Effect of Phenylacetic Acid Addition on Productivity of *Penicillium chrysogenum* in Penicillin G Production Using Pilot Scale Reactor

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Abstract :

Effect of penicillin's precursor addition, phenylacetic acid (PAA), was done by addition of PAA in several concentrations in a pilot scale reactor batch culture to produce penicillin G with fungi *Penicillium chrysogenum*. Culture was analyzed by morphology observation and PAA was analyzed by HPLC. High concentration of PAA showed decreasing of biomassa and production of penicillin G that had contribution in increasing of cellular autolysis. However, *Penicillium chrysogenum*'s morphology analysis did not show significant effect in autolysis and decreasing of biomassa. Low concentration of PAA showed low production of penicillin G and low effect in biomassa or autolysis. Those effects of PAA addition need an exploitation to induce the phenomenon in *Penicillium chrysogenum*'s culture.

Keywords: penicillin G, *Penicillium chrysogenum,* phenylacetic acid, reactor

1. Introduction

Betalactam antibiotics especially penicillin and its derivates still widely used in threatment for gram-positive bacterial's infections. Fermentation of *Penicillium chrysogenum* is the most effective procedure to produce penicillin. Optimization of fermentation can be done by observing the effect of phenylacetic acid (PAA) addition during the fermentation process. PAA is the 'toxic' penicillin precursor **[1]**.

Feeding PAA to the culture requires an exact regime to ensure efficient convertion to product, as PAA can be up to 11% of the raw material cost **[2]**, but also to minimize toxic effects on the organism **[3,4]**. The optimum range for PAA concentration is said to be 0.1 to 1.0 g l^{-1} **[2]**, above which, PAA inhibits growth and penicillin biosynthesis, and is reported to be particularly toxic to fragmented, aged hyphae **[5]**. It is also essential to control precursor levels to prevent the oxidation of PAA to p-hydroxy-PAA, which would render the precursor unavailable for penicillin biosynthesis **[5]**. The toxicity of PAA (a weak acid), manifests as the dissipation of the trans-membrane pH gradient, since the protonated, lipid soluble PAA will transport protons across the membrane. PAA also inhibits ATP formation during electron transport **[2,6]**, reducing the energy available to the organism. Given such toxic effects, PAA clearly has the potential to influence autolysis in *Penicillium chrysogenum* **[1]**.

2. Materials and Methods

2.1. Vegetative stage

Strain of *Penicillium chrysogenum,* which spored in rice, was supplied as 400 ml liquid spore suspensions and used to inoculate 3,6 l of sterile vegetative medium to give 10% inoculants concentration in vegetative medium. Vegetative medium comprised of : corn steep liquor (219,6 g), sucrose (72 g), and $CaCO_3$ (18 g). A reactor BioFlo® (New Brunswick Scientific, USA) was used in this study, with an operating volume of 4 l, total volume 7,5 l. Vegetative culture conditions were maintained at pH 6,1, agitation rate 450 rpm, aeration rate 4 lpm, and temperature 25^{o}C throughout the processes. Foaming was controlled by addition of adekanol. Vegetative stage was occurred for 36 hours.

2.2. Fermentative stage

After 36 hours in vegetative stage, vegetative culture was transferred into a pilot scale reactor. 4 l of vegetative culture was used to inoculate 36 l of sterile fermentative medium to give 10% inoculants concentration in fermentative medium. As a fed batch culture, fermentative medium comprised of : corn steep liquor (3,2 kg), $NaH_2PO_4.2H_2O$ (180 g), glucose (180 g), sodium sulphate (9,6 g), ammonium sulphate (195,2 g), $Ca(OH)_2$ (48 g), $CaCO_3$ (30 g), 10,3% PAA (68,4 ml), and adekanol (4 ml). A pilot scale reactor was used in this study, with an operating volume of 40 l, total volume 75 l. Fermentative culture conditions were maintained at pH 5,9 – 6,6, agitation rate 250 – 450 rpm, and temperature 25^{o}C throughout the processes. Foaming was controlled by addition of adekanol. Reducing sugar was maintained at concentration 0,8 – 2,5 % and controlled by addition of 37% sterile glucose solution. Ammonium level was controlled by addition of 15% sterile ammonium sulphate solution. pH was controlled by addition of 28% ammonia. Dissolved oxygen was maintained at 30% or above. Fermentative stage was stopped at 229,5 h.

2.3. Phenylacetic acid addition

Sterile phenylacetic acid solution was prepared by dissolved 161,65 g of PAA 99% in 131,25 ml of 32% NaOH solution, stirred well, pH of this solution must be 7,0 or above. Add this solution with RO water to 1,9 l. Make adjustment to pH 6,0 with addition of 32% NaOH solution. Add the solution with RO water to 2 l. The 10,3% of phenylacetic acid solution was sterilized in 121^{o}C for 15 minutes. PAA addition was started at 43 h to produced extracellular PAA concentration from optimal level (100 – 1000 ppm) to high concentration (more than 1000 ppm).

2.4. Analysis

Biomass determination

Biomass was measured by PMV (Packed Mycelium Volume). 10 ml aliquots of fermentative culture was sentrifuged in 4000 rpm for 20 minutes and volume of concentrate was measured.

Reducing sugar assay

The reducing sugar concentration in fermentative culture filtrates was measured by spectrophotometry using dinitrosalysic acid (DNS) with standards prepared using glucose in a concentration range between 200 – 400 ppm, with appropriate sample dilutions.

Ammonium assay

Ammonium concentration in fermentative culture filtrates was measured by distillation followed by titration.

Penicillin and PAA assay

Both of penicillin and PAA concentration in fermentative culture filtrates was measured by HPLC method with using of standard penicillin and PAA in a concentration range between 200 – 1000 ppm for penicillin and 20,61 – 103,04 ppm for PAA.

Morphological analysis

Morphological characteristics of fermentative culture was examined by optical microscope with 50 times magnification.

3. Results and Discussion

This study was designed to give low PAA concentration to high PAA concentration, approximately 2 times the recommended level of 1,0 g l^{-1} **[2]**, during the fermentative stage in a pilot scale reactor. As a fed batch culture, the extracellular concentration of PAA was started at 308 ppm, and the PAA addition was started at 43 h when extracellular concentration of PAA was decreased to 183 ppm. The extracellular concentration of PAA was controlled at 100 – 1000 ppm until 54 h. The extracellular concentration of PAA was increased up to approximately 2000 ppm since 60 h.

The results of the processes are shown in Figure 1 – 4 and summarized in Table 1.

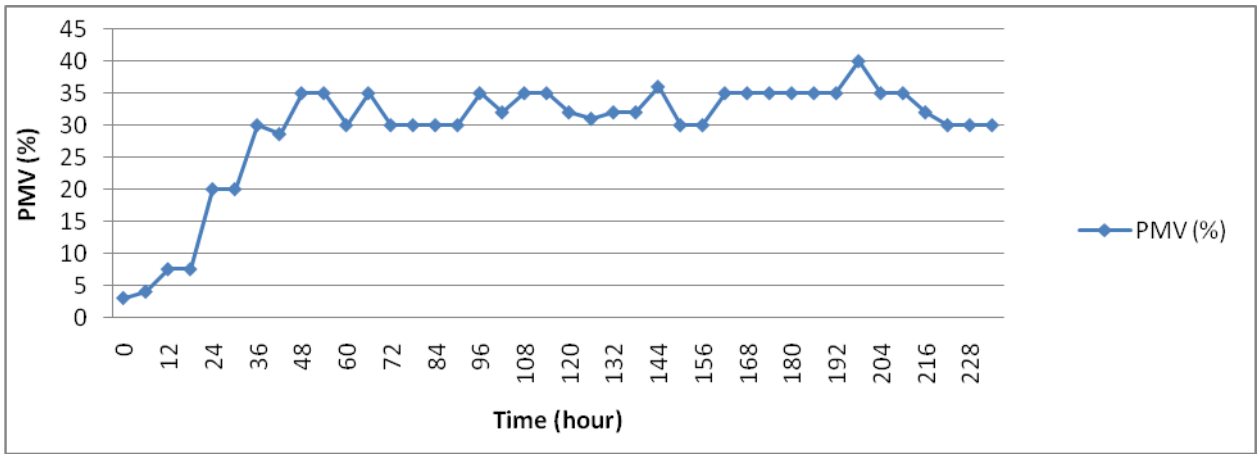

Figure 1. Graphic of biomassa condition during fermentative stage.

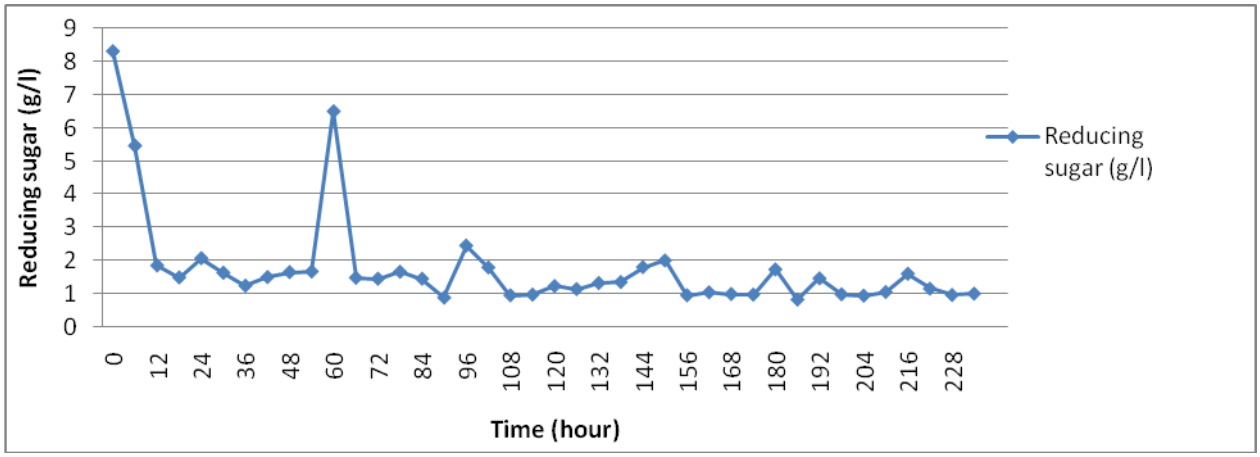

Figure 2. Graphic of reducing sugar condition during fermentative stage.

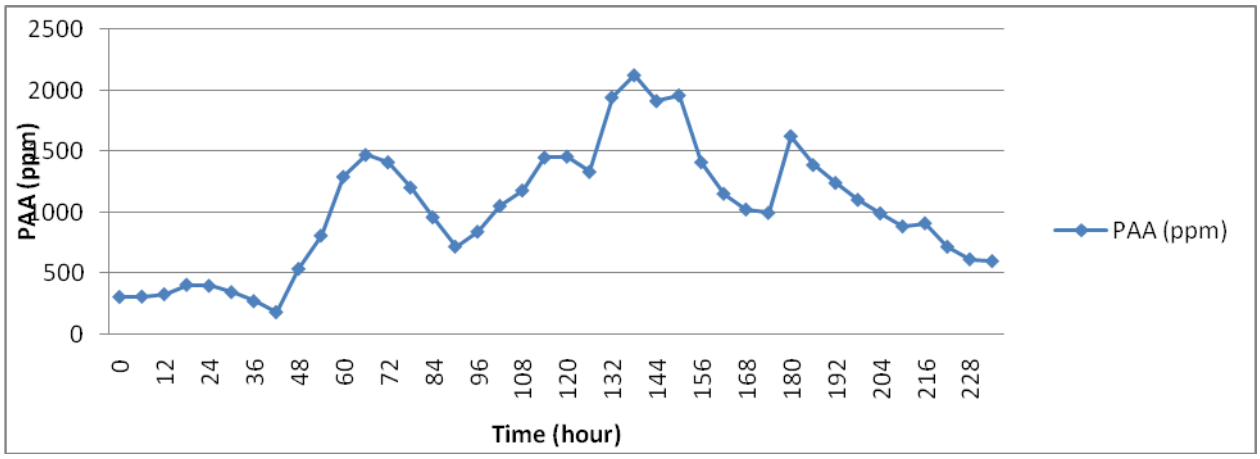

Figure 3. Graphic of extracellular concentration of PAA during fermentative stage.

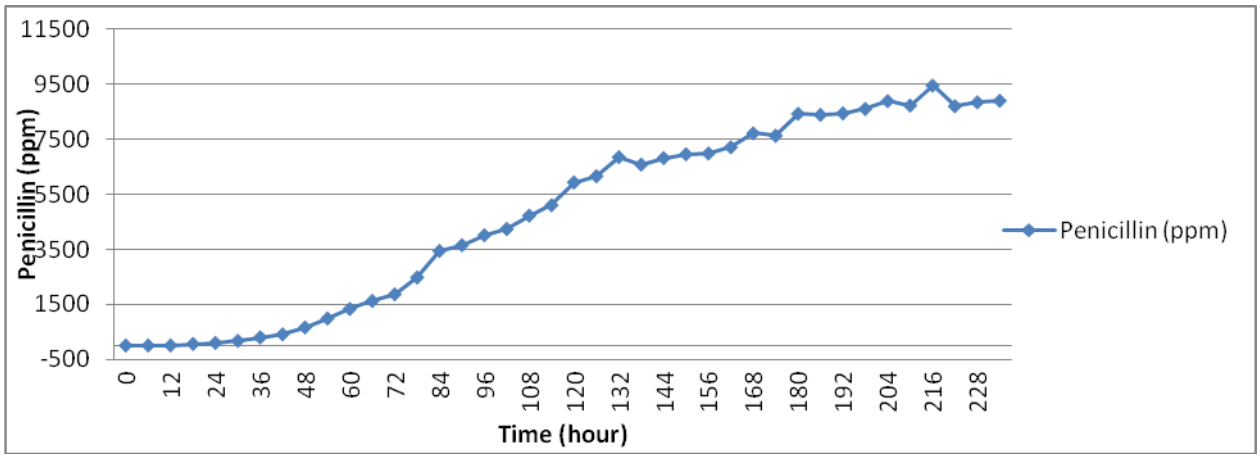

Figure 4. Graphic of penicillin producted during fermentative stage.

Table 1. Fermentative stage conditions.

Hour	PMV (%)	Reducing Sugar (g/l)	Ammonium (%)	PAA (ppm)	Penicillin (ppm)
0	3	8.32	0.2	308	0
6	4	5.47	0.2	311	0
12	7.5	1.86	0.16	330	3
18	7.5	1.5	0.22	408	57
24	20	2.08	0.23	399	94
30	20	1.64	0.21	347	169
36	30	1.25	0.21	273	296
42	28.6	1.51	0.16	183	406
48	35	1.66	0.13	538	657
54	35	1.68	0.11	811	983
60	30	6.51	0.05	1294	1331
66	35	1.49	0.04	1474	1621
72	30	1.46	0.03	1413	1861
78	30	1.67	0.04	1206	2474
84	30	1.46	0.05	962	3440
90	30	0.89	0.04	721	3651
96	35	2.46	0.04	843	4013
102	32	1.8	0.04	1055	4241
108	35	0.96	0.04	1182	4725
114	35	0.98	0.04	1452	5104
120	32	1.24	0.04	1460	5935
126	31	1.15	0.04	1335	6160
132	32	1.33	0.04	1946	6854
138	32	1.36	0.04	2128	6583
144	36	1.81	0.04	1916	6822
150	30	2.01	0.04	1963	6959
156	30	0.96	0.04	1413	6987
162	35	1.05	0.04	1155	7212

168	35	1	0.04	1026	7721
174	35	0.98	0.04	998	7631
180	35	1.74	0.05	1628	8431
186	35	0.83	0.06	1391	8391
192	35	1.47	0.04	1244	8447
198	40	0.99	0.06	1105	8607
204	35	0.95	0.05	994	8891
210	35	1.06	0.05	887	8731
216	32	1.61	0.05	913	9457
222	30	1.16	0.06	720	8707
228	30	0.97	0.05	617	8848
229.5	30	1.01	0.05	602	8905

During the first 54 hours of fermentative stage, where extracellular concentration of PAA was in low concentration (100 – 1000 ppm), there was a biomassa increasing from 3% to 35% in PMV value. This condition was followed by low rate of penicillin biosynthesis. In 54 hours, 983 ppm of penicillin was produced. This was because of the fermentative culture was in growth phase. In addition, the excess of ammonium can reduce penicillin biosynthesis **[5]**, and PAA uptake **[7]**.

At 60 h onward, in production phase of penicillin, extracellular concentration of PAA was raised above 1000 ppm by addition of 10,3% sterile PAA solution. This addition caused decline of biomassa. It also gave low rate of penicillin production. This phenomenon was caused by increasing of cellular autolysis. High PAA levels inhibited growth and increased the percentage of autolysed regions **[1]**. Using of glucose as carbon source in carbon feeding might cause the cellular autolysis related to its effect on autolysis. Low levels of readily metabolisable sugars, such as glucose, have been shown to induce rapid, severe autolysis **[8]**. Low concentration of extracellular penicillin could also caused by degradation of penicillin that had been found to be concomitant with autolysis, via the action of penicillin acylase **[9]**, while chemical degradation to penicilloic acid can occur **[10]**.

PAA addition during the fermentative stage to give both low concentration and high concentration of PAA in extracellular give 8905 ppm concentration of penicillin in the harvest time. This value was a low productivity of penicillin.

4. Conclusion

Above the optimal level of extracellular concentration, PAA had toxicity effect on *Penicillium chrysogenum* that decline both biomassa and productivity rate of penicillin biosynthesis in a pilot scale reactor. This phenomenon related to the cellular autolysis activity during fermentative stage.

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