Effect of Aeration on Growth and Production of Violacein by Chromobacterium violaceum using a Bubble Column Reactor

Mary Anupama Palukurty

Senior Assistant Professor
Dept. of Chemical Engineering and Biotechnology
ANITS, Sangivalasa, Visakhapatnam
Guru Mahesh Darsi, Subba Rao Somalanka

Dept. of Chemical Engineering, ANITS, Sangivalasa, Visakhapatnam

Abstract- Violacein is a pigment that has diverse applications in the field of medicine. It is majorly produced by Chromobacterium violaceum, and submerged production of the dye using bubble column reactor is the present nature of study. The present investigation is done using Optimized medium whose composition is Wheat bran, soya granules powder and few salt supplements in a bubble column reactor. The medium is aerated at 5, 7.5 and 10 liters per hour growth and production is monitored. Highest yield of 1.82g/L of violacein is obtained. FTIR studies have revealed that the product is a mixture of deoxy- and oxy-violacein.

Key words: Violacein, Chromobacterium violacein, Bubble column reactor, Production medium and FITR studies.

1. INTRODUCTION

Violacein is a bisindole dye, produced by the opportunistic pathogen bacterium Chromobacterium violaceum. This purple colored pigment is produced by many bacterial species, while the best source is identified as Chromobacterium violaceum [1]. Tryptophan has been identified as the major carbon source and the other physical variables that contribute to production of violacein are pH 7, temperature 28^{o}C and time of production is 24hours [2, 3]. Previous studies on violacein production using alternative and economically viable sources were conducted and optimization of production parameters was done by submerged studies [4]. Studies were done by various researchers on various methods of production of violacein which include both solid state and submerged methods [5,6]. Oxygen supply is known to increase violacein production [7] and it has been proved that production in bioreactor gave better results than in shake flask studies [8]. Hence in the present investigation effect of aeration on violacein production was studied using a fabricated bubble column reactor using final optimized medium.

2. MATERIALS AND METHODS

2.1 Microorganism chosen and its maintenance:

The bacterial strain of Chromobacterium violaceum MTCC No: 8071 obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India was used throughout the study. The culture is maintained in nutrient agar slants and is sub cultured once in a fortnight and stored at 4℃ until futher use.

2.2 Preparation of seed culture:

Nutrient broth is used for the production of seed culture of Chromobacterium violaceum was prepared in nutrient broth. To a slant of the organism, 10ml water was added to disperse the bacterium. 1ml of the cell suspension is added to 100ml of sterilized nutrient broth and left for 24hr at 28℃ and 150rpm. The 24h aged culture is used as seed for studies using bubble column reactor.

2.3 Optimized production medium:

The components of final optimized medium used for production of violacein has few inexpensive components which include- Wheat bran- 4.5, Soya granules powder- 4, Ammonium sulphate- 0.1, NaCl- 8, $FeSO_4$- 0.008, $MgSO_4$ – 0.8, K_2HPO_4- 3, $ZnSO_4$ – 0.25 and $CaNO_3$ – 0.3. Same conditions are maintained as seed culture production studies except that the inoculum levels are maintained at 4.4% which is from optimization studies.

2.4 Reactor design:

A 5 liter bubble column reactor was fabricated for violacein production. The working volume can be kept at 3 liters and appropriate arrangements were made to regulate aeration, for addition of sterilized medium, inoculation of seed, and intermittent sample withdrawl. The figure 1 below shows reactor with aeration set up that was sued in the present studies.

2.5 Aeration set up:

Aeration was given from the bottom of the reactor. A compressor is attached to the rotameter whose outlet is connected to Whatman HEPA CAP 75 filter. This line filter device that will retain 99.97% of all the particles, ≥ 0.3µm. The outlet of the HEPA CAP 75 (consists of sterilized air) is in turn attached to the hose, that is at the bottom of the reactor. To connect all the equipment in a sequential manner, silicon tubing is used, which are always autoclaved at 121˚C, at 15 lb pressure for 15 min for sterilization.

The whole setup was mounted on a steel frame. The product is collected at the end of every 12h to estimate colony count and violacein. Aeration was given as follows, 5 Liters per hour, 7.5 liters per hour and 10 liters per hour.

2.6 Extraction of Violacein:

At the end of 24h, 50ml of production medium was taken to which 50 ml of ethanol was added and left for 30 minutes in shaking. This leads to dissolution of cell wall of the bacteria to release the dye. Then 25 ml of ethyl acetate is added and left for solvent separation. The dye comes into ethyl acetate layer of

which 2 ml is used to used for estimation of violacein. The rest of the ethyl acetate extract of violacein is subjected to solvent recovery at 50^{o}C using Soxhlet apparatus. At 95% concentrate, the thick slurry was poured into petriplate and left in oven at 40^{o}C for the residual solvent to evaporate. The powder which is left in the petriplate is violacein collected, weighed and used for characterization.

Characterization of violacein:

Characterization of violacein was done by FTIR studies. The Fourier transform infrared spectroscopy studies help to identify the sample and this is done based on the finger print generated. It helps in chemical identification of the samples. The FTIR uses infra red radiation of about 10,000 to 100 cm^{-}1. The resulting signal at the detector presents as a spectrum, typically from 4000cm^{-1}to 400 cm^{-1} representing the pigment structure. The FTIR studies are done for 7.5 LPH and 10 LPH.

Preparation of KBr pellet for FTIR:

About 125mg of the solid sample is taken and 250mg of KBr is added and mixed thoroughly using mortar and pestle. The compressed pellets of the same were taken for FTIR studies and scanned for 45 min. The graph thus obtained is between % transmission and cm^{-1}would be obtained which can be interpreted for purity of violacein.

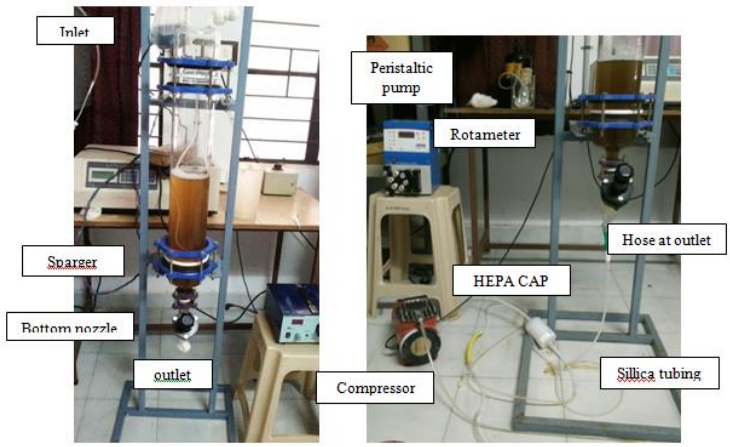


Figure 1. Bubble column reactor and Aeration setup

3. RESULTS AND DISCUSSION

3.1 Production media in bubble column reactor:

Effect of aeration on growth and production of the secondary metabolite was studied. Variation in aeration was shown to effect increase in production of the secondary metabolite. The images that are shown in figure 2 gives a comparative picture of effect of no aeration and when 7.5 Lph aeration was given at zero time and at the end of 24 hours. Optimal production of violacein was observed at 7.5 LPH for the organism Chromobaterium violaceum under the specified conditions among the chosen range (5LPH, 7.5LPH and 10LPH).

OD was taken for samples at the end of every 24 hours and the results are presented in table 1. When compared to zero aeration (0LPH), there is a systematic increase in production of violacein till 7.5 LPH.

From then on a decrease was noticed, which indicates that excess aeration can be harmful to the organism. Along with tryptophan, oxygen levels also contribute to increase in violacein which is reflected in the results. While further increase in aeration led to increase in froth associated with fast pigment release was noticed with decrease in net violacein production. Shearing of cells at high aeration also leads to decrease in production of the secondary metabolite. These indicate that 7.5LPH of aeration resulted in better yield. When 10LPH was given, no such increase in color was observed rather intense foaming was noticed.

Figure 2: Images representing effect of no aeration and aeration at 7 LPH on violacein production

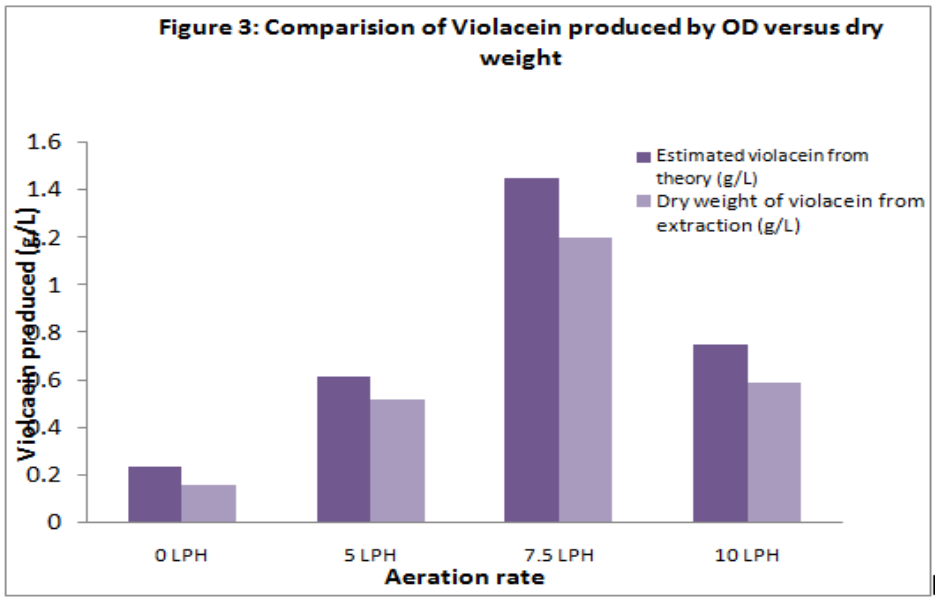
<table>
<tr>
<td>No aeration, seed percentage-4.4%, 0th hour
  </td>
<td>No aeration, seed percentage-4.4%,24th hour
  </td>
</tr>
<tr>
<td>7.5 liters per hour aeration,
seed percentage-4.4%,
0th hour
  </td>
<td>7.5 liters per hour aeration,
seed percentage-4.4%,
24th hour
  </td>
</tr>
</table>

Table 1: Effect of aeration on violacein production:

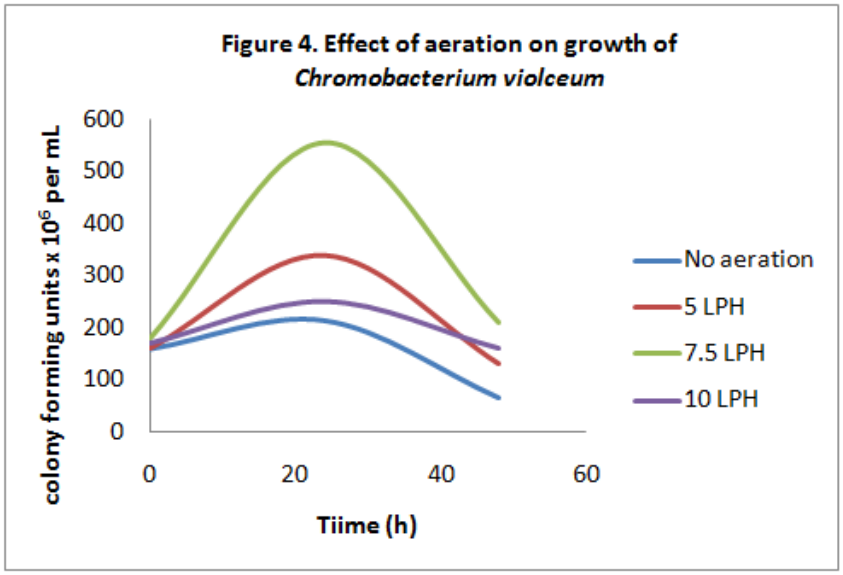
S.No	Seed%	Aeration(LPH)	Violacein (g/L)
1.	4.4	No aeration	0.24
2.	4.4	5.0	0.613
3.	4.4	7.5	1.4525
4.	4.4	10.0	0.75

Comparison of Violacein produced:

Comparison of violacein results obtained by using OD and the dry weight of extracted product is presented in figure 3. .The percentage variations for the data obtained for no aeration, 5 LPH, 7.5 LPH and 10 LPH are – 33%, 15.4%, 17.2% 16% respectively. The variation could be due to problem in collecting dried violacein powder from petriplate. While in increased levels of loss for no aeration method cannot be explained with the same reason.



Effect of aeration on growth of Chromobacterium violcaeum: The following table represents the variation in cell count for production medium. A steady increase is observed till 7.5 LPH while further increase in aeration has reduced the cell count due to its negative effects.



FTIR Results:

Studies on samples obtained from bubble column reactor aerated at 7.5 LPH, containing wheat bran and soya granules flour as tryptophan contributors was done. Presence of violacein should result in sharp decrease in % of transmittance between 2800-3100 cm^{-1} due to the presence of C-H bond, while C=O bond would result in depression between 1000 to 1800 cm^{-1}, O-H bond at 3300-3600 cm-^{1}. A C-C or C-N bond would show a sharp downfall at 2200-2100 cm^{-1} and C-O shows at 1200- 1300 cm^{-1}.

A sharp peak at hydroxyl group in Figure 5, indicates presence of violacein that has hydroxyl groups. More concentration of deoxyviolacein must have been present as compared to Violacein as indicated from the decrease in transmittance, hence it may be concluded that it is a mixture of violacein and deoxyviolacein. Bacteria are known to produce both the forms of violacein [9] and deoxyviolacein is also known for its potential applications [10].

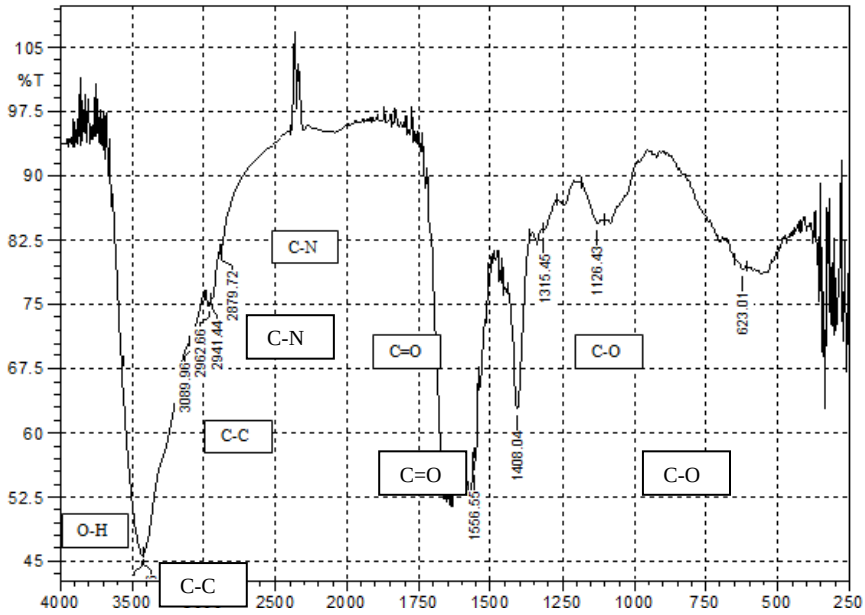


Figure 5: FTIR results of violacein obtained at 7.5LPH aeration

4. CONCLUSION

Bubble column reactor studies for production of the potential antimicrobial and anticancerous agent, Violacein, indicate that aeration plays vital role in the production of this secondary metabolite. Inoculation of 4.4% (v/v) seed culture was ideal for violacein production and an aeration of 7.5 Litres per hours has resulted in 1.82g/L of violacein production. The dye was evaluated for its purity by FTIR which indicated that it is a mixture of violacein and deoxyviolacein.

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