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Effect of medium composition and cultural condition on cellulase production by *Aspergillus terreus*

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The effect of medium composition and environmental condition on the production of cellulase by *Aspergillus terreus* was investigated using shake flask culture with oil palm empty fruit bunch (OPEFB) as substrate. The highest activity of FPase (0.76 U ml^{-1}), CMCase (8.64 U ml^{-1}) and β-glucosidase (6.81 U ml^{-1}) was obtained in medium containing 6 g L^{-1} yeast extract and 10 g L^{-1} delignified OPEFB fiber. In fermentation with the addition of Tween 80 (2 ml L^{-1}) as surfactant, the production of cellulase was increased by two-fold as compared to fermentation without surfactant. Cellulase production by *A. terreus* was also enhanced with the addition of calcium chloride (3 mM) and magnesium sulfate (5 mM) in the medium. Optimum pH and temperature for cellulase production by *A. terreus* was 5.5 and 28°C, respectively. Cellulase production in agitated shake flask fermentation at 200 rpm was four times higher as compared to fermentation in static flask.

Key words: Optimization, *Aspergillus terreus*, submerged fermentation, cellulase, oil palm empty fruit bunch.

INTRODUCTION

Industrial interest in cellulase is high due to its wide application in various industries such as animal feed production, starch processing, malting and brewing, grain alcohol fermentation, extraction of fruit and vegetable juices, as well as manufacture of pulp, paper and textiles (Adsul et al., 2007; Kaur et al., 2007). Enzymatic hydrolysis of cellulosic materials is achieved by a sequence of reactions with the main components of cellulase complex enzymes, which include FPase, CMCase and β-glucosidase. The characteristics of all these three component of cellulase complex are the main factors that influence the application of enzyme-based bioconversion technology. Therefore, research has been directed to discover new microorganisms that have capability to produce cellulolytic enzymes with high specific activity

and characteristics that favour the use in industrial reaction conditions (Johnvesly et al., 2002). Among the cellulolytic fungi, *Trichoderma* spp. and *Aspergillus* spp. have been widely studied for their ability to secrete high levels of cellulose-degrading enzymes (Zhou et al., 2008). *Aspergillus* spp. is the major agents of decomposition and decay and as such produce a broad range of enzymes, including cellulase. Cellulase characteristics and production by *Aspergillus* spp. have been well documented in the literature (Lockington et al., 2002; Ong et al., 2004; Wang et al., 2006). However, only a few reports are available on the production of cellulase by *Aspergillus terreus* (Emtiazi et al., 2001; Gao et al., 2008; Pushalkar and Rao, 1998; Singh et al., 1996), and in many cases, have not been studied in depth.

The use of expensive substrate is one of the main problems in cellulase production by fermentation. Reduction of the cost of the substrate may be possible by the modification of cellulosic materials using microorganisms that have the ability to produce high activity of cellulase (Kotchoni and Shonukan, 2002). Reduction in the production cost and improvement in cellulase yield

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Abbreviations: **OPEFB**, Oil palm empty fruit bunch; **CMCase**, carboxymethylcellulase; **FPase**, filter-paper-hydrolysing activity.

could also be achieved using appropriate and low cost carbon and nitrogen sources in the formulation of fermentation medium (Beg et al., 2000; Senthilkumar et al., 2005). Large volume of lignocellulosic materials generated by the palm oil plantation has not been effectively utilized and it appears to be a viable alternative as a cheap source of substrate for various fermentation processes. For example, the generation of oil palm empty fruit bunch (OPEFB), obtained after stripping the palm oil fruit from the bunch, is about 7.3×10^6 tonnes annually (Chua, 1991). Since OPEFB is available in large quantities and has fairly high cellulose content with an average of 50% based on an oven dried basis (Kume et al., 1990; Husin et al., 1985), it appears to be a potential biomass for cellulase fermentation.

The objective of this study was to investigate the effect of medium component and culture conditions on cellulase production by *A. terreus* using OPEFB fibers as carbon source. The use of different types and concentrations of nitrogen sources on the enhancement of cellulase production was first investigated. Subsequently, the effect of cultural conditions such as initial pH, temperature, shaking speed and addition of surfactant to the culture on growth of *A. terreus* and cellulase production was investigated.

MATERIALS AND METHODS

Cellulosic materials

The OPEFB fibers obtained from an oil palm processing factory (Sri Ulu Langat Palm, Dengkil, Selangor, Malaysia) were first washed with water, then dried and shredded by grinding in a hammer mill (Mill Powder Tech Solutions, Taiwan) to obtain the fibers with an average of 1 mm length. The fibers were delignified by soaking in phosphoric acid and then exposed to hydrothermal treatment at 160°C for 10 min. The pretreated OPEFB fibers were filtered and washed with distilled water until no traces of acid could be detected and then dried in an oven at 95°C for 2 days (Shahriarinour et al., 2011b). In all experiments, 10 g L⁻¹ of dry pretreated OPEFB fiber was used as the carbon source in basal medium.

Microorganism

The fungus *A. terreus*, isolated from the compost of oil palm empty fruit bunch (OPEFB) waste at a local oil palm processing factory (Sri Ulu Langat Palm, Dengkil, Selangor, Malaysia) was used in this study as cellulase producer. Details of the method of isolation and identification of this fungus were described in our previous paper (Shahriarinour et al., 2011a).

Optimization of culture conditions for maximum cellulase production

Effect of medium composition

The basal medium described by Mandels and Weber (1969) was used for all fermentations. To investigate the effect of nitrogen sources on cellulase production, different types of nitrogen source [$(NH_4)_2SO_4$, peptone, urea and yeast extract] at different

concentrations (3, 6, 9, and 12 g L⁻¹) were used separately in the medium formulation. To investigate the effect of surfactant, different concentrations (0, 1, 2, 3 and 4 ml L⁻¹) of Tween 80 were added to the fermentation medium. The effects of different concentrations of calcium chloride (0, 1, 3, 5, 7 and 10 mM) and magnesium sulfate (0, 1, 3, 5, 7 and 10 mM) on growth on *A. terreus* and cellulase production were also investigated.

Effect of culture conditions

The effect of initial culture pH on growth of *A. terreus* and cellulase production was performed by adjusting the medium pH, ranging from 4.5 to 7.5 using either 1 N HCl or 1 N NaOH. To investigate the effect of temperature and agitation speed, the flasks were incubated at different temperatures (24, 28, 32, 36 and 40°C) in an incubator shaker agitated at different shaking speeds (0, 100, 200, and 300 rpm).

Inoculum preparation and fermentation

Potato dextrose agar (PDA) plate incubated at 30°C for 7 days was used for the production of *A. terreus* spores. Spores were harvested using 10 ml of sterile 0.01% (v/v) Tween 80 with the aid of wire loop. In all fermentations, 100 ml of the medium (pH 5.5) was dispensed into a 500 ml shake flask and sterilized at 121°C for 15 min. The flasks were inoculated with 5 ml of spore suspension containing approximately (6×10^6 spores/ml) and then incubated at 28°C on a rotary shaker (Certomat, B. Braun, Germany) agitated at 200 rpm for 12 days. Each experiment was carried out in triplicate.

Analytical procedure

During the fermentation, the culture samples were withdrawn at 24 h intervals. The cultures were centrifuged at 15,000 rpm at 4°C for 15 min using a refrigerated ultracentrifuge (SORVALL RT7 PLUS). The supernatant was used for the determination of soluble protein and all the three main component of cellulase activities. The cell pellet was used for the estimation of cell concentration.

The physical separation of mycelium from the OPEFB fibres for measurement of mycelium concentration was not possible. Thus, the chemical method based on the measurement of glucosamine was adopted for the estimation of mycelium concentration (Khan and Strange, 1975). This method involved the production of chitosan from fungal chitin and liberation of glucosamine from chitosan through a chemical reaction. Chitin is an insoluble liner polymer of α-1, 4-linked-N-acetylglucosamine units produced by most fungi but not found in plant tissues. Absorbance of the colour developed from the reaction was measured with spectrophotometer (Model Shimadzu, UV-1601 PC) at 650 nm. Glucosamine concentration in the mycelia of *A. terreus* was found to be proportional to the mycelial weight and remained constant throughout the growth phases.

Endoglucanase or carboxymethylcellulase (CMCase) activity was determined by measuring spectrophotometrically the reducing sugars produced from 2% (w/v) carboxymethylcellulose, while filter-paper-hydrolysing (FPase) activity was determined by estimating the reducing sugars liberated from the filter paper (Wood and Bhat, 1988). Both reactions were carried out in 0.05 M sodium acetate buffered at pH 5 and incubated at 50°C. The reaction time was 30 and 60 min for CMCase and FPase, respectively. One unit of CMCase or FPase activity is defined as 1 µmol reducing sugar released/ml enzyme/min. Meanwhile, β-glucosidase was determined using the method described by Wood and Bhat (1988). In this method, p-nitrophenol released from p-nitrophenyl-β-D-glucopyranoside (Fluka) was measured using a spectrophotometer

Table 1. Effect of different nitrogen sources on growth of *A. terreus* and cellulase production.

Nitrogen source	Concentration* (g L^{-1})	X*(Cell concentration) (g L^{-1})	FPase* (U ml^{-1})	CMCase* (U ml^{-1})	β-glucosidase* (U ml^{-1})	Yield (U g^{-1} cellulose)	Protein (mg ml^{-1})
(NH$_4$)$_2$SO$_4$	3	2.3±0.5	0.05±0.01	2.85±0.1	0	7.88	0.75±0.03
	6	2.2±0.1	0.05±0.03	1.74±0.2	0	7.88	0.77±0.01
	9	2.1±0.3	0.04±0.02	0.82±0.1	0	6.30	0.78±0.01
	12	2.0±0.2	0.03±0.03	0.61±0.3	0	4.73	0.79±0.02
Yeast extract	3	4.6±0.1	0.61±0.04	6.57±0.1	5.40±0.04	96.21	1.05±0.04
	6	5.1±0.2	0.76±0.01	8.64±0.3	6.81±0.01	119.87	1.16±0.09
	9	5.1±0.4	0.64±0.02	7.59±0.2	4.95±0.02	100.94	1.17±0.05
	12	5.2±0.3	0.58±0.01	5.51±0.3	4.48±0.07	91.48	1.20±0.08
Peptone	3	4.5±0.2	0.53±0.03	6.16±0.1	4.93±0.03	83.59	1.06±0.01
	6	5.0±0.4	0.69±0.05	7.41±0.2	6.37±0.02	108.83	1.09±0.02
	9	5.0±0.1	0.56±0.01	7.36±0.5	4.86±0.02	88.32	1.11±0.02
	12	5.1±0.2	0.51±0.03	5.14±0.2	4.15±0.01	80.44	1.12±0.03
Urea	3	3.7±0.1	0.31±0.02	4.08±0.1	2.46±0.03	48.89	0.85±0.06
	6	3.3±0.3	0.28±0.01	3.11±0.3	0.84±0.02	44.16	0.88±0.03
	9	3.3±0.1	0.21±001	1.23±0.1	0.65±0.04	33.12	0.91±0.02
	12	3.2±0.2	0.10±0.03	0.93±0.2	0.13±0.01	15.77	0.92±0.01

Values are means of triplicate ± standard deviation.*Maximum concentration obtained during fermentation.

(Shimadzu, UV-1601 PC). One unit of β-glucosidase activity is defined as 1 μmol p-nitrophenol liberated/ml of enzyme/min, while the specific activity is defined as unit/mg protein. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

The results were compared by one-way analysis of variance (one-way ANOVA) and multiple range tests to find the differences between the measurement means at 5% (0.05) significant level. The data were analyzed using Minitab 14 statistical software (Minitab, PA, USA).

RESULTS AND DISCUSSION

Effect of nitrogen source

Among the nitrogen sources studied, yeast extract gave the highest production of all three main components of cellulase complex by *A. terreus*, followed by peptone, urea and (NH$_4$)$_2$SO$_4$. The activity of β-glucosidase was not detected when (NH$_4$)$_2$SO$_4$ was used as a nitrogen source. Yeast extract and peptone concentrations of above 9 g L^{-1} were inhibitory to cellulase production. Cellulase production was greatly inhibited at a urea and (NH$_4$)$_2$SO$_4$ concentration of above 3 g L^{-1}. The maximum activities of FPase, CMCase and β-glucosidase obtained

with 6 g L^{-1} yeast extract as nitrogen source were 0.76, 8.64 and 6.81 U ml^{-1}, respectively (Table 1).

Effect of Tween 80 concentration

Cellulase production by *A. terreus* was enhanced with the addition of Tween 80 in the culture (Table 2). The highest cellulase yield was obtained in medium containing 2 ml L^{-1} of Tween 80. At higher concentrations of Tween 80 (> 2 ml L^{-1}), the cellulase yield did not increase. The stimulatory effect of surfactants may be a consequence of its action on cell membranes causing increased in permeability by promoting the release of cell-bound enzymes (Rege et al., 2002).

Different concentrations of Tween 80 did not affect the growth of *A. terreus*. However, the addition of Tween 80 to the culture medium led to a substantial increase in the activity of cellulase by *A. terreus*. As the concentration of Tween 80 increased, the production of cellulase was also increased until it reached the maximum activity at 2 ml L^{-1} of Tween 80. Cellulase production in fermentation with the addition of 2 ml L^{-1} of Tween 80 was improved by 1.6 times as compared to fermentation without surfactant.

The effect of surfactant on growth of fungi and cellulase production is well known (Pardo, 1996). The use of Tween 80 is beneficial because it does not denature the enzymes. Tween 80 at a concentration of 2 ml L^{-1} was

Table 2. Effect of Tween 80 concentration on growth of *A. terreus* and cellulase production.

Tween 80 (ml l⁻¹)	X* (Cell concentration) (g L⁻¹)	FPase* (U ml⁻¹)	CMCase* (U ml⁻¹)	B-glucosidase* (U ml⁻¹)	Yield (U g⁻¹ cellulose)	Protein (mg ml⁻¹)
0	3.47 ± 0.12^a	0.37 ± 0.04^a	5.74 ± 0.20^a	4.98 ± 0.05^a	58.35	0.73 ± 0.01
1	4.89 ± 0.15^b	0.48 ± 0.03^b	7.13 ± 0.15^b	7.81 ± 0.12^b	75.70	0.95 ± 0.06
2	4.91 ± 0.09^b	0.60 ± 0.02^c	9.98 ± 0.08^c	8.84 ± 0.07^c	94.63	1.02 ± 0.07
3	4.85 ± 0.13^b	0.52 ± 0.05^d	8.45 ± 0.13^d	7.96 ± 0.19^d	83.59	0.98 ± 0.03
4	4.93 ± 0.11^b	0.44 ± 0.03^e	7.49 ± 0.16^e	7.24 ± 0.14^e	69.40	0.96 ± 0.05

Values are means of triplicate ± standard deviation. [a-e] Means values in the same column with different superscripts are significantly different ($P < 0.05$). *Maximum concentration obtained during fermentation.

optimum for the production of cellulase and β-glucosidase by mixed culture of *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure (Wen et al., 2005). Cellulase production by *Trichoderma reesei* strain QM-9414 and *Streptomyces flavogriseus* was also enhanced with the addition of 1 and 0.2 % (v/v) of Tween 80, respectively (Hari Krishna et al., 2000).

Since the initial stage of saccharification depends on the adsorption of enzyme to insoluble cellulosic substrates, the addition of the surfactant would increase cellulose conversion. Adsorption and orientation of the surfactant molecules at the solid-liquid interface should render the substrate readily wettable by the enzyme solution, bringing the substrate quickly into intimate with the enzyme and allowing the enzymes to reach the substrate. Otherwise, accessible substrate site to enzyme is very poor. Furthermore, interactions between the surfactant molecules, the substrates, and the adsorbed enzymes might result in a decrease in the strength, in which, the enzymes are retained on the substrates. According to Castanon and Wilke (2004), the activity of endoglucanase was retained for longer period when the enzyme was in the liquid phase. Singh et al. (2007) reported that the surfactants are amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or insoluble organic compounds. Thus, it appears that for a variety of reasons, Tween 80 has the potential for reducing the cost of enzymatic hydrolysis of cellulosic materials.

Effect of calcium chloride

The addition of calcium chloride to the basal medium enhanced the growth of *A. terreus* and cellulase production (Table 3). As the concentration of calcium chloride increased, growth and cellulase production were also increased. The highest cell concentration and cellulase activity were obtained at 3 mM calcium. Enhancement of cellulase production with increasing con-centration of calcium chloride in the culture of *T. reesei*

Rut C-30 has also been reported (Chen and Wayman, 1992). The reason for the positive effect of calcium chloride on cellulase production is not known. Calcium may be responsible for some changes in the permeability of the cell wall that result in a more rapid excretion of the enzymes, which in turn, improves cellulase synthesis (Chen and Wayman, 1992).

Effect of magnesium sulfate

Cellulase activities of *A. terreus* were increased with the presence of magnesium in the culture (Table 4). Among the concentration of magnesium investigated, the highest growth and cellulase activity were observed at 5 mM. Metal cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Co^{2+} and Zn^{2+} were necessary for cellulase synthesis by *Trichoderma viride* QM6a (Mandel and Reese, 1999). The effect of balance between different metal ion concentrations could be more important than their individual effects. For example, magnesium is needed for cellulase production, but it is also inhibitory at high concentrations and the inhibition is counteracted by calcium (Mandel and Reese, 1999). It has been hypothesized that the metal may prevent some components necessary for induction from leaking out of the cells.

Effect of initial culture pH

Figure 1 shows that *A. terreus* was able to secrete all the three main components of cellulase over a broad pH, ranging from 4.5 to 7.5. Significant activity of cellulase was detected at initial culture pH ranging from 5 and 6; and the highest activities of all the three component of cellulase (FPase, CMCase and β-glucosidase) were obtained at pH 5.5. Production of FPase and CMCase by *A. terreus* reached maximum values after about 144 h of fermentation, while β-glucosidase activity reached a maximum value after about 192 h. Reduction in cellulase activities by about 10 to 20% and 40 to 50% was observed with increased in culture pH to 6 and 7.5, respectively (Figure 1). The optimum pH obtained from

Table 3. Effect of calcium chloride concentration on growth of *A. terreus* and cellulase production.

CaCl$_2$ (mM)	X*(Cell concentration) (g L^{-1})	FPase* (U ml^{-1})	CMCase* (U ml^{-1})	B-glucosidase* (U ml^{-1})	Yield (U g^{-1} cellulose)	Protein (mg ml^{-1})
0	2.63±0.26[a]	0.24±0.19[a]	2.35±0.12[a]	3.65±0.14[a]	37.85	0.81±0.02
1	4.15±0.09[a]	0.69±0.02[a]	8.64±0.17[a]	7.04±0.11[a]	108.83	1.02±0.05
3	4.23±0.11[b]	0.77±0.05[b]	9.23±0.14[b]	7.58±0.15[b]	121.45	1.08±0.08
5	4.47±0.13[b]	0.59±0.07[c]	7.72±0.11[c]	6.72±0.10[c]	93.05	0.97±0.03
7	4.78±0.05[d]	0.53±0.01[d]	6.56±0.12[d]	6.31±0.14[d]	83.59	0.91±0.02
10	3.47±0.12[e]	0.48±0.03[e]	3.77±0.15[e]	4.60±0.12[e]	75.70	0.84±0.07

Values are means of triplicate ± standard deviation. [a-e] Means values in the same column with different superscripts are significantly different ($P < 0.05$).*Maximum concentration obtained during fermentation.

Table 4. Effect of magnesium sulfate concentration on growth of *A. terreus* and cellulase production.

MgSO$_4$ (mM)	X* (Cell concentration) (g L^{-1})	FPase* (U ml^{-1})	CMCase* (U ml^{-1})	β-glucosidase* (U ml^{-1})	Yield (U g^{-1} cellulose)	Protein (mg ml^{-1})
0	3.62±0.15[a]	0.31±0.21[a]	4.76±0.17[a]	3.82±0.11[a]	48.89	0.83±0.01
1	4.13±0.12[a]	0.44±0.09[a]	6.44±0.13[a]	5.24±0.14[a]	69.40	0.91±0.05
3	4.78±0.08[b]	0.60±0.11[b]	7.62±0.18[b]	6.38±0.12[b]	94.63	1.03±0.04
5	4.96±0.11[c]	0.74±0.15[c]	9.05±0.10[c]	7.01±0.15[c]	116.71	1.12±0.05
7	5.12±0.14[d]	0.65±0.06[b]	8.13±0.12[d]	6.71±0.12[d]	102.52	1.09±0.04
10	4.65±0.07[e]	0.53±0.08[e]	7.05±0.11[e]	5.49±0.11[e]	83.59	0.97±0.02

Values are means of triplicate ± standard deviation.[a-e] Means values in the same column with different superscripts are significantly different ($P < 0.05$).*Maximum concentration obtained during fermentation.

this study was in the range of those reported for cellulase production by *T. reesei* (Tangnu et al., 2004). Cellulase production by *Aspergillus niger* MS82 was production by *Aspergillus niger* MS82 was maximal when the initial culture pH was adjusted to 6.0 or 7.0 (Sohail et al., 2009). On the other hand, Juhasz et al. (2004) claimed that the maximum cellulase production was obtained at pH ranging from 3.0 to 5.0. Generally, the pH of the culture increased during the first two days of cellulase fermentation by fungi due to utilization of yeast extract, hemicellulose and amorphous cellulose from ligno-cellulosic materials for growth. After an active growth was achieved, the culture pH decreased due to the formation of carboxylicgroups and carbonic acids from lignin (Portjanskaja et al., 2006). At this stage, the fungus started to utilize the crystalline portion of cellulose and starts secreting cellulase. The significant changes in culture pH, as observed in cellulase fermentation by *A. terreus* carried out in this study, were due to the use of weak buffering capacity (2 g L^{-1} of KH$_2$PO$_4$).

During the fermentation, the culture pH was reduced to acidic when cellulose was consumed by the fungi. Reduction in culture pH was due to the absorption of ammonium ions by the fungal mycelium (Cornejo et al., 2009). After cellulose has been completely consumed, the culture pH was increased possibly due to the consumption of organic acids accumulated in the culture

during the earlier stages of fermentation. In cellulase fermentation by *T. reesei*, organic acid is produced in direct relation to the amount of cellulose consumed (Mandels et al., 1974). Under favorable conditions, *T. reesei* would produce large quantities of cellulase, while acids with the presence of which would cause a drastic decrease in culture pH from 5.5 to 3.0 (Mandels et al., 1974). At culture pH of below 3.0, inhibition of growth and inactivation of cellulases occurred. Therefore, appropriate pH control strategy is necessary for the enhancement of cellulase production.

Effect of temperature

Figure 2 shows the effect of incubation temperature on growth of *A. terreus* and cellulase production. Good growth of *A. terreus* was observed over a temperature ranging from 24 to 32°C. Optimal growth was observed at 28°C. The highest CMCase, FPase and β-glucosidase activities were also obtained at 28°C and the activities were significantly decreased when the cultures were incubated at 24°C. In fermentation carried out at 28°C, cellulase activity was increased gradually and reached a maximum value after about 144 h of fermentation. Although, *A. terreus* was able to grow at high temperature (40°C) but the final cell concentration was only
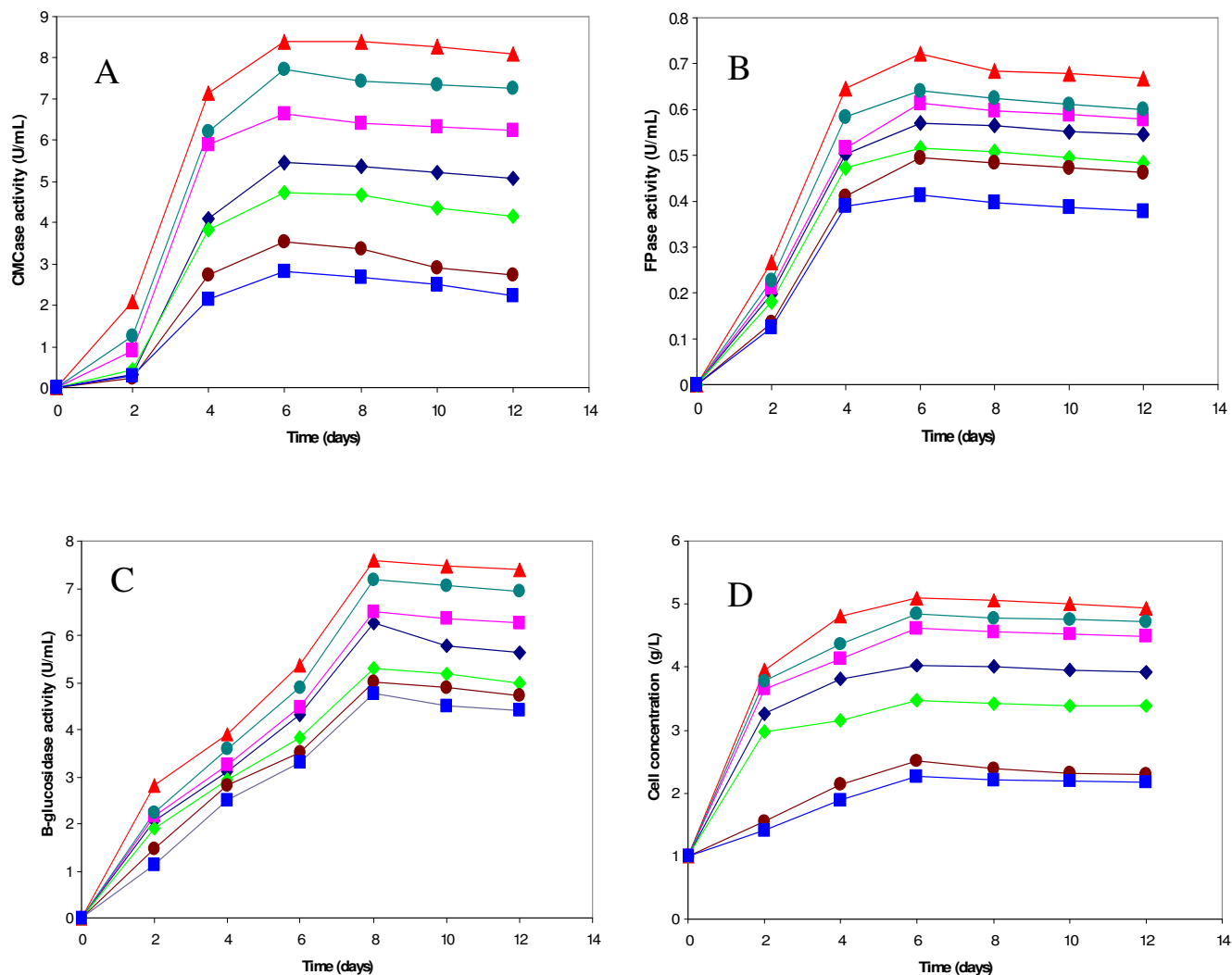
Figure 1. Effect of pH on growth of *A. terreus* and cellulase production. Note; (A) CMCase, (B) FPase, (C) β-glucosidase and (D) Cell concentration. Cultures were grown in basal medium with 10 g L^{-1} OPEFB at different initial culture pH. Data are means of three replicates with ± standard error of 2% of measured values. Symbols represent; (♦) pH 4.5, (■) pH 5.0, (▲) pH 5.5, (●) pH 6.0, (♦) pH 6.5, (●) pH 7.0, (■) pH 7.5.

about 15% of that obtained at 28°C. At higher temperatures, 36 and 40°C, cellulase production was reduced by about 40 to 50% as compared to that obtained at optimal temperature (28°C).

Most work concerning the effect of incubation temperature on growth of filamentous fungi supports the finding that is within limits, increased incubation temperature results in increased growth rate (Brown et al., 1987; Mandels et al., 1974). However, in most published works the effect of incubation temperatures on cellulase is not fully discussed. Reduced activity of FPase and β-glucosidase in fermentation by *penicillium pinophium* strain NTG III/6 was observed with increased in incubation temperature from 28 to 35°C (Brown et al., 1987). The optimum temperature (28°C) for growth and cellulase

production by *A. terreus*, as reported in this study, was similar to those observed in *T. ressei* RUT C30 (Juhasz et al., 2004). The optimum temperature for cellulase production by *T. ressei* was 25 to 28°C (Ahmed et al., 2005) while the optimum temperature for cellulase production by *Trichoderma harzianum* Rut-C 8230 was 28 °C (Kocher et al., 2008).

Effect of shaking speed

The production of all three main components of FPase, CMCase and β-glucosidase was varied with shaking speed (Table 5). In general, cellulase production was increased with increasing shaking speed. This might be
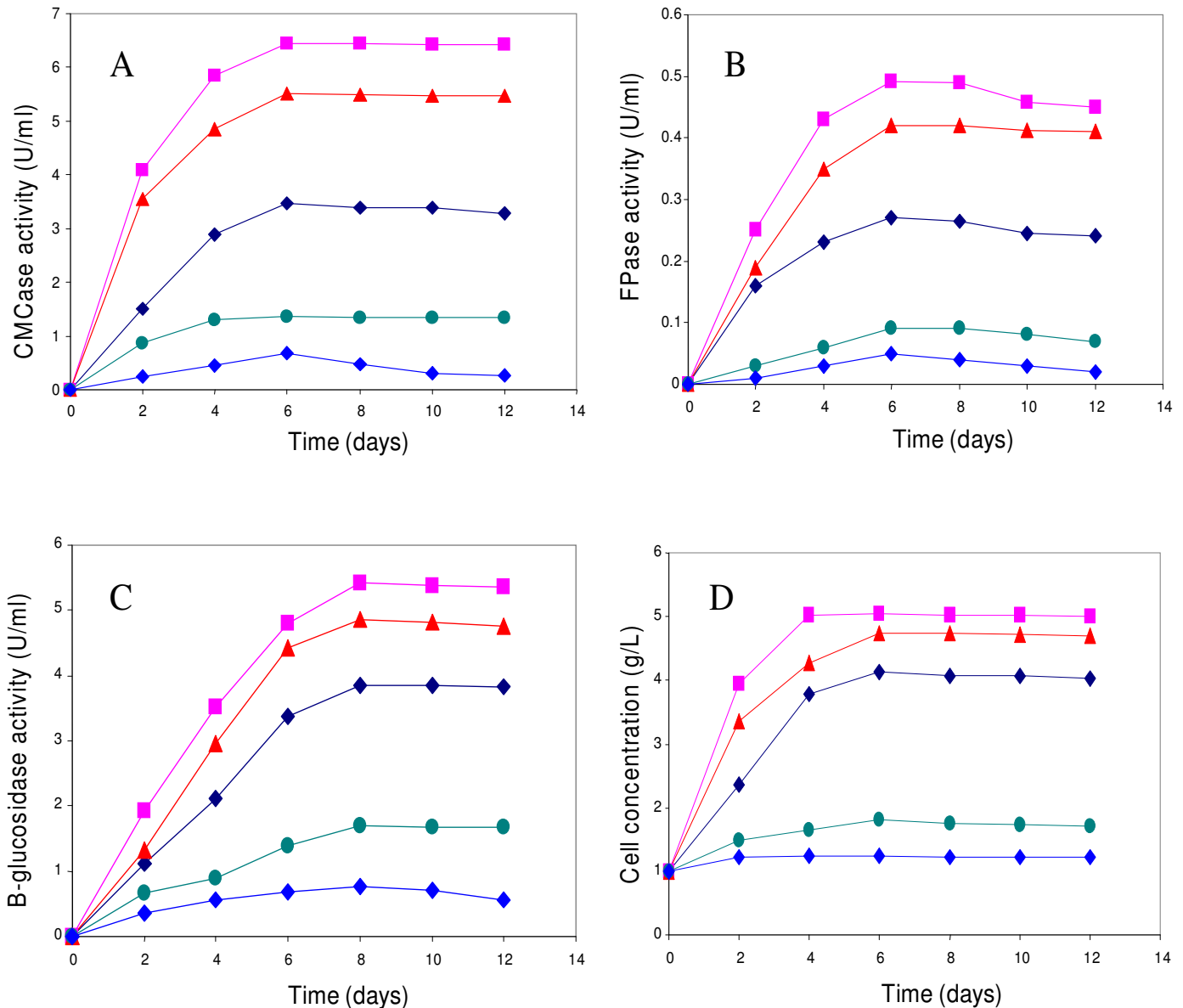
Figure 2. Effect of temperature on growth of *A. terreus* and cellulase production. Note: (A) CMCase, (B) FPase, (C) β-glucosidase and (D) Cell concentration. Cultures were grown in basal medium with 10 g L^{-1} OPEFB at different incubation temperatures. Symbols represent; (■) 28°C than at (♦) 24°C, (▲) 32°C, (●) 36°C , (♦) 40°C.

explained by the fact that the shaking speed increased the dissolved oxygen in the culture, which is necessary for cell membrane components and uniform distribution of the medium contents such as nutrients and catabolites (Rajagopalan and Krishnan, 2008). FPase activity obtained in the static culture of *A. terreus* was only 0.09 U ml^{-1} and the activity was increased to 0.45 U ml^{-1} in culture agitated at 200 rpm. In cultures agitated at 200 rpm, growth of *A. terreus* (4.90 g L^{-1}) was about 3 times higher than those obtained in static culture (1.40 g L^{-1}). On the other hand, CMCase (5.81 U ml^{-1}) and β-glucosidase (4.20 U ml^{-1}) activities were about five times

higher than those obtained in static cultures (1.20 and 1.10 U ml^{-1}, respectively). In the static culture, a layer of mycelium grew at the top of the culture while the OPEFB fiber remained at the bottom of the flask, which significantly reduced the contact time and area between the fungal cells and substrates. This is another possible explanation for reduced in the growth and cellulase production in static culture as compared to agitated culture.

The production of all the three main component of cellulose by *A. terreus*, as reported in this study, was comparable with other fungal strains reported in the

Table 5. Effect of shaking speed on growth of *A. terreus* and cellulase production.

Shaking speed (rpm)	X[*] (Cell concentration)(g L^{-1})	FPase[*] (U ml^{-1})	CMCase[*] (U ml^{-1})	B-glucosidase[*] (U ml^{-1})	Yield (U g^{-1} cellulose)	Protein (mg mL^{-1})
0	1.40±0.19[a]	0.09±0.02[a]	1.20±0.05[a]	1.10±0.16[a]	14.19	0.47±0.01
100	2.71±0.15[b]	0.18±0.03[b]	2.42±0.09[b]	1.70±0.12[b]	28.39	0.68±0.03
200	4.90±0.11[c]	0.45±0.07[c]	5.81±0.11[c]	4.21±0.10[c]	70.97	0.97±0.02
300	4.78±0.12[c]	0.43±0.08[c]	5.49±0.13[d]	4.02±0.13[d]	67.82	0.95±0.04

Values are means of triplicate ± standard deviation.[a-d] Means values in the same column with different superscripts are significantly different ($P < 0.05$).*Maximum concentration obtained during fermentation.

literature. For example, CMCase, FPase and β-glucosidase produced by *T. reesei* was ranging from 3.5 to 6.5 to U/ml, 0.18 to 0.63 U/ml, 3.5 to 5.8 U/ml, respectively (Gomes et al., 2008; Rashid et al., 2009; Rodriguez and Pioneros, 2007).

Conclusions

Results from this study have indicated that *A. terreus* is capable of producing high activities among all the three main component of cellulase (FPase, CMCase and β-glucosidase) in batch fermentation using OPEFB, an agriculture waste, as substrate. Yeast extract was the preferred nitrogen source as compared to peptone, urea and $(NH_4)_2SO_4$ for cellulase production. Cellulase production by *A. terreus* was improved by two-fold with the addition of Tween 80 at a concentration of 2 ml L^{-1} to the culture. Addition of calcium chloride (3 mM) and magnesium sulfate (5 mM) also enhanced cellulase production by *A. terreus*. The preferred initial culture pH and temperature for cellulase production by *A. terreus* was 5.5 at 28°C, respectively. Agitated shake flask culture improved cellulase production by about four times higher than a static culture, indicating that oxygen supply is the critical factor that determined the performance of the fermentation process. Since this newly isolated strain (*A. terreus*) has the ability to produce high activity of all three main components of cellulase in low cost substrate, it has great potential to be used as industrial strain for cellulase production. Development of large scale fermentation process in stirred tank bioreactor is the subject of our current research, emphasizing on the selection of various control strategies such as dissolved oxygen tension and pH for improvement of the production.

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