Abstract

Constitutive protein expression in *Pichia pastoris* using the *GAP* promoter to avoid the use of methanol is an effective alternative to the inducible *AOX1* promoter. *GAP* promoter is a strong constitutive promoter that shows high expression when cells are grown on glucose. When *P. pastoris* are grown on glycerol, the non-fermentable sugar, small amount of ethanol can be detected. Ethanol has been reported to repress the *AOX1* promoter resulting in lower expression level of recombinant protein. However, no literature has reported on the production of ethanol by-product when *P. pastoris* are grown on glucose or the effect of ethanol on the expression of *GAP* promoter. In this work, first, the effect of ethanol on the growth and expression of *GAP* promoter were investigated using β-glucosidase as a reporter protein. The present of ethanol in culture medium decreased the cell concentration, specific growth rate (μ) and β-glucosidase activity. These results indicated that ethanol inhibit the cell growth and also the expression of the *GAP* promoter. Then, the influence of initial glucose concentration was studied under oxygen non-limit condition (DOT > 25 %). The optimum initial glucose concentration of 20 g/L provides $\mu = 0.20\ h^{-1}$ and lag time of 3.99 h. The increase of initial glucose concentration to 60 and 80 g/L resulted in the accumulation of ethanol, which decrease μ and increased the lag time. These results indicated that under oxygen non-limit condition, the ethanol was produced *via* overflow metabolism. Finally, 4 fed-batch systems were designed base on the effect of glucose concentration and DOT to find the optimum fed-batch control (system I: glucose and oxygen limit, system II: glucose limit and oxygen non-limit (DOT > 25%), system III: glucose non-limit ([glu] ≈ 10 g/L) and oxygen limit), and system IV: glucose and oxygen non-limit). The results showed that the oxygen limitation had a major drawback effect to both cell growth and expression of the *GAP* promoter. More ethanol by product was accumulated under the oxygen limited condition (13.5-14.0 g/L) compare to the oxygen non-limited condition (0.6 g/L). The higher ethanol concentration results in the decrease of μ about 35% and 63% when compared system I with system II and system III with system IV, respectively. Furthermore, about 7 times

higher specific production rate was observed under non-oxygen limit condition compared with oxygen limitation. The best results were obtained when system IV was used, however, the system IV is very difficult to control under high cell density culture. Therefore, we recommended system II of glucose limit and oxygen non-limit for the constitutive expression process of *P. pastoris* using glucose as substrate.