harvested with centrifugation at 6000 rpm for 15 min at room temperature after cultivation. The resulting filtrates included including crude extract Pen G, extracellular bioproduct, without extracting the organic solution's culture sample. A stock of crude extract Pen G was filtered, sterilized (0.25 µm), and stored in aliquots at-4°C. Afterward, the Pen G concentration in the sample filtrates was assessed by injecting the crude extract Pen G into the HPLC instrument. The HPLC criteria: HPLC Shimadzu, detector UV 220 nm, C-18 column, with methanol: phosphate buffer 10 mM (80:20) as the mobile phase, at room temperature and flow rate of 1 mL/min. The standard Pen G used was penicillin G sodium salt with 1000 mg L^{-1} [20]. To obtain Pen-G concentration was used a single standard method: C1/A1 = C2/A2, each defined as

C1= standard concentration of Pen G (1 g/L), A1= standard peak area of Pen G, C2= concentration of the sample, A1= peak area of the sample [21].

The Pen-G's antibacterial activities from the fungi cultures were assessed by employing the disk diffusion method. The disks were put onto the NA medium previously inoculated with each 0.3 mL of pathogenic bacteria (*S. aureus*, *B. subtilis*, and *S. typhi*). 30 µL of Pen-G samples were pipetted onto each disk and incubated for 24 hours at room temperature. Subsequently, the developed clear zones were analyzed. Positive (penicillin G sodium salt) and negative (methanol) controls were included in each test. The developed clear zones showed the strength of Pen G activities [22, 23].

The presence of Pen G spots was confirmed by the Thin Layer Chromatography (TLC) method. Prior, the filtrated samples that contain Pen G were extracted with Chloroform solvent (1:1 v/v). The Pen G extracts were then obtained by evaporating the organic solvent. The Pen G extracts were then applied to the TLC plate and eluted with ethyl acetate: distilled water: acetic acid (60:20:20) as the mobile phase. After that, the TLC was dried by heat until the spots were visible on top of the TLC plates. Prediction of the compound was carried out under UV light at 254 nm [24].

2.7. Data Analysis

All data performed were as mean ± SD Data, analyzed using ANOVA and tested with Tukey HSD (Honestly Significant Difference) using software IBM SPSS Statistics 25.

3. Results and Discussion

3.1. The Growth Medium Influence on Pen G Production

The Initial medium to observe endophytic fungus *P. chrysogenum* presence and growth were carried out using the PDA standard medium. The observation showed that the unique fungus characteristics, green-colored in the upper part, and pale-yellow colored in the hyphae's basal part (Figure 2). Further, the penicillin metabolite's production from the fungus was done in the PDB medium and various carbon media (Table 1). As mentioned before, the analyzed Pen-G product was the crude extract (extracellular bio-product), without extracting the sample. Subsequently, the product was compared with the standard. The assignment of samples' concentration was referred as the spectrum of standard pen-G employing HPLC. Observation from the standard Pen G (Pen G sodium salt) employing C-18 column HPLC showed the presence of standard (as positive control) peak spectrum retention at 2.738 minutes (Figure 3a). The confirmation of penicillin production from the *P. chrysogenum* fungus was referred to the same retention time from the standard, 2.737 minutes (3b).

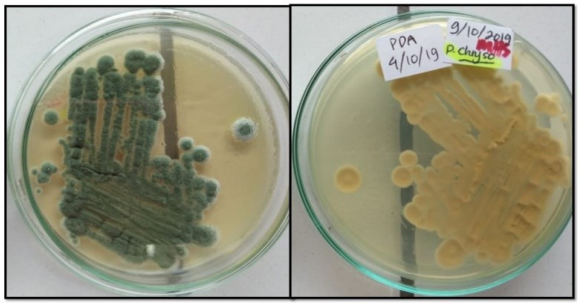

Figure 2. *P. chrysogenum* fungus' isolate in a PDA standard medium at room temperature.

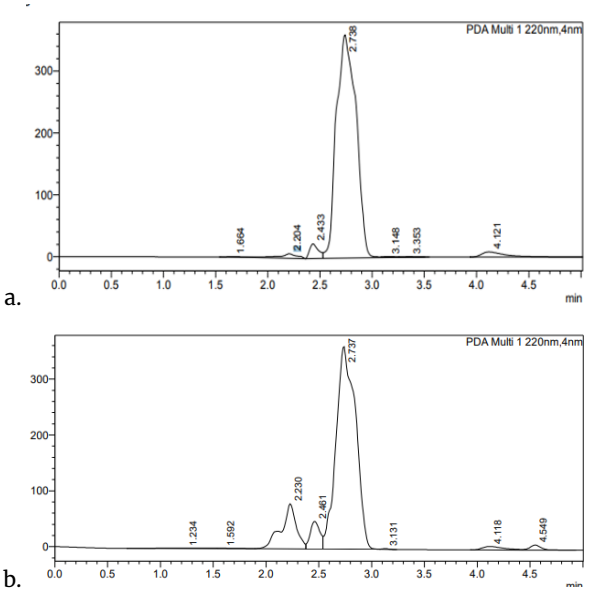

Figure 3. The standard peak of penicillin G sodium salt as a positive control (a) and a peak of penicillin-G from bioproduction (b).

Based on the produced Pen G metabolite's analysis and result declare as average ± SD (n=4), the concentration varied from 0.107 gL^{-1} to 0.425 gL^{-1} as the highest concentration produced (Figure 4). Referenced from Figure 4, the highest Pen G metabolite produced is 0.425 gL^{-1} from the PDB medium. That result was higher than the lactose medium, with only 0.107 gL^{-1} of Pen G produced and the other carbon sources. The results showed that proper nutrition supply heavily influences the characteristics of *P. chrysogenum*. The *P. chrysogenum* fungus cultured in the PDB medium has a higher concentration of Pen G produced than the other carbon sources, viz. lactose, maltose, sucrose, and glucose. The PDB medium contains complex sugar (polysaccharide), contains nutrition intake as the complex sugar needed to be hydrolyzed first by enzymes into simple sugars [25]. The mentioned condition caused the fungus to work harder to produce the required enzymes [15]. The enzyme's mechanism caused the fungus to create other secondary products, which was the penicillin antibiotic [26].

In contrast to glucose, a simple sugar, it can be readily absorbed by the fungus [27]. The smoother the absorption occurs, the higher it is for primary metabolites to be produced [28]. Onyegeme-Okerenta *et al.* [15]

reported that Pen G production from *P. chrysogenum* fungus was affected by many factors, including carbon nutrition. The carbon source used in fungus' growth can be monosaccharide (glucose, fructose, galactose), disaccharide (sucrose, lactose, maltose), and/or complex carbon like starch [29, 30].

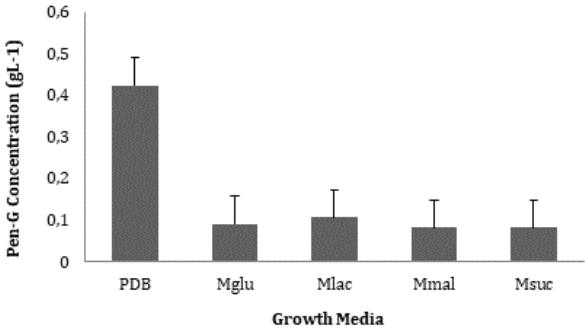

Figure 4. The Pen G production from *P. chrysogenum* fungus in various media (PDB: Potato Dextrose Broth, Mglu: Pen G production medium with glucose, Mlac: Pen G production medium with lactose, Mmal: Pen G production medium with maltose, Msuc: Pen G production medium with sucrose). This result declares as average ± SD (n=4)

3.2. Addition of Phenylacetic Acid (PAA) towards the Penicillin G Production

Pen G production from *P. chrysogenum* fungus in the production medium with PAA addition gave a significantly different result. Pen G production in the PAA-added lactose medium was increased by 0.6 gL^{-1}, compared to the standard PDB medium with similar PAA concentration added (Figure 5). The tendency of Pen G concentration to increase is linearly attributed to the rise of PAA concentration in the lactose culture medium. A decreasing trend occurred at 0.3 gL^{-1} addition of PAA, although the trend recovered in the next higher concentration. This phenomenon can be caused by the medium's unstable pH, which led to cells' autolysis. The increasing trend of Pen G in the lactose medium with 0.6 gL^{-1} PAA addition achieved the optimal concentration around 2.460 gL^{-1} until the final concentration of 2.565 gL^{-1}, whilst in the PDB medium, the highest Pen G produced is 0.470 gL^{-1}. The use of precursors is significant for Pen G production [31]. PAA is a precursor and the central of Pen G metabolism [32]. If PAA is supplied into the fermentation media of *P. chrysogenum*, the Pen G would be secreted into the medium [33].

In industries of Pen G production, the addition of precursor is needed. With the precursor, the higher the chances for microbes to produce various types of penicillin depending on available precursor, e.g., penicillin F and K, which contain 3-hexanoic acid and octanoic acid. Phenoxy acetic acid is the precursor of phenoxymethylpenicillin (penicillin V), while PAA acts as the hydrophobic side chain of benzylpenicillin or Pen G [34]. On average, the Pen G produced with varied carbon sources has a far lower concentration than in the media with PAA addition. PAA precursors can support the optimum Pen G production if given proper doses [35]. However, PAA can be contra-productive for the growth and production of fungus because of its acidic property. This acidic property is toxic to the fungus culture [36]. Pen G production can decrease in high PAA concentration because of the increased rate of cells' autolysis. Insufficient PAA concentration can also decrease Pen G produced, although it has insignificant effects on the cells' autolysis [37].

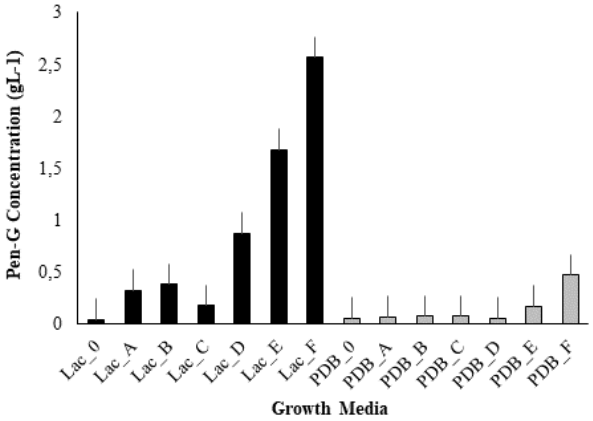

Figure 5. The Pen G production from *P. chrysogenum* fungus with the various concentration of phenylacetic acid (PAA) precursor in Pen G production contain lactose (Lac) and Potato Dextrose Broth (PDB) medium. Vertical bars show the standard error of the mean based on four independent measurements. Each block of difference color shows the real difference at the ($P < 0.05$) level, based on the Tukey HSD test.

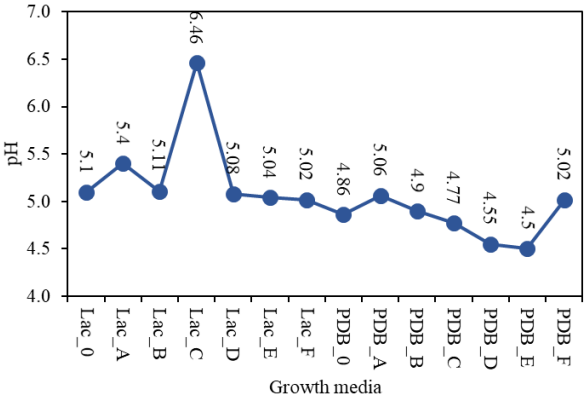

Figure 6. Graphic of final fermentation pH from *P. chrysogenum* fungus with the various concentration of phenylacetic acid (PAA) precursor in Pen G production contain lactose (Lac) and Potato Dextrose Broth (PDB) medium. This result declares as average ± n=2.

Based on the study done by Wagner *et al.* [38], PAA concentration below 0.25 gL^{-1} could cause an unstable condition in the reactor, whereas PAA concentration above 0.5 gL^{-1} could inhibit the fungus' growth. Other than that, referenced from [37], PAA's addition above 1 gL^{-1} could inhibit penicillin's biosynthesize. The pH of the fermented filtrates in both lactose and PDB medium were analyzed at room temperature. The filtrate from the lactose medium was known to have a stable pH in the range of 4.5, while filtrate from the PDB medium is in the range of pH 5.4 (Figure 6). The different condition was shown from the lactose medium with 0.3 gL^{-1} PAA addition, in which the pH was increased up to pH 6.46. This extreme pH escalation caused the stability of *P.*

chrysogenum growth to be disturbed, furthered with a decreasing trend of pen-G concentration in the filtrate. In the previous study, pH above 6.0 caused the fungus' growth to be unstable, subsequently affecting the Pen G production (decreasing). In contrast, the culture's pH below 4.0 disturbed *P. chrysogenum* growth [39].

3.3. Analysis of Penicillin G Bioproduction

The antibacterial activities assessment from the potential strain of *P. chrysogenum* for Pen G production was done employing the disk diffusion method. The bioactivity of Pen G was shown to be potent against both the Gram-positive (*S. aureus* and *B. subtilis*) and negative (*S. typhi*) bacteria (Table 2). The inhibition zone from the samples actively inhibits the tested pathogenic bacteria, shown by the presence of clear zones, compared to the positive control (penicillin 1000 mg L^{-1}).

Table 2. Inhibition zone of Pen-G filtrated samples against tested bacteria; *S. aureus*, *S. typhi*, and *B. subtilis*

No.	Filtrate sample	Zone of inhibition against tested bacteria (mm)		
		S. aureus	*S. typhi*	*B. subtilis*
1	Lac_0	9	7.5	6.5
2	Lac_A	8.5	11	10
3	Lac_B	13	10	11
4	Lac_C	12	9	12
5	Lac_D	8	11	11.5
6	Lac_E	8.5	8	12
7	Lac_F	15	11	15
8	+ control (1 g L^{-1})	15	8.5	13
9	- control (chloroform)	0	0	0
9	PDB_0	7	8.5	9
10	PDB_A	8.5	7	11
11	PDB_B	12.5	7	10
12	PDB_C	11	8.5	8
13	PDB_D	7	7	9
14	PDB_E	6.5	7	9
15	PDB_F	11	8	9

Notes: Lac= Lactose, Glu= Glucose, Suc= Sucrose, Mal= Maltose and PDB as standard medium. PAA Concentration Variation (0, A, B, C, D, E, F). 0= 0 gL^{-1}; A= 0.1 gL^{-1}; B= 0.2 gL^{-1}; C= 0.3 gL^{-1}; D= 0.4 gL^{-1}; E= 0.5 gL^{-1}; F= 0.6 gL^{-1}

In the beginning, Pen G was known only effectively to inhibit Gram-positive bacteria [40]. Therefore, a derivative of penicillin has been developed, which has a broader spectrum of Gram-positive and Gram-negative bacteria, one of which is amoxicillin. However, after updated studies, Pen G was shown to have the ability against Gram-negative bacteria such as *S. typhi*. This phenomenon may be caused by the nutrition source combined with the production medium of Pen G. The application of semi-synthetic material conjoined with enzymatic reactions can shift Pen G production into different targets [11].

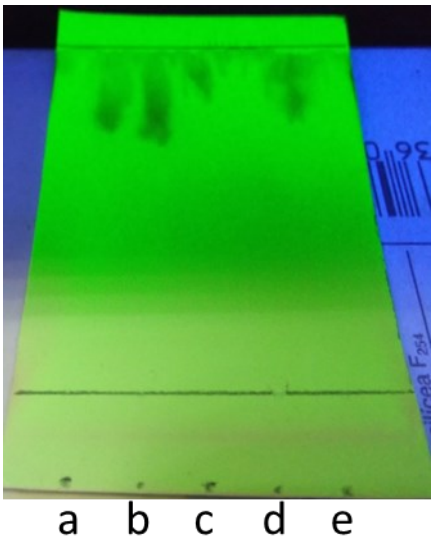

Figure 7. Spots from Pen G elution in ethyl acetate: distilled water: acetic acid (60: 20: 20); (a) Pen G standard (1 gL^{-1}); (b)-(c) spots from lactose media; (d)-(e) spots from PDB media in UV 254 nm

Identification of potential Pen G spots on the ethyl acetate: distilled water: acetic acid (60:20:20) mobile phase was done with the Thin Layer Chromatography method (Figure 7). Pen G spots can be seen under the UV light after dried by heat and does not require any markers. Prediction of the compound was identified under 254 nm UV light. The TLC method usage was to see the presence of Pen-G spots in extracts after being separated with appropriate organic solvents. Afterward, the spots can be migrated and identified. The similarity between the observed standard-derived and sample-derived Pen G spots showed the presence of spots at Rf 0.65 cm.

4. Conclusion

This study confirmed that proper nutrition and PAA precursor addition could increase the Pen G bioproduction from *P. chrysogenum* fungus. The addition of 0.6 gL^{-1} PAA in the lactose sourced medium increased the production of Pen G up to 2.46 gL^{-1}.

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Contribution

The authors declare that Martha Sari and Dirayanti (main contributors), Eris Septiana, Bustanussalam, and Zainal Apon (other contributors), are responsible for all activities, including the concept development, design, laboratory, and data analysis, writing, as well as revisions its publication.

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