Experimental correlation of volumetric oxygen transfer coefficient (K_La) with pyocyanin production at bench scale level from *Pseudomonas aeruginosa* MH038270

Rani Alka, Singha Arun, Gyala Deepak and Azmi Wamik*
Department of Biotechnology, Himachal Pradesh University, Shimla-171005, INDIA
*wamikazmi@rediffmail.com

Abstract

Pyocyanin is a water soluble bluish green pigment secreted by the Pseudomonas aeruginosa MH038270 isolated from clinical sample of hospital. Shikimic acid pathway is used for the biosynthesis of pyocyanin, an extracellular pigment which is only produced by P. aeruginosa. Pyocyanin has gained increasing importance due to its vast applications in various field viz. agriculture, pharmaceutical, food and aquaculture industry. The response of agitation and aeration on production of pyocyanin in submerged fermentation is extremely important. The cumulative effect of aeration and agitation rate was studied on the production of pigment from P. aeruginosa in a laboratory fermenter by applying the Response Surface Methodology (RSM). A 36.8% increase in concentration of pyocyanin production was achieved in fermenter.

Moreover, the fermentation time reduced to 72h in fermenter from 120h in shake flask which subsequently reduced the production cost. The volumetric oxygen transfer coefficient (K_La) was used as parameter for the evaluation of aeration efficiencies. K_La value for most favourable fermentation condition aiding maximum pyocyanin production was 0.38 min^{-1}.

Keywords: *Pseudomonas aeruginosa*, Pyocyanin, RSM, aeration rate, agitation rate, K_La.

Introduction

Pyocyanin (5-N-methyl-1hydroxy phenazine) is an aromatic molecule containing nitrogen; fig. 1 comes under the tricyclic phenazine class of compounds produced extracellularly by *P. aeruginosa*. Pyocyanin is a zwitterion, the weak acidic characteristic of zwitterions is due to a phenol group attached to it (pKa of 4.9). It is believed that pyocyanin easily permeates the cell membranes because of its low molecular weight and zwitter ionic properties[8]. Pyocyanin is the most thoroughly studied among all the phenazine pigments[5]. Pyocyanin is produced from chorismic acid via the phenazine pathway (shikimic acid pathway), nine proteins encoded by a gene cluster. The two steps catalyzed by enzyme PhzM and PhzS are responsible for the conversion of phenazine-1-carboxylic acid to pyocyanin. It has been shown that PhzM is only active in the physical presence of PhzS which suggests that a protein-protein interaction is involved in pyocyanin formation[9].

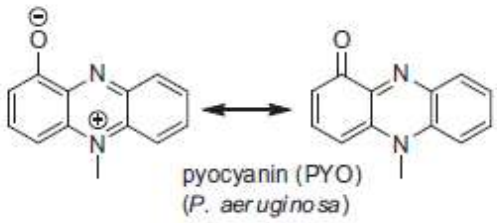

Fig. 1: Structure of pyocyanin

For *P. aeruginosa*, pyocyanin has been considered both a quorum sensing (QS) signalling molecule as well as a virulence factor. The *P. aeruginosa* also employs QS to control the formation of biofilms[11]. To fight with bacteria which are multi antibiotic-resistant, disruption of QS system is a better way. Pyocyanin is regulated by quorum sensing to induce the generation of reactive oxygen species (ROS) which results in increased oxidative stress. High levels of ROS may be incompatible with cellular survival and ROS may exert cytotoxic effects, thereby promoting cell death and suppressing cancer progression[7].

However, *P. aeruginosa* has ability to protect themselves against pyocyanin production with high amount of superoxide dismutase and catalase[18]. Pyocyanin has a characteristic property of inhibiting many of bacterial and fungal growth both *in vivo* and *in vitro* condition[16].

No other species of gram-negative non-fermenting bacteria produce pyocyanin which makes its presence helpful in identifying the organism[13]. It has various applications *viz.* antimicrobial, antioxidant, anticancerous and antibiofilm besides helping in pathogenicity of *P. aeruginosa*. *E. coli, Salmonella paratyphi, Klebsiella* sp and *Alternaria* sp were found sensitive to pyocyanin pigment produced from *P. aeruginosa*[21]. Pyocyanin, as a versatile and multifunctional phenazine pigment, has been applied as bio-control agent against many pathogens[3]. It has also got application in biosensors as a redox compound for carrying out electron transfer between enzyme molecules and the electrode material.

Therefore, the biosensors based on pyocyanin were also expected to come up to cater the need of different fields such as agricultural, medicine and environment[19]. Pyocyanin can be used as electron shuttle in microbial fuel cells by enabling bacterial electron transfer towards the microbial fuel cells (MFC) anode[4]. It was observed that the addition of pyocyanin to MFC-containing *Brevibacillus* sp. PTH1

doubled the rate of electron transfer[20]. Antimicrobial resistance is an alarming issue to deal with, as most of the pathogens are becoming resistant to their common treatable antibiotic.

Previous studies reported that *S. aureus*[23] and *E. coli* are multidrug resistant bacteria[22] and the inhibition of these and others like pathogens expresses the importance and potentiality of this molecule as an antimicrobial agent. To treat infections caused by these pathogenic bacteria, pyocyanin can be used as pharmaceutical agent. The compound also showed good antibiofilm activity against multiple antibiotic-resistant food pathogens. This can be used to control several other potent food pathogens if used in the food industry.

The phenazine-based pyocyanin pigment has a particular usefulness for its capability to generate reactive oxygen species (ROS). Phenazines are associated with antitumor activities[10] and the cells that are actively respiring such as tumor cells, appear to be more susceptible to reactive oxygen species (ROS) generation caused by pyocyanin. Additionally, it interferes with topoisomerase I and II activities in eukaryotic cells[3]. The development of pyocyanin as anticancerous drug will find key important place in cancer biotherapy.

The compound showed very high antioxidant activity at very minute concentrations which is a positive indication for the safe use of compound[12]. Moreover, the pyocyanin showed no cytotoxic effects on red blood cells of human[17] and in cultured L929 cells[2]. The cytotoxic effect of pyocyanin on human pancreatic cell line (Panc-1) has also been reported. There was inhibition of 98.69±0.23 and 89.88±1.86% of 6mg/mL of pyocyanin extracted from clinical and soil isolates of *P. aeruginosa* respectively[14]. A study has evaluated the anti-corrosion property of the bioactive pyocyanin produced by *P. aeruginosa*. The researchers explored a new antibacterial property of pyocyanin produced by *Pseudomonas* sp. TBH2 in controlling the biocorrosive bacterial biofilm formed by *Bacillus* sp.[15]. This study gains importance as the production of pyocyanin from the indigenous bacteria is not an expensive process and moreover, it is environment friendly.

Batch fermentation is the simplest mode of operation and is often used in the laboratory to obtain substantial quantities of cells or product(s) for further analysis. A batch fermentation is a closed system, where all of the nutrients required for the organism's growth and product formation are contained within the vessel at the start of the fermentation process.

Batch fermentation is the simplest mode of operation and is often used in the laboratory to obtain substantial quantities of cells or product(s) for further analysis. A batch fermentation is a closed system where all of the nutrients required for the organism's growth and product formation are contained within the vessel at the start of the fermentation process.

The batch fermentation is the simplest way to cultivate microbial cell and often used in laboratory to obtain product for further investigation. It is a closed setup in which all the nutrients are required for the growth of bacteria and production of pigment contained in a vessel at the beginning of fermentation. Keeping in view the importance of microbial pyocyanin in the medical, agriculture and food industries, this study focused evaluation of cumulative effect of aeration and agitation on bench scale fermentation of *P. aeruginosa* for the production of pyocyanin.

Material and Methods

Microorganism and Inoculum preparation: The pyocyanin producing culture was isolated from clinical sample procured from IGMC, Shimla and identified as *Pseudomonas aeruginosa* MH038270 by 16s rRNA sequencing. The production medium (pH 6.5) contained: peptone 0.5 (%, w/v), beef extract 0.25 (%, w/v), NaCl 0.875 (%, w/v) and glycerol 2 (%, v/v). *P. aeruginosa* MH038270 was maintained on nutrient agar medium (pH 7.0). The maintained culture of *P. aeruginosa* MH038270 was transferred to autoclaved seed medium of same composition excluding agar for the preparation of the inoculum.

The flasks containing *P. aeruginosa* MH038270 inoculated seed medium were incubated at 50 rpm in an orbital shaker at 37°C and the exponential phase cell mass (24h age) was used as inoculum for the production of pyocyanin. The size of the inoculum was in accordance with the previously optimized value (3%, v/v) for maximum pyocyanin production.

Sterilization and inoculation of the production medium: Fermentation of *P. aeruginosa* was carried out in 14L laboratory fermenter (Scigenics India Pvt. Ltd.) at 8L working volume in batch mode. The fermenter is well equipped with pH, temperature, agitation, aeration, dissolved oxygen and antifoam sensors as well as controls. For the development of a laboratory inoculum, the seed was prepared in conical flasks containing the seed medium. The seed medium was inoculated with loopful culture of *P. aeruginosa* and incubated at 37°C for 24h on a rotary shaker (50rpm).

As the fermenter has inbuilt facility of sterilization (*in situ* sterilization), 8L production medium (pH 6.5) was loaded into the fermenter. The sterilization of the production medium was carried out at 121°C for 15min. After the completion of sterilization cycle, the temperature of the medium was adjusted to 37°C by cooling. The 8L of production medium was supplemented with 3% of 24h old seed (v/v) through the inoculation port by peristaltic pump attached to a feed bottle and 2mL silicone oil as antifoam agent.

The pH, temperature and dissolved oxygen were monitored throughout the operation. However, temperature was maintained at 37°C for the entire course of fermentation with the help of inbuilt heater and chiller. After inoculation, periodical sampling (at 12h interval) was carried out and samples were analyzed for growth and pyocyanin production by *P. aeruginosa* cells.

Estimation of cell mass: The cell mass is estimated by using dry cell weight (DCW) method. The known dried cell weight corresponding to their optical density was recorded and a standard graph was plotted between dry cell weight and A_{600}.

Pyocyanin quantitation assay: Pyocyanin was extracted from culture supernatant and measured based on the absorbance of pyocyanin in acidic solution at 520nm[5]. The fermentation broth was centrifuged at 10000 rpm for 10min. The culture supernatants were transferred into new test tubes and extracted with chloroform (1:2) and the aqueous phase was removed. The bottom layer was re-extracted with 1ml of 0.2N HCl until color change was observed. Following this, the absorbance of the pigment solution was measured using spectrophotometer at 520nm.

The concentration was calculated as microgram pyocyanin pigment produced per milliliter of culture supernatant. The optical density at 520nm was multiplied by 17.072 to determine the concentration of pigment[24] (extinction coefficient).

Central Composite Design: The growth of *P. aeruginosa* and concentration of pyocyanin were measured under different conditions of agitation and aeration by adopting the statistical tool RSM. The RSM measures the interaction between the response (pyocyanin concentration) and the independent variables and defines the effect of the independent variables either alone or in combination. A central composite design (CCD) of response surface methodology was selected to study the combined effect of agitation speed (RPM) and aeration rate (VVM) towards pyocyanin production, pH, dissolved oxygen (DO, % saturation), cell mass.

The parameters were represented at two levels, high (+1) and low (-1) i.e. agitation speed at 100 and 50rpm; aeration rate of 0.8 and 0.25 vvm. A total of 13 experimental runs were designed by CCD with different combination of agitation and aeration rate (Table 1). Each experiment was conducted in laboratory fermenter and pyocyanin concentration (µg/mL) was taken as the response in each run. The statistical software "Design-Expert 11.0" (StatEase) was used to analyze the experimental results.

Determination of Volumetric Oxygen Transfer Coefficient (K_La): Dynamic method for K_La determination was used and this method is based upon the dynamic oxygen balance in a batch culture.

$$dC_L/dt = K_La\,(C^* - C_L) - Q_{o2}X \quad (1)$$

where K_La = Volumetric oxygen transfer coefficient, C* and C_L=Saturation and actual dissolved oxygen concentration in the liquid medium, respectively and Q_{o2}=Rate of oxygen consumption per unit mass of cells (cellular respiration) ($mMO_2g^{-1}h^{-1}$).

Rearranging the equation 1, we get:

$$C_L = C^* - 1/K_La\,(Q_{o2}X + dC_L/dt) \quad (2)$$

Table 1
CCD table showing the combinations of varying rate of agitation speed and aeration rate on the pyocyanin production by *P. aeruginosa*

Run	Agitation Speed (rpm)	Aeration Rate (vvm)
1	62.5 (set at 63)	0.0757359 (set at 0.08)
2	62.5 (set at 63)	0.5
3	100	0.8
4	62.5 (set at 63)	0.5
5	62.5 (set at 63)	0.5
6	25	0.2
7	62.5 (set at 63)	0.5
8	115.533 (set at 115.5)	0.5
9	62.5 (set at 63)	0.5
10	25	0.8
11	100	0.2
12	62.5 (set at 63)	0.924264 (set at 0.9)
13	9.46699 (set at 9.5)	0.5

In order to determine K_La, the air supply to fermenter has been stopped at a certain time when fermentation was in active stage and decrease in dissolved oxygen concentration (C_L) was measured as a function of time for determination of oxygen uptake rate ($Q_{o2}X$). Air supply was established again and increase in the dissolved oxygen concentration was also observed as a function of time. Then the values of $Q_{o2}X$ and (dC_L/ dt) and K_La were calculated from C_L versus time curve and C_L versus $Q_{o2}X$ + [(dC_L/ dt)] curve respectively.

Role of K_La on growth and pyocyanin production by *Pseudomonas aeruginosa*: The effect of K_La was determined by comparing the K_La values of different fermentation batches of *P. aeruginosa* with respect to the biomass and pyocyanin production. The results were demonstrated by K_La versus growth and K_La versus pyocyanin concentration curve.

Results and Discussion

In the present study, the combined effect of aeration rate and agitation speed on cell growth, pyocyanin production and other parameters such as pH, DO was determined during the course of fermentation of *P. aeruginosa* by applying RSM. The reports of bench scale production of pyocyanin were not abundantly available in literature.

However, these studies assume immense importance if the biochemical and other industrial applications of this pigment are to be realized at commercial scale. A central composite design (CCD) of response surface methodology was chosen to study the combined effect of agitation speed (RPM) and aeration rate (VVM) towards pyocyanin production.

The optimum values of the two components as obtained experimentally by RSM were calculated to be as 63rpm of agitation speed and 0.9vvm of aeration rate at which maximum production of pyocyanin has been obtained (58.4µg/mL) (Table 2).

The results were examined by using ANOVA (analysis of variance) suitable for analysis of the designed experiment (Table 3).

Table 2
CCD table showing the effect of varying rate of agitation speed and aeration rate on the pyocyanin production by *P. aeruginosa*

Run	Agitation Speed (rpm)	Aeration Rate (vvm)	Pyocyanin concentration (µg/mL)
1	62.5 (set at 63)	0.0757359 (set at 0.08)	12.0
2	62.5 (set at 63)	0.5	40.0
3	100	0.8	52.6
4	62.5 (set at 63)	0.5	37.6
5	62.5 (set at 63)	0.5	38.1
6	25	0.2	5.0
7	62.5 (set at 63)	0.5	37.0
8	115.533 (set at 115.5)	0.5	26.0
9	62.5 (set at 63)	0.5	36.6
10	25	0.8	24
11	100	0.2	15
12	62.5 (set at 63)	0.924264 (set at 0.9)	58.4
13	9.46699 (set at 9.5)	0.5	1.2

Table 3
ANOVA for Response Surface Quadratic model Analysis of variance table

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3666.56	5	733.31	271.86	<0.0001	Significant
A-Agitaion rate	11.31	1	11.31	4.19	0.0798	
B-aeration rate	1756.74	1	1756.74	651.29	< 0.0001	
AB	86.49	1	86.49	32.06	0.0008	
A^2	1034.14	1	1034.14	383.39	< 0.0001	
B^2	13.49	1	13.49	5.00	0.0604	
Residual	18.88	7	2.70			
Lack of Fit	11.85	3	3.95	2.25	0.2251	Not significant
Pure Error	7.03	4	1.76			
Cor Total	3685.44	12				

The ratio of mean square regression and mean square residual depicted the model F-value. The model F-value of 271.86 implies that the model is significant. The model P-value (<0.0001) less than 0.0500 indicated that model terms are significant. Values greater than 0.1000 indicate that the model terms are not significant. "Lack of fit" is not significant which proves our model to be fit i.e. significant.

3D graph was generated for regression analysis of CCD design using pair wise combination of two factors for pyocyanin production. These 3D response surface plots described the effects of the independent variables and combined effects of each independent variable upon the response i.e. pyocyanin concentration.

Fig. 2 represents the interaction between agitation speed and aeration rate where the shape of the response surface indicates the effect of these two variables. The increase in the agitation speed from 25 to 63rpm and prolonged aeration rate from 0.2 to 0.9vvm led to increase in pyocyanin concentration. However, further increase in agitation speed beyond 63rpm decreases the concentration of pyocyanin slowly, although the increase in aeration rate from mean point results in increase in concentration of pyocyanin.

The maximum pyocyanin concentration obtained by performing suggested experiment was 58.4µg/mL which is more than predicted value 56.68µg/mL calculated by ANOVA and shown in Predicted vs. Actual plot (fig.3). In the perturbation plot (fig.4), the effect of agitation speed and aeration rate at the optimum run conditions in the design space was compared. A nearly steep curvature line in case of agitation speed (A) showed that the response of the pyocyanin concentration was very sensitive to this factor while there was no curvature in aeration which showed that concentration of pyocyanin increases with increase in aeration.

Evaluation of combined effect of aeration and agitation on *P. aeruginosa* cell mass, pyocyanin production and dissolved oxygen: Oxygen must be supplied to all aerobic cultures to satisfy the need of oxygen for growth and production.

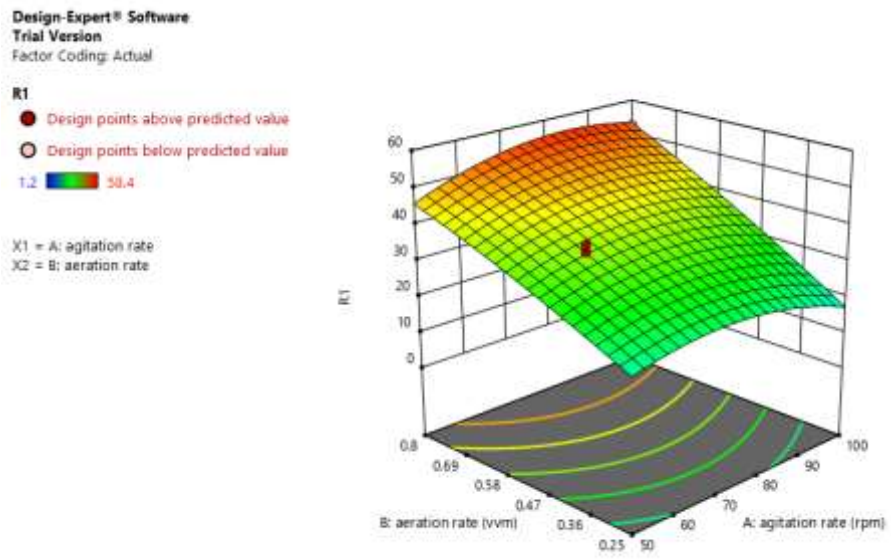

Fig. 2: 3D Response surface plot

Table 4
Summary of results obtained during interactive experimental design

Batch	Agitation (rpm)	Aeration (vvm)	Pyocyanin concentration (µg/mL)	Biomass (mg/mL)	K_La (min^{-1})
1.	62.5 (set at 63)	0.5	37.8	11.00	0.38
2.	100	0.2	15.0	4.96	0.43
3.	62.5 (set at 63)	0.924264 (set at 0.9)	58.4	20.0	0.38
4.	100	0.8	52.6	16.4	0.24
5.	62.5 (set at 63)	0.0757 (set at 0.08)	12.0	4.5	0.17
6.	115.533 (set at 115.5)	0.5	26.0	9.8	0.42
7.	25	0.8	24.0	9.5	0.17
8.	9.466 (set at 9.5)	0.5	1.2	9.6	0.49

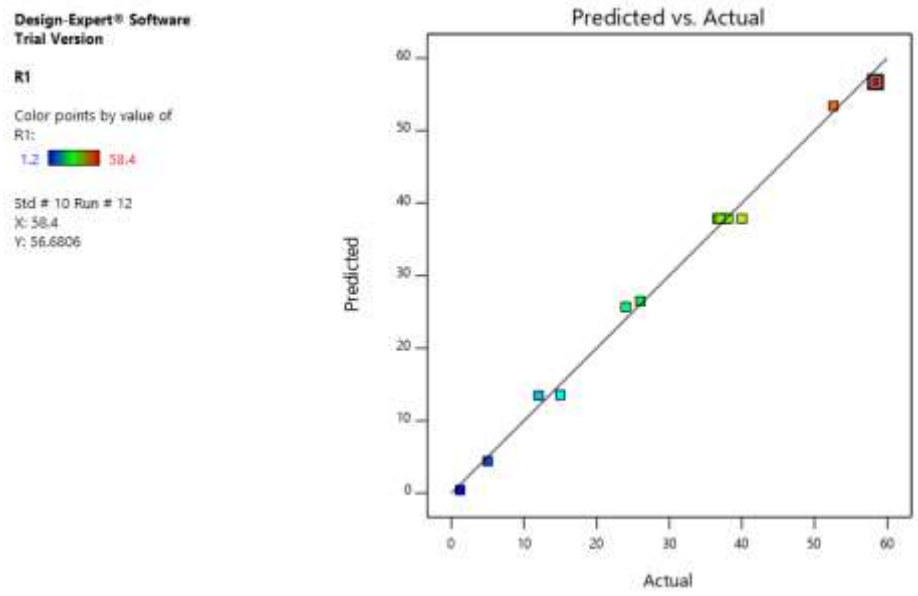

Fig. 3: Predicted vs. Actual plot for different experimental runs in CCD

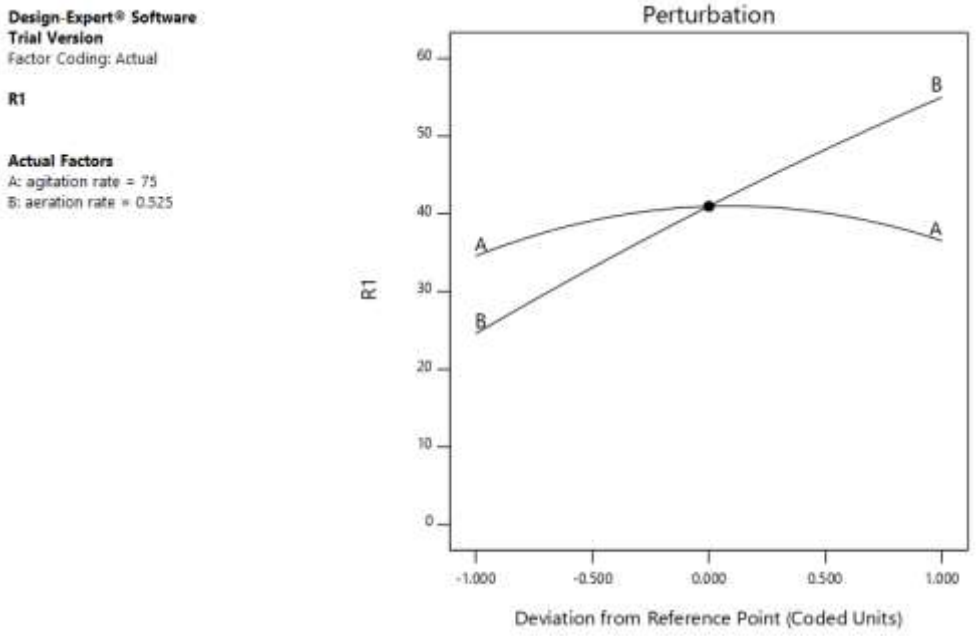

Fig. 4: Perturbation plot showing the optimum value for different variable

In fermentor, this is mainly obtained by correct set up of aeration and agitation leading to the transfer of a sufficient amount of oxygen to each cell[6]. Fermentation was carried out for 84h in batch mode. The selection of optimum combination of rate of agitation and aeration was essential for the production of pyocyanin of *P. aeruginosa*. Hence these cells were grown at varying combination of agitation speed (9.5, 25, 63, 100 and 115.5rpm) and aeration rate (0.08, 0.2, 0.5, 0.8 and 0.9vvm).

The maximum cell mass (20.0mg/ml) of *P. aeruginosa* was obtained at 48h of fermentation at 63rpm and 0.9vvm aeration which was higher than the cell mass attained under over all fermentation. The increase in the agitation speed (from 9.5 to 63rpm) and aeration rate (from 0.08 to 0.9vvm) proved to be beneficial for the growth of the *P. aeruginosa* (fig. 5).

The maximum biomass (4.9mg/ml) at 100rpm agitation and 0.2vvm aeration was obtained at 36thh of fermentation and nearly constant or at slight decrease with further incubation. In case of 100rpm agitation speed, 0.8vvm aeration resulted in maximum biomass (16.4mg/mL) as compared to 0.2vvm (4.9mg/mL). In contrast at 63rpm agitation speed, maximum growth yield was obtained at 0.9vvm aeration rate (20.0mg/ml) as compared to 0.08vvm (4.5mg/ml) and 0.5vvm (11.0mg/ml). Further increase in agitation rate from 63rpm to 115.5rpm decreases the growth. This might be due to the shearing forces operative at high agitation rate. The 63rpm of agitation speed at 0.9vvm aeration was found to be the most optimum for the cultivation of *P. aeruginosa*.

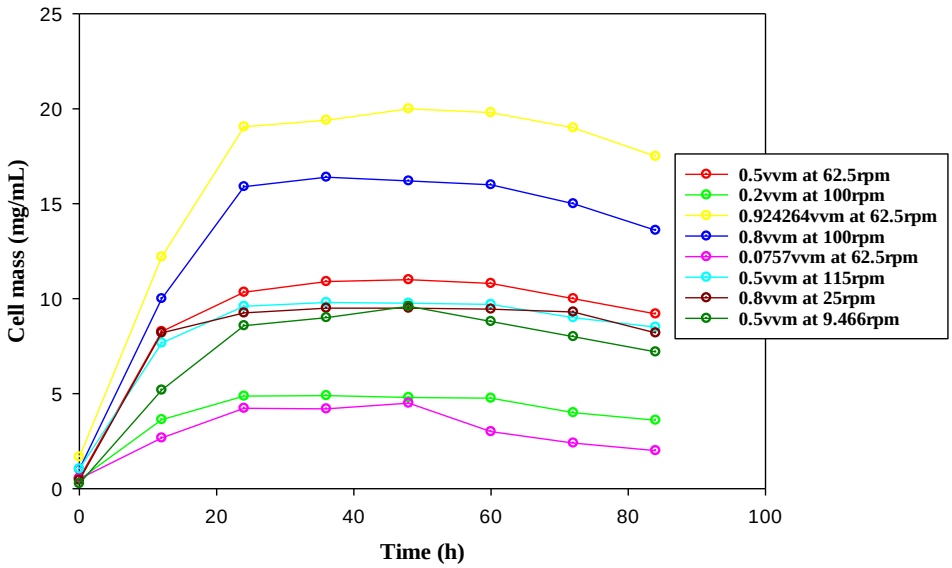

Fig. 5: Effect of agitation speed and aeration rate on growth of *P. aeruginosa* in fermenter

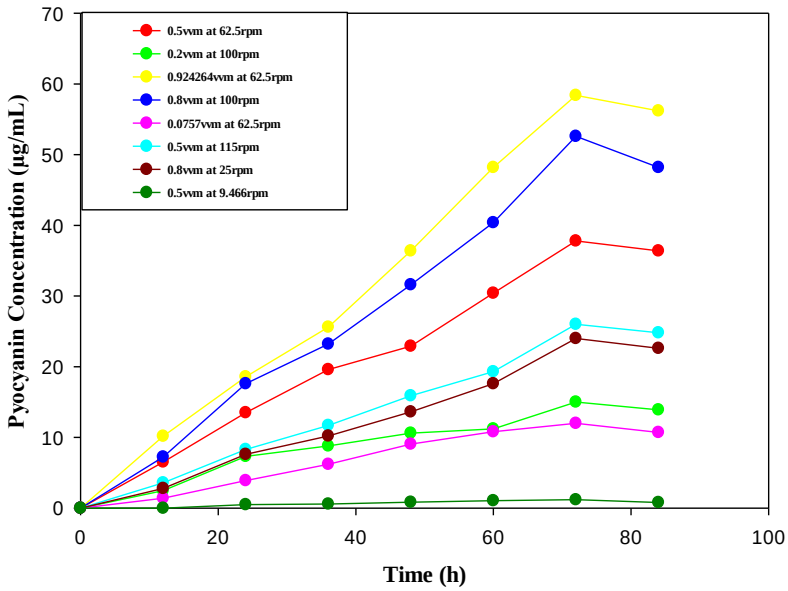

Fig. 6: Effect of agitation speed and aeration rate on pyocyanin production by *P. aeruginosa* in fermenter

The optimum combination of rate of agitation and aeration was essential for the hyper production of pyocyanin. The maximum pigment concentration of 58.4µg/mL was obtained in 72h at 63rpm agitation and 0.9vvm aeration (fig. 6) followed by agitation speed of 100rpm and aeration rate of 0.8vvm at 72h of fermentation (52.6µg/mL). The increase in the agitation speed (from 9.5 to 63rpm) and aeration rate (from 0.08 to 0.9vvm) proved to be beneficial for the pyocyanin concentration of the *P. aeruginosa*.

100rpm agitational speed at 0.8vvm aeration resulted in maximum pyocyanin concentration (52.6µg/mL) as compared to that at 0.2vvm (15.0µg/mL). In contrast at 63rpm agitational speed, maximum pyocyanin production was obtained at 0.9vvm aeration rate (58.4µg/mL) as compared to 0.08vvm (12.0µg/mL) and 0.5vvm (38.1µg/mL).

In case of 63 and 115.5rpm, at 0.5vvm aeration, the pigment concentration was found to be 38.1µg/mL and 26.0µg/mL respectively at 72thh of fermentation. Hence it could be concluded that with increase in agitation speed, there was increase in pyocyanin concentration upto optimum level and then decreased slowly with increase in further agitation. This might be due to the inhibitory effect of shearing forces that becomes operative at higher speed of stirrer.

The dissolved oxygen profiles of the fermentation broth under different rates of agitation and aeration reveal that depletion in the dissolved oxygen was insignificant at the higher rate of agitation and aeration (fig.7). There is decline

in DO from 100% saturation to 2.0, 2.4 and 3.9% during first 48h of the course of fermentation at 9.5rpm (0.5vvm), 63rpm (0.5vvm) and 63rpm (0.9vvm) respectively and the DO level at higher aeration rate (0.9vvm) and 63rpm first dropped below 3% during first 48h and then started to increase from 60h and reached to 100% at 72thh of fermentation and remained constant at 84thh.

Further increase in agitation speed 100rpm (0.2vvm), 100rpm (0.8vvm) and 115.5rpm (0.5) dropped the DO level to 3.6 and 2.2, 1.6% at 24thh and 36thh of fermentation respectively and increased continuously during further course of fermentation.

This might be due to the reason that culture used in the experiment was completely microaerophilic which means it needs continuous supply of oxygen as it cannot ferment or respire anaerobically and after attaining the stationary phase of growth, there was no further utilization of dissolved oxygen.

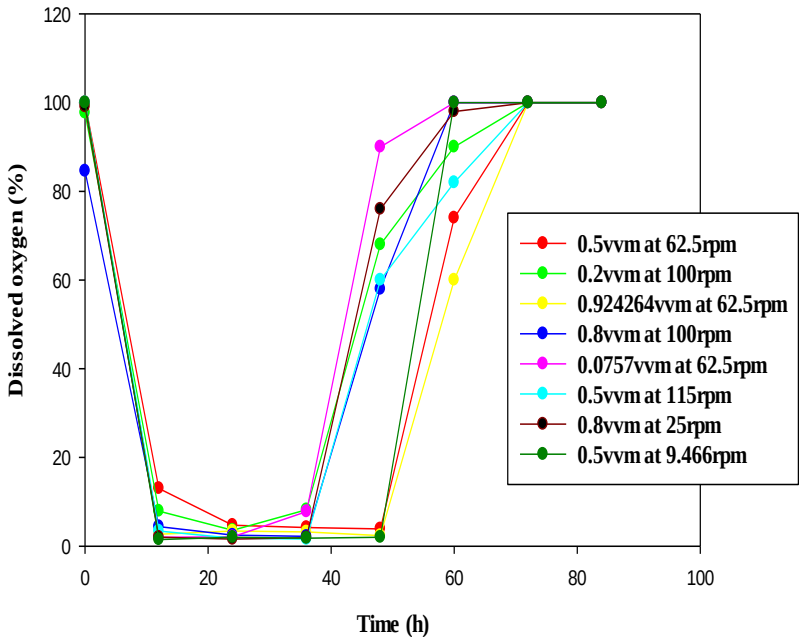

Fig. 7: Effect of agitation speed and aeration rate on dissolved oxygen during growth of *P. aeruginosa* in fermenter

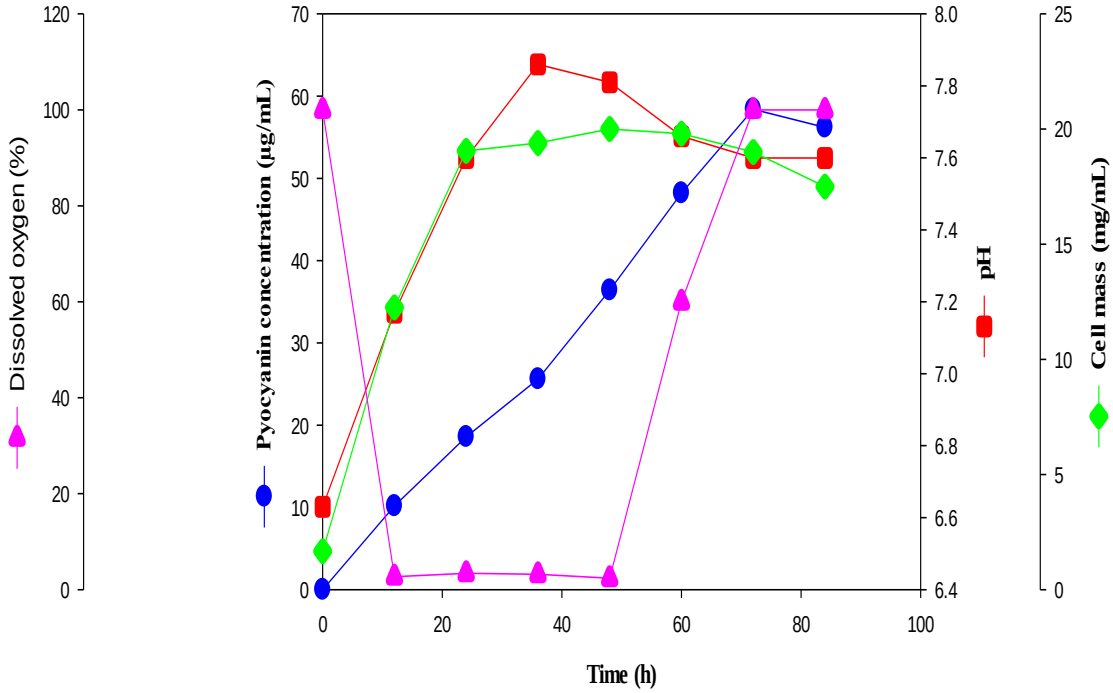

Fig. 8: The course of fermentation by *P. aeruginosa* for the production of pyocyanin

Course of fermentation of *P. aeruginosa* for the maximum biomass and pyocyanin production: In the present study, fermentation of *P. aeruginosa* cells for pyocyanin production was carried out in the optimized medium (pH 6.5) at 0.9vvm aeration and 63rpm agitation speed (fig.8). The cells of *P. aeruginosa* started growing exponentially after an initial lag period. The maximum cell mass (20.0mg/ml) of *P. aeruginosa* was observed at 48h of fermentation.

The maximum pyocyanin concentration obtained was 58.4µg/mL at 72h of incubation up to the initial 12h of incubation, the pyocyanin production was less which thereafter increased gradually. The increase in cell mass of *P. aeruginosa* leads to the rapid utilization of oxygen which reaches to minimum at 48thh of fermentation. The rapid utilization of dissolved oxygen coincides with the exponential phase of *P. aeruginosa* growth. Once the *P. aeruginosa* reached the stationary phase, the concentration of dissolved oxygen started increasing after 48h of fermentation and reached 100%.

P. aeruginosa JY21 cultivated in 7L bench top bioreactor (Bioflow 310, New Brunswich, NJ, USA) at a constant temperature of 30°C with constant shaking at 200rpm. The amount of biomass achieved was 1.36mg/mL during death phase in 81h and concentration of pyocyanin reached its maximum value of 614µg/mL during the death phase at 81h post-inoculation[1].

Role of K_La in growth and pyocyanin production by *P. aeruginosa:* The volumetric oxygen transfer coefficient (K_La) is one of the most important scale-up factors in fermentations. K_La value affected the growth rate and pyocyanin concentration in *P. aeruginosa* for the production of pyocyanin. In the present study, the growth of *P. aeruginosa* increased with increase in K_La from 0.17 to 0.38min^{-1} and thereafter decreased suddenly with further increase in K_La from 0.42 to 0.49min^{-1} (fig.9). Maximum biomass (20.0mg/ml) was obtained at 0.38min^{-1} K_La. These results suggest that oxygen transfer upto certain level in bioreactor favoured the growth of *P. aeruginosa*. However, at higher oxygen transfer rate, the growth might decrease due to oxygen toxicity or shear generated during the course of increase in oxygen transfer rate.

Similar pattern was observed in case of pyocyanin production by *P. aeruginosa*. Initially enzyme activity increased with increase in K_La up to a limit and then decreased (fig. 9). This might be due to the inhibitory effect of the dissolved oxygen concentration during the course of fermentation for pyocyanin production by *P. aeruginosa* cells. Maximum pyocyanin concentration (58.4µg/ml) was obtained at 0.38min^{-1} K_La. This exactly coincides with the profile obtained for the growth of *P. aeruginosa*. So, it could be concluded from the above data that higher oxygen transfer rate adversely affect the growth of *P. aeruginosa* which in turn results in less pyocyanin production.

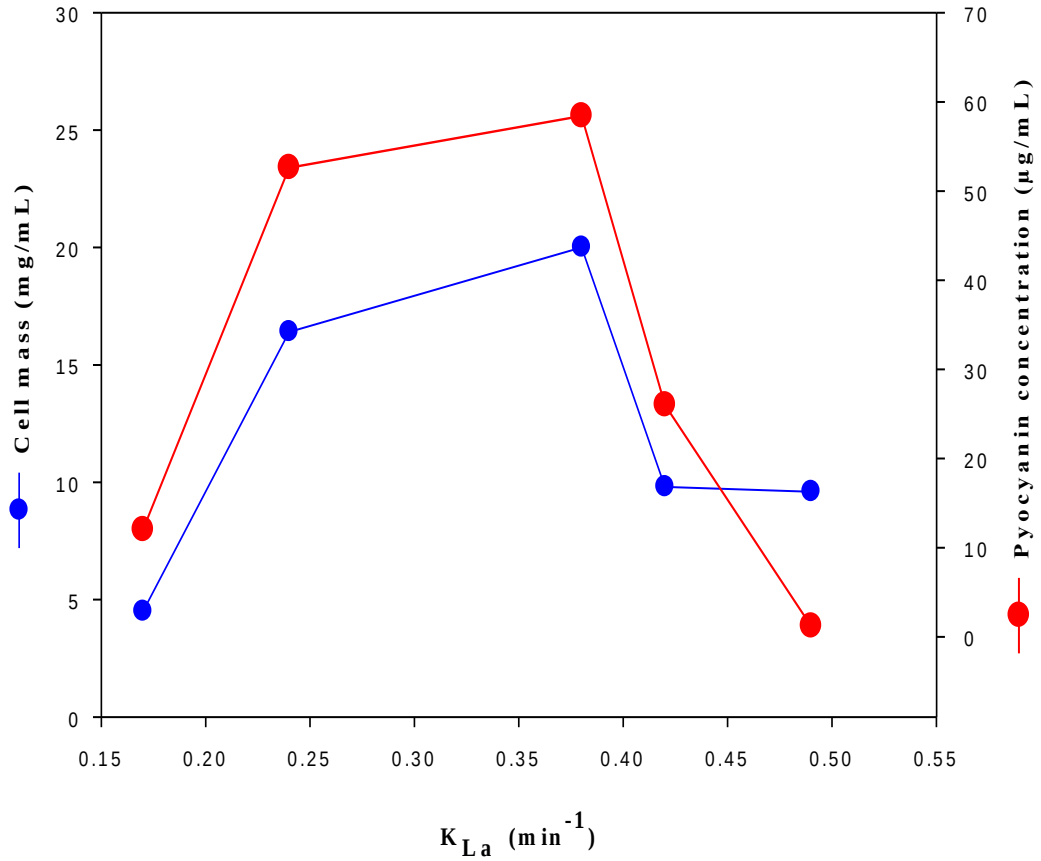

Fig. 9: Effect of K_La on growth of *P. aeruginosa* and pyocyanin production.

In previous studies, the effects of process variables (*viz.* speed of impeller, flow of oxygen and impeller geometry) on the volumetric oxygen mass transfer coefficient, K_La, in a biocalorimeter (Bio-RC1) were studied. They obtained the maximum K_La value of 0.559min^{-1} under the agitation and aeration condition of 200rpm and 1L min^{-1} for growth of *P. aeruginosa*.

According to their study, further increase in impeller speed to 250rpm caused a significant decrease in growth observed which may be due to inefficient oxygen transfer under high turbulence conditions and high foaming formation at 250 rpm was also observed. Turbine-type impeller showed maximum transfer of oxygen when compared with Rushton-turbine type. This may be due to the increase in viscosity of broth media due to biomass growth and the inefficiency of Rushton-turbine type to break the bubbles effectively for enhanced mass transfer. Excessive foaming was also observed when Rushton-Turbine type impeller was used[25].

Conclusion

The desired combination of agitation and aeration rate is necessary for growth and production of pyocyanin by *P. aeruginosa* in a fermenter. The K_La was found to be a critical factor in fermentation. The sets of fermentation batches were designed by RSM to enhance the production of pyocyanin by *P. aeruginosa* at minimum cost and time.

The production of pyocyanin pigment from *P. aeruginosa* has been improved after the scale up. The production time was also lowered to 72 from 120h which favors the bioprocess economics. This suggests that pyocyanin production by *P. aeruginosa* holds the potential for the commercial production of pyocyanin.

Acknowledgement

The author Alka Rani gratefully acknowledges the senior research fellowship from Indian Council of Medical Research, Govt. of India, New Delhi.

References

1. Abo-Zaid G.A., Wagih E.E., Matar S.M., Ashmawy N.A. and Hafez E.E., Scaling-up production of pyocyanin from *Pseudomonas aeruginosa* JY21 as biocontrol agent against certain plant pathogenic fungi, *Int. J. Chem Tech Res.*, **8(9),** 213 **(2015)**

2. Arung E.T., Wicaksono B.D., Handoko Y.A., Kusuma I.W., Yulia D. and Sandra F., Anti-cancer properties of diethylether extract of wood from sukun (*Artocarpus altilis*) in human breast cancer (T47D) cells, *Trop. J. Pharm. Res.*, **8(4),** 317 **(2009)**

3. Barakat K.M., Mattar M.Z., Sabae S.Z., Darwesh O.M. and Hassan S.H., Production and characterization of bioactive pyocyanin pigment by marine *Pseudomonas aeruginosa* OSh1, *Res. J. Pharm. Biol. Chem. Sci.*, **6(5)**, 933 **(2015)**

4. Boon N., De Maeyer K., Höfte M., Rabaey K. and Verstraete W., Use of Pseudomonas species producing phenazine-based metabolites in the anodes of microbial fuel cells to improve electricity generation, *Appl. Microbial. Biotechnol.*, **80(6),** 985 **(2008)**

5. Essar D.W., Eberly L.E.E., Hadero A. and Crawford I.P., Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications, *J. Bacteriol.*, **172(2)**, 884 **(1990)**

6. Fenice M., Barghini P., Selbmann L. and Federici F., Combined effects of agitation and aeration on the chitinolytic enzymes production by the Antarctic fungus *Lecanicillium muscarium* CCFEE 5003, *Microbial Cell Factories*, **11(12)**, 12 **(2012)**

7. Fruehauf J.P. and Meyskens F.L., Reactive oxygen species: a breath of life or death?, *Clin. Cancer Res.*, **13(3)**, 789 **(2007)**

8. Hall S., McDermott C., Anoopkumar-Dukie S., McFarland A., Forbes A., Perkins A., Davey A., Chess-Williams R., Kiefel M., Arora D. and Grant G., Cellular effects of pyocyanin, a secreted virulence factor of *Pseudomonas aeruginosa*, *Toxins*, **8(8)**, 236 **(2016)**

9. Hassani H.H., Hasan H.M., Al-Saadi A., Ali A.M. and Muhammad M.H., A comparative study on cytotoxicity and apoptotic activity of pyocyanin produced by wild type and mutant strains of *Pseudomonas aeruginosa*, *Euro. J. Exper. Biol.*, **2**, 1389 **(2012)**

10. Hussein S.S., Hashim N. and Mamdoh A., Cytotoxic effect of pyocyaninon leukemic cells *in vitro*, *Iraqi J. Cancer*, **2**, 73 **(2009)**

11. Lau G.W., Hassett D.J., Ran H. and Kong, F., The role of pyocyanin in *Pseudomonas aeruginosa* infection, *Trends Mol. Med.*, **10(12),** 599 **(2004)**

12. Liyana-Pathirana C.M. and Shahidi F., Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions, *J. Agric. Food Chem.*, **53(7)**, 2433 **(2005)**

13. Meyer J.M., Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent Pseudomonas species, *Arch. Microbial.*, **174(3),** 135 **(2000)**

14. Moayedi A., Nowroozi J. and Sepahy A.A., Cytotoxic effect of pyocyanin on human pancreatic cancer cell line (Panc-1), *IJBMS*, **21(8)**, 794 **(2018)**

15. Narenkumar J., Sathishkumar K., Sarankumar R.K., Murugan K. and Rajasekar A., An anticorrosive study on potential bioactive compound produced by *Pseudomonas aeruginosa* TBH2 against the biocorrosive bacterial biofilm on copper metal, *J. Mol. Liq.*, **243**, 706 **(2017)**

16. Pai S.S., Anas A., Jayaprakash N.S., Priyaja P., Sreelakshmi B., Preetha R., Philip R., Mohandas A. and Singh I.S.B., *Penaeus monodon* larvae can be protected from Vibrio harveyi infection by pre-emptive treatment of a rearing system with antagonistic or non-antagonistic bacterial probiotics, *Aqua. Res.*, **41(6),** 847 **(2010)**

17. Park E.J., Oh H., Kang T.H., Sohn D.H. and Kim Y.C., An isocoumarin with hepatoprotective activity in Hep G2 and primary hepatocytes from *Agrimonia pilosa*, *Arch. Pharm. Res.*, **27(9),** 944 **(2004)**

18. Price-Whelan A., Dietrich L.E. and Newman D.K., Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14, *J. Bacterial.*, **189(17),** 6372 **(2007)**

19. Priyaja P., Pyocyanin (5-methyl-1-hydroxyphenazine) produced by *Pseudomonas aeruginosa* as antagonist to vibrios in aquaculture: Overexpression, downstream process and toxicity **(2012)**

20. Rabaey K., Boon N., Höfte M. and Verstraete W., Microbial phenazine production enhances electron transfer in biofuel cells, *Environ. Sci. Technol.*, **39(9),** 3401 **(2005)**

21. Rani A., Chauhan S. and Azmi W., Production and antimicrobial, antioxidant and anticancer applications of pyocyanin from isolated *Pseudomonas aeruginosa*, *Sci Fed J. Fermen. Microbial Technol.*, **1**, 2 **(2018)**

22. Sabaté M., Prats G., Moreno E., Ballesté E., Blanch A.R. and Andreu A., Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater, *Res. Microbiol.*, **159(4),** 288 **(2008)**

23. Salman M.T., Khan R.A. and Shukla I., Antimicrobial activity of *Nigella sativa* Linn. seed oil against multi-drug resistant bacteria from clinical isolates, *Nat. Prod. Radiance*, **7(1),** 10 **(2008)**

24. Sarkisova S., Patrauchan M.A., Berglund D., Nivens D.E. and Franklin M.J., Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms, *J. Bacteriol.*, **187(13),** 4327 **(2005)**

25. Sivaprakasam S., Mahadevan S. and Gopalaraman S., Oxygen mass transfer studies on batch cultivation of *P. aeruginosa* in a biocalorimeter, *Electron. J. Biotechn.*, **11(1),** 80 **(2008)**.

(Received 21st September 2019, accepted 20th November 2019)