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Effect of Methanol Feeding Rate on the Expression of *Glomerella cingulata* Cutinase Using Recombinant *Pichia pastoris* in Shake Flask

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Abstract: In this study, we focused on the impact of different methanol feeding rates to the expression of WP_4-tagged *Glomerella cingulata* cutinase in shake flask cultivation of recombinant *P. pastoris* (Mut^+). Methanol serving as the inducer for cutinase expression was fed in fed-batch mode to avoid its accumulation in the culture. The fed-batch feeding rates were varied at constant volumetric rate of 0.160, 0.236, 0.317 and 0.633 mL MeOH/L/h throughout the 48 h of induction phase. Final biomass concentrations in all flasks showed an increase from 35 g L^{-1} (wet cell weight) to a range of 41-50 g L^{-1}. The maximum unit activities acquired after 48 h induction phase were 56, 163, 169 and 336 U mL^{-1} respectively. Therefore, the best methanol fed-batch feeding rate in shake flask is 0.633 mL MeOH/L/h, which produced maximum cutinolytic activity with an exponential trend. Next, we demonstrated cultivation in 7.5 L fermenter with 3 L working volume that implemented similar constant linear feeding rate for the 0.633 mL MeOH/L/h. In the first 24 h of cultivation in fermenter, the volumetric productivity was increased 10-fold relative to shake flask cultivation. However, for the next 24 h, cutinase activity was significantly dropped from 260 to 178 U mL^{-1}. The loss in cutinolytic activity was possibly due to proteolytic degradation caused by proteases dissolved in the culture as the total protein concentration was reduced from 73 to 56 mg L^{-1}.

Key words: *Pichia pastoris*, cutinase, fed-batch cultivation, methanol feeding rate, WP_4-tagged

INTRODUCTION

Cutinases (3.1.1.74), known as flexible hydrolytic enzymes that are able to catalyse cutinolytic, lipolytic and esteriolytic reactions. The capability of cutinases to catalyse these reactions has made it available for many industrial applications such as detergent formulation, ester synthesis, biodiesel production and etc. (Dutta *et al*., 2009).

Production of cutinase in bench top scale fermenter using wild-type strains has been reported by many authors (Chen *et al*., 2007; He *et al*., 2009). However, application of recombinant techniques for cutinase production offers several advantages in terms of high level expression and simplification of purification steps (Calado *et al*., 2002).

The most outstanding expression system that has been studied by many researchers since last a few decades is *P. pastoris* expression system. *P. pastoris* is methylotrophic yeast that is able to metabolise methanol as its sole carbon source due to the existence of alcohol oxidase (AOX) gene (Cereghino and Cregg, 2000). There are numerous advantages offered by *P. pastoris* as a host of recombinant protein production; its ability to grow on cheap fully-defined nutrient, its ability to grow to high cell density thus extensively increasing the production of recombinant proteins up to grams per litre (Sreekrishna *et al*., 1997).

One of the pivotal parameters in optimisation of heterologous protein production is methanol feeding rate as it affects specific productivity (Cunha *et al*., 2004). Zhang *et al*. (2000) reported that insufficient methanol supply might not allow the expression system to activate transcription of recombinant protein whereas high residual methanol concentration (>5 g L^{-1}) in *P. pastoris* culture can be toxic to the growth of the cells.

The recombinant protein levels in shake flask cultivation were typically low compared to the

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recombinant protein levels in fermenter. Among the reasons are unfavourable environment in shake flask due to limited oxygen transfer and uncontrolled pH during cultivation (Larentis *et al*., 2004). Another considerable factor is lack of intensive study conducted on the methanol feeding rate from shake flask to fermenter (Guarna *et al*., 1997).

Therefore in this study, we focused on the effect of methanol feeding rate on the expression of *Glomerella cingulata* cutinase in shake flask. Finally, we demonstrated a fermentation run in 7.5 L fermenter by applying the best methanol feeding rate from shake flask level.

MATERIALS AND METHODS

Microorganism: *P. pastoris* (Mut$^+$) clone pPicZáC-Cut-Q-WP_4 used in this study was obtained from the Department of Microbiology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

Shake flask cultivation: A single colony of *P. pastoris* was inoculated from YPD agar plate into an Erlenmeyer flask containing 30 mL BMGY medium. The YPD agar consist of (per L) 10 g yeast extract, 20 g peptone, 20 g dextrose and 20 g agar. While, the BMGY medium contains (per L) 10 g yeast extract, 20 g peptone, 100 mL of 1 M potassium phosphate buffer pH 6, 13 g Yeast Nitrogen Base (YNB) with $(NH_4)_2SO_4$, 2 mL of 500×biotin and 12.6 g glycerol.

The culture was incubated for 16-20 h at 30°C and 250 rpm. Once its OD_{600} reached to the range 6-10, the entire culture was transferred into a 2 L Erlenmeyer flask containing 270 mL BMGY and incubated at same operating conditions. After 20 h cultivation, pure methanol was fed according to its respective feeding rate.

Cultivation in 7.5 L fermenter: The 2.7 L of Basal Salt Medium (BSM) that contains (per L) 40 g glycerol, 26.7 mL H_3PO_4, 4.13 g KOH, 0.93 g $CaSO_4$, 18.2 g K_2SO_4 and 14.9 g $MgSO_4.7H_2O$ was prepared in a 7.5 L fermenter and autoclaved at 121°C for 15 min. Next, the temperature and aeration rate were fixed to 30°C and 1 vvm, respectively. The pH of the medium was adjusted to 5.5 using undiluted ammonia solution. Finally, 13 mL of PTM_1 was added aseptically into the medium by using a 0.2 μm syringe filter. The PTM_1 solution contains (per L) 6 g $CuSO_4.5H_2O$, 0.08 g NaI, 3 g $MnSO_4.H_2O$, 0.2 g $Na_2MoO_4.2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4.7H_2O$, 0.2 g biotin and 5 mL H_2SO_4.

The primary and secondary inocula were prepared same as in shake flask cultivation. After 20-24 h incubation of the secondary inoculum, the whole 300 mL culture with 10% inoculum was inoculated into the fermenter. The Dissolved Oxygen (DO) was maintained above 40% saturation by varying agitation speed.

The cultivation with a glycerol batch phase was continued in the fermenter for 18-20 h. At the end of the glycerol phase, a rapid increase in dissolved oxygen level to 100% saturation appeared. This indicates that microbe undergo starvation phase as glycerol is depleted. As the batch phase ended, methanol induction phase was started immediately by setting the speed of a calibrated peristaltic pump to the desired methanol feeding rate. The duration on methanol induction phase was fixed to 48 h.

Sample analysis

Biomass: Cell growth was measured in term of Wet Cell Weight (WCW). 1 mL of culture sample was centrifuged at 6000 rpm for 5 min. Supernatant was decanted into a microcentrifuge tube for protein and cutinase assay analysis while the weight of its pellet (biomass) was measured.

Protein assay: Total protein concentration was determined by miniaturised Bradford method. 500 μL of Bradford reagent was vortexed with 50 μL of 0.15M NaCl added with 50 μL of protein sample. The mixture was incubated at 25°C for 30 min. Absorbance of the protein assay at 595 nm wavelength was determined using BIOMOLECULAR Microplate Reader device.

Miniaturised cutinase assay with pNP-Laurate as substrate: Cutinase activity was measured based on its esteriolytic activity. 190 μL of 0.5 mM phosphate buffer, 5 μL of 20 μM pNP-laurate and 5 μL of enzyme sample were mixed well and incubated at 25°C. After 20 min, the assay was chilled at -20°C for 8 min. Concentration of p-nitrophenol was determined based on its absorbance at 405 nm wavelength.

RESULTS AND DISCUSSION

Effect of methanol feeding rate in shake flask level: The study of batch cultivation in shake flask was performed using BMGY medium to generate sufficient biomass to express WP_4-tagged cutinase. Biomass concentration added to all shake flasks at the beginning of methanol induction phase was measured to be approximately 35 g L^{-1} (Fig. 1).

In the first 12 h after induction phase, no cutinase activity was observed in any flasks. This might be due to the adaptation time taken by the cells to shift from

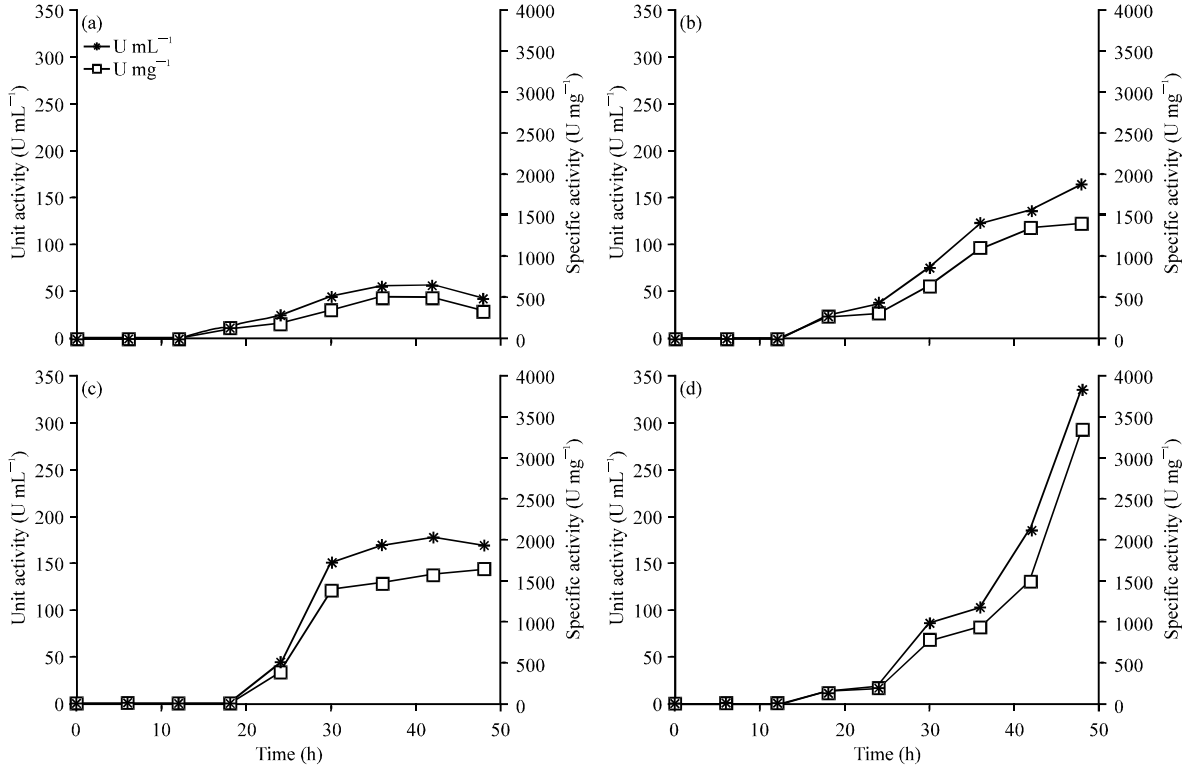

Fig. 1(a-d): Plots of enzymatic unit activity (U mL^{-1}) and specific activity (U mg^{-1}) against time at different methanol feeding rate, (a) 0.160 mL MeOH/L/h, (b) 0.236 mL MeOH/L/h, (c) 0.317 mL/MeOH/L/h and (d) 0.633 mL MeOH/L/h

glycerol to methanol metabolic pathway. Final biomass concentration in all flasks increased to a range of 41-50 g L^{-1} after 48 h induction phase which indicated the utilization of methanol for cell growth. The summary of overall results is tabulated in Table 1. 1 U is defined as the amount of enzyme required to hydrolyse 1 nmol of pNP-laurate per minute.

The volumetric feeding rate of 0.633 mL MeOH/L/h during shake flask cultivation was equivalent to 0.014 g MeOH/g WCW/h. This value is still comparatively low than the maximum specific methanol consumption rate claimed by Zhang *et al.* (2000) for *P. pastoris* (Mut^+) which corresponded to a value of 0.068 g MeOH/g WCW/h.

Production in 7.5 L fermenter: Based on the shake flask results, we performed cultivation in fermenter by implementing the best methanol feeding rate. Although the volumetric feeding rate was maintained at 0.633 mL MeOH/L/h in fermenter, its specific methanol consumption rate was different as the biomass concentration at the end of glycerol batch phase in fermenter was almost two-fold compared to the shake flask. The initial specific methanol consumption rate in fermenter corresponded to a value of 0.007 g MeOH/g WCW/h.

Table 1: Summary of WP_4-tagged cutinase in all shake flasks

	MeOH feeding rate (mL L^{-1} h^{-1})			
Parameters	0.160	0.236	0.317	0.633
Cutinase unit activity (U mL^{-1})	56	163	169	336
Cutinase specific activity (U mg^{-1} total protein)	505	1405	1641	3345
Cutinase volumetric productivity (U mL^{-1} h^{-1})	1.6	4.5	4.7	9.3
Cutinase specific formation rate (U g^{-1} biomass h^{-1})	43	131	136	274

In the first 24 h of induction, volumetric productivity of cutinase showed 10-fold increase compared to shake flask cultivation. Furthermore, the specific productivity increased from 51 U g^{-1} WCW/h in the shake flask to 162 U g^{-1} WCW/h in the fermenter.

However, after 24 h of induction phase, cutinase activity started to drop while the total protein concentration fluctuated and showed degradation of certain protein (data not shown). There are 2 main explanations suggested by researchers regarding proteolysis of recombinant protein in *P. pastoris*; limited

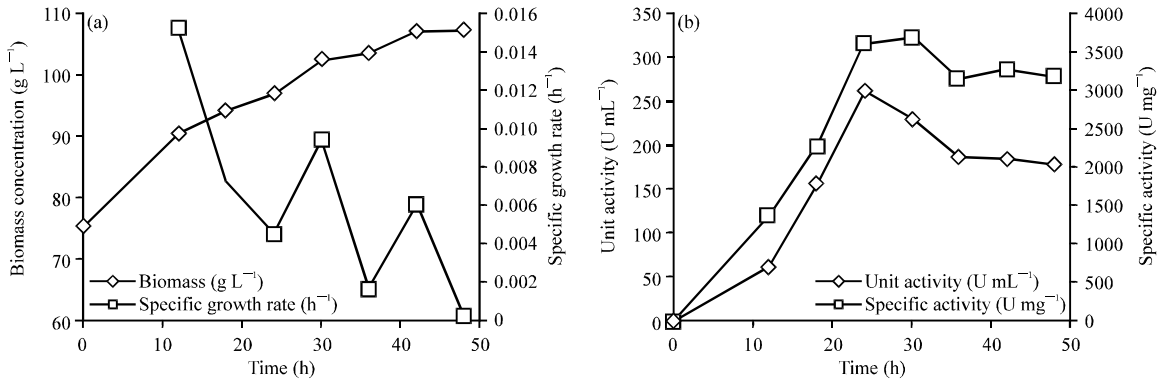

Fig. 2(a-b): Plots of biomass concentration, specific growth rate, unit activity and specific activity against time in fermenter

nitrogen source and secretion of intracellular proteases due to cell lysis (Macauley-Patrick *et al*., 2005).

Zhou and Zhang (2002) reported that the decrease in specific growth rate during methanol induction phase causes proteolytic degradation of heterologous protein in *P. pastoris*. They also proved that protease activity can be significantly reduced by maintaining specific growth rate between 0.020-0.047 h^{-1}.

Constant linear type of feeding rate resulted in the decrease of specific growth rate (Fig. 2). As the biomass concentration increased over the time due to cell growth, its specific substrate consumption rate decreased. As specific substrate consumption rate is a linear function of specific growth rate (Hang *et al*., 2008), decrease in specific substrate consumption rate results in the decrease of specific growth rate. Typically, specific growth rate can be maintained throughout the methanol fed-batch phase by designing an exponential feeding rate (Zhang *et al*., 2000).

CONCLUSION

In our study, maximum cutinase activity in shake flask (336 U mL^{-1}) was achieved at the feeding rate of 0.633 mL MeOH/L/h. However, cultivation in fermenter at similar feeding rate showed detrimental effect on cutinase activity after 24 h of induction time. Therefore, the methanol feeding rate in fermenter must be further studied in order to get higher productivity and this result can be used as the guide for the future optimization study.

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REFERENCES

Calado, C.R., M. Mannesse, M. Egmond, J. Cabral and L.P. Fonseca, 2002. Production of wild-type and peptide fusion cutinases by recombinant *Saccharomyces cerevisiae* MM01 strains. Biotechnol. Bioeng., 78: 692-698.

Cereghino, J.L. and J.M. Cregg, 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS. Microbiol. Rev., 24: 45-66.

Chen, Z., C.F. Franco, R.P. Baptista, M.S. Cabral, A.V. Coelho, C.J. Rodrigues and E.P. Melo, 2007. Purification and identification of cutinases from *C. kahawae* and *C. gloeosporiodes*. Applied Microb. Biotechnol., 73: 1306-1313.

Cunha, A.E., J.J. Clemente, R. Gomes, F. Pinto and M. Thomaz *et al*., 2004. Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*. Biotechnol. Bioeng., 86: 458-467.

Dutta, K., S. Sen and V.D. Veeranki, 2009. Production, characterization and applications of microbial cutinases. Process Biochem., 44: 127-134.

Guarna, M.M., G.J. Lesnicki, B.M. Tam, J. Robinson and C.Z. Radziminski *et al*., 1997. On-line monitoring and control of methanol concentration in shake-flask cultures of *Pichia pastoris*. Biotechnol. Bioeng., 56: 279-286.

Hang, H.F., W. Chen, M.J. Guo, J. Chu, Y.P. Zhuang and S. Zhang, 2008. A simple unstructured model-based control for efficient expression of recombinant porcine insulin precursor by Pichia pastoris. Korean J. Chem. Eng., 25: 1065-1069.

He, G., G. Huo, L. Liu, Y. Zhu, G. Du and J. Chen, 2009. Enhanced cutinase production of *Thermobifida fusca* by a two-stage batch and fed-batch cultivation strategy. Biotechnol. Bioprocess Eng., 14: 46-51.

Larentis, A.L., M.S. Almeida, K.M.S. Cabral, L.N. Medeiros, E. Kurtenbach and M.A.Z. Coelho, 2004. Expression of *Pisum sativum* defensin 1 (Psd1) in shaking flasks and bioreactor cultivations of recombinant *Pichia pastoris* at different pHs. Brazil. J. Chem. Eng., 21: 155-164.

Macauley-Patrick, S., M.L. Fazenda, B. McNeil and L.M. Harvey, 2005. Heterologous protein production using the *Pichia pastoris* expression system. J. Yeast, 22: 249-270.

Sreekrishna, K., R.G. Brankamp, K.E. Kropp, D.T. Blankenship and J.T. Tsay *et al*., 1997. Strategies for optimal synthesis and secretion of heterologous protein in the methylotrophic yeast *Pichia pastoris*. Gene, 190: 55-62.

Zhang, W., M.A. Bevins, B.A. Plantz, L.A. Smith and M.M. Meagher, 2000. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnol. Bioeng., 70: 1-8.

Zhou, X.S. and Y.X. Zhang, 2002. Decrease of proteolytic degradation of recombinant hirudin produced by *Pichia pastoris* by controlling the specific growth rate. Biotechnol. Lett., 24: 1449-1453.