Xylanase Production by *Penicillium citrinum* in Laboratory-scale Stirred Tank Reactor

G. Ghoshal,[a,*] U. C. Banerjee[b] and U. S. Shivhare[a]

[a]Dr. S. S. Bhatnagar University Institute of Chemical Engineering and Technology, Panjab University, Chandigarh – 160014, India

[b]Department of Pharmaceutical Technology, National Institute of Pharmaceutical Education and Research, SAS Nagar-160062, Panjab, India

doi: 10.15255/CABEQ.2013.1885

Original scientific paper
Received: November 11, 2013
Accepted: August 31, 2014

Xylanase constitutes an important class of hydrolases, and is used in numerous industrial applications. The aim of the present work was to study the production of xylanase from *Penicillium citrinum* MTCC 9620 in a 5 L stirred tank bioreactor. Effect of various process parameters; pH, temperature, aeration, agitation rates, substrate concentration, and, dissolved oxygen (DO) concentration on xylanase production were studied. Combination of all the optimized parameters resulted in 2.5 times higher enzyme activity as compared to the shake flask fermentation after 96 h. Effect of varying agitation and aeration on the volumetric mass transfer coefficient (K_La) was determined. It revealed that K_La is influenced by both aeration and agitation. Growth kinetics of *P. citrinum* MTCC 9620 in bioreactor was studied using Monod, Moser, Contois and Edward equation. Based on R^2, SE and pattern of residuals, the microbial growth kinetics of *P. citrinum* MTCC 9620 was effectively represented by Moser equation.

Key word:
xylanase, *Penicillium citrinum*, sugarcane bagasse, volumetric mass transfer coefficient, kinetics models

Introduction

Xylanase is the group of enzymes that degrades xylan. Xylans are the major components of the hemicellulosic fraction of lignocellulosic biomass. Xylanase is mainly composed of endoxylanases that cleave the internal xylosidic linkages on xylan backbone and β-xylosidases that release xylosyl residues by endwise attack of xylooligosaccharide.[1,2] Xylanases have significant applications in the paper and pulp industry; bioconversion of lignocellulosic residues to alcohol, enzymes, etc.; clarification of fruit juices and beer; bakery manufacturing; and, improvement of digestibility of animal feed stocks.[2–5]

Usually, processed or refined substrates like sugars, cellulose and pure xylans are used in xylanase production. These substrates are however expensive in manufacturing xylanase at commercial level. Utilization of low cost agricultural residues not only reduce the environmental pollution problem but also add value to the waste materials in manufacturing useful products like enzymes.[6,7] The aim of the present study was to utilize cheaper lignocellulosic agro-industrial waste materials for xylanase production in a laboratory-scale bioreactor (fermenter). Aeration and agitation during fermentation have positive effects in xylanase production whether it is bacterial or filamentous fungal fermentation.[8] Determination of K_La is important to interpret the relative influence of mass transfer on chemical reaction rate. Volumetric mass transfer coefficient (K_La), indicates that the mass transfer rate is dependent on agitation and aeration.

Moreover, compared to the experimental studies modelling studies on the production of xylanase has been scanty. Kinetics of enzyme production can be characterized by modelling the kinetic data. Combining experimental results with mathematical modelling, it is feasible to represent a meaningful explanation of the results to analyze new aspects of microbial physiology. Model formulation, identification and estimation of the parameters and solutions of the equations are difficult in all stages of model development. In this present study, modelling of growth kinetics of fermenter data was represented using Monod,[9] Moser,[10] Contois[11] and Edward[12] equation.

The specific objectives were to investigate: (i) The dependence of growth of microorganisms and xylanase production by *Penicillium citrinum* MTCC 9620 on pH, temperature, aeration, agitation, dis-

*Corresponding author: gghoshal@yahoo.com; phone +91-(0172)2534908 (O), +91–9872749308 (M); Fax: +91–0172–2779173

solved oxygen concentration, substrate concentration (alkali treated sugarcane bagasse) during fermentation in controlled atmosphere in stirred tank reactor; (ii) Effect of aeration and agitation on volumetric oxygen transfer coefficient (K_La); and (iii) Modelling of the growth kinetics of *P. citrinum*.

Materials and methods

Chemicals and substrates

All the chemicals used were of AR grade. Birchwood xylan, *N*-Acetyl-D-glucosamine and *N*-*N*-dimethyl-*p*-aminobenzaldehyde were purchased from Sigma-Aldrich, Seelze, Germany. Sugarcane bagasse was purchased from local market of Chandigarh, India. Sugarcane bagasse was treated with 0.1 mol L^{-1} alkali, washed with distilled water to neutralize, dried and used as substrate.[13]

Microorganisms and growth conditions

Fungal strain *P. citrinum* was isolated from paper pulp industry waste (Bilt Paper Mill, Yamunagar, Haryana). Minimal salt media (MSM) containing 0.5 g L^{-1} KCl, 0.5g L^{-1} $MgSO_4 \cdot 7H_2O$, 5.0 g L^{-1} NH_4NO_3, 0.1 g L^{-1} $MnSO_4$, H_2O, 0.1 g L^{-1} $FeSO_4 \cdot 7H_2O$ was used for isolation of organism. 1 g of paper pulp waste was mixed with 10 mL sterilized distilled water to make slurry and the slurry was used as inocula. Spread plate method was used to isolate fungal strains. The inoculated plates were incubated at 30 ºC for 72 h. Identical larger colonies were selected and were further grown in Czapek yeast extract media containing 3.0 g L^{-1} $NaNO_3$, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.01 g L^{-1} $FeSO_4 \cdot 7\ H_2O$, 1 g L^{-1} K_2HPO_4, 5 g L^{-1} yeast extract, 30 g L^{-1} sucrose and 1 % birchwood xylan in 250 mL conical flask containing 50 mL media. The isolate was preserved at 4 ºC on slants. The organism was maintained in the Czapek yeast extract agar media. The seed culture for fermenter was grown in two stages. In the first stage, organism was grown in 100 mL conical flask containing 20 mL medium and in the second stage in 500 mL conical flask containing 100 mL medium. Both the cultures were allowed to grow at an agitation rate of 200 rpm at 30 ºC for 24 h. Freshly-grown colonies on the plates were used for inocula and seed culture development.

Xylanase production

Batch culture for the growth and production of xylanase by *P. citrinum* MTCC 9620 was carried out in a 5-L stirred tank reactor (fermenter of 3-L working volume, B-Lite, Sartorious, Bangalore, India). Initially, the media composition and physicochemical parameters were optimized in a shake flask. The fermenter (3-L) containing the media was sterilized in the autoclave and inoculated aseptically with 10 % (v/v) inoculum. Foaming was controlled using minimum amount of refined soybean oil. The culture conditions varied were pH, temperature, agitation, aeration, dissolved oxygen concentration (%), and substrate (sugarcane bagasse, SB) concentration.

Dissolved oxygen (DO) was measured with a membrane-covered polarographic oxygen electrode which was heat-sterilizable (Metteler Toledo, MA, USA). The ``zero'' was realized by sparging nitrogen into the sterilized medium. The full scale was established by sparging air so that a dissolved oxygen concentration of "100 %" corresponded to a medium saturated with air. DO level was varied from 5 to 20 %.

Conditions of standard fermentation were 30 ºC, initial pH 6.0 (as standardized in shake flask), and 144 h of fermentation time. Four pH (uncontrolled) levels (4, 5, 6, 7), three levels of temperature (25 ºC, 30 ºC, 35 ºC), four levels of agitation speeds (200, 300, 400, and 500 rpm), three levels of aeration rates (0.5, 1.0, 1.5 vvm), four DO levels (5, 10, 15, 20 %), and three substrate concentrations (5, 10, 15 g L^{-1}) were used for fermenter run. Sugarcane bagasse was cleaned using distilled water, treated with 0.1 mol L^{-1} NaOH, washed with distilled water, dried, ground to ≤ 0.5 mm particle size and used as substrate.[13] Samples were withdrawn aseptically after every 24 h, centrifuged at 10,000 rpm for 20 minutes and filtrate was used for xylanase activity, extracellular protein, and sugar content. Retentate was dried at low temperature and used for biomass estimation and residual substrate estimation.

During fermentation, pH was maintained at desirable pH using 6 mol L^{-1} HCL and 1 mol L^{-1} NaOH. Product yield was calculated with respect to substrate consumed ($Y_{p/s}$) and to dry biomass ($Y_{p/x}$). Dry biomass yield was calculated with respect to substrate consumed ($Y_{x/s}$). Where, p is enzyme activity (U L^{-1}), s is substrate consumed (g L^{-1}), and X is dry biomass (g L^{-1}).

Estimation of total sugar

Total sugar content of the centrifuged supernatant of culture broth was estimated following Somogyi's method using Anthrone reagent.[14] The absorbance was measured at 625 nm against a reagent blank. Standard curve using glucose stock solution was prepared in 10–100 µg mL^{-1} range. The sugar content was expressed in terms of g L^{-1}.

Estimation of protein content

Extracellular protein concentration was estimated by Lowry method using bovine serum albumin (BSA) as standard.[15] Standard curve was prepared using BSA (1 mg mL^{-1}) dissolved in distilled water. All the assays were carried out in triplicate along with appropriate buffer and reagent control. The absorbance was monitored at 680 nm.

Estimation of biomass

Biomass estimation was carried out during the course of fermentation. Biomass was estimated using an indirect method by estimating glucosamine content after acid hydrolysis of the dry fermented substrate. 1 g of dry fermented substrate was suspended in 10 mL of 6 mol L^{-1} HCL in screw cap tubes. The tubes were heated in a boiling water bath for 4 h, cooled to room temperature, and the solids were separated. Supernatants were neutralized with 2 mol L^{-1} NaOH and used for glucosamine assays. Glucosamine content in the supernatant was determined by Erhlich's reagent (1.6 g *N*-*N*-dimethyl-*p*-aminobenzaldehyde in a 1:1 mixture of ethanol and concentrated HCl) from standard glucosamine plot. The biomass glucosamine was calculated by measuring glucosamine content in fermented dry substrate minus glucosamine content of the inoculated substrate. The conversion of glucosamine to biomass was adopted according to Said.[16] The conversion factor for glucosamine content to dry biomass was calculated by estimating glucosamine content of the dry biomass in submerged fermentation. Based on the data of the submerged culture, where the entire nutrient was in soluble condition, 120 mg of glucosamine was found equivalent to 1 g dry fungal biomass. Fungal biomass content of each batch of fermentation condition was expressed in g L^{-1}.

Enzyme assay

Xylanase activity was measured using 1 % birchwood xylan solution as a substrate.[17] The release of reducing sugars in 5 minutes at 50 °C, ph 5.3 (0.05 mol L^{-1} citrate buffer) was measured as xylose equivalent using dinitrosalicylic acid method.[18] One unit of xylanase activity (U) is defined as the amount of enzyme liberating 1 μmol of xylose per minute.

Filter paper cellulase activity (U) was measured according to IUPAC recommendations employing filter paper (Whatman No.1) as substrate.[19] Release of reducing sugar in 60 minutes at 50 °C at ph 4.8 (0.05 mol L^{-1} citrate buffer) was measured as glucose equivalent using dinitrosalicylic acid method. One unit of filter paper cellulase activity is measured as the amount of enzyme liberating 1 μmol of glucose per minute.

Determination of volumetric oxygen transfer coefficient ($K_L a$)

Volumetric oxygen transfer coefficient (K_La) of the laboratory-scale aerated, stirred tank reactor was determined using the dynamic gassing out method.[20] The dynamic gassing out method uses the respiratory action of the growing culture to vary oxygen concentration in the broth due to the respiratory activity of the cell mass. After few minutes, the air supply was switched on and oxygen concentration increased again, until it returned to the initial steady state value. K_La was computed using eq. (1).

$$dC_L/dt = K_L a(C_L^* - C_L) - XQ_{O_2} \quad (1)$$

where, $K_L a(C_L^* - C_L)$ is oxygen transfer rate (OTR, kg L^{-1} h^{-1}), XQ_{O_2} is oxygen uptake rate (OUR, kg L^{-1} h^{-1}), Q_{o_2}, is specific rate of oxygen consumption (h^{-1}) and X, is the biomass concentration (kg L^{-1}).

Rearranging eq. (1) yields

$$C_L = -1/K_L a[dC_L/dt + XQ_{O_2}] + C^* \quad (2)$$

A plot of C_L vs $[dC_L/dt + XQ_{o_2}]$ gives a straight line, with slope ($-1/K_L a$) and C^* as the intercept.

Modeling of microbial growth

Kinetics of microbial growth was analyzed from the dry fungal biomass measured under different conditions in due course of fermentation in the fermenter using the following rate equations.

$$\mu = \mu_m s/(K_s + s) \text{ (Monod equation)} \quad (3)$$

$$\mu = \mu_m (ks)^{Bi} / \left(1 + (ks)^{Bi}\right) \text{ (Moser equation)} \quad (4)$$

$$\mu = \mu_m \frac{1}{\left[1 + \frac{K_k x}{s}\right]} \text{ (Contois equation)} \quad (5)$$

$$\mu = \mu_m \frac{s}{K_s + s + \left(s^2/K_1\right)} \text{ (Edward equation)} \quad (6)$$

where,

Bi = Empirical coefficient, (dimensionless)

k = Biological rate equation coefficient, M^{-1} L^3

K_1 = Inhibition constant, M L^{-3}

K_k = Empirical constant in Contois equation, (dimensionless)

s = Substrate concentration, M L^{-3}

X = Dry fungal biomass per unit volume of batch culture, M L^{-3}

μ = Specific growth rate, h^{-1}

μ_m = Maximum specific growth rate, h^{-1}

K_s = Limiting substrate concentration when $\mu=\mu_m/2$, (dimensionless).

Statistical analysis

All the experiments were carried out in triplicate. Modelling and statistical parameters calculation (coefficient of determination, standard error, analysis of variance (ANOVA) were carried out using STATISTICA version 6.0 (Statsoft Inc., 2002, Tulsa, USA).

Results and discussion

The isolate was characterized by colonies with restricted growth and blue green on malt extract agar (MEA), reverse bright yellow with the same pigmentation diffusing into agar. The isolate was identified as *Penicillium citrinum* MTCC 9620 by the Institute of Microbial Technology (IMTECh), Chandigarh, India and deposited with an accession number of MTCC 9620. Optimum conditions for cultivation such as pH, temperature, agitation rate, medium composition and inoculation parameters for xylanase production by *P. citrinum* MTCC 9620 in shake flask was investigated.

Biomass growth and enzyme production by *P. citrinum* in the bioreactor is shown in Fig. 1. Fermentation was carried out at 30 ºC temperature, initial pH of 6.0 (uncontrolled), 300 rpm agitation and 1.0 vvm aeration for 144 h. In shake flask fermentation, optimum growth and enzyme production were obtained after 120 h of fermentation period with maximum activity of 156 U mL^{-1}, whereas in laboratory-scale bioreactor maximum fungal biomass (16.09 g L^{-1}) and maximum xylanase activity (246.53 U mL^{-1}) were obtained after 96 h. Protein content was also found maximum (0.965 mg mL^{-1}) after 96 h. From Fig. 1 it is apparent that sugar utilization was slower, which may have been due to the presence of sugarcane bagasse as substrate. An earlier report[21] established that the initial rate of substrate consumption was very high after that substrate consumption rate had decreased to almost constant in the early phase of fermentation when pure xylan was used as substrate. In the present study, the substrate utilization rate was not very fast, which may have been due to the presence of lignocellulosic substrate in the media, causing primary solubilization of insoluble non-starch polysaccharides into the medium, after which the microorganism can utilize that substrate for xylanase production. That xylanase acts as biocatalyst for solubilization of insoluble non-starch polysaccharide to soluble form in the media, therefore, the rate of consumption is almost constant giving the gradually decreased total sugar concentration plot with fermentation period. In shake flask fermentation, optimum pH was 6.0. Therefore, initial pH in fermenter was set to 6.0. As the growth progressed, the pH of the culture broth changed to 5.0 within 60 h and stabililized with subsequent increase of xylanase activity, which supports the results of the sugar consumption rate. As the rate of sugar consumption was almost constant, pH of the media was also almost constant. Earlier reports[3,22,23] revealed that, pH also changed with consumption of substrate. Dissolved oxygen level falls to 10 % within 60 h when % DO level was not maintained further % DO level decreased gradually to the level which was undetectable by the probe. Combination of agitation rate at 300 rpm and aeration rate at 1.0 vvm might not be sufficient to maintain oxygen demand of the microorganism in the media during exponential phase. Rate of transfer of oxygen from the gas phase to liquid phase was less than the rate of consumption of oxygen by microorganisms from liquid phase. Enzyme production was 2.5 times higher in bioreactor as compared to shake flask.

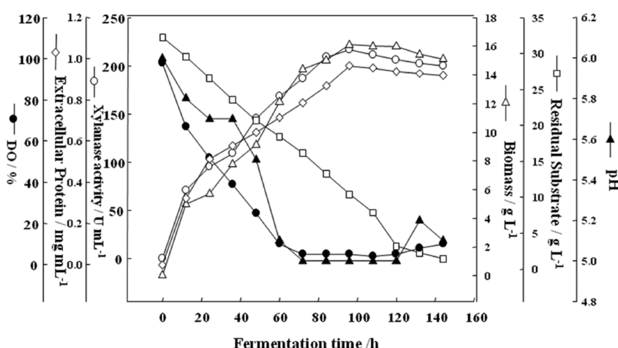

Fig. 1 – *Growth curve of P. citrinum MTCC 9620 for the production of xylanase in a laboratory scale stirred tank bioreactor. Batch fermentation run was taken in Czapek yeast extract media at initial pH 6 (uncontrolled), 30 ºC with aeration and agitation of 1.0 vvm and 300 rpm respectively.*

Effect of substrate concentration

The time to achieve maximum enzyme activity as well as maximum biomass growth decreases as compared to the case where lignocellulosic wastes are used instead of pure glucose or pure xylan as substrate. As the structure of lignocellulosic substrates is complex in nature, microorganisms need some time to degrade the substrate and solubilize in the media and then it can be easily utilized by the organism. In our recent study as the basal media contain sucrose, in the initial phase of fermentation the microorganisms utilized sucrose, and sugarcane bagasse was utilized after complete consumption of sucrose. Previous studies demonstrated that the optimum biomass growth was achieved in the fermen-

ter within 24 to 48 h when pure sugars were used, whereas in our study, 72–96 h was the optimum fermentation time range for optimum biomass growth and enzyme activity using lignocellulosics as carbon source.

Though the optimum fermentation period was higher, the process was economic since low cost lignocellulosic residues were used as substrate in fermenter. Total fermentation time to achieve maximum xylanase activity and biomass growth had decreased with increased concentration of substrate. The corresponding optimum fermentation times for 5, 10 and 15 g L^{-1} substrate concentrations were 96, 72 and 84 h, respectively. At substrate concentration of 10 g L^{-1}, $Y_{x/s}$ was higher compared to other concentrations, whereas $Y_{p/s}$ and $Y_{p/x}$ were lower but overall productivity was highest at this substrate concentration. It may therefore be inferred that 10 g L^{-1} is the optimum substrate concentration for maximum xylanase activity (223.43 U mL^{-1}).

Effect of aeration

In our study, we had chose three levels of aeration (0.5, 1.0 and 1.5 vvm). From Table 1 it is clear that biomass increased with aeration, and the highest growth was obtained with 1.0 vvm aeration at 72 h, whereas, maximum enzyme production was obtained with 1.0 vvm aeration at 96 h of fermentation. Maximum $Y_{p/x}$, $Y_{p/s}$ and $Y_{x/s}$ were obtained at 1.0 vvm aeration at 96 h. It can therefore, be inferred that higher aeration rate supports the fungal growth but not the enzyme production. At lower aeration, the fermenter may suffer O_2 depletion due to poor mixing of the fermenter content and eventual foam formation and thus the movement of fungal biomass may be hampered. At higher aeration in the fermenter vessel, the air flow along the shaft increases which results in the flooding of the impeller, and this is not desirable. Therefore, to maximize product formation, the optimum aeration level is requisite. When an impeller is encircled by air bubbles, the air bubbles detach the impeller from the contact of liquid causing poor mixing, reduced air dispersion, and diminished oxygen transfer[24] along with variation of temperature in the medium. In such conditions, the DO level is insufficient for microbial growth at higher agitation. Therefore, along with aeration, DO level optimization is important to avoid undesirable changes in the fungal morphology and rheological properties of the medium.

Table 1 – *Effect of different pH, temperature, agitation, aeration, % DO, and substrate concentration on xylanase activity, protein content, specific activity, biomass, productivity,* $Y_{p/s}$, $Y_{x/s}$, $Y_{p/x}$

Sl. No.	Parameter and level	Xylanase activity (· 10^3 U L^{-1})	Extracellular protein (g L^{-1})	Specific activity (U g^{-1})	Biomass (g L^{-1})	Productivity (U L^{-1} h^{-1})	$Y_{p/x}$ (· 10^3 U g^{-1})	$Y_{p/s}$ (· 10^3 U g^{-1})	$Y_{x/s}$
1	Substrate 5 g L^{-1}	174.23	0.98	178.52	13.32	1814.94	13.07	6.94	0.53
2	10 g L^{-1}	223.43	1.15	193.62	19.82	3104.22	11.27	11.54	1.02
3	15 g L^{-1}	209.22	0.99	209.85	14.56	2490.69	14.36	9.41	0.66
4	Aeration 0.5 vvm	188.62	0.98	193.26	19.29	1964.81	9.77	8.11	0.83
5	1.0 vvm	249.99	1.18	212.09	22.12	2604.03	11.48	10.42	1.21
6	1.5 vvm	159.90	1.12	142.34	19.69	1665.64	8.36	7.33	1.22
7	Temperature 25 ºC	198.34	0.94	210.29	19.26	2066.06	8.66	8.53	0.98
8	30 ºC	258.67	0.96	267.95	24.55	2694.53	11.34	10.78	0.95
9	35 ºC	207.68	0.88	236.94	17.25	2163.31	12.04	9.53	0.79
10	Agitation 200 rpm	121.23	0.90	135.15	13.66	1262.81	8.87	–	–
11	300 rpm	157.89	0.93	169.72	19.17	1644.76	7.79	6.79	0.94
12	400 rpm	267.86	1.25	213.74	22.54	2790.26	8.86	11.16	1.24
13	500 rpm	178.99	1.12	159.67	19.40	1864.45	9.23	8.21	0.89
14	% DO 5	226.79	0.96	234.91	18.88	3779.77	12.01	15.69	1.52
15	10	279.21	1.12	248.63	22.23	3877.96	12.55	15.59	0.94
16	15	205.54	1.01	204.11	16.88	2446.93	10.99	10.99	0.95
17	20	184.49	0.99	185.24	15.34	2196.41	11.64	8.29	0.79
18	pH 4	244.82	0.99	245.95	16.03	2550.23	15.27	9.18	0.60
19	5	299.51	1.14	263.64	22.53	3119.85	13.69	12.48	0.91
20	6	246.53	0.96	255.36	16.09	2567.99	15.31	11.31	0.74
21	7	205.48	0.94	217.85	09.19	2140.39	22.36	8.18	0.36

Effect of temperature

Optimum temperature for the growth and maximum xylanase production in the fermenter was found to be 30 ºC. Stability of any biocatalyst is dependent on temperature. At 25 and 35 ºC, the growth of organism was less as compared to that at 30 ºC. Table 1 shows that maximum cell growth was achieved at 30 ºC in 72 h. But maximum xylanase production was obtained at 30 ºC in 96 h. The growth continued for a longer period with lower magnitude (0.0219 g L^{-1} h^{-1}) of growth rate. Therefore, optimum xylanase production and biomass growth were achieved at 30 ºC with lower specific growth rate. The corresponding time to achieve maximum biomass and xylanase production at 30 ºC in fermenter was 72 h and 96 h respectively.

Effect of agitation

Agitation results in the proper mixing of the fermentation broth and influences the productivity of the system.[25] From Fig. 1 it is clear that the DO level decreased to 10 % at 300 rpm at 56 h of fermentation. Effect of agitation and aeration on different parameters is reported in Table 1.

Agitation conditions were varied from 200 to 500 rpm. At 200 and 300 rpm, xylanase activity and biomass growth were found relatively lower compared to higher agitation rates. This might be due to the improper mixing (at lower agitation rate) in the medium, where the microorganism experienced a deficiency of oxygen concentration. Maximum xylanase activity was obtained at 400 rpm after 96 h of fermentation with the early generation of biomass after 72 h. At 500 rpm, maximum biomass growth was obtained at 96 h of fermentation. At 300 rpm, maximum biomass growth was obtained after 84 h but the biomass concentration was lower than at 400 and 500 rpm. From Table 1, it is apparent that biomass growth and enzyme production decreased with increased agitation, which indicates that higher agitation results in greater shear stress, consequently reducing the cell growth. Similar observations have been reported by Manolov (1992) and Shioya *et al.* (1999) during the cultivation of *Aspergillus clavatus* and *Streptomyces virginiae*, respectively.[26,27] Panda (1989) reported a decrease in xylanase production when agitation was raised from 100 to 400 rpm.[28] He inferred that higher agitation results in hyphal disruption of mycelia and eventually lower xylanase production. In the present study, *P. citrinum* shows two morphological growth patterns. Diameter of the spherical cells decreased with increased agitation, whereas, thread-like mycelia and their length reduced. Higher agitation may cause dispersion of mycelia. The change in the growth pattern also affected the rheology and viscosity of the broth. With increasing agitation, a change in viscosity of the medium was also observed. Similar observations have been reported for various filamentous fungi.[22,29–32]

Effect of dissolved oxygen (DO) concentration

In the present study, four levels of dissolved oxygen were used to investigate the effect of DO on xylanase production and biomass growth. The effects of DO on the biomass growth and enzyme production are represented in Table 2. At 5 % DO level, both enzyme activity and biomass were at their maximum at 60 h of fermentation, whereas, at 10 % DO level both maximum enzyme activity and biomass were achieved at 84 h, which was 23 % higher than with 5 % DO level. Aeration at 1.0 vvm and DO at 10 % saturation have some synergistic effect on the activity and cell mass production, therefore the values of $Y_{p/x}$, $Y_{x/s}$, and $Y_{p/s}$ were higher at 10 % DO level.

Data on the effect of varying dissolved oxygen concentrations and oxygen availability on the production of xylanase by fungi are limited in scientif-

Table 2 – *Effect of agitation and aeration on oxygen transfer coefficient (K_La)*

Sl. no.	Agitation (rpm)	Aeration (vvm)	K_La (h^{-1})	Xylanase activity ($\cdot 10^3$ U L^{-1})	Extracellular protein content (g L^{-1})	Specific activity (U g^{-1})	Biomass (g L^{-1})	Productivity (U L^{-1} h^{-1})
1	300	0.50	11.68	109.02	0.79	142.25	17.02	1135.59
2	300	1.00	20.65	157.90	0.93	169.72	19.17	1644.76
3	300	1.50	30.29	148.31	0.88	168.16	16.89	1544.94
4	400	0.50	43.36	188.62	0.97	193.26	19.29	1964.81
5	400	1.00	46.84	249.99	1.18	212.09	22.11	2604.03
6	400	1.50	52.59	159.90	1.12	142.32	19.69	1665.64
7	500	0.50	55.98	138..01	0.99	138.61	18.05	1437.66
8	500	1.00	57.29	178.99	1.12	159.67	19.39	1864.45
9	500	1.50	62.06	158.33	1.00	155.42	17.51	1649.31

ic literature. *Trichoderma ressei* Rut C-30 was grown on 1 % cellulose or on xylan at oxygen level of 10–50 % saturation. Enzyme levels and extracellular proteins were not affected by oxygen levels of 20 % or above, but were severely reduced at 10 % oxygen saturation. Purkarthofer *et al.,* (1993), in xylanase production by *T. lanuginosus* DSM 5826, reported that a lower level of dissolved oxygen or even oxygen limitation had no adverse effect on enzyme production.[23] By varying the aeration rate used in laboratory fermentation of *T. lanuginosus* RT9 from 0.5 – 1.5 vvm, Hoq *et al.* (1994) showed a slight increase in enzyme activity was observed when the aeration rate was increased from 1.0 to 1.5 vvm.[33]

When DO level was increased above 10 %, an unfavorable effect was observed on both the growth and enzyme activity, which reflected on the morphology and metabolic pattern of cell growth. At higher DO level and lower agitation rate, the diameter of the cells was greater than at lower DO level. With the combination of 15 or 20 % DO level and higher agitation rate, cell growth was adversely affected and resulted in breakage of cells. A similar observation has been reported by Robinson (1984) and Rodriguez *et al.* (1991).[34,35] In the present study, the combination of 400 rpm, 1.0 vvm aeration and 10 % DO level yielded maximum enzyme activity and biomass growth.

Effect of initial pH

Initial pH of the medium was varied from 4 to 7 using 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH. pH was not controlled during fermentation. Effect of initial pH on the growth, xylanase production, protein content, and specific activity were observed in the bioreactor (Table 1). Maximum xylanase activity was obtained at pH 5.0 after 96 h, while maximum biomass growth was obtained after 72 h at pH 5.0. These results are in contrast with the results obtained in shake flask fermentation of *P. citrinum* MTCC 9620. In shake flask, optimum pH was 6.0, whereas in bioreactor the optimum pH was found to be 5.0. With the increased initial pH, both the enzyme activity and cell growth decreased. Irrespective of initial pH, the final pH changed within the range of 4.5 to 5.5 after 144 h fermentation. It may therefore be inferred that the optimum pH zone for the organism to grow in exponential phase is between the pH 4.5 – 5.5. However, the change in pH was very slow and fermentation was carried out without pH control. Fermentation with controlled pH, also showed no significant difference in maximum activity and biomass growth, compared to fermentation study without pH control. It can be inferred that control of pH at the selected level exhibited no beneficial effect on xylanase production and biomass growth. Xylanase yield with respect to biomass ($Y_{p/x}$) was calculated. $Y_{p/x}$ value was found maximum at pH 7.0 at 96 h of fermentation, and at pH 4.0 after 48 h. This may be due to the decrease in the corresponding biomass at lower pH, and therefore, $Y_{p/x}$ increased. Although the enzyme production and biomass production were maximum at pH 5.0, $Y_{p/x}$ was lower that with other pHs, whereas yield of xylanase with respect to substrate consumed, ($Y_{p/s}$) and dry biomass yield with respect to substrate consumed, ($Y_{x/s}$) were maximum at pH 6.0 after 96 h, and at pH 5.0. One important change in morphological behavior was observed at higher initial pH 6.0 and 7.0. The average diameter of the light yellow colored cells was 4.5 to 5.0 mm whereas at lower pH the average diameter of the dark yellow cells was 2 to 3 mm, along with thread-like 2 mm wide, 1 mm thick variable length mycelia with bluish spongy outer surface. It may therefore be inferred that by increasing pH, the inherent metabolism of the organisms had altered.

Effect of agitation and aeration on K_La

Different agitation speeds (300, 400 and 500 rpm) and different aeration rate (0.5, 1.0 and 1.5 vvm) were used in the bioreactor to investigate the effect of agitation and aeration on oxygen transfer coefficient K_La.

Fig. 2 shows that at a constant aeration level with increasing agitation, the degree of increase of K_La follows the same trends for all the aeration levels. From 300 to 400 rpm K_La increased sharply but from 400 to 500 rpm increase of K_La was not proportional. This trend was similar for all the aeration conditions. With the increase in aeration at a particular agitation rate the increase of K_La (Fig. 3) was very slow at higher agitation of 400 and 500 rpm but at lower agitation (300 rpm) K_La increased sharply. Oxygen transfer coefficient K_La increased with both agitation and aeration (Fig. 2 and Fig. 3). A similar observation has been reported by several

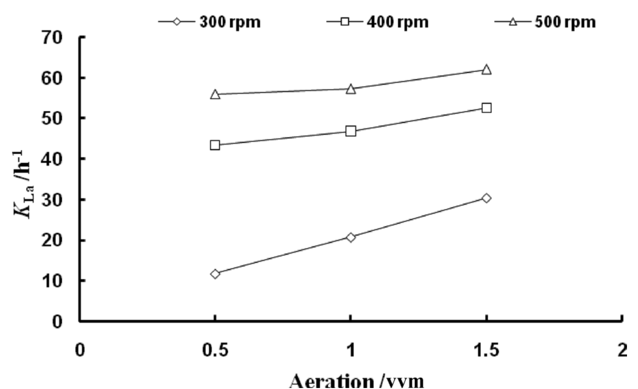

Fig. 2 – *Effect of aeration on the volumetric mass transfer coefficient K_La*

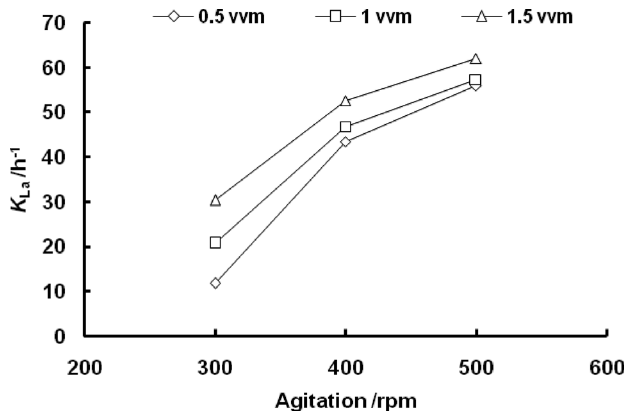

Fig. 3 – *Effect of agitation on the volumetric mass transfer coefficient K_La*

authors.[8,34,36] However, the increase was more rapid with variation in agitation than with aeration in the present study. It may therefore be inferred that agitation had a more pronounced effect than aeration.

Sugarcane bagasse can be used as a substrate for the production of xylanase in commercial scale which would render the process cost effective, although the fermentation period is longer (the microorganism took some time to respond initially) compared to pure xylan. Optimum conditions for xylanase production in laboratory-scale fermenter were media pH 5.0, temperature 30 °C, 400 rpm agitation, 1.0 vvm aeration, 10 % DO level and 10 g L^{-1} substrate (sugarcane bagasse) concentration. The optimum conditions resulted 2.5 fold higher xylanase activity than in shake flask conditions.

Modelling of microbial growth in fermenter

Growth kinetics of *P. citrinum* MTCC 9620 were studied in laboratory-scale fermenter. The effect of different parameters, such as substrate concentration (g L^{-1}), aeration (vvm), temperature (°C), agitation (rpm), dissolved oxygen concentration (%), and media pH on xylanase production (U mL^{-1}), dry biomass (g L^{-1}) were measured, varying one parameter at a time and keeping other parameters constant in the course of fermentation. Data were analyzed and modeled using Monod, Moser, Contois and Edward equation (eqs. 3–6). Adequacy of fit of each model was established considering the calculated values of the coefficient, coefficient of determination (R^2), and standard error (SE). Based on R^2, SE, and pattern of residuals, both Moser and Contoise model were found to describe adequately the growth kinetics of *P. citrinum* in laboratory-scale fermenter (Tabel 3 and Fig. 4). The coefficient of Moser mod-

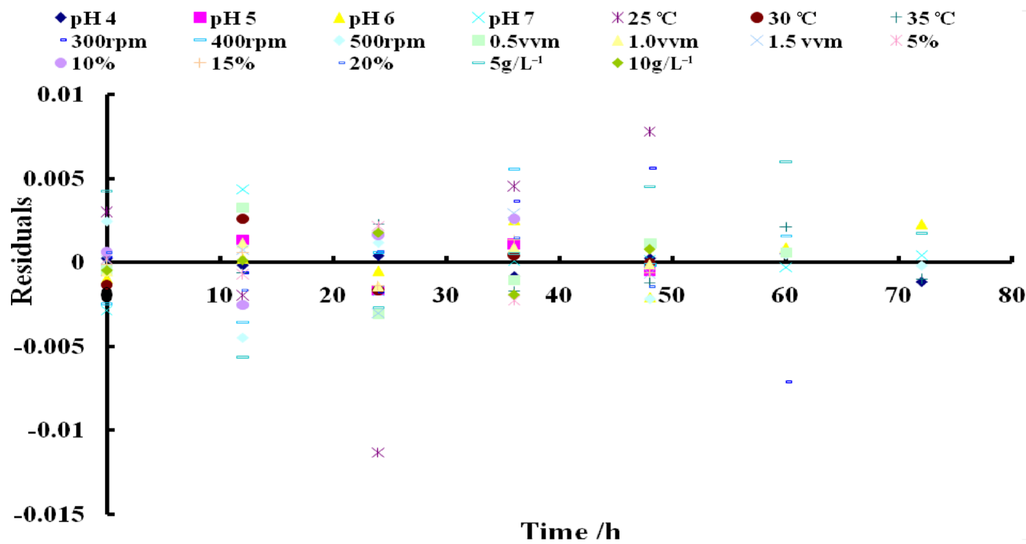

Fig. 4 – *Residual plot of Moser model*

Table 3 – *Range of values of coefficients of eq. (3 – 6)*

Monod		Moser		Contois		Edwards	
coefficients	value range	coefficients	value range	coefficients	value range	coefficients	value range
μ_m (h^{-1})	0.2 to 15	μ_m (h^{-1})	0.02 to 0.201	μ_m (h^{-1})	0.02 to 0.17	μ_m (h^{-1})	0.70 to $2.9 \cdot 10^4$
k_s (g L^{-1})	1.31 to 210	k	0.002 to 0.18	k_k	0.11 to 31.09	k_s (g L^{-1})	$4.6 \cdot 10^2$ to very high
R^2	0.24 to 0.83	Bi	0.003 to 13.69	R^2	0.01 to 0.99	k_1 (g L^{-1})	0.1 to very high
SE	0.19 to 8.4	R^2	0.64 to 0.98	SE	$1.2 \cdot 10^{-4}$ to $7.9 \cdot 10^{-4}$	R^2	0.07 to 0.78
		SE	0.0001 to 0.80			SE	$2.8 \cdot 10^{-4}$ to $9.1 \cdot 10^{-4}$
Residuals	Patterened	Residuals	Random	Residuals	Random	Residuals	Patterened

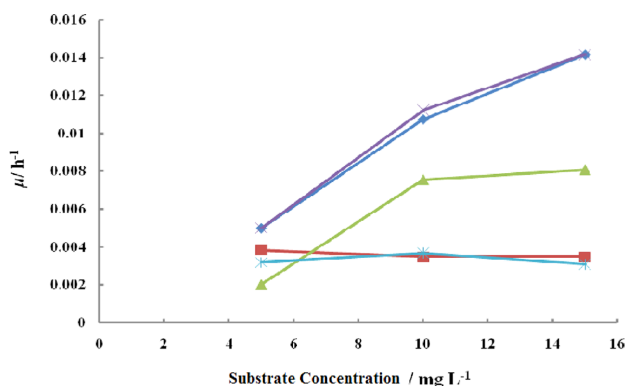

Fig. 5 – *Comparison of calculated specific growth rate found from equations (3–6) with experimental*

el, along with the other tested models is presented in Tabel 3. The range of coefficients μ_m, k, and Bi of Moser model reported between 0.02 to 0.201 (h^{-1}), 0.002 to 0.18, and 0.003 to 13.69, respectively. These values are in agreement with the practical values. From Fig. 5 it is clear that the calculated values of specific growth rate (μ) from Moser model were very close to experimental value. Compared with other calculated coefficient values, R^2 and SE values, the Moser model was found to describe adequately the kinetic data of xylanase production in laboratory-scale fermenter.

Conclusion

Lignocellulosic residue, like sugarcane bagasse, is a potential substrate for the commercial-scale production of xylanase which renders the process economically feasible. Although the fermentation period is longer, the use of a cheaper agro-industrial residue lowers the process cost of fermentation effectively. Optimum conditions for xylanase production in laboratory-scale fermenter were 5.0 pH, 30 °C temperature, 400 rpm agitation, 1.0 % aeration, 10 % DO level and 10 g L^{-1} substrate (sugarcane bagasse) concentration. Combination of all the parameters resulted in 2.5 times higher enzyme activity compared to shake flask conditions. The Moser model was found to describe adequately the kinetics of xylanase production.

ACKNOWLEDGEMENT

GG and USS are thankful to AICTE, New Delhi, India for providing financial support to carry out this work.

Abbreviations:

rpm – Revolutions per minute
vvm – Volume per volume per minute
OUR – Oxygen uptake rate, kg L^{-1} h^{-1}
OTR – Oxygen transfer rate, mmol O_2 L^{-1} h^{-1}

Nomenclature:

a – Gas-liquid interfacial area, cm^2 cm^{-3}
C_L – Concentration of oxygen in liquid phase, mmol L^{-1}
C_L^* – Saturated dissolved oxygen transfer coefficient, h^{-1}
K_L – Oxygen transfer coefficient, cm h^{-1}
K_La – Volumetric oxygen transfer coefficient, h^{-1}
Q_{O_2} – Specific oxygen consumption rate, h^{-1}
X – Biomass concentration, M L^{-3}
Bi – An empirical coefficient, (dimensionless)
k – Biological rate equation coefficient, M^{-1} L^3
K_1 – Inhibition constant, (dimensionless)
K_k – An empirical constant in Contois equation, (dimensionless)
s – Substrate concentration, M L^{-3}
X – Mass of cell per unit volume of batch culture, M L^{-3}
μ – Specific growth rate, h^{-1}
μ_m – Maximum specific growth rate, h^{-1}
K_s – Limiting substrate concentration when $\mu = \mu_m/2$

References

1. *Biely*, *P.*, *Mislovicova*, *D.*, *Toman*, *R.*, Anal. Biochem. **144** (1985) 142. http://dx.doi.org/10.1016/0003–2697(85)90095–8
2. *Subramaniyan*, *S.*, *Prema*, *P.*, Crit. Rev. Biotechnol. **22** (2002) 33. http://dx.doi.org/10.1080/07388550290789450
3. *Haltrich*, *D.*, *Nidetzky*, *B.*, *Kulbe*, *K. D.*, *Steiner*, *W.*, *Zupancic*, *S.*, Biores. Technol. **58** (2) (1996) 137. http://dx.doi.org/10.1016/S0960–8524(96)00094–6
4. *Wong*, *K. K. Y.*, *Tan, L. U. L.*, *Saddler*, *J. N.*, Microbiol. Rev. **52** (1988) 305.
5. *Ghoshal*, *G.*, *Shivhare*, *U. S.*, *Banerjee*, *U. C.*, J. Food. Quality. **36** (3) (2013) 172. http://dx.doi.org/10.1111/jfq.12034
6. *Ghoshal*, *G.*, *Banerjee*, *U. C.*, *Chisti*, *Y.*, *Shivhare*, *U. S.*, Chem. Biochem. Eng. Q. **26** (1) (2012) 61.
7. *Ghoshal*, *G.*, *Banerjee*, *U. C.*, *Shivhare.*, *U. S.*, Brit. Biotechnol. J. **3** (4) (2013) 509. http://dx.doi.org/10.9734/2013/4135
8. *Palma*, *M. B.*, *Milagres*, *A. M. F.*, *Prata*, *A. M. R.*, *Mencilha*, *I. M. D.*, Proc. Biochem. **31** (1996) 141. http://dx.doi.org/10.1016/0032–9592(95)00042–9
9. *Monod*, *H.*, Ann. Rev. Microbiol. **3** (1958) 371. http://dx.doi.org/10.1146/annurev.mi.03.100149.002103
10. *Moser*, *H.*, Carnegie Inst., Pub. No. 614, Washington 1958.
11. *Contois*, *D. E.*, J. Gen. Microbiol. **21** (1959) 40. http://dx.doi.org/10.1099/00221287–21–1-40
12. *Edwards*, *V. H.*, Biotechnol. Bioeng. **12** (1970) 679. http://dx.doi.org/10.1002/bit.260120504
13. *Ghoshal*, *G.*, Ph.D thesis of Panjab University, Chandigarh, India, **58** (2012) 97.

14. *Somogyi*, *M.*, J. Biol. Chem. **160** (1945) 61.
15. *Lowry*, *O. H.*, *Roseborough*, *N. J.*, *Farr*, *A. L.*, *Randall*, *R. J.*, J. Biol. Chem. **193** (1951) 265.
16. *Said*, *F. M.*, *Chisti*, *Y.*, *Brooks*, *J.*, Int. J. Env. Sci. Dev. **1** (2010) 1.
17. *Bailey*, *M. J.*, *Biely*, *P.*, *Poutanen*, *K.*, J. Biotechnol. **23** (1992) 257. http://dx.doi.org/10.1016/0168–1656(92)90074-J
18. *Miller*, *G. L.*, Anal. Chem. **31** (1959) 426. http://dx.doi.org/10.1021/ac60147a030
19. *Ghosh*, *T.*, "Measurement of cellulose activities",In Commission on Biotechnology. *IUPAC*, (1994) 1.
20. *Stanbury*, *P.*, *Whitaker*, *A.*, Pragamon Press, Oxford, 2nd Edition. (1986) 169.
21. *Illias*, *M.*, *Hoq*, *M. M.*, World. J. Microbiol. Biotechnol. **14** (1998) 765. http://dx.doi.org/10.1023/A:1008837410130
22. *Bailey, M. J.*, *Viikari L.*, World. J. Microbiol. Biotechnol. **9** (1993) 80. http://dx.doi.org/10.1007/BF00656523
23. *Purkarthofer*, *H.*, *Sinner*, *M.*, *Steiner*, *W.*, Biotechnol. Lett. **15** (1993) 405. http://dx.doi.org/10.1007/BF00128285
24. *Doran*, *P. M.*, Bioprocess engineering principles. London: Academic Press Limited (1995) pp. 316.
25. *Milagres*, *A. M. F.*, M.Sc. Thesis of Federal University Vicosa, Vicosa, Brazil 1988.
26. *Manolov*, *R. J.*, Appl. Biochem. Biotechnol. **33** (1992) 157. http://dx.doi.org/10.1007/BF02921831
27. *Shioya*, *S.*, *Marikawa*, *M.*, *Kajihara*, *Y.*, *Shimizu*, *H.*, Appl. Microbiol. Biotechnol. **51** (1999) 164. http://dx.doi.org/10.1007/s002530051377
28. *Panda, T.*, Proc. Biochem. **24** (1989) 104.
29. *Mitard*, *A.*, *Riba*, *J. P.*, Biotechnol. Bioeng. **32** (1988) 835. http://dx.doi.org/10.1002/bit.260320617
30. *Mukataka*, *S.*, *Kobayashi*, *N.*, *Sato*, *S.*, *Takahashi*, *J.*, Biotechnol. Bioeng. **32** (1988) 760. http://dx.doi.org/10.1002/bit.260320606
31. *Smith*, *D. C.*, *Wood*, *T. M.*, Biotechnol. Bioeng. **38** (1991) 883. http://dx.doi.org/10.1002/bit.260380810
32. *Yang*, *J. D.*, *Wang*, *N. S.*, Biotechnol. Bioeng. **40** (1992) 806. http://dx.doi.org/10.1002/bit.260400708
33. *Hoq*, *M. M.*, *Hempel*, *C.*, *Deckwer*, *W. D.*, J. Biotechnol. **37** (1994) 49.
34. *Robinson*, *P. D.*, Biotechnol. Lett. **6** (1984) 119. http://dx.doi.org/10.1007/BF00127301
35. *Rodriguez*, *H.*, *Ponce*, *T.*, *De La Torre, M*, *Enriquez*, *A.*, Biotechnol. Lett. **13** (1991) 563. http://dx.doi.org/10.1007/BF01033410
36. *Bhattacharya*, *M. S.*, *Singh*, *A.*, *Banerjee*, *U. C.*, Biores. Technol. **99** (2008) 8765. http://dx.doi.org/10.1016/j.biortech.2008.04.035