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Xylanase production from *Phanerochaete chrysosporium* using response surface methodology and its validation in a bioreactor

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***Abstract*— The parameters affecting the production of xylanase from *Phanerochaete chrysosporium* were studied using three factor central composite design for optimization. MEB was considered as the best suited media for the growth. The factors considered were agitation rate (0-200 rpm), temperature (25-35°C) and pH (3.5-6.5). The most significant factors influencing enzyme production were temperature and pH. The second order polynomial regression model obtained was fitted and found adequate, with an R^2 of 0.9725 ($p < 0.0001$). Xylanase activity of 6.72 U ml^{-1} was obtained in 144 h of cultivation at agitation rate of 150 rpm, temperature 30°C and pH 4.5. The validation of the optimized result was carried out in a 7.5 L stirred-tank bioreactor at an aeration and agitation rates in the range of 0.5-1.0 vvm and of 50-100 rpm respectively. The culture was continued for 96 h with an initial pH of 4.5 and 30°C temperature. Xylanase activity of 7.2 U ml^{-1} in 53 h was attained in the bioreactor at 0.5vvm which was 20% higher than that of the shake flask cultivation study. A significant reduction of 63% in the cultivation time at the bioreactor was recorded. The k_La value for the bioreactor as determined by dynamic gassing out method was 0.127 h^{-1}.**

***Index Terms*— Bioreactor, Optimization, *Phanerochaete chrysosporium*, RSM, Xylanase**

I. INTRODUCTION

In recent years, extracellular enzyme, xylanase extracted from basidiomycetes group of fungi has awaken enormous interest for its wide range of industrial applications such as wood pulp bio-bleaching, manufacture of food and beverages, animal nutrition and bioethanol production. Our interest in the present research is the bio-bleaching ability of xylanase. Xylanases have the capability to hydrolyse hemicellulose present in the pulp without affecting the cellulose. The major structural component of plant cell wall comprising 20-35% dry weight of wood and agricultural wastes is the hemicellulosic polysaccharide called xylan [1]. When treated with cellulosic pulps, xylanase can selectively remove residual xylan dissolved in the pulp and thereby improve the pulp quality. Xylan forms an inter-phase between lignin and other polysaccharides. It is likely that xylan molecules covalently link with phenolic residues of lignin and also interact with polysaccharides, such as pectin and glucan. In simplest form, xylans are linear homopolymers that contain D-xylose monomers linked through β-1, 4–glycosyl bonds. Xylanase hydrolyses β-1, 4 xylan by cleaving β-1, 4 glycosidic linkages randomly, and xylose and xylo-oligosaccharides like xylobiose are produced [2]. Pulp and paper industries are considered to be highly polluting industries in the world [3]. Utilization of microbial xylanase in these industries will reduce the chlorine consumption, absorbable organic halogens (AOX), chemical oxygen demand (COD) and make the pulp and paper industries more environmental friendly [4].

Phanerochaete chrysosporium was advantageously utilized for the production of xylanase [5], [6]. The cultivation involves many factors, such as temperature, pH and agitation rate, which are important and influence the growth and productivity. It is difficult to find the most important factors and therefore necessary to optimize the conditions for improving the production of xylanase. Response surface methodology (RSM) is an experimental strategy for seeking the optimum conditions for a multivariable system [7]. The aim of this research is to apply the central composite design for examining and optimizing the culture conditions for production of xylanase from *Phanerochaete chrysosporium* in shake flask and validate in a bioreactor at 3 L level.

II. MATERIALS AND METHODS

A. Organism

The fungal strain *Phanerochaete chrysosporium* (MTCC 787) was taken from CSIR-IMTECH, Chandigarh, India. Cultures of the strain were maintained on MEA (Malt extract agar) (in g/l: malt 30.0; peptone 5.0 and agar 15.0) slants at 4°C by transferring to a fresh medium at regular interval.

B. Chemicals and reagents

All chemicals used were of AR grade. Birch wood xylan and chemicals for the enzyme assay were from Sigma Aldrich, USA. Malt, peptone and agar were from HiMedia, Mumbai.

C. Detection of xylanase

The strain *P. chrysosporium* was tested for its ability to produce xylanase on selective xylan agar media [8]. The agar plate was incubated at 30°C for 24 h. The plates were then stained with congo red solution composed of 0.5% congo red and 5% ethanol for 15 min and de-stained with 1M NaCl. Formation of yellow zone around the colony indicates xylanase positive.

D. Xylanase assays

Xylanase assay was studied both in extracellular and intracellular materials. In extracellular study, the clear

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supernatant obtained by centrifugation (10000 rpm, 10 min) of the culture-broth was used for estimating the xylanase activity. Mycelial biomass was collected and subjected to cell lysis through sonication (SONICS & Materials, Inc., 40 kHz, USA) to extract the intracellular xylanase present. Xylanase assay was determined by measuring released reducing sugar from birch wood xylan with 3, 5-dinitrosalicylic acid (DNS) [9] with xylose (0-500 µg) as the standard. The reaction mixture consists of acetate buffer (0.2 M, pH 5.0), 0.3 ml of 1% (w/v) xylan (substrate) and 0.3 ml of enzyme solution. After 30 min of incubation, the reaction was arrested by adding 1 ml DNS 1% (w/v) and again incubated in a boiling water bath for 15 min. The absorbance of the colour intensity was recorded at 540 nm. An auto zero was set in UV-VIS spectrophotometer (Lambda 35, Perkin Elmer, USA) using blank solution. The intracellular xylanase activity was observed to be in the range of 0.066 to 0.749 U ml^{-1} which contributed only 1% of the total xylanase produced. One unit of the xylanase activity was defined as the amount of enzyme that release 1 µmol of reducing sugar (expressed as xylose equivalent) per min under the above assay conditions.

E. Inoculum preparation

The fungal strain was cultivated on three media for production of xylanase viz. Potato dextrose broth (PDB), malt extract broth (MEB) and Mandel & Reese [10]. The PDB contained (in g/l) 20.0 dextrose, 200.0 potato extract; The MEB contained (in g/l): 30.0 malt, 5.0 peptone; Medium of Mandel & Reese contained 1% proteose peptone, 1.4 g/l $(NH_4)_2SO_4$, 2.0 g/l KH_2PO_4, 0.3 g/l urea, 0.3 g/l $CaCl_2$, 0.3 g/l $MgSO_4.7H_20$, 5.0 mg/l $FeSO_4.7H_2O$, 1.6 mg/l $MnSO_4.H_2O$, 1.4 mg/l $ZnSO_4.7H_2O$, 2.0 mg/l $CoCl_2$. All these media were adjusted to pH 4.5 and autoclaved. The inoculum was prepared by transferring a loopful of spores from 7 days old cultures into 250 ml flasks containing 50 ml of the above media under sterilized conditions in laminar air flow (Ikon Instruments, Delhi) and incubated at 30°C in shaking (NBS, INNOVA 44R Orbital shaker) conditions at 150 rpm for 9 days. Preliminary investigation revealed that in comparison the xylanase production was low in the medium of Mandel & Reese (1.986 U ml^{-1}) and PDB (0.072 U ml^{-1}). Therefore, MEB was selected as the growth media for the shake flask studies.

F. Shake flask experiments

The effect of temperature (25-35°C) and initial pH (3.5-6.5) on the xylanase production under varying conditions were studied. Flasks were taken out from the shaker at regular interval of 24 h over a period of 24 to 216 h as per the experimental design. The broth from each flask was centrifuged at 10,000 rpm for 10 min and the clear supernatant was used for the enzyme assay. The final biomass expressed as dry cell weight (mg ml^{-1}) was determined by means of gravimetric method. 5ml fermentation broth was filtered through pre-weight Whatman No. 1 filter paper, followed by drying to constant weight at 40°C for approximately 2 h.

G. Optimization experimental design

Three independent variables; temperature (25-35°C), initial pH (3.5-6.5) and fermentation time (24-216 h) were investigated for their influences on the production of xylanase. A central composite design (CCD) for a three-variable (k=3), five combinations coded -1.682, -1, 0, +1, +1.682 was employed to study the combined effect of these independent variables which generated 20 treatment combinations. The 20 sets of randomized experiments included three factorial points, five central points and four extra axial points. Table I shows the actual factor levels corresponding to the coded levels. The treatment combinations and the actual values for response are given away in Table II. All analyses were carried out in triplicate and the average mean values were taken. The variables were coded according to the following equation:

$$x_i = \frac{(X_i - X_i^*)}{\Delta X_i} \qquad \ldots\ldots \quad (1)$$

Where, x_i is the coded value of the i^{th} independent variable, X_i is the corresponding natural value of the i^{th} independent variable, X_i^* is the natural value of the i^{th} independent variable at the centre point of the considered domain and ΔX_i is the step change value of the i^{th} independent variable. Using the aforesaid design a second order polynomial equation regression model (2) is proposed for the generation of the response surface. The experimental design used in this research is Central Composite Rotatable Design (CCRD). The design was proposed by Box and Wilson [11]. CCRD with the rotatable property is conducted by choosing an appropriate axial distance [12]. Rotatable property is important for a second-order design to possess a reasonably stable distribution of scaled prediction variance throughout the experimental design region. The data was subjected to analysis of variance (ANOVA) and multiple regressions using the Design-Expert version 9.0.1.0 software (State-Ease Inc., Minneapolis, MN 55413, USA)

$$Y = \beta_0 + \sum_{i=1}^{k} X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \ \ldots\ldots \quad (2)$$

Where, Y is the predicted response for xylanase production, X_i and X_j are the levels of independent variables, β_0 is the intercept term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient and k is the number of factors optimized in the study.

H. Bioreactor geometries and experiment

The bioreactor experiments were carried out in a 7.5 L stirred-tank reactor (Model NBS Bioflo110, New Brunswick Inc., NJ, USA) in an operating volume of 3 L. The bioreactor is constructed of jacketed glass and the L/D ratio is 1.8. A spin filter attachment with marine type impellers was used for the experiments. The design details of the bioreactor vessel are as follows: Internal diameter 17.5 cm; Height 32.5 cm; Number of impellers 3; Type of impeller disc marine; Impeller diameter 9 cm; Spin filter height 17 cm, diameter 10 cm; Location of bottom of impeller from bottom of vessel 5.5 cm; The head plate of the bioreactor has 16 ports on use for pH, dissolved oxygen and temperature probes, air sparging, sampling and ventilation etc. Air was sparged into the bioreactor from an air compressor (EC Medicair 102 S/R, Italy) with control valve. The compressed air was sparged through a 0.2 µm (PTFE) membrane filter and a rotameter.

The seed culture for the bioreactor experiment was grown separately in 250 ml Erlenmeyer flask in an incubator orbital shaker (NBS, INNOVA 44R, New Brunswick Inc., USA) at an agitation rate of 150 rpm and 30°C for 48 h. The reactor vessel containing the media was sterilized in an autoclave and inoculation was carried out aseptically with 7% (v/v) inoculum. The experiment was continued for 96 h and during the process the agitation (50-100 rpm) and aeration (0.5-1.0 vvm) rates were varied according to the investigational plan. For the studies on influence of DO on the enzyme production, three different sets of experiments with 0.25, 0.5, 1.0 vvm of air supply was carried out. Samples of 5.0 ml aliquots were withdrawn at every 4 h interval for analysis. The culture samples were centrifuged (Sigma 6-16K, Sigma Laborzentrifugen GmbH, Germany) at 10,000 rpm and 4^0C for 5 min and were assayed for dry cell mass and enzyme activities. The change in dissolved oxygen concentration during the course of the fermentation was also recorded throughout the incubation period. The experiments were repeated three times and the presented values are the average of three independent experiments, the standard deviation for each value is less than 5%.

III. Results and Discussion

A total of 20 experiments with different combinations of the three variables were carried out. The experimental design and the results are shown in Table I and II. The highest xylanase activity (6.72 U ml^{-1}) was observed at run number 17, where the factors viz. fermentation time, initial pH and experimental temperature were used at levels of 144 h, 5.0 and 30°C respectively. This activity was observed to be more than twice the lowest value that was recorded at run number 13, where the related factors were used at lower level for X_3 (-1). As per the analysis, the xylanase production (Y) by *Phanerochaete chrysosporium* can be expressed in terms of the following regression model equation:

$$Y = 6.53 + 0.025\,X_1 - 0.17\,X_2 + 0.33\,X_3 - 0.64\,X_1^2 - 0.66\,X_2^2 - 1.01\,X_3^2 - 0.079\,X_1X_2 - 0.0001\,X_1X_3 + 0.32\,X_2X_3 \quad \ldots\ldots \quad (3)$$

where, X_1 =fermentation time in h, X_2= initial pH, X_3= experimental temperature in °C

Table III shows the analysis of the variance (ANOVA) and the regression coefficients indicate high significance of the model. The high R^2 value 0.9725 showed good agreement between the theoretical values predicted by the model and the experimental results [13]. The value of R^2 indicated that only 2.75% of the total variations were not explained by the model. The R^2 value was always between 0 and 1 and closer the R^2 was to 1.0, the stronger the model for better prediction of the response [14]. The value of the adjusted determination coefficient (adj R^2=0.9478) was also reasonably high to advocate for a high significance of the model [14]. A lower value of coefficient of variation (CV=5.44%) showed the experiments conducted were precise and reliable [15].

Table I: Process variables used in the CCRD (k=3) with actual factor levels corresponding to the coded

Run	Coded variable factors			Mean Response,
	X_1	X_2	X_3	Y (U ml^{-1})
1	-1	-1	-1	4.121
2	-1	-1	1	4.046
3	-1	1	-1	3.657
4	-1	1	1	4.721
5	1	-1	-1	4.414
6	1	-1	1	4.203
7	1	1	-1	3.5
8	1	1	1	4.694
9	-1.682	0	0	4.755
10	1.682	0	0	4.803
11	0	-1.682	0	5.328
12	0	1.682	0	4.107
13	0	0	-1.682	2.995
14	0	0	1.682	4.475
15	0	0	0	6.604
16	0	0	0	6.229
17	0	0	0	6.72
18	0	0	0	6.447
19	0	0	0	6.706
20	0	0	0	6.468

Table II: Treatment combinations and mean responses

Variable	Code#	Actual factor level at coded factor levels of					ΔΞ
		-1.682*	-1	0	1	1.682	
Time	X_1	22.91	72	144	216	265.09	24
pH	X_2	2.48	3.5	5	6.5	7.52	1.5
Temp	X_3	21.59	25	30	35	38.41	5

Code level limits based on preliminary investigations and also to reflect what is done in practice. X_1= (time 144)/24; X_2= (temperature 30)/5.0; X_3= (pH 5)/1.5

* Level based on Central Composite Rotatable Design

Table III: Analysis of variance for the evaluation of second order model in xylanase production

Variance Source	*df*	Sum of squares	Mean square	*F*-ratio	*p*-value
Model	9	25.62	2.85	39.29	< 0.0001
Residual	10	0.72	0.072		
Total	19	26.34			
R^2 = 0.9725; adj R^2 = 0.9478; pred R^2 = 0.8313; CV = 5.44%					

The significance of the coefficients was determined by their *p*-values which were listed in Table IV. The ANOVA analysis of the optimization study indicated that X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 were significant than the effect of X_1, X_2 and X_1X_2. Temperature indicated an affirmative influence on the xylanase production and in comparison, the fermentation

time and initial pH did not show much significance. Positive interaction was also shown between pH and temperature at the $p<0.01$ probability level. The coefficient of the interactions between X_1X_3 and X_1X_2 were not found to be significant.

Table IV: Estimated regression coefficients for xylanase production

Model term	Coefficient	Standard error	Significance level (p)
X_1	0.0254	0.073	0.7346
X_2	-0.1659	0.073	0.0460*
X_3	0.3265	0.073	0.0012**
X_1X_2	-0.0793	0.095	0.4244
X_1X_3	-0.0001	0.095	0.9918
X_2X_3	0.3183	0.095	0.0074**
X_1^2	-0.6425	0.071	< 0.0001**
X_2^2	-0.6642	0.071	< 0.0001**
X_3^2	-1.0116	0.071	< 0.0001**

*** highly significant: $p<0.01$; *significant: $p<0.05$*

It is clearly seen from the analysis that there is strong synergy between the fermentation time and temperature, as well as between the initial media pH and xylanase production. The response was represented by the polynomial quadratic (3), containing nine estimated coefficients with six significant at $p < 0.05$. The F-test for the regression was significant at a level of 39.29 at $p < 0.0001$ and the model did not show lack of fit and presented a high determination coefficient with R^2 value of 0.9725 explaining 97% variability of the response.

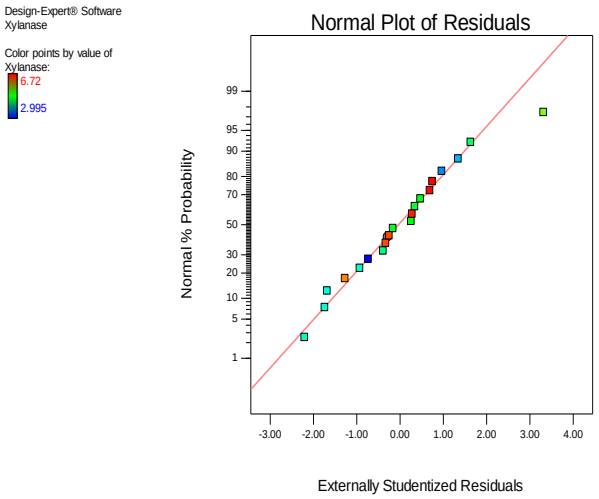

Fig. 1: Probability plot of residuals

The normal probability plot of residuals showing the normality of the residuals was presented in Fig. 1. The data were distributed in close proximity to the straight line indicating normal distribution for the residuals.

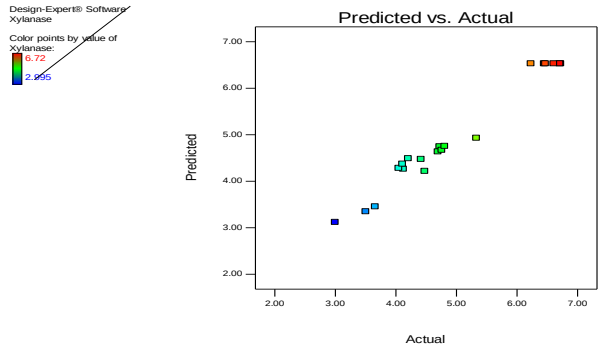

Fig. 2: Plot of predicted versus actual yields

The correlation between the actual and predicted values of the yields showing the linear agreement between the two values indicating the measure of reasonable accuracy among the results was presented in Fig. 2.

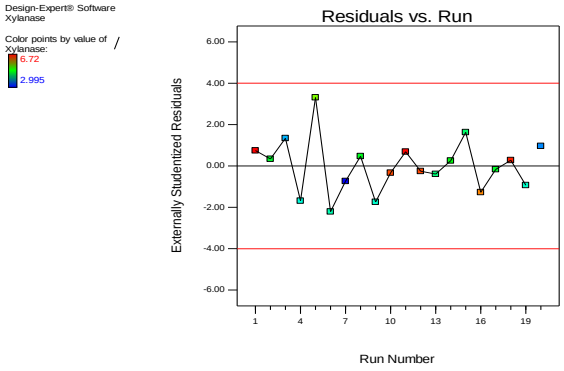

Fig. 3: Observation order versus residual plot

The observation order versus residual plot was illustrated in Fig. 3. The data were randomly scattered around zero-centred line. It indicated that the residual was independently random variable and uncorrelated.

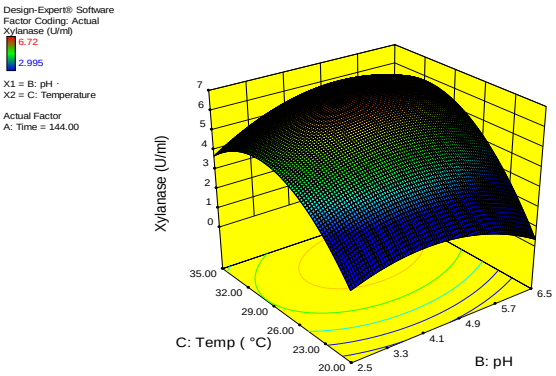

Fig. 4: Response surface plot of the combined effect of pH and temperature on xylanase production

The surface plot presented in Fig. 4 shows the interaction of initial pH and temperature on xylanase production. The plot shows an increment of xylanase production with the increase of pH and temperature up to a critical point. However, beyond these optimum condition the yield decreases, indicating the influence of thermal denaturation to inactivate the growth process in the fermentation. It is likely that the pH interferes with hydrogen and ionic bonds that hold together the enzyme and thereby decreases the rate of reaction for its production [16].

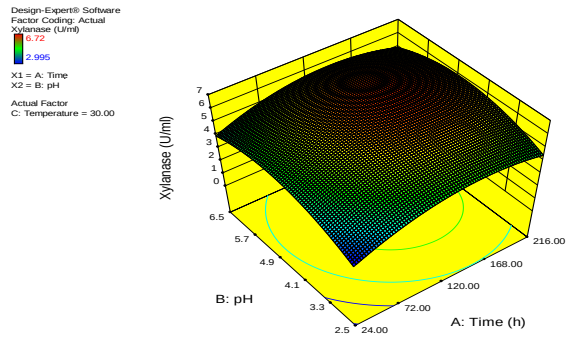

Fig. 5: Response surface plot of the combined effect of pH and fermentation time on xylanase production

Affect of the time course in the fermentation and initial pH values on the yield of xylanase was presented in Fig. 5. It was observed that xylanase increases gradually from 80-200 h of the fermentation and reaches a maximum value of 6.72 U ml^{-1} at 144 h. The illustration also indicates the interaction of pH and fermentation time, which had been observed as not highly significant in affecting the yield of xylanase.

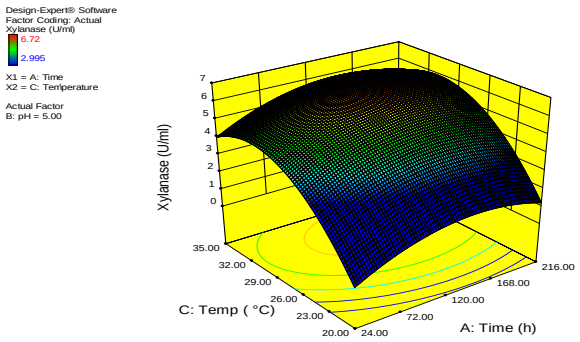

Fig. 6: Response surface plot of the combined effect of temperature and fermentation time on xylanase production

In this work maximum xylanase production of 6.72 U ml^{-1} was observed when temperature was maintained at 30°C and initial pH was kept at 4.5. It was observed that temperature influences the xylanase production (Fig. 6). With rise in temperature, the internal energy of the molecules in the system increases. This internal energy involving chemical bonding of the molecules as well as non-covalent interactions like hydrogen bonds and Vander Waals forces may convert the thermal energy into chemical potential energy which sever the weak bonds that determine the three dimensional shape of the active proteins. This could lead to a thermal denaturation of the protein and thus inactivate the growth process in the fermentation at higher temperatures [17].

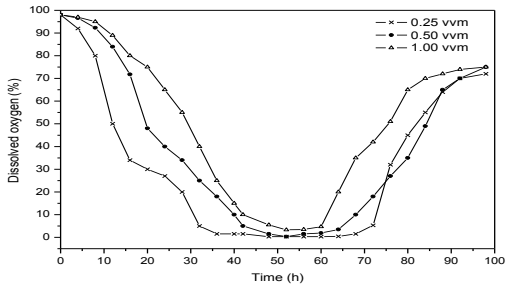

Fig. 7: Effect of aeration rate on dissolved oxygen saturation

The optimum conditions obtained through RSM for xylanase production from *P. chrysosporium* was verified in the bench scale bioreactor. Aeration inside the reactor influences the availability of the dissolved oxygen in the culture broth and thereby enhances the growth of the cell mass as well as the enzyme production [18]. The operating parameters during the course of the experiment was maintained by gradual increase of agitation from the normal operating value of 50 rpm so that the dissolved oxygen does not fall below 15% of the saturation value.

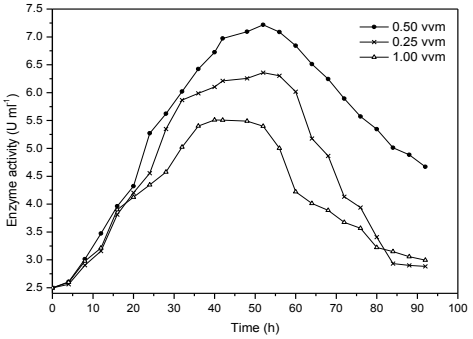

Fig. 8: Effect of aeration rate on Xylanase activity

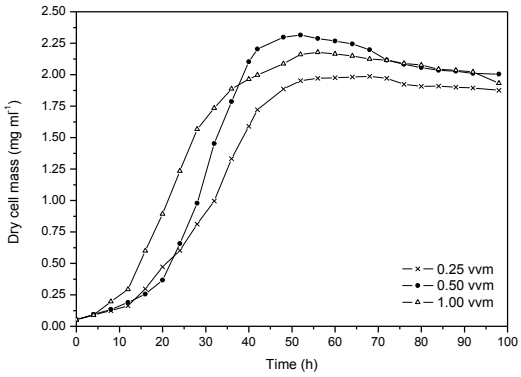

Fig. 9: Effect of aeration rate on cell mass growth

From the experiments carried out at three different aeration rates it was observed (Fig. 8 & 9) that the characteristic variation in the aeration rates did not have much influence on the growth of cell mass and xylanase production. With higher and lower aeration rates, beyond 0.5 vvm there was decrease in the xylanase production. The time requirement to achieve the maximum cell mass and the xylanase production remained reasonably the same. The reason may be attributed to the availability of sufficient dissolved oxygen concentration inside the reactor irrespective of variation of aeration rates in the experimental range. This can be observed in Fig. 7 which indicated that the influence of aeration rate was insignificant to the DO profile. The maximum dry cell mass of 2.315 mg ml^{-1} and xylanase production of 7.2 U ml^{-1} was obtained at 0.5 vvm over a period of 53 h of fermentation at 100 rpm agitation. It was also observed that during this period, the culture reached for the maximum yield and remained reasonably constant for about 70 h. Beyond this point of the cultivation, the production indicated a decline. It so happens that the growth enters a lethal phase wherein the availability of the nutrients diminishes and the concentration of the metabolites in the culture reaches such a level that cell autolysis might occur.

Volumetric oxygen mass transfer coefficient (k_La) of the bioreactor with spin filter marine blade impeller assembly was determined by a dynamic gassing out method [19] at 0.5 vvm air flow and 100 rpm agitation and was observed at 0.127 h^{-1}. With achievement of 20% higher yield than the shake flask results, along with significant decrease of 63% in the fermentation time, the model obtained can be considered valid and potential for scaled up production.

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