การพัฒนากระบวนการหมักสำหรับการผลิตรีคอมบิเนนท์โปรตีน โดย *Pichia pastoris*

นายเทพปัญญา เจริญรัตน์

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FERMENTATION PROCESS DEVELOPMENT FOR RECOMBINANT PROTEIN PRODUCTION BY *Pichia pastoris*

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FERMENTATION PROCESS DEVELOPMENT FOR RECOMBINANT PROTEIN PRODUCTION BY *Pichia pastoris*

Suranaree University of Technology has approved this thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เนื่องจากยีสต์ *Pichia pastoris* มีอัตราการสร้างผลผลิตจำเพาะ (specific productivity) ต่ำ เป็นเหตุให้กระบวนการผลิตรีคอมบิเนนท์โปรตีนโดยยีสต์ชนิดนี้โดยมากจะใช้กระบวนการเพาะเลี้ยงแบบเติมสารอาหาร (fed-batch culture) ซึ่งทำให้มีความหนาแน่นของเซลล์สูง การเพาะเลี้ยงภายใต้ความหนาแน่นของเซลล์สูงนั้นส่งผลให้ความต้องการออกซิเจนสูงตามไปด้วย ดังนั้นอัตราการถ่ายเทออกซิเจนจึงเป็นตัวแปรที่สำคัญมากตัวหนึ่ง ในการทดลองนี้ได้ทำการออกแบบกระบวนการที่สะดวกและประหยัดในการเพิ่มอัตราการถ่ายเทออกซิเจนสองแบบ และทำการเปรียบเทียบประสิทธิภาพของกระบวนการทั้งสองแบบกับกระบวนการอ้างอิง คือ กระบวนการเพาะเลี้ยงแบบเติมเมธานอลในอัตราที่จำกัด (methanol limited fed-batch process) ภายใต้ความดันรวม 1.2 บาร์ และ DOT (dissolved oxygen tension) เท่ากับ 25 เปอร์เซ็นต์ อากาศอิ่มตัว (กระบวนการแบบ MLFB) สำหรับกระบวนการแรกที่ทำการออกแบบเพื่อเพิ่มอัตราการถ่ายเทออกซิเจน คือ กระบวนการเพาะเลี้ยงแบบเติมเมธานอลภายใต้สภาวะจำกัดออกซิเจน (oxygen limited fed-batch process) ภายใต้ความดันรวม 1.2 บาร์ (กระบวนการแบบ OLFB) กระบวนการนี้จะทำการเพาะเลี้ยงภายใต้สภาวะที่ออกซิเจนจำกัดส่งผลให้อัตราการถ่ายเทออกซิเจนสูงขึ้นประมาณ 35 เปอร์เซ็นต์ กระบวนการที่สอง คือ กระบวนการเพาะเลี้ยงแบบเติมเมธานอลในอัตราที่จำกัด ภายใต้ความดันรวม 1.9 บาร์ และ DOT เท่ากับ 25 เปอร์เซ็นต์ อากาศอิ่มตัว (กระบวนการแบบ HPFB) ซึ่งการเพิ่มความดันส่งผลให้ความสามารถในการละลายของออกซิเจนในน้ำหมักสูงขึ้น ทำให้อัตราการถ่ายเทออกซิเจนสูงขึ้นประมาณ 59 เปอร์เซ็นต์

ถึงแม้ว่าปริมาณเมธานอลที่ถูกใช้ไปทั้งหมดจะเพิ่มขึ้นในอัตราส่วนที่ใกล้เคียงกับอัตราการเพิ่มขึ้นของอัตราการถ่ายเทออกซิเจน แต่การเพิ่มของชีวมวล (biomass) นั้นน้อยกว่าอัตราส่วนของอัตราการถ่ายเทออกซิเจน คือ เพิ่มขึ้นเพียง 7 และ 12 เปอร์เซ็นต์ ในกระบวนการแบบ OLFB และกระบวนการแบบ HPFB ตามลำดับ สาเหตุเกิดจากการตอบสนองของ *P. pastoris* ต่อการเพาะเลี้ยงภายใต้ความดันสูงและสภาวะที่ออกซิเจนจำกัดโดยการเพิ่มค่าสัมประสิทธิ์การบำรุงรักษาเซลล์ (maintenance coefficient) ส่งผลให้ผลได้ของชีวมวลจากเมธานอล (biomass yield) ลดลง อย่างไรก็ตาม ผลผลิตของเอนไซม์เบต้ากลูโคสิเดสทั้งหมดในกระบวนการเพาะเลี้ยงแบบ

OLFB และกระบวนการแบบ HPFB ยังคงเพิ่มขึ้นประมาณ 41 และ 50 เปอร์เซ็นต์ ตามลำดับ ซึ่งเป็นค่าที่ใกล้เคียงกับอัตราส่วนการเพิ่มขึ้นของอัตราการถ่ายเทออกซิเจน

โดยการอ้างอิงถึงการวิเคราะห์สมดุลมวลของคาร์บอนในแต่ละกระบวนการ พบว่าในทุกกระบวนการยีสต์ *P. pastoris* ไม่มีการใช้เมธานอลโดยเมตาบอลิซึมรูปแบบอื่น ยิ่งไปกว่านั้น ในทุกกระบวนการยังแสดงค่าร้อยละของเซลล์ที่มีชีวิตใกล้เคียงกัน เป็นการแสดงให้เห็นว่าทั้งกระบวนการแบบ OLFB และกระบวนการแบบ HPFB สามารถเพิ่มประสิทธิภาพในการผลิตรีคอมบิเนนท์โปรตีนได้ โดยที่กระบวนการที่มีประสิทธิภาพสูงที่สุด คือ กระบวนการแบบ HPFB

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา_____

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

THEPPANYA CHAROENRAT : FERMENTATION PROCESS DEVELOPMENT FOR RECOMBINANT PROTEIN PRODUCTION BY *Pichia pastoris*. THESIS ADVISOR : ASST. PROF. MARIENA KETUDAT-CAIRNS, Ph.D. 184 PP. ISBN 974-533-499-5

OXYGEN TRANSFER RATE/OXYGEN-LIMITATION/HIGH PRESSURE CULTIVATION/β-GLUCOSIDASE

Due to its low specific productivity, high cell density fed-batch culture is often used for recombinant protein production by *Pichia pastoris*. High cell density causes the process to have high oxygen demand which makes the oxygen transfer rate (OTR) a key limiting parameter. In this work, two economical and simple process strategies were designed to increase the OTR and were compared with the reference methanol limited fed-batch technique of 1.2 bar total air pressure and DOT = 25% air saturation (MLFB process). The oxygen limited fed-batch technique at 1.2 bar total air pressure (OLFB process) was run under oxygen limited conditions, which could increase the OTR about 35%. The methanol limited fed-batch technique at 1.9 bar total air pressure and DOT = 25% (high pressure fed-batch, HPFB process) used an elevated total air pressure to increase the oxygen solubility, which increased the OTR about 59%.

The total methanol consumption increased almost in proportion to the OTR, but the biomass increased much less. Only 7% and 12% increases in biomass were produced in the OLFB and HPFB processes, respectively. This was due to the

response of *P. pastoris* to the oxygen limitation and high pressure condition by increasing the maintenance coefficient (q_m), which resulted in the decrease of the biomass yield ($Y_{x/s}$). However, total product formation per process increased about 41% and 50% in OLFB and HPFB, respectively, which is close to proportional to the increase in OTR.

The carbon mass balance analysis of all processes indicated that *P. pastoris* did not apply any alternative metabolism during oxygen limitation. Furthermore the percent of cell viability in all processes were similar. Thus, both OLFB and HPFB can improve the *P. pastoris* process efficiency without any major negative effect. The highest efficiency was obtained in the HPFB process.

School of Biotechnology

Academic Year 2005

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Advisor's Signature

Co-advisor's Signature

Co-advisor's Signature N. Vanichsriratana

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LIST OF ABBREVIATIONS

a	specific air bubble area per volume (m^{-1})
AOX	alcohol oxidase enzyme
aox1	alcohol oxidase 1 gene
C	oxygen concentration in bulk liquid outside the film (mol L^{-1})
CAT	catalase enzyme
C*	oxygen concentration in gas-liquid interface (mol L^{-1})
C^*_{ref}	C* under 100% DOT electrode calibration condition (mol L^{-1})
CPR	carbon dioxide production rate (mol h^{-1})
DHA	dihydroxy acetone
DHAP	dihydroxy acetone phosphate
DHAS	dihydroxy acetone syntase enzyme
DH1	formaldehyde dehydrogenase enzyme
DH2	formate dehydrogenase enzyme
DOT	dissolved oxygen tension (%)
F	substrate feed rate (g h^{-1} or ml h^{-1})
F0	initial substrate feed rate (g h^{-1} or ml h^{-1})
F6P	fructose-6-phosphate
F1,6BP	fructose-1,6-bisphosphate
GAP	glyceroldehyde-3-phosphate
GF	glycerol feed medium

LIST OF ABBREVIATIONS (Continued)

GSCH2OH	S-hydroxymethylglutathione
GSCHO	S-formylglutathione
HPFB	high pressure fed-batch
K_L	oxygen transfer coefficient (m h^{-1})
K_La	volumetric oxygen transfer coefficient (h^{-1})
K_La_{ref}	K_La under 100% DOT electrode calibration condition (h^{-1})
K_s	saturation constant (g L^{-1})
MCR	methanol consumption rate (mol L^{-1} or g L^{-1})
MCS	multi cloning sites
MF	methanol feed medium
MLFB	methanol-limited fed-batch
OLFB	oxygen-limited fed-batch
OTR	oxygen transfer rate (mol h^{-1})
OUR	oxygen uptake rate (mol h^{-1})
P	air pressure inside the bioreactor (bar)
P	product concentration (g L^{-1} or U L^{-1})
PI	propidium iodide
P_{ref}	P under 100% DOT electrode calibration condition (bar)
P_{tot}	total air pressure (bar)
Q	gas flow rate (L h^{-1} or L min^{-1})
q_m	maintenance coefficient (g g^{-1} h^{-1})
q_O	specific oxygen uptake rate (g g^{-1} h^{-1} or mol g^{-1} h^{-1})

LIST OF ABBREVIATIONS (Continued)

q_p	specific production rate (U g h^{-1} or g g h^{-1})
q_s	specific substrate consumption rate (g g h^{-1})
r_O	oxygen uptake rate (mol h^{-1})
ROS	reactive oxygen species
RQ	respiratory quotient
RRR	relative rate of respiration
r_S	substrate consumption rate (g h^{-1} or mol h^{-1})
V	medium volume (L)
Vm	molar volume of gas (L mol^{-1})
X	biomass concentration (g L^{-1})
Xu5P	xylulose-5-phosphate
$Y_{CO2/S}$	carbon dioxide yield from methanol (g g^{-1} or mol mol^{-1})
Yem	biomass yield on methanol, exclusive the maintenance (g g^{-1})
$Y_{O/S}$	oxygen requirement per methanol uptake (g g^{-1} or mol mol^{-1})
$Y_{P/S}$	production yield on methanol (U g^{-1})
$Y_{x/s}$	biomass yield on methanol (g g^{-1})
μ	specific growth rate (h^{-1})
δ	separation factor

Subscripts

an	anabolism
en	energy metabolism

LIST OF ABBREVIATIONS (Continued)

i	inlet
max	maximum value
meth	methanol
o	outlet

CHAPTER I

INTRODUCTION

Recombinant protein expression by *Pichia pastoris* is an excellent expression system (Cregg et al., 2000; Lin Cereghino and Cregg, 2000). Several *P. pastoris* fermentation strategies have been applied to improve both product quality and productivity such as *i*. optimization of the methanol concentration (Kupcsulik and Sevella, 2004; Mayson et al., 2003; Zhang et al., 2000; Katagura et al., 1998), *ii*. increasing of oxygen concentration in inlet air (Trentmann et al., 2004; Lee et al., 2003; Jahic et al., 2002), *iii*. pH optimization (Larentis et al., 2004; Jahic et al., 2003a) and *iv*. temperature optimization (Jahic et al., 2003a; Hong et al., 2002; Li et al., 2001). Many publications have reported that the temperature and the pH often affect the recombinant protein productivity, recombinant protein stability and protease activity. However, the effects really depend on the type of the protein produced (Jahic et al., 2003a; Hong et al., 2002; Li et al., 2001; Cassland and Jönsson, 1999; Chiruvolue et al., 1998; Sreekrishna et al., 1997). On the other hand, the increase of the oxygen transfer rate (OTR) in the *Pichia* system has been reported to enhance the oxygen uptake rate (OUR) due to the increase of the methanol (substrate) consumption rate (MCR), cell growth and productivity of many recombinant proteins. Thus, the OTR is more general parameter involved in productivity and seem to be independent to the type of protein product (Trentmann et al., 2004; Lee et al., 2003; Jahic et al., 2002). The addition of pure oxygen in inlet air flow has been shown to be

an effective technique to increase the OTR (Trentmann et al., 2004; Jahic et al., 2002), however, the supply and handling of pure oxygen is expensive and can be unsafe.

Therefore, in this research the emphasis was to develop the *P. pastoris* fermentation process by designing new techniques to increase the OTR. Two fermentation techniques were designed based on the general OTR model [$OTR = K_L a(C^* - C)$]. OTR is affected by three parameters: *i.* the oxygen transfer coefficient (K_L), *ii.* the specific air bubble area per volume (*a*) and *iii.* driving force (C*-C). The first technique was designed to run the process under oxygen limited conditions due to decreasing the oxygen concentration in liquid outside the film ($C \approx 0$), thereby resulting in an increase in the driving force. This technique is called the oxygen limited fed-batch or OLFB technique. The second technique was designed to increase the oxygen solubility in equilibrium with the gas phase (C*) by increasing the total air pressure (P_{tot}) in the bioreactor. This technique is called the high pressure fed-batch or HPFB technique.

However, both OTR increasing techniques (OLFB and HPFB techniques) might generate some biological stresses that might be detrimental to the cells and product. Thus, the growth, viability and productivity from these two new techniques were compared with the traditional methanol limited fed-batch (MLFB) technique. The recombinant Thai rosewood β-glucosidase was used as a model protein in this study.

1.1 Research objectives

The main task of this work is to improve the process efficiency in recombinant protein production in *P. pastoris*. Two process strategies to increase the oxygen transfer rate were designed by running the process under oxygen limitation or increasing the total air pressure. The influence of both oxygen limitation and air pressurization during *P. pastoris* culture on methanol was studied.

1.2 Publications and main contributions

The thesis work has contributed to two major publications. The list of papers is followed and full texts are presented in Appendix IV.

Paper I

Charoenrat, T., Ketudat-Cairns, M., Stendahl-Andersen, H., Jahic, M. and Enfors, S.-O. (2005) Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes. Bioprocess Biosystem Engineering. 27(6): 399-406.

The paper presents a new fermentation process design based on increasing the OTR for recombinant protein production by *P. pastoris*. An oxygen limited fed-batch (OLFB) technique was designed to increase the oxygen transfer. The presence of residue methanol in culture broth makes the oxygen demand higher than the oxygen

transfer, until the oxygen available is limited. Thus, in this technique, the maximum oxygen transfer capacity was used.

Paper II

Charoenrat, T., Ketudat-Cairns, M., Jahic, M., Veide, A. and Enfors, S.-O. (2006) Increased total air pressure *versus* oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process. Biochemical Engineering Journal. *In press*.

In this paper an alternative technique to increase the oxygen transfer was applied in the *P. pastoris* fermentation process. Elevation of the total air pressure was used to increase the oxygen solubility in the liquid phase, which resulted in an increased oxygen transfer rate. The process efficiency was enhanced by this technique.

CHAPTER II

LITERATURE REVIEW

2.1 *Pichia pastoris*

Some functionally active proteins that cannot be expressed efficiently in bacteria, *Saccharomyces cerevisiae* or insect cells have been successfully produced in *Pichia pastoris* under the control of the *alcohol oxidase 1* (*aox1*) promoter (Lin Cereghino et al., 2001). The methylotrophic yeast *P. pastoris* can be genetically engineered to express foreign proteins (Cregg et al., 1985). It is easily manipulated at the molecular genetic level (e.g. gene targeting, high-frequency DNA transformation, cloning by functional complementation). Foreign proteins may be expressed both intracellularly and/or extracellularly, at high levels, and protein modifications, such as glycosylation, disulfide-bond formation, and proteolytic processing are performed (Cregg et al., 2000; Lin Cereghino and Cregg, 2000).

P. pastoris can be grown up to >150 g L^{-1} dry weight (DW) (Jahic et al., 2003b). The alcohol oxidase 1 (*aox1*) promoter in *P. pastoris* is a strong promoter used to drive the expression of foreign genes. This system has been shown to be simple, easy to scale-up and cost-effective for industrial fermentation (Cregg et al., 1993). Using the high cell density fermentation technique coupled with the

advantage of a strong *aox1* promoter, the processes can produce as much as 22 g L^{-1} intracellular product (Hasslacher et al., 1997) and 14.8 g L^{-1} of secreted protein in the clarified supernatant (Werten et al., 1999).

2.1.1 Classification of *P. pastoris*

P. pastoris belongs to the class Hemiascomycetes, which is generally present in the vegetative haploid state. It has a predominantly unicellular talus that may produce pseudomycelium. They reproduce asexually primarily by multilateral budding and produce hat- or saturn-shaped ascospores in a free ascus that originates either from a zygote or parthogenetically from a single somatic cell. The classification of *P. pastoris* is shown in Table 2-1.

Table 2-1 Classification of *P. pastoris*

Kingdom	Fungi
Phylum	Ascomycota
Class	Hemiascomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	Pichia
Species	Pichia pastoris

Note: Adapted from Alexopoulos et al. (1996) and Kurtzman and Fell (1998)

2.1.2 Methanol metabolism in *P. pastoris*

The methanol metabolism of *P. pastoris* is summarized in Fig. 2-1. The alcohol oxidase (AOX) enzyme is the first enzyme in the methanol utilization pathway, which oxidizes methanol to formaldehyde inside the peroxisome by a reaction coupled with catalase (CAT) (Veenhuis et al., 1976). Formaldehyde is used for two objectives. One is for energy metabolism (dissimilation) by oxidation to formate and carbon dioxide by two cytoplasmic dehydrogenases reactions, formaldehyde dehydrogenase (DH1) and formate dehydrogenase (DH2) (Fukui et al., 1975). The other is for anabolism (assimilation) by assimilation to form cellular constituents by a cyclic pathway that starts with the condensation of formaldehyde with xylulose-5-monophosphate (Veenhuis et al., 1983). This reaction is catalyzed by a third peroxisomal enzyme dihydroxyacetone synthase (DHAS) (Douma et al., 1985).

2.1.3 *Alcohol oxidase 1* (*aox1*) promoter

In *P. pastoris* grown on methanol, two alcohol oxidase genes, *aox1* and *aox2*, are involved in alcohol oxidase (AOX) enzyme production. The *aox1* gene has been shown to be the major AOX enzyme producing gene (Cregg et al., 1989). It is a strong promoter used to generate a large amount of AOX enzyme to compensate for the poor affinity of the enzyme to oxygen. In methanol-grown cells, approximate 5% of poly(A)$^+$ RNA is from the *aox1* promoter (Cregg and Madden, 1988) and more than 30% of soluble proteins inside the cell is detected as AOX enzyme (Couderc and

Baratti, 1980). However, both the *aox1* poly(A)$^+$ RNA and the AOX enzyme are undetectable in cells grown on high concentrations of most other carbon sources (e.g., glucose, glycerol or ethanol) (Cregg and Madden, 1988; Roggenkamp et al., 1984; Couderc and Baratti, 1980). The AOX1 enzyme has a molecular weight of 675 kDa and is composed of eight identical 80 kDa subunits (Couderc and Baratti, 1980).

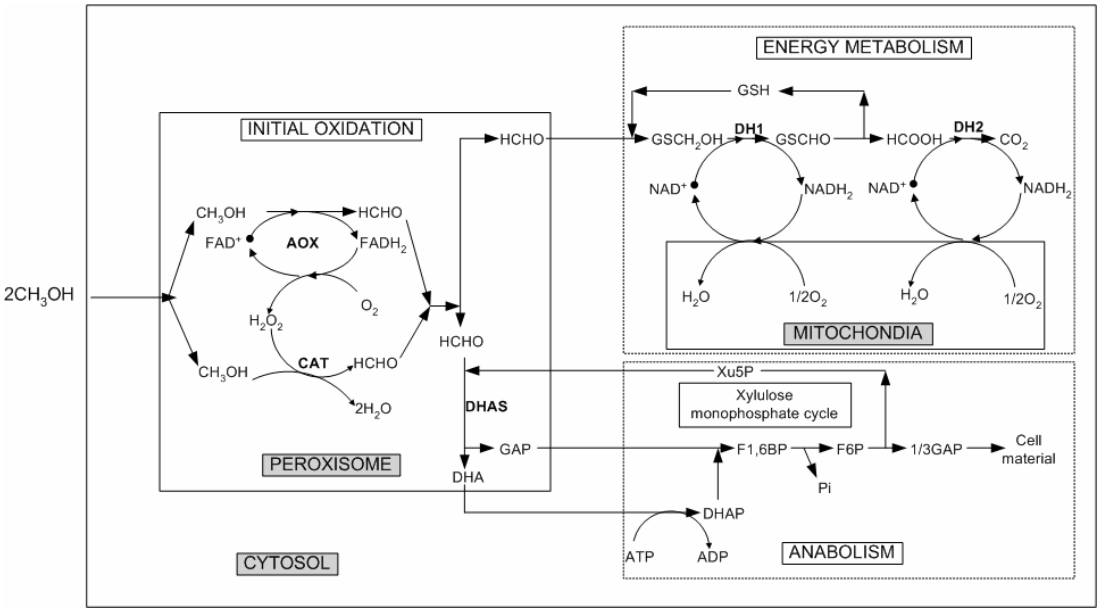

Figure 2-1 Methanol metabolism in *P. pastoris*. AOX, alcohol oxidase; CAT, catalase; DH1, formaldehyde dehydrogenase; DH2, formate dehydrogenase; GSCH2OH, S-hydroxymethylglutathione; GSCHO, S-formylglutathione; GAP, glyceraldehyde-3-phosphate; DHA, dihydroxyacetone; DHAS, dihydroxyacetone syntase; DHAP, dihydroxyacetone phosphate; F1,6BP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; Xu5P, xylulose-5-phosphate. Adapted from Douma et al. (1985).

P. pastoris expression of the *aox1* gene is controlled at the transcriptional level. The regulation of the *aox1* gene involves two mechanisms: repression/derepression and induction (Egli et al., 1980). The repression occurs when the cell is grown at moderate levels of repressing substrate. On the other hand, this gene is derepressed when the repressing carbon source is limited (Jahic et al., 2002; Egli et al., 1980) and powerfully induced by addition of methanol (Lin Cereghino and Cregg, 2000). The properties of this *aox1* promoter make it useful as a promoter to control foreign gene expression.

2.1.4 Construction of *P. pastoris* expression strains

Three basic steps for the construction of *P. pastoris* foreign gene expression strains have been summarized by Lin Cereghino and Cregg (2000). First is insertion of the foreign gene into an expression vector. Then, the expression vector with the foreign gene in an expression cassette is introduced into the *P. pastoris* genome. Finally, potential expression strains are selected for a selectable marker and expression the foreign gene product. Nowadays, a variety of *P. pastoris* expression vectors and host strains are available. In this literature review I will concentrate only on *P. pastoris* Y-11430 host strain and pPICzαB plasmid which was used in this research.

P. pastoris Y-11430 is a wild-type strain (Lin Cereghino and Cregg, 2000). Thus, neither antibiotic resistance gene nor metabolic gene mutation is present, which

allows for selection of expression vectors containing any appropriate antibiotic resistance gene selectable marker upon transformation.

pPICzαB has been designed as an *E. coli-P. pastoris* shuttle vector, which contains an origin of replication for plasmid maintenance in *E. coli* and a zeocin resistance gene selectable marker, which is functional in both organisms. The pPICZαB has an expression cassette composed of a 0.9 kb fragment of the 5' promoter sequences of the *aox1* gene and a part of the *aox1* transcription termination sequence. Between the promoter and terminator sequences are the multiple cloning sites (MCS) for insertion of foreign coding sequence. Prior to the MCS, the sequences for the secretion signal sequence of *S. cerevisiae* α-mating factor is placed. This α-secretion signal will promote the secretion of target protein. In addition, this vector contains a *c-myc* epitope and 6His tag, which can be incorporated in the protein if inframe fusion is made with the c-terminal sequence of the target protein (Lin Cereghino and Cregg, 2000; Invitrogen® catalog).

2.2 Fermentation techniques for *P. pastoris*

The *P. pastoris* protein expression system can be developed to produce heterolo- gous proteins in shake flask culture. However, production in a fermentor has several advantages over shake flask: the fermentor media of *P. pastoris* (containing biotin, salts, trace elements and glycerol or methanol) are economical and well defined, and parameters such as pH, aeration and carbon source feed rate can be

controlled. Using a fermentor also makes it possible to achieve ultra high cell densities (Lin Cerghino et al., 2002).

Many fermentor cultures processes have been developed for expression of foreign proteins in *P. pastoris* under the control of the *aox1* promoter. *P. pastoris* is slow growing yeast when grown on methanol, thus, a high cell density is desired before the process switch to methanol feeding. The suggested carbon substrate for growth phase is glycerol, since it produces a higher growth rate and biomass yield compared with methanol (Chiruvolu et al., 1998; Brinkmann et al., 1990). In the production phase, methanol is added to drive the expression of both the AOX enzyme and foreign protein.

Table 2-2 Standard method for *P. pastoris* fermentation according to Jahic et al. (2002)

Stage	Approximate time (h)	Mode	Substrate	Feed substrate*
1) Growth	0-24	Batch	Glycerol	None
2) Growth	24-27.5	Fed-batch	Glycerol	50% Glycerol
3) Induction	27.5-30	Fed-batch	Methanol	99% Methanol
4) Production	30-…	Fed-batch	Methanol	99% Methanol

* All feed solution contained 12 ml L^{-1} of PTM1 trace salts.

A high efficiency *P. pastoris* fermentation protocol has been developed by Jahic et al. (2002). This protocol contains two stages for cell growth and two stages for protein production, as shown in Table 2-2. Each stage has been designed for different objective. First is the glycerol batch phase which is used to increase cell

density. Second is the glycerol fed batch phase which provides maximum cell density and derepression of the *aox1* promoter (Chiruvolu et al., 1997). Third is the methanol induction phase in which methanol is introduced at a low concentration into the fermentor. During this phase, the *P. pastoris* is adapted to use methanol as carbon substrate. Last, the protein production phas, is the phase where the methanol feed rate is increased to increase the expression of the *aox1* promoters. The methanol feed control of this phase is very important. It can lead to success or failure. The methanol flow rates must be controlled to provide a methanol concentration that is just enough for protein synthesis but not in excess, which can cause toxicity (Cino, 1999).

2.3 Process control during the protein production phase

The process control during the protein production phase is the most important variable in the *P. pastoris* processes. The methanol feeding strategie which related to the methanol concentration needs to be adjusted to avoid methanol accumulation since methanol is very high toxic to the cells especially at high concentrations (Ellis et al., 1985). To avoid methanol toxicity, the methanol must be maintained at low concentration (Jahic et al., 2002).

2.3.1 Methanol limited fed-batch (MLFB) process

The MLFB process is a standard process described by Jahic et al. (2002). The methanol feed is controlled as proportional integral differential (PID) indirect

feedback regulation by using a DOT signal to avoid oxygen limitation. The pump speed is increased when the DOT is higher than the set point and decreased when the DOT is equal to or lower than the set point. The methanol feed control in the MLFB process is illustrated in Fig. 2-2. A similar strategy as MLFB is called dissolved oxygen tension stat (DOT-stat) by other authors (Lim et al., 2003; Lee et al., 2003; Chung, 2000).

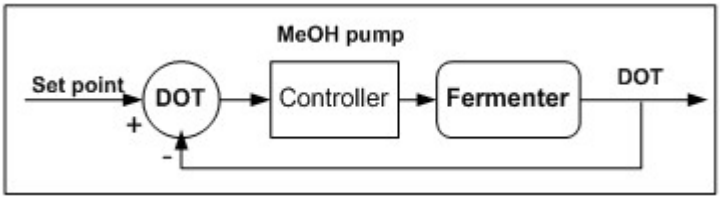

Figure 2-2 Methanol feed regulation by DOT signal in the MLFB process.

2.3.2 Methanol-stat process

Although the DOT-stat has provided high process efficiency in several protein production systems (Lim et al., 2003; Lee et al., 2003; Jahic et al.,2002; Chung, 2000), higher efficiency has been reported when the methanol concentration was controlled by methanol-stat, which is based on online monitoring of methanol concentration (Minning et al., 2001). The methanol feed is controlled as PID feed-back regulation by using a methanol signal to maintain the methanol concentration at the set point. The methanol feed rate is increased when the methanol concentration is lower than the set point and decreased when the methanol concentration is equal to or higher than the set point Fig. 2-3.

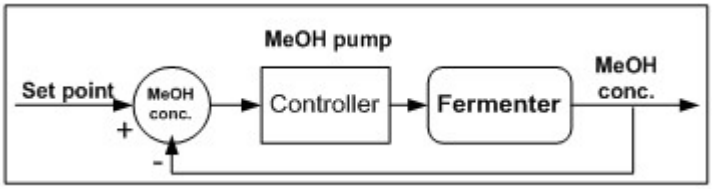

Figure 2-3 Methanol feed regulation by the methanol signal for methanol-stat process

In many publications used this strategy to control methanol feed (Damas-ceno et al., 2004; Ramon et al., 2004; Minning et al., 2001), but none has reported about the DOT during the cultivation. In this thesis, the methanol-stat was applied as the process control for production of the β-glucosidase. High cell density cultivation with the presence of methanol residue resulted in a decrease of the DOT to zero. The alternative name, the oxygen limited fed-batch (OLFB) process was used for the methanol-stat control in oxygen limited cultivation.

2.3.3 Temperature limited fed-batch (TLFB) process

The TLFB process for *P. pastoris* fermentation was designed by Jahic et al. (2003b). It is an alternative fed-batch technique which controls both the methanol concentration and DOT at the same time. The methanol concentration was controlled to be constant at about 0.5 g L^{-1} with PID direct feedback regulation as in the methanol-stat process (Fig. 2-3). The temperature was decreased to reduce not only methanol metabolism, but also the oxygen uptake rate (Fig. 2-4). Therefore, the DOT was unlimited even at high cell density.

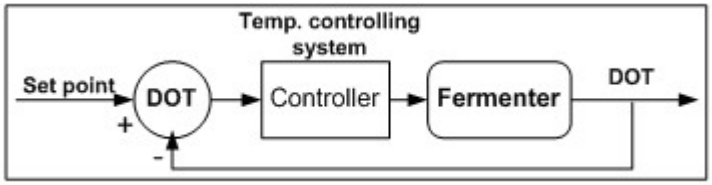

Figure 2-4 DOT regulation by temperature for TLFB process

To avoid DOT limitation in the TLFB process, the rate of cooling water circulation is increased when the DOT is lower than the set point to decrease temperature and decreased to increase temperature when the DOT is equal to or higher than the set point. The heater may also be switch on if DOT is lower than the set point (Fig. 2-4).

2.4 Parameters that affect *P. pastoris* protein expression process

They are many parameters that affect the efficiency of the recombinant protein production by *P. pastoris* process, for example, the methanol concentration, methanol uptake, DOT, oxygen transfer rate, cultivation temperature and cultivation pH. However, in this thesis, the fermentation processes were developed based on the methanol concentration, methanol uptake, DOT and oxygen transfer, so these are the most emphasized parts.

2.4.1 Influence of methanol concentration

Normally, general microorganisms are sensitive to methanol. The methylotrophic yeast *P. pastoris* is more methanol tolerant, and methanol below the

critical concentration (about 3-5 g L^{-1}) does not inhibit growth, which follows Monod's type kinetic. However, methanol above the critical concentration exhibited substrate inhibition, which followed an uncompetitive inhibition model (Kupcsulik and Sevella, 2004; Kobayashi et al., 2000; Zhang et al., 2000; Katagura et al., 1998). Increasing the methanol concentration resulted in increasing the expression level up to the optimum methanol concentration, then it showed an inhibition effect with a decrease in expression level (Mayson et al., 2003; Katakura et al., 1998).

The optimum methanol concentration for cell growth optimization and heterologous protein production varies depending on the methanol utilization phenotype (Mut^+, Mut^s and Mut^-), host strain (wild type or mutant) and type of protein. However, it has been observed that the optimum methanol concentration for cell growth differed significantly from the optimum methanol concentration for production (Mayson et al., 2003) and the specific product formation rate (q_p) does not correlate to the specific growth rate (μ) (Kupcsulik and Sevella, 2004).

The relationship between the specific methanol consumption rate (q_s) and the specific growth rate (μ) was linear, with an increase in μ in proportion to q_s (Zhang et al., 2000). Furthermore, the oxygen uptake rate (OUR) also increased with a resulting decrease in dissolved oxygen tension (DOT) (Jahic et al., 2002). During the methanol-inhibited growth (methanol concentration higher than 5 g L^{-1}), it has been observed that biomass yield ($Y_{x/s}$) decreased and the maintenance coefficient (q_m) increased. In the methanol-limited condition, the $Y_{x/s}$ is higher, while the q_m is lower, compared to those in methanol-inhibited growth (Zhang et al., 2000).

It could be summarized that the effect of methanol concentration on *P. pastoris* culture is that excess methanol inhibits growth of the host cells, while an insufficient amount of energy source and/or methanol starvation leads to poor growth and production. Although the specific growth rate and methanol consumption rate decreased with an increase of methanol concentration above the growth inhibiting threshold (3-5 g L^{-1}), the specific production rates increased, indicating that the energy for the production competed with that for cell growth (Kupcsulik and Sevella, 2004; Mayson et al., 2003; Kobayashi et al., 2000; Zhang et al., 2000; Katakura et al., 1998).

In some cases, a limiting methanol supply does not affect the product concentration (Trinh et al., 2003). However, in most cases when methanol is limited, cells die and release proteases into the medium, resulting in a decline in the product yield and quality (Curvers et al., 2001). At higher methanol concentrations, the volumetric product yield can be higher (Guarna et al., 1997), the production rate faster (Katagura et al., 1998), and degradation rate slower (Zhou and Zhang, 2002).

An alternative observation was seen in recombinant laccase expression. Fivefold higher volumetric laccase activity was obtained when the methanol concentration was maintained at 0.5% instead of 1.0%. This effect may be attributed to lower laccase stability, higher proteolytic activity and folding problems due to the higher growth rate in 1.0% methanol. Only 1% of methanol can denature 10% of laccase in 24 h (Hong et al., 2002).

2.4.2 Influence of dissolved oxygen tension and oxygen uptake rate

In a stirred tank bioreactor, dissolved oxygen tension (DOT) can be controlled by manipulating several parameters: the air flow rate, the oxygen concentration in the incoming air, the total air pressure inside the reactor, or the stirring speed. However, *P. pastoris* high cell density cultures with high methanol concentration cannot be run without oxygen limitation. The temperature limited fed-batch process was (low temperature cultivation,) designed to overcome the oxygen limitation in high methanol concentration and high cell density (Jahic et al., 2002).

The high oxygen demand of methanol metabolism makes oxygen supply a major control parameter in *P. pastoris* culture. In cyclic fed-batch cultivation of human serum albumin, the specific product formation depends linearly on the DOT in quasi steady states with no product accumulation at DOT below 15% saturation (Bushell et al., 2003). In consideration of the methanol metabolism pathways in *P. pastoris*, oxygen limitation is generally supposed to negatively affect the foreign gene expression and cell growth (Lin Cereghino and Cregg, 2000). It is considered useful to supplement the air with oxygen gas (Jahic et al., 2002), reduce the oxygen uptake rate (OUR) by maintaining lower cell densities in continuous culture (Lee et al., 2003); decreasing methanol feed rate, or decreasing the cultivation temperature (Jahic et al., 2003b). The product yield is increased at higher DOT set points (Lee et al., 2003) and with a dosage of pure oxygen supplied (Jahic et al., 2002).

Although many reports have presented the influence of DOT in *P. pastoris* culture, only a few papers have report the effect of OUR. Jahic et al. (2002) reported the results of two methanol limited fed-batch (MLFB) processes (DOT-stat with 30% DOT set point), with and without pure oxygen supply. In the oxygen-enriched culture, the methanol feed rate was about 2 times higher than standard culture, because the OUR was about 2 times higher. This resulted in higher maximum cell density and recombinant protein product concentration.

2.4.3 Influence of other parameters

Cultivation pH: *P. pastoris* is able to grow in a wide range of pH (3-7) with minimal effect on growth rate. However, this variable may affect recombinant protein stability and protease activity (Jahic et al., 2003a; Chiruvolue et al., 1998; Sreekrishna et al., 1997). pH was the most significant factor affecting production of recombinant anticoagulant peptide (Inan et al., 1999). The optimal pH for human serum albumin production was 6.0 (Kobayashi et al., 2000), but pH 3.0 was optimal for insulin-like growth factor-I production (Brierley et al., 1994). Decreasing the cultivation pH from 6.0 to 4.0 in defensin 1 (rPsd1) production can enhance its productivity (Larentis et al., 2004). In the same way, decreasing the fermentation pH from 5.0 to 4.0 resulted in an increase of the fraction of full-length CBM-CALB fusion protein produce from 40% to 90% (Jahic et al., 2003a), and also reduced the proteolysis problem in xyloglucan endotransglycosylase production (Jahic et al., 2003). In recombinant merozoite surface protein 3 (MSP3) production at a

cultivation pH of 6.8, 484 mg L^{-1} protein product was obtained, but it could not be detected when the cultivation pH was lower 5.0 (Wang et al., 2005).

Cultivation temperature: The production of functional recombinant protein depends on the temperature. Cultivation temperature can influence degradation of recombinant proteins. Protein misfolding often occurs at elevated temperatures either due to direct heat damage or to an increase in the recombinant protein synthesis rate, which leads to a limitation in folding dynamics. This may render a protein susceptible to proteolysis. In this case, lowering the temperature may prevent proteolysis (Hong et al., 2002; Li et al., 2001). However, recombinant proteins are alien proteins for the host cells, so they might be recognized as targets for proteases even in their correctly folded form. In this case, lower cultivation temperature can influence yields of recombinant proteins just for pure thermodynamic reasons (Jahic et al., 2003a). About 16-fold higher laccase activity was found when the cultivation temperature was controlled at 19^{o}C instead of 28^{o}C (Cassland and Jönsson, 1999). In CBM-CALB lipase production by a methanol limited fed-batch (MLFB) process at 30^{o}C, about 1 g L^{-1} of product, which was considerably degraded by protease(s), was observed. However, in the temperature limited fed-batch (TLFB) process, which controlled the cultivation temperature at about 12^{o}C, the yield of full-length protein was increased to 2 g L^{-1}. Furthermore, cell death, observed by flowcytometry, was lower in TLFB compared with MLFB (Jahic et al., 2003a; Jahic et al., 2003b).

Proteolysis: Proteolytic degradation of recombinant proteins during its production is a severe problem. However, *P. pastoris* extracellular proteases have not

been documented (Ogrydziak, 1993), and very low amounts of endogenous protein is secreted (Lin Cereghino et al., 2001). Thus, the proteolysis of secreted recombinant proteins can be affected by cell bound proteases (Kang et al., 2000) and/or intracellular proteases from lysed cells. Several techniques have been applied to control the proteolysis of protein product. Protease-deficient strains, such as SMD1168 and KM71, have been successfully used to increase expression levels of several recombinant proteins (Boehm et al., 1999; Brankamp et al., 1995). Modification of the amino acid sequence in the protein product is one possible strategy to control the proteolysis by avoiding the amino acid sequences recognized by the proteases (Gustavsson et al., 2001). The control of an ammonium ions (NH_4^+) in the culture broth at optimum concentration improved both hurudin productivity and quality (less hudurin degradation) (Yang et al., 2004). Addition of amino acid rich supplements, such as peptone or casamino acids, to the culture medium has been show to reduce product degradation (Clare et al., 1991a). The yield of the anticoagulant-antimetastatic protein ghilanten and merozoite surface protein 3 (MSP3) were improved by supplementation of the media peptone and casamino acids (Wang et al., 2005; Brankamp et al., 1995). However, the addition of a rich medium to compete as substrate for protease had no effect on the proteolysis of CBM-CALB fusion protein (Jahic et al., 2003a).

2.5 Kinetic models of fermentation processes

Mathematical models can be used to describe and understand the performance of biochemical reactors. Modeling is used to investigate the key parameters by

sensitivity analysis. For instance, the effect of recirculation of biomass or the effect of flow rate on the outlet concentration of substrate components in continuous processes may be visualized. Thus, modeling is a tool for optimization of processes. It is also important as a teaching aid to demonstrate the performance of bioreactors. Furthermore, mathematical models are important in development of advanced control strategies (Enfors and Häggström, 2000).

2.5.1 General mass balance of the reactor

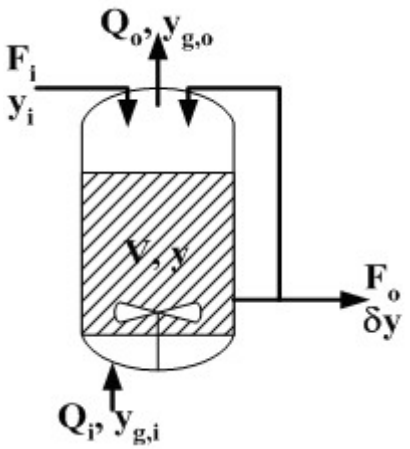

Figure 2-5 A perfectly mixed reactor with medium volume (V) and the concentration (y) of a component in the reactor. Medium flow rates are designated as F and gas flow rates as Q. Concentration in gas phase is labeled with sub-script *g*. The subscripts *i* and *o* refer to parameters of inlet and outlet flows, respectively. A separation factor (δ) specifies the concentration of y in the outlet medium.

The schematic diagram of a homogeneous reactor with a liquid volume, V (L) is shown in Fig. 2-5. The concentration of a dissolved compound is expressed as y (g L^{-1}). This state variable will later on be replaced by concentration of biomass, X (g L^{-1}), substrate, S (g L^{-1}), product, P (g L^{-1}) and DOT (%). The liquid flows are designated as F (L h^{-1}) and gas flow rates are designated as Q (L h^{-1}). The subscripts *i* and *o* refer the parameter to inlet and outlet flow, respectively.

A mass balance (g L^{-1}) of a component with concentration y in the reactor can be written as:

Change = Inlet - Outlet + Reaction (g h^{-1})

$$\frac{d(Vy)}{dt} = (F_i y_i + Q_i y_{gi}) - (F_o \delta y + Q_o y_{go}) + V r_y \qquad (2\text{-}1)$$

where r_y is the volumetric reaction rate (g L^{-1} h^{-1}) for production ($r_y > 0$) or consumption of the component ($r_y < 0$) with concentration y (g L^{-1}). It is assumed that the component is not transported via the gas phase. However, if the component is transported via the gas such as oxygen, the mass balance equation must be complemented with inlet/outlet terms of component transport via the gas flow.

Assuming that V and y are independent variables, differentiation of the left hand side of *eq. 2-1* gives:

$$\frac{d(Vy)}{dt} = V\left(\frac{dy}{dt}\right) + y\left(\frac{dV}{dt}\right) \qquad (2\text{-}2)$$

The rate of volume change, dV/dt is defined as:

$$\frac{dV}{dt} = F_i - F_o \qquad (2\text{-}3)$$

Inserting the *eq. 2-2* and *2-3* into *eq. 2-1* gives a general mass balance equation that describes the change of y with time:

$$\frac{dy}{dt} = \frac{F_i}{V}(y_i - y) + \frac{F_o}{V}(y - \delta y) + r_y + \frac{Q_i}{V} y_{g,i} - \frac{Q_o}{V} y_{g,o} \qquad (2\text{-}4)$$

Note that the sign of term $F_o/V(y-\delta y)$ is positive. The mode of process operation is determined by setting the values of F_i, F_o, δ and Q_i. The expressions for the biological reaction rate (r_y) and for the gas-liquid mass transfer rate, $(Q_i y_{g,i} - Q_o y_{g,o})/V$, must be inserted in the mass balance equation.

2.5.2 The mass balance equation for biomass and substrate

In fed-batch operation, the culture media is not taken during the process, thus the term of F_o disappears from the mass balance equation (*eq. 2-4*). Then, insertion of the state variables (X and S) into *eq. 2-4* and replacing the reaction rate term yields:

For cells,

$$\frac{dX}{dt} = \frac{F_i}{V}(-X) + \mu X \qquad (g\ L^{-1}\ h^{-1}) \qquad (2\text{-}5)$$

For substrate (glycerol, methanol),

$$\frac{dS}{dt} = \frac{F_i}{V}(S_i - S) - q_s X \qquad (g\ L^{-1}\ h^{-1}) \qquad (2\text{-}6)$$

where μ (h^{-1}) is the specific growth rate and q_s (g g h^{-1}) is the specific substrate consumption rate.

The mass balance equation for oxygen and carbon dioxide can also be written:

For oxygen,

$$OUR = q_o XV \qquad (g\ h^{-1} \text{ or mol } h^{-1}) \qquad (2\text{-}7)$$

For carbon dioxide,

$$CPR = q_c XV \qquad (g\ h^{-1} \text{ or mol } h^{-1}) \qquad (2\text{-}8)$$

where OUR (g h^{-1} or mol h^{-1}) is the oxygen uptake rate, q_o (g g^{-1} h^{-1}) is the specific oxygen uptake rate, CPR (g h^{-1} or mol h^{-1}) is the carbon dioxide production rate and q_c (g g^{-1} h^{-1}) is the specific carbon dioxide production rate.

The respiratory quotient (RQ) is the ratio between molar production of carbon dioxide and molar consumption of oxygen.

$$RQ = \frac{CPR}{OUR} \qquad (\text{mol mol}^{-1}) \qquad (2\text{-}9)$$

2.5.3 Partitioning of anabolism and energy metabolism in *P. pastoris*

Models of growth, substrate consumption, oxygen consumption and carbon dioxide production are based on the metabolic flux of substrate carbon and the associated consumption of molecular oxygen for oxidation. In yeasts growing on methanol, the oxygen molecule is not only required for respiration but also for initial oxidation of methanol to formaldehyde. Formaldehyde can be separated into two fluxes corresponding to consumption for incorporation of the elements C, O and H into biomass, *i.e.* anabolism ($q_{s,an}$) and combustion for energy production ($q_{s,en}$). The latter can be further split into a flux used for maintenance (q_m) and another used for growth ($q_{s,en,growth}$) (Fig. 2-6).

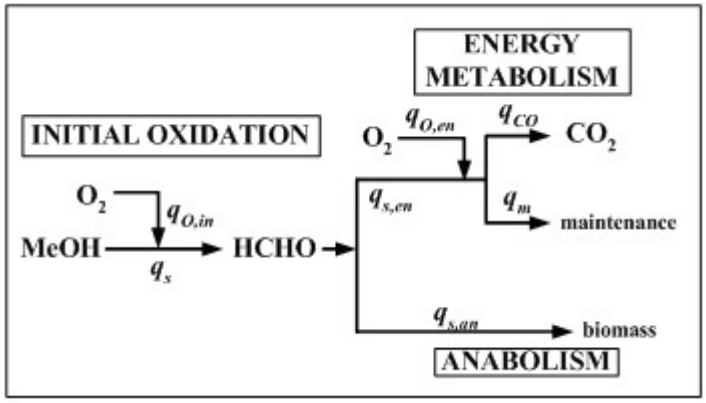

Figure 2-6 Partitioning of carbon flux for *P. pastoris* growth on methanol (Adapted from Jahic et al., 2002)

From Fig. 2-6 the methanol metabolism by *P. pastoris* can be separated into three parts corresponding to initial oxidation, anabolism and energy metabolism. The methanol consumption rate (q_s) can be expressed as:

$$q_s = q_{s,an} + q_{s,en,growth} + q_m \qquad (g\ g^{-1}\ L^{-1}) \qquad (2\text{-}10)$$

Initial oxidation: All methanol molecules need to enter the initial oxidation step. They are oxidized to formaldehyde by a coupled reaction of two enzymes, alcohol oxidase (AOX) and catalase (CAT) in the peroxisome (Fig. 2-1). These reactions use molecular oxygen as the electron acceptor and give the net stoicheometry:

$$\mathbf{CH_3OH + 0.5O_2 \longrightarrow HCOH + H_2O} \qquad (2a)$$

which gives the stoicheometric conversion coefficient in the initial oxidation, $Y_{o/s,in}$ = 0.5 (mol mol^{-1}).

At a low methanol concentration or methanol limitation, q_s can be assumed to follow the Monod's model (*eq. 2-11*) that is often used to explain the kinetics in systems without substrate inhibition.

$$q_s = q_{s\max}\frac{S}{S+K_s} \qquad (g\ g^{-1}\ h^{-1}) \qquad (2\text{-}11)$$

The specific oxygen uptake rate for the initial oxidation ($q_{o,in}$) can also be followed:

$$q_{o,in} = q_s Y_{o/s,in} \qquad (g\ g^{-1}\ h^{-1}) \qquad (2\text{-}12)$$

The formaldehyde flux is then partitioned into anabolism and energy metabolism.

Anabolism: In anabolism the oxygen demand is considered insignificant and methanol has already oxidized to formaldehyde in the initial oxidation. So the oxygen uptake per methanol consumption is close to zero, $Y_{o/s,an} \approx 0$ (mol mol^{-1}).

The partitioning of methanol (in the form of formaldehyde) to anabolism can be obtained from two carbon mass balance equations, if the carbon content in cells (C_x, g g^{-1}), the carbon content in the substrate (C_s, g g^{-1}) and Y_{em} are known:

$$\text{Flux of carbon in to anabolism} = C_s q_{s,an} \qquad (\text{g g}^{-1}\ \text{h}^{-1}) \qquad (2\text{-}13)$$

$$\text{Flux of carbon converted to biomass} = C_X\left(q_s - q_m\right)Y_{em} \qquad (\text{g g}^{-1}\ \text{h}^{-1}) \qquad (2\text{-}14)$$

These fluxes are equal (*eq. 2-13 = eq. 2-14*), the anabolic flux can be obtained as:

$$q_{s,an} = \left(q_s - q_m\right)Y_{em}\frac{C_X}{C_S} \qquad (\text{g g}^{-1}\ \text{h}^{-1}) \qquad (2\text{-}15)$$

Energy metabolism: The remaining part of the methanol flux is used for energy metabolism in the form of formaldehyde. In this step formaldehyde is oxidized by NAD^+ to carbon dioxide and water with molecular oxygen acting as the electron accepter. The net stoicheometry of this reaction is

$$\mathbf{HCHO + O_2 \longrightarrow CO_2 + H_2O} \qquad (2b)$$

which gives stoicheometric conversion coefficient of oxygen in energy metabolism $Y_{o/s,en}$ = 1 (mol mol^{-1}).

The methanol flux (in the form of formaldehyde) to energy metabolism is

$$q_{s,en} = \left(q_s - q_{s,an}\right) \qquad (g\ g^{-1}\ h^{-1}) \qquad (2\text{-}16)$$

The specific oxygen consumption rate for energy metabolism, $q_{o,en}$ (g g^{-1} h^{-1}) is as follows:

$$q_{o,en} = q_{s,en} Y_{o,en} \qquad (g\ g^{-1}\ h^{-1}) \qquad (2\text{-}17)$$

The specific oxygen consumption rate of the whole process, q_o (g g^{-1} h^{-1}) can be calculated from $q_{o,in}$ and $q_{o,en}$, which correspond to stoicheometric conversion coefficients:

$$q_o = q_{o,in} + q_{o,en} = q_s Y_{o,in} + q_{s,en} Y_{o,en} \qquad (g\ g^{-1}\ h^{-1}) \qquad (2\text{-}18)$$

The specific carbon dioxide production rate, q_{co} (g g^{-1} h^{-1}) can be obtained:

$$q_{CO} = q_{s,en} Y_{CO/s,en} = q_s Y_{CO/s} \qquad (g\ g^{-1}\ h^{-1}) \qquad (2\text{-}19)$$

where $Y_{CO/s,en}$ is the yield coefficient of carbon dioxide from methanol in energy metabo-lism and $Y_{CO/s}$ is the yield coefficient of carbon dioxide from methanol in all processes.

The specific growth rate is obtained from the yield coefficient exclusive maintenance (Y_{em}) and the difference between the specific methanol consumption rate (q_s) and maintenance (q_m):

$$\mu = (q_s - q_m)Y_{em} \qquad (h^{-1}) \qquad (2\text{-}20)$$

2.6 Oxygen transfer and oxygen demand

The oxygen demand of a fermentation process is normally satisfied by aerating and agitating. It can be expressed as

$$r_o = Xq_o = X\frac{\mu}{Y_{x/o}} \qquad (g\ L^{-1}\ h^{-1} \text{ or } mol\ L^{-1}\ h^{-1}) \qquad (2\text{-}21)$$

However, oxygen availability is often limited, which affects the efficiency of the process in microbial cells. The oxygen requirement is mostly depend on the carbon source. A high degree of reduction of the carbon results in a high oxygen requirement for oxidation (Darlington 1964; Johnson 1964). From the stoichiometric analysis of respiration on glucose and methanol, the oxygen requirement for methanol (1.5 $g_{O2}\ g_{meth}^{-1}$) is higher than for glucose (1.07 $g_{O2}\ g_{glu}^{-1}$). In high cell density fed-batch culture of *P. pastoris*, very high oxygen demand is generated. Furthermore, when it grows on methanol, the oxygen molecule is used for both initial oxidation and respiration (Fig. 2-1 and Fig. 2-6). These factors make the oxygen transfer a major parameter which affects the process efficiency.

A common model showing parameters that influence the OTR is shown in *eq. 2-22*

$$OTR = K_L a(C^* - C) \qquad (g\ L^{-1}\ h^{-1} \text{ or } mol\ L^{-1}\ h^{-1}) \qquad (2\text{-}22)$$

In *eq. 2-22*, OTR is shown to be determined by three parameters: *i.* the oxygen transfer coefficient (K_L), *ii.* the specific air bubble area per volume (*a*) and *iii.* driving force (C*-C). Since *a* is difficult to measure, the two terms, K_L and *a* are combined in the $K_L a$. $K_L a$ is called the volumetric oxygen transfer coefficient, which can be used to determine the aeration capacity of a fermentor. The $K_L a$ value depends on the design and operation conditions of fermentor, which is affected by many variables such as agitation rate, aeration rate, and impeller design. These variables affect K_L by determining the resistances to transfer, and affect *a* by changing the number, size and residence time of air bubbles. To increase OTR in *P. pastoris* processes beyond what is achieved by just increasing the aeration rate and the stirrer speed, which increases the volumetric oxygen transfer coefficient ($K_L a$), several strategies have been used to increase the driving force (C*-C).

The C*-C can easily be increased by decreasing of the oxygen concentration in the liquid outside the film (C). However, this cannot be applied for processes with facultatively anaerobic organisms like *Escherichia coli* and *Saccharomyces cerevisiae*, which generally switch to fermentative metabolism and accumulate toxic metabolites such as alcohol, acid and endotoxin under oxygen limiting conditions.

Therefore, such organisms are mostly cultivated at 20-30% air saturation. However, documentation of the *P. pastoris* response to oxygen limitation when growing on methanol has not been studied extensively been described.

The oxygen solubility in equilibrium with the gas phase (C*) can be increased either by introducing higher oxygen concentration in the inlet air or by increasing the total pressure (P_{tot}) in the bioreactor. The addition of pure oxygen in the inlet air flow is effective on a small scale. It increased the methanol feed rate and the produc-tivity (Trentmann et al., 2004; Jahic et al., 2002). However, on a large scale the supply and handling of pure oxygen can be very costly and unsafe. Alternatively, an oxygen enriching polysulphone membrane can be used for separation of regular air into nitrogen-enriched retentate and oxygen-enriched permeate (Ettouney et al., 1998). An oxygen-enriched (35-38% oxygen partial pressure) stream obtained with a polysulphone membrane has been used to increase the DOT, which enhanced production of elastase inhibiting peptide by recombinant *P. pastoris* (Lee et al., 2003). Increase of the total air pressure, P_{tot}, has been applied to increase C* in fermentation of other yeast (Pinheiro et al., 2003; Pinheiro et al., 2000), but not in *P. pastoris*.

However, all these methods may have biological side-effects that have to be considered. The use of oxygen limitation may theoretically result in elevated formal-dehyde concentrations in the cell, if it generates an imbalance between the initial oxidation of methanol, resulting in formaldehyde, and the formaldehyde consuming reactions in anabolism and catabolism. High air pressure increases not only the

partial pressure of oxygen, but also that of carbon dioxide. The metabolism of most organisms also yields carbon dioxide. The increased carbon dioxide partial pressure has been reported to result in decreased pH (Thiering et al., 2001). Furthermore, increasing the oxygen equilibrium concentration C* by high pressure may result in toxic side-effects of molecular oxygen. In the aerobic process, during the reduction of molecular oxygen to water through acceptance of four electrons, reactive oxygen species (ROS), such as the superoxide anion radical ($O_2^{\bullet -}$), H_2O_2 and hydroxyl radicals ($HO^{\bullet}$), are generated (Izawa et al., 1995). The initial oxidation of methanol by methylotrophic yeast is another reaction which generates H_2O_2 (Tani et al., 1972). These ROS attack almost all cell components, DNA, proteins and lipid membranes (Izawa et al., 1995; Toyokuni et al., 1995). Hence, the effects of ROS must be observed when a new *Pichia* process is developed. Therefore, in this research, flowcytometry was used to detect cell viability, which could be adversely affected by production of ROS.

2.7 β-glucosidase

β-glucosidases (β-glucoside glucohydrolases; EC 3.2.1.21) are members of a widely distributed group of O-glycosyl hydrolases (EC 3.2.1.-). β-Glucosidases hydrolyse β-glucosidic bonds at the non-reducing terminal between D-glucose residues or between a D-glucose and a non-carbohydrate moiety (i.e. aglycone) (Reese, 1977). They are found in microorganisms, plants and animals (Woodward and Wiseman, 1982). These enzymes have a variety of roles, which are different in

each organism. The role of each enzyme must be compatible with the physiological function and location of the enzyme.

The basidiomycetes, *Phanerochaete chrysosporium*, produces and secrete β-glucosidase to degrade cellobiose to glucose and use it as carbon substrate (Yoshida et al., 2004). One of the functions of *Saccharomyces cerevisiae* β-glucosidase located at the cell surface is presumably to enable the yeast to utilize glucose as a carbon and energy source (Rath and Srinivasan, 1978).

Several human β-glucosidase isozymes have been found, and the role and physiological function have been described (Berrin et al., 2002). For example, the acid β-glucosidase which is located in the lysosomal membranes hydrolyzes glucosphingolipids (Neufeld, 1991). Lactase-phlorizin hydrolase in the brush-border of the small intestine, hydrolyzes lactose (Hollox et al., 2001).

In plants, several β-glucosidases have been classified, mainly based on their specificity towards the substrates. Some are involved in oligosaccharide degradation of cell wall β-glucans (Wood and Baht, 1988). Others have been related to the physiological functions of the aglycone moiety of their natural substrates. The cyanogenic glucosides are natural substrates which are involved in a defense mechanism against plant pathogens and herbivores (Poulton, 1990). In the infected cyanogenic plants the cyanogenic β-glucosidases hydrolyze cyanogenic glucosides which resulted in the release of the toxin hydrogen cyanide. This biological response have been found in variety of species, for example cassava (*Manihot esculenta* Crantz) (Hughes et al., 1992) and sorghum (*Sorghem bicolor* L. Moench) (Cicek and

Esen, 1998). Most phytohormones are produced as inactive forms of phytohormone glycosides. The hydrolysis reaction of these compounds by β-glucosidase has been shown to activate the activity of abcissic acid, auxins, cytokinins, and gibberellins (Falk and Rask, 1995; Brzobohaty et al., 1993; Schliemann, 1984). β-Glucosidases are also involved in the release of plant flavors. Ordourless glycone conjugated aromatic compounds, such as mono- or diglycosides of fruits flavors, require hydrolysis for release the flavor form (Winterhalter and Skouroumounis, 1997; Vasserot et al., 1995).

2.7.1 β-glucosidase applications

β-glucosidases are widely used in biotechnological processes (Wood and Baht, 1988). Cellulose containing material, for example wood shavings and paper waste, can be used as a renewable resource by hydrolysis to sugar. β-Glucosidases are one of the hydrolytic enzymes involved in cellulose hydrolysis. Cellulose hydrolysates have been directly used as sugar products (Woodwards and Wiseman, 1982) and also used as raw materials for other industries such as production of ethanol-based fuels (Bothast and Saha, 1997).

The β-glucosidase function of releasing flavor compounds from flavorless substrates has been applied to improve the taste of many products. In muscadine grape juice, the enzymatic hydrolysis of glycoside aroma compounds by β-glucosidase leads to release of fragrant flavor (Baek and Cadwallader, 1999). For wine processes, a free volatile form of grape juice aroma arises during fermentation

and aging. This aromatic quality can be rapidly improved by the use of immobilized β-glucosidase for hydrolysis of an odorless compound in wine (Gueguen et al., 1997). Furthermore, β-glucosidases have also been reported to solve the problem of the bitterness taste in citrus fruit which results from the glycosidic compound, naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside). α-Rhamnosidase and β-glucosidase is required to hydrolyze this compound to the non-bitter aglycone, naringinin (4',5,7-trihydroxyflavanone) (Roitner et al., 1984).

Bio-surfactants are of interest for both basic research and application. Alkyl-glucosides are a group of nonionic bio-surfactants. They are non-toxic, not-irritant and have also an antimicrobial activity (Mutsumura at al., 1990). The hydrophilicity of the sugar unit gives the alkyl-glucosides molecule, the surface-active properties (Folmer, 2003). Some of them have already been used for deter-gents, foods and pharmaceuticals (Hughes et al., 1970). Several techniques have been applied for synthesis of alkyl-glucosides: *a)* esterification of fatty acid and glucose by lipases (Gao et al., 1999; Otto et al., 1998) and *b)* condensation and transglycosylation of alcohol and glucose by β-glucosidases (Ducret et al., 2002; Panintrarux et al., 1995). In the process of screening thirteen various glucosidases for the synthesis of octyl glycoside surfactant by Ducret et al. (2002), the maximum yield of octyl glucoside from each glycosidase varied from 0-51%. This might be related to substrate specificity due to the hydrophobicity and hydrophilicity of the substrate binding site each β-glucosidase.

2.7.2 Thai Rosewood β-glucosidase

The seeds of 50 Thai plants were screened for glycosyl hydrolases, with the objective to use them for oligosaccharide and glycoside synthesis (Surarit et al., 1995). *Dalbergia cochinchinensis* Pierre (Thai Rosewood) was found to contain the highest β-glucosidase and β-fucosidase activity. After purification and characterization, both activities were shown to be present in the same protein. The native form of this protein is composed of four subunits with a molecular mass of approximately 330 kD, and the molecular mass of each subunit determined by SDS-PAGE is about 66 kD (Srisomsap et al., 1996; Surarit et al., 1996).

In 2000, Ketudat-Cairns et al. cloned the cDNA encoding Thai Rosewood β-glucosidase and concluded that it is a member of glycosyl hydrolase family 1. The expression of this recombinant enzyme was first performed in *E. coli*, but only insoluble, nonfunctional protein was produced. However, the active protein has been successfully expressed in *P. pastoris* (Ketudat-Cairns et al., 2000). Charoenrat et al. (2004a) scaled up the production process of this recombinant enzyme from shake flask to two-liter fermentor culture. The effect of pH on this enzyme production by *P. pastoris* was studied, and it was found that pH 5.0-5.25 was the optimal pH. The same group of researchers also studied the effect of temperature during the protein production phase. The results show that both productivity and yield of β-glucosidase activity from the cultivation at 30^{o}C was higher than that obtained at 18^{o}C (Charoenrat et al., 2004b). However, further work is needed to be able to explain the higher productivity at 30^{o}C.

CHAPTER III

MATERIALS AND METHODS

3.1 Organism

The methylotrophic yeast *Pichia pastoris* strain Y-11430 is a wild-type (Mut^+ and His^+) strain, which was a gracious gift from J. Lin Cereghino (Lin Cereghino and Cregg, 2000). The *β-glucosidase* cDNA from *Dalbergia cochinchinensis* Pierre (Thai Rosewood) (Ketudat-Cairns et.al., 2000) was clone into the pPICZαB *P. pastoris – E. coli* shuttle vector. This vector uses the zeocin resistance gene as a selectable marker, not only in *P. pastoris*, but also in *E. coli* (Invitrogen®). The pPICZαB with the *β-glucosidase* gene was linearized by restriction enzyme (Sac I) digestion at the *aox1* promoter fragment, then integrated into *P. pastoris* Y-11430 at the *aox1* promoter to create a Mut^+ phenotype.

3.2 Inoculum preparation

Primary inoculum was prepared by picking a colony of *P. pastoris* from YPD agar containing 100 μg ml^{-1} zeocin (Appendix I) into 20 ml YPD broth containing 100 μg ml^{-1} zeocin. The culture was incubated at 30°C, with 250 rpm rotary shaking for 24 h.

The secondary inoculum was prepared by transfering the entire 20 ml of primary inoculum into a 500 ml shake flask that contained 80 ml glycerol BMGY medium pH 5.0 (Appendix I). The culture was incubated under the same conditions as the primary inoculum.

3.3 Standard protocol for *P. pastoris* fermentation process

Fed-batch fermentation was carried out in a 10 L fermentor (Belach Bioteknik, Stockholm, Sweden). The fermentor was sterilized *in situ* and the glycerol basal salt (GBS) medium added using a 0.2 μm AcroPak™ 20 Filter (Pall Life Sciences, Ann Arbor, MI) to sterilize this medium. The PTM1 trace salt was sterilized using a 0.2 μm syringe filter (Sartorius, Goettingen, Germany) and added separately. The temperature, pH, pO_2, aeration rate, pressure, pump speed, agitation rate and antifoam level were automatically controlled. All parameters and the signals for fermentor weight, feed reservoir weight, NH_3 reservoir weight, accumulated antifoam pump run time, and outlet gas composition (CO_2, O_2 and MeOH) were monitored and logged. Foaming was automatically control by means of a level electrode. Antifoam A (A-5758, Sigma) was used as antifoam. The fermentation process was divided into four stages described in Table 3-1.

I. Glycerol batch phase: The inoculum (100 ml and $OD_{600} \approx 3$) was transferred into a fermenter containing 3.00 L of GBS medium with 4.35 ml L^{-1} of PTM1 trace salts. The fermentation condition was controlled at 30°C, 6 L min^{-1} aeration, 1.2 bar pressure, 1,000 rpm agitation and pH 5.0. NH_4OH 25% was used as

an alkaline to control the pH. The batch culture was grown until the glycerol was completely consumed, which was indicated by a sharp increase in the dissolve oxygen tension (DOT). The final cell concentration after this phase was over about 24 g L^{-1}.

Table 3-1 Standard protocol for *P. pastoris* fermentation process

Stage	Approximate time (h)	Mode	Substrate	Feed substrate*
1) Growth	0-24	Batch	Glycerol	None
2) Growth	24-27.5	Fed-batch	Glycerol	50% Glycerol
3) Induction	27.5-30	Fed-batch	Methanol	99% Methanol
4) Production	30-…	Fed-batch	Methanol	99% Methanol

* All feed solution contained 12 ml L^{-1} of PTM1 trace salts.

II. Glycerol fed-batch phase (Derepression phase): After the glycerol was completely consumed in the glycerol batch phase, glycerol feed (GF) medium (Appendix I) was added to the fermentor with an exponential feed rate of 0.18 h^{-1} (Appendix III), as suggested by Jahic et al. (2002). The fermentation condition was controlled as in the glycerol batch phase. When the cell density reach 40 g L^{-1} (OD_{600} = 120), which took about 3.0-3.5 h, the process was switched to the next stage. Only the sample at the end of this phase was taken and analyzed for cell growth (OD_{600} and dry cell weight), total protein concentration and β-glucosidase activity.

III. Methanol induction phase: The methanol feed (MF) medium was fed at a very low flow rate to allow enough time for *P. pastoris* to produce the enzymes in the

methanol metabolism pathway. The fermentation condition was controlled as in glycerol batch phase. Samples were analyzed for cell growth (OD_{600} and dry cell weight), total protein concentration and β-glucosidase activity assays and SDS-PAGE were performed.

IV. Protein production phase: The MF medium feed strategies and total air pressure were varied depending on the control techniques, which will be described later. The other fermentation conditions were controlled as in the glycerol batch phase. Samples were analyzed for cell growth (OD_{600} and dry cell weight), total protein concentration, and β-glucosidase activity, and SDS-PAGE was also performed.

3.4 Techniques for recombinant protein production in *P. pastoris*

3.4.1 Methanol limited fed-batch technique

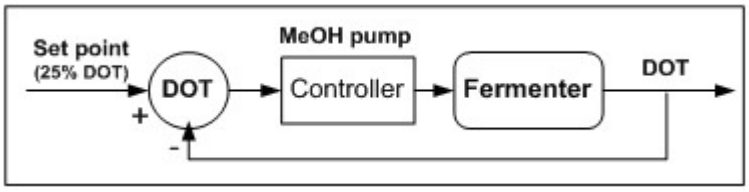

Figure 3-1 Block diagram of DOT regulation for the methanol feed control in the MLFB technique.

The MF medium feed during the protein production phase of the methanol limited fed-batch (MLFB) technique was controlled as indirect feedback regulation of

DOT (Fig 3-1). The DOT was controlled at 25% with a PID cascade controller regulating the pump. In this case, the pump flow rate was increased when the DOT was higher than the set-point and decreased when it was equal to or lower than the set-point. The total air pressure inside the fermentor was controlled at 1.2 bar.

3.4.2 Oxygen limited fed-batch technique

In the oxygen limited fed-batch (OLFB) technique, the MF medium feed was controlled as direct feedback regulation (Fig 3-2). The methanol concentration signal from outlet gas analyzer was applied to keep the methanol concentration in the culture broth at about 350 mg L^{-1} by regulating the feed pump with a PID cascade controller. The total air pressure inside the fermentor of this technique was control at 1.2 bar. The effect of this new MF medium feed strategy was compared with the standard MLFB technique.

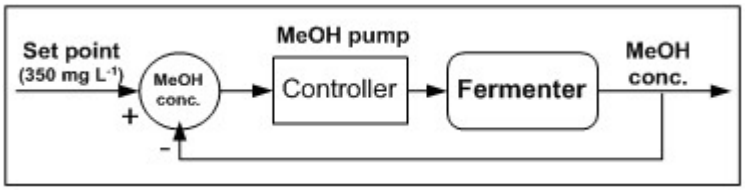

Figure 3-2 Block diagram of methanol-signal regulation for the methanol feed control in the OLFB technique.

3.4.3 High pressure fed-batch technique

Air pressurization of a bioreactor can be applied to increase DOT in culture medium (Enfors and Häggström, 2000). The high pressure fed-batch (HPFB) technique was designed to increase the oxygen transfer rate (OTR) by increasing the total air pressure inside the fermentor. The MF medium feed was controlled as in MLFB technique. The operating pressure was controlled at 1.9 bar with a regulatory valve in the exit gas line. The influence of pressurized air in the fermentor was compared to the standard MLFB technique.

In this work, the methanol limited fed-batch (MLFB) technique (Jahic et al., 2002) was run as a standard technique. The oxygen limited fed-batch (OLFB) technique and high pressure fed-batch (HPFB) technique are the novel techniques performed with the objective to increase the oxygen transfer rate. The performance, advantage and disadvantage of these two new techniques were compared with MLFB.

3.5 Analytical methods

3.5.1 Cell concentration

Cell concentration was determined by optical density at the absorbance of 600 nm (OD_{600}), and cell dry weight (Appendix II).

3.5.2 Cell viability

Cell viability was measured by staining cells with propidium iodide (PI; P-4170 Sigma, St Louis, MO, USA). A Partec PAS flowcytometry (Partec GmbH, Münster, Germany) equipped with a 488 nm argon laser was used. Samples taken from the fermentor were diluted with PBS (0.16 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4 and 0.001 M KH_2PO_4, pH 7.3). For staining, 25 µl of a stock solution containing 200 µg ml^{-1} of PI dissolved in water was added to 975 µl of diluted sample at room temperature. Samples were then analysed, at a data rate of about 1500 counts sec^{-1}. A total count of 50,000 was collected for each sample. The measurement was calibrated by using 3 µm diameter fluorescent beads (Standard 05-4008, Partec GmbH, Germany). PI positive cells were considered as dead and the PI negative cells were considered as viable.

3.5.3 Methanol concentration and outlet gas analysis

The concentrations of oxygen, carbon dioxide and methanol in the outlet air were continuously analyzed using an outlet gas analyzer, Industrial Emissions Monitor Type 1311 (Brüel & Kjær, Innova, Denmark), as described previously (Jahic et al., 2002). The methanol signal was calibrated (at the stirrer speed of 1000 RPM; aeration rate, 6 L min^{-1}; and temperature 30^{o}C in liquid phase, applied in the process) by addition of aliquots of methanol to the fermentor in a 1 g L^{-1} NaCl solution before fermentation (Appendix II).

3.5.4 Protein concentration

The total protein concentration in the supernatant was analyzed according to Bradford (Bradford, 1979). Bovine serum albumin was use as a protein concentration standard. The range of sensitivity was 25-200 μg ml^{-1} (Appendix II).

3.5.5 β-glucosidase activity assay

β-glucosidase activity was assayed by the method of Evans (1985). This method used a spectrophotometric assay to measure the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenol-β-D glucopyranoside by β-glucosidase. One unit of enzyme was defined as the amount of enzyme releasing 1μmol *p*NP per minute at 30^{o}C, pH 5.0 (Appendix II).

3.5.6 Alcohol oxidase activity

The intracellular alcohol oxidase activity was assayed with the method described by Jahic et al. (2002). Alcohol oxidase units were expressed as μmol of methanol oxidized per minute (Appendix II).

3.5.7 SDS-PAGE analysis

The sample, containing 60 μl of supernatant, 25 μl of sample buffer (NuPAGE LDS 4x sample buffer, Invitrogen, CA, USA), 10 μl of 0.5 M

dithiothreitol and 5 μl of 3.5% PMSF in ethanol was incubated for 10 minutes at 95^{o}C. SDS-PAGE was performed on NuPAGE® Novex 4-12% Bis-Tris Gel (1.0 mm x 10 well) (Invitrogen, CA, USA) using MOPS running buffer. Ten microliters of prepared sample was loaded to the well and run at 200 V for 60 minutes. The gel was stained with Coomassie Blue R-250 (Merck, Darmstadt, Germany) for 30 minutes and destained (destain solution: methanol 100 ml l^{-1} and glacial acetic acid 100 ml l^{-1}) for 1-2 hours (Appendix II).

3.5.8 DOT electrode calibration

The calibrations of DOT electrode for all processes were performed under the same conditions. The 100% air saturation was calibrated in 3 liters of glycerol basal salts medium, pH 5.0, at the temperature of 30^{o}C, aeration at 6 vvm, agitation at 1000 rpm and pressure of 1.2 bar. For the zero calibration, the nitrogen gas was fed into the fermentor instead of air.

3.6 Calculations

3.6.1 Calculation of the methanol feed

The signal from a balance on which the feed solution was placed was used for calculation of the experimental methanol feed rate. Since the accumulation in the medium and the evaporation of methanol were insignificant in comparison to the

feed, the integrated value of this feed rate can be assumed as the total methanol consumption.

3.6.2 Calculation of the relative respiration rate and carbon mass balances

The oxygen uptake rate (OUR) (mol h^{-1}) and carbon dioxide production rate (CPR) (mol h^{-1}) were calculated from:

$$CPR = \frac{Q_o CO2_{,o} - Q_i CO2_{,i}}{100 V_m} \quad (3\text{-}1)$$

$$OUR = \frac{Q_i O2_{,i} - Q_o O2_{,o}}{100 V_m} \quad (3\text{-}2)$$

$$Q_o = \frac{Q_i \left(100 - O2_{,i} - CO2_{,i}\right)}{\left(100 - O2_{,o} - CO2_{,o} + H2O_{,o}\right)} \quad (3\text{-}3)$$

where Q is the air flow rate (L h^{-1}), O_2 is the oxygen concentration in air (% v/v), CO_2 is the carbon dioxide concentration in air (% v/v), H_2O is the water concentration in air, and V_m is molar volume of the gas at the analysis temperature (24.04 L mol^{-1}). The subscripts *i* and *o* refer to inlet and outlet gas, respectively.

The respiratory quotient (RQ) is the ratio between the molar production of carbon dioxide and the molar consumption of oxygen that can be calculated from:

$$RQ = \frac{CPR}{OUR} \tag{3-4}$$

The relative rate of respiration (RRR) is a ratio between the observed specific oxygen uptake rate (q_o) (g g^{-1} h^{-1}) and the maximum observed specific oxygen uptake rate ($q_{o,max}$) (g g^{-1} h^{-1}).

$$RRR = \frac{q_o}{q_{o,\max}} \tag{3-5}$$

where q_o was obtained from:

$$q_o = \frac{OUR}{XV} \tag{3-6}$$

in which X is cell concentration (g L^{-1}) and V is culture volume (L). $q_{o,max}$ was obtained from the maximum q_o value before methanol or DOT became limiting.

For the carbon mass balance calculations, a previously analyzed carbon concentration in *P. pastoris* (0.396 g g^{-1} DW) was used (Jahic et al., 2002).

CHAPTER IV

RESULTS

Three *P. pastoris* fermentation techniques have been performed in this work. The methanol limited fed-batch (MLFB) (Jahic et al., 2002) was used as a standard technique to compare with two new process designs: the oxygen limited fed-batch (OLFB) technique and the high pressure fed-batch (HPFB) technique. Both techniques were intended to increase the oxygen transfer rate (OTR). In this chapter, the results obtained from each technique are presented and discussed briefly. The indepth discussion of such details as the performance, advantage and disadvantage of each technique will be treated separately in Chapter V.

As mention in Chapter III (Materials and Methods), a four-stage *P. pastoris* fermentation protocol (Jahic et al., 2002) was applied. This protocol was designed to avoid methanol toxification during the transition phase from repressing substrate (glycerol) to inducing substrate (methanol), based on the repression/derepression and induction mechanisms of the *aox1* promoter (Jahic et al., 2002; Chiruvolu et al., 1997). In the first three fermentation stages (glycerol batch phase, glycerol fed-batch phase and methanol induction phase), the control systems were the same in all three techniques (MLFB, OLFB and HPFB), thus only the results of one experiment will be shown (Fig. 4-1) and explained in detail for each stage. For the last stage (protein

production stage), the control system varied for each technique, therefore the results will be described case by case.

In the glycerol fed-batch phase, glycerol was used as the carbon and energy source, since it gives rapid growth and high biomass yield (Chiruvolu et al., 1998). This phase was run till a sharp increase in the DOT was observed, which presumable meant that the glycerol was completely consumed (Fig 4-1). At the end of this phase, the cell dry weight was about 24 g L^{-1} in all experiments.

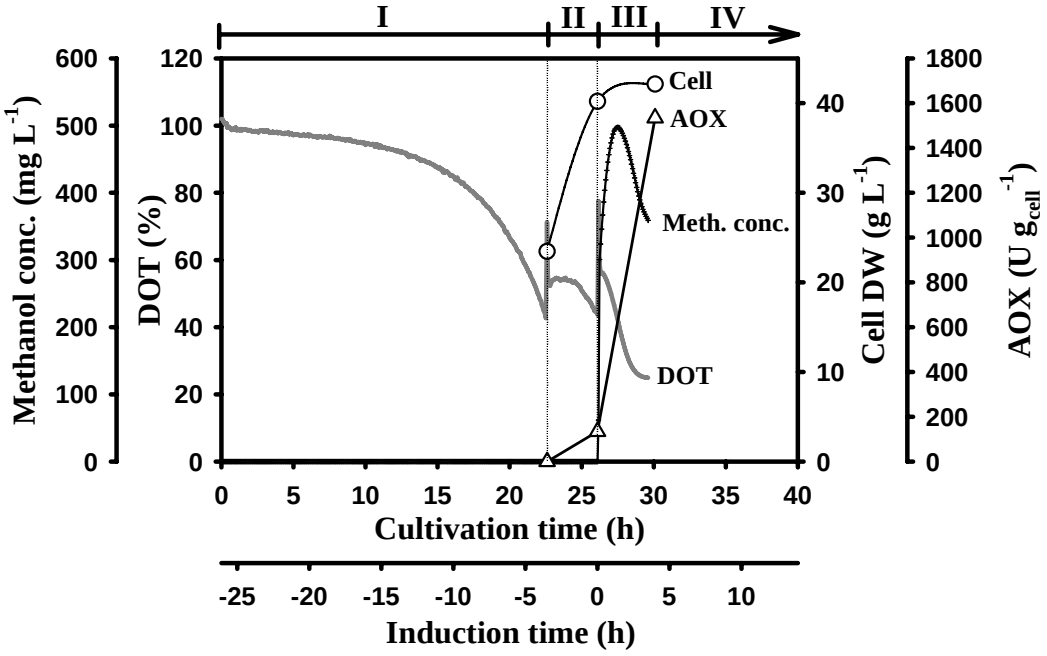

Figure 4-1 Experimental data (Methanol concentration, DOT, Cell DW and AOX) of *Pichia pastoris* culture following a four-stage fermentation protocol. I: glycerol batch phase, II: glycerol fed-batch phase, III: methanol induction phase, and IV: protein production phase

The glycerol fed-batch phase was started by feeding glycerol feed (GF) medium into the fermentor. The regulation of the *aox1* promoter appears to involve two mechanisms, an induction mechanism and a repression/derepression mechanism (Chiruvolu et al., 1997). Derepression is a phenomenon where transcription of the gene occurs without any inducer in the presence of low repressor concentration. The intention of this phase is not only to derepress the *aox1* promoter but also to increase cell density. Thus, the GF medium feed rate has to be controlled to achieve a low residual glycerol concentration in the culture broth, which is high enough to increase the cell concentration. The perfect control during this phase results in an exponential increase in the cell density, while derepression of an *aox1* promoter can be detected.

The glycerol feed profile can be calculated from the mass balance of glycerol (Jahic et al., 2002),

$$\frac{dS}{dt} = \frac{F}{V}(S_i - S) - \frac{\mu}{Y_{x/s}}X \qquad (4\text{-}1)$$

where S is the glycerol concentration in culture broth (g L^{-1}), t is time (h), F is the feed rate (L h^{-1}), V is the culture volume (L), S_i is the glycerol concentration in the GF medium (g L^{-1}), μ is the specific growth rate (h^{-1}), $Y_{x/s}$ is the biomass yield coefficient (g g^{-1}), and X is the cell concentration (g L^{-1})

Under substrate limited conditions, the glycerol concentration in the culture broth can be assumed to be insignificant compared to the glycerol concentration in

the GF medium, thus, S≈0. The starting feed rate (F_0; L h^{-1}) can be obtained by reformation of *eq. (4-1)*. Under the condition dS/dt = 0, F_0 is corresponds to the substrate consumption rate for a certain amount of biomass (X_0V_0) at the time of the feed start.

$$F_0 = \frac{\mu}{Y_{x/s}} X_0 V_0 \qquad (4\text{-}2)$$

F_0 calculated for $\mu = 0.18\ h^{-1}$ (Jahic et al., 2002) and the biomass (X_0V_0) at the end of glycerol batch phase (24*3 = 72 g). Where Yx/s = 0.7 g g^{-1} and Si = 500 g L^{-1}.

$$F_0 = \frac{0.18}{0.7 \cdot 500} \cdot 72 = 0.037 \quad L\ h^{-1}$$

From F_0, the GF medium feed profile was set to

$$F(t) = F_0 e^{\mu t} \qquad (4\text{-}3)$$

where t is time (h) after the start of the exponential feed.

This phase was run until the cell concentration was approximately 40 g L^{-1} (which took about 3.5 h). At the end of this phase, a low amount of AOX enzyme activity (135 U g_{cell}^{-1}) and β-glucosidase activity (56 U L^{-1}) were detected even in the

absence of methanol (Fig 4-1), which was assumed to reflect the derepression phenomena of the *aox1* promoter.

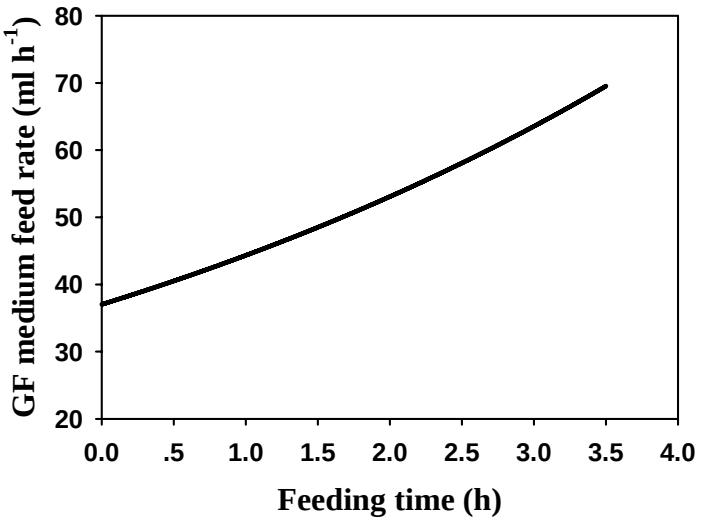

Figure 4-2 GF medium feed profile for the glycerol fed-batch phase

The methanol induction phase was then applied by feeding the methanol feed (MF) medium into the fermentor at a very low flow rate (about 3.5 ml L^{-1} h^{-1}) so that the cells would have enough time to activate the enzymes in the methanol metabolism pathway. AOX, formaldehyde dehydrogenase and formate dehydrogenase are some of the enzymes in this pathway; (Rose and Harrison, 1989). A rapid increase of AOX enzyme from 135 to 1534 U g_{cell}^{-1} was detected in this phase (Fig. 4-1), which indicates that the *P. pastoris* were adapting to the use of methanol as carbon and energy source. Thus, the process was then shifted to the protein production phase. The MF medium was fed into the fermentor at an increasing feed rate. The process control in this phase was varied depended on the control technique (MLFB, OLFB and HPFB).

4.1 Methanol limited fed-batch technique

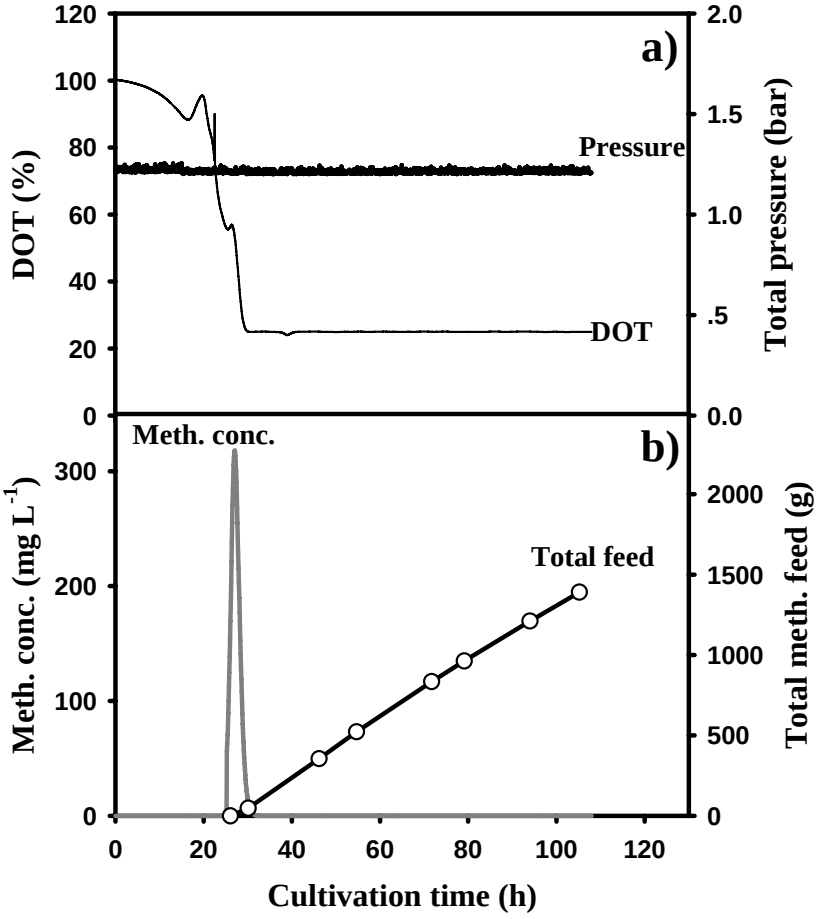

Figure 4-3 Fed-batch fermentation with MLFB technique: a) pressure (thick line) and DOT (thin line), b) methanol concentration (thick line) and total methanol feed (-o-)

By manipulation of the methanol feed rate with the DOT signal in the MLFB technique, it is possible to avoid both methanol toxication and oxygen limited condition (Jahic et al., 2002). Pressure was controlled constant at 1.2 bar total air pressure (Fig. 4-3a). During the protein production phase, the DOT was constant at 25% air saturation (Fig. 4-3a), while the methanol concentration was lower than the

detection sensitivity of the gas analyzer (Fig. 4-3b). At the end of the process, about 1390 g of methanol has been used (Fig. 4-3b).

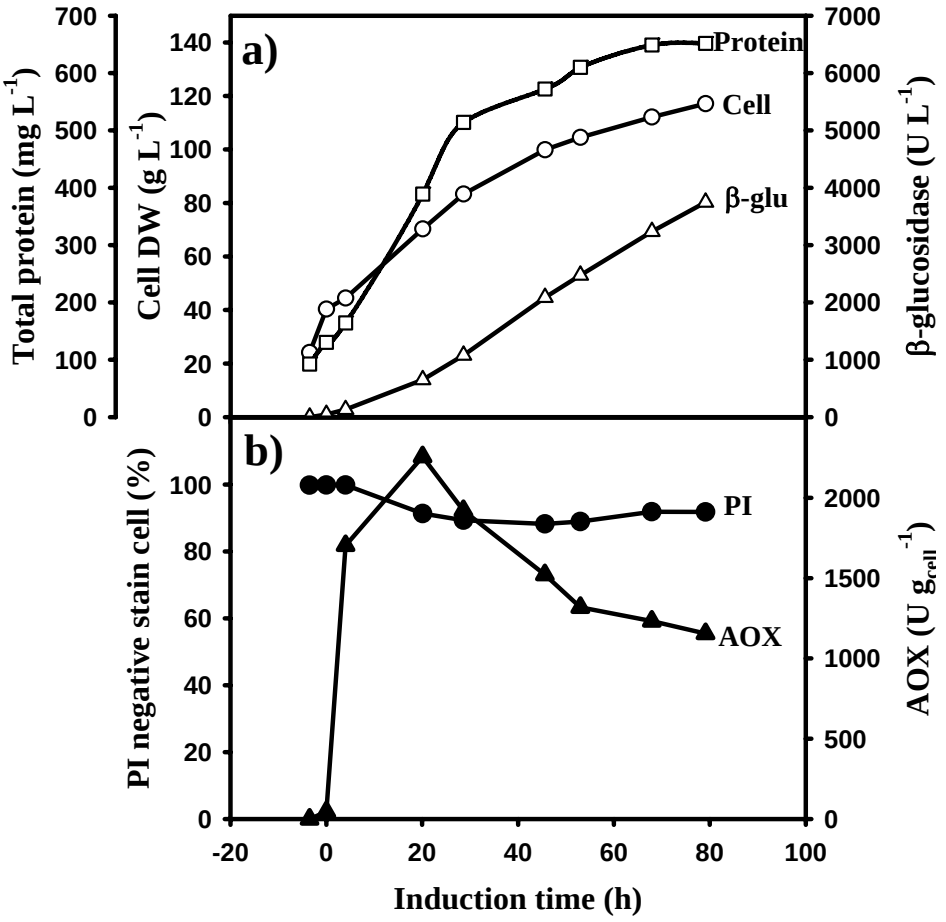

Figure 4-4 Process profile of MLFB technique a) cell (–○–), total protein (–□–), β-glucosidase (–△–); b) PI negative stain cell (–●–), AOX (–▲–)

At the beginning of the protein production phase, the cell density and total protein concentration increased rapidly. However, the rate of increase declined after the methanol started to be limiting at about 30 h of induction (Fig. 4-4a). On the other hand, the β-glucosidase activity increase slowly at the initiation of induction and increase rapidly when methanol was limiting (Fig. 4-3b and Fig. 4-4a). PI is

often used to determined viability by flowcytometry. The PI stain cells are considered dead and PI negative stain cells are considered viable. Percent of healthy cell decreased from about 100% at the beginning of the protein production phase to about 90% in 25 h of induction. After that, the percent of viability maintained at about 90% until the end of the process (Fig. 4-4b).

The β-glucosidase production was controlled by the same promoter as the AOX enzyme. This AOX enzyme is very important to the cell even in the maintenance stage, because it is the first enzyme in the methanol metabolism pathway (Rose and Harrison, 1989). The specific AOX production increased rapidly during the methanol induction phase and highest at about 2250 U g_{cell}^{-1} at 20 h of induction, and then declined to about 1150 U g_{cell}^{-1} at the end of the process (Fig. 4-4b).

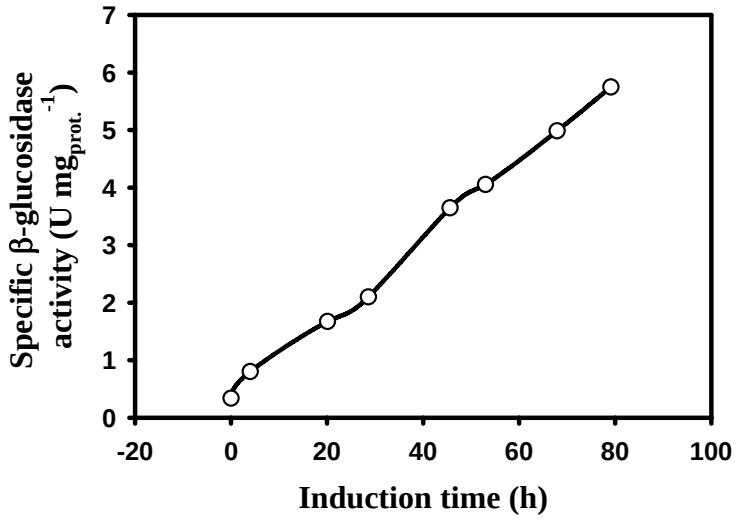

Figure 4-5 The specific β-glucosidase activity of MLFB process

The specific enzyme activity can be used as a simple and easy technique to determine enzyme purity. The specific β-glucosidase activity in the MLFB process increased in proportion to the methanol uptake, even in the growth limited condition. The specific activity was about 5.7 U mg_{prot}^{-1} at the end of the process (Fig. 4-5).

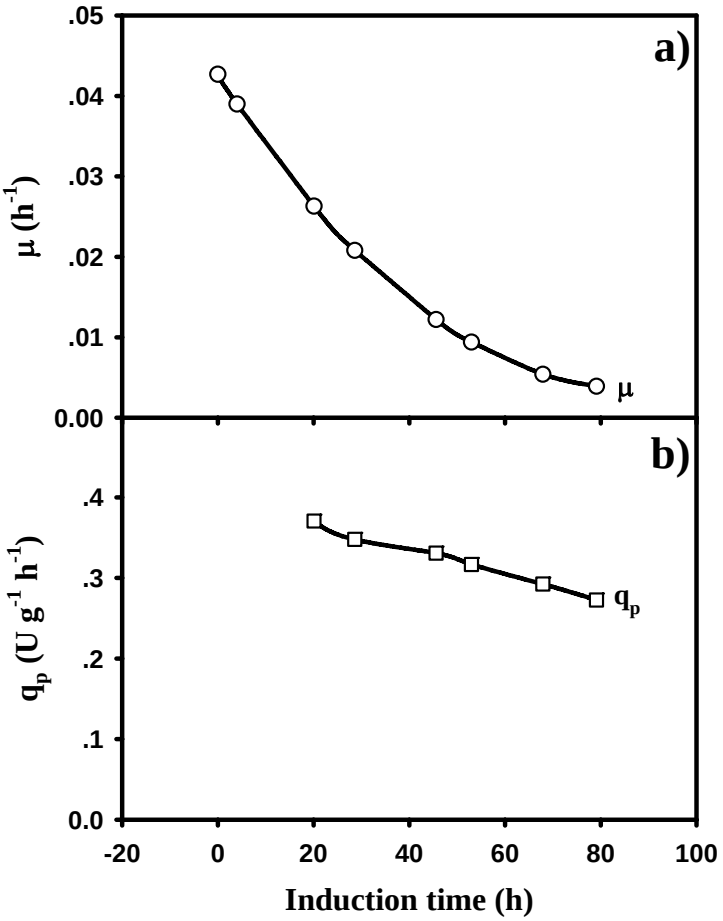

Figure 4-6 Specific rate of MLFB process: a) specific growth rate (μ; -○-) and b) specific β-glucosidase production rate (q_p; -□-)

Cell growth and product formation are usually presented as the specific rates with respect to biomass (X). They are normally used to compare the efficiency of the various fermentation processes. The specific growth rate is μ = 1/X*dX/dt, where X

is the cell concentration and dX/dt is cell growth rate. The specific production rate is $q_p = 1/X*dP/dt$, where dP/dt is the product formation rate. In the MLFB process, both μ and q_p decreased, however, a rapid decline was seen in μ, while a slower rate of decline was observed in q_p (Fig. 4-6). These might be due to a stronger effect of methanol limitation on growth than on product formation.

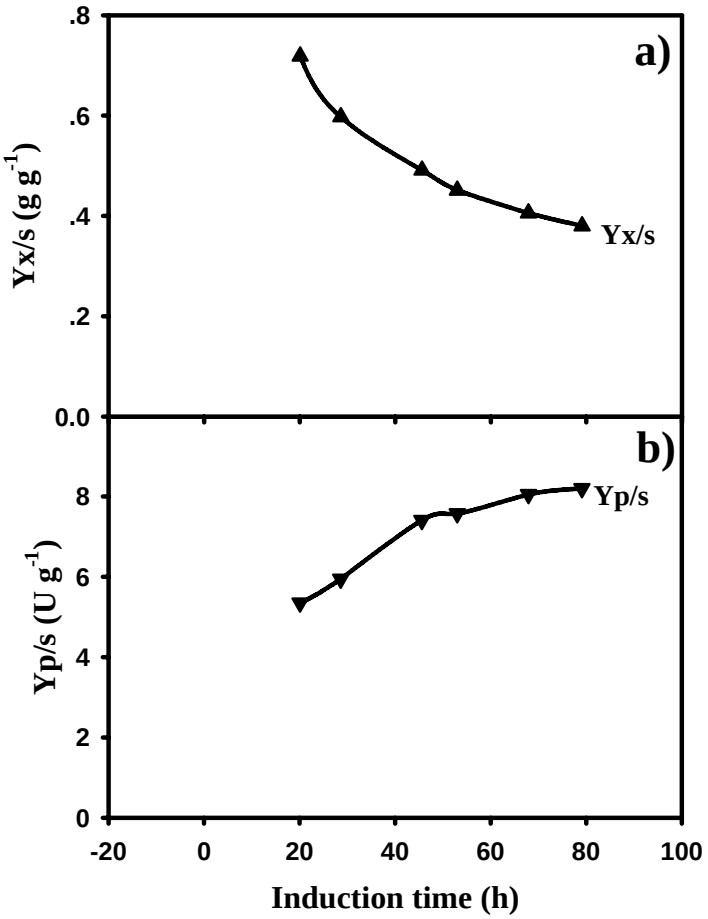

Figure 4-7 Substrate yield from MLFB process: a) biomass yield (Yx/s; –▲–) and b) product yield (Yp/s; –▼–)

Normally, the substrate is a main cost of the fermentation process which makes the profit directly depend on the product yield (Yp/s). Thus, increasing Yp/s is

one objective in process optimization. The high Yx/s was presented at the beginning and declined with time, while the Yp/s increased rapidly after methanol limitation (Fig 4-7). This result might be due to the use of methanol for cell growth with excess methanol. But when methanol is limited, a larger proportion of the methanol was used for maintenance. This is related to a rapid increase in the biomass at the beginning and decline during methanol limitation. In the other hand, the β-glucosidase accumulation increased rapidly after methanol became limiting (Fig. 4-3b and Fig. 4-4a).

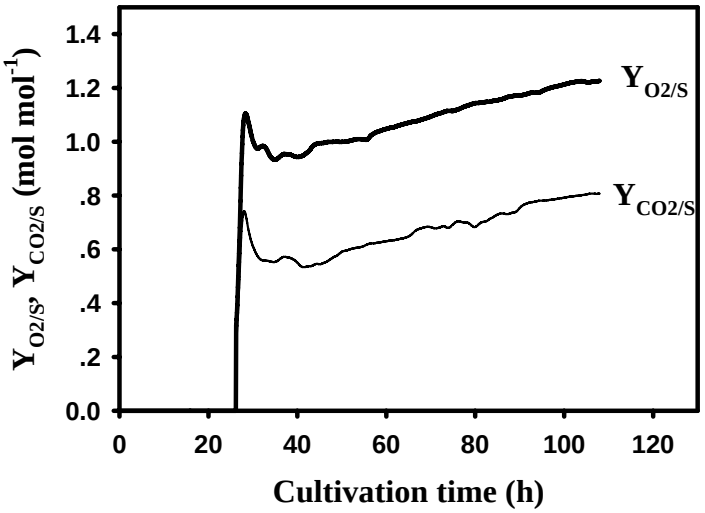

Figure 4-8 The oxygen use per mole of methanol in methanol metabolism ($Y_{O2/S}$; thick line) and yield of carbon dioxide from methanol ($Y_{CO2/S}$; thin line) in MLFB process

The oxygen use per mole of methanol in methanol metabolism ($Y_{O2/S}$) and yield of carbon dioxide from methanol ($Y_{CO2/S}$) can be used to measuring the carbon flux in this process. According to the model of methanol metabolism in *P. pastoris*

(Fig. 2-1), the increase of $Y_{O2/S}$ from 0.95 to 1.23 mol mol^{-1} and $Y_{CO2/S}$ from 0.54 to 0.81 mol mol^{-1} indicated an increase of methanol flux in energy metabolism. This resulted in decreasing of both biomass accumulation and biomass yield, which was in agreement with the experimental data, as showed in Fig. 4-4 and Fig. 4-7a.

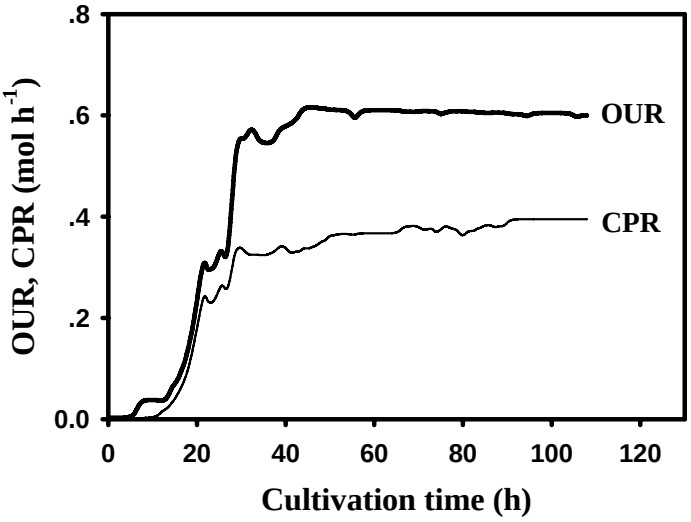

Figure 4-9 The oxygen uptake rate (OUR; thick line) and carbon dioxide production rate (CPR; thin line) of the MLFB process

After 50 h of cultivation, oxygen uptake rate (OUR) and carbon dioxide production rate (CPR) were almost constant at 608 mmol h^{-1} and 385 mmol h^{-1}, respectively (Fig. 4-9). The respiratory quotient, RQ = CPR/OUR is an alternative parameter for measuring the maintenance demand. In the MLFB process, RQ was about 0.6 mol mol^{-1} (Fig. 4-10). The relative rate of respiration, RRR = q_o/q_{omax} is a parameter that can be used to describe the degree of both oxygen and carbon substrate limitation. During the induction phase, the process was non-limited with respect to both oxygen and methanol and RRR was 1. However, this parameter declined rapidly

in the protein production phase and approached about 0.3 at the end with respect to methanol limitation (Fig. 4-10).

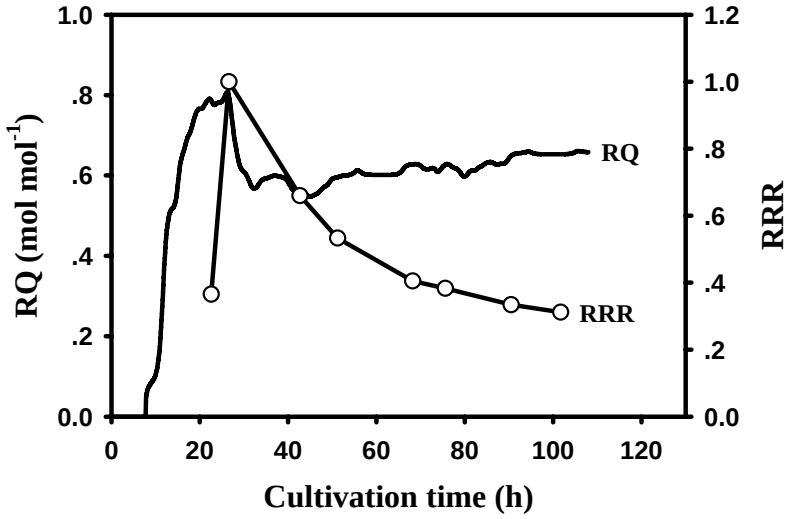

Figure 4-10 Relative rate of respiration (RRR; -○-) and respiratory quotient (RQ; line) in the MLFB process

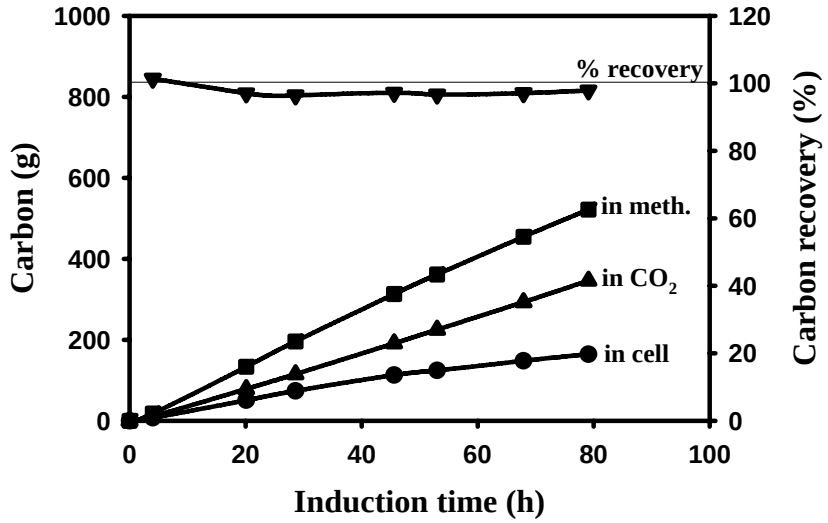

Figure 4-11 Carbon mass balance: carbon input in methanol (-■-), carbon output in cells (-●-) and CO_2 (-▲-) and percent of carbon recovery (-▼-) from the MLFB process

The carbon mass balance based on carbon input from methanol and carbon output from cell and carbon dioxide presented good agreement with the carbon recovery of 98-99% (Fig. 4-11). Thus, the methanol metabolism of *P. pastoris* in the MLFB process did not produce any major by-product.

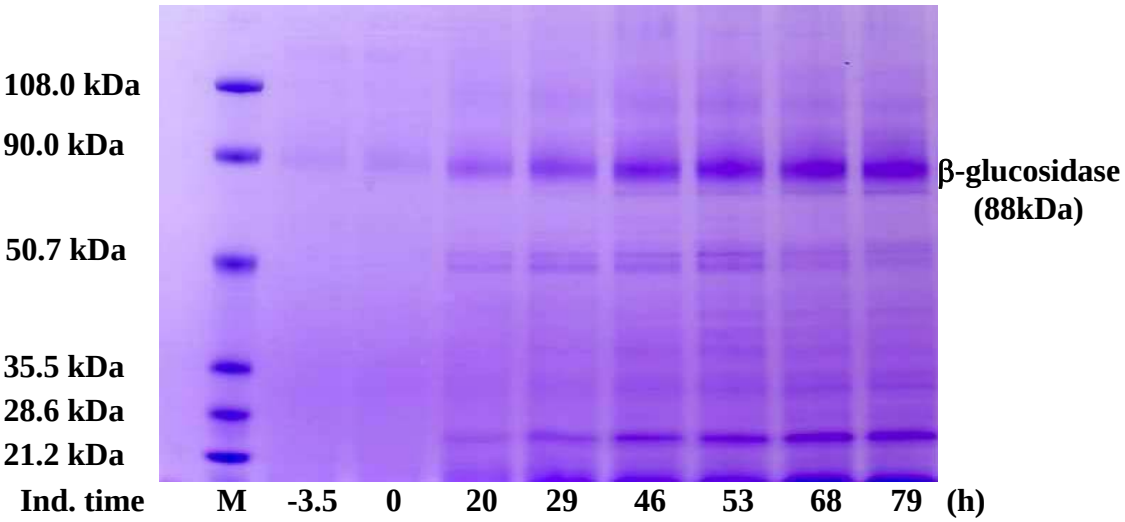

Figure 4-12 SDS-PAGE with Coomassie blue stain of fermentation supernatant at increasing time profile from MLFB process (M; protein marker)

SDS-PAGE of culture medium from MLFB process showed the accumulation of β-glucosidase over time. The estimate molecular weight of this recombinant β-glucosidase under denaturing condition is approximately 88 kDa (Fig. 4-12) which is larger than the natural enzyme purified from Thai rosewood seeds (60 kDa) (Srisomsap et al., 1996). This is possibly due to over glycosylation of the recombinant protein in *P. pastoris*, which has been reported to occur to many proteins produced in yeast in the literature (Bretthauer and Castellino, 1999).

4.2 Oxygen limited fed-batch process

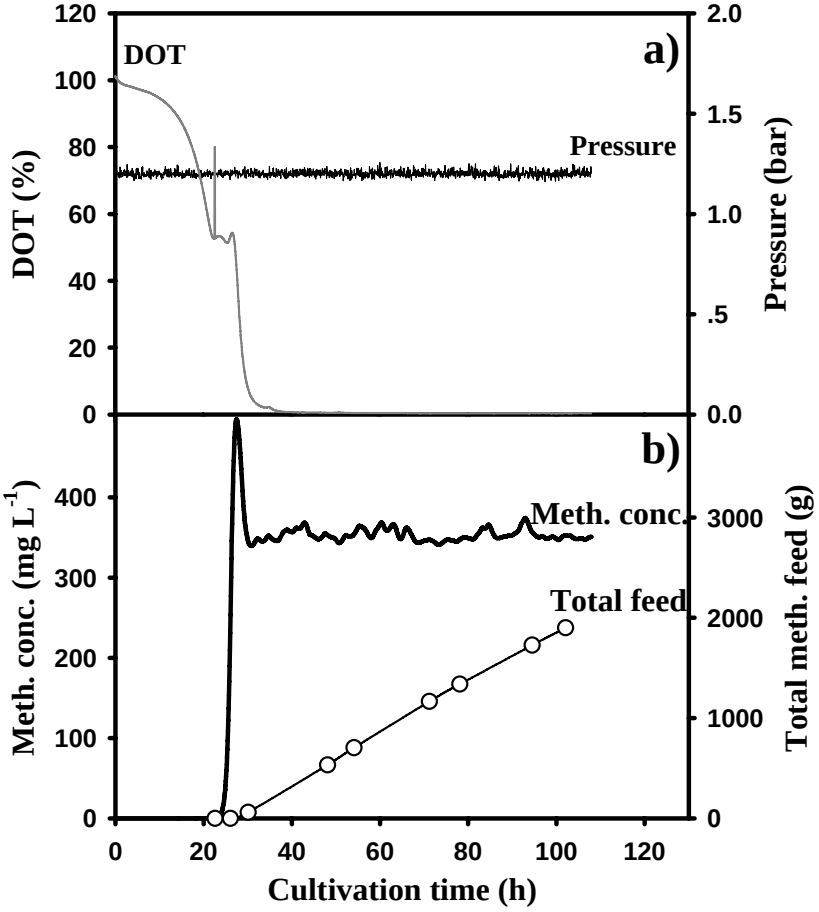

Figure 4-13 Fed-batch fermentation with OLFB technique: a) pressure and DOT, b) methanol concentration and total methanol feed

The DOT was reported to be important for several recombinant protein processes (Bushell et al., 2003). However, the presence of residue methanol has been shown to be more important in others (Mayson et al., 2003; Katakura et al., 1998). Increasing the residue methanol concentration resulted in increasing the expression level of single-chain antibody fragment (scFv), even in oxygen limited condition (Khatri and Hoffmann, 2005). Thus, the OLFB process was applied to increase the

residue methanol concentration and OTR, which are related to the expression level. The pressure in the OLFB process was also controlled constant at 1.2 bar total air pressure (Fig. 4-13a). The residue methanol during the protein production phase was maintained at 350 mg L^{-1}(Fig. 4-13b) by regulation of the methanol feed rate with the methanol signal from a gas analyzer. The DOT decreased until closed to zero in 4 h after the start of this phase (Fig. 4-13a). At the end, about 1900 g of methanol was used (Fig. 4-13b).

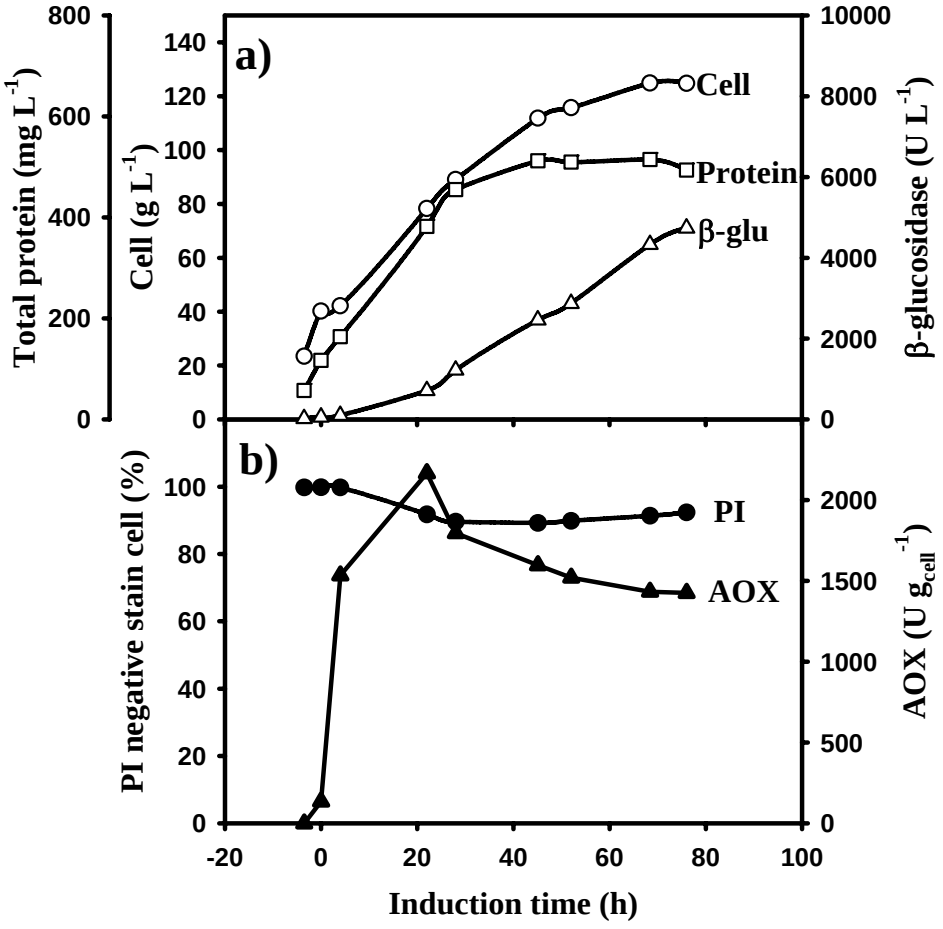

Figure 4-14 Process profile of the OLFB technique a) cell DW (-○-), total protein (-□-), β-glucosidase (-△-); b) PI negative stain cell (-●-), AOX (-▲-)

The pattern of cell growth, total protein production and β-glucosidase accumulation in the OLFB process (Fig. 4-14a) were similar to the results from the MLFB process (Fig. 4-4a). However, higher values were attained in the OLFB process. The final cell, total protein and β-glucosidase accumulation were about 125 g L^{-1}, 500 mg L^{-1} and 4750 U L^{-1}, respectively (Fig. 4-14a). Cell viability was higher than 90% through out the process (Fig. 4-14b). The specific AOX production increased rapidly and reached its highest value at about 2150 U g_{cell}^{-1} during the methanol induction phase, then declined to about 1400 U g_{cell}^{-1} at the end (Fig. 4-14b).

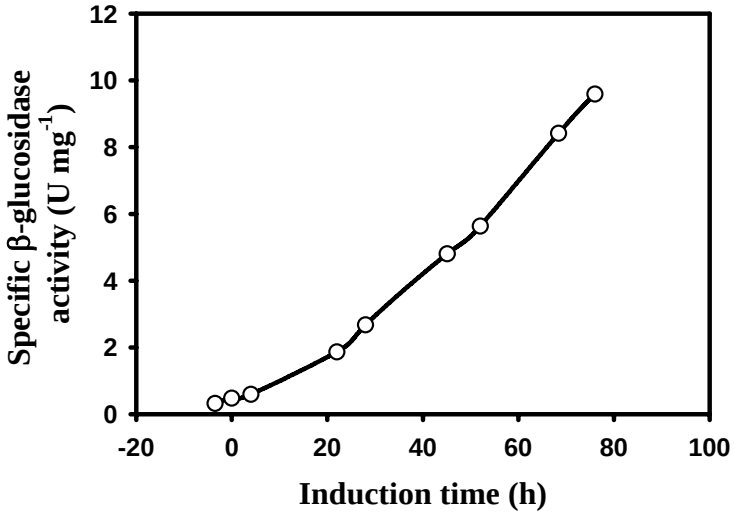

Figure 4-15 The specific β-glucosidase activity of OLFB process

The specific β-glucosidase activity in OLFB process increased with induction time and reached about 9.6 U mg_{prot}^{-1} at the end of the process (Fig. 4-15).

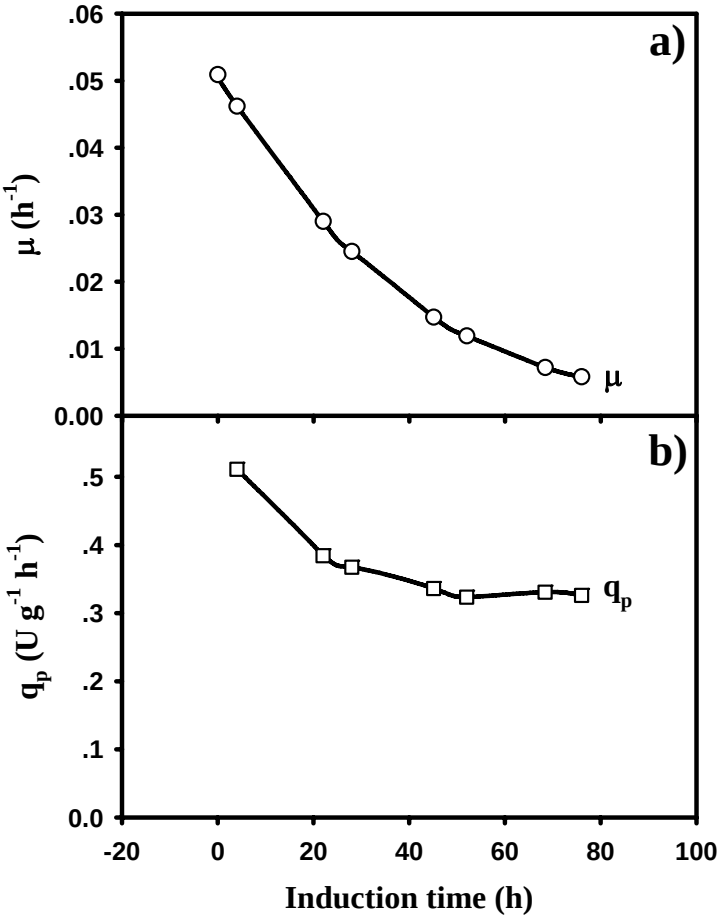

Figure 4-16 Specific rates of the OLFB process: a) specific growth rate (μ; –○–) and b) specific β-glucosidase production rate (q_p; –□–)

In the OLFB process, initially both μ and q_p decreased rapidly, however, after 40 h of induction the rate of decline was slower. At the end, μ and q_p were about 0.006 h^{-1} and 0.33 U g^{-1} h^{-1}, respectively (Fig. 4-16).

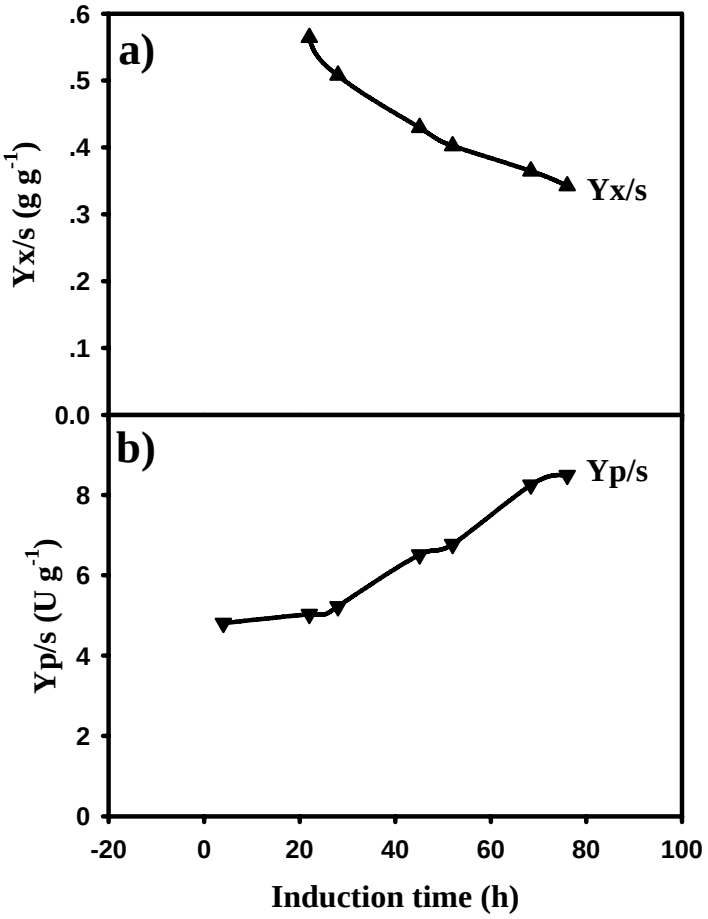

Figure 4-17 Substrate yield from OLFB process: a) biomass yield (Yx/s; —▲—) and b) product yield (Yp/s; —▼—)

The biomass yield, Yx/s, of the OLFB process decreased at almost a constant rate and reached 0.34 g g^{-1} at the end (Fig. 4-17a). On the other hand, Yp/s increased slowly at the beginning, but after 30 h of induction, the rate of increase was faster. The final, maximum Yp/s was about 8.5 U g^{-1} (Fig. 4-17b).

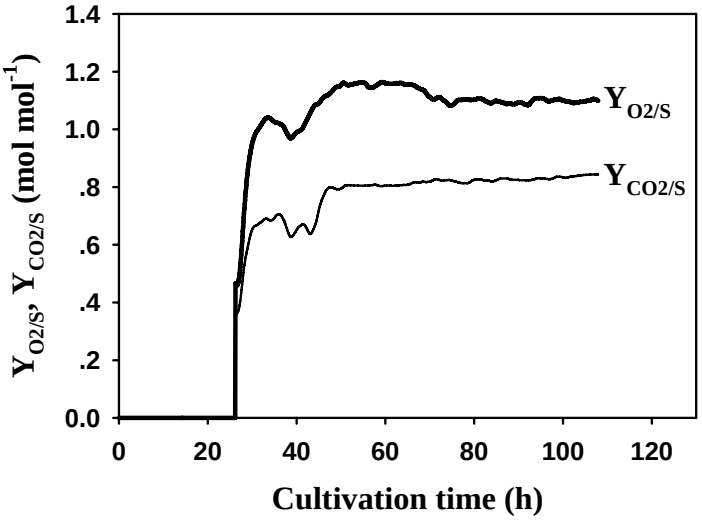

Figure 4-18 The oxygen use per mole of methanol in methanol metabolism ($Y_{O2/S}$; thick line) and yield of carbon dioxide from methanol ($Y_{CO2/S}$; thin line) of the OLFB process

In the OLFB process, after 50 h of cultivation $Y_{O2/S}$ and $Y_{CO2/S}$ were almost constant at 1.10 and 0.84 mol mol^{-1}, respectively (Fig. 4-18).

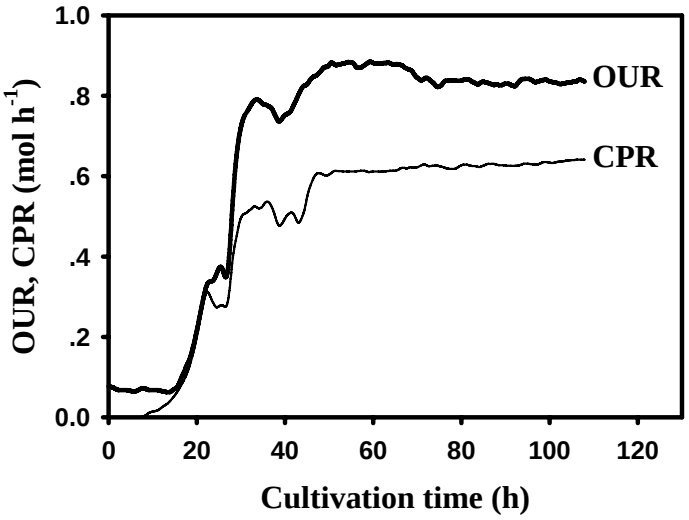

Figure 4-19 The oxygen uptake rate (OUR; thick line) and carbon dioxide production rate (CPR; thin line) of the OLFB process

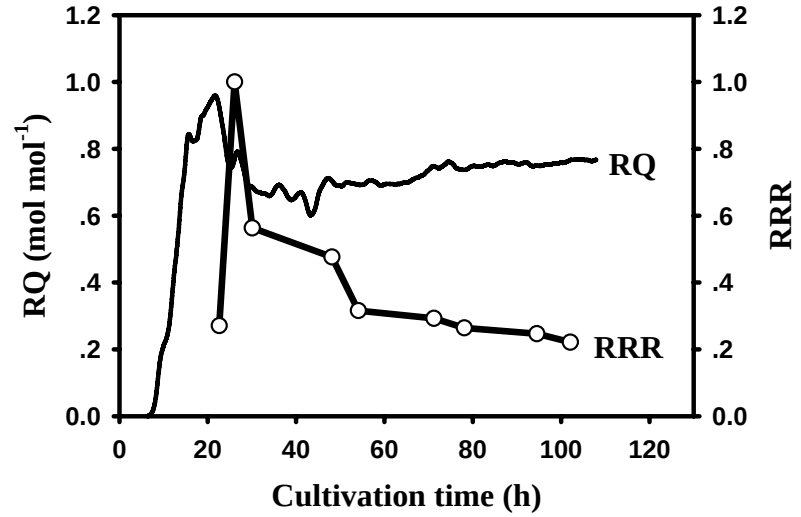

Figure 4-20 Relative rate of respiration (RRR; –○–) and respiratory quotient (RQ; line) in the OLFB process

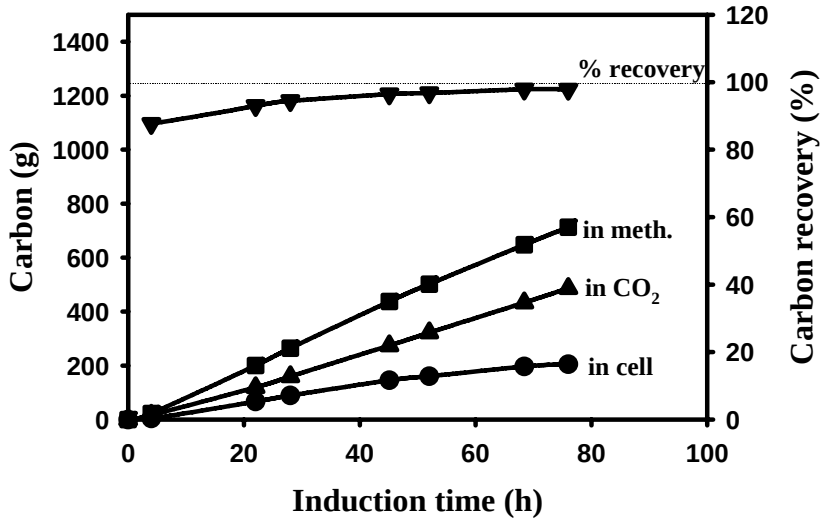

Figure 4-21 Carbon mass balance: carbon input in methanol (–■–), carbon output in cell mass (–●–) and CO_2 (–▲–) and percent of carbon recovery (–▼–) from the OLFB process

The oxygen uptake rate (OUR) and carbon dioxide production rate (CPR) of OLFB were nearly constant at 840 mmol h^{-1} and 640 mmol h^{-1}, respectively (Fig. 4-

19). RQ was about 0.75 mol mol^{-1} (Fig. 4-20). RRR was 1 during the methanol induction phase, which indicated that there was no limitation of either methanol or oxygen. Then in the protein production phase, the RRR decreased rapidly to about 0.22 at the end with respect to oxygen limitation (Fig. 4-20).

The carbon recovery in the OLFB process was about 98-99% (Fig. 4-21). This values indicated that methanol metabolism in *P. pastoris* did not generate any major by-product, even under oxygen limitation.

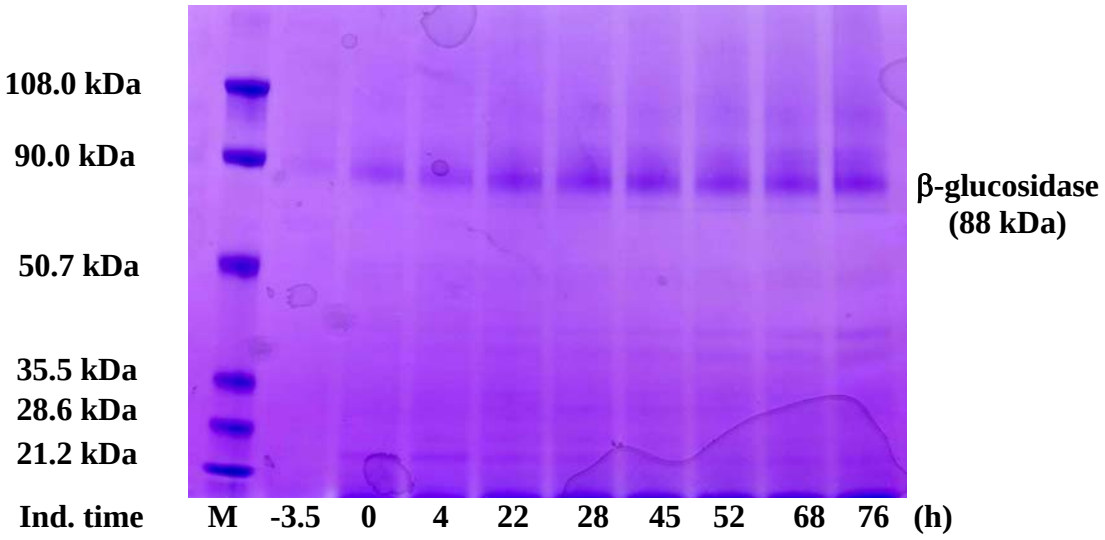

Figure 4-22 SDS-PAGE with Coomassie blue stain of fermentation supernatant at increasing time profile from the OLFB process

SDS-PAGE of OLFB culture medium showed the accumulation of β-glucosidase over time. It was a major product present on the gel with an approximate molecular weight of 88 kDa (Fig. 4-22).

4.3 High pressure fed-batch process

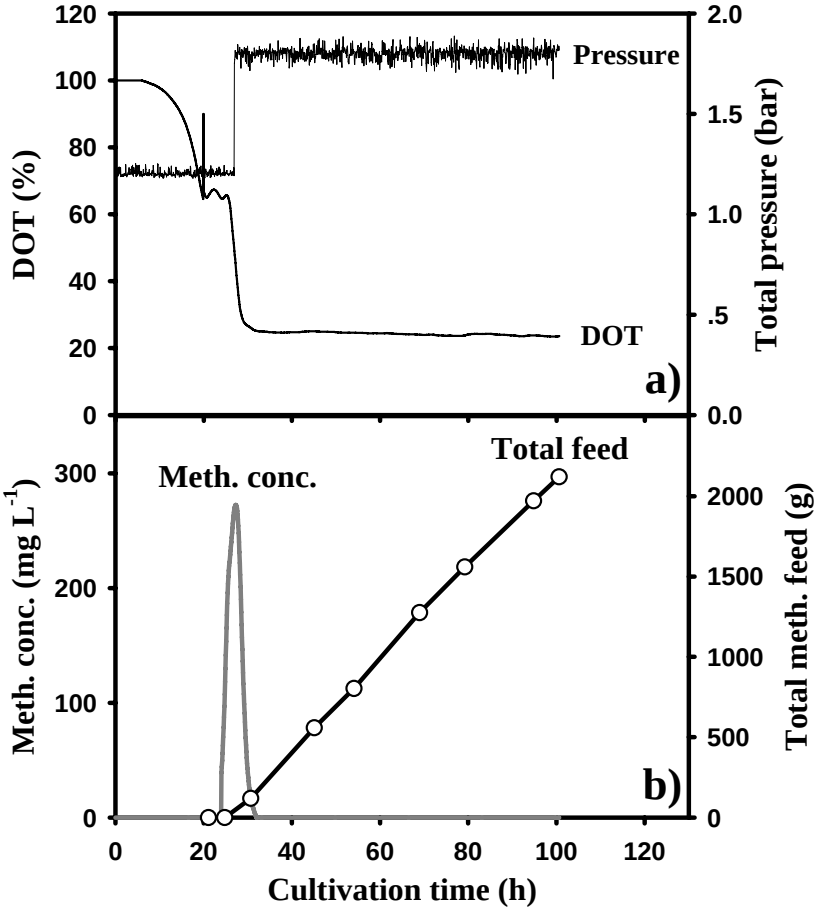

Figure 4-23 Fed-batch fermentation with the HPFB technique: a) pressure and DOT, b) methanol concentration and total methanol feed

In high cell density *P. pastoris* fermentation, the maximum achievable oxygen transfer is normally lower than the oxygen demand. An increase of oxygen transfer can enhance both growth and recombinant protein production (Lee et al., 2003; Jahic et al., 2002). Elevation of the total air pressure is an easy and economical technique to increase the OTR. The combination of the normal MLFB technique with an increase in the total air pressure, high pressure fed-batch (HPFB) process was applied

for recombinant β-glucosidase production by *P. pastoris*. After the methanol induction phase, the total air pressure was increased from 1.2 to 1.9 bar (Fig. 4-23a). During the protein production phase, the DOT was controlled at a constant 25% air saturation (Fig. 4-23a), while the methanol concentration was also lower than the detection sensitivity (Fig. 4-23b), as observed in the normal MLFB process. At the end, about 2120 g of methanol was used (Fig. 4-23b).

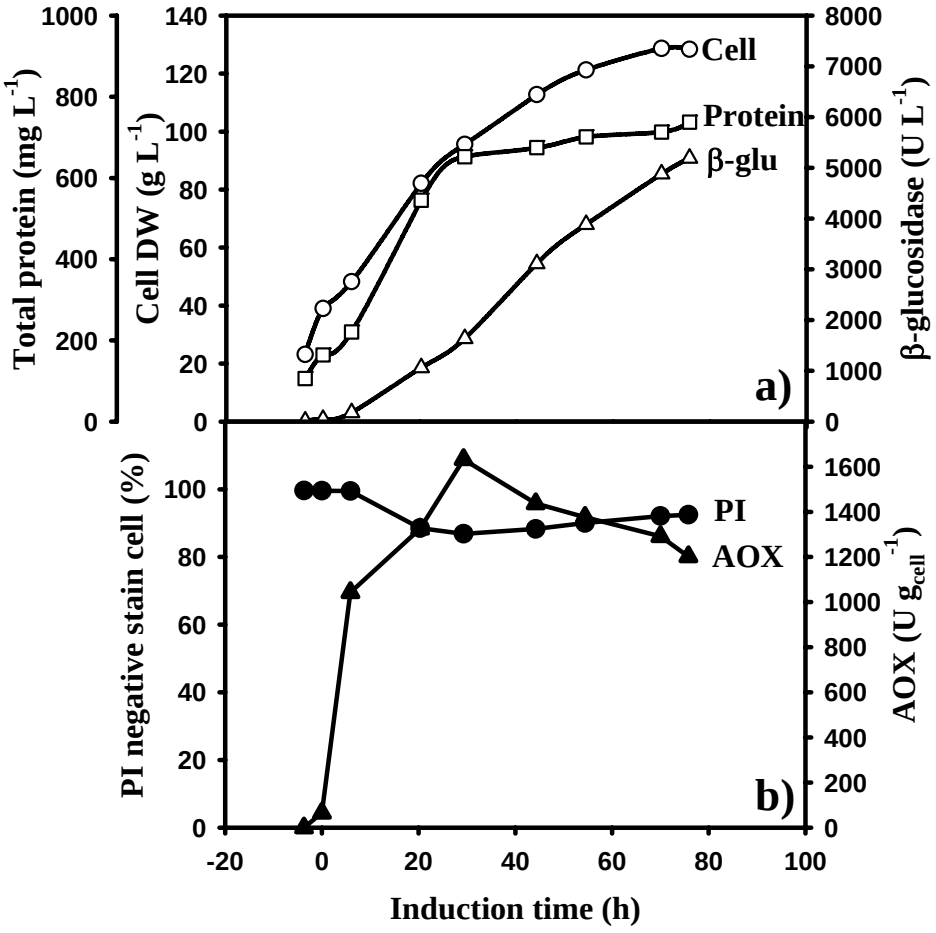

Figure 4-24 Process profile of HPFB technique a) cell DW (-○-), total protein (-□-), β-glucosidase activity (-△-); b) PI negative stain cell (-●-), AOX (-▲-)

Even though cell growth, total protein production and β-glucosidase accumulation in three processes (Fig. 4-4a, Fig. 4-14a and Fig. 4-24a) were similar, the highest values were present in the HPFB process (Fig. 4-24a). The maximum cell DW, total protein and β-glucosidase accumulation in the HPFB process were about 130 g L^{-1}, 740 mg L^{-1} and 5200 U L^{-1}, respectively (Fig. 4-24a). The combination of flowcytometry with PI was also applied for measuring cell death under high total air pressure. More than 85% viable cells were collected in the HPFB process. It was not much different from both MLFB and OLFB processes, which gave values of about 90%. Thus, an increase of total air pressure in the range of 1.2-1.9 bar does not affect the cell viability significantly (Fig. 4-24b). The specific AOX production increased rapidly during the methanol induction phase and then continued to increase to its highest value of about 1630 U g_{cell}^{-1} in the early protein production phase. After that, it slowly decreased to 1200 U g_{cell}^{-1} at the end (Fig. 4-24b).

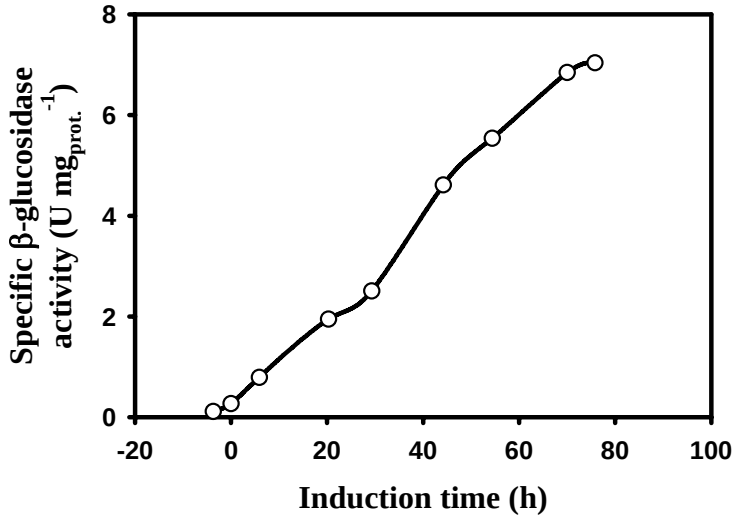

Figure 4-25 The specific β-glucosidase activity of HPFB process

The specific activity of β-glucosidase in the HPFB process increased with induction time. The highest specific β-glucosidase activity was obtained at the end of the process, which was about 7.0 U mg_{prot}^{-1} (Fig. 4-25).

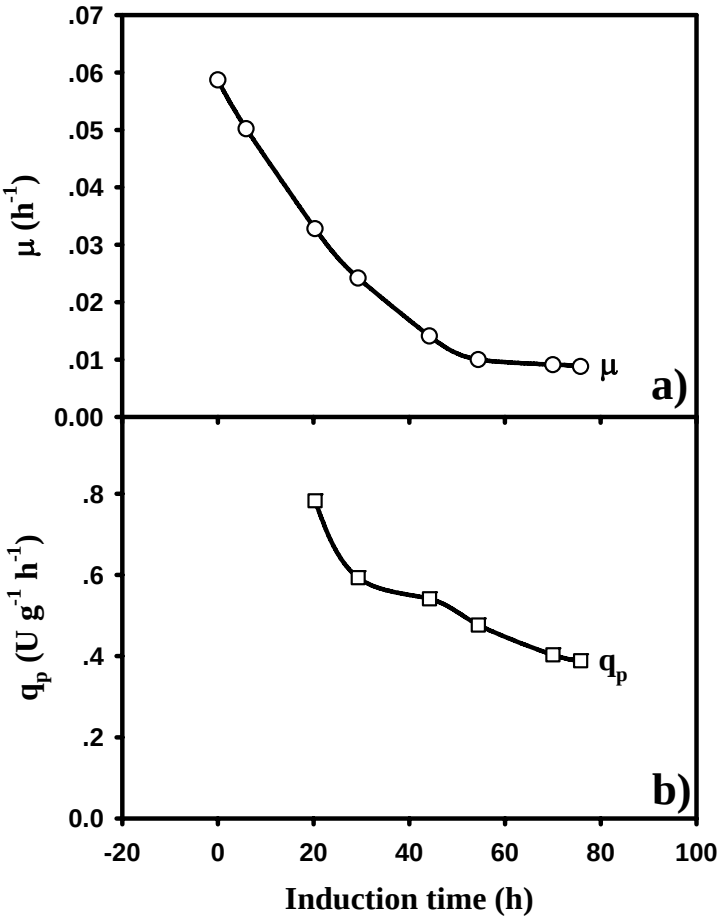

Figure 4-26 Specific rates in the HPFB process: a) specific growth rate (μ; -○-) and b) specific β-glucosidase production rate (q_p; -□-)

In the HPFB process, μ decreased rapidly and then remained constant at 0.009 h^{-1} after 50 h of induction. q_p continuously decreased and reached about 0.40 U g^{-1} h^{-1} at the end of the process (Fig. 4-26).

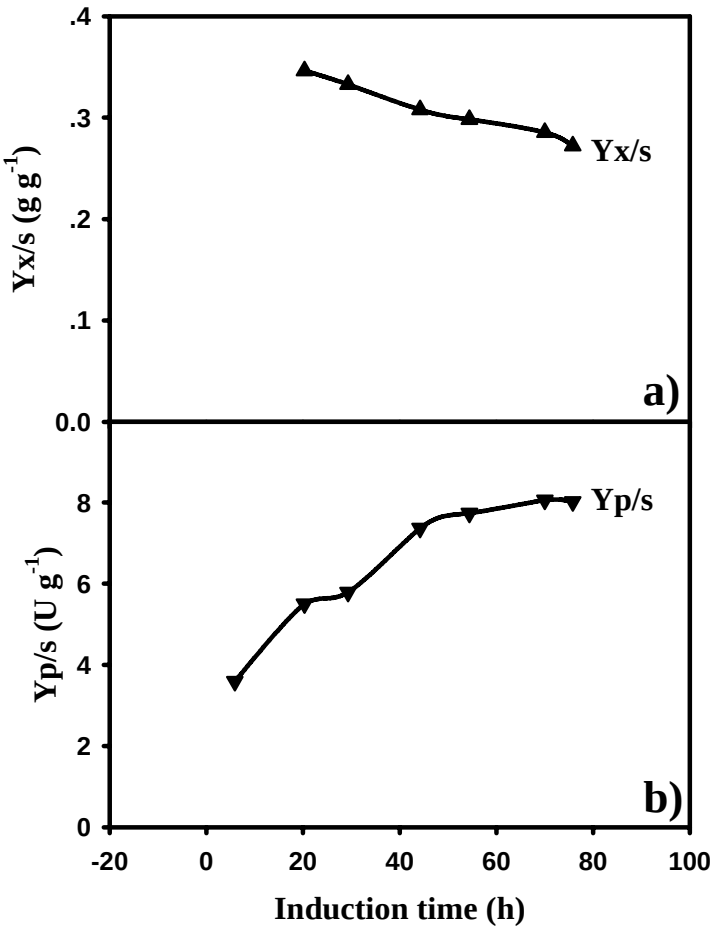

Figure 4-27 Substrate yield from the HPFB process: a) biomass yield (Yx/s; –▲–) and b) product yield (Yp/s; –▼–)

In the HPFB process, Yx/s decreased over the time and gave a value of 0.27 g g^{-1} at the end (Fig 4-27a). In contrast, Yp/s continuously increased to a maximum value of about 8.0 U g^{-1} at the end (Fig 4-27a).

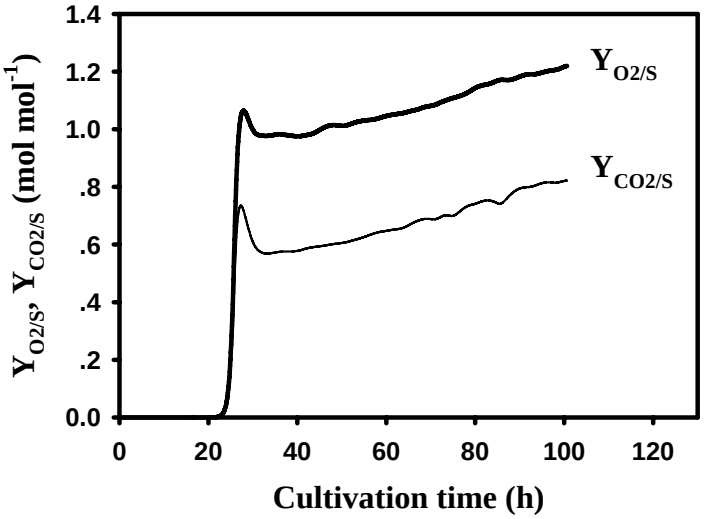

Figure 4-28 The oxygen use per mole of methanol in methanol metabolism ($Y_{O2/S}$; thick line) and yield of carbon dioxide from methanol ($Y_{CO2/S}$; thin line) in the HPFB process

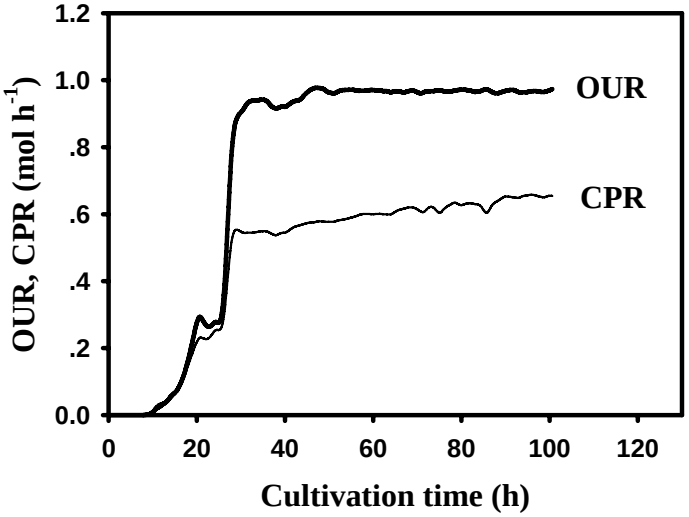

Figure 4-29 The oxygen uptake rate (OUR; thick line) and carbon dioxide production rate (CPR; thin line) of the HPFB process

At 40 h of cultivation $Y_{O2/S}$ and $Y_{CO2/S}$ were 0.98 and 0.58 mol mol^{-1}, and then increased to 1.22 and 0.92 mol mol^{-1} at the end, respectively (Fig. 4-28). This indicated the increase of methanol flux in energy metabolism, which was reflected in decrease in both biomass accumulation and biomass yield (Fig. 4-14 and Fig. 4-27a).

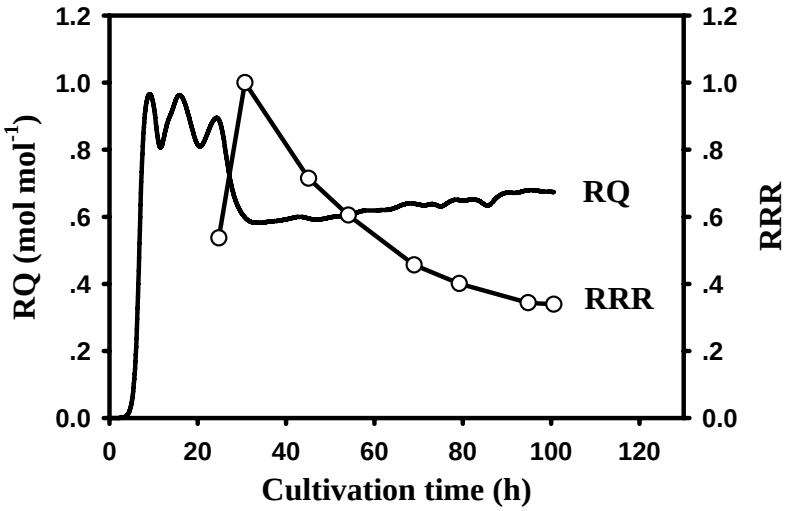

Figure 4-30 Relative rate of respiration (RRR; –○–) and respiratory quotient (RQ; line) from the HPFB process

In the HPFB process, the OUR and CPR were almost constant at 970 mmol h^{-1} and 630 mmol h^{-1}, respectively (Fig. 4-29). RQ was about 0.65 mol mol^{-1} (Fig. 4-30). RRR presented a similar profile as in MLFB process. Before 6 h of induction, the RRR was 1 which indicated there was no limitation of either oxygen or methanol. After that, it continuously declined to about 0.34 at the end due to methanol limitation (Fig. 4-30).

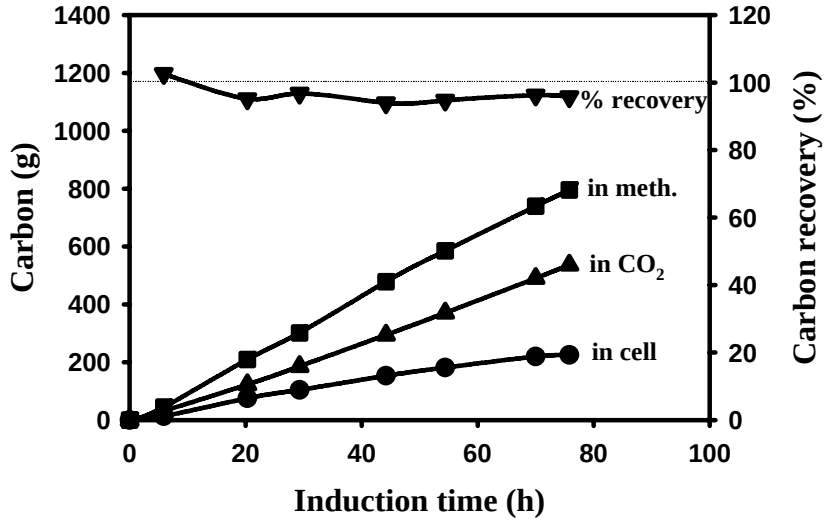

Figure 4-31 Carbon mass balance: carbon input in methanol (–■–), carbon output in cell (–●–) and CO2 (–▲–) and percent of carbon recovery (–▼–) from HPFB process

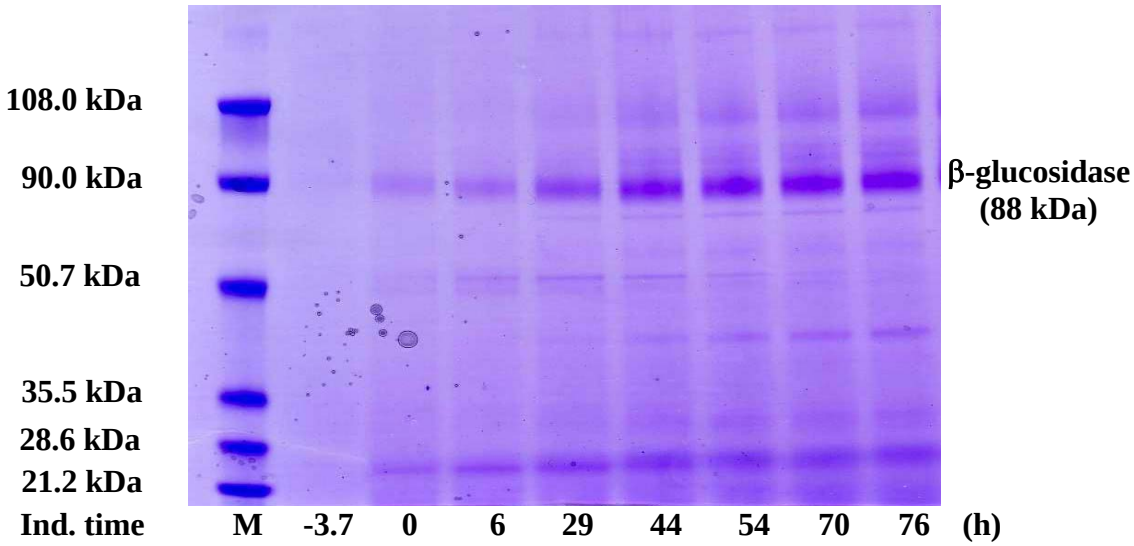

Figure 4-32 SDS-PAGE with Coomassie blue stain of fermentation supernatant at increasing time profile from HPFB process

In the HPFB process, more than 95% carbon was recovered (Fig. 4-31). Thus, higher pressure does not appear to stimulate any alternative by-product producing pathway in the methanol metabolism of *P. pastoris*.

SDS-PAGE of culture medium from the HPFB process showed β-glucosidase accumulation. It was the major product presented on the gel. The estimated molecular weight of this recombinant β-glucosidase under denaturing condition was approximately 88 kDa (Fig. 4-32).

CHAPTER V

DISCUSSION

The reference methanol limited fed-batch process at 1.2 bar air pressure and DOT = 25% air saturation (MLFB process) of β-glucosidase production with *P. pastoris* was first investigated. It showed that increasing the OTR might result in increasing the methanol uptake rate (MUR), which could possibly enhance the product formation. Two process techniques, the oxygen limited fed-batch process at 1.2 bar air pressure (OLFB process) and the methanol limited fed-batch process at 1.9 bar pressure and DOT = 25% air saturation (HPFB process) were designed to increase the OTR. The cell growth, cell viability and product formation from the two new fermentation techniques were compared with the traditional MLFB process.

5.1 The oxygen transfer rate

The OTR is the rate of oxygen transport from the gas bubble to bulk liquid. A general model showing parameters that influence the OTR is given by:

$$OTR = K_L a(C^* - C) \qquad (5\text{-}1)$$

K_L is a mass transfer coefficient (m h^{-1}) and *a* is the air bubble surface area per volume unit (m^{-1}). The combination of the two terms K_L and *a* is mostly used as a parameter for characterization of the oxygen transfer capacity of a reactor. K_La is called the volumetric oxygen transfer coefficient. C* is the oxygen concentration at the gas-liquid interface and C is the oxygen concentration in bulk liquid outside the film. The concentration difference between C* and C is the driving force of the diffusion ($\Delta C = C^*-C$; mol m^{-3}). Alternatively, the oxygen transfer capacity is expressed as K_La C*, which is mostly given the units mmol L^{-1} h^{-1} (Enfors and Häggström, 2000).

According to Henry's law, the oxygen concentration in the OTR model can be expressed in terms of the DOT

$$OTR = \frac{K_La}{H}(DOT^* - DOT) \qquad (5\text{-}2)$$

where H is a Henry's law derived constant that relates the oxygen concentration in g L^{-1} unit to the DOT in percent air saturation unit. DOT* is the maximum dissolved oxygen tension.

$$H = 100\frac{k_H}{P_{O2,cal}} \qquad (5\text{-}3)$$

where $P_{O2,cal}$ is the oxygen partial pressure in the air during conditions when the electrode was calibrated to 100%. k_H is Henry's constant, which is about 26 bar g^{-1} for pure water at 25°C and is equals to 8 mg L^{-1} or 0.25 mM oxygen solution.

In all processes, the DOT electrode was calibrated as 100% air saturation in 3 liters of glycerol basal salts medium pH 5.0 at the temperature of 30°C, aeration at 6 vvm, agitation at 1000 rpm and pressure of 1.2 bar.

During the protein production phase of the MLFB process, the DOT was controlled at 25% air saturation, which gave OTR as

$$OTR_{MLFB} = \frac{K_L a}{H}(100 - 25)$$

$$OTR_{MLFB} = 75 * \frac{K_L a}{H} \qquad (5\text{-}4)$$

In the OLFB process, the methanol feed rate during the protein production phase was controlled directly by the methanol signal to maintain the methanol concentration at 350 mg L^{-1}. This resulted in the reduction of the DOT to zero due to the increased driving force. Thus, the OTR in OLFB can be presented as

$$OTR_{OLFB} = \frac{K_L a}{H}(100 - 0)$$

$$OTR_{OLFB} = 100 * \frac{K_L a}{H} \qquad (5\text{-}5)$$

In the HPFB process, the total air pressure was increased from 1.2 to 1.9 bar at the beginning of the protein production phase and maintained at this pressure value until the end of the process. The DOT was controlled at 25% air saturation. To determine the effect of pressure on the DOT, after the DOT electrode was calibrated at 100%, the pressure was increased in the range of 1.2 to 2.0 bar. The DOT value was collected every 0.2 bar and plotted *versus* the total air pressure. The linear regression this data is presented in Fig. 5-1 which gives a DOT at 1.9 bar equal to 164% air saturation.

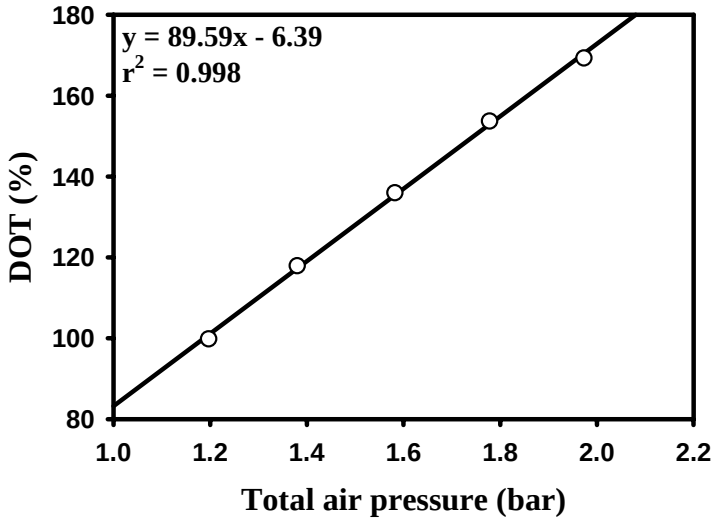

Figure 5-1 The relationship between total air pressure and DOT

From Fig. 5-1, DOT* was equal to 164% air saturation, so the OTR can be calculated as

$$OTR_{HPFB,eq.5-2} = \frac{K_L a}{H}(164 - 25)$$

$$OTR_{HPFB,eq.5-2} = 139 * \frac{K_L a}{H} \qquad (5\text{-}6)$$

The observed OUR during the protein production phase from the three processes are shown in Fig. 5-2 and Table 5-1. The highest value was observed in the HPFB process, followed by the OLFB process and the lowest was found in the MLFB process.

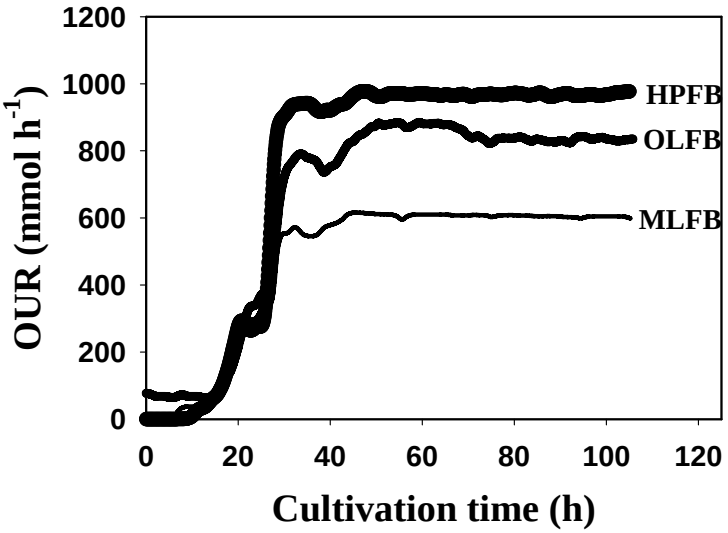

Figure 5-2 Oxygen uptake rate (OUR) in the reference process (MLFB), the oxygen-limited process (OLFB), and the high-pressure process (HPFB).

In the fermentation processes, the oxygen uptake rate (OUR, $q_o x$) can be presented as the OTR when the DOT is constant as

$$\frac{dDOT}{dt} = \frac{K_L a}{H}(DOT^* - DOT) - q_o x \qquad (5\text{-}7)$$

when the DOT is constant dDOT/dt = 0, thus

$$q_o x = \frac{K_L a}{H}(DOT^* - DOT) \qquad (5\text{-}8)$$

This can be used to confirm that the OTR in all processes (MLFB, OLFB and HPFB) followed *eq. 5-2*. The relative values calculated from *eq. 5-2* were compared with the relative observed OUR (Table 5-1).

The relative OTR calculated from *eq. 5-2* compared to the reference process (MLFB) were 133% and 185% for the OLFB and HPFB processes, respectively (Table 5-1). In the OLFB process, the relative value calculated from *eq. 5-2* (133%) was similar to the relative value from the experimental data (135%). Thus, increasing the OTR by increasing the driving force in OLFB process followed the general oxygen transfer rate model (*eq. 5-2*). However, in the HPFB process, the relative value calculated from *eq. 5-2* (185%) was much higher than the relative observed value (159%). This indicated that the elevation of the total air pressure does not only effect an increase in oxygen solubility, but also affects other parameters (Table 5-1 and *eq. 5-9)*.

Table 5-1 The OTR-values calculated from *eq. 5-2* and *eq. 5*-9 and experimental data expressed as absolute values and values relative to the control culture for the three process control strategies.

Parameters	**MLFB**		**OLFB**		**HPFB**	
	Observed values	Relative values (%)	Observed values	Relative values (%)	Observed values	Relative values (%)
OTR (*eq.5-2*)	75(K_La/H)	100	100 (K_La/H)	133	139(K_La/H)	185
OTR (*eq.5-7*)	-	-	-	-	121(K_La/H)	161
OTR experiment	608 ± 2 (mmol h^{-1})	100	821 ± 6 (mmol h^{-1})	135	966 ± 3 (mmol h^{-1})	159

The effect of air pressure on the OTR have been investigated by Yang and Wang (1992), who showed that the OTR both measured in sulfite solution and in *E. coli* culture was approximately directly proportional to the total pressure in the range of 1.06-2.72 bar. Thus, the K_La was not affected by air pressurization. On the other hand, Pinheiro et al. (2003) found that the OTR, determined by the sulfite oxidation method, increased less than in proportion about 30% and 42% when pressure was increased from 1.2 to 4 and 6 bar, respectively. This is in agreement with the observed OTR from the HPFB process, in which the OTR increased less than the proportionally to the increase driving force caused by the increase in oxygen solubility. The compressed air in the HPFB process might cause a counter-balancing effect by decreasing the specific air bubble area (a, m^{-1}) in K_La. If the gas volume compression is taken into account, the K_La should be proportion to the cubic root of the square of the pressure ratio. Then, the OTR becomes:

$$OTR = \frac{K_L a_{ref}}{H}\left(\frac{P_{ref}}{P}\right)^{2/3}\left(PDOT^{*}_{ref} - DOT\right) \qquad (5\text{-}9)$$

Thus, the OTR of the HPFB process when the change of gas volume compression is taken into account is as follows:

$$OTR_{HPFB,eq.5-7} = \frac{K_L a_{ref}}{H}\left(\frac{1.2}{1.9}\right)^{2/3}\left(1.9*100 - 25\right)$$

$$OTR_{HPFB,eq.5-7} = 121\frac{K_L a_{ref}}{H} \qquad (5\text{-}10)$$

The new OTR of the HPFB process calculated from *eq. 5*-9 is presented in *eq. 5-10*. The new obtained relative OTR value (161%) was similar to the experimental OTR (159%) (Table 5-1). This suggested that the air compression affect the *a* which caused a decrease in $K_L a$. Obviously, different investigations have resulted in different conclusions regarding the effect of air pressure on $K_L a$. The reason may be that the parameter *a* depends also on the air dispersion and bubble coalescence, which are likely to depend on the medium properties and fluid dynamic situation.

5.2 Total methanol uptake

The higher OTR in the OLFB and HPFB process compared with the reference MLFB process is reflected in a higher methanol consumption rates, which in each

process was approximately constantly during the 80 h of methanol feeding (Fig. 5-3). The total methanol uptake in the MLFB, OLFB and HPFB processes were 1414 ± 73, 1887 ± 11 and 2224 ± 104 g, respectively. The relative values of the total methanol uptake were almost proportional to the OTR, with 133% and 157% of that in the reference MLFB process in the OLFB and HPFB processes, respectively (Table 5-2). Thus, the increases of OTR obtained by the use of both the OLFB and HPFB techniques resulted not only in higher OUR, but also in a correspondingly higher methanol consumption rate. This was in agreement with recent work on mouse endostatin production (Trinh et al., 2003) and scFv production (Trentmann et al., 2004) where the methanol-saturation condition was applied for *P. pastoris* expression.

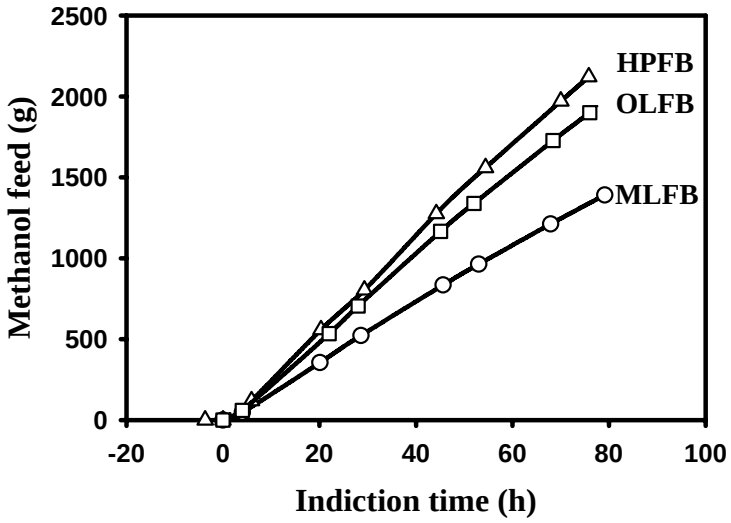

Figure 5-3 Comparison of the total methanol uptake in the MLFB process (—○—), OLFB process (—□—) and HPFB process (—△—)

Table 5-2 The experimental OTR from Table 5-1 and total methanol uptake expressed as absolute values and relative values in three process control strategies.

Parameters	MLFB		OLFB		HPFB	
	Observed values	Relative values (%)	Observed values	Relative values (%)	Observed values	Relative values (%)
OTR experiment	608 ± 2 ($mmol\ h^{-1}$)	100	821 ± 6 ($mmol\ h^{-1}$)	135	966 ± 3 ($mmol\ h^{-1}$)	159
Total meth. uptake	1414 ± 73 (g)	100	1887 ± 11 (g)	133	2224 ± 104 (g)	157

5.3 Cell growth

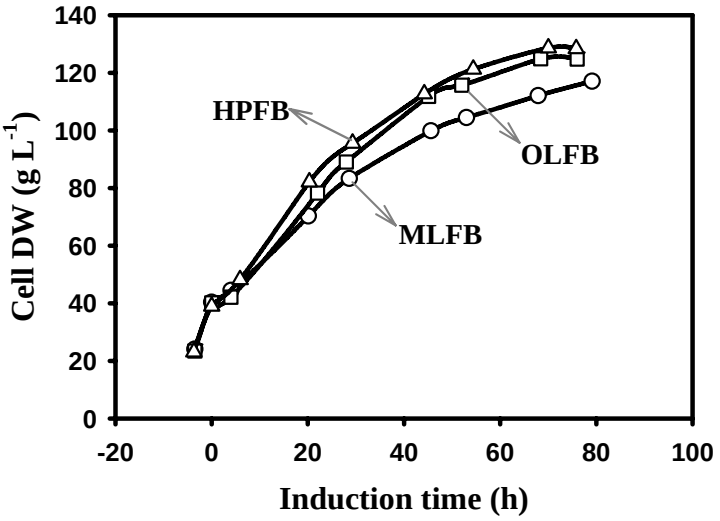

Figure 5-4 Comparison of the cell density in the MLFB (-○-), OLFB (-□-) and HPFB (-△-) processes

The biomass concentration in all processes resulted in similar profiles. The final cell density was highest in the HPFB process (131 ± 1 g L^{-1}), followed by the

OLFB process (125 ± 1 g L^{-1}). The lowest final cell density was seen in the MLFB process (116 ± 1 g L^{-1}) (Fig. 5-4 and Table 5-3). This was related to the specific growth rate (μ), as shown in Fig. 5-5. The highest value of μ was observed in the HPFB process and the lowest was seen in MLFB process.

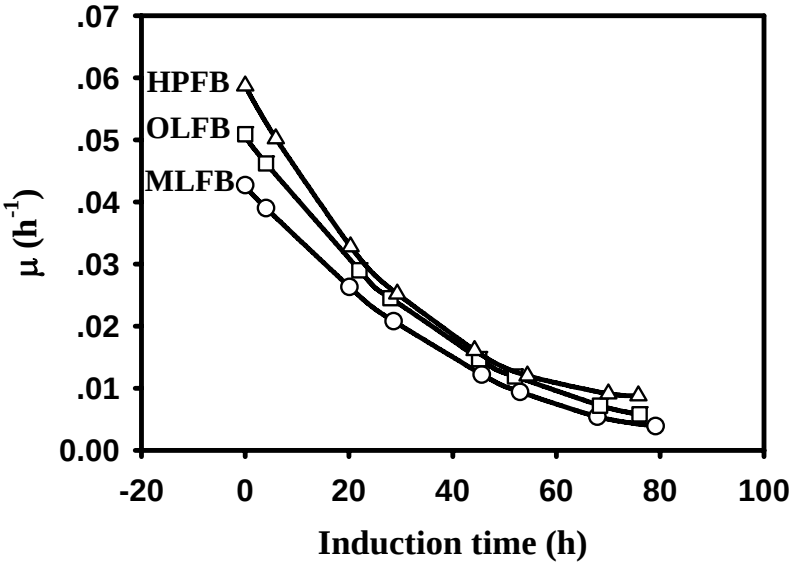

Figure 5-5 Comparison of the specific growth rate (μ) in the MLFB (-○-), OLFB (-□-) and HPFB (-△-) processes

Even though the cell density increased in both the OLFB and the HPFB processes compared to the standard MLFB process, it did not fully respond to the increased methanol utilization at higher OTR. Only 7% and 12% higher final cell mass was obtained in the OLFB and HPFB processes, respectively (Fig. 5-4 and Table 5-3). This indicates that some sort of stress was induced under the oxygen limiting (methanol toxicity and formaldehyde accumulation) and high air pressure conditions (reactive oxygen species, ROS).

Table 5-3 The experimental data of total methanol uptake from Table 5-2, maximum cell density, biomass yield, β-glucosidase accumulation and specific β-glucosidase activity expressed as absolute values and relative values in three process control strategies.

Parameters	**MLFB**		**OLFB**		**HPFB**	
	Observed values	Relative values (%)	Observed values	Relative values (%)	Observed values	Relative values (%)
Total meth. uptake	1414 ± 73 (g)	100	1887 ± 11 (g)	133	2224 ± 104 (g)	157
Cell DW	116 ± 1 ($g\ L^{-1}$)	100	125 ± 1 ($g\ L^{-1}$)	107	131 ± 3 ($g\ L^{-1}$)	112
Yx/s	0.37 ± 0.01 ($g\ g^{-1}$)	100	0.33 ± 0.01 ($g\ g^{-1}$)	89	0.29 ± 0.01 ($g\ g^{-1}$)	78
β-glucosidase	3761 ± 16 ($U\ L^{-1}$)	100	4807 ± 72 ($U\ L^{-1}$)	127	5204 ± 16 ($U\ L^{-1}$)	138
Total product yield	11404 ± 72 ($U\ process^{-1}$)	100	16125 ± 195 ($U\ process^{-1}$)	141	17082 ± 87 ($U\ process^{-1}$)	150
Specific activity	6.04 ± 0.29 ($U\ mg^{-1}$)	100	9.06 ± 0.53 ($U\ mg^{-1}$)	150	7.28 ± 0.24 ($U\ mg^{-1}$)	121
q_m	0.022 ($g\ g^{-1}\ h^{-1}$)	100	0.027 ($g\ g^{-1}\ h^{-1}$)	123	0.035 ($g\ g^{-1}\ h^{-1}$)	159

5.4 Biomass yield

The biomass yields (Yx/s) in all processes decreased over time. The final Yx/s in the MLFB, OLFB and HPFB processes were 0.37 ± 0.01, 0.33 ± 0.01 and 0.29 ± 0.01, respectively (Fig. 5-6 and Table 5-3). The reduced Yx/s may reflect an altered metabolism, such as formation of by-products from the carbon source, which

is often the case when cells are subject to oxygen limitation. However, *P. pastoris* is not known to metabolise methanol in the absence of oxygen. Further investigation to measure by-product formation can be performed by carbon mass balance.

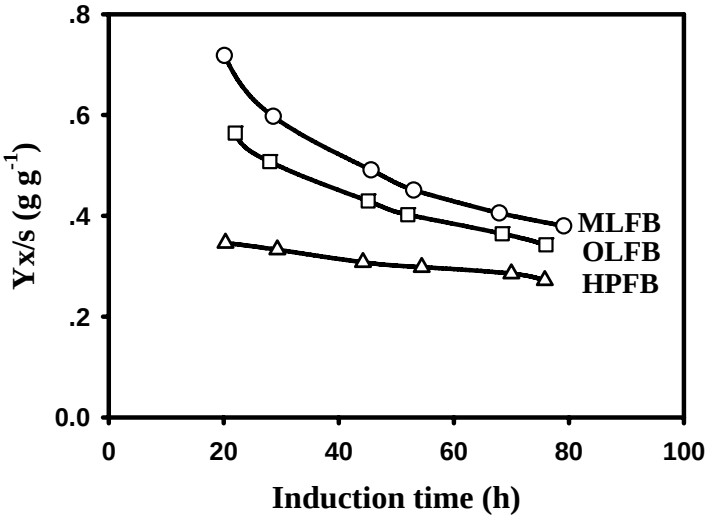

Figure 5-6 Comparison of the biomass yield from the MLFB (-○-), OLFB (-□-) and HPFB (-△-) processes.

Carbon mass balances based on methanol input and outputs of biomass and carbon dioxide in the medium were in good agreement in all processes as presented in chapter 4. During the main part of the process time, the carbon recovery was 98-99% (Fig. 5-7). The extracellular protein in the medium accounted for less than 0.4 % of the total carbon. Thus, no major by-products from the methanol metabolism were produced.

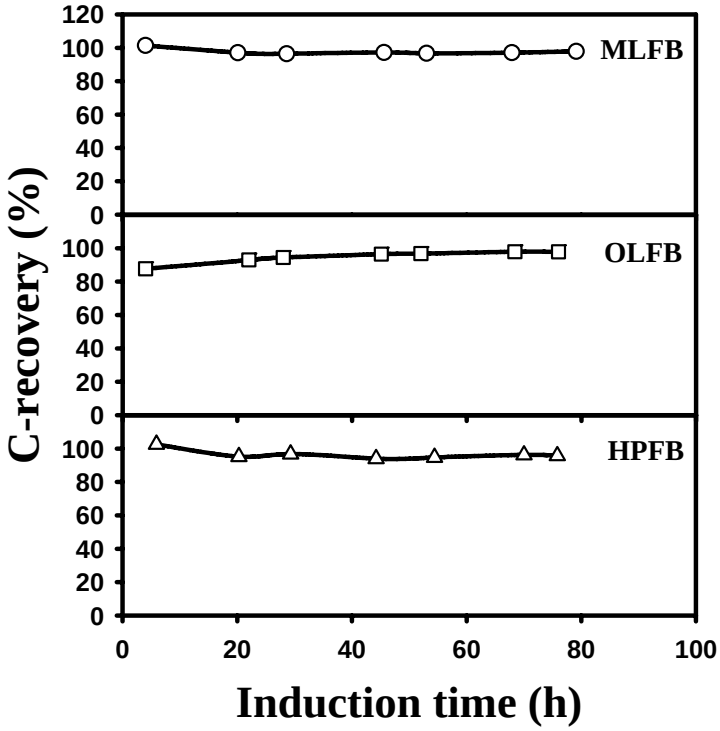

Figure 5-7 Comparison of the carbon recovery from the MLFB (upper panel), OLFB (middle panel) and HPFB (lower panel) processes

Alternatively, the reduction of the biomass yield might be due to increased maintenance demand, which was expressed as increased combustion of the energy source. The maintenance coefficient (q_m) has been suggested to be decisive for determining the cell density reached in fed-batch processes with constant feed (Jahic et al., 2002). Simulations of the biomass concentration profiles obtained in the present investigation were performed with the experimentally obtained methanol feed rates, using the model presented in Chapter II.

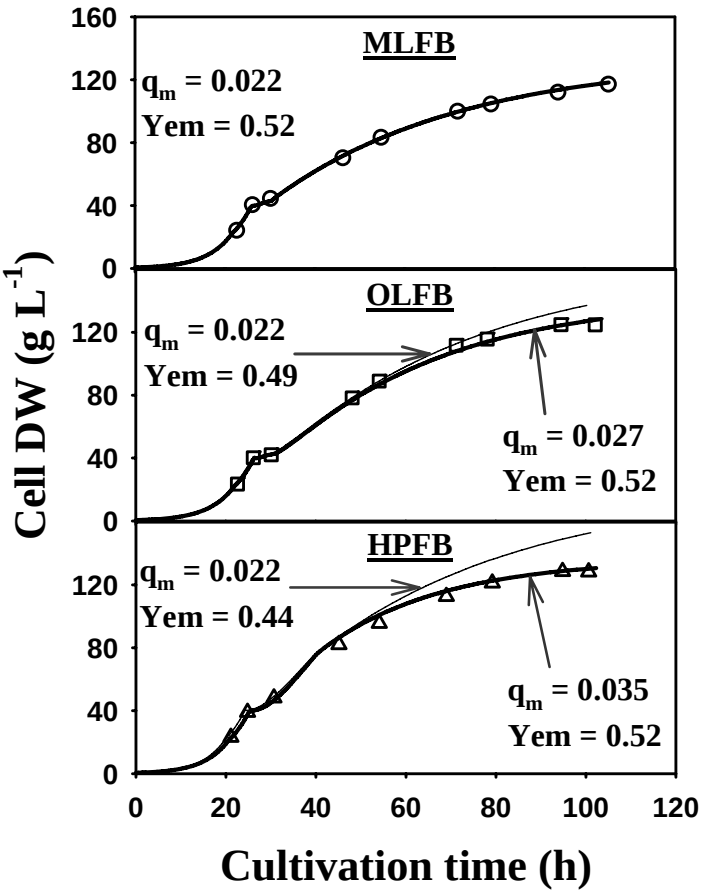

Figure 5-8 Comparison of the experimental cell growth (symbols) and simulation (thick lines) in the MLFB process (upper panel), the OLFB process (middle panel) and the HPFB process (lower panel). The thin continuous lines in the middle and lower panels represent simulations in which the maintenance coefficient (q_m) is kept unchanged and only the yield coefficient exclusive maintenance (Yem) is reduced to fit the data.

In the simulations of the biomass concentration profiles, all parameters were first fixed constant, and only q_m was changed to fit the data (thick lines in Fig. 5-8). The simulation data of all three processes fit well with the experimental data when the q_m were 0.022, 0.027 g g^{-1} h^{-1} and 0.035 g g^{-1} h^{-1} in the MLFB, OLFB and HPFB

processes, respectively (Fig. 5-8). However, q_m is not the only parameter affects biomass formation, since the biomass yield exclusive maintenance (Y_{em}) also affects biomass. Thus, the second simulation was performed by varying only Y_{em} to fit the data (thin lines). The alternative curves derived by varying Y_{em} did not fit the experimental data well (thin line in middle and lower panel of Fig. 5-8). The explanation is that the lower Yx/s is caused by the higher q_m. These results are in comparable with the previous observation in heavy-chain fragment C of boturinum neurotoxin production, where the Yx/s decreased and q_m increased with increased methanol concentration (Zhang et al., 2000).

5.5 Cell viability

Methanol is toxic to many organisms; however, methylotrophic organisms are more resistant to methanol. The methylotrophic yeast *P. pastoris* can with stand 10 g L^{-1} methanol with only a slight reduction of its specific growth and methanol uptake rates. But, the growth was almost completely inhibited when the methanol concentration reached 30 g L^{-1} (Katakura et al., 1998). However, the 350 mg L^{-1} of methanol in the OLFB process (Fig. 5-9, middle panel) should not show any inhibitory effect.

However, the low oxygen concentration in the OLFB might cause an accumulation of intracellular methanol, if it limits the oxidation rate of methanol. This is especially intriguing since *in vitro* assays of the AOX kinetics showed a very high K_m (22.4 mg L^{-1}) for oxygen at 10 mM (320 mg L^{-1}) methanol (Couderc and

Baratti, 1998), even though the oxygen solubility in pure water at 25°C is only 8 mg L^{-1}.

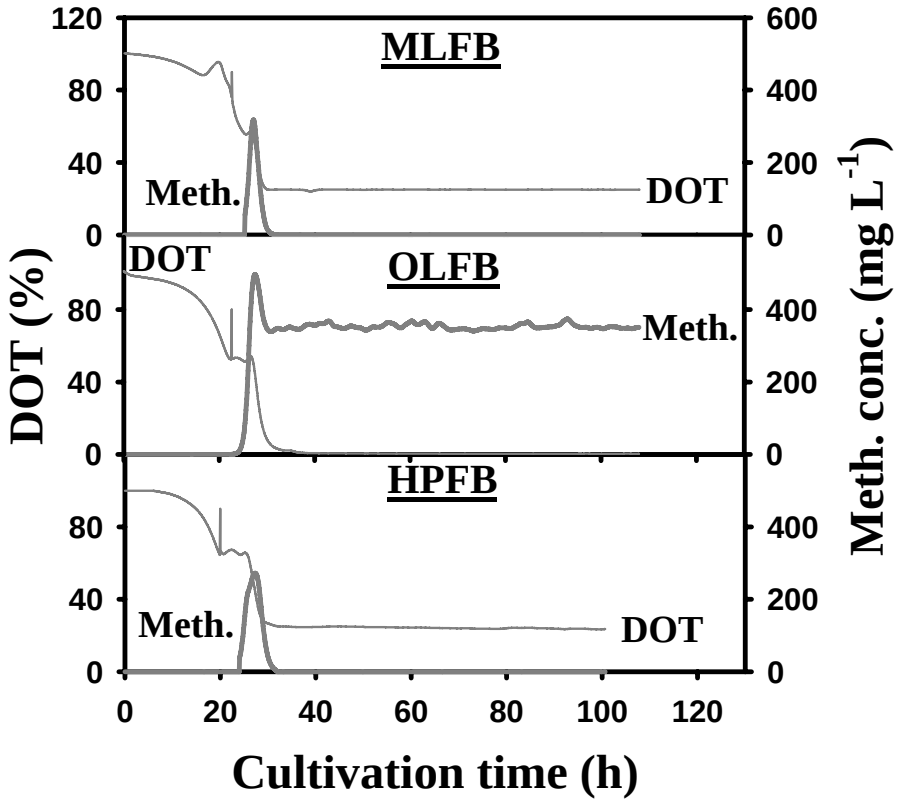

Figure 5-9 Comparison of the DOT (thin lines) and methanol concentration (thick lines) in the MLFB process (upper panel), OLFB process (middle panel) and HPFB process (lower panel)

Increasing the air pressure in the fermentation process increases not only the partial pressure of oxygen but also the carbon dioxide partial pressure. The increases in the carbon dioxide partial pressure would have resulted in increased carbon dioxide solubility, which would cause the reduction of the pH (Thiering et al., 2001). Furthermore, increasing C* by high pressure could perhaps have resulted in increasing toxic side-effects of reactive oxygen species (ROS) such as $O_2^{\bullet-}$, H_2O_2 and

$HO^{\bullet}$ (Izawa et al., 1995). These ROS attack almost all cell components, DNA, proteins and lipid membranes (Izawa et al., 1995; Toyokuni et al., 1995).

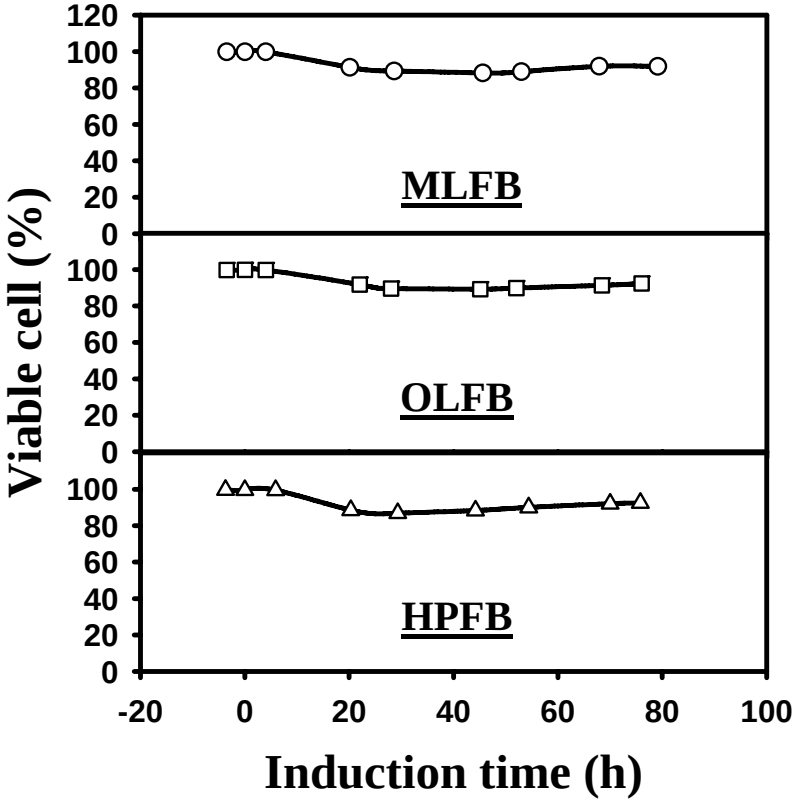

Figure 5-10 Comparison of the percentage of viable cells in the MLFB (upper panel), OLFB (middle panel) and HPFB (lower panel) processes.

The effect of both oxygen limitation in the OLFB process and the effect of ROS in the HPFB process may have some biological side-effects. Thus, in this research flowcytometry was used to observe the cell viability. This method has previously been used to reveal large differences in viability of *P. pastoris* cultures under different process conditions (Jahic et al., 2003). However, non-significant differences in percent viability were observed in all processes (Fig. 5-10). The frequency of viable cells dropped to about 90% during the first 22 h of induction, but

then stayed within 90-95% throughout the processes. Thus, the percent of cell death was significantly influenced by neither oxygen limitation nor high pressure condition.

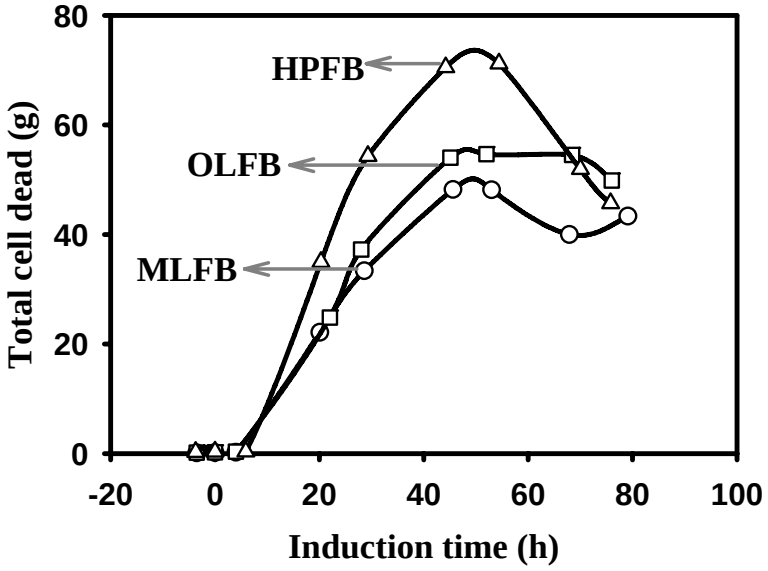

Figure 5-11 Total amount of dead cells during the methanol feed phase of the MLFB process (-○-), OLFB process (-□-) and HPFB process (-△-)

However, these viable cell values do not include the dead cells that lysed, which cannot be detected by the flowcytometry. Therefore, the total mass of dead cells was calculated from the percent of dead cell and cell dry weight data. The total amount of dead cells in all processes was highest at about 45 h after induction and then stabilized or decreased (Fig. 5-11). Only in the HPFB process was the total amount of dead cells significantly decreased, which means that cells must have lysed at least in this process, though it cannot be excluded that cell lysis also took place in the other processes.

5.6 Product formation

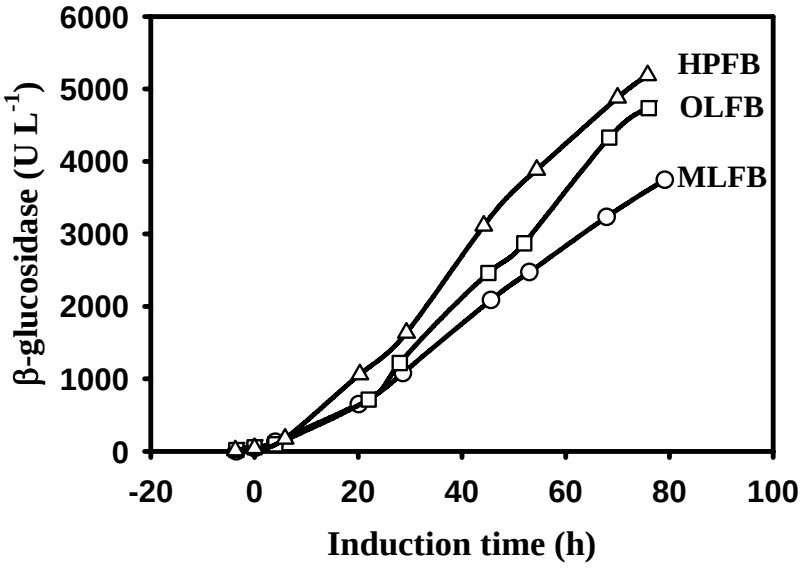

Figure 5-12 β-Glucosidase accumulation during the methanol feed phase of the MLFB process (-○-), OLFB process (-□-) and HPFB process (-△-)

The maximum β-glucosidase accumulation in the MLFB, OLFB and HPFB processes were 3761 ± 16, 4807 ± 72 and 5204 ± 16 U L^{-1}, respectively (Fig. 5-12 and Table 5-3). The product accumulation at different time period can also be visualized by SDS-PAGE analysis (Chapter IV). The β-glucosidase seems to be the main protein accumulated in the medium. The relative values of productivity responded more favorably to the increased OTR, while cell growth increased only slightly. The final enzyme yield (U $process^{-1}$) increased with 41% and 50% in the OLFB and HPFB processes, respectively (Table 5-3).

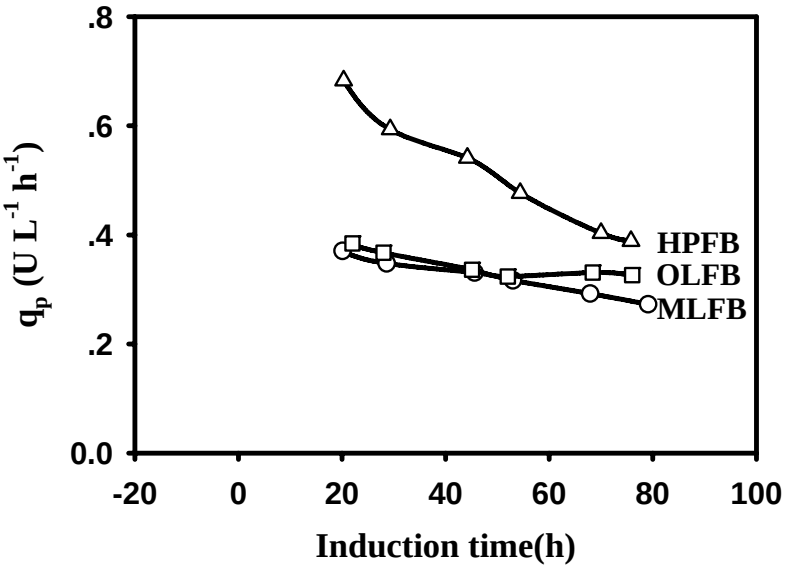

Figure 5-13 The specific β-Glucosidase production rate (q_p) during the methanol feed phase of the MLFB (-○-), OLFB (-□-) and HPFB (-△-) processes

In the MLFB and OLFB processes, the specific β-glucosidase productivity (q_p) declined slowly during the process, but in the HPFB process the decline rate was faster (Fig. 5-13). However, the q_p at the end of all processes was still high (0.27-0.39) compared with the initial values. This indicated that the process can be extended, even if the cell density does not increase so much. Similar observations have been seen in earlier studies on production of a fusion protein between a cellulose binding module and a lipase with *P. pastoris,* in which the specific productivity was even better preserved during 160 h (Jahic et al., 2002). Thus, both the stresses from oxygen limitation and high pressure conditions did not inhibit β-glucosidase accumulation. Similar results with the OLFB process have also been observed in production of other proteins (Trentmann et al., 2004; Hellwig et al., 2001).

5.7 Specific β-glucosidase activity

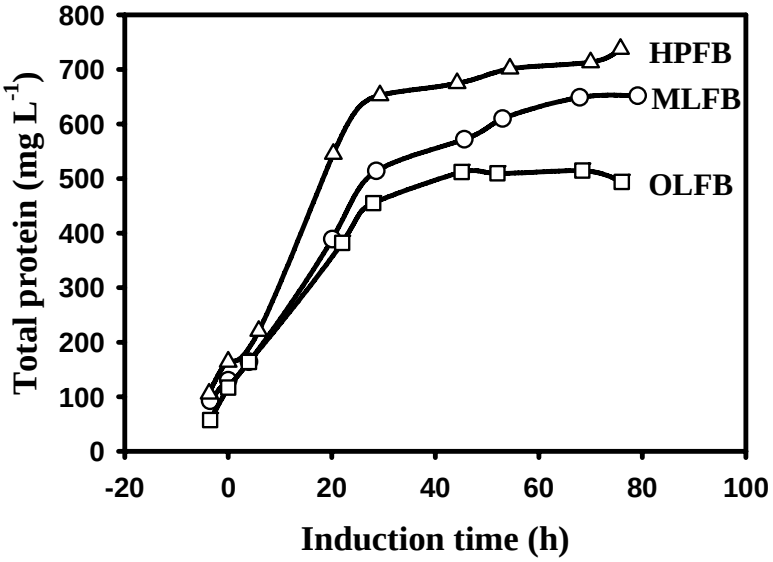

Figure 5-14 The total protein accumulation in the MLFB (-○-), OLFB (-□-) and HPFB (-△-) processes.

The specific enzyme activity is an alternative value that can be used to monitor the purity of the enzyme. The specific activity of the β-glucosidase in the broth was calculated from the β-glucosidase activity (Fig. 5-12 and Table 5-3) and the total protein concentration (Fig. 5-14). The specificity was 6.04 ± 0.29, 9.06 ± 0.53 and 7.28 ± 0.24 U mg^{-1} at the end of the MLFB, OLFB and HPFB processes, respectively (Fig. 5-15 and Table 5-3). The highest specific β-glucosidase activity was seen in the OLFB process, even though the highest β-glucosidase activity was present in the HPFB process. This was because of the lower total protein accumulation in the OLFB process. It cannot be ruled out that there is a difference in secretion of endogenous proteins from *P. pastoris* under the different process

conditions. A possible explanation is that there was less endogenous protein from cell lysis in the OLFB process (Fig. 5-11).

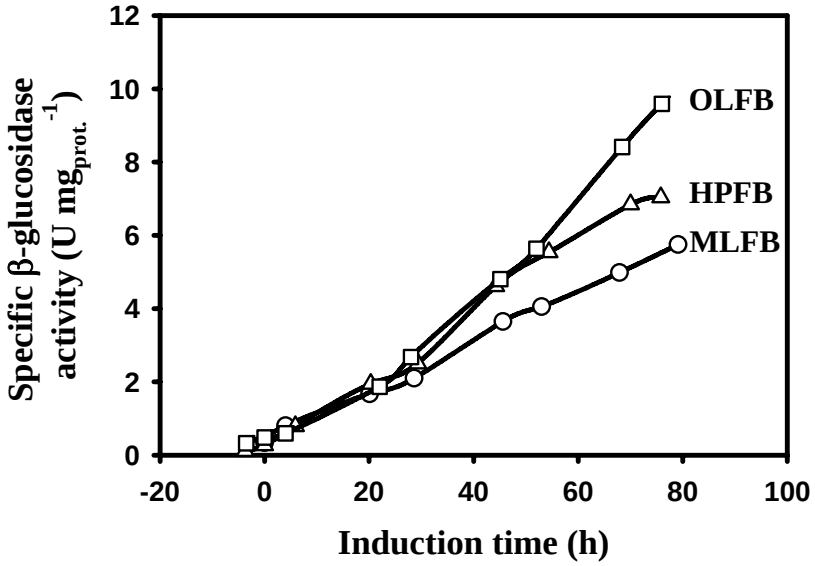

Figure 5-15 The specific β-Glucosidase activity in the MLFB (-○-), OLFB (-□-) and HPFB (-△-) processes.

5.8 The relative rate of respiration

The relative respiration rate (RRR) is the current respiration rate (q_O) divided by the maximum respiration rate ($q_{O,\ max}$). It is a parameter that can be used to describe the degree of energy limitation. For *P. pastoris*, which is obligately aerobic and has no alternative pathways for the methanol metabolism, RRR can be used to measure both the degree of oxygen and methanol limitation. The analysis showed that all processes were quite similar with respect to the degree of energy limitation (Fig. 5-16). At the beginning of production phase, all cultures were non-limited with respect to oxygen and methanol and RRR was 1. After that, this parameter dropped

rapidly and approached about 0.25-0.34, with a slightly lower value (i.e. more degree of energy limitation) for the OLFB process.

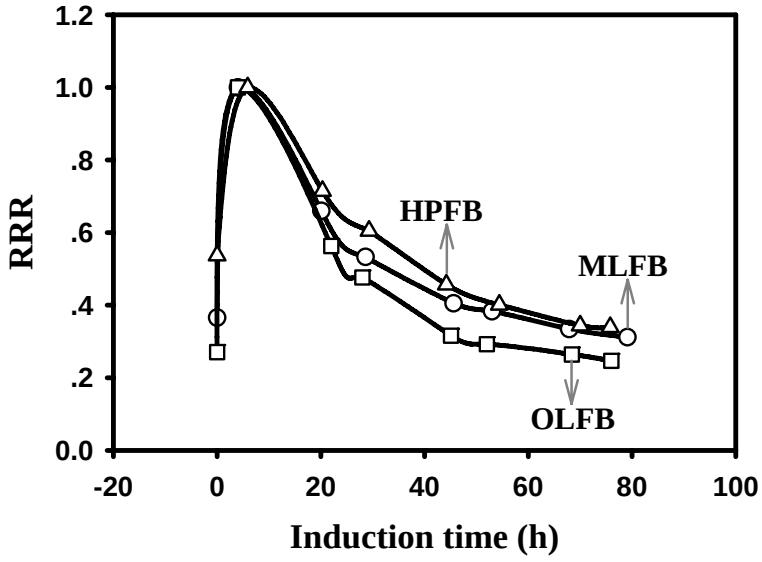

Figure 5-16 The relative rates of respiration (RRR) in the MLFB (-○-), OLFB (-□-) and HPFB (-△-) processes.

CHAPTER VI

CONCLUSION

This work shows that the oxygen transfer rate (OTR) is very important in processes of production of recombinant protein by *Pichia pastoris*. By comparison with the reference, the MLFB process, the OTR increased about 35% and 59% when the oxygen limitation in the OLFB process and elevated total air pressure in the HPFB process were applied, respectively. The total methanol consumption increased in proportion with the increase in the OTR. On the other hand, the biomass yield (Yx/s) diminished with increasing of the OTR, which resulted in less proportion increase of final biomass concentration. Only 7% and 12% biomass increased in the OLFB and the HPFB processes, respectively. The explanation is that the oxygen limitation and high pressure conditions led to an increase in the maintenance coefficient (q_m). The carbon mass balance analysis suggests that both oxygen limitation and high pressure conditions did not generate much alternative metabolism since no major by-product was detected by this analysis under these conditions. Furthermore, similar cell viability was observed in all processes. Product formation increased with the elevation of the OTR. The highest product accumulation was also attained in the HPFB process, which indicated it gave the highest process efficiency.

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APPENDICES

APPENDIX I

Culture medium

1. YPD (Yeast extract peptone dextrose) medium

Yeast extract	10 g
Peptone	20 g
Dextrose	20 g

Adjusted the pH of the solution to 5.5 with NaOH or H_3PO_4 and bring the volume up to 1 L with distilled water. Autoclaved for 20 minutes at 15 lb $sq.in^{-1}$. Let cool to 55°C and add desired antibiotics at this point. If the agar medium is desired, 15 g L^{-1} agar is added before autoclaving.

2. Buffered glycerol complex (BMGY) medium

Yeast extract	10 g
Peptone	20 g
Glycerol	10 g

Dissolve in 100 mM potassium phosphate buffer, pH 6.0, to the final volume of 1 L. Autoclaved for 20 minutes at 15 lb $sq.in^{-1}$. Let cool to 55°C and add desired filter-sterilized antibiotics at this point.

3. Glycerol basal salt (GBS) medium

H_3PO_4 85%	(26.7 ml)
$CaSO_4$	0.93 g
K_2SO_4	18.2 g
$MgSO_4.7H_2O$	14.9 g
KOH	4.13 g
Glycerol	40.0 g

Adjust the pH of the solution to 5.5 with NH_4OH and bring the volume up to 1 L with distilled water. Autoclave for 20 minutes at 15 lb $sq.in^{-1}$. After autoclaving add 12 ml of PTM1 trace salt.

4. Glycerol feed (GF) medium

Dissolve 500 g of 99.5% glycerol with distilled water and adjust the volume to 1 L. Autoclave for 20 minutes at 15 lb $sq.in^{-1}$. After autoclaving add 12 ml of PTM1 trace salts.

5. Methanol feed (MF) medium

Add 12 ml of PTM1 trace salts into 1 L of 99.9% methanol.

6. PTM1 trace salts

$CuSO_4.5H_2O$	6.0 g
KI	0.08 g
$MnSO_4.H_2O$	3.0 g
$Na_2MoO_4.2H_2O$	0.2 g
H_3BO_3	0.02 g
$ZnCl_2$	20.0 g
$FeCl_3$	13.7 g
$CoCl_2.6H_2O$	0.9 g
H_2SO_4	5.0 ml
Biotin	0.2 g

Dissolve in distilled water and adjust the volume to 1 L. Filter sterilization with a 0.45 μm sterile and store at room temperature.

APPENDIX II

Analytical methods

1. Cell concentration

Turbidity in the fermentation broth was measured with a spectrophotometer (Novaspec, LKB Biochrom) at 600 nm.

Table AII-1. Correlation of cell dry weight and OD_{600}

Cell dry weight (g L^{-1})	**OD_{600}**
0	0
1.620	3.82
3.113	6.70
4.427	12.30
5.740	15.10
6.807	16.10
7.840	19.30
13.897	32.70

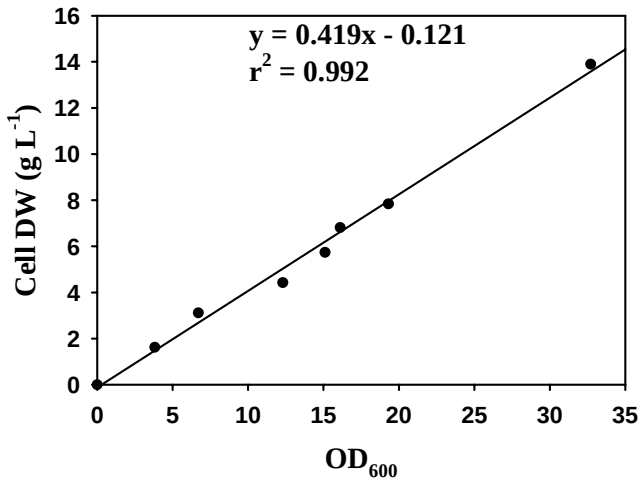

Figure AII-1. Standard curve of cell dry weight *versus* OD_{600}

The dry cell weight was determined from a 5 ml culture sample, which was centrifuged at 4000 rpm for 10 min. The supernatant was collected for other analyses, and the pellet was washed with distilled water once and dried at 105 °C for 24 h (each sample was taken in duplicate). Cell dry weight was calculated using the following equation.

$$\text{Cell DW (g L}^{-1}\text{)} = \frac{\text{weight of tube and dry cell weight (g)} - \text{weight of tube (g)}}{\text{volume of sample (ml)} \times 10^{-3}\text{(L ml}^{-1}\text{)}}$$

2. Methanol concentration and outlet gas analysis

The concentrations of oxygen, carbon dioxide and methanol in the outlet air were continuously analyzed using an outlet gas analyzer, Industrial Emissions

Monitor Type 1311 (Brüel & Kjær, Innova, Denmark), as described in Jahic et al. (2002). The oxygen and carbon dioxide concentration are presented in percentage of each gas in outlet air.

Table AII-2. Relationship between methanol concentration and analyzer signal

Methanol (g L^{-1})	**Analyzer signal**
100	11.47
200	19.98
300	30.88
400	41.55
500	50.53

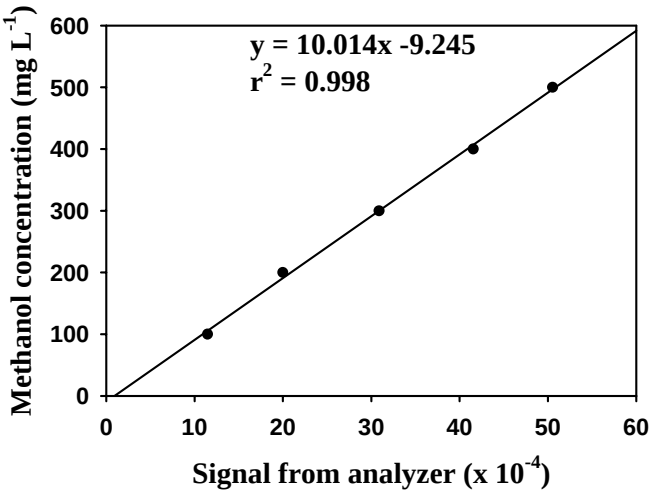

Figure AII-2. Standard curve of methanol concentration and gas analyzer signal

For the methanol concentration in culture broth, the calibration curve between methanol concentration in the water phase and the signal from the analyzer was required. The methanol signal was calibrated (at the stirrer speed, 1000 rpm; aeration rate, 6 L min^{-1}; and temperature 30^{o}C in liquid phase, applied in the process) by addition of aliquots of methanol to the fermentor in a 1 g L^{-1} NaCl solution before fermentation, as shown in Table AII-2 and Figure AII-2.

3. Protein concentration by Bradford assay (Bradford, 1976)

1. Reagents

1.1 Serva Blue G Dye

1.2 Bovine serum albumin (BSA) 1 mg ml^{-1}

1.3 Ethanol 95%

1.4 Phosphoric acid 85%

2. Solutions

2.1 Bradford stock solution

Ethanol 95%	100 ml
Phosphoric acid 85%	200 ml
Serva Blue G Dye	350 mg

Stable indefinitely at room temperature

2.2 Bradford working buffer

Distilled water	425 ml
Ethanol 95%	15 ml

Phosphoric acid 85%	30 ml
Bradford stock solution	30 ml

Filter through Whatman No. 1 paper, store at room temperature in brown glass bottle. Usable for several weeks, but may need to be refiltrated.

3. Assay

3.1 Pipet protein solution 300 μl in to tube.

3.2 Add 3 ml Bradford working buffer and vortex.

3.3 Read OD_{595} after 2 minutes but before 1 hour.

4. Generating a standard curve

Prepare the bovine serum albumin standard by diluting the 0.2 mg L^{-1}ml bovine serum albumin stock with distilled water, following Table AII-3.

Table AII-3. Dilution factor for preparation of standard solutions of BSA

BSA conc. (mg L^{-1})	**Standard BSA 0.2 mg ml^{-1} (ml)**	**Distilled water (ml)**
0	0	10
40	2	8
80	4	6
120	6	4
160	8	2
200	10	0

Table AII-4. Relationship of protein concentration and OD_{595} in Bradford assay

BSA conc. (mg L^{-1})	**$OD_{595}^{average}$**
0	0
40	0.114
80	0.223
120	0.339
160	0.434
200	0.517

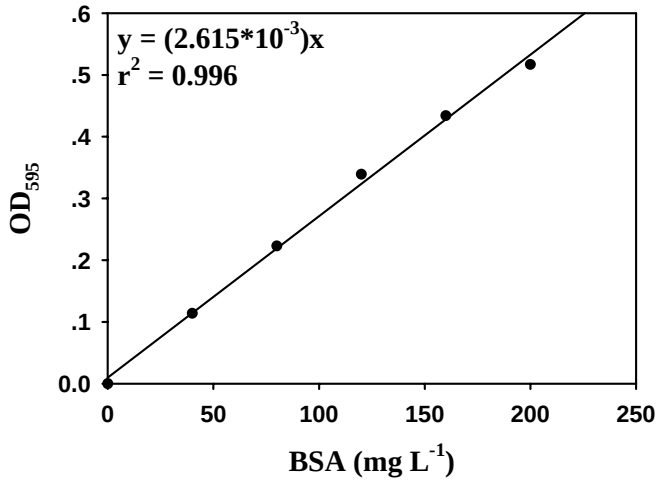

Figure AII-3. Standard curve of OD_{595} *versus* BSA concentration

4. β-glucosidase activity assay

β-glucosidase activity was assayed by the method of Evans (1985). This method uses a spectrophotometric assay to measure the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenol-β-D glucopyranoside (*p*NP-Glu).

4.1 Reagent

4.1.1 *p*-nitrophenol-β-D glucopyranoside (*p*NP-Glu)

4.1.2 CH_3COONa

4.1.3 CH_3COOH

4.1.4 Na_2CO_3

4.1.5 *p*-nitrophenol (*p*NP)

4.2 Solution

A. CH_3COONa 0.5 M: Dissolve 68.02 g of CH_3COONa with distillate water and adjust the volume to 1.0 L.

B. CH_3COOH 0.5 M: Dissolve 28.65 ml of CH_3COOH with distillate water and adjust the volume to 1.0 L.

C. Na-acetate buffer 0.1 M pH 5.0: Mix 14.1 ml of solution A and 5.9 ml of solution B and add distilled water to 100 ml.

D. *p*NP-Glu 3.3 mM: Dissolve 0.10 g of *p*NP-Glu with 100 ml of solution C.

E. Na_2CO_3 2 M: Dissolve Na_2CO_3 211.98 g with distilled water and adjust the volume to 1.0 L.

F. 0.1927 mM *p*NP: Dissolve *p*NP 0.0134 g in 500 ml of 0.01 M NaOH.

4.3 Assay

4.3.1 Incubate 0.9 ml of solution D at 30^oC.

4.3.2 Add 0.1 ml of sample (enzyme), incubate at 30^oC for 45 min

4.3.3 Add 2.0 ml of solution E to stop the reaction.

4.3.4 Under alkali conditions, the liberated *p*NP could be quantitated spectrophotometrically by measuring the absorbance at 400 nm.

4.4 Calculation

$$\text{Enzyme activity} = \frac{[p\text{NP}]}{45 \times 0.10 \times 1000} \quad (\mu\text{mol min}^{-1}\ \text{ml}^{-1} \text{ or U ml}^{-1})$$

Table AII-5. *p*NP standard solutions

***p*NP concentration (μM)**	**0.01 M NaOH (ml)**	**0.1927 mM *p*NP stock solution (ml)**
0	5	0
19.27	4.5	0.5
38.53	4	1
77.07	3	2
115.60	2	3
192.68	0	5

4.5 Generating the standard curve of *p*NP

The *p*NP standard solution is prepared following Table AII-5. Then add 2.0 ml of 2 M Na_2CO_3 before measured at OD_{400}.

Table AII-6. Stand curve of *p*NP

*p*NP concentration (μM)	OD_{400}
0	0
19.27	0.131
38.53	0.254
77.07	0.501
115.60	0.744
192.68	1.213

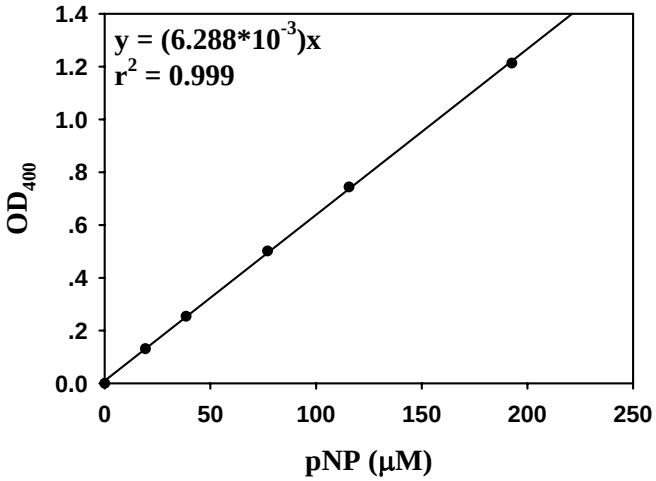

Figure AII-4. Standard curve of *p*NP

$$[pNP] = \frac{OD_{400} \text{ x dilution factor}}{\text{Slope}}$$

5. SDS-PAGE

5.1 Materials for SDS-PAGE

5.1.1 PMSF (0.871% w/v in ethanol)

5.1.2 DTT (0.5 M)

5.1.3 NuPAGE® Novex 4-12% Bis-Tris Gel (1.0 mm x 10 well) (Invitrogen)

5.1.4 Protein loading buffer (NP 0007, Invitrogen)

5.1.5 Protein marker (161-0305, Biorad)

5.1.6 Protein samples

5.1.7 Running buffer (MOPS buffer)

5.2 Solutions preparation

5.2.1 Running buffer (MOPS buffer) (X20)

MOPS	104.6	g
Tris-base	60.6	g
SDS	10.0	g
EDTA	3.0	g

Store at $4^{o}C$

5.2.2 Loading buffer mixer

Protein loading buffer (NP 0007, Invitrogen)	250	µl
DTT (0.5 M)	100	µl

PMSF (0.871% w/v in ethanol)	50	µl
Total volume	400	µl

5.2.3 Coomassie blue R-250 staining solution

Coomassie blue R-250	0.24	g
Methanol	400	ml
Glacial acetic acid	70	ml

Adjust volume to 1 L with distilled water.

5.2.4 Coomassie destain solution

Methanol	100	ml
Glacial acetic acid	100	ml

Adjust volume to 1 L with distilled water.

5.3 SDS-PAGE

5.3.1 Sample preparation

Loading buffer mixer	20	µl
Sample	30	µl
Total volume	50	µl

Heat at 95^{o}C for 10 min. Spin down at 4000 rpm for 30 s.

5.3.2 Gel preparation

-Rinse the gel with DI-water.

-Peel tape and comb out.

-Rinse each well with running buffer 3 times.

5.3.3 Set up running apparatus

Add 1X MOPS buffer in inner chamber.

5.3.4 Load each sample 10 μl in each well (marker 5 μl).

5.3.5 Add MOPS buffer in outer chamber.

5.3.6 Run gel at 200 V constant about 45-60 minute.

5.3.7 Stain gel with Coomassie Blue R-250 stained solution for 30 minutes.

5.3.8 Destained with destain solution for 1-2 hours.

6. Viable cells

Cell viability was determined with a Partec PAS flow cytometer (Partec GmbH, Mu”nster, Germany) equipped with a 488 nm argon laser.

6.1 Reagents

6.1.1 Propidium Iodide (PI) (P 4170, Sigma)

6.1.2 Fluorescent beads 3 μm diameter (Standard 05-4008, Partec GmbH, Germany)

6.2 Solutions

6.2.1 Dulbeccos buffer saline (DBS) (1 l)

NaCl	9.35	g

KCl	0.224 g
Na_2HPO_4	1.423 g
KH_2PO_4	0.136 g

Adjust pH to 7.3 with HCl.

6.2.2 Stock solution PI 200 μg ml^{-1} in distilled water.

6.3 Methods

6.3.1 Dilute the samples with DBS to a concentration about 10^6 cell ml^{-1}.

6.3.2 Transfer 975 μl of sample to a sample tube.

6.3.3 Add 25 μl PI stock solutions.

6.3.4 Mix gentle by vortex.

6.3.5 Analyze with flow cytometer (For *P. pastoris* a sample speed of 1-1.5 μl s^{-1} and count rate 1000-1500 cell s^{-1}.), a total count of 50,000 were collected for each sample. Measurement was calibrated using 3 μm diameter fluorescent beads. PI positive cells were considered as dead and the PI negative cells were considered as viable.

7. Alcohol oxidase activity

Alcohol oxidase (AOX; EC 1.1.3.13) was assayed with the method described by Jahic et al. (2002). Samples from the fermentation (5 ml) were centrifuged at

4200 rpm (1400 x *g*), 4^{o}C for 10 min. The volume of the supernatant was measured. The cell pellet was washed once with 5 ml of 0.1 M potassium-phosphate buffer, pH 7.5, and resuspended in the same buffer with the original supernatant volume. The cells were then disintegrated in a French press (SLM Aminco, USA) at 800 bars. One millilitre of 0.1 M potassium-phosphate buffer, pH 7.5, with 2000 units of catalase (from bovine liver, Sigma-Aldrich, Sweden) and 10 µl of cells homogenate were mixed. The AOX is inhibited by, hydrogen peroxide (H_2O_2), which is produced. Therefore, to assure that the H_2O_2 produced does not inhibit the AOX reaction, the H_2O_2 was converted to molecular oxygen and water by catalase. This makes the stoichiometry of the reaction 1 mole O_2 per 2 moles of CH_3OH. The reaction was started by adding 10 µl of 10 M methanol. Oxygen consumption was assayed polarographically with a Clark type oxygen electrode (Medelco AB, Sweden) at 37^{o}C in air-saturated buffer. Alcohol oxidase units were expressed as a µmol of methanol oxidised per minute. Specific AOX activity of the enzyme was expressed as units per gram of cell DW.

APPENDIX III

Glycerol feed profile

1. Peristaltic pump calibration

Table AIII-1. Relationship of pump speed and GF medium feed rate

Pump speed (%)	GF medium feed rate (ml h^{-1})
10	18.2
15	36.3
20	54.6
25	72.8
30	88.8

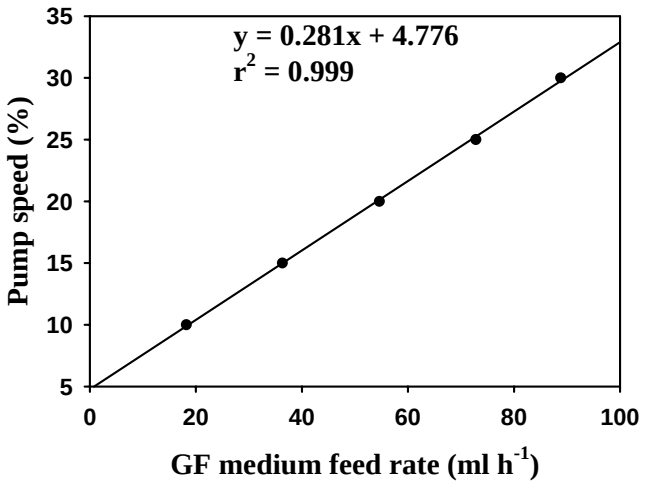

Figure AIII-1. Calibration curve of pump speed and GF medium feed rate

2. Glycerol feed profile

From

$$X_t = X_0 e^{\mu t}$$

$$F_t = F_0 e^{\mu t}$$

$$F_0 = (\mu X_0 V_0)/(Y_{x/s} S_i)$$

Where μ $= 0.18\ h^{-1}$ X_0 $= 25.21\ g_{cell}\ L^{-1}$

V_0 $= 3\ L$ $Y_{x/s}$ $= 0.7\ g_{cell}\ g_{glycerol}^{-1}$

S_i $= 500\ g_{glycerol}\ L^{-1}$

So that

F_0 $= (0.18 \times 25.21 \times 3)/(0.7 \times 500) = 38.89\ ml\ h^{-1}$

F_1 $= 38.89 \times e^{(0.18 \times 1)} = 46.56\ ml\ h^{-1}$

$\%_0$ $= (0.2812 \times 38.89) + 4.7758 = 15.712\%$

$\%_1$ $= (0.2812 \times 46.56) + 4.7758 = 17.868\%$

Table AIII-2. Glycerol feed profile

Time (h)	Cell (g L^{-1})	Glycerol feed (ml h^{-1})	% pump
0	25.51	38.89	15.71
1	30.18	46.56	17.87
2	36.13	55.74	20.45
3	43.26	66.74	23.54
4	51.79	79.90	27.24

APPENDIX IV

Publication I

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ORIGINAL PAPER

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Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes

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Abstract An oxygen-limited fed-batch technique (OLFB) was compared to traditional methanol-limited fed-batch technique (MLFB) for the production of recombinant Thai Rosewood β-*glucosidase* with *Pichia pastoris*. The degree of energy limitation, expressed as the relative rate of respiration ($q_O/q_{O,max}$), was kept similar in both the types of processes. Due to the higher driving force for oxygen transfer in the OLFB, the oxygen and methanol consumption rates were about 40% higher in the OLFB. The obligate aerobe *P. pastoris* responded to the severe oxygen limitation mainly by increased maintenance demand, measured as increased carbon dioxide production per methanol, but still somewhat higher cell density (5%) and higher product concentrations (16%) were obtained. The viability was similar, about 90–95%, in both process types, but the amount of total proteins released in the medium was much less in the OLFB processes resulting in substantially higher (64%) specific enzyme purity for input to the downstream processing.

Keywords Oxygen-limited fed batch (OLFB) · Methanol-limited fed batch (MLFB) · *Pichia pastoris* · β-glucosidase

List of symbols

AOX	Enzyme alcohol oxidase
AOX1	Alcohol oxidase gene 1
CPR	Carbon dioxide production rate (mol h^{-1})
DOT	Dissolved oxygen tension (%)
MLFB	Methanol limited fed-batch
OLFB	Oxygen limited fed-batch
OUR	Oxygen uptake rate (mol h^{-1})
PI	Propidium iodide
q_o	Specific oxygen uptake rate (mol g_{cell}^{-1} h^{-1})
$q_{o,max}$	Maximum specific oxygen uptake rate (mol g_{cell}^{-1} h^{-1})
q_p	Specific β-*glucosidase* productivity (U g_{cell}^{-1} h^{-1})
Q_i	Inlet air flow rate (L h^{-1})
Q_o	Outlet air flow rate (L h^{-1})
RRR	Relative rate of respiration
V	Medium volume (L)
V_m	Molar volume of gas (L mol^{-1})
X	Biomass concentration from dry weight (g L^{-1})
$Y^C_{CO2/S}$	Carbon yield coefficient of carbon dioxide from methanol (mol mol^{-1})
$Y^C_{X/S}$	Carbon yield coefficient of biomass from methanol (mol mol^{-1})

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Introduction

Pichia pastoris is a methylotrophic yeast that is often genetically engineered to express proteins [1]. It is suited for foreign protein expression for three main reasons: it can be easily manipulated at the molecular genetic level; it can express and secrete proteins at high levels; and it can perform many of the 'higher eukaryotic' protein modifications such as glycosylation, disulfide-bond formation and proteolytic processing [2].

Pichia pastoris can be grown to very high cell densities (more than 130 g dry cell weight L^{-1}) [3, 4]. It also contains a tightly methanol-controlled alcohol oxidase (*AOX1*) promoter that is induced by methanol and can be used to drive expression of foreign genes [5]. The strong promoter, coupled with the high cell-density fermentation, has allowed production of recombinant product at very high levels. Product concentrations can

reach 22 g L^{-1} for intracellular production [6] and 14.8 g L^{-1} of clarified supernatant for secretion protein production [7].

In recombinant protein production by *P. pastoris*, a four-stage fermentation protocol has been suggested [4]. After an initial batch phase on glycerol to produce biomass, an exponential glycerol feed with glycerol-limiting concentration is applied for a short period to derepress the *AOX1* promoter [8]. Then a low but increasing methanol feed is applied matching the increasing AOX activity caused by the induction [9]. This results in increasing oxygen uptake rate, and when DOT reaches about 25% air sat., the methanol feed rate is kept constant during the main production phase to avoid oxygen limitation. Under these conditions the concentration of methanol is very low and growth-rate-limiting.

Several papers describe the effect of methanol concentration on *P. pastoris*. At low concentration (< 3–5 g L^{-1}) the specific growth rate exhibits typical Monod kinetics [10], but at higher concentrations substrate inhibition is observed [10–12]. However, in spite of the methanol-inhibiting effects on growth and substrate uptake observed by Katakura et al. [12], the specific rate of production of a human β_2-glycoprotein *I* domain *V* fragment increased considerably. This might be due to a higher *AOX1* promoter activity at the higher methanol concentration but no AOX data were presented in this paper. However, high methanol concentration cannot be kept in high cell-density cultures without oxygen limitation or temperature limitation [9]. Facultative organisms like *Saccharomyces cerevisiae* and *E. coli* switch to anaerobic metabolism and accumulate toxic metabolites when exposed to oxygen limitation. Such responses have been suggested to play a major role in the scale-up responses of such organisms [13]. The information of *P. pastoris* response to oxygen limitation when growing on methanol is limited. Trentmann et al. [14] compared two *P. pastoris* cultivation techniques, methanol-limited and methanol-satzurated, with oxygen limitation cultures. The recombinant scFv protein quality and productivity were higher in the methanol-saturated processes. On the other hand, no significant difference was found when both techniques were applied for mouse endostatin production [15].

The methanol metabolism of *P. pastoris* has been reviewed by Lin Cereghino and Cregg [16] and the methanol and oxygen consumption has been modeled by Jahic et al. [4]. The pathways summarizing the metabolism of methanol in the methylotrophic yeast *P. pastoris* are shown in Fig. 1. Molecular oxygen is not only used for the respiration but also for the initial oxidation of methanol to formaldehyde. This reaction, catalyzed by the AOX enzyme, generates hydrogen peroxide that can also be used by AOX for methanol oxidation. Thus, two potentially toxic metabolites are generated in the cell during the initial methanol oxidation [5, 17, 18]. Of these components, formaldehyde could be expected to accumulate in the cells with detrimental effect when the cells are exposed to oxygen limitation.

In this work we investigate the possibility to run fed-batch cultures with higher methanol concentration under oxygen limitation. Oxygen-limited fed-batch (OLFB) was compared with methanol-limited fed-batch (MLFB) for the production of the Thai Rosewood β-*glucosidase*.

Materials and methods

Strain and plasmid

The *P. pastoris* strain Y-11430 (wild-type strain) was a gift from J. Lin Cereghino [16]. β-*glucosidase* cDNA gene from Thai Rosewood (*Dalbergia cochinchinensis* Pierre) [20] was cloned into the *pPICzα B* vector (Invitrogen). The *pPICzα B* with the β-*glucosidase* gene was then integrated in to *P. pastoris* Y-11430 at the *AOX1* promoter.

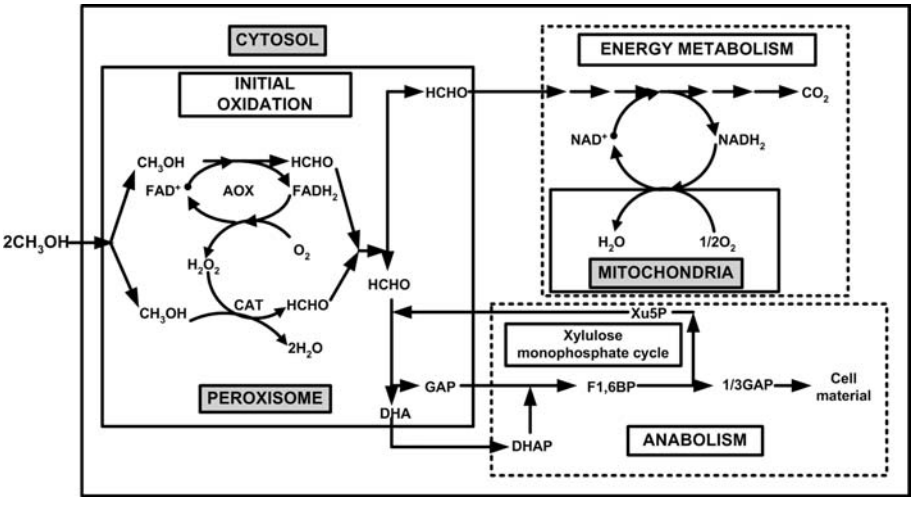

Fig. 1 Methanol metabolism in *P. pastoris*: *AOX* alcohol oxidase; *CAT* catalase; *GAP* glyceraldehyde-3-phosphate; *DHA* dihydroxyacetone; *DHAP* dihydroxyacetone phosphate; *F1,6BP* fructose-1,6-bisphosphate; *Xu5P* xylulose-5-phosphate. Adapted from Douma et al. [19]

Inoculum preparation

The first inoculum culture was prepared from one colony of *P. pastoris* on YPD agar (yeast extract 10 g, peptone 20 g and dextrose 20 g in 1 L of deionized water) containing 100 μg zeocin ml^{-1} suspended in 20 ml YPD broth containing 100 μg zeocin ml^{-1}. The culture was incubated at 30° C, in a 100-ml baffled shake-flask on rotary shaker with 200 rpm for 24 h. A second inoculum culture was prepared by transferring the entire 20 ml of first inoculum into 1,000-ml baffled shake-flask that contained 80 ml BMGY medium (yeast extract 10 g, peptone 20 g and glycerol 10 g; dissolved in 1 L of 0.1 M potassium phosphate buffer pH 6.0). The culture was then incubated under the same condition as the first inoculum culture for 24 h.

Fed-batch fermentation

The fed-batch fermentation was carried out in a 10-L stirred tank bioreactor (Belach Bioteknik AB, Stockholm) which contained 3.0 L of glycerol basal salts (GBS) medium (containing H_3PO_4 85% 26.7 ml; $CaSO_4$ 0.93 g; K_2SO_4 18.2 g; $MgSO_4.7H_2O$ 14.9 g; KOH 4.13 g; glycerol 40.0 g; PTM1 trace salts 4.35 ml in 1 L of deionized water). The PTM1 trace salts contained: $CuSO_4.5H_2O$ 6.0 g; KI 0.08 g; $MnSO_4.H_2O$ 3.0 g; $Na_2MoO_4.2H_2O$ 0.2 g; H_3BO_3 0.02 g; $ZnCl_2$ 20.0 g; $FeCl_3$ 13.7 g; $CoCl_2.6H_2O$ 0.9 g; H_2SO_4 5.0 ml; biotin 0.2 g in 1 L of deionized water. The fermentation was controlled under the following conditions: temperature 30° C, aeration 6 L min^{-1}, agitation 1,000 rpm and pH 5.0. Ammonia solution 25% was used to control pH and addition of antifoam A (A-5758, Sigma) was controlled by a level electrode.

A four-stage fermentation protocol was used in this study: The first stage was a glycerol batch phase. About 24 h after inoculation when the glycerol was completely consumed as indicated by the DOT signal, the process was switched to glycerol fed batch with a glycerol feed (GF) medium (glycerol 500 g L^{-1} and PTM1 trace salts 12 ml L^{-1}) added to the bioreactor at an exponentially increasing rate of 0.18 h^{-1} starting with 35.5 ml h^{-1}. After 3–3.5 h when the cell density reached 40 g L^{-1} ($OD_{600} = 80$), the process was switched to the third phase (methanol induction phase) by replacing the GF medium with the methanol feed (MF) medium (12 ml PTM1 trace salts per liter of methanol). The initial MF medium feed rate was constant at about 10 ml h^{-1} for 2–3 h until the production phase. The MF medium was then fed into the bioreactor with different strategies (MLFB or OLFB).

Methanol feed control

In the MLFB, the DOT was kept at 25% air sat. by means of a feedback control of the methanol feed rate based on the DOT signal (Fig. 2a). In the OLFB, the methanol concentration was kept at 350 mg L^{-1} by means of feedback control of the methanol concentration from methanol analysis (Fig. 2b).

Analyses

Cell concentration and viability

Cell concentration was monitored by measuring the optical density at 600 nm (OD_{600}). Dry cell weight was determined by centrifugation of 5 ml of culture broth at 4,500 rpm for 10 min, and the supernatant was collected for the analysis of other compounds. The pellet was washed with distilled water once and dried at 105° C, till constant weight.

The viability was measured by staining with propidium iodide (PI; Sigma, P-4170). A Partec PAS flow cytometry (Partec GmbH, Münster, Germany) equipped with a 488-nm argon laser was used for this analysis. Samples taken from the fermentor were diluted with PBS (0.16 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4 and 0.001 M KH_2PO_4, pH 7.3). For staining, 25 μl of a stock solution containing 200 μg ml^{-1} of PI dissolved in water was added to 975 μl of diluted sample at room temperature. Samples were then analyzed, at a data rate of about 1,500 counts s^{-1}. A count of 50,000 was collected in each measurement. The measurement was calibrated by using 3 μm diameter fluorescent beads (Standard 05–4008, Partec GmbH). PI-positive cells were considered as dead and the PI-negative cells were considered as viable.

Total protein concentration

The total protein concentration in the supernatant was analyzed according to Bradford [21]. Bovine serum albumin was used as standard protein.

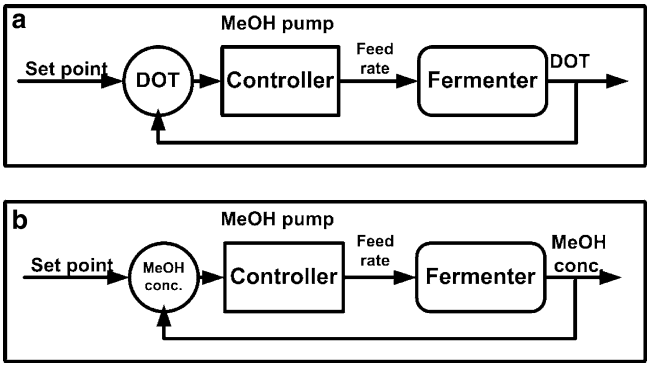

Fig. 2 Block diagram of the methanol feed control: **a** DOT regulation for a methanol-limited fed-batch (*MLFB*) process and **b** methanol concentration regulation for an oxygen- limited fed-batch (*OLFB*) process

β-glucosidase activity

β-glucosidase activity was assayed by Evans method [22]. This method used spectrophotometric assay to measure the release of *p*-nitrophenol (pNP) from *p*-nitrophenol-β-D glucopyranoside (pNP-Glu) by β-glucosidase reaction. The 3.3 mM pNP-Glu in 0.1 M sodium acetate buffer pH 5.0 was used as a substrate. One unit of enzyme was defined as the amount of enzyme releasing 1 µmol pNP per minute at 30° C, pH 5.0.

Methanol feed

Methanol feed rate was calculated from the signal of a balance on which the feed solution was placed. The integrated value of this feed rate was used as the total methanol consumption since the accumulation in the medium and the evaporation of methanol were insignificant when compared to the feed.

Outlet gas analysis

The concentrations of oxygen, carbon dioxide and methanol in the outlet air were continuously analyzed using Industrial Emissions Monitor Type 1311 (Brüel& Kjær, Innova, Denmark). The methanol signal was calibrated (at the stirrer speed, 1,000 rpm; aeration rate, 6 L min^{-1}; and temperature 30° C in liquid phase, applied in the process) by addition of aliquots of methanol to the fermentor in a 1 g L^{-1} NaCl solution before fermentation, as described recently [9].

Calculation of relative respiration rate and carbon mass balances

The oxygen uptake rate (OUR) (mol h^{-1}) and carbon dioxide production rate (CPR) (mol h^{-1}) were calculated from:

$$\mathrm{CPR} = \frac{Q_\mathrm{o}\,\mathrm{CO}_{2,\mathrm{o}} - Q_\mathrm{i}\,\mathrm{CO}_{2,\mathrm{i}}}{100\,V_\mathrm{m}} \quad (1)$$

$$\mathrm{OUR} = \frac{Q_i\,\mathrm{O}_{2,\mathrm{i}} - Q_\mathrm{o}\,\mathrm{O}_{2,\mathrm{o}}}{100\,V_\mathrm{m}} \quad (2)$$

$$Q_\mathrm{o} = \frac{Q_\mathrm{i}\left(100 - \mathrm{O}_{2,\mathrm{i}} - \mathrm{CO}_{2,\mathrm{i}}\right)}{\left(100 - \mathrm{O}_{2,\mathrm{o}} - \mathrm{CO}_{2,\mathrm{o}}\right)}, \quad (3)$$

where Q is air flow rate (l h^{-1}), O_2 is oxygen concentration in air (% v/v), CO_2 is carbon dioxide concentration in air (%v/v), and V_m is molar volume of the gas at the analysis temperature (24.04 l mol^{-1}). Subscripts i and o refer to inlet and outlet gas, respectively.

Relative rate of respiration (RRR) is a ratio between the observed specific oxygen uptake rate (q_o; g g^{-1} h^{-1}) and the maximum specific oxygen uptake rate ($q_{o,max}$; g g^{-1} h^{-1}).

$$\mathrm{RRR} = \frac{q_\mathrm{o}}{q_{\mathrm{o,max}}}, \quad (4)$$

where q_o was obtained from:

$$q_\mathrm{o} = \frac{\mathrm{OUR}}{XV}, \quad (5)$$

in which X is cell concentration (g L^{-1}) and V is culture volume (l). $q_{o,max}$ was obtained from the maximum q_o value before methanol or DOT became limiting.

For the carbon mass balance calculations, a previously analyzed carbon concentration in *P. pastoris* (0.396 g g^{-1}) was used [4].

SDS-PAGE analysis

The sample, containing 60 µl of supernatant, 25 µl of sample buffer (NuPAGE LDS 4x sample buffer, Invitrogen), 10 µl of 0.5 M dithiothreitol and 5 µl of 3.5% PMSF in ethanol, was incubated for 10 min at 95° C. SDS-PAGE was performed on NuPAGE Novex 4–12% Bis-Tris Gel (1.0 mm×10 well; Invitrogen) using MOPS-running buffer. Ten µl of prepared sample was loaded to each well and run at 200 V for 60 min. The gel was stained with Coomassie Blue R-250 for 30 min and destained (with destain solution; 100 ml L^{-1} methanol and 100 ml L^{-1} glacial acetic acid in distillate water) for 1–2 h.

Alcohol oxidase activity

Alcohol oxidase was assayed with the method described recently [9]. Sample from the fermentation (5 ml) were centrifuged at 4,200 rpm, 4° C for 10 min. The volume of supernatant was measured. The cell pellet was washed once with 5 ml of 0.1 M potassium-phosphate buffer pH 7.5 and finally suspended in the same buffer to the original supernatant volume. The cells were then disintegrated in a French press (SLM Aminco, USA) at 800 bars. One milliliter of 0.1 M potassium-phosphate buffer pH 7.5 with 2,000 units of catalase (from bovine liver, Sigma-Aldrich, Sweden) and 10 µl of cells homogenate were mixed. Catalase was added to assure that the hydrogen peroxide produced was converted to molecular oxygen, making the stoichiometry of the reaction 1 mol O_2 per 2 mol of CH_3OH. The reaction was started by adding 10 µl of 10 M methanol. Oxygen consumption was assayed polarographically with a Clark type oxygen electrode (Medelco AB, Sweden) at 37° C in air-saturated buffer. Alcohol oxidase units were expressed as a µmol of methanol oxidized per minute.

Results and discussion

Cell growth and product accumulation

The duplication processes of OLFB and MLFB processes showed the good reproducibility. The biomass

concentration profiles were quite similar for the two control strategies, but with a higher maximum value for the OLFB (129 g L^{-1}) than for the MLFB (122 g L^{-1}; Fig. 3a). Also the β-glucosidase accumulation was higher in the OLFB with total 6,000 U L^{-1} supernatant fluid, compared to 5,170 U L^{-1} in the MLFB process. The specific β-glucosidase productivity (q_p) declined slowly during the process, but it was still about 52% of the initial value after 120 h of induction (Fig. 3b), indicating a capacity for extending the process even if the cell density does not increase. This is in agreement with earlier results on production of a fusion protein between a cellulose binding module and a lipase with *P. pastoris*, when the specific productivity was even better preserved during 160 h [4]. Thus, the oxygen limitation did not inhibit β-glucosidase accumulation which is similar to other protein productions [14, 23].

Effect of feed strategy on total methanol consumption and OUR

The methanol feed in the MLFB was regulated with the DOT electrode keeping the DOT constant at 25% air sat. (Fig. 4a). After a transient accumulation of methanol to 700 mg L^{-1}, the concentration decreased below the detection limit (about 25 mg L^{-1}) in 5 h (Fig. 4d). In the OLFB, the methanol concentration was automatically controlled at 350 mg L^{-1} (Fig. 4d), and it resulted in a rapid drop of DOT below the detection limit (Fig. 4a). In the OLFB, the OUR was about 40% higher than in the MLFB (Fig. 4b) and it was kept relatively constant in both processes.

Correspondingly, the total methanol consumption was about 40% higher in OLFB (Fig. 4d). Thus, the increased oxygen transfer rate obtained by the use of the OLFB technique resulted in higher OUR and correspondingly higher methanol consumption rate. This was similar to a recent work on mouse endostatin production [15] and scFv production [14] when the methanol-saturation condition was applied for *P. pastoris* expression system.

The relative respiration rate (RRR), i.e., the current respiration rate divided by the maximum respiration rate is a parameter that can be used to describe the degree of oxygen limitation. It should also be a measure of the degree of methanol limitation, provided the organism is obligately aerobic and has no alternative pathways for the methanol metabolism. This analysis showed that both processes were quite similar with respect to degree of energy limitation (Fig. 4c). Initially, after the induction, both cultures were nonlimited with respect to oxygen and methanol and RRR was 1, but then this parameter dropped rapidly in both processes and approached about 0.20–0.25 with slightly a higher value (i.e., less degree of energy limitation) for the MLFB.

Maintenance demand and viability

Taking into account that considerably more methanol was consumed in the OLFB process but only slightly

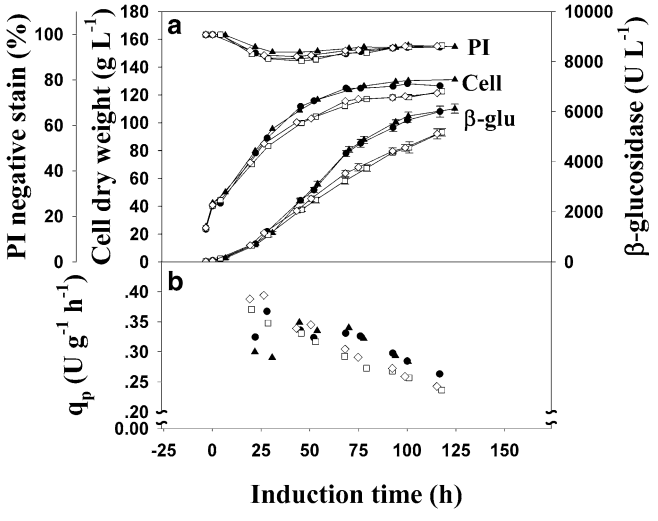

Fig. 3 **a** Fermentation profile of β-glucosidase production in MLFB (*open symbols, thin lines*) and OLFB (*close symbols, thick lines*); cell dry weight, β-glucosidase activity and PI-negative-stained cell **b** specific productivity (q_p)

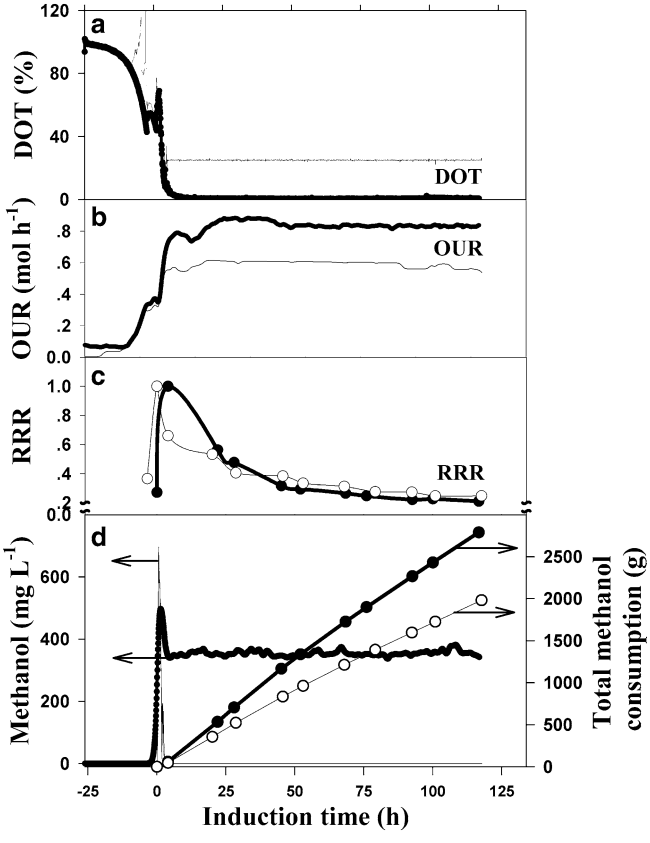

Fig. 4 Comparison of **a** DOT, **b** OUR, **c** RRR and **d** methanol concentration (*lines*) and total methanol uptake (*symbols*) in MLFB (*open symbols, thin lines*) and OLFB (*close symbols, thick lines*)

more cells were produced, the carbon yield coefficient of biomass from methanol ($Y^{C}_{X/S}$) must have been lower in the OLFB process. This was further investigated by carbon mass balances.

Carbon mass balances based on methanol input and outputs of biomass, carbon dioxide and protein in the medium showed good agreement in both processes. The methanol loss in the outlet air is insignificant since the methanol analyzer signal from 500 mg L^{-1} in the fermentor is kept stable for at least 2 days under calibration condition (data not shown). During the main part of the process time the carbon recovery was 98–99% (Fig. 5). The extracellular protein in the medium accounted for less than 0.4 % of the total carbon (data not shown). Thus, no major by-products from the methanol metabolism were produced. The $Y^{C}_{X/S}$ (molC $molC^{-1}$) gradually declined from 0.33 to 0.24 for the OLFB and from 0.38 to 0.27 for the MLFB (Table 1). Corresponding carbon yield coefficients of carbon dioxide from methanol ($Y^{C}_{CO2/S}$) increased from 0.60 to 0.74 in the OLFB and from 0.59 to 0.72 in the MLFB. The decreasing biomass yield and the increasing carbon dioxide yield reflects the increasing total maintenance demand at declining specific growth rate [4] but it also shows that the maintenance demand is larger in the OLFB.

Methanol is known to be toxic for many species. However, *P. pastoris* is quite resistant to methanol and 10 g L^{-1} methanol exhibited only a slight reduction of the specific growth and methanol uptake rates [12]. Not until the methanol concentration reached 30 g L^{-1} was the growth almost completely inhibited. Therefore the higher methanol concentration in the OLFB process (350 mg L^{-1}) was not expected to be inhibitory. However, the low oxygen concentration in the OLFB might cause an accumulation of intracellular methanol if it limits the oxidation rate of methanol. This is especially intriguing since in vitro assays of the AOX kinetics showed a very high K_m (0.7 mM) for oxygen at 10 mM methanol [5].

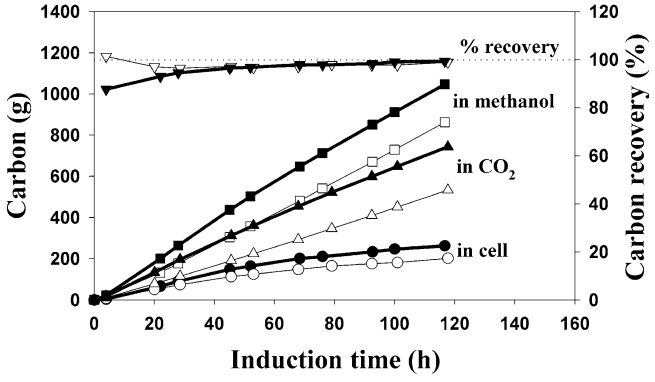

Fig. 5 Carbon mass balances of a MLFB (*open symbols*) and an OLFB (*close symbols*): carbon in cell (*circle*), carbon in CO_2 (*triangle*), carbon in methanol (*square*) and carbon recovery (*inverted triangle*)

The possible toxic effect of methanol in the OLFB process was investigated by comparing the viability analyzed as frequency of dead cells according to propidium iodide (PI) staining. This method has previously been used to reveal large differences in viability of *P. pastoris* cultures under different process conditions [9]. However, no difference in viability was observed between the OLFB and the MLFB processes (Fig. 3a). In both cases the frequency of viable cells dropped to about 90% during the first 22 h of induction but then it stayed within 90–95% throughout the processes.

The intracellular AOX activity

The β-glucosidase accumulation during the transition phase after the induction was similar to both cultivation techniques, but after 20 h higher level of β-glucosidase accumulation was observed in OLFB process (Fig. 3a). To investigate whether there were differences in the activity of the AOX promoter, the intracellular AOX activity was compared. Figure 6 shows that the AOX activity initially showed a similar rapid increase to a maximum of about 2,000–2,300 U g^{-1}_{cell} after 20 h of induction in both techniques. Then during the main production phase a gradual decrease of the AOX activity was observed in both processes, but the decrease was less pronounced in the OLFB and at the end of the process, the OLFB exhibited 37% higher AOX activity. This might be due to the fact that the methanol concentration was higher and not growth-limiting in the OLFB. It is also plausible that the higher β-glucosidase production, at least partly, could be due to a higher activity of the *AOX1* promoter since the deviation between the q_p curve (Fig. 3b) and the AOX curve for the two processes follows a similar pattern after about 50 h (Fig. 6).

Product purity

One of the advantages of the *P. pastoris* system for the production of recombinant proteins is that it often permits secretion of the product to a defined mineral salt medium contaminated with only a few host proteins. At the end of the processes, at about 120–130 g L^{-1} cell dry weight, the β-glucosidase activity was higher in an OLFB process (6,000 U L^{-1}) than in a MLFB process (5,200 U L^{-1}). On the contrary, the total protein in the medium (data not shown) was lower in the OLFB (final concentration about 470 mg L^{-1}) than MLFB (about 660 mg L^{-1}). The combined effect of these parameters was that the specific activity of the β-glucosidase became much higher in the OLFB processes (Fig. 7).

Conclusions

The obligately aerobic *P. pastoris* did not respond to severe oxygen limitation with major negative responses.

Table 1 Carbon yield coefficient of biomass and carbon dioxide for MLFB and OLFB

MLFB			OLFB		
Induction time (h)	$Y^{C*}_{X/S}$ (molC molC^{-1})	$Y^{C**}_{CO2/S}$ (molC molC^{-1})	Induction time (h)	$Y^{C*}_{X/S}$ (molC molC^{-1})	$Y^{C**}_{CO2/S}$ (molC molC^{-1})
20.1	0.38	0.59	22.0	0.33	0.60
28.6	0.38	0.59	28.0	0.34	0.60
45.6	0.36	0.61	45.1	0.33	0.63
53.0	0.34	0.62	52.0	0.32	0.64
67.9	0.33	0.65	68.4	0.3	0.67
79.1	0.32	0.66	76.0	0.29	0.68
92.4	0.29	0.68	92.6	0.27	0.71
100.9	0.28	0.70	100.0	0.26	0.72
117.7	0.27	0.72	116.7	0.24	0.74

** $Y^{C}_{CO2/S}$ Carbon yield coefficient of carbon dioxide from methanol

* $Y^{C}_{X/S}$ Carbon yield coefficient of biomass from methanol

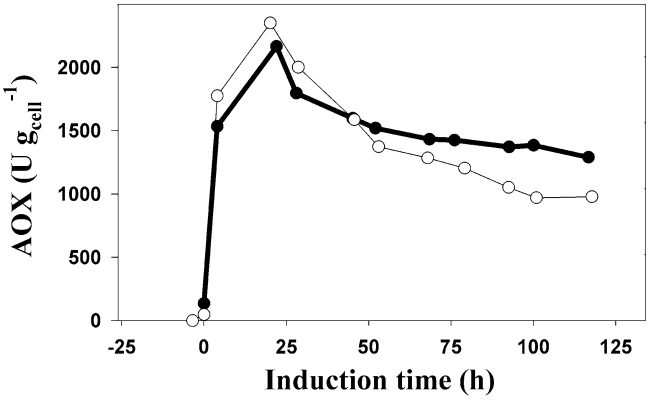

Fig. 6 Comparison of the AOX activity in MLFB grown cells (*open symbols*) and OLFB grown cells (*close symbols*)

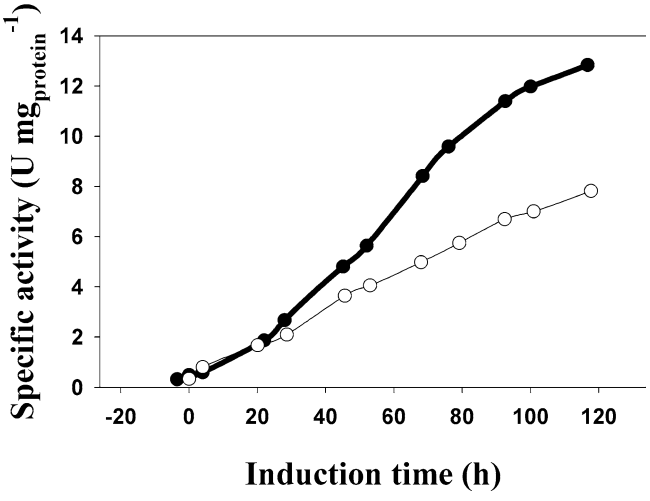

Fig. 7 Specific activity of β-glucosidase in the culture supernatant from one MLFB (*open symbols*) and OLFB (*close symbols*) process

Therefore, an oxygen-limited fed-batch technique could be used to improve the oxygen transfer rate and productivity. An additional advantage was that less total proteins were released to the medium making the specific product concentration in the broth much higher. On the other hand, the oxygen limitation caused an increasing maintenance demand which resulted in a lower biomass yield per methanol.

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Publication II

Title: Increased total air pressure *versus* oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process

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Increased total air pressure *versus* oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process

(former Ms. Ref. no.: BEJ-D-05-00407)

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Key words: aeration; oxygen transfer; dissolved oxygen tension; enzyme production; β-glucosidase; oxygen-limited fed-batch

Abstract

Two strategies to increase the productivity of secreted Thai Rosewood β-glucosidase in *Pichia pastoris* processes were evaluated. Both methods were based on increasing the oxygen transfer rate (OTR) in the process by simple means. Increasing the driving force for the diffusion from the air bubbles to the medium by elevating the air pressure, from 1.2 to 1.9 bar increased the oxygen uptake rate (OUR) by 59% while increasing the driving force by accepting oxygen limitation increased the OUR by 35%. The OTR increased less than in proportion to the increased solubility in the high-pressure process, which indicates that air bubble compression reduces the volumetric oxygen transfer coefficient (K_La). Even though the methanol consumption increased almost in proportion to the OTR in both processes the biomass production did not increase as much. This is explained as a higher maintenance demand for methanol in the oxygen limited (0.027 g g h^{-1}) and high pressure processes (0.035 g g h^{-1}), compared to 0.022 g g h^{-1} in the methanol limited reference process. However, in spite of the low effect of increasing OTR on the biomass production the total β-glucosidase yield increased almost in proportion to the increased methanol consumption and reached highest value in the high-pressure process, while the β-glucosidase purity was highest in the oxygen-limited process due to release of less contaminating proteins.

List of symbols

AOX	enzyme alcohol oxidase
aox1	alcohol oxidase 1 gene
C	oxygen concentration in bulk liquid outside the film (mol L^{-1})
C*	oxygen concentration in gas-liquid interface (mol L^{-1})
C^*_{ref}	C* under 100% DOT electrode calibration (mol L^{-1})
CPR	carbon dioxide production rate (mol h^{-1})
DOT	dissolved oxygen tension (%)
K_La	volumetric oxygen transfer coefficient (mol L^{-1} h^{-1})
K_La_{ref}	K_La under 100% DOT electrode calibration condition (mol L^{-1} h^{-1})
MLFB	methanol-limited fed-batch
OLFB	oxygen-limited fed-batch
OTR	oxygen transfer rate (mol h^{-1})
OUR	oxygen uptake rate (mol h^{-1})
P	air pressure inside the bioreactor (bar)
PI	propidium iodide
P_{ref}	P under 100% DOT electrode calibration (bar)
q_m	maintenance coefficient (g g^{-1} h^{-1})
ROS	reactive oxygen species
Y_{em}	biomass yield on methanol, exclusive the maintenance (g g^{-1})
$Y_{x/s}$	biomass yield on methanol (g g^{-1})

1. Introduction

The methylotrophic yeast *Pichia pastoris* is a common host for production of recombinant proteins. It is mostly used with the strong alcohol oxidase 1 (*aox1*) promoter to control the production of the recombinant protein, but this promoter also controls synthesis of the alcohol oxidase (AOX) enzyme that is used for oxidation of the carbon- and energy-source methanol [1,2]. The initial oxidation of methanol by the AOX enzyme is dependent on molecular oxygen as an electron acceptor resulting in formaldehyde and hydrogen peroxide (H_2O_2) [3]. *Pichia* processes are typically associated with relatively low specific product formation rate but this is compensated for by the low maintenance demand [4] that permits cultivations at very high-cell-density, typically in the range of 130-150 g L^{-1} cell dry weight [4, 5]. However, the high-cell-density and the use of the reduced energy source methanol emphasize the demand for high oxygen transfer rate (OTR).

A common model showing parameters that influence the OTR is

$$OTR = K_L a(C^* - C) \quad (1)$$

To increase the OTR in *P. pastoris* processes beyond what is achieved by just increasing the aeration rate and the stirrer speed, which increases the volumetric oxygen transfer coefficient (K_La), several strategies have been used for increasing the driving force (C*-C).

The driving force for oxygen transfer is increased if the process is permitted to run under oxygen limitation ($C \approx 0$). This may have serious drawbacks in processes with facultatively anaerobic organisms like *Escherichia coli* and *Saccharomyces*

cerevisiae, which switch to fermentative metabolism under oxygen limiting conditions. Therefore such organisms are mostly cultivated at 20-30% air saturation. *P. pastoris* is however an obligately aerobic organism when grown on methanol. It can not employ alternative metabolic reactions for methanol under oxygen limitation (DOT ≈ 0). Therefore, oxygen-limited fed-batch processes can be utilized [5-7]. About 33% OTR increase was obtained when oxygen-limited *P. pastoris* processes were compared with methanol-limited processes run at 25% DOT. Both growth and recombinant protein production was increased in the oxygen limited process [5].

Increased dissolved oxygen concentration in equilibrium with the gas phase (C*) can be achieved either by using higher oxygen concentration in the inlet air or by increasing the total air pressure (P_{tot}) in the bioreactor. Mixing pure oxygen in the inlet air flow is effective on the small scale and it has been utilised to increase the methanol feed rate and the productivity [4, 6] but on a large scale the supply and handling of pure oxygen can be unsafe. Alternatively, an oxygen enriching polysulphone membrane can be used for separation of regular air into nitrogen-enriched retentate and oxygen-enriched permeate [8]. An oxygen-enriched (35-38% oxygen) stream obtained with a polysulphone membrane has been used to increase the DOT which enhanced production of elastase inhibiting peptide by recombinant *P. pastoris* [9]. Increase of the total air pressure, P_{tot}, has been applied in other yeast processes [10, 11].

However, all these methods may have biological side-effects. The use of oxygen limitation in *Pichia* processes may theoretically result in elevated formaldehyde concentrations in the cell if it generates an imbalance between the initial oxidation of

methanol, resulting in formaldehyde, and the formaldehyde consuming reactions in anabolism and catabolism. The high air pressure increases not only the partial pressure of oxygen but also that of carbon dioxide which is a common antimicrobial agent [12]. Furthermore, increasing the oxygen equilibrium concentration C* by high pressure may result in toxic side-effects of molecular oxygen. In the aerobic process, during the reduction of molecular oxygen to water through acceptance of four electrons, reactive oxygen species (ROS) such as the superoxide anion radical ($O_2^{\bullet -}$), H_2O_2 and hydroxyl radicals ($HO^{\bullet}$) are generated [13]. The initial oxidation of methanol by methylotrophic yeast is another reaction which generates H_2O_2 [3]. These ROS attack almost all cell components, DNA, protein and lipid membrane [13, 14].

In the present work, we investigate two simple methods for increasing the productivity of *Pichia* processes by increasing the OTR. Increased total air pressure is compared with the use of oxygen limitation to increase the driving force for oxygen transfer in a process for recombinant Thai Rosewood β-glucosidase production with *P. pastoris*. The growth, viability and product formation were compared with a traditional methanol-limited fed-batch process.

2. Materials and methods

2.1 Strain and plasmid

The *β-glucosidase* cDNA gene from Thai Rosewood (*Dalbergia cochinchinensis* Pierre) [15] was cloned into the pPICzαB vector (Invitrogen) and then integrated into *P. pastoris* Y-11430 (wild-type strain) at the *aox1* promoter. A Mut^+, $zeocin^+$

transformant was selected and characterized for extracellular β-glucosidase production.

2.2 Inoculum preparation

The inoculum culture was prepared from one colony of *P. pastoris* on YPD agar (1 L contained 10 g yeast extract, 20 g peptone, 20 g dextrose, and 15 g agar) containing 100 μg zeocin ml^{-1} suspended in 100 ml BMGY medium (10 g yeast extract, 20 g peptone, and 10 g glycerol dissolved in 1 L 0.1 M potassium phosphate buffer with pH 6.0). The culture was incubated at 30°C, with 200 rpm for 24 h.

2.3 Fed-batch fermentation

The fed-batch fermentations were carried out in a 10 L stirred tank bioreactor (Belach Bioteknik AB, Stockholm) with 3.0 L of glycerol basal salts (GBS) medium (containing per litre: H_3PO_4 85% 26.7 ml, $CaSO_4$ 0.93 g, K_2SO_4 18.2 g, $MgSO_4*7H_2O$ 14.9 g, KOH 4.13 g, glycerol 40.0 g and PTM1 trace salts 4.35 ml). The PTM1 trace salt solution contains per litre: $CuSO_4*5H_2O$ 6.0 g, KI 0.08 g, $MnSO_4*H_2O$ 3.0 g, $Na_2MoO_4*2H_2O$ 0.2 g, H_3BO_3 0.02 g, $ZnCl_2$ 20.0 g, $FeCl_3$ 13.7 g, $CoCl_2*6H_2O$ 0.9 g, H_2SO_4 5.0 ml, and biotin 0.2 g. The fermentations were controlled at the following conditions: temperature 30°C, aeration 6 L min^{-1}, agitation 1000 rpm and pH 5.0. Ammonia solution 25% was used to control pH and addition of antifoam A (A-5758, Sigma) was controlled by a level electrode.

A four stage fermentation protocol was used in this study: The first stage was a glycerol batch phase. About 24 h after inoculation when the glycerol was completely consumed, as indicated by the DOT signal, the process was switched to glycerol fed-

batch by feeding 500 g L^{-1} glycerol plus 12 ml L^{-1} PTM1 trace salts to the bioreactor at an exponentially increasing rate of 0.18 h^{-1} starting with 35.5 ml h^{-1}. After 3-3.5 h when the cell density reached 40 g L^{-1} ($OD_{600} \approx 80$), the process was switched to the methanol induction phase by replacing the glycerol feed with a feed of methanol plus 12 ml L^{-1} PTM1 trace salts. The initial methanol feed rate was constant at about 10 ml h^{-1} for 2-3 h. In methanol limited processes (designated ML below) the methanol was fed into the bioreactor by means of feed-back control to keep DOT at 25% air sat.. The operating pressure was controlled with a regulatory valve in the exit gas line. The values of total air pressure studied were 1.2 bar (reference process ML1.2) and 1.9 bar (high-pressure process ML1.9). In the oxygen-limited fed-batch processes (OL1.2) the stirrer speed and aeration rate were the same as in the processes described above, but the methanol concentration was kept at 350 mg L^{-1} by feed rate control from the methanol analyser. The data in Table 1 are based on two processes of each kind.

2.4 Analyses

Analysis of cell dry weight, β-glucosidase activity in the medium and SDS-PAGE of the medium were performed with conventional methods as described previously [5]. Cell viability was analysed by flow cytometry counting of the frequency of propidium iodide (PI) stained (= dead) cells, as described earlier [5, 6]. Total protein concentration in the medium was analysed according to Bradford [16] with BSA as reference.

Methanol feed rate: The methanol feed rate was calculated from the signal of a balance on which the feed solution was placed. The integrated value of this feed rate

was used as the total methanol consumption, since the accumulation in the medium and the evaporation of methanol were insignificant in comparison to the feed.

Gas analyses: The concentrations of oxygen, carbon dioxide and methanol in the outlet air were continuously analyzed using Industrial Emissions Monitor Type 1311 (Brüel & Kjær, Innova, Denmark). The methanol signal was calibrated by addition of aliquots of methanol to the bioreactor in a 1 g L^{-1} NaCl solution before the fermentation. The inlet air flow rate was measured with a mass flow meter, and the outlet air flow rate was calculated from the inlet flow and the outlet oxygen and carbon dioxide concentrations, based on the mass balance of the inert nitrogen in the gas flow.

DOT electrode calibration: The calibration of the DOT electrode was performed under the same conditions for all experiments: The 100% air sat. point was calibrated in 3 litres of glycerol basal salts medium at pH 5.0 and 30°C, with aeration at 6 L min^{-1}, agitation at 1000 rpm, and a total air pressure of 1.2 bar. For the zero calibration, nitrogen gas was flown through the bioreactor instead of air.

3. Results and discussion

Three modes of operation with different maximum oxygen transfer rates were investigated: the reference methanol-limited process at 1.2 bar air pressure and DOT=25% air saturation (ML1.2), the oxygen-limited process at 1.2 bar air pressure (OL1.2) and the methanol-limited process at 1.9 bar pressure and DOT = 25% air saturation (ML1.9). Due to the mode of calibration, the DOT at 1.9 bar was 164% air

sat. before the inoculation. According to *eq.1*, the relative oxygen transfer capacities compared to the reference process (ML1.2) are 133% and 185% for the oxygen-limited (OL1.2) and high-pressure (ML1.9) processes, respectively (Table 1). The assumed value 185% at 1.9 bar air pressure and DOT=25% is obtained if only the pressure effect on the partial pressure of oxygen is taken into account at the elevated pressure.

TABLE 1 HERE

The resulting OUR in the three processes are shown in (Fig. 1). Under the quasi-steady state condition during the methanol feed phase (50-100 h), the OUR also represents the OTR, which was 608 ± 2, 821 ± 6, and 966 ± 3 mmol h^{-1}, for ML1.2, OL1.2 and ML1.9, respectively. This gives the observed OTR 135% in the oxygen-limited process and 159% in the high-pressure process compared to the reference process (Table 1). Thus, while the OTR for the oxygen-limited process increased approximately according to the increased driving force (see Table 1), the corresponding values for the process with higher air pressure did not increase according to the increased driving force caused by higher oxygen solubility (*eq. 1* and Table 1)

Fig. 1 HERE

An explanation to this may be the effect of air pressure on the parameter *a* (specific air bubble area m^{-1}) in K_La which may be reduced due to the gas compression. If the gas volume compression is taken into account K_La should be proportional to the cubic root of the square of the pressure ratio. Then the oxygen transfer rate becomes:

$$OTR = K_L a_{ref} \left(\frac{P_{ref}}{P} \right)^{2/3} (PC^*_{ref} - C) \quad (2)$$

The effect of air pressure on the OTR was investigated by Yang and Wang [17] who showed that in the pressure range of 1.06 – 2.72 bar, the OTR both in sulfite solutions and in *E. coli* cultures was approximately directly proportional to the total pressure, indicating that K_La was not influenced by the air pressure. On the other hand, Pinheiro et al. [10] found that the sulfite oxidation rate increased only to 255% and 350% of the reference value at 1.2 bar when the pressure was increased by 333 and 500% (4 and 6 bar). This means that the OTR increased less than in proportion to the driving force increase caused by the increased oxygen solubility. This is in agreement with the measured OTR in ML1.9 in which the measured OTR (159%) agrees well with the value calculated by taking the air compression of the bubble interface into account (161%) (Table 1 and *eq. 2*). Obviously, different investigations have resulted in different conclusions regarding the effect of air pressure on K_La. The reason may be that the parameter *a* depends also on the air dispersion and bubble coalescence, which are likely to depend on the medium properties and fluid dynamic situation.

Fig. 2 HERE

The increased OTR was reflected by a correspondingly increased methanol consumption rate, which was approximately constant during the 80 h of methanol feeding (Fig. 2a). The relative values of total methanol consumption were almost proportional to the OTR, with 133% and 157% in the OL1.2 and ML1.9 processes, compared to the reference process (Table 1). However, the biomass production did

not fully respond to the increased methanol utilization at higher OTR. Only 7% and 12% higher final cell mass was obtained in the OL1.2 and ML1.9 processes, respectively (Fig. 2b and Table 1). This indicates some sort of stress induced under the oxygen limiting and high air pressure conditions.

Fig. 3 HERE

The reduced biomass yield per methanol may reflect an altered metabolism, *e.g.* formation of by-products from the carbon source. This is often the case when cells are subject to oxygen limitation, but *P. pastoris* is not known to metabolise methanol in absence of oxygen. Furthermore, the biomass yield per methanol dropped even more in the high-pressure process (78%) than in the oxygen limited process (89%). Alternatively, an increased maintenance demand, expressed as increased combustion of the energy source may reduce the obtained biomass. The maintenance coefficient has been suggested to be decisive for the level of cell density reached in fed-batch processes with constant feed [4]. Simulations of the biomass concentration profiles obtained in the present investigation were performed with the experimentally obtained methanol feed rates, using a model presented previously [4]. The simulation results show that the maintenance demand (q_m) increased from 0.022 g g^{-1} h^{-1} in the reference process (ML1.2) to 0.027 g g^{-1} h^{-1} in the high-pressure process (ML1.9) and to 0.035 g g^{-1} h^{-1} in the oxygen limited (OL1.2) process (Fig. 3). The alternative, that the biomass composition or the yield exclusive maintenance (Yem) changes does not give a fit to the experiments (thin line in Fig. 3b and Fig. 3c).

Cell death that is followed by cell lysis may be interpreted as low biomass yield and high maintenance demand. *Pichia* cultures are especially subject to cell death during the transition to the methanol phase [18, 19]. Furthermore, lysis of dead cells can be responsible for substantial proteolytic degradation of products secreted to the medium [19, 20]. In the present investigation, though, the percent of cell death was not influenced by neither oxygen limiting nor high-pressure conditions. From an initial value of 100% viable cells during the glycerol batch phase, the value declined to about 90% after transition to the methanol phase in all processes. During the methanol phase the viability remained constant and similar in all processes (Fig. 4a). However, this does not exclude that some of the dead cells lysed and escaped the flow cytometry count. Therefore also the total mass of dead cells was calculated from the flow cytometry and cell dry weight data. The total amount of dead cells was in all processes highest at about 45 h after induction and then stabilized or decreased (Fig. 4b). A decrease of total amount of dead cells, which was significant only in the high pressure process, means that cells must have lysed at least in this process, though it can not be excluded that cell lysis also took place in the other processes.

Fig. 4 HERE

An indication that lysis of dead cells in the initial methanol feed phase may be involved to different degree is obtained from the data on the specific enzyme activity. Fig. 5 shows the accumulation of β-glucosidase activity and total protein in the medium. While the β-glucosidase activity accumulated in a pattern resembling that of cell growth, the total protein accumulated much faster during the first hours after start of the methanol feed, when the cell viability dropped. Unfortunately the specific

activity of pure β-glucosidase is not available and therefore it is not possible to translate the activity curve (Fig. 4a) into a mass curve. However, SDS-PAGE analyses show that the product seems to be the main part of the total protein in the medium (data not shown).

Fig. 5 HERE

Even though the biomass production increased only slightly when the methanol consumption was increased, the enzyme productivity responded more favorably to the increased oxygen transfer. The final enzyme yield (U process^{-1}) increased with 41% and 50% in the OL1.2 and ML1.9 processes, respectively (Table 1).

The specific activity of the β-glucosidase in the broth was calculated from the β-glucosidase activity and total protein data, (Fig. 6 and Table 1). It showed that the specific activity was highest in the oxygen-limited process even though the total activity was highest in the high-pressure process. One possible explanation is that the higher specific activity is caused by less cell lysis in the oxygen limited process but it can not be ruled out that there is a difference in secretion of endogenous proteins from *P. pastoris* under the different process conditions.

Fig. 6 HERE

4. Conclusion

This work shows that the cell productivity of *P. pastoris* processes can be improved by increasing the oxygen transfer rate by two simple and inexpensive means: application of elevated air pressure and/or accepting oxygen limitation. The increased oxygen transfer rate resulted in approximately correspondingly increased methanol consumption and total product yield, while the biomass yield did not increase in proportion to the increased methanol consumption rates. The latter is actually an advantage, since the high biomass concentration in *Pichia* processes complicates the downstream processing.

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Legends of the figures

Figure 1. Oxygen uptake rate (OUR) in the reference process (ML1.2), the oxygen-limited process (OL1.2), and the high-pressure process (ML1.9).

Figure 2. Comparison of a) accumulated methanol uptake and b) cell growth in the reference process (ML1.2, –○–), the oxygen limited process (OL1.2, –□–) and the high-pressure process (ML1.9, –●–).

Figure 3. Comparison of the experimental cell growth (symbols) and simulation (lines) in the reference process (ML1.2; upper panel), the oxygen limited process (OL1.2; middle panel) and the high-pressure process (ML1.9; lower panel). The thin continuous lines in the middle and lower panels represent simulations in which the maintenance coefficient (q_m) is kept unchanged and only the yield coefficient exclusive maintenance (Yem) is reduced to fit the data.

Figure 4. a) Viability and b) total amount of dead cells during the methanol feed phase of the reference process (ML1.2, ○), the oxygen-limited process (OL1.2, □) and the high-pressure process (ML1.9, –●–)

Figure 5. Accumulation of total protein and β-glucosidase in the *P. pastoris* processes. a) β-glucosidase activity in the medium, b) total protein in medium Reference process: ML1.2 (–○–), oxygen-limited process (OL1.2, –□–) high-pressure process (ML1.9, –●–)

Figure 6. Specific activity of the β-glucosidase (U $mg^{-1}_{prot.}$) in the reference process (ML1.2, –○–), oxygen-limited process (OL1.2, –□–) and the high-pressure process (ML1.9, –●–)

Legends of the table

Table 1. The relative OTR-values calculated from *eq. 1* and *eq. 2* and experimental data expressed as absolute values and values relative to the control culture for the three process control strategies. Each experimental value is an average from two similar processes.

Parameters	**ML1.2**		**OL1.2**		**ML1.9**	
	Observed values	Relative values (%)	Observed values	Relative values (%)	Observed values	Relative values (%)
OTR (*eq.1*)	-	100	-	133	-	185
OTR (*eq.1+2*)	-	100	-	133	-	161
OTR observed	608 ± 2 ($mmol\ h^{-1}$)	100	821 ± 6 ($mmol\ h^{-1}$)	135	966 ± 3 ($mmol\ h^{-1}$)	159
Total meth. consumption	1414 ± 73 (g)	100	1887 ± 11 (g)	133	2224 ± 104 (g)	157
Cell DW	116 ± 1 ($g\ L^{-1}$)	100	125 ± 1 ($g\ L^{-1}$)	107	131 ± 3 ($g\ L^{-1}$)	112
Yx/s	0.37 ± 0.01 ($g\ g^{-1}$)	100	0.33 ± 0.01 ($g\ g^{-1}$)	89	0.29 ± 0.01 ($g\ g^{-1}$)	78
β-glucosidase	3761 ± 16 ($U\ L^{-1}$)	100	4807 ± 72 ($U\ L^{-1}$)	127	5204 ± 16 ($U\ L^{-1}$)	138
Specific activity	6.04 ± 0.29 ($U\ mg^{-1}_{prot.}$)	100	9.06 ± 0.53 ($U\ mg^{-1}_{prot.}$)	150	7.28 ± 0.24 ($U\ mg^{-1}_{prot.}$)	121
Total product yield	11404 ± 72 ($U\ process^{-1}$)	100	16125 ± 195 ($U\ process^{-1}$)	141	17082 ± 87 ($U\ process^{-1}$)	150

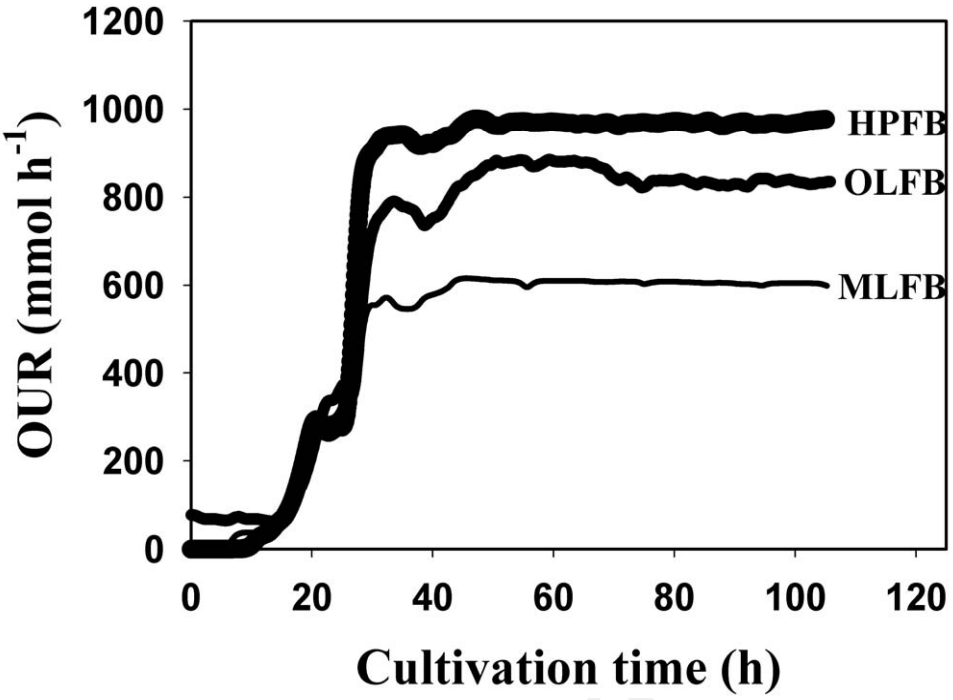

FIG 1

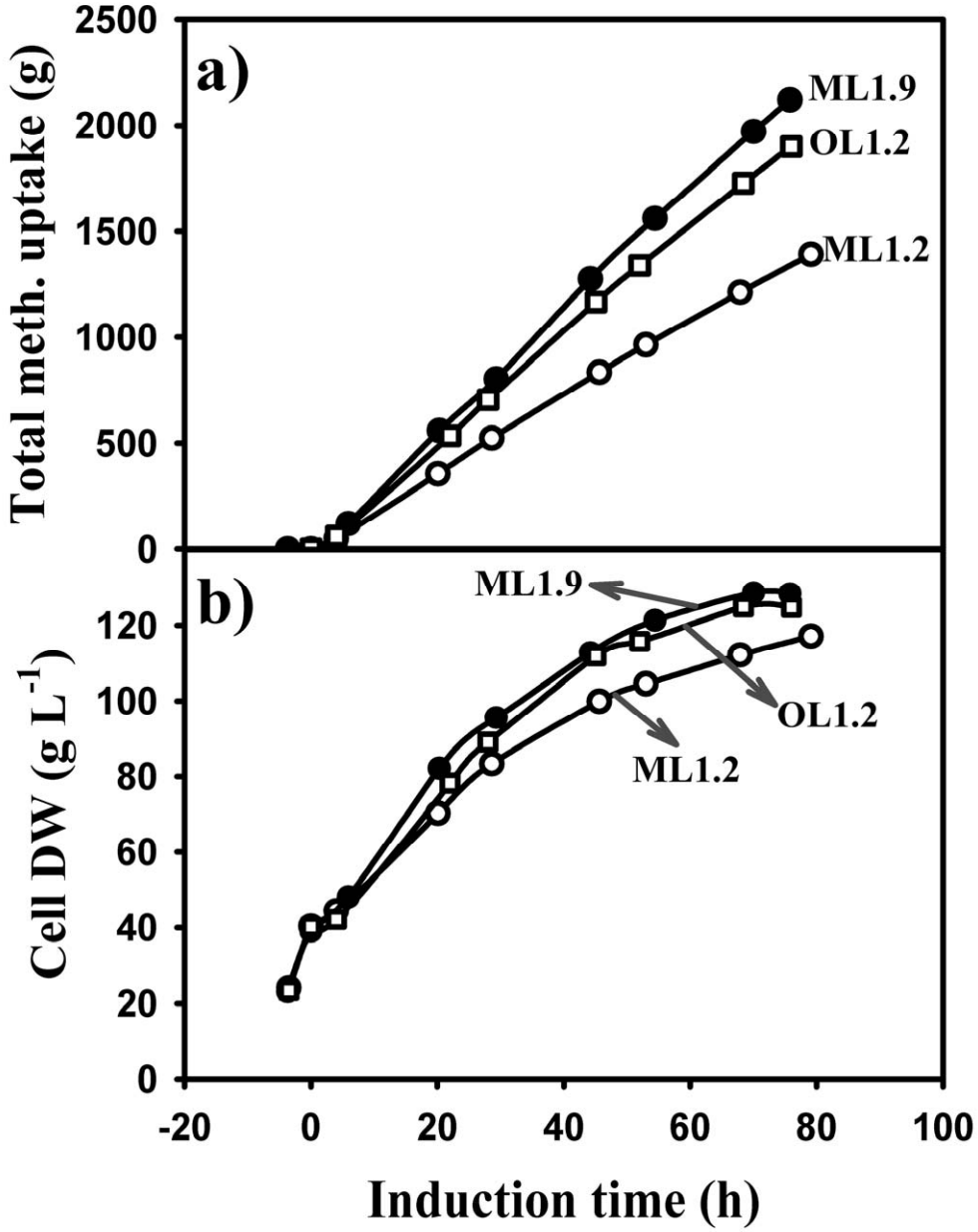

FIG 2

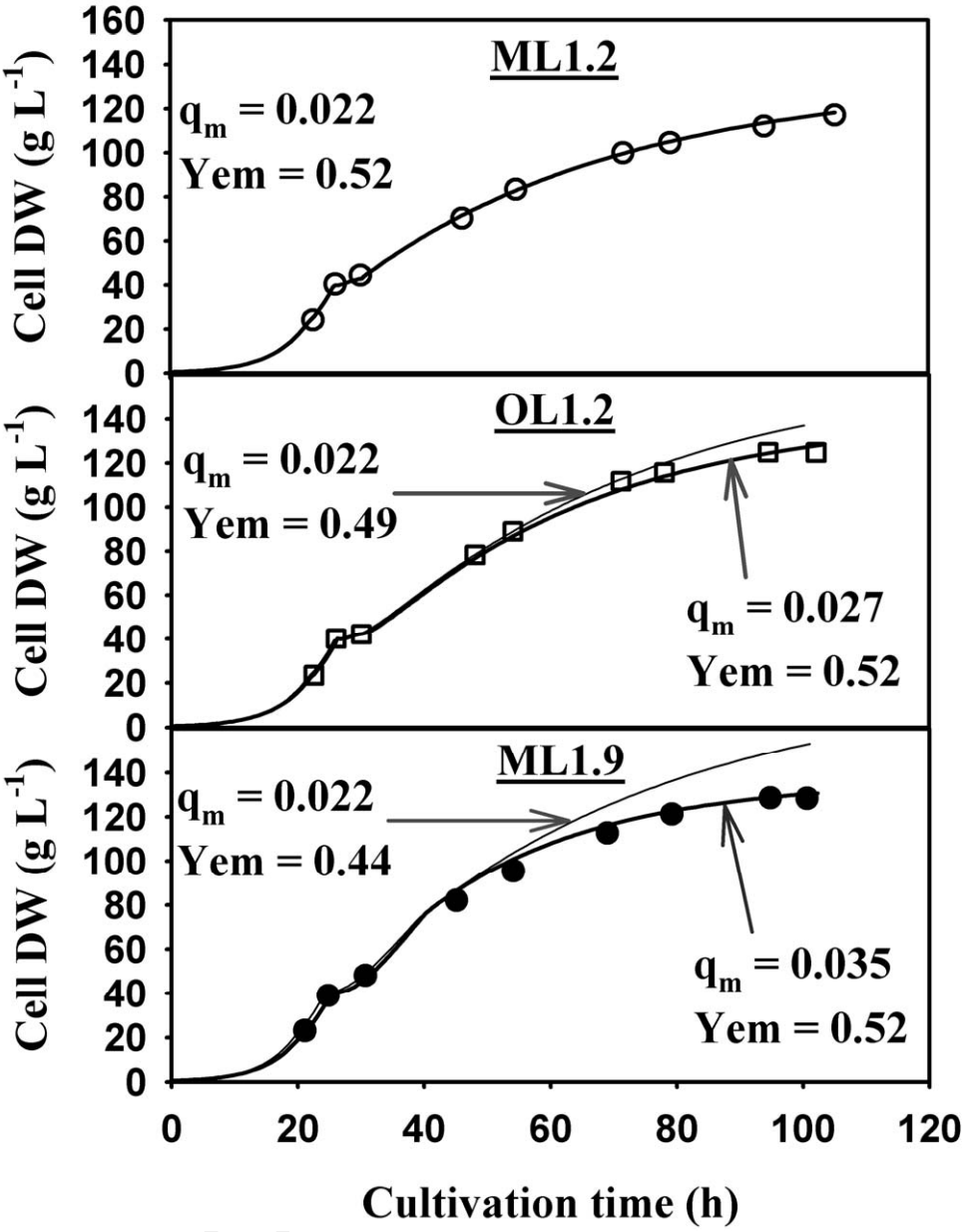

FIG 3

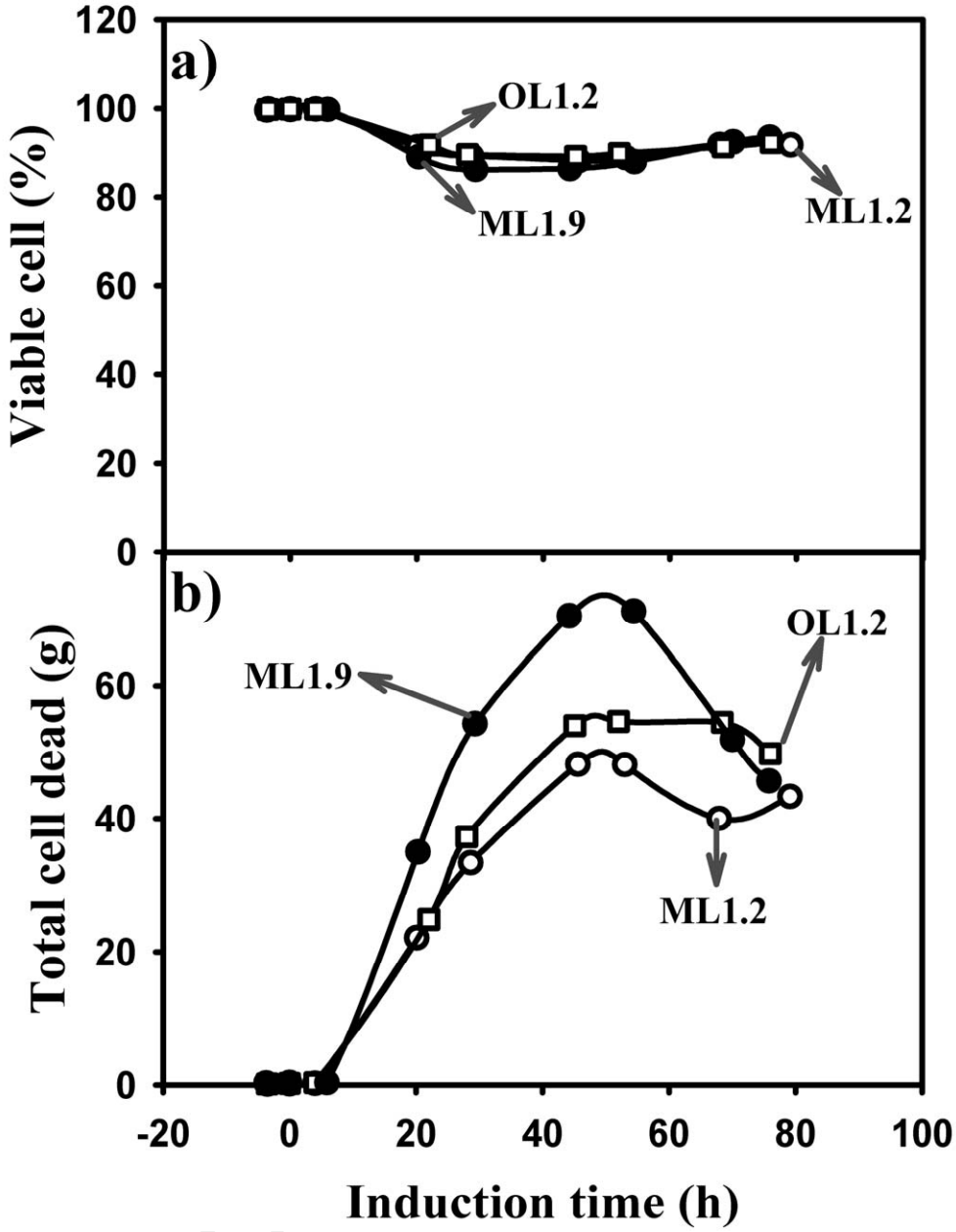

FIG 4

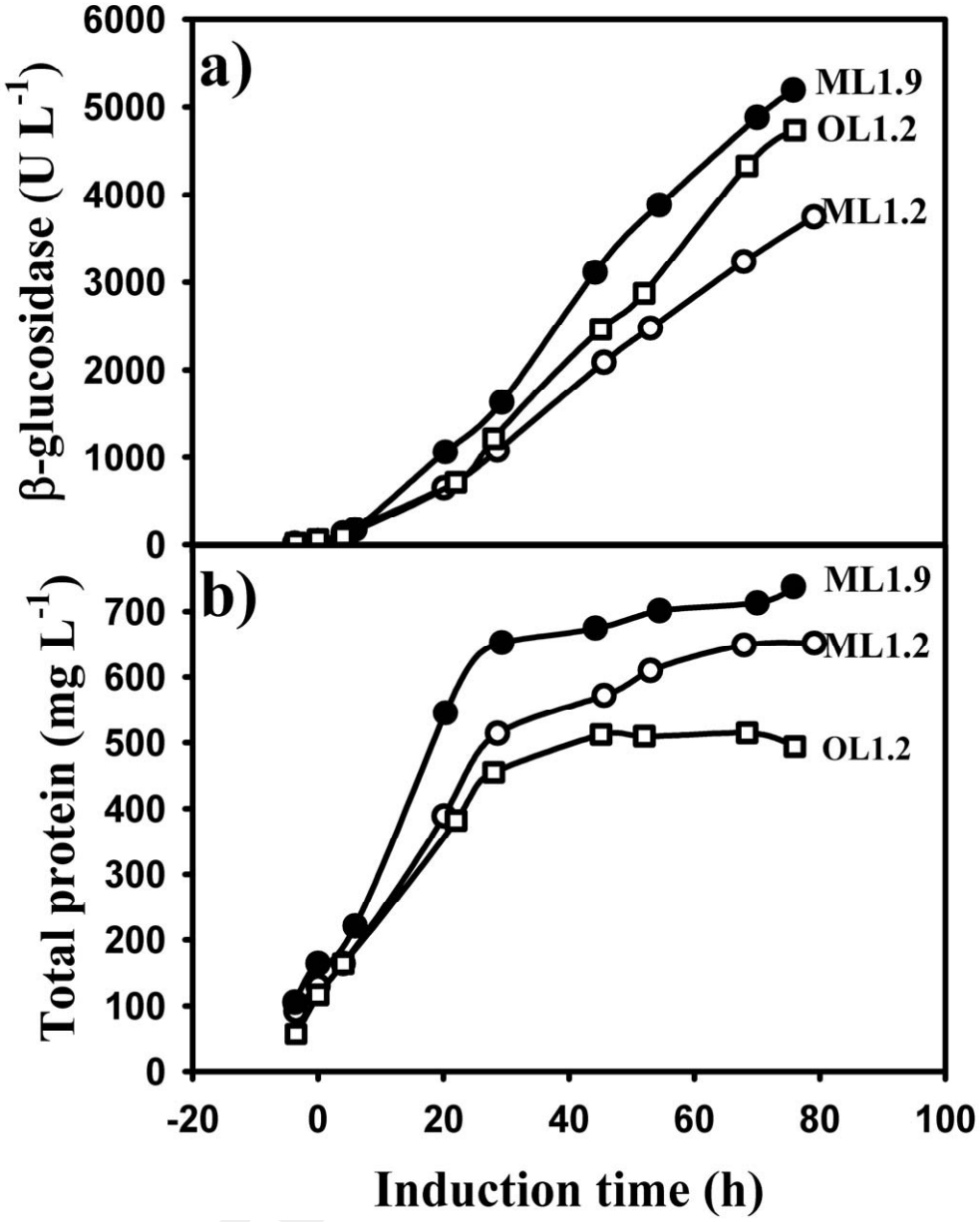

FIG 5

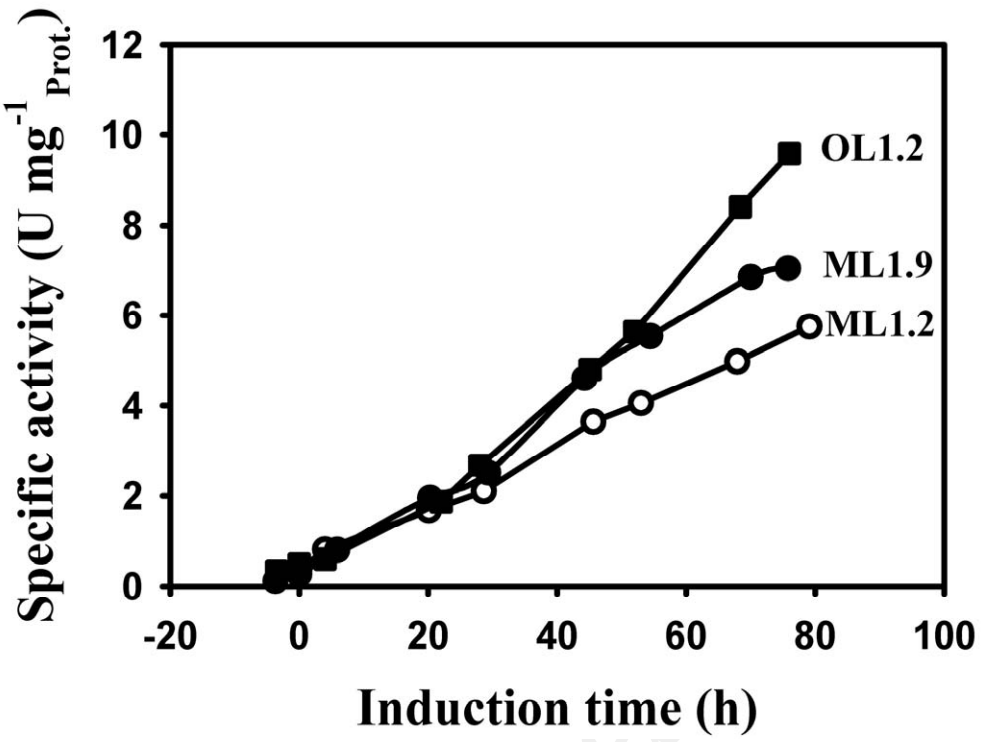

FIG 6

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Charoenrat, T., Ketudat-Cairns, M., Jahic, M., Veide, A. and Enfors, S.-O. (2006) Increased total air pressure *versus* oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process. Biochemical Engineering Journal. *In press*.

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Charoenrat, T., Ketudat-Cairns, M., Jahic, M.**,** Enfors, S.-O. and Veide, A. (2005) Comparison of novel type Streamline Direct CST1 adsorbent with Streamline SP for recovery of recombinant β-glucosidase from *Pichia pastoris* high cell density culture broth. (Poster presentation) The 13th International Conference on Biopartitioning and Purification (BPP2005) During June 20 - 24, 2005 at Amsterdam, Netherland.

Nilsson-Välimaa, K., Vasi, J., Belew, M., **Charoenrat, T.**, Lundström, C.S., Veide, A. and Magnusson, R. (2005) Purification of a β-glucosidase in two parallel processes - An integrated process application for scale-up and modelling. Cap'05; Initial recovery and capture techniques. 22nd-25th May, 2005, Phoenix, Arizona, USA.

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Charoenrat, T. and Ketudat-Cairns, M. (2003) Influence of pH on Recombinant β-glucosidase Production by *Pichia pastoris*. (Poster presentation) BioThailand 2003: Technology for Life, 17-20 July, 2003, Pattaya, Thailand.

Charoenrat, T., Wanchaitanawong, P., Kitpreechavanich, V. and Vanichsriratana, W. (2001) Fed-batch ethanol production from waste cassava starch by pH-dependent substrate feed. (Poster presentation) BioThailand 2001: From Research to Market, November, 2001, Bangkok, Thailand**.**

PATENT:

Charoenrat, T. and Vanichsriratana, W. (2002) Ethanol Production from Cassava Waste Hydrolysate. Thai Patent