FINAL REPORT OF UGC – MRP
MRP-MAJOR-BIOT-203-9237

TITLE: OPTIMIZATION OF PRODUCTION PARAMETERS, EXTRACTION AND CHARACTERIZATION OF A MEDICINALLY IMPORTANT DRUG VIOLACEIN BY SOLID STATE FERMENTATION

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1st July 2015 to 30th June, 2018

PROJECT REPORT

Title of Project: Optimization of production parameters, extraction and characterization of a Medicinally important Drug Violacein by Solid state fermentation

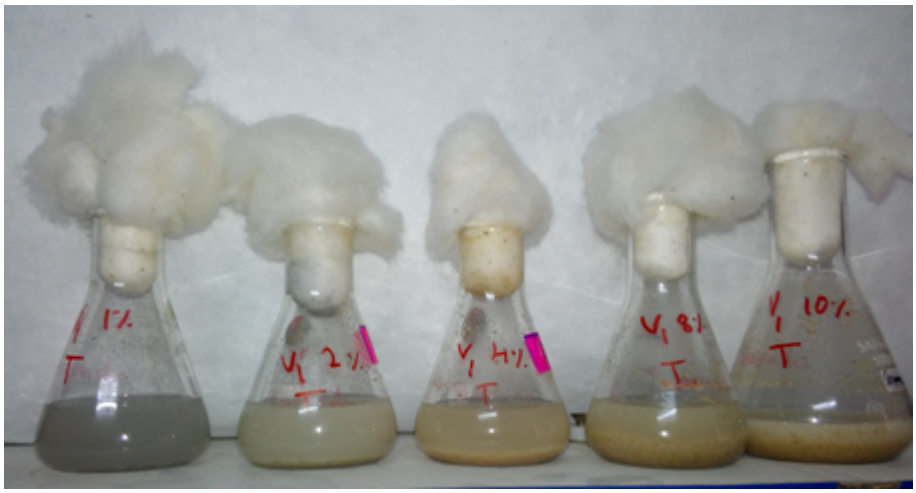
Objectives of Project work:

- Screening of few alternative nitrogen supplements
- Optimization of variables for Solid substrate fermentation by RSM
- Scale up studies
- Extraction and evaluation of product

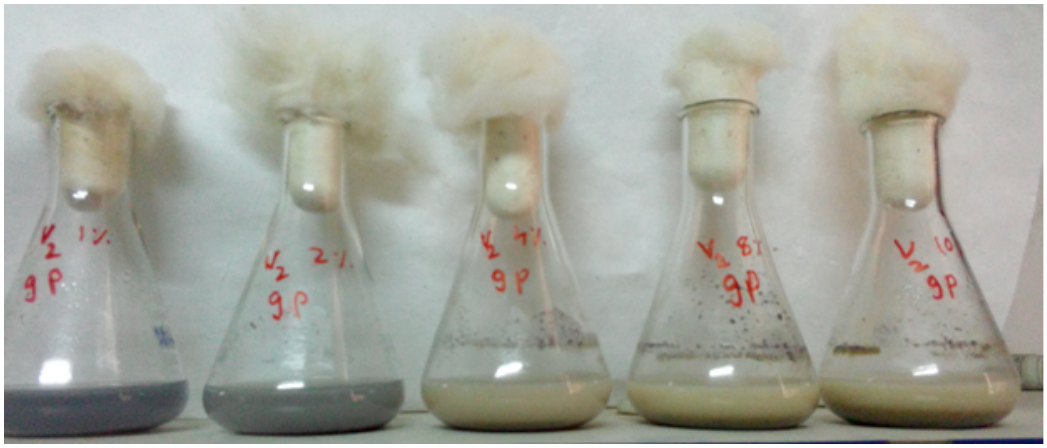
1. Screening of alternative nutrients:

Plant based materials tested- Rice bran, Wheat bran, corn flour, Green gram bran, Black gram bran. Wheat gram bran was found to be better among the natural ones.

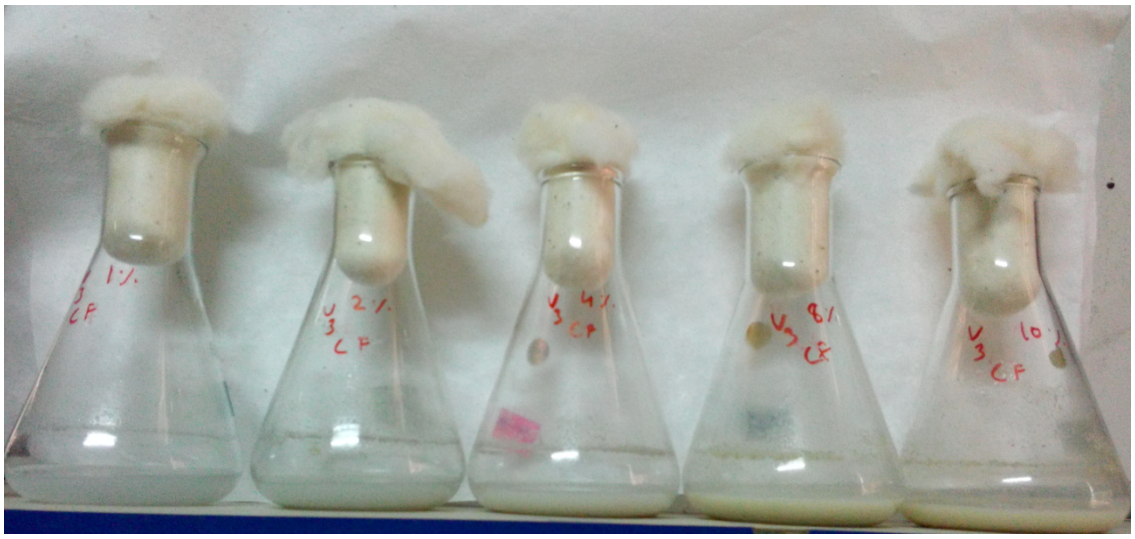
Effect of Rice bran (% w/v) : 1, 2, 4, 8, 10



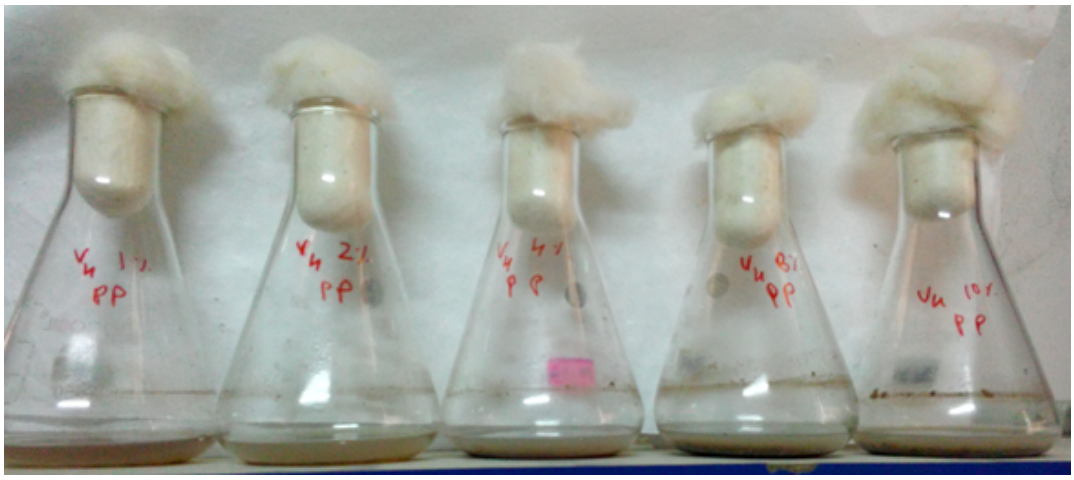
Effect of Wheat Bran (% w/v) : 1, 2, 4, 8, 10



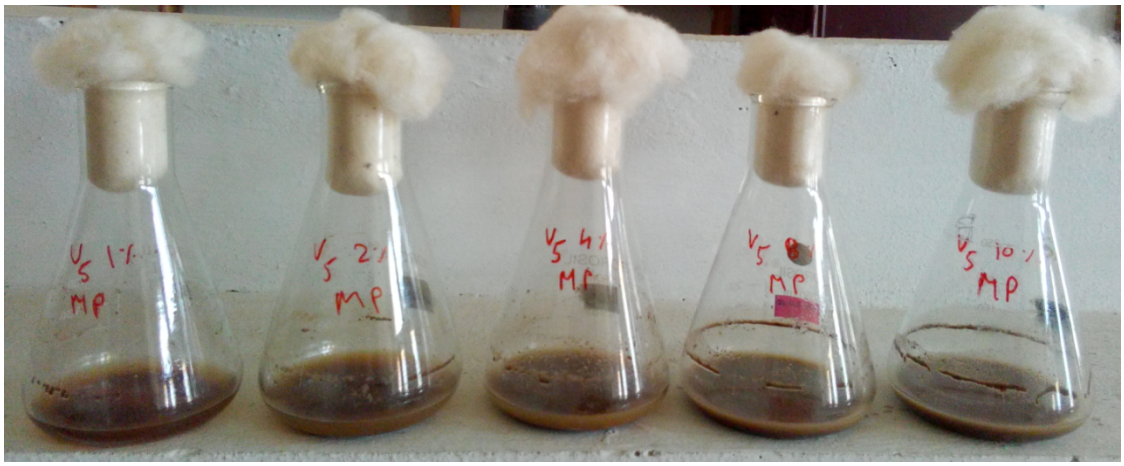
Effect of Corn Flour: (% w/v) : 1, 2, 4, 8, 10



Effect of green gram bran (% w/v) : 1, 2, 4, 8, 10

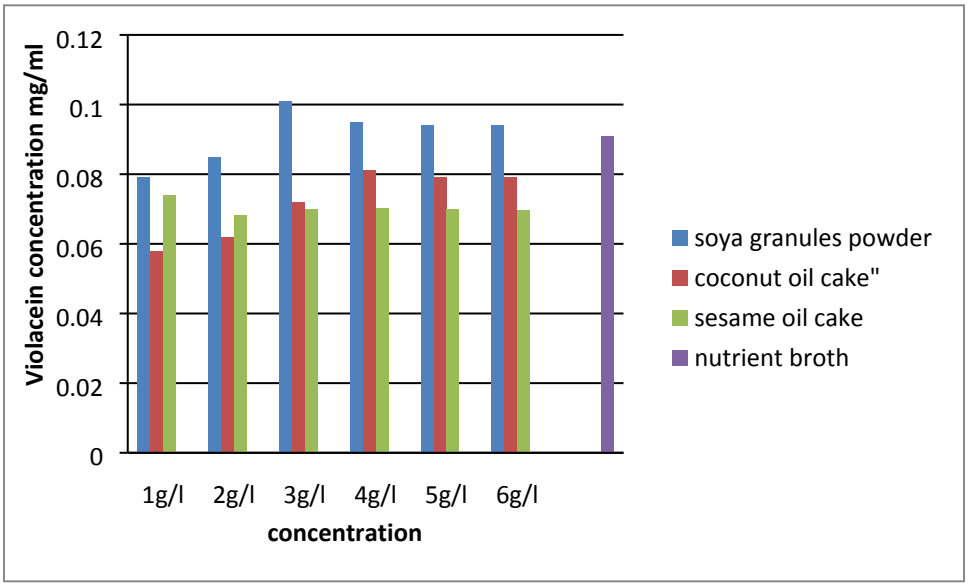


Effect of Black gram (% w/v) : 1, 2, 4, 8, 10

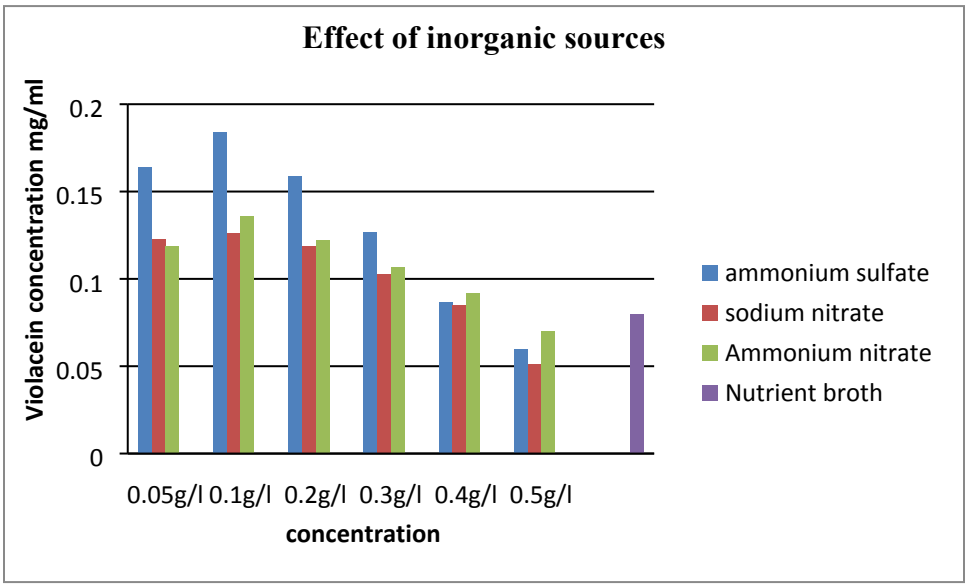


This was compared with Ground nut fodder, Sesame oil cake, coconut oil cake, soya granule powder, the natural ones with Yeast extract, Peptone, Malt extract and beef extract.

Wheat bran, Coconut oil cake, sesame oil cake, Soya granules powder were found to be significant. While Yeast extract and malt extract were found to be very effectively contributing to violacein production.

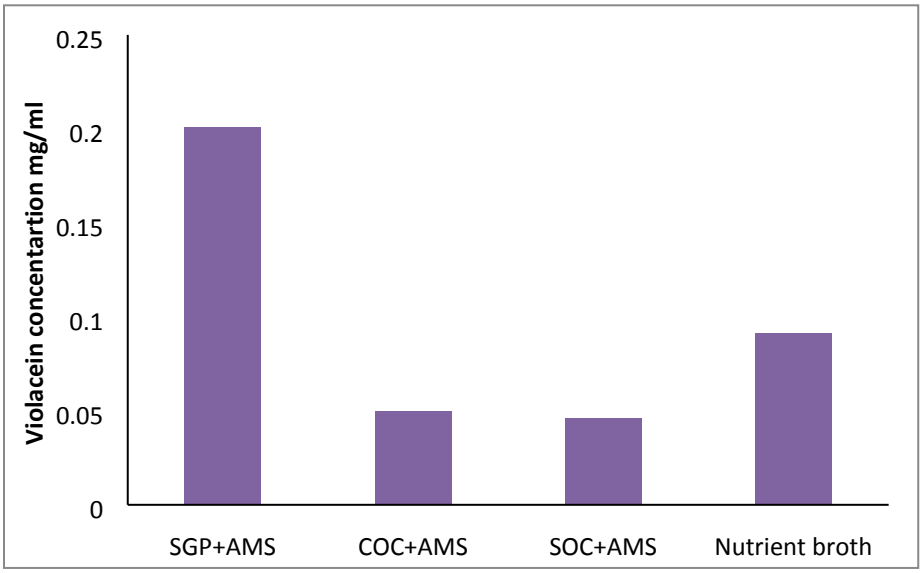


Inorganic nitrogen supplements were also tested in combination with organic ones, when added to wheat bran as the primary carbon and nitrogen source, Ammonium sulphate has resulted in good Violacein production.



Effect of combination of organic and inorganic nitrogen sources:

Though the soya granules powder and ammonium sulfate were observed as the best nitrogen sources for the production of violacein the other nitrogen sources were also tested in combination with the ammonium sulfate (AMS) but the combination of soya granules powder(SGP) and ammonium sulfate was observed as the best. (COC- Coconut oil cake, SOC – Seasmum oil cake)



Combination of Wheat bran, Soya granules powder and Ammonium sulphate was tried. Wheat bran with soya granules powder and 1mg/L Ammonium sulphate present in the first flask has shown better violcaien formation.

Effect of Mineral supplements: From the literature various elements were found to contribute to increased production of violacein. In order to screen these Plackett-burman design was used. The range of variables studied and amount of vioaclein produced in shake flasks is given in the table below.

Screening of mineral supplements was done from literature using Plackett-Burman design.

	NaCl (g/L)	$Ca(NO_3)_2$ (g/L)	$FeSO_4$ (g/L)	$MgSO_4$ (g/L)	$Na_2HPO_4.12H_2O$	K_2HPO_4 (g/L)	$ZnSO_4.7H_2O$
1	2.000000	0.300000	0.008000	0.800000	6.500000	9.000000	0.250000
2	8.000000	0.300000	0.008000	0.200000	0.500000	9.000000	0.750000
3	2.000000	0.900000	0.008000	0.200000	6.500000	3.000000	0.750000
4	8.000000	0.900000	0.008000	0.800000	0.500000	3.000000	0.250000
5	2.000000	0.300000	0.016000	0.800000	0.500000	3.000000	0.750000
6	8.000000	0.300000	0.016000	0.200000	6.500000	3.000000	0.250000
7	2.000000	0.900000	0.016000	0.200000	0.500000	9.000000	0.250000
8	8.000000	0.900000	0.016000	0.800000	6.500000	9.000000	0.750000
9	5.000000	0.600000	0.012000	0.500000	3.500000	6.000000	0.500000

Except for Na_2HPO_4 rest of the elements were found to be significant after analysis using statistical software.

2. Optimization of conditions

Systematic optimization of physical parameters like, pH, inoculum age and inoculum level was followed by screening of other variables. This was done by submerged method and applied to surface cultivation method. A paper was published on the first set of studies, "Analyzing Alternative Nutrient Supplements and Optimization of Production Parameters for Violacein using Central Composite Design" International Journal of Scientific & International Research, vol.7, Issue 7, July-2016, pp. 294-300. (Enclosed), another paper is in the process of publication.

3. Reactor design:

Necessity- from the previous studies it was noticed that product formation is influenced by dissolved oxygen levels. Few publications exist on culturing the organism on cotton mats, on slats, in petri plates and so on. This is supposed to contribute to better oxygen availability than submerged fermentation. Hence various reactors were designed and the aim during this process was to have ideal utilization of available area and to obtain maximum surface area for culturing the organism after coating with nutrient agar. This would be followed by coating the optimized medium. At the same tine a bubble column reactor was fabricated to see the effect of aeration for comparison. The bubble column reactor is a 5 litre column reactor whose working volume is kept at 3 litres. The reactor has two inlets, one for medium and the other for airflow. The nozzle at the base can be regulated and aeration set up was attached to this for sterile air flow. Its aspect ratio is 10:1.

Outcome: The following paper incorporates the designs prepared, available surface area and assumed product concentration.

"Perspective Approach on Reactor design for Surface Cultivation of *Chromobacterium violaceum*". International Journal of Innovative Research in Science and Engineering , vol 2, Issue 8, August 2016: 153-160. ISSN-2347- 3207

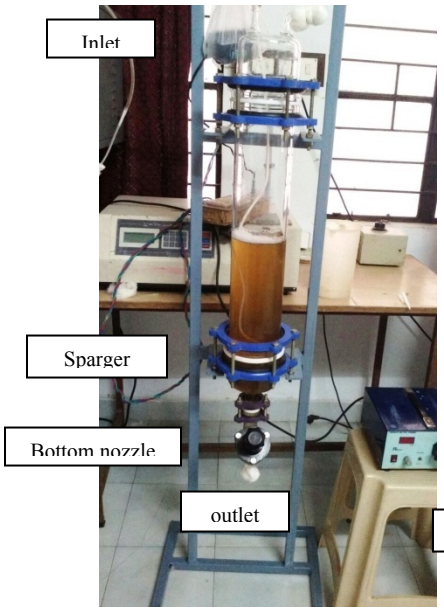
Patent has been filed on the reactor

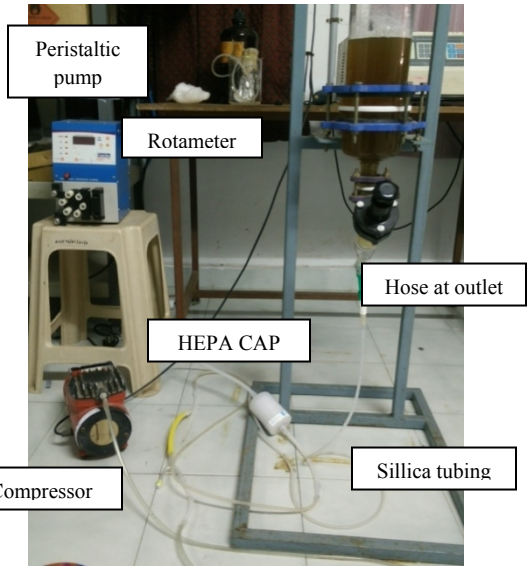
4. Reactor studies: These were done by submerged cultivation method and also by surface cultivation method.

a) Studies using Bubble column reactor:

The bubble column is surface sterilized while the medium is autoclaved, The temperature was maintained at around 28˚C by insulating the walls of the column. Seed percentage is maintained as 4.4% (v/v) as per preliminary studies. For studies using nutrient broth as well as optimized medium conditions remain same. Samples were withdrawn every 12h to see product formation.

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. The parameter was varied as follows- No aeration, 5 Liters per hour, 7.5 liters per hour and 10 liters per hour.





Bubble column reactor setup **Aeration setup**

b) Studies using Novel reactor: Studies were carried out by coating the surfaces of the reactor with nutrient broth initially and then production medium. After coating the medium the reactor is left for dye production. Results evaluation was done at the end of 24h.

Studies were done using both nutrient agar and optimized medium in this reactor. Care for taken to maintain sterile conditions.

5. Evaluation of results: At the end of 24h, 50ml of sterilized water is added to the reactor 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 which separates from ethanol and come out to the surface in the separating funnel. 2 ml of this same is ethyl acetate extract is used for checking OD.

The rest of the ethyl acetate extract of violacein is subjected to solvent recovery at 50^{o}C using Soxhlet apparatus. As the extract concentrates and around 95% of solvent is recovered, slurry is left in the round bottom flask. This is then 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 which is weighed and used for characterization.

6. Characterization of violacein:

The following are the characterization tests done- solubility tests, FTIR and antimicrobial assays.

a. Solubility tests:

The solubility tests were done to check the minimal amount of pigment that can be dissolved in the solvent system. Solvent systems used include acetone, ethyl alcohol and ethyl acetate and their concentration is varied as follows (violacein w/v)- 0.1 mg/mL, 0.05mg/mL, 0.025 mg/mL, 0.0125mg/mL, 0.00625 mg/mL. The optical density is measured by a spectrophotometer. UV-VIS spectrophotometer is used. The absorbance is measured at 560 nm.

b. FTIR studies: 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:

The solid sample from the production runs were taken for FTIR analysis. About 1/8th of the solid sample is taken with a micro spatula. About 0.25g of KBr is taken. They were mixed thoroughly in a mortar while grinding with pestle. If the sample consists of large crystals, then it can be ground separately before adding KBr. This mixture is then placed on the bottom of the pellet die. Then a pressure of 5000-10000 psi is applied on the die. A pellet would be formed. Carefully remove the pressed sample from the die and place it in the FTIR sample holder. The pressed disc should be nearly clear. If it is translucent, it has to be regrinded and repressed.

The pellet is scanned for 45 min and the graph between % transmission and cm^{-1}would be obtained. From the graph, interpretations can be done, whether the obtained pigment is violacein or not.

C. Antimicrobial activity:

The sensitivity of the bacterial isolates to violacein was tested on nutrient agar media. The micro organisms tested against violacein are: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Bacillus cereus*. The concentration of violacein varied from 0.1 mg/mL to 0.025 mg/mL in ethanol and 15 micro liters is loaded into wells. Pure ethanol is kept as control.

7. Evaluation of growth kinetics:

Microbial growth is an autocatalytic process. Viable cell count was determined by serial dilution method and the rate of growth. The bacterial growth refers exponential increase in the cell count

in the organism. The growth kinetics is the relation between specific growth rate (μ) and concentration of substrate (K_s).

Determination of K_s:

Ks is the saturation constant. For determination of the saturation constant, Monod's kinetics were employed.

$$\mu = \frac{\mu \max S}{Ks + s}$$

where, μ is the specific growth rate (1/time) for the culture

μ_{max} is the maximum growth rate (1/ time) for the culture

S is the substrate concentration,

K_s is the half saturation constant (g/L), which is also known as the affinity constant.

To determine the saturation constant, a graph is plotted between colony forming units/ mL and the time taken to achieve that growth.

Then the slope of the curve is calculated. This is the specific growth rate of the culture. This is noted down as μ. The maximum specific growth rate (μ_{max}) is given as half of the specific growth rate.

S is the amount of L-Tryptophan that is consumed in the process of production of violacein.

Then the K_s(w/v) is calculated from all the above data. K_s is described as Monod's constant. Rate of enzymatic reactions can be determined from this basic calculation. Ks is the substrate concentration, that supports half the maximum of nutrient uptake or growth.

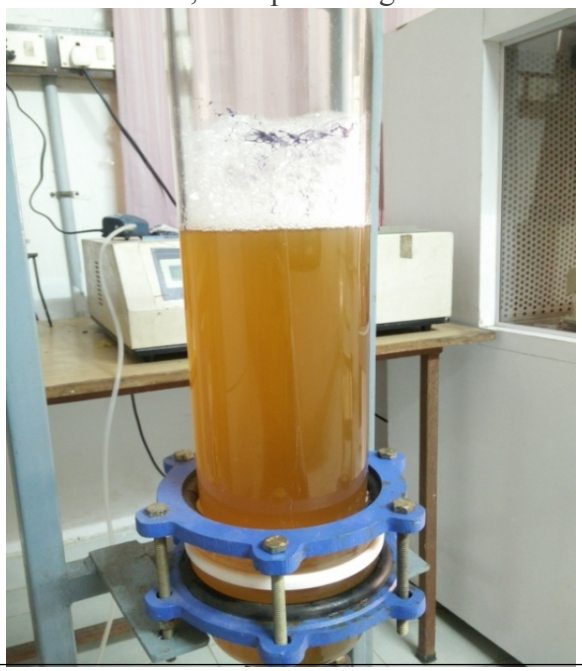
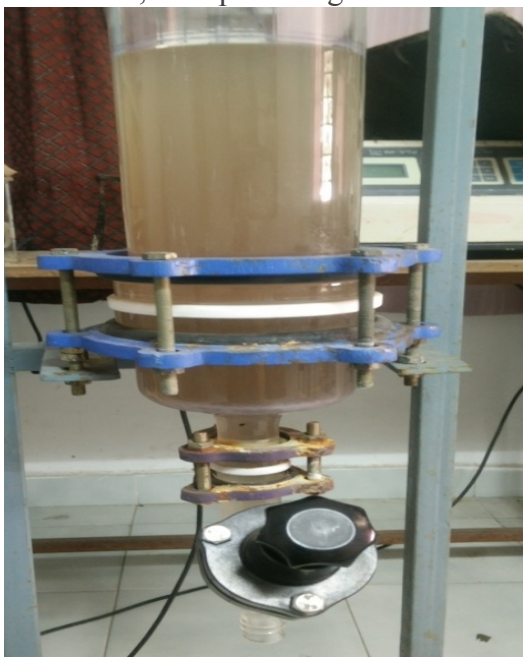
RESULTS

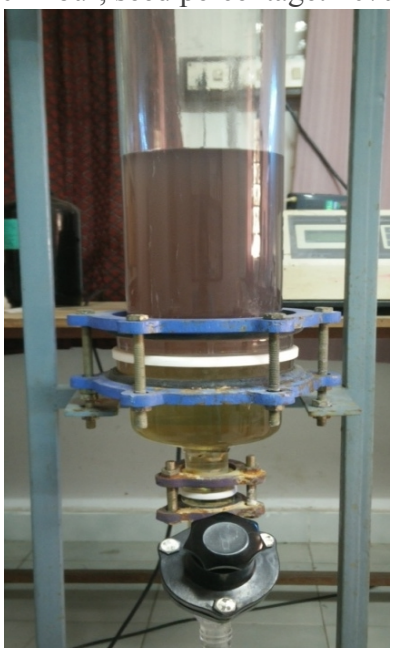
BUBBLE COLUMN REACTOR

Results of Production using nutrient broth:

Effect of Aeration:

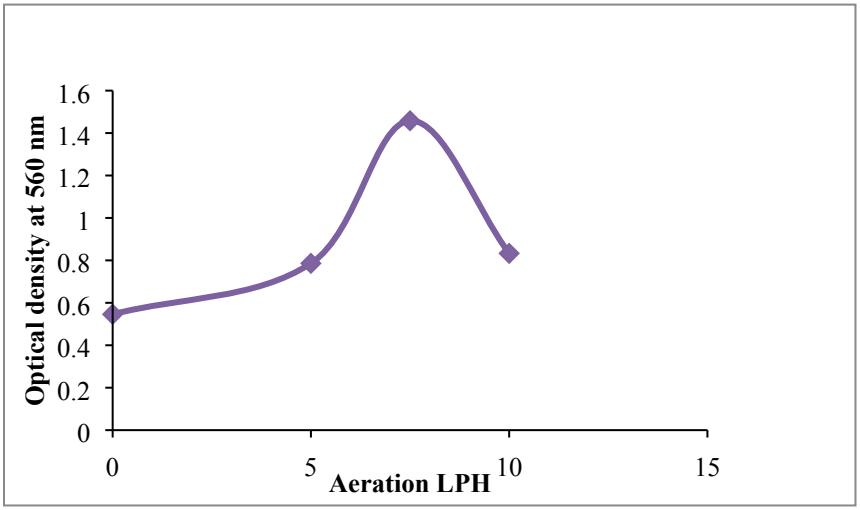
The following are the figures representing all modes of aeration.

No aeration mode: 0^{th} hour, seed percentage: 10% 	**No aeration mode:** 24^{th} hour, seed percentage :10% 
5.0 litres per hour of aeration: 0^{th} hour, seed percentage: 10% 	**5.0 litres per hour of aeration:** 24^{th} hour, seed percentage: 10% 

7.5 litres per hour of aeration: 0th hour, seed percentage: 10% 	**7.5 litres per hour of aeration:** 24th hour, seed percentage: 10% 
10.0 litres per hour of aeration: 0th hour, seed percentage: 10% 	**10.0 litres per hour of aeration:** 24th hour, seed percentage: 10% 

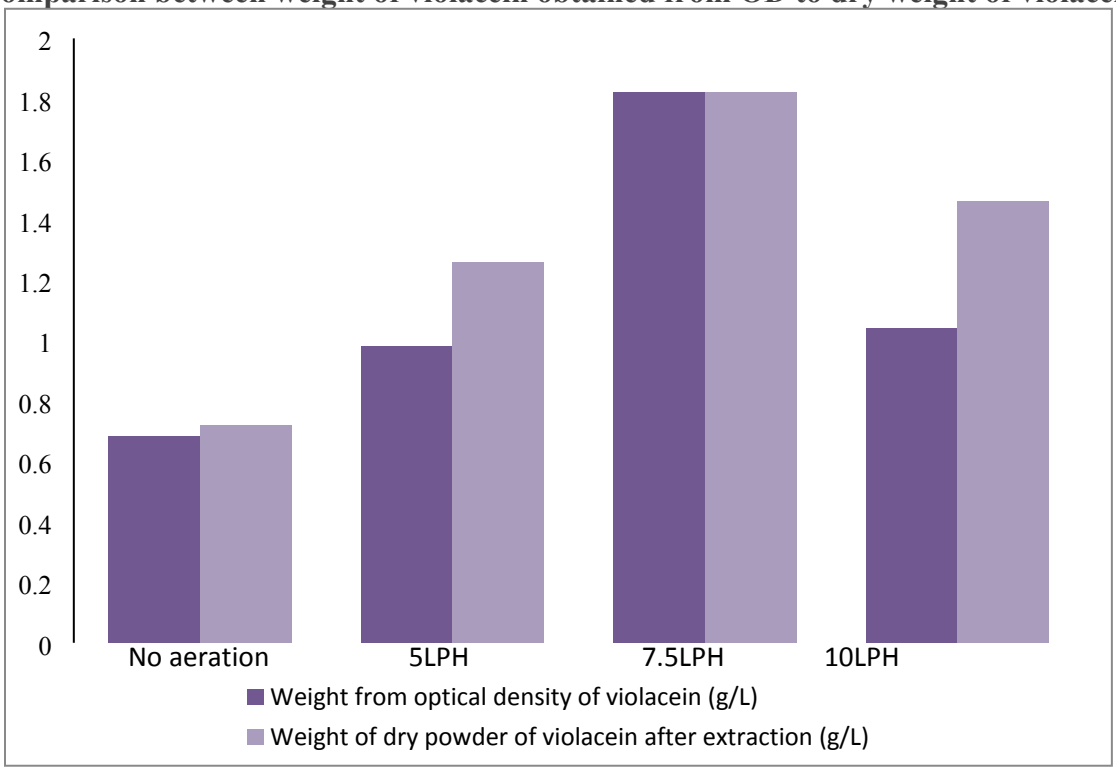
As the aeration increased from no aeration to 10.0 Liters per hour, the optical density gradually increased from no aeration mode to 7.5 liters per hour. But there was a sharp decrease observed at 7.5 liters per hour. As minimal amount of oxygen supply leads to high production of violacein.

Effect of aeration levels on optical density on violacein



Dry weight of violacein after extraction is shown in the table below: The values are high which maybe reflection that there can be other soluble intermediates. This comparison is done to know the difference between the actually obtained values from the dry weight to the calculated weight of violacein that is obtained from the optical density.

Comparison between weight of violacein obtained from OD to dry weight of violacein



Results of seed percentage on production of violacein in bubble column reactor:

The seed percentage was varied as follows (v/v)- 5%, 10%, 20% and 30%. The reactors and variations in OD are noted in the following table. The variance in seed percentage is noted down. The best optical density was seen for 10 percent seed with an aeration range of 7.5 litres per hour. from 30 percent seed. The lowest optical density is recorded for 5 percentage of seed used.

Effect of seed percentage on optical density

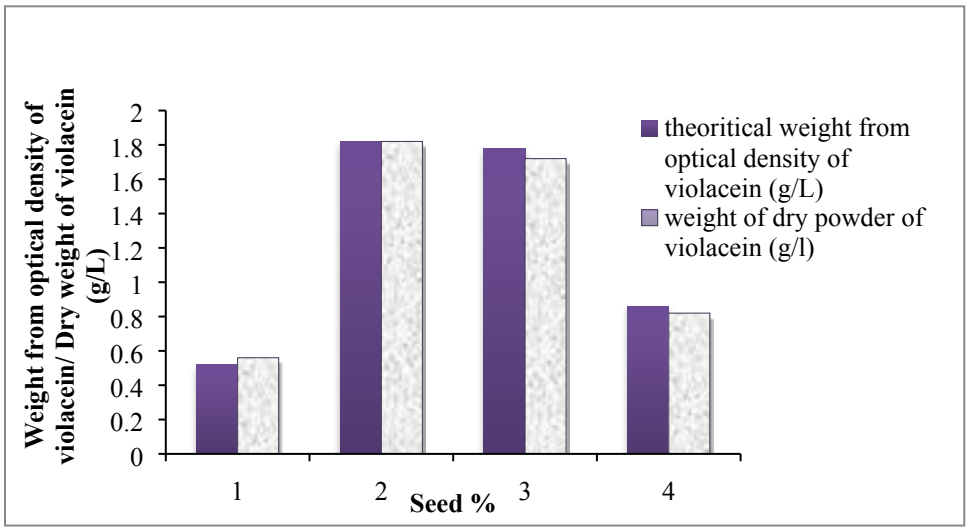
S no.	Seed %	Optical density at 560 nm
1.	5	0.416
2.	10	1.424
3.	20	1.286
4.	30	0.693

Effect of seed percentage on colony forming units/mL:

The highest colony forming units were formed at 10% seed at 24 hours (790 x 10^6cfu/ml) . Moderate growth was seen for 20% of seed used. Low colony forming units are seen for 30% of seed and 5% of seed.

Comparison of dry weights: Dry weights of violacein obtained from solvent extraction to calculated value of violacein from optical density

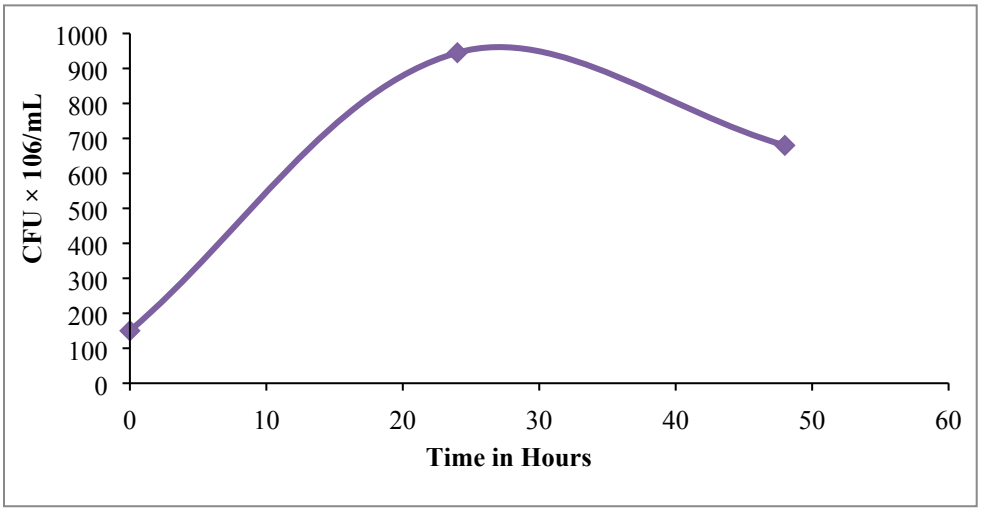
Comparison between dry weight of violacein and calculated value from optical density



Determination of K_s:

Ks, the saturation constant is determined for the highest yielded production run, that is with the conditions 7.5 litres per hour of aeration and at 10 percentage seed level. The graph is plotted between the colony forming units/mL and time, that is taken to form those colony forming units/mL. Ks was determined from graph

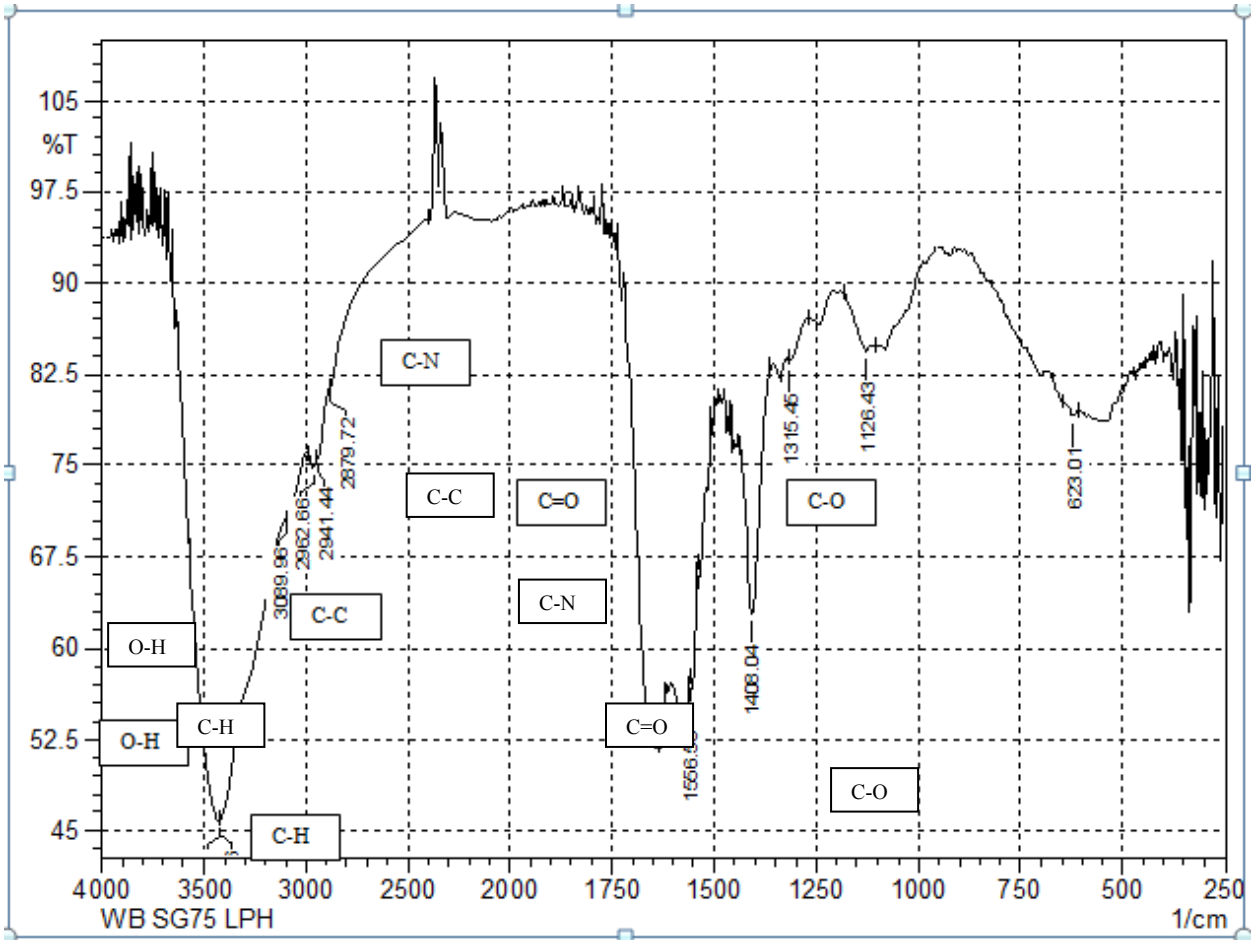
Effect of aeration, seed culture on colony forming units/mL in accordance to time



FTIR Analysis:

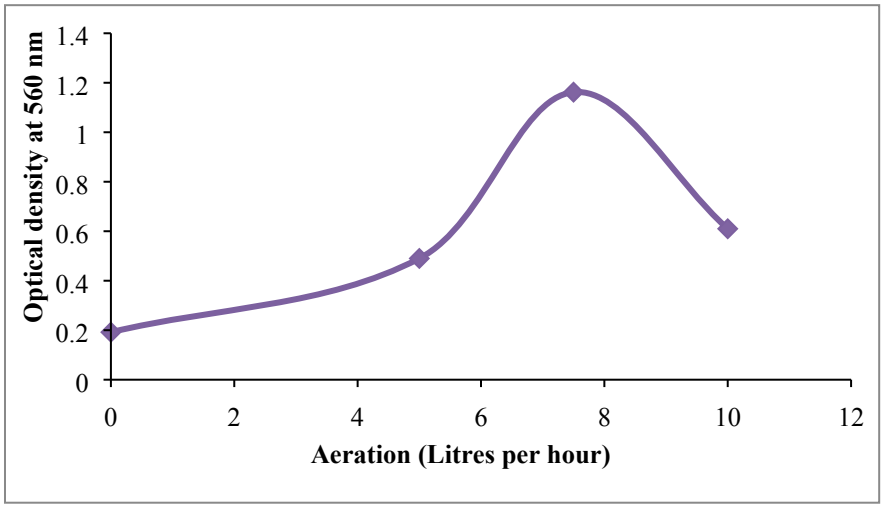
FTIR analysis is done for the conditions- 7.5 litres per hour with 10 percent seed in it and for 10 litres per hour of aeration, with 10% seed in it. Broader peak at hydroxyl group indicates that the product may be a mixture of violacein and deoxy violacein. The region between C=O to C-O absorption also indicated that the product must be having many intermediates for10LPH.

FTIR analysis for 7.5 litres per hour, 10% seed

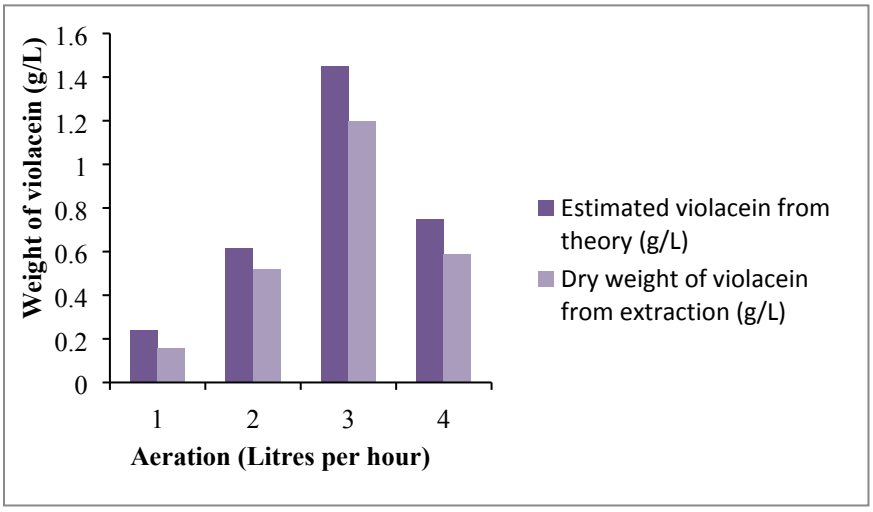


Production media in bubble column reactor: Optmized medium was used with percentage of inoculum fixed at 4.4(v/v). at an aeration of 7.5LPH maximum production was observed

Effect of aeration on optical density of violacein

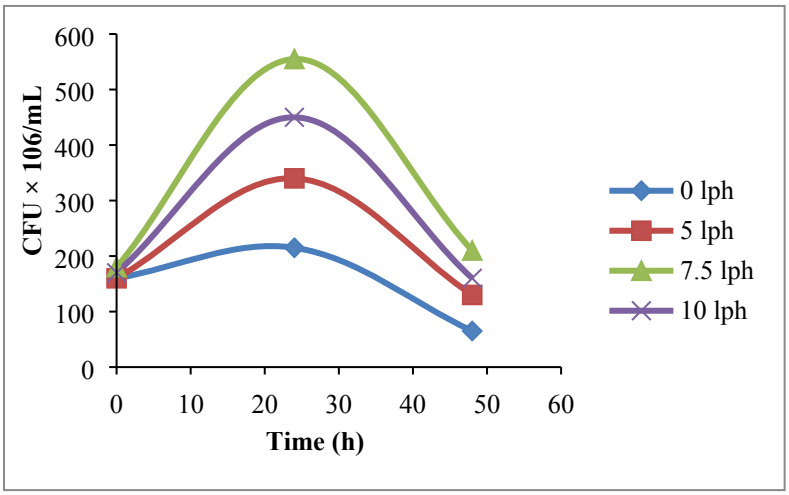


Comparison between weight of violacein from optical density and dry weight of violacein



Effect of aeration on cell count (cfu/ml): A steady increase in cell count was observed till 7.5 LPH while further increase in aeration has reduced the cell count due to its negative effects.

Effect of aeration on colony forming units/mL



Growth kinetics: These were determined for production media

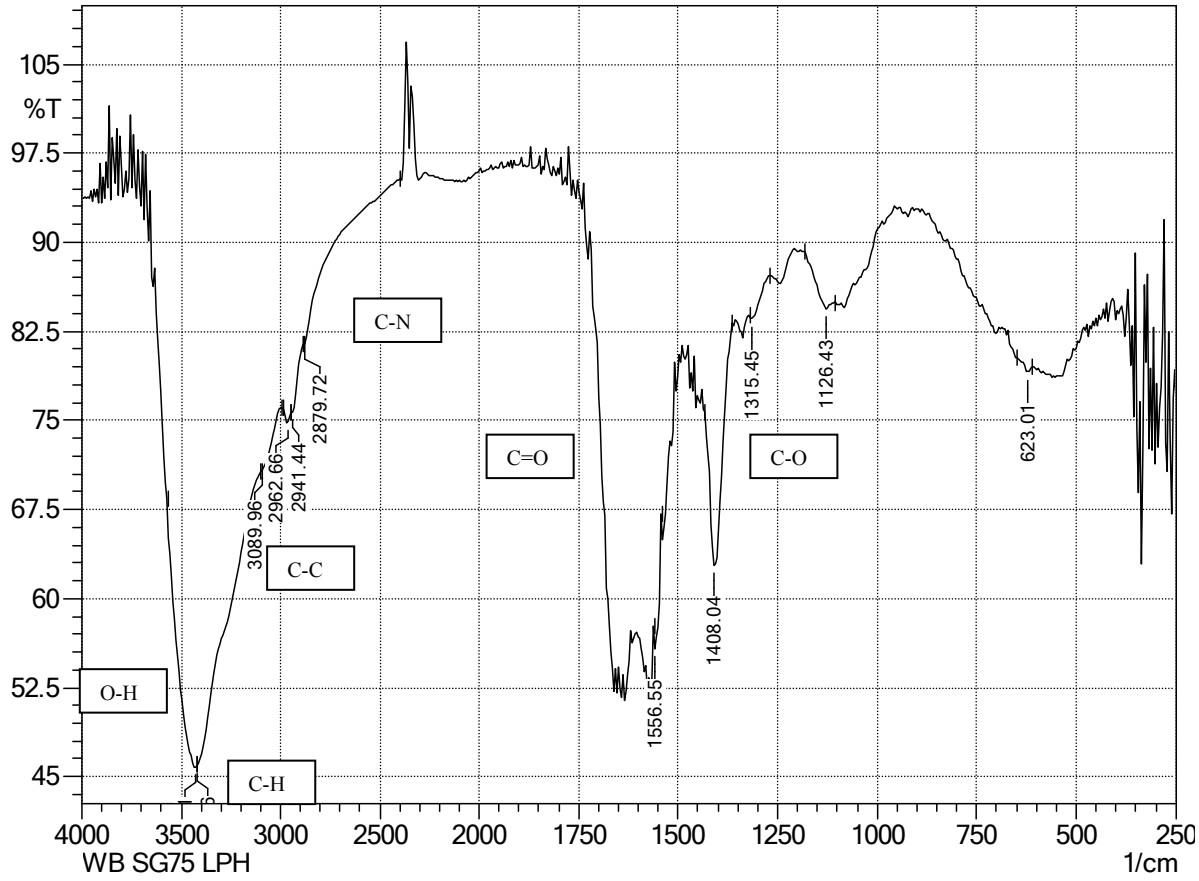
According to Monod's Kinetics:

$\mu=\frac{\mu\max S}{Ks \times S}$, by substituting all the above parameters and calculating Saturation constant

Ks = 1.2 g/L

FTIR Results:

Production media: Bubble column reactor, 7.5 LPH, wheat bran and soya granules flour as tryptophan contributors. The results are as follows: A sharp peak at hydroxyl group indicates presence of violacein that has hydroxyl groups. More concentration of violacein must have been present as compared to Violacein as indicated from the decrease in transmittance. As indicated previously it may be concluded that it is a mixture of violacein and deoxyviolacein.

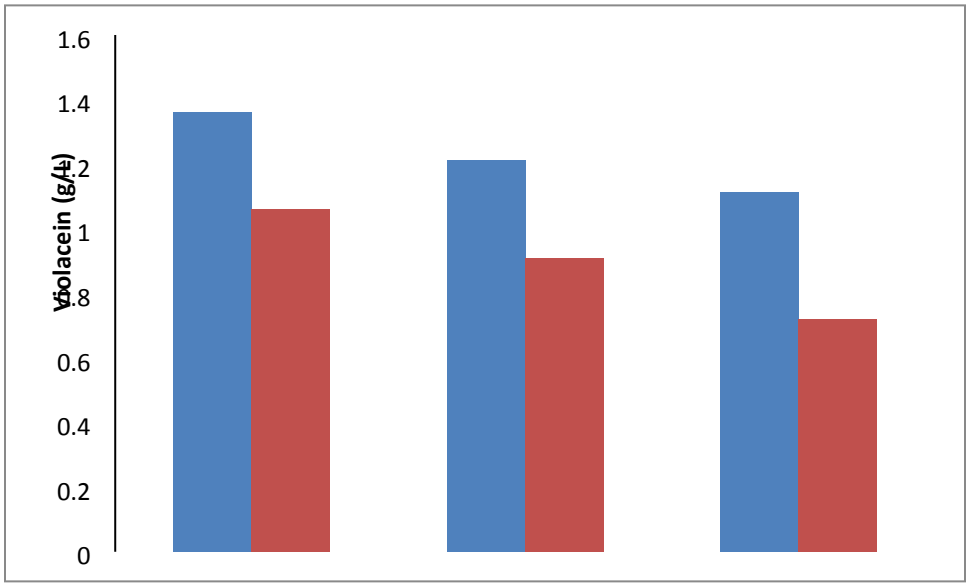


RESULTS IN NOVEL REACTOR

Results using nutrient broth: Violacein extraction and its concentration determination was done just as in submerged studies

A comparison of violacein produced from optical density and dry weight is given in the figure bellow.

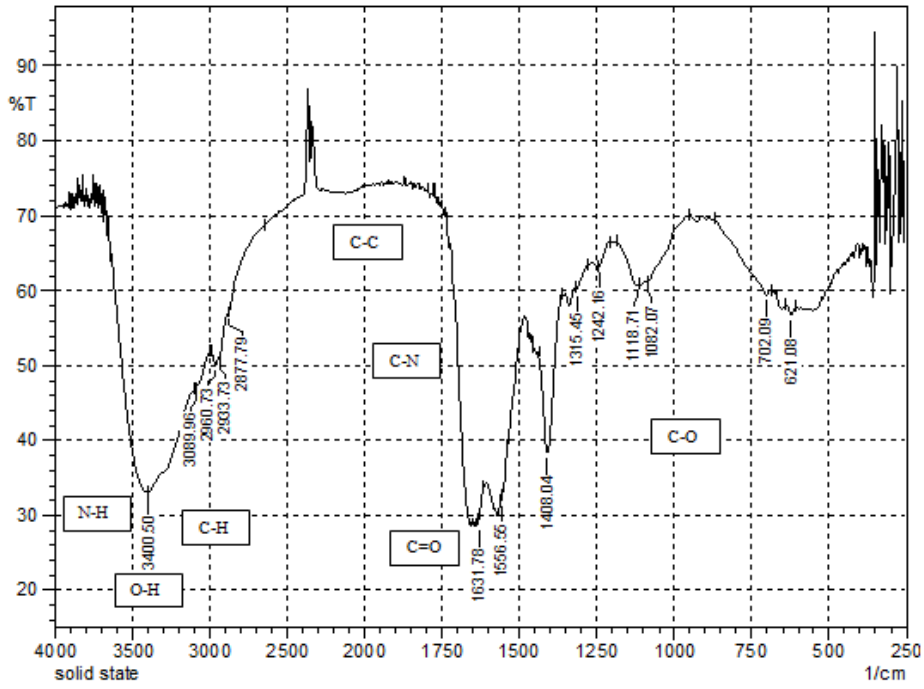
Comparison of dry weight and weight obtained from optical density of violacein



Using nutrient agar the net value of violacein produced is 3.684g/L while that for optimized medium is 3.33g/L.

FTIR analysis for tube in tube reactor: (Production media)

A sharp depression at –OH is not observed. Hence it may be concluded that the product obtained is a mixture of Violacein and Deoxyviolacein, but has more quantity of violacein.



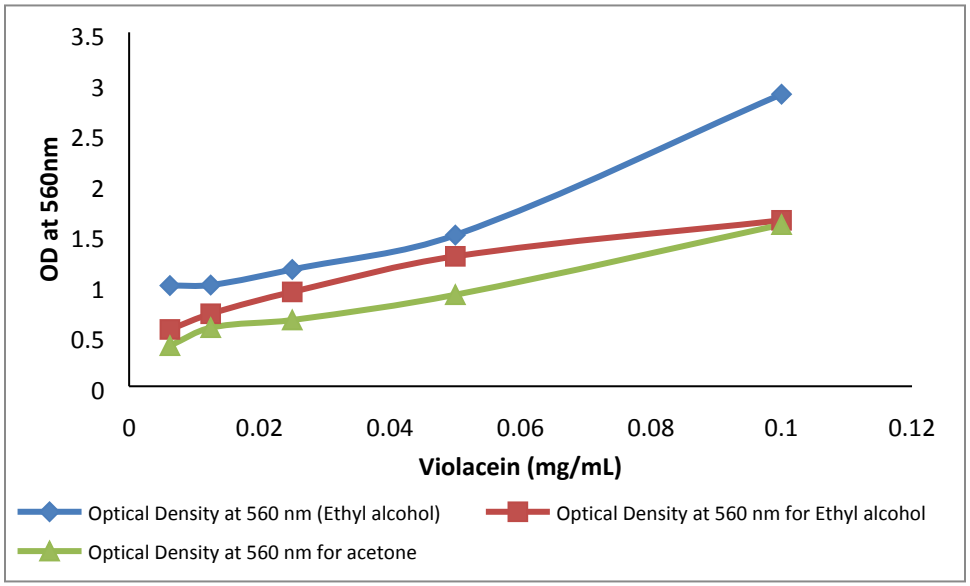
Growth kinetics for Novel reactor (production media): Growth of cell was evaluated and the Ks obtained is 1.360 g/L.

Characterization of violet pigment:

Solvent solubility tests:

Below is the graph showing solubility curves for the three solvents, acetone, ethylalcohol and ethyl acetate (0.1, 0.05, 0.025, 0.0125, 0.00625mg/mL). Among all ethyl acetate resulted in high optical densities for all the concentrations of violacein.

Graph showing results of solubility test for violcaein



Anti microbial tests: Results with four different organisms namely *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Bacillus cereus* chosen for the study are presented in the pictures below. For *Bacillus cereus* highest inhibition zones are seen , and next for *Escherichia coli*.S

PRODUCTION COST OF VIOLACEIN

Cost analysis for submerged fermentation:

Cost of Nutrient broth: 500g bottle is Rs. 482/-

Cost per litre = Rs. 12.50 (13g/L)

Cost of Optimized Production medium: was Rs.6/-

Cost of solvents used in extraction: Rs.87.64

Other charges: Rs. 211.72

Total charges for production with Nutrient broth for one run for 3L = Rs. 524/-
Total charges for production with optimized medium (3L) = Rs. 505/-

Cost analysis for surface cultivation:
Cost of Nutrient agar 500g: Rs. 3000/-
Cost of 19.6g of Nutrient agar = Rs. 117.60
Expenses on solvents = Rs. 5.46
Other charges = Rs.110.72

Total expenditure for production using nutrient agar by surface cultivation = Rs. 233.78

Cost analysis for surface cultivation using optimized medium:
Cost of Optimized medium with agar = Rs. 120.38

Expenses for submerged studies and surface cultivation:

Type of study	**Submerged studies**		**Surface cultivation studies**	
	Nutrient broth	**Optimized medium**	**Nutrient agar**	**Optimized medium**
Expenses per liter (Rs)	174.66	168.33	224	120
Violacein and or deoxyviolacein (g/L)	1.82	1.2	1.864	1.36
Productivity (g/l/h)	0.0758	0.05	0.077	0.056
Cost per mg of mixture (Rs.)	0.1	0.14	0.12	0.09
Cost of Violacein(100% pure) = Rs. 30,000 per mg				

This analysis states that use of optimized medium has produced very high levels of the mixture of violacein and deoxyviolacein. As purification cost of pure viocalein from the mixture is not incorporated, cost comparison cannot be done.

SUMMARY

- Preliminary studies confirmed usage of wheat bran, soya granule powder and Ammonium sulphate can be used as nutritional supplements.
- Screening of mineral elements and optimization of production variables using Response surface methodology has resulted in production medium.
- Bubble column reactor studies using nutrient broth and using optimized production medium have shown that aerating the medium contributes to better yield. Nutrient broth studies have revealed that an inoculum level of 10% and aeration of 7.5LPH resulted in good yield. While optimized medium required 4.4% inoculums and 7.5 LPH aeration.
- Product concentrations in bubble column studies are 1.3g/L for nutrient broth and 1.5g/L for optimized medium respectively.
- Surface cultivation confirmed that it would contribute to better production of violacein.
- Growth kinetics and Ks values were determined indicating better utilization of substrate in surface cultivation.
- Solubility tests revealed that acetone is a better solvent.
- Antimicrobial activity studies confirmed that violacein is a broad range antimicrobial agent and it has exhibited good activity again *Bacillus cereus* followed by *Escherichia coli* among the chosen organisms.
- Cost estimation was done for the media used. The cost of optimized production medium along with the other charges account to 50% to that of the cost of production using Nutrient agar and 45% to that of nutrient broth.
- Purity of violacein cannot be authenticated using the methods used and hence cost comparison was not done for product.

CONCLUSION

The objective for identification, optimization and production of violacein using surface cultivation method is accomplished. The fabricated reactors in the project were effective in contributing to increased production of violacein. Bubble column studies reveal role of aeration, and surface cultivation method helped to come up with a novel reactor for which IPR was filed.