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Statistical Response to Different Fermentation Parameters in Rapid Production of Cellulases by *Penicillium purpurgenium* MA1 in Solid State Fermentation of Rice Hulls

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ABSTRACT

Time is critical value in biotechnological production of enzymes. Rapid production not only save time and money but also avoid physiological complication of long fungal cultivation. In this study, Plackett-Burman design was employed for screening the effect of 15 nutritional and cultivation parameters in production of CMCase, FPase and β-glucosidase by *P. purpurgenium* MA1 via solid state fermentation of rice hulls after 30, 36 and 42 h of incubation. The obtained data showed a great variation with different nutrients and with different times. The coefficient of determination (R^2) and the adjusted R^2 values of the test responses are in reasonable agreement recording ≥0.9. For CMCase, moisture level showed positive effect after 42 h where, pH ranged from negative to slightly positive effect. Inorganic nitrogen sources as $(NH_4)_2SO_4$ and NH_4NO_3 have a little positive effect after 30 and 36 h where it recorded high negative effect after 42 h. Yeast extract have a pronounced positive effect in the three tested times. For FPase, Tween 80, pH and $CaCl_2$ have a negative effect in enzyme production. Inorganic nitrogen sources have a positive effect after 42 h in opposite manner and the three organic nitrogen sources have a negative effect. For β-glucosidase, inorganic nitrogen sources have a weak effect as well as peptone where yeast extract have relatively stronger effect, pH have a negative effect after 30 and 36 h, where it have a positive effect one after 42 h.

Key words: Cellulases, *Penicillium purpurgenium*, solid state fermentation, rice hulls

INTRODUCTION

Cellulases are complex mixture of enzyme proteins with different specificities to hydrolyze glycosidic bonds which are mainly of three types; endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (Gao *et al*., 2008). Cellulases which produced by many fungi provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen *et al*., 2005) and have wide applications in various industries, such as textile industry (Nierstrasz and Warmoeskerken, 2003), food and feed industry (Ogel *et al*., 2001), pulp and paper industry (Ikeda *et al*., 2006) and in research development (Gao *et al*., 2008).

The most sophisticated method in production of fungal cellulases is Solid State Fermentation (SSF), in which an insoluble substrate is fermented with sufficient moisture but without free water. This method is more economic due to lower capital investment, lower operating expenses, ease of use, use of simpler fermentation media, reduced production of waste water and easier control of bacterial contamination (Pandey *et al*., 1994; Panagiotou *et al*., 2003; Yang *et al*., 2004). Moreover, enzyme titer is higher than that in submerged fermentation (SMF) (Gowthaman *et al*., 2001; Viniegra-Gonzalez *et al*., 2003).

The cost of the media has been estimated to be the major contribution to cellulase production cost, lignocellulosic agrowastes providing low cost substrate rather than expensive pure cellulose. Moreover, production time is another critical factor. This study aimed to reduce the cost and the time of cellulase production by application of Plackett-Burman statistical design in studying the variation in effect of different nutrients and fermentation parameters of low cost waste (rice hulls, RH) in early production of cellulases (after less than 48 h) by selected and tested fungal isolate, *P. purpurgenium* MA1 via SSF.

MATERIALS AND METHODS

Fungal isolate: *Penicillim purpurgenium* MA1 was isolated from deteriorated RH sample by placing it in sterile petri plates lined with sterile filter papers wetted with sterile H_2O. The plates incubated at 23±2°C for 7 days. Hyphal-tip and single spore isolation techniques were used to obtain pure cultures which maintained on Potato Dextrose Agar (PDA) slants for identification and further studies.

Solid state fermentation: In 100 mL glass flask, 1 g of dried RH moisten with 1 mL of basal salt solution (g L^{-1}) KH_2PO_4, 15; $(NH_4)_2SO_4$, 5; $MgSO_4.7H_2O$, 0.6; $ZnSO_4.7H_2O$, 0.14; $MnSO_4.6H_2O$, 0.16; $CaCl_2.6H_2O$, 0.37 and peptone, 2; (pH 4.8), autoclaved at 120°C for 20 min. Then inoculated with 0.5 mL of fungal spore suspension (106 spore mL^{-1}) of the tested species and incubated at 28±2°C.

Elution of cellulases enzymes: The crude enzyme was eluted after cultivation by adding 10 mL of saline solution (1:10 w/v) mixed with Berj 35 (0.25%). After shaking at 200 rpm for 30 min, the contents were then filtered through a nylon cloth and centrifuged for 15 min at 10,000 rpm to remove cells and residual RH and the filtrate was used for cellulases assay.

Enzyme assays

Carboxy methyl cellulose (1.4 -β-D-glucan glucano hydrolase EC 3.2.1.4): A mixture of 0.5 mL of substrate (0.5 w/v of carboxymethyl cellulose, sigma) in 0.2 mol acetate buffer, pH 4.8+0.5 mL of enzyme was incubated for 30 min at 50°C (Magnelli and Forchiassin, 1999). One unit of endoglucanase activity is the amount of enzyme required to release reducing sugars equivalent to 1 μmole glucose per min under the above experimental conditions.

Filter paper activity (FPase): This method estimates the overall cellulolytic activity. It was assayed by a modification of the method of Ghose (1987) incubating 1 mL of supernatant of culture with 50 mg filter paper Whatman No. 1 (1.0×6.0 cm) in 1 mL of 0.2 mol acetate buffer (pH 4.8) at 50°C for 60 min. One unit of FPase activity corresponding to 1 μmole of glucose equivalent released per minute under the experimental assay conditions.

β-glucosidase (cellobiase or β D-glucoside glucohydrolase EC 3.2.1.21): β-glucosidase activity against cellibiose was determined by 0.1 mL of culture supernatant to 0.5 mL of cellibiose in 0.2 mol acetate buffer (pH 4.8). The reaction mixture was incubated at 50°C for 30 min. One β-glucosidase activity unit is equivalent to 1 μmole of glucose per minute under the above experimental conditions.

The released reducing sugars of three enzymes were measured by glucose oxidase kit using glucose as standard (Spinreact company, Spain).

Table 1: Independent variables with their codes and corresponding levels used in Plackett-Burman design

Variables	Code	Low level (-1)	High level (+1)
Weight (of RH) (g)	X1	1	2
Moisture content (mL g^{-1})	X2	0.5	1.5
pH	X3	4.8	5.4
Inoculums size	X4	4×10^6	8×10^6
Sand	X5	0	0.25 g g^{-1} RH
$(NH_4)_2SO_4$	X6	0	5 g L^{-1} = 0.02 g g^{-1} RH
NH_4NO_3	X7	0	1.5 g L^{-1} = 0.0015 g g^{-1} RH
$NaNO_3$	X8	0	2.6 g L^{-1} = 0.0026 g g^{-1} RH
Peptone	X9	0	2 g L^{-1} = 0.002 g g^{-1} RH
Yeast extract	X10	0	1 g L^{-1} = 0.001 g g^{-1} RH
Corn steep liquor	X11	0	1 g L^{-1} = 0.001 g g^{-1} RH
Wheat bran	X12	0	1 g L^{-1} = 0.001 g g^{-1} RH
$MgSO_4$	X13	0	0.6 g L^{-1} = 0.0006 g g^{-1} RH
$CaCl_2$	X14	0	0.6 g L^{-1} = 0.0006 g g^{-1} RH
Tween 80	X15	0	1 g L^{-1} = 0.001 g g^{-1} RH

Plackett-Burman design: The Plackett-Burman experimental design (Plackett and Burman, 1946) was used in this study to demonstrate the relative importance of medium components which added to RH on CMCase, FPase and β-glucosidase production of *P. purpurgenium* MA1.

In the experimental design, each row represents an experiment and each column represents an independent variable. The positive and negative signs represent high and low levels of the given independent variable (Table 1 and 2). Each scenario (an assemblage of upper and lower values for the parameters) will produce output (CMCase, FPase and β-glucosidase). The number of output variables will depend on the model being analyzed.

All trials were performed in triplicate and each experiment was repeated twice, with the mean considered the response. Microsoft Excel, was used for the determination of variable significance.

RESULTS

Time course of cellulases production of *P. purpurgenium* MA1 on RH via SSF: As shown in Fig. 1, the cellulolytic activity of *P. purpurgenium* MA1 starting before 6 h of incubation and increasing with time from 10 U g^{-1} for CMCase to more than 14 U g^{-1} after 30 h incubation time, Fpase starting with low activity, increasing gradually with time and flocculated around 3 U g^{-1} up to 48 h, where β-glucosidase increasing steeply and recorded its highest activity after 42 and 48 h. Generally, the maximum productivity level was recorded after 30-42 h of incubation and tended to decline with increase in time.

Variation in response of cellulases production by *P. purpurgenium* MA1 to variation in medium component and time based on experimental design of Plackett-Burman matrix: Plackett-Burman design has been adopted at two levels for studying the effect of 15 variables namely weight of RH, moisture content, pH, inoculum size, sand particles, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, peptone, yeast extract, Corn Steep Liquor (CSL), wheat bran, $MgSO_4$, $CaCl_2$ and Tween 80 (Table 3) on celluases production for three enzymes CMCase, FPase and β-glucosidase after 30, 36 and 42 h of incubation.

There is a considerable variation in cellulases production in the same time with variation in fermentation parameters as well as variation in cellulases production under the same fermentation parameters with variation in time.

Table 2: Plackett-Burman experimental design matrix for evaluation of relative importance of selected nutrients in production of cellulases by *P. purpurgenium* MA1

Trials	Weight	Moisture	pH	Inoculum	Sand particls	$(NH_4)_2SO_4$	$NaNO_3$	NH_4NO_3	Peptone	Yeast	Corn steep liquor	Wheat bran	$MgSO_4$	$Cacl_2$	Tween 80
1	1	1	-1	1	-1	1	-1	1	1	1	-1	-1	-1	1	-1
2	-1	1	1	-1	1	-1	1	-1	1	1	1	-1	-1	-1	1
3	1	-1	1	1	-1	1	-1	1	-1	1	1	1	-1	-1	-1
4	-1	1	-1	1	1	-1	1	-1	1	-1	1	1	1	-1	-1
5	-1	-1	1	-1	1	1	-1	1	-1	1	-1	1	1	1	-1
6	-1	-1	-1	1	-1	1	1	-1	1	-1	1	-1	1	1	1
7	1	-1	-1	-1	1	-1	1	1	-1	1	-1	1	-1	1	1
8	1	1	-1	-1	-1	1	-1	1	1	-1	1	-1	1	-1	1
9	1	1	1	-1	-1	-1	1	-1	1	1	-1	1	-1	1	-1
10	-1	1	1	1	-1	-1	-1	1	-1	1	1	-1	1	-1	1
11	1	-1	1	1	1	-1	-1	-1	1	-1	1	1	-1	1	-1
12	-1	1	-1	1	1	1	-1	-1	-1	1	-1	1	1	-1	1
13	1	-1	1	-1	1	1	1	-1	-1	-1	1	-1	1	1	-1
14	-1	1	-1	1	-1	1	1	1	-1	-1	-1	1	-1	1	1
15	1	-1	1	-1	1	-1	1	1	1	-1	-1	-1	1	-1	1
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

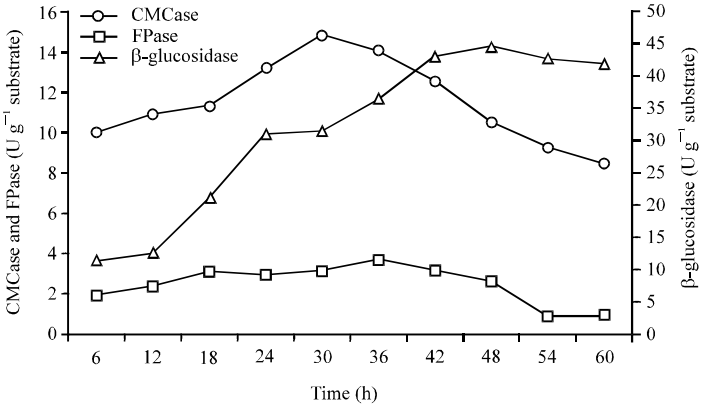

Fig. 1: Time course (h) of cellulases production of *P. purpurgenium* MA1 on RH via SSF

Table 3: Response of cellulases production by *P. purpurgenium* MA1 for fermentation parameters and regression statistics of ANOVA based on experimental design of Plackett-Burman matrix

	CMCase (U g^{-1})			FPase (U g^{-1})			β-glucosidase (U g^{-1})		
Run	30 h	36 h	42 h	30 h	36 h	42 h	30 h	36 h	42 h
1	10.58	8.63	13.66	2.34	1.30	5.03	31.84	26.46	26.67
2	9.57	7.62	12.65	0.44	4.39	2.94	32.82	40.44	63.89
3	11.59	9.64	14.67	2.18	2.93	4.98	46.25	40.63	42.38
4	9.44	10.74	7.53	2.56	5.15	4.82	43.02	46.25	56.15
5	8.43	9.73	6.52	1.74	2.03	3.32	16.35	22.54	31.62
6	10.45	11.75	8.54	1.79	2.23	1.54	41.95	44.10	27.75
7	10.61	7.63	15.42	2.32	3.24	3.03	57.01	38.72	27.53
8	9.60	6.62	14.41	1.85	2.54	4.17	55.72	51.23	68.19
9	11.62	8.64	16.43	2.24	2.52	3.73	23.45	20.44	46.68
10	5.06	7.66	17.40	1.62	3.25	1.86	27.53	30.00	40.01
11	4.05	6.65	16.30	1.32	2.67	1.57	24.74	40.23	29.90
12	6.07	8.67	18.50	1.31	4.16	4.13	32.27	42.38	57.44
13	8.70	8.24	7.50	1.92	1.57	4.13	16.56	20.63	43.55
14	7.60	7.23	6.40	1.15	3.64	6.11	29.69	46.47	60.45
15	9.80	9.25	8.60	3.25	1.08	2.99	22.23	24.95	41.95
16	12.69	9.67	4.35	1.44	3.09	3.94	18.28	17.64	31.19
R^2	0.999	0.998	1.000	0.991	0.998	0.999	1.000	1.000	0.997
Adj R^2	0.999	0.999	0.999	0.987	0.998	0.998	1.000	0.999	0.996
St Er	0.110	0.066	0.109	0.073	0.053	0.057	0.161	0.420	0.925

The coefficient of determination (R^2) and the adjusted R^2 values of the testes responses are in reasonable agreement recording ≥0.9. However, the higher the R^2, the more accurate of the mode. The values of R^2 of these responses are more than 0.9 i.e., the model can explain more than 90% of variation in any of the different responses. The adjusted R^2 is measures of how well the fitness of the data. This value can help in selection the model with the best fit. It should be noted that a R^2 value greater than 0.75 indicates the aptness of the model, however, the higher of their values, the more accuracy of the relationships between the variables (factors) and the response.

Effect of different medium components on rapid production of *P. purpurgenium* MA1 CMCase: Generally and as shown in Fig. 2, 3 and 4, if the effect is positive a higher concentration than the indicated high value (+) concentration was required and vise versa if the effect is negative a lower concentration than the indicated low value (-) concentration was required during further optimization studies.

In Table 3 and Fig. 2 there are a pronounced variation of the effect of different medium components and a difference in the effect in different time in each one. For example, weight and Tween 80 have a little negative effect after 30 and 36 h, where it recorded high positive effect after 42 h, moisture showed positive effect after 42 h where pH ranged from negative to slightly positive effect. Inorganic nitrogen sources as $(NH_4)_2SO_4$ and NH_4NO_3 have a little positive effect after 30 and 36 h where it recorded high negative effect after 42 h where peptone and CSL have weak effect on the rapid production of the enzyme. Yeast extract have a pronounced positive effect on the three tested times.

Effect of different medium components in rapid production of *P. purpurgenium* MA1 FPase: The data in Fig. 3 showed that the effect of RH weight differ from positive to negative to positive with time where, the effect of moisture changed from negative to slightly positive to strong

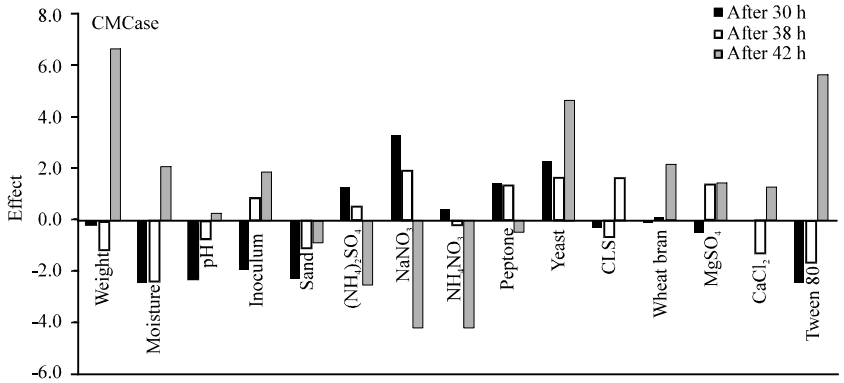

Fig. 2: Effects of the tested parameters on CMCase production

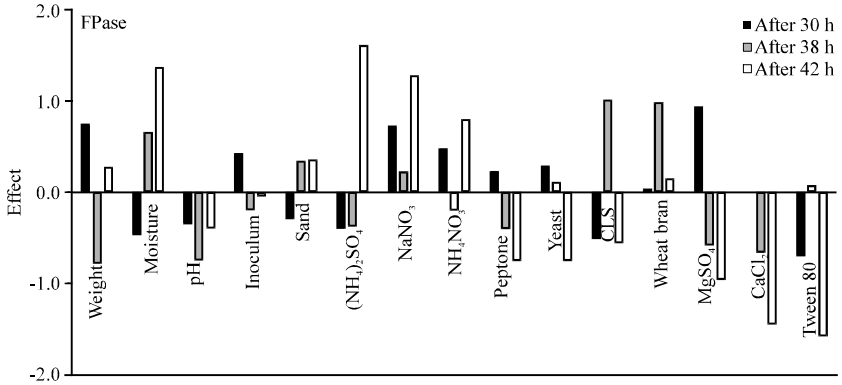

Fig. 3: Effects of the tested parameters on FPase production

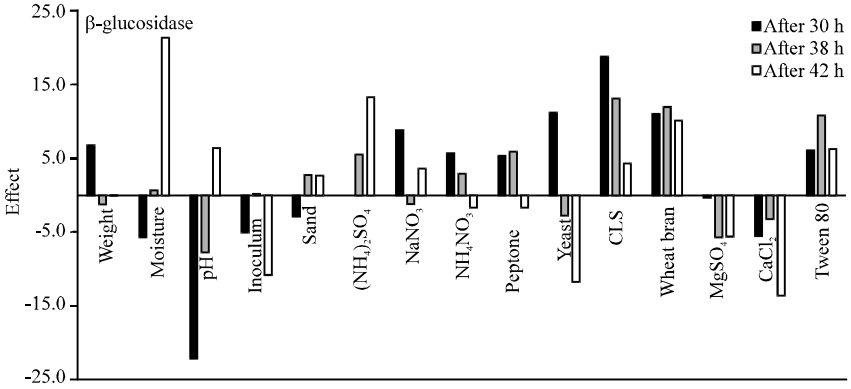

Fig. 4: Effects of the tested parameters on **β**-glucosidase production

positive. Tween 80, pH and $CaCl_2$ have a negative effect on enzyme production. Inorganic nitrogen sources differ in its effect after 30 and 36 h but they have a positive effect after 42 h in opposite manner the three organic nitrogen sources have a negative effect after 42 h.

Effect of different medium components in rapid production of *P. purpurgenium* MA1 β-glucosidase: The activity of **β**-glucosidase depends on the activity of CMCase and FPase as the produced cellobiose hydrolyzed by **β**-glucosidase. As shown in Fig. 4, inorganic nitrogen sources have a weak effect as well as peptone where CSL and yeast extract have relatively stronger effect, wheat bran as in CMCase and Fpase have a positive effect, weight and sand have a weak effect, pH have a negative effect after 30 and 36 h where it have a positive one after 42 h.

DISCUSSION

Agrowaste cellulose is good and cheap source for cellulase production. RH was selected as a low cost agrowaste containing cellulose and hemicelluloses as a major carbohydrates which induced the cellulases production (Muthuvelayudham *et al*., 2005; Brijwani *et al*., 2010). The selection of *P. purpurgenium* MA1 is based in previous study which confirm the capability of *Penicillim* in production of a complex group of endoglucanases and exoglucanases (Picart *et al*., 2007). However, the fungal physiological state, during the process of fermentation, may not always be kept at its optimum for enzyme production, the physiological balance may be set back from productive to non- productive cell growth or forward to unwanted sporulation, leading in both cases to decreased enzyme productivity (Li *et al*., 2010). For the above reasons, after the selection of high cellulases producing fungal isolate, the main task is the development and maintenance of conditions for maximal enzyme production.

Rapid production in short time is very critical. Generally, the time of the highest cellulase productivity depends upon the substrate and fungus so, we found a great variation in the production time in the previous studies, many of them concluded that maximum cellulases production often after more than 5 d whereas other researchers are in agreement with our record in rapid production as Ojumu *et al*. (2003), who recorded highest level of cellulase activity at the 12^{th} h of fermentation by *Aspergillus flavus* on sawdust, bagasse and corncob as substrates. Alam *et al*. (2005) found that the highest level of cellulose activity using *Trichoderma harzianum* occurred on the 2^{nd} day using palm oil biomass as a substrate (Maeda *et al*., 2013).

This rapid production can be explained under our condition of SSF by porous and good aerated RH medium enhancing the fast growth of secondary mycelium and branching of apical and subapical hyphal regions, as cellulase production is cell growth phase and mycelia morphology dependent (De Nicolas-Santiago *et al*., 2006; Shafique *et al*., 2009).

This relatively rapid production decreased with time until the end of the fermentation period which can be explained by delignification produces aromatic water-soluble products production of by- products resulting from microbial metabolism, as well as nutrient depletion.

In the study, the values of CMCase activity were higher than FPase all time intervals studied during the fermentation period. This fact have been reported in various studies on cellulolytic fungus and with different substrate (Singh *et al*., 2009) as well as the high ratio of β-glucosidase activity to FPase is important in simultaneous saccharification and fermentation process to avoid accumulation of the cellobiohidrolases inhibitor (cellobiose) in the reaction medium (Maeda *et al*., 2011).

Cellulase production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources and physical factors such as pH, temperature, initial substrate concentration, inoculums concentration and culture time. To investigate the effect, One Variable At a Time (OVAT) search is laborious and time consuming, especially for a large number of variables and it does not ensure desirable conditions. In order to overcome the limitations in OVAT, the statistical method are very useful for examining the simultaneous, systematic and efficient variations of all the components, the cumulative interactive effect present on the system and to predict the optimal conditions for a given operating system. Therefore, for screening more than five factors, the Plackett–Burman design is recommended (Shanmugaprakash *et al*., 2014).

The statistical design based on the addition of various nutrients is a good way to improve cellulases production (Gao *et al*., 2008; Kachlishvili *et al*., 2006). But the amount and interaction of these nutrients with time is very important to maximize the production and in the same time, minimize the time. In this connection the effect of RH weight ranged between negative to positive with incubation time. At lower substrate/enzymes ratio, these exo and endo acting enzyme are relatively close to one another as compared to higher substrate/enzyme ratio. Thus, considering the case of randomly associated exo and endo acting enzymes, synergy will be reduced as substrate concentrations increase above that required for maximum enzyme adsorption (Fenske *et al*., 1999). A high ratio will, thus, give similarly high surface coverage, leading to a reduction of the average distance between cellobiohydrolases and available chain end created by endoglucanase (Sarkar *et al*., 1998). The cellulases have been proposed to be laterally diffused on the cellulose surface (Huang and Penner, 1991).

The moisture content of the growth medium is critical variable affecting the solid-state fermentation, with fungal growth the effect of moisture differ with different incubation times, low moisture leads to reduced solubility of nutrients, substrate swelling while, increase in moisture level in solid state fermentation showed increase in enzymes productivity to a limit after which the enzyme production decrease which may be attributed to particles sticking, limited gas exchange and higher vulnerability to bacterial contamination (Hamidi-Esfahani *et al*., 2004).

In regarding to nitrogen sources, many of previous studies concluded that organic nitrogen sources, yeast extract and peptone gave more biomass with least cellulase activity, whereas the inorganic nitrogen sources, NH_4NO_3 and urea supported least biomass and higher cellulase activity. This may be attributed to the fact that complex substances like amino acids and vitamins in organic

nitrogen sources could trigger the biomass production, thus making it unnecessary for the fungus to produce cellulase (Kalogeris *et al*., 2003; Wen *et al*., 2005; Daroit *et al*., 2007). But in this study, we noted the variation on the effect of organic and inorganic nitrogen sources from positive to negative with time, therefore the ability to utilize particular nitrogen sources to produce cellulases varied with species difference and as well as with time. In the other hand, addition of wheat bran as a nutrient-richer byproduct of the wheat processing industry supply microorganisms with ample nutrition such as protein, hemicellulose, iron, manganese, zinc and copper (Oberoi *et al*., 2010) mostly gave positive effect on all enzymes at the three times intervals.

The pH in this study have negative effect in early production and can be probably due to low pH of the medium (4.8) which with the growth of the fungus and secretion of organic acids such as citric, acetic and lactic acids which will derived the pH to more decrease. Hendy *et al*. (1984) reported that performing the fermentation above pH 5.0, resulted in a significant loss of cellulase production by *T. reesei*. This indicates that cellulase properties and the optimal condition for their performance are different even with the same species, on the other hand, Tangnu *et al*. (1981) observed no significant effect on the production rate and final cellulase yield by *T. reesei* in the pH range of 4.0-6.0 but β-glucosidase production was greatly influenced and considerably enhanced when pH was controlled at 6.0.

Application of surfactant give mainly positive effect which is probably due to increase in the permeability of the cell membrane, allowing more rapid secretion of enzymes (Ahamed and Vermette, 2008) and this permeability differ from enzyme to other.

The decrease in cellulase production with further increase in inoculum might be due to depletion of nutrients by the enhanced biomass which resulted dwindle in metabolic activity (Kashyap *et al*., 2002). A balance between the increasing biomass and accessible nutrient would yield an optimal production of enzyme (Ramachandran *et al*., 2004).

CONCLUSION

Variation in effect of different nutritional and fermentation parameters changed in rapid manner in narrow time intervals. In biotechnological applications, this variation must be fully studied.

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