ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Optimization of phenyl acetic acid concentration during the growth phase of Penicillin-G production in pilot plant.

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ABSTRACT

Penicillium chrysogenum is a filamentous fungus used for the production of penicillin. It is able to synthesize penicillin with specific side chains when the appropriate precursor is fed to the production medium. Phenyl acetic acid (PAA) is the only precursor for the production of penicillin G, which is fed in small amounts to avoid toxic side effects. When PAA was added during the growth phase, the organism to its toxic effects, adapts itself to the environment. 0.0, 0.2, 0.4, 0.6 g/l were the different concentrations of phenyl acetic acid added to the growth phase in the seed fermenter (70 liters), where the organism multiplies. The organism from the seed tank were then transferred to the fermenter (1000 liters) containing the media. In the fermenter Sugar, Ammonium sulphate, Ammonium hydroxide and PAA were added based on the requirement of the organism. During production the samples were tested in the microbiology and quality control laboratories for the levels of residual sugar, nitrogen, activity, residual PAA, sterility, viability, packed mycelia volume and pH. After the production the product were extracted using techniques such as filtration, purification, distillation, etc. The activity during production was monitored using high performance liquid chromatography (HPLC). The weight of the final product indicates that the production increases when 0.4 g/l PAA were added in the seed fermenter.

Keywords: Penicillin-G, seed fermenter, HPLC, filamentous fungi.

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ISSN: 0975-8585

INTRODUCTION

Penicillin is a group of antibiotics derived from *Penicillium* fungi. Penicillin G is produced when the R group in the ring is replaced by a benzyl group from the precursor (phenyl acetic acid). Penicillin G is also called as benzyl penicillin. For this production, mutant strain of *Penicillum chrsogenum* is used.

Penicillin is a secondary metabolite of fungus *Penicillium* that is produced when growth of a fungus is inhibited by stress. It is not produced during active growth. Production is limited by negative feedback inhibition in the synthesis pathway of penicillin. (Luengo *et al*.,1989). The ketoglutarate combines with acetylcoenzyme A to give homocitrate which in turn produces aminoadipic acid from which the antibiotic is synthesised. The byproduct L-Lysine inhibits the production of homocitrate, so the presence of exogenous lysine should be avoided in penicillin production. The amount of carbon available is limited for the growth of microorganism which is achieved by creating a cabohydrate stress. As a result, the production of lysine decreases which in turn increases the production of the antibiotic.

Various concentrations of phenyl acetic acid were added to the seed stage so that the toxicity limit of the organism gets enhanced and the production of penicillin G increases. 0.0, 0.2, 0.4, 0.6 g/l were the various concentrations of phenyl acetic acid added to a seed fermenter during production as 0.5 g/l is the toxicity limit of the organism.

MATERIALS AND METHODS

MATERIALS:

CULTURE USED: *Penicillium chrysogenum*

MEDIA USED: Viable count media, nutrient agar, nutrient broth, sabouraud's dextrose agar.

GLASS WARES: 1 l and 500ml wide mouth conical flask was used. 10ml, 5ml and 1ml pipette, measuring jar, beaker, test tube, petriplate, and autoclave

SUBSTRATE USED: Basmati rice

CHEMICAL USED: Corn steep liquor, potassium chloride, potassium dihydrogen phosphate, sodium nitrate, potassium dihydrogen phosphate, peptone, yeast extract, isopropyl alcohol (IPA), tween-60.

INSTRUMENTS USED: Weighing machine, vortex mixer, autoclave, magnetic stirrer, microscope, fridge, lyophilizer, etc…

CHAMBERS USED: Laminar airflow chamber, thermostatic chamber, media preparation room, etc…

METHODS:

There are four steps involved in this process and they are

1. Sporulation
2. Seed fermentation
3. Fermentation
4. Recovery

SPORULATION

The organism must multiply before introducing them into the seed tank. Rice is used as a substrate in a flask for sporulation.

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RICE FLASK PREPARATION

125g of basmati rice was soaked in 500ml of water for 25mins. Under room temperature the water is drained off and the excess water was air dried on filter paper for few minutes (rice should not stick with one another). After partial drying the net weight was measured for moisture calibration.

MOSITURE CALIBRATION

The moisture content of the rice flask was calibrated to adjust the moisture content to 23%

$$\% \text{ of moisture} = \frac{\text{Weight difference} * 100}{\text{Total weight of rice after soaking}}$$

Weight difference = (weight after soaking – weight before soaking)

50g of rice was then transferred to separate 500ml conical flasks. The flasks were stopped with a cotton plug and covered with brown paper and the weight of the individual flasks before sterilization was determined.

STERILIZATION

Autoclave method sterilizes all the prepared materials. Sterilization details were noted for every batch of sterilization. After sterilization moisture lost will be there in the rice flask and has to be calculated.

MOISTURE LOSS CALIBRATION

There will be moisture loss during sterilization that can be calculated by,
Moisture loss = Difference in net weight before and after sterilization

Water is added to the rice to prevent the loss of moisture during sterilization.

INOCULATION

During inoculation hands are cleaned with isopropyl alcohol (IPA). The mouths of all flasks were heated by keeping mouth near the flame. In front of the flame, water and inoculums were added one by one to the flasks as per requirement. Then the mouth of all the flasks were kept in flame and covered with cloth and shaken well.

INCUBATION

The flasks were incubated at 24°C-25°C and 70-80% relative humidity for 14 days with regular checking every day.

HARVEST OF SPORES

After 14 days of incubation, the rice flask was harvested by adding tween60 in sterile condition for breaking the spore chain for easier collection the flask was shaken for 2-3 minutes and the spore suspension alone was transferred aseptically to a sterile flask and stored at low temperature. Before adding tween 60 for doing total count, 5gm of rice was taken from the rice flask under sterile conditions. To this 5 gm of rice 45 ml of tween 60 were added in separate flasks. The flasks were kept at 175 rpm for 20 minutes in shaker. The entire sample was observed in haemocytometer.

TOTAL COUNT

The spores were counted after 14 days, by total count method using heamocytometer.

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STERILITY TEST

This test was done to check bacterial and other fungal contamination.

1 ml of spore suspension from the harvested flask was added to nutrient agar slants, nutrient broth flasks, sabouraud's dextrose agar (SDA) slants and sabouraud's dextrose broth flasks in sterile condition. The nutrient broth and agar slants were incubated at 37°C for 24-48 hours. SDA slants and broth were incubated at 37°C.

VIABILITY TEST

This test was done to check the viability of the spores in the suspension. The viable count media was prepared and the spore suspension was serially diluted to 10^5 dilutions. From each dilution 0.1 ml of the solution was plated on 3 viable count media plates under aseptic condition by spread plate method.

The plates were incubated at 25°C for 5 days. After incubation, the colonies were counted to check the viability of the spores in the suspension.

$$\% \text{ of viability} = \frac{\text{viable count}}{\text{Total count}} * 100$$

SEED FERMENTATION

FERMENTER

Stainless steel seed fermenter with a capacity of 70 litres was used for fermentation. These were cleaned with caustic soda before media charging.

MEDIA COMPOSITION

Synthetic medium along with the different concentrations of PAA such as 0.0, 0.2, 0.4, 0.6 mg/ml (in separate batches) was mixed and its pH was adjusted to 6.5 with the help of caustic soda. These concentrations were selected based on the toxicity limit of the organism.

STERILIZATION AND COOLING

After the addition of medium and pH adjustment sterilization was done by increasing the temperature up to 121°C. This was done by first passing steam into outer jacket and then directly into the sparger due to the decrease in the heat transfer rate.

AIR FILTRATION AND SPECIFICATION

Instrument air which does not have any moisture content was used for this process normally. The pressure of the air was regulated and was passed through a 0.1 micron size filter to filter all the microorganisms.

SEED INOCULATION

The harvested spore suspended in the surfactant was brought from the microbiology lab to be inoculated into the medium aseptically.

PARAMETERS

The parameters to be monitored were temperature, pressure, aeration and agitation which were mostly stable. The dissolved oxygen content must be increased initially to 100% by aerating. The organism utilizes this oxygen for their growth during the process. The initial pH of the medium is kept at 6.5 with the help of caustic soda. The samples were withdrawn for every four hour interval to check the pH and Packed

Mycelial Volume (PMV). The normal growth characteristics were estimated based on the seed maturation criteria such as age, pH and PMV.

FERMENTATION

FERMENTER

Production fermenters with a size of 1000 litres were utilized for the fermentation. It was cleaned prior to media charging.

MEDIA STERILIZATION

The steam was passed in the outer jacket up to 90°C and then into the sparger up to 121°C at 1.2kg.m/s^2.

COOLING

The cold water at 5°C was passed through the outer jacket to cool the medium up to 25°C. Once cooling was achieved the fermenter was maintained under positive pressure to maintain the sterility.

SEEDING

When the batch in the seed tank gets matured i.e. the required PMV of 10-14% was achieved, it was transferred to the fermenter aseptically.

PARAMETERS

1) pH – 6.5

The initial pH was maintained at 6.5 by using Ammonium hydroxide (AMH) and it was maintained between 6.2 – 6.4 during the production.

2) Air flow– 1vvm

The aeration was maintained at 1vvm (volume of broth, volume air / min). It was increased with respect to the decreased in dissolved oxygen concentration.

3) Dissolved oxygen (DO)

The DO was maintained at 50-60% of the maximum level. It was dependent on the rate of aeration and agitation. DO decreases with increase in viscosity during the addition of sugar.

4) Temperature

By passing cold water in the outer jacket of the fermenter, the temperature was maintained at 25ºC

5) Residual PAA

The residual PAA was maintained at 0.5% of the total broth volume as the toxicity limit of the microorganism was 0.8%. The addition of PAA was based on the residual PAA value which was analysed once in 4hrs.

NUTRIENT ADDITION

Peristaltic pumps were used for the addition of nutrients such as

1) Sugar

Sugar may be in any form usually a sugar solution of concentration 700g/l solution was sterilized and added when required. A higher concentration of the sugar solution was added to prevent the overflow of the fermenter.

2) Phenyl acetic acid (PAA)

The sodium salt of phenyl acetic acid at a concentration of 500g/l was added to the fermenter. The amount added was based on the residual PAA value. It acts as a precursor for the production of Penicillin G.

3) Ammonium sulphate (AMS)

Ammonium sulphate solution of 40% concentration w/v was added to fulfil the nitrogen requirement of microorganisms. This AMS also supplies the sulphate required for the synthesis of Penicillin G.

4) Ammonium hydroxide (AMH)

20% w/v ammonium hydroxide solution was added for the pH adjustment and maintenance.

PROCESS DESCRIPTION

All the media components were added to the fermenter and sterilized in the fermenter itself. Then the microorganism was added from the seed tank which had a concentration of 7000 spores/ml. The seed volume should be 5% of the total volume of the media. The microorganisms grow up to the phase where the nutrients were added so that the production gets started.

Temperature, airflow, agitation are maintained through the supervisory control and data accuracy system (SCADA), a software which is directly connected to the hardware in the fermenter.

The SCADA systems, monitors the pH and DO variation which has to be adjusted manually. All these processes go on for 180 hrs and in every 8hrs samples were taken to be checked in the microbiology & quality control laboratory.

MICRO BIOLOGY TESTING

1. Sterility

This test was done to check bacterial and other fungal contamination.1 ml of spore suspension from the harvested flask was added to nutrient agar slants, nutrient broth flasks, Sabourauds Dextrose Agar (SDA) slants and sabourauds dextrose broth flask in sterile condition.

2. Viability test

This test was done to check the viability of the spores in the suspension. The viable count media was prepared and the spore suspension was serially diluted to 10^5 dilutions. Spread Plate Method under aseptic condition is used for each dilution on 3 viable count media plates

The plates were incubated at 25°C for 5 days. After incubation, the colonies were counted to check the viability of the spores in the suspension.

$$\% \text{ of viability} = \frac{\text{viable count}}{\text{Total count}} * 100$$

QUALITY CONTROL TESTING

1. **Residual PAA and activity**

Residual PAA and activity were measured with the help of High performance liquid chromatography system (HPLC). The stationary phase used was octadecyl silane and the mobile phase was a mixture of acetonitrile and water.

Stationary phase: Octadecyl silane

Mobile phase consists of 80% 0.1M potassium dihydrogen phosphate buffer with 20% acetonitrile.

2. Residual sugar

This was measured using Fehling's reagent. To the freshly prepared Fehling's reagent 30% of 10ml KI and 28% of 10ml Sulphuric acid was added along with the sample. This mixture was titrated with 0.1N sodium thiosulphate. From the titre value the percentage of sugar was calculated. Starch was used as an indicator and the end point was the appearance of white colour.

3. Nitrogen

Nitrogen was estimated using Kjeldahl's method. In this, the liberated ammonia was converted to ammonium tetra borate and was titrated with 0.05 N HCl. The amount of nitrogen present was expressed in terms of parts per million.

4. pH

The probe must be calibrated using standard buffers before measuring the broth pH. A pH meter with a calomel glass electrode was used for this purpose. The variation in pH assists in addition of ammonium hydroxide.

TERMINATION

After 180 hours when the activity shows a decreasing trend, 0.5% formaldehyde was added to the fermenter to kill microorganisms. The broth was then transferred for recovery.

2.2.4. RECOVERY

Down stream processing technique is used for recovery of product. This was done to get the stable form of product. This process of extraction was based on the elevation of boiling point.

PROCESS DESCRIPTION

Initially the broth pH and wet broth activity were checked and the broth was diluted. The pH of the diluted broth was adjusted to 2 ± 0.2 using 15% sulphuric acid and the volume of acid consumed was noted. 3% of demulsifier was added to form a distinct layer between butyl acetate and the broth. Half the broth volume of butyl acetate was added to the broth with simultaneous agitation for 10 minutes. The mixture was poured into a separating funnel and allowed to stand for 15 minutes and then the spent broth was decanted. The Penicillin rich BAC was collected separately. The Penicillin rich BAC was then washed with equal volume of water. 7.5 ml of 3% carbon slurry solution was added to 100 ml of penicillin rich BAC. They were stirred well and filtered through a whatman no: 5, filter paper. This was done till the penicillin rich BAC gets decolourised. The decolourised Penicillin rich BAC was taken in a separating funnel and 13% potassium carbonate solution was added.

The solution was allowed to settle for 10 mins and the rich aqueous were collected. The pH of the Penicillin rich aqueous layer was between 7.5-8.2.The pH of the rich aqueous was brought down to 6.4-6.7 using acetic acid. One volume of the aqueous phase was mixed with three volumes of butanol. This was then taken in a round bottom flask and crystallized using a rotary evaporator. After some time, the mixture comes to a semisolid form which was filtered in a Buchner funnel. The Penicillin G crystals were then washed with butanol and dried. The amount of Penicillin G was finally weighed after drying.

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RESULTS AND DISCUSSION

SPORULATION

After 14 days of incubation, a green layer appears over the surface of the rice in the flask. The appearance of green colour indicates the growth of the organism. The spores are then harvested from the rice flask using a surfactant (tween 60) and are inoculated in to the seed fermenter.

SEED FERMENTATION

The fermentation in the seed tank was terminated only when the following conditions are satisfied

1. 60 hours of age is reached.
2. Packed Mycelial Volume (PMV) is greater than 12%.
3. pH starts to decrease

When these conditions are satisfied the seed fermenter constituents are transferred to the production fermenter.

FERMENTATION

Trail 1: Normal batch

The final broth activity for the normal batch was obtained as 59710 IU/ml.

The activity shows an increasing trend throughout the fermentation process (fig 1). When the percentage of increase in activity decreases the process was terminated.

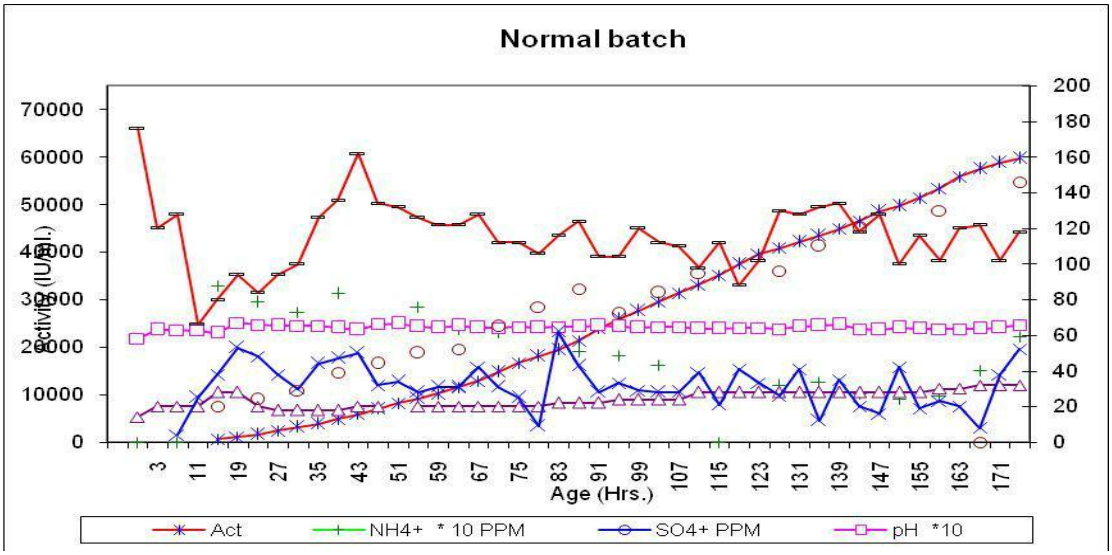

Figure 1: Parameter graph for trial 1

Trial 2 – (0.2 g/l) PAA induction

The final broth activity before termination was 63840 IU/ml. 6% increase in the production than the normal batch was observed (fig 2).

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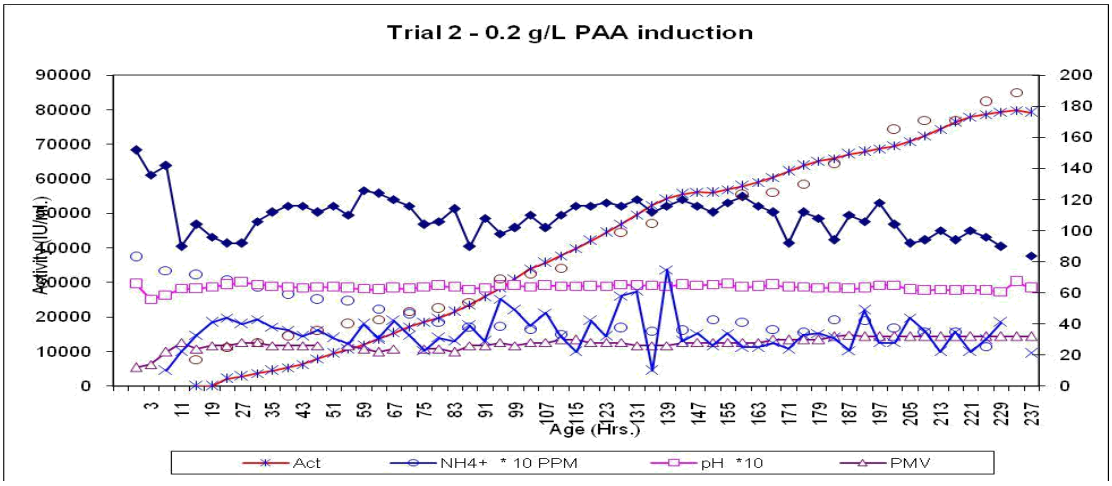

Figure 2: Parameter graph of trial 2

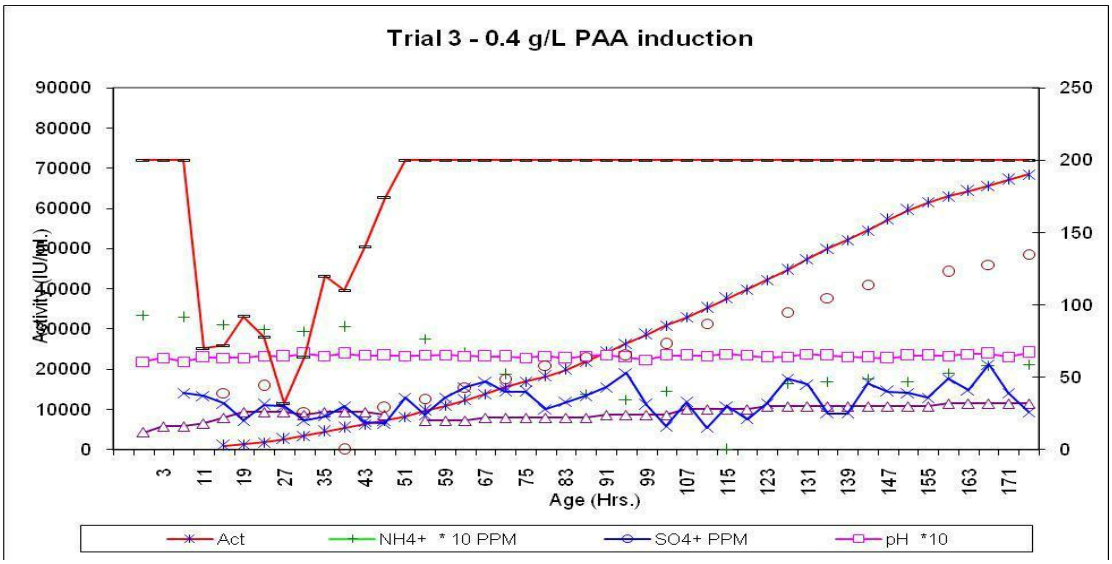

Figure 3: parameter graph for trial 3

Trial 3 – (0.4g/l) PAA induction

The final broth activity was obtained as 68550 IU/ml which was 12% greater than that of the normal batch. It was evident that the mutational effect in this trial (fig 3) was more significant than the previous trial.

Trial 4 – (0.6 g/l) PAA induction.

The final broth activity was obtained as 57620 IU/ml (fig 4). This value was less than that of the final activity of the normal batch.

The profiles depict a detailed description of the process, including all the additions as well as the removals. The aertion and agitation are mostly kept to a maximum value. pH was maintained constant by adding ammonium hydroxide. In the third trial the dissolved oxygen probe posed a major problem and recorded as 100% throughout the process.

The activity shows different trends in various concentrations. The activity considerably increases when 0.4 mg/ml of PAA is added. The production was thus, maximum at this concentration.

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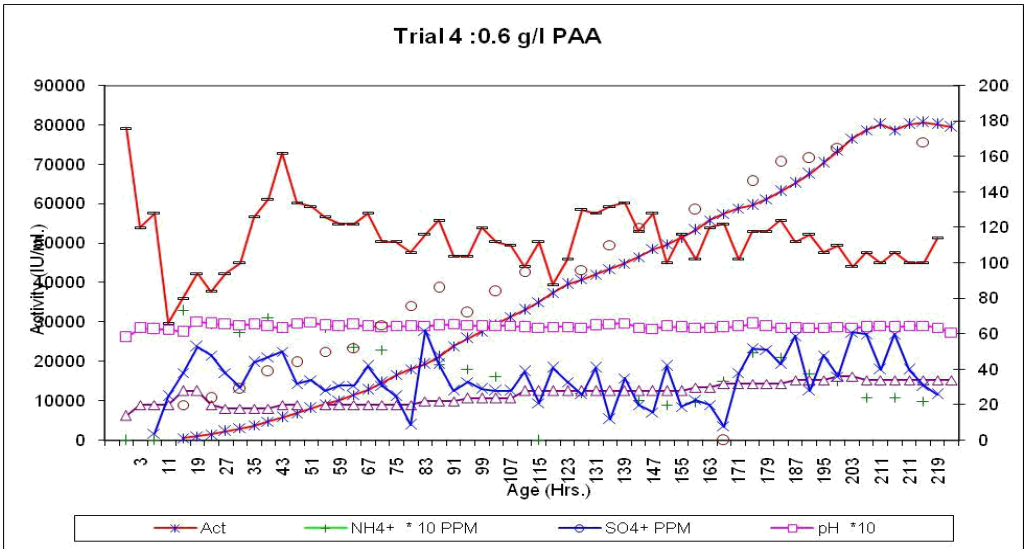

Figure 4: Parameter graph for trial 4

There was a 12% increase in the production of penicillin G in the trial of concentration 0.4 g/l compared to the normal batch. This increase was due to the development of resistance against phenyl acetic acid by the organism. The organism got accustomed to the PAA containing environment at this stage which was responsible for the increase in production of penicillin G.

Table 1: Activity vs concentration profile

Trial No.	Total volume of the fermenter (litres)	Age (hours)	Concentration of PAA(g/l)	Activity (IU/ml)	Total amount of Penicillin G produced (BU)
1.	1000	175	0.0	59710	45.67
2.	1000	175	0.2	63840	48.83
3.	1000	175	0.4	68550	52.44
4.	1000	175	0.6	57620	44.07

Table 2: Amount of penicillin G produced

Trial no	Concentration of PAA added (g/l)	Amount of penicillin G produced(g)
1	0.0	31.6
2	0.2	32.9
3	0.4	39.7
4	0.6	31.1

RECOVERY

The amount of penicillin G obtained after recovery is tabulated in Fig 2. The amount of penicillin G crystallized is directly proportional to the activity of final broth. Thus, the amount of penicillin G obtained is higher in the trial of concentration 0.4 g/l of PAA.

CONCLUSION

A good increase in the total penicillin G yield was obtained in submerged cultures of *penicillium chrysogenum* with the addition of 0.4 g/l of phenyl acetic acid (PAA) in a seed fermenter media. The addition of phenyl acetic acid during the growth phase reduces the effect of initial shock experienced and increases the

production. The organism develops maximum resistance to phenyl acetic acid at a concentration of 0.4 g per liter of the media.

Thus the optimum concentration of phenyl acetic acid to be added for penicillin production in the seed fermenter can be 0.4 g of PAA per liter of the medium. In SPIC, phenyl acetic acid (PAA) was not one of the media constituents in the seed tank, but the addition of 0.4 g/l of PAA can be recommended to increase production of penicillin G.

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