African Journal of Biotechnology Vol. 5 (20), pp. 1873-1881, 16 October, 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

Fermentative production and kinetics of cellulase protein on *Trichoderma reesei* using sugarcane bagasse and rice straw

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Accepted 25 August, 2006

Cellulase a multienzyme made up of several proteins finds extensive applications in food, fermentation and textile industries. *Trichoderma reesei* is an efficient producer of cellulase protein. The comparative study was made on various carbon sources on the production of cellulase using strains of *T. reesei* QM 9414, 97.177 and Tm3. Pretreatment of sugarcane bagasse and rice straw offers very digestible cellulose and potentially less inhibition. Cellulase production was enhanced by multiple carbon sources because of diauxic pattern of utilization of substrates. This is the first attempt of combining the synthetic substrate (xylose, lactose) with natural substrate (sugarcane bagasse, rice straw). The mixture of substrates produced the highest maximal enzyme activity on cellulose with xylose, cellulose with lactose, bagasse with xylose, bagasse with lactose, rice straw with xylose and rice straw with lactose. In addition Monod growth kinetics and Leudeking piret product formation kinetics were studied using *T. reesei* with optimized medium under optimized conditions of inoculum concentration, D.O. level, agitator speed, temperature and pH.

Key words: Glucose, Xylose, Lactose, Cellulose, Bagasse, Rice Straw, cellulose.

INTRODUCTION

Cellulose is the world's most abundant natural biopolymer and a potentially important source for the production of industrially useful materials such as fuels and chemicals. Enzymatic hydrolysis is an economic process in the conversion of cellulose to easily fermentable low cost sugars. Cellulase was identified as one of the key enzyme degrading cellulose (Kotchoni et al., 2003). Biosynthesis of cellulase protein was found to be most expensive process (Solomon et al., 1997). Therefore, several approaches including chemical mutations, UV irradiations and genetic engineering to obtain enhanced cellulase producing strains have been given a high priority in the last decade. Strains that are genetically improved for high level cellulase production have been successfully used in a number of applications including animal feed, pharmaceutical and textile industries (Aristidou and Penttilä, 2000). Although a large number of microorganisms are capable of degrading cellulose, only few of them produce significant quantities of cell-free cellulase capable of completely hydrolyzing crystalline cellulose *in vitro*. The genus *Trichoderma,* filamentous ascomycetes are widely used in industrial applications because of high secretory capacity and inducible promoting characteristics (Mach and Zeilinger, 2003). *T. reesei* was selected as the best cellulase producing strain. Morphology studies were carried out on *T. reesei* QM 9414 in submerged culture (Lejeune et al., 1995). Hypercelluloytic mutant strains secrete large amounts of cellulases (Ilmen et al., 1997). A cellulosic enzyme system consists of three major components: exo-1,4-β-D-glucan cellobiohydrolases, which cleave cellobiosyl units from the ends of cellulose chains; endo-1,4-β-D-glucanses which cleave internal glucosidic bonds and 1,4-β-D-glucosidase, which cleaves glucose units from cellooligosaccharides (Jorgensen et al., 2003). Biosynthesis of cellulase was made on *T. reesei* QM 9414 using cellulose as carbon source. The production of cellulase was carried out by culture

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Abbreviations: S, Substrate concentration in growth stage (g/l); μ_{max}, maximum growth rate $(day)^{-1}$; μ, specific growth rate $(day)^{-1}$; K_s, Monod constant; P, product concentration (g/l); α and β, Leudeking Piret constants; and X, substrate concentration (g/l).

using *T. reesei* Rut C-30 and *T. reesei* NG-14. The maximum growth of *T. reesei* C5 and the production of cellulase enzyme were obtained with lactose as carbon source (Muthuvelayudham et al., 2004). The detailed study was made on the production of cellulase using mutants of *T. reesei* (Muthuvelayudham and Viruthagiri, 2003). Fermen-tative production of cellulase was made on substrates cellulose, xylose and lactose using *T. reesei* (Muthuvelayudham et al., 2005). The synthesis of cellulase using *T. reesei* QM 9414 (Muthuvelayudham et al., 2006) increa-sed using a mixture of cellulose with lactose. Cellulase production was improved in the mixture substrates of cellulose with xylose using *T. reesei*. Cellulase protein has also been produced from sugar-cane bagasse by *Aspergillus niger* and *T. reesei* (Aguiar, 2001; Muthuvelayudham and Viruthagiri, 2005). Biosyn-thesis of cellulase was also made on lignocellulo-sic materials such as sugar beet pulp and alkaline extracted sugar beet pulp and cellulose (Olsson et al., 2003). Cellulose and hemi cellu-lose-degrading enzyme was produced on wet-oxidized wheat straw using various filamentous fungi (Thygesen et al., 2003). In this study, cellulase protein production was made on substrates glucose, xylose, lactose, cellulose, sugarcane bagasse, rice straw and mixture of carbon sources using *T. reesei* QM 9414, 97.177 and Tm3. The enzymatic activity of exoglucanase, endoglucanse and β-glucosidase were determined as a function of time. Optimization of D.O. level, pH, temperature, agitator speed and medium composition were also made. The growth kinetics of *T. reesei* 97.177 and the product formation kinetics for cellulase protein production were investigated.

MATERIALS AND METHODS

Materials

T. reesei QM9414 was procured from NCIM, National Chemical Laboratories (Pune, India) and it was maintained on potato dextrose agar slants for 6 days at 28°C. *T. reesei* 97.177 and Tm3 were obtained from the M.T.C.C, Institute of Microbial Technology (Chandigarh, India) and they were maintained on malt extract agar slants for 5 days at 25ºC. Sugarcane bagasse collected from MRK sugar factory, Chidambaram and rice straw collected from nearby area of Annamalainagar, India.

Pretreatment

100 g of the washed and ground sugarcane bagasse was treated separately with 2000 ml of 4% solution of sodium hydroxide, autoclaved at 121°C for 30 min. The material recovered by filtration was washed with distilled water until pH 7 and dried at 65°C to constant weight. One portion of the sugarcane bagasse obtained was neutralized with phosphoric acid and filtered. The material recovered was then dried at 65°C to constant weight. The equal volume of distilled water was added to the sugarcane bagasse and heated at 121°C for 30 min. The suspension was filtered and the solid material dried at 65°C to constant weight (Aguiar, 2001). The same procedure was followed for rice straw.

Cellulase production

The basal medium for the growth of *T. reesei* and production of cellulase is as follows (g/l): KH_2 PO_4: 2.0, Urea: 0.3, $MgSO_4.7H_2O$, 0.3; $CaCl_2.2H_2O$, 0.3 and (mg/l): $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2.6H_2O$, 2.0. In addition peptone (0.1%), Tween 80 (polyoxyethylene sorbitan monoleate, 0.1%) and one of the carbon substrate were added to the medium to induce cellulase production. The pH was controlled using 2 N HCl and 2 N NaOH. Medium was autoclaved for 30 min and seeded with a suspension of *T. reesei* spores. The submerged culture was run for 10 days at 28°C and at pH 4 on Applicon Modular Fermentor at 220 rpm.

Analytical methods

In culture filtrate 20–90% ammonium sulfate ($(NH_4)_2SO_4$) was added and precipitated. Precipitates were separated by centrifugation and redissolved in citrate buffer (0.05 M) and centrifuged (Harikrishna et al., 2000). CMCase or endo-1,4-β-D-glucanses activity was assayed by carboxymethyl cellulose as substrate. Reaction mixtures containing 50 mM sodium acetate pH 5, 4 g CMC/L and culture supernatant, were incubated for 10 min at 50°C. The formation of reducing sugar was measured by DNS method (Thygesen et al., 2003). Cellulase activity was measured as Filter Paper Units (FPU). 0.5 ml culture filtrate was diluted to an appropriate concentration in sodium citrate buffer and then incubated with a 32 mg dry What man No.1 filter paper for 60 min at 50°C. After incubation, reducing sugars liberated were measured by the DNS method. β-glucosidase activity was assayed by the glucose oxidase peroxidase method. 0.1 ml of the culture supernatant was incubated with 0.5 ml of 0.05 M acetate buffer containing 2.5 mg cellobiose (Zaldivar et al., 2001). Cellulase protein was measured by modified Lowry method using bovine serum albumin as a standard. Estimation of reducing sugar was carried out by dinitro salicylic acid (DNS) method (Sadasivam and Manickam, 1997). Substrate concentration was determined by measuring the reducing sugars (DNS method) in the filtered fermentation medium. Culture filtrate was diluted prior to the addition of DNS reagent in sodium citrate buffer to yield an absorbance range 0.0 to 0.5 at 540 nm. Substrate concentrations were ascertained using standard curves. For cellulose evaluation, anthrone method was implemented. 1 ml of sample, 8 ml of 2% anthrone solution and 4 ml of distilled water, being this solution incubated in boiling water for 15 min. The spectrophotometric measuring was made against curve cellulose to 620 nm (Aguiar, 2001). For cell mass estimation, 5 ml portion of culture both was centrifuged for 20 min and the supernatant was discarded. The resulting pellets were dried and dry weight was estimated.

RESULTS

In this study, the influence of various carbon substrates on cellulase protein production was investigated using glucose, xylose, lactose, cellulose, sugarcane bagasse and rice straw. In addition to these materials, different mixtures of substrates were utilized as carbon sources for the cultivation with *T. reesei* QM 9414, 97.177 and Tm3.

Cell mass

T. reesei QM9414 grew well with sole carbon sources like glucose, xylose, lactose, cellulose, bagasse and rice straw (Figure 1A). *T. reesei* 97.177 and Tm3 showed increased cell mass than *T. reesei* QM 9414 on the single

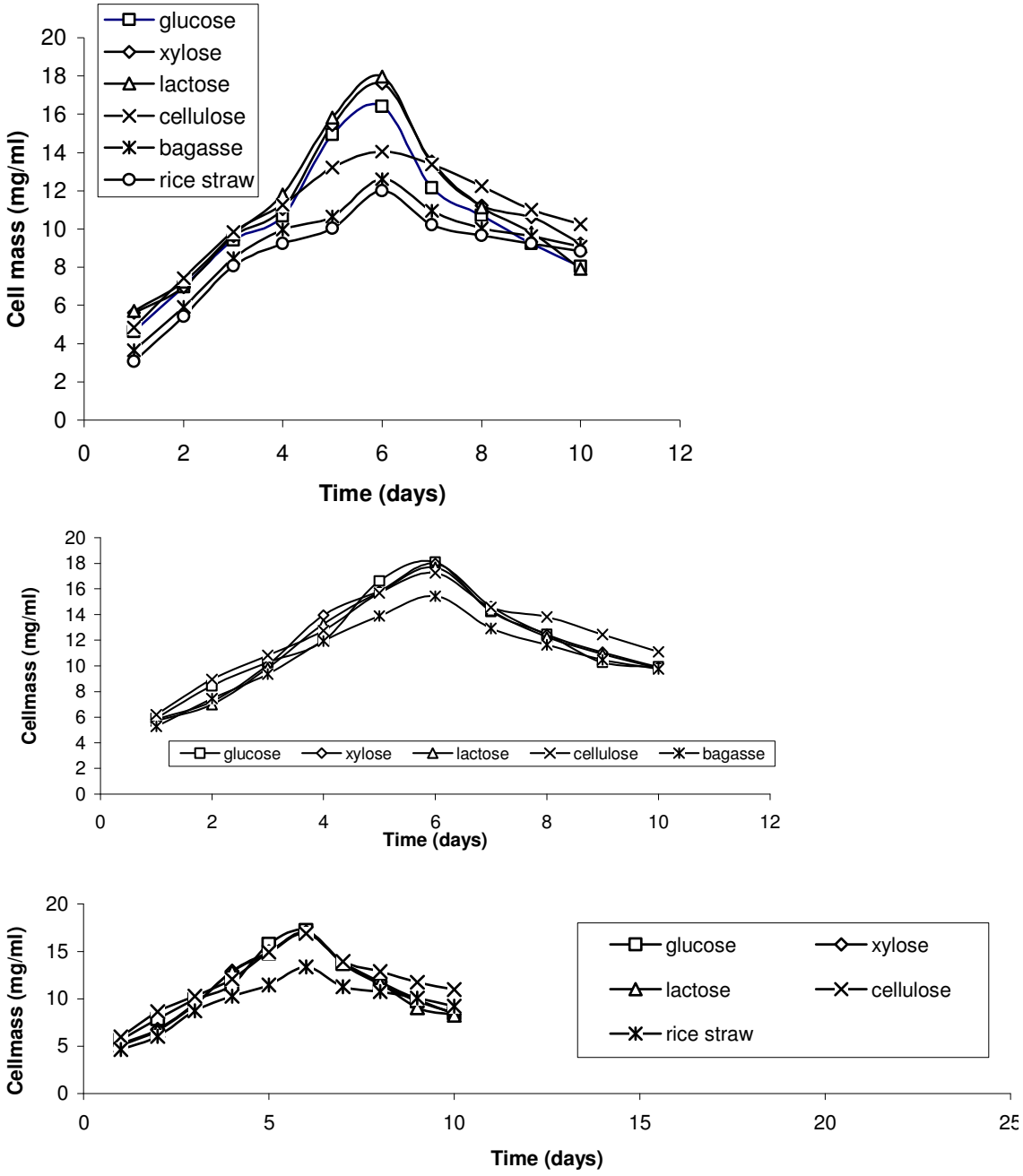

Figure 1. Change in cell mass with fermentation time for single carbon sources using *Trichoderma reesei* QM 9414, 97.177 and Tm3.

carbon substrates (Figures 1B and 1C). The decanting method of separating mycelial growth from undigested cellulose may have resulted in the slightly higher cell mass estimates for the bagasse and rice straw sample. Growth patterns of *T. reesei* QM 9414, 97.177 and Tm3 were similar in all media containing multiple carbon sources (Figures 2a, b and c) with maximum biomass achieved in 5-6 days, followed by a gradual decline thereafter. This decline has often been observed in batch fermentation processes when substrate levels fall below concentration required to support cell maintenance, resulting in cell death and lysis.

Substrate

Media containing sole carbon source showed a favorable utilization of substrate during fermentation of using *T. reesei* QM 9414, 97.177 and Tm3 (Figure 3a, b and c). But the utilization of polysaccharide substrates like cellu-

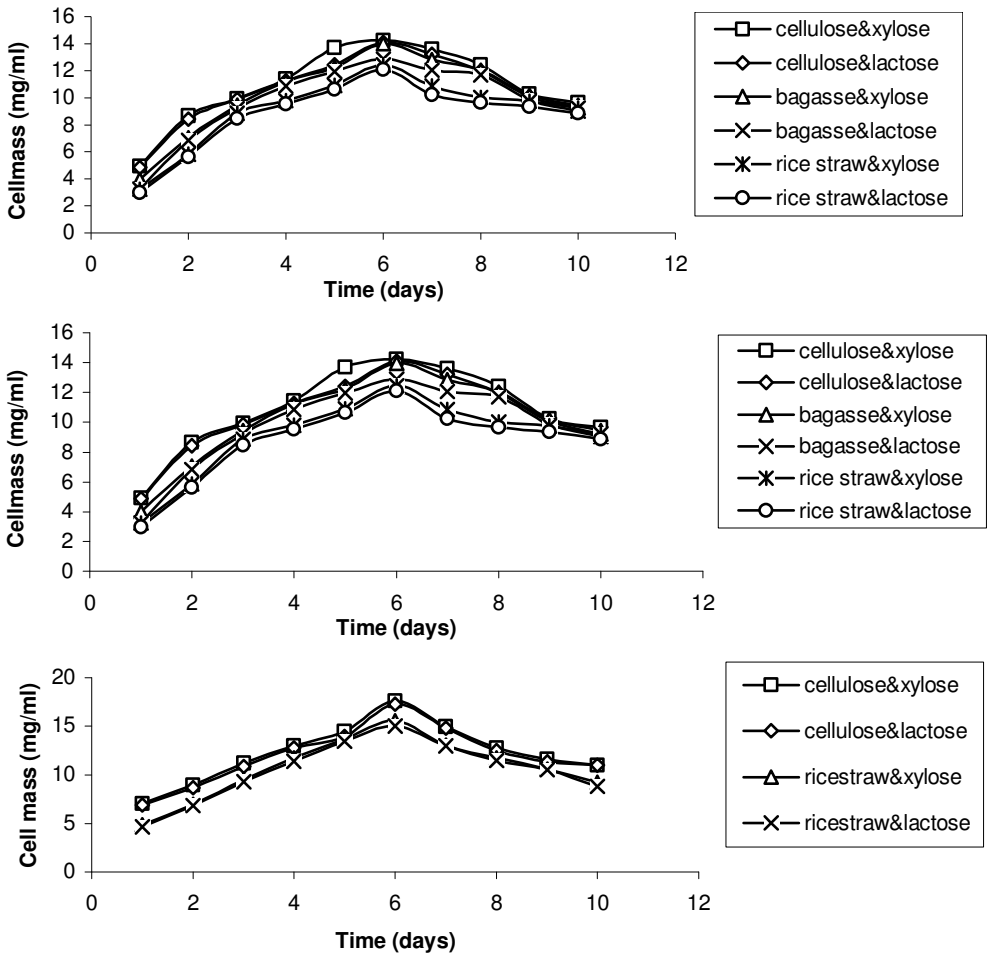

Figure 2. Change in cell mass with fermentation time for multiple carbon sources using *Trichoderma reesei* QM 9414, 97.177 and Tm3.

lose, sugarcane bagasse and rice straw showed poor results. In mixture of substrates the concentration was optimized by means of various trials. Media having mixture of carbon sources exhibited a considerable utilization of substrate during product formation period of *T. reesei* QM 9414, 97.177 and Tm3 (Figure 4a, b and c). The medium containing a cellulose carbon source could not be evaluated by this technique of DNS method, since the quantity of reducing sugar groups per unit weight was very low. A slight increase in reducing sugar groups in the cellulose-containing sample indicated that some hydrolysis of the cellulose polymer might have occurred. Cellulose amount was evaluated by anthrone reagent method.

Enzyme

Biosynthesis of cellulase was found to be high in the medium of sole carbon source like xylose, lactose, cellulose and bagasse of *T. reesei* QM 9414, 97.177 and Tm3 (Figures 5a, b and c). But the cellulase activity was found to be less when glucose was used as carbon source because of inhibition. In media containing multiple substrates (Figures 6a, b and c) of *T. reesei*, cellulase production was enhanced. *T. reesei* 97.177 showed a better yield of carboxymethyl cellulase and β-glucosidase than *T. reesei* QM 9414 and Tm3. The yield of cellulase protein, CMCase or exoglucanase, β-glucosidase and FPA of *T. reesei* QM9414, 97.177 and Tm3 were shown in the Tables 1a, b and c.

Optimization

The influence of cultural conditions upon the enzymatic profile is studied for the production of cellulase from *T. reesei* (Cochet, 1991). Temperature, pH, inoculum concentration and dissolved oxygen (D.O.) are the parameters that have been considered for the growth and product formation. A D.O.level of less than 10% as reported to adversely affect the enzyme yields of mutant *T. reesei* QM 9414 (Rakshit and Sahai, 1991). In the case of mutant *T. reesei* 97.177 and Tm3, a D.O. level of greater

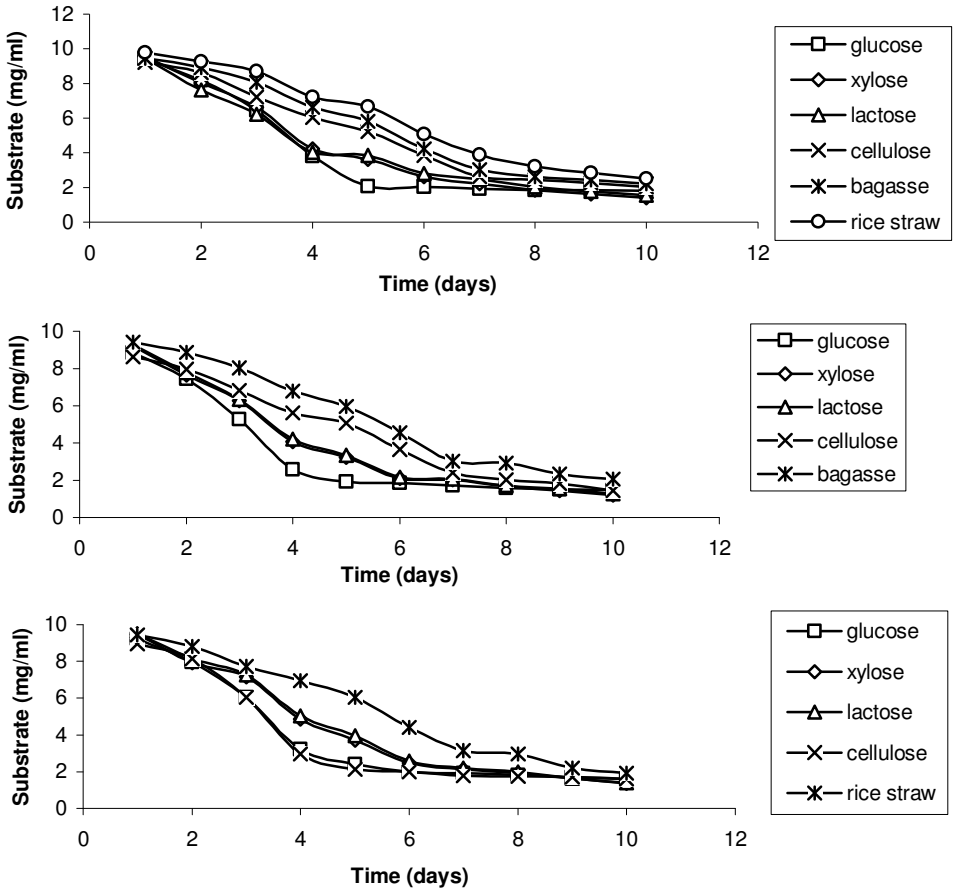

Figure 3. Change in substrate concentration with fermentation time for single carbon sources using *Trichoderma reesei* QM 9414, 97.177 and Tm3.

Table 1a. Cellulase protein of *T. reesei* 94.144 in batch culture.

Substrate	Extra cellular protein (mg/ml)	CMCase (U/ml)	FPA (U/ml)	β-glucosidase (U/ml)
Glucose	1.78	24	1.94	0.92
Xylose	8.02	28	4.06	2.02
Lactose	7.98	28	3.98	1.94
Cellulose	9.74	78	6.4	3.20
Sugarcane bagasse	7.94	68	5.66	2.80
Rice straw	7.16	44	5.26	2.60
Cellulose and xylose	10.02	74	7.02	3.46
Cellulose and lactose	10.00	70	6.78	3.34
Bagasse and xylose	9.24	72	6.4	3.18
Bagasse and lactose	9.02	70	6.2	3.12
Rice straw and xylose	8.24	50	5.58	2.72
Rice straw and lactose	8.00	48	5.32	2.60

ter than 15% was found to be sufficient. Enzyme activities are found to be higher with the mycelia inocu-lum compared to the spore inoculum (Harikrishna et al., 2000). Inoculum age is also found to be important in the protein production. The effect of high and low temperature on the synthesis of cellulase protein from *T. reesei* was studied. Fermentation was carried out at different temperatures (26, 28, 30 and 32°C). Maximum cellulase activity was obtained at temperature 28°C. The optimum

Table 1b. Cellulase protein of *T. reesei* 97.177 in batch culture.

Substrate	Extra cellular protein (mg/ml)	CMCase (U/ml)	FPA (U/ml)	β-glucosidase (U/ml)
Glucose	2.42	34	2.02	0.82
Xylose	8.98	34	4.62	2.30
Lactose	8.82	30	4.52	2.24
Cellulose	28.22	376	23.60	10.42
Sugarcane bagasse	14.20	84	7.20	3.50
Cellulose and xylose	29.98	386	24.86	12.34
Cellulose and lactose	29.62	378	23.42	11.44
Bagasse and xylose	16.42	88	9.60	4.52
Bagasse and lactose	16.04	82	9.04	4.42

Table 1c. Cellulase protein of *T. reesei* Tm3 in batch culture.

Substrate	Extra cellular protein (mg/ml)	CMCase (U/ml)	FPA (U/ml)	β-glucosidase (U/ml)
Glucose	2.08	30	1.88	0.72
Xylose	8.68	28	4.08	2.14
Lactose	8.60	26	4.06	2.12
Cellulose	27.42	364	22.42	9.84
Rice straw	12.24	72	6.84	3.06
Cellulose and xylose	28.94	364	23.48	11.96
Cellulose and lactose	28.46	360	23.04	10.96
Rice straw and xylose	14.58	74	8.88	4.06
Bagasse and lactose	14.16	70	8.76	3.96

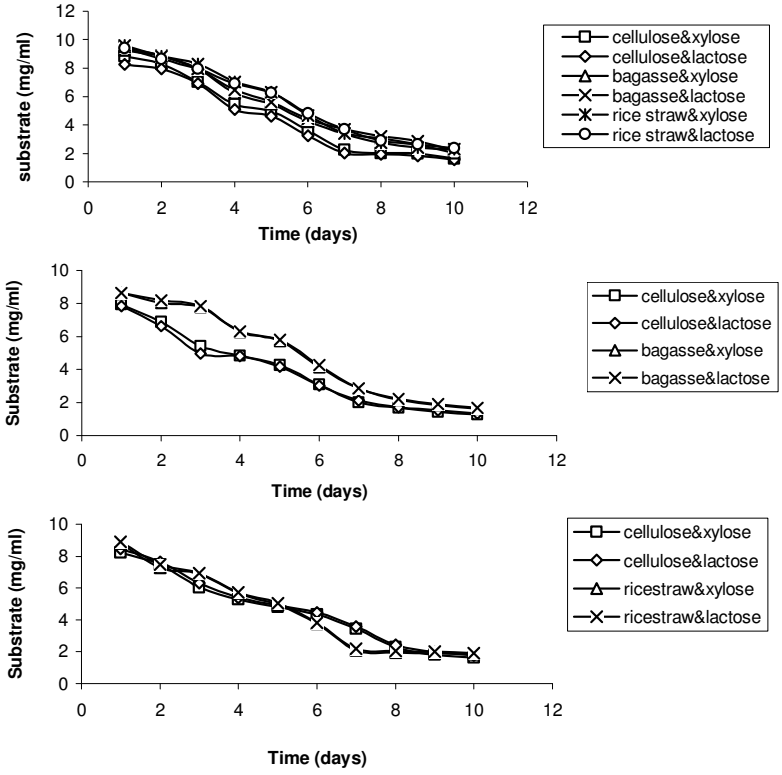

Figure 4. Change in substrate concentration with fermentation time for multiple carbon sources using *Trichoderma reesei* QM 9414, 97.177 and Tm3.

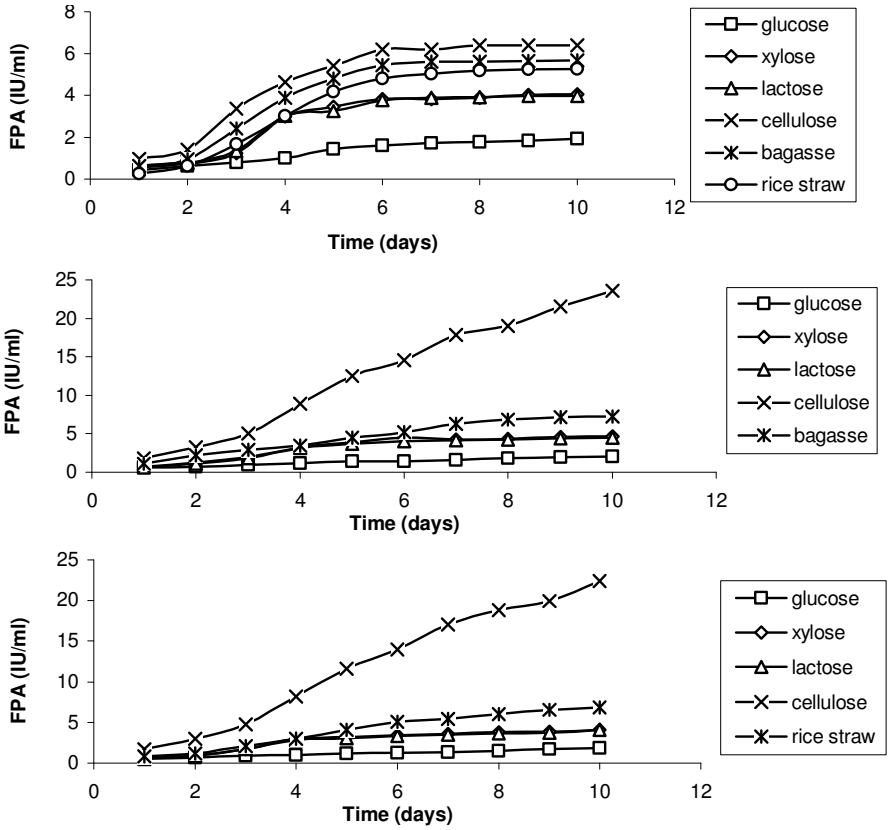

Figure 5. Cellulase production on fermentation for single carbon sources using *Trichoderma reesei* QM 9414, 97.177 and Tm3.

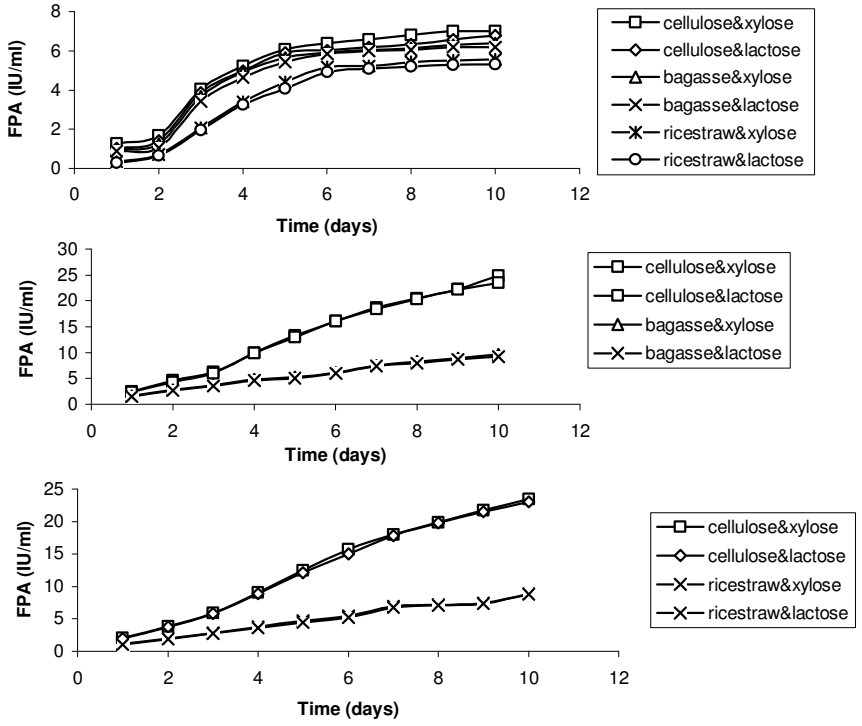

Figure 6. Cellulase production on fermentation for multiple carbon sources using *Trichoderma reesei* QM 9414, 97.177 and Tm3

Table 2a. Kinetic Parameters of cellulase production from *T. reesei* 97.177.

Substrate	Monod kinetics value		Leudeking piret kinetic values	
	K_s (mol/l)	μ_{max} (hr^{-1})	α	β
Glucose	37.5	0.260	0.067	0.002
Xylose	38.3	0.240	0.321	0.003
Lactose	37.2	0.240	0.211	0.003
Cellulose	32.6	0.200	0.200	0.002
Sugarcane bagasse	22.4	0.120	0.200	0.002
Cellulose and xylose	34.8	0.220	0.260	0.004
Cellulose and lactose	34.0	0.240	0.280	0.004
Bagasse and xylose	30.2	0.180	0.240	0.003
Bagasse and lactose	29.6	0.160	0.220	0.003

Table 2b. Kinetic Parameters of cellulase production from T.reesei Tm3.

	Monod kinetics value		Leudeking piret kinetic values	
Substrate	K_s (mol/l)	μ_{max} (hr^{-1})	α	β
Glucose	37.0	0.240	0.058	0.001
Xylose	38.0	0.220	0.300	0.003
Lactose	37.2	0.220	0.280	0.002
Cellulose	31.8	0.200	0.200	0.002
Rice straw	22.8	0.100	0.180	0.002
Cellulose and xylose	34.8	0.220	0.240	0.004
Cellulose and lactose	34.0	0.210	0.240	0.004
Rice straw and xylose	28.2	0.140	0.200	0.003
Rice straw and lactose	28.0	0.140	0.200	0.003

pH 3.5 is fixed in all previous runs was chooses based on reports from mutant *T. reesei* QM 9414 (Rakshit and Sahai, 1991). The best control point of pH varies from strain to strain. The medium, with nutrients and inoculums, were adjusted and controlled at different pHs (3.5, 4.0, 4.5 and 5.0) and incubated for 10 days. Maximum activity was reached at pH 4. In three strains, *T. reesei* 97.177 and Tm3 showed better results than that of *T. reesei* QM 9414.

Kinetics

Monod growth kinetics (Rakshit and Sahai, 1991) was developed for the carbon sources like glucose, xylose, lactose, sugarcane bagasse and rice straw respectively. Monod Kinetics for growth rate is given as:

$$\mu = \frac{\mu_{\max} S}{K_s + S} \qquad (1)$$

The graph was plotted between time versus cell mass and substrate concentration. Monod constants were calculated using slope values.

For the cellulase enzyme production, the Leudeking piret model (Rakshit and Sahai, 1991) was developed from various carbon sources like glucose, xylose, lactose, cellulose and bagasse. Leudeking Piret model for product formation is:

$$\frac{dp}{dt} = \alpha \frac{dx}{dt} + \beta X \qquad (2)$$

From the graph between time versus cell mass and time versus product concentration the Leudeking piret model values were calculated. Monod constant and Leudeking piret constant values were given in Tables 2a and b for *T. reesei* 97.177 and Tm3.

DISCUSSION

The enzymatic degradation of waste cellulose by the fungal enzymes has been suggested as a feasible alternative for the conversion of lignocellulosics into fermentable sugars and fuel ethanol (Szengyel et al., 2000). However the most widely used enzymatic system, namely *T. reesei* cellulases has not successful one. Several disadvantages were low enzymatic yield, low spe-

cific activity, and end product inhibition of the enzymes. Mutant strain has the capability to overcome these problems. The morphological state of *T. reesei* 97.177 and Tm3 were known to influence the growth and protein production. In the present study, the inoculum size was the same in all cultivations. Growth characteris-tics depended on the carbon source used in the particular cultivations. It was found that the resulting enzyme activities were generally higher during growth on mixed substrates compared to when only single substrate was used. The rate of production depended on the composition of the carbon source. The general trend is that more cellulose in the mixture results in higher levels of endoglucanase (Olsson et al., 2003). But *T. reesei* in general exhibits poor β-glucosidase activity. However, mutant strain 97.177 and Tm3 showed enhanced β-glucosidase yield than *T. reesei* QM 9414. β-glucosidase activity can also be induced by elaborately controlling culture conditions. CMCase, FPU activities exhibited a pH optimum of approximately 4, while the pH optimum of β-glucosidase was between pH 5 and 6. pH adjustment was therefore investigated with the objective of uniformly inducing all three activities. Culturing at pH 4 for 2 days, followed by a shift to either pH 5 or 6 on the third day, resulted in a marked increase in the β-glucosidase activity of *T. reesei* 97.177. In addition, when urea was used as nitrogen source, the β-glucosidase yield was further improved.

In conclusion, the present investigation enlightened the influence of various carbon substrates in the production of cellulase protein using *T. reesei* 97.177 and Tm3. The production of β-glucosidase rich cellulase protein was increased by *T. reesei* 97.177 and Tm3 strains. Cellulase shows maximum yield of cellulose as synthetic source. In natural source sugarcane bagasse, contain 41% cellulose holds better for the production of cellulase. Similarly rice straw, had 35% cellulose;this shows good yield of cellulase. Due to diauxic pattern of substrate utilization, the mixture of substrates such as bagasse with either xylose or with lactose and rice straw with neither xylose nor lactose exhibited better yield. Kinetic studies were also made for the growth and production using Monod equation and Leudeking Piret model respectively.

ACKNOWLEDGEMENT

The Authors are thankful to the authorities of Annamalai University, Annamalainagar for permitting them to carry out the research work.

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