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Review

Carbon source feeding strategies for recombinant protein expression in *Pichia pastoris* and *Pichia methanolica*

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Pichia pastoris **and** ***Pichia methanolica*** **have been used as expression systems for the production of recombinant protein. The main problems of the production are the slow hierarchic consumption of ethanol and acetate which cause toxicity problems due to methanol accumulation when this surpasses 0.5 gl^{-1}. In some cases, the laboratory scale cultures does not show the methanol accumulation problems because the cells are usually washed before changing the depleted carbon source culture media, by a fresh one, supplemented with methanol; a strategy that is clearly inapplicable in the industrial productions. Other authors use to feed the methanol before the depletion of glycerol or D-glucose, but this practice does not guaranty the ethanol and acetate fast consumption; leading to methanol accumulation and toxicity problems. It was concluded that pre-induction stage strategy should be studied in detail and that it is very important to start the induction stage with a concentration of biomass as great as possible. On the other side, it is essential that there should be a monitoring of ethanol and acetate until reaching non-toxic methanol stable-concentration and these conditions should be maintained till the end of the process.**

Key words: Carbon source, protein expression, *Pichia pastoris*, *Pichia methanolica*.

INTRODUCTION

Yeasts are beneficial microorganisms which are used to produce food by employing them in fermentation process, like in the production of goods such as wine, beer, bread, among other leavened products. Recently, these micro organism have been used for production of compounds such as single cell protein (SCP), recombinant vaccines or recombinant proteins, enzymes, growth factors, ethanol and many other biochemical compounds (Cinza et al., 1991; Nigam, 2001; Vassileva et al., 2001; Sevo et al., 2002; Teramoto et al., 2005; Cordoba-Ruiz et al., 2009).

These microorganisms are one of the host cells most frequently used for heterologous proteins expression. Initially the utilization of yeasts for this purpose was focused on *Saccharomyces cerevisiae*, although other species like *Pichia* sp., *Kluyveromyces* sp. or *Hansenula* sp. have been considered up to date (Gellisen and Hollenberg, 1997; Kang et al., 2001; Wang et al., 2007). Different yeast genera, except *S. cerevisiae* and *Schizosaccharomyces pombe*, were classified by Wolf, as non-conventional yeasts (Wolf, 1996).

Methylotrophic yeasts, like *Pichia pastoris*, *Pichia angusta* (*Hansenula polymorpha*), *Pichia methanolica*

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Abbreviations: SCP, Single cell protein; **Mut pathway,** methanol utilization pathway; **AO,** alcohol oxidase; **MO,** methanol oxidase, **PTS,** peroxisomal targeting signals; **DHAS,** dihydroxyacetone synthase, **CAT,** catalase; ***MOD2 (AUG2)***, alcohol utilizing genes; **AOX,** alcohol oxidase genes; **Mut^S,** slow methanol growth phenotype; **Mut$^+$,** wild type; **Mut$^-$,** negative methanol utilization.

(*Pichia pinus*) or *Candida boidini* among other methylotrophic yeasts, were included in the non-conventional group according to their capacity to use methanol as sole carbon and energy source, which is given by the presence of genes coding enzymes related with the methanol utilization pathway (Mut pathway), (Poutou et al., 2005b; Hartner and Glieder, 2006).

Several studies have reported that methanol metabolism in methylotrophic yeasts requires the expression of alcohol oxidase (AO) (E.C.1.1.3.13) or methanol oxidase (MO) from *P. pastoris*, *P. methanolica* or *P. angusta* (Couderc and Baratti, 1980; Nakagawa et al., 1996). The AO and MO promoters and transcription termination sequences has been used to develop different protein expression systems (Kang et al., 2001; Guo et al., 2006; Landazuri et al., 2009) to regulate the heterologous protein expression with the influence of the carbon source feeding strategies (Chauhan and Khanna, 1999; Cordoba et al., 2003).

This review summarizes the carbon source addition strategies for the pre-induction stage when *P. pastoris* and *P. methanolica* were used for the expression of recombinant proteins. There is the necessity of deeper studies of the pre-induction stage's metabolites as ethanol and acetate.

METHYLOTROPHIC YEASTS

Essential steps of the Mut pathway, occurs in the peroxisomes. Consequently yeasts experiments a process called peroxisome biogenesis, when methanol is present in the culture media, increasing the peroxisomes numbers for detoxifying the cell from H_2O_2 generated from the methanol metabolism (Subramani, 1993; Wiemer et al., 1996; Johnson et al., 1999; Snyder et al., 1999).

Peroxisome and methanol metabolism

Peroxisomes are typical organelles present in almost all eukaryotic cells, characterized because they contain oxidative enzymes such as catalase (CAT) (E.C.1.11.1.7) and oxidase, which is the enzyme responsible of generating hydrogen peroxide (H_2O_2). All the peroxisomal enzymes are codified in the nuclear DNA and imported into the organelle after the synthesis of proteins. The peroxisomal targeting signals (PTS) are marks for importation into the peroxisome and it have to be present in each one of the immature proteins that should be imported (de Hoop and Ab, 1992; Glover et al., 1994; Rachubinski and Subramani, 1995; Johnson et al., 1999).

Targeting of peroxisomal proteins

Two peroxisomal targeting signals (PTS) have been studied up to date, the most common, the PTS1 [(**S**/A/V/G)(**K**/R/H)(**L**/I/M/V/Y/F)] is located at the C-terminus region of many peroxisomal proteins in animals, plants and yeasts (van der Klei et al., 2006). Other peroxisomal proteins (named as PST2) is a nonapeptide containing a signal at the N-terminus [(**R**/K) (**L**/V/I) ($\mathbf{X_5}$) (**H**/**Q**) (**L**/A)], which is commonly found in mammalians and yeasts (the most frequent amino acid are shown in bold for both PTS1 and 2), (Poutou et al., 2005b; van der Klei et al., 2006). Several peroxisomal proteins, called peroxins, which participate in the process of importing proteins, have been associated with the cytosolic face of the peroxisome membrane or cytosol containing PTS1 or PTS2 receptors (also know as putative receptors or shuttle-receptors). These receptors are responsible of selective recognition of proteins PTS and to allow importation (Marzioch et al., 1994; Rachubinski and Subramani, 1995; Zhang and Lazarow, 1996; Johnson et al., 1999; Koller et al., 1999; Snyder et al., 1999; Ozimek et al., 2003; van der Klei et al., 2006).

In methylotrophic yeasts, the peroxisomes biogenesis is induced abundantly, when the cells grow in methanol, pectin or oleic acid as sole carbon and energy source (Nakagawa et al., 2005; Poutou et al., 2005b); being clear that the importation of AO, CAT and the oxidase into the organelle has occurred, but when the methylotrophic yeasts cells grows in D-glucose or ethanol the peroxisome´s active degradation is promoted through autophagy process (mediated by proteolysis), (Holzer, 1976; Veenhuis et al., 1978; Yuan et al., 1997; Guan et al., 2001; Stromhaug et al., 2001).

Methanol metabolism in methylotrophic yeast

Methylotrophy in eukaryotic cells refers to the ability of an organism to use C_1-compounds like methanol as energy sources, having the capacity to derive all energy and cell carbon from reduced molecules that have no C - C bond. The general mode to assimilated methanol is to convert three C_1 molecules into a C_3 compound via a cyclic pathway (Poutou et al., 2005b; van der Klei et al., 2006).The methanol metabolism inside peroxisomes starts with the oxidation of methanol into CO_2 by AO, resulting in the formation of formaldehyde and H_20_2 and the decomposition of hydrogen peroxide into H_2O and O_2 by the action of CAT. The peroxisomal dihydroxyacetone synthase (DHAS) (E.C.2.2.1.3) catalyses the formaldehyde metabolism forming two C_3 compounds (dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (GAP)) which are derived from the condensation reaction between formaldehyde and xylulose-5-P (Xu-5p), (Figure 1-I). It is important to mention that other enzymes required for methanol utilization are cytosolic (dissimilation pathway) in yeast, which includes the enzymes for the oxidation of formaldehyde to CO_2 (that is, formaldehyde dehydrogenase (E.C.1.2.1.1), S-formylglutathione hydrolase (E.C.3.1.2.12)

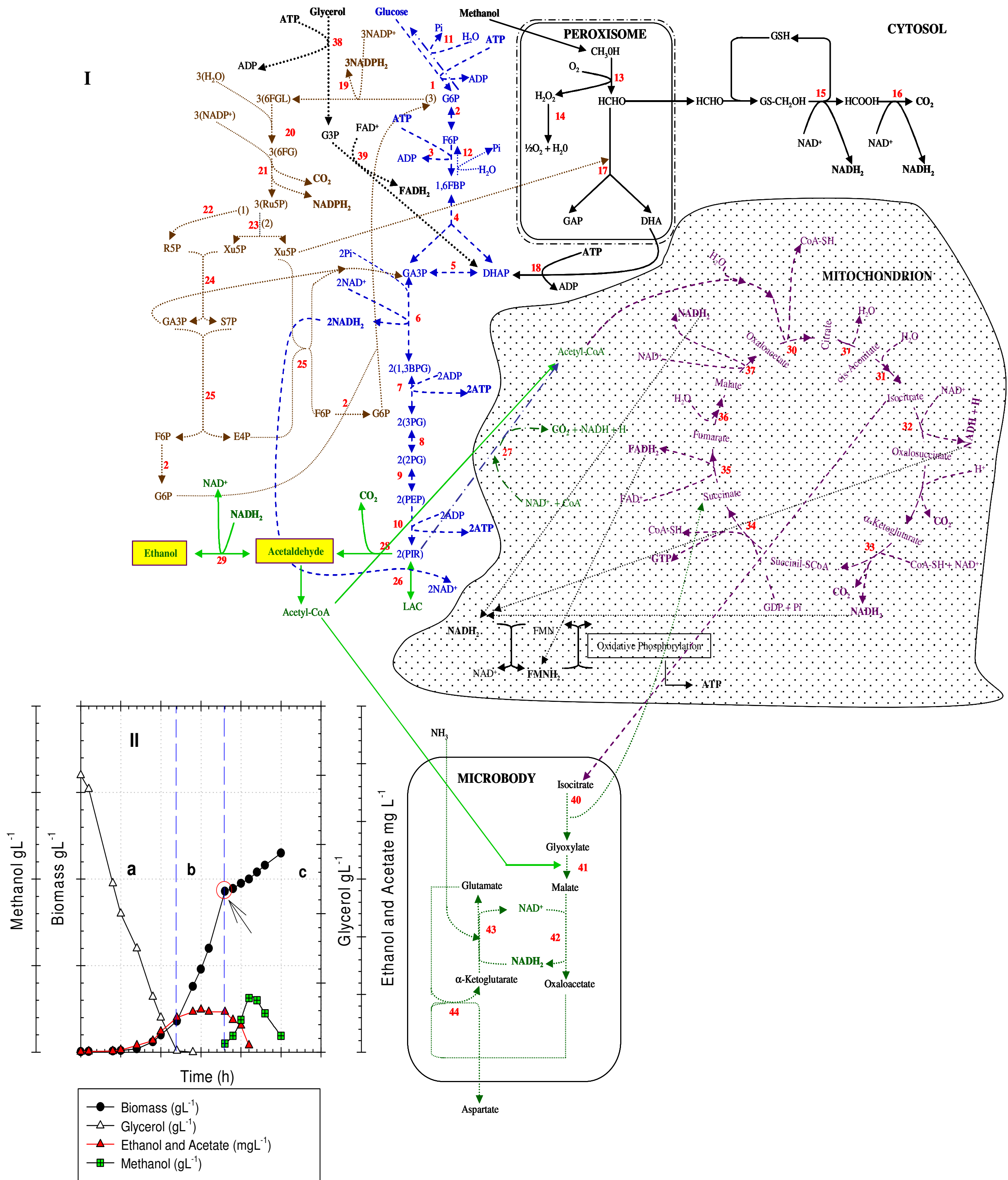

Figure 1. **I**) Metabolic pathway for *Pichia* sp. (Numbers in red): enzymes of the pathway. (Cytosolic line in blue): Glycolysis and Gluconeogenesis Pathways, 1: hexokinase (E.C.2.7.1.1), 2: glucose phosphate isomerase (E.C.5.3.1.9), 3: 6-phosphofructokinase (E.C.2.7.1.11), 4: aldolase (E.C.4.1.2.13), 5: triose phosphate isomerase (E.C.5.3.1.1), 6: glyceraldehyde 3-phosphate dehydrogenase (E.C.1.2.1.12), 7: phosphoglycerate kinase (E.C.2.7.2.3), 8: phosphoglycerate Mutase (E.C.2.7.5.3), 9: enolase (E.C.4.2.1.11), 10: pyruvate kinase (E.C.2.7.1.40), 11: glucose 6-phosphatase (E.C.3.1.3.9), 12: fructose 1,6-biphosphatase (E.C.3.1.3.11). (Cytosolic line in black): Methanol Pathway, 13: alcohol oxidase (E.C.1.1.3.13), 14: catalase

Figure 1. Continues: (E.C. 1.11.1.7), 15: formaldehyde dehydrogenase (E.C.1.2.1.1), 16: formate dehydrogenase (E.C.1.2.1.2), 17: dihydroxyacetone synthase (E.C.2.2.1.3), 18: dihydroxyacetone kinase (E.C. 2.7.1.29). (Cytosolic line in brown): Pentose Phosphate Pathway, 19: glucose 6-phosphate dehydrogenase (E.C.1.1.1.49), 20: lactonase (E.C.3.1.1.45), 21: 6-phosphogluconate dehydrogenase (E.C.1.1.1.43), 22: ribose phosphate isomerase (E.C.5.3.1.6), 23: ribulose phosphate 3-epimerase (E.C.5.1.3.1), 24: transketolase (E.C.2.2.1.1), 25: transaldolase (E.C.2.2.1.2). (Cytosolic line in green): Other cytosolic reactions 26: lactate dehydrogenase (E.C.1.1.1.27), 27: pyruvate dehydrogenase complex (pyruvate dehydrogenase, dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase), 28: pyruvate decarboxylase (E.C.4.1.1.1), 29: alcohol dehydrogenase (E.C.1.1.1.1). (Mitochondrial line in dwelled): Krebs Cycle, 30: citrate synthase (E.C. 4.1.3.7), 31: aconitase (E.C.4.2.1.3), 32: isocitrate dehydrogenase (E.C.1.1.1.41), 33: α-ketoglutarate dehydrogenase (E.C.1.2.4.2), 34: succinyl-CoA synthetase (E.C.6.2.1.4), 35: succinate dehydrogenase (E.C.1.3.99.1), 36: fumarase (E.C. 4.2.1.2), 37: malate dehydrogenase (E.C. 1.1.1.37). (Mitochondial line in black): Oxidative Phosphorylation. (Cytosolic dash line in black): Glycerol metabolism, 38: glycerol kinase (E.C.2.7.1.30), 39: glycerol 3-phosphate dehydrogenase (E.C.1.1.99.5). (Microbody dash line in green): Glyoxylate Cycle, 40: isocitrate lyase (E.C. 4.1.3.1), 41: malate synthase (E.C. 2.3.3.9), 42: malate dehydrogenase (E.C. 1.1.1.37), 43: glutamate dehydrogenase (E.C. 1.4.1.2), 44: glutamate oxaloacetate transaminase (E.C. 2.6.1.1). **II)** Schematical representation of *Pichia* sp., culture: a: first stage (batch culture), b: pre-induction stage (feed-batch culture), c: methanol induction stage (feed-batch culture). Taken and modify from (Sulter et al., 1991; Cereghino and Cregg, 2000; Schuller, 2003; Poutou et al., 2005b; Sola et al., 2007).

and formate dehydrogenase (E.C.1.2.1.2)), (Poutou et al., 2005b; van der Klei et al., 2006). van der Klei et al. (2006) have mention the importance of the localization of AO, CAT and DHAS peroxisomes of cells grown in batch cultures on methanol (Cereghino and Cregg, 2000; van der Klei et al., 2006).

P. pastoris and *P. methanolica* similarities and differences

P. methanolica and *P. pastoris* offer many advantages in the expression of eukaryotic systems, such as protein processing and protein folding, making them capable of being manipulated in the laboratory as *Escherichia coli* or *S. cerevisiae* offering high levels of proteins expression levels (10 to 100-fold times). Additionally, there is also cross-complementation between Saccharomyces genes such as *HIS3*, *HIS4*, *LEU2*, *ARG4*, *TRP1*, *URA3* or *ADE2* and *LEU2* from *P. pastoris* or *P. methanolica,* respectively (Yong et al., 1992; Hiep et al., 1993; Paifer et al., 1994; Raymond et al., 1998).

These strains have two genes encoding for AO, *AOX1* and *AOX2* genes (alcohol oxidase) in *P. pastoris* and *MOD1 (AUG1)* and *MOD2 (AUG2)* genes (alcohol utilizing gene) in *P. methanolica* (Nakagawa et al., 1996; Raymond et al., 1998). *AOX1* and *AUG1* genes appear to be responsible for the majority of AO activity inside the cell. The promote genes have been used to trigger the recombinant expression of the genes encoding the heterologous protein (Invitrogen, 1996a,b, 2009; Hartner and Glieder, 2006).

The expression of Mut pathway genes (*AOX* or *MOD*) are repressed by D-glucose, glycerol or ethanol and strongly induced by methanol through two distinct repression mechanisms. In *P. pastoris*, carbon sources such as D-glucose and ethanol-glycerol mixture cause the repression of both *AOX1* and *AOX2* genes, while in *P. methanolica* the *MOD1* and *MOD2* genes are repressed by D-glucose and ethanol. *MOD1* is de-repressed by glycerol (~60 to 70%), although *MOD2* showed no activity when the cell was grown in glycerol cultures. However, it is important to mention that glycerol-methanol mixture induces the activity of both genes, *MOD1* and *MOD2* (100 and 70% respectively) (Hartner and Glieder, 2006).

In *P. pastoris*, the homologous recombination is predominant. Thus when the expression vector integrates at cell genomic DNA, two different His^+ phenotypes are possible. When *AOX1* gene was knockout, it resulted in a slow methanol growth phenotype (Mut^S), which is a slow utilization of methanol (it contains *AOX2* but lacks *AOX1* gene), while the growth of *AOX2* knockout gene in methanol was very similar to the wild type (Mut^+) (positive utilization of methanol that contains both *AOX1* and *AOX2* genes). Double knockout strains, can not grow on methanol (Mut^-), (negative methanol utilization) as sole carbon and energy source due to the lack of *AOX1* and *AOX2* genes, (Invitrogen, 1996b, 2009; Raymond et al., 1998; Hartner and Glieder, 2006).

When *P. methanolica* was transformed, non-homologous recombination events predominate (> 90%). Therefore, the expression cassette is integrated at several places, resulting in a Mut^+ phenotype, (Hiep et al., 1993; Raymond et al., 1998). Two different phenotypic classes of Ade^+ transformants can be obtained, Mut^+ and Mut^S. Mut^S phenotype appear because *MOD1* (*AUG1*) gene knockout, while a Mut^+ behavior is obtained when *MOD2* (*AUG2*) gene is knockout. This behavior was described by Raymond et al. (1998) using low concentration of methanol (1% v/v). Although, at concentration of 2% v/v of methanol both mutants (Mut^+ or Mut^S) grow as Mut^S, being better than the *mod1D* strain at 0.25% v/v of methanol. The growth deficiency of the *mod1D* variant is still more severe than the one of the *mod2 D* strain. The growth of mutants at 3% v/v of methanol was similar to the one observed for Mut^+ (wild type), due to the gene compensation; restoring a wild-type growth rate and biomass yield (Hartner and Glieder, 2006; Nakagawa et al., 2006).

In terms of glycosylation pattern, *P. pastoris* do not

Table 1. Some heterologous proteins expressed in *P. pastoris* or *P. methanolica*.

Protein	Protein origin	Host cell	Reference
Surface Antigen	Hepatitis B Virus	*P. pastoris*	(Hardy et al., 2000)
Rennin (E.C. 3.4.23.4)	*M. pusillus*		(Beldarraín et al., 2000)
11 β-hydroxysteroid Dehydrogenase 1/carbonyl Reductase (E.C. 1.1.1.146)	*H. sapiens*		(Blum et al., 2000)
Human α-Galactosidase A (E.C. 3.2.1.22)	*H. sapiens*		(Chen et al., 2000)
Dextranase (E.C. 3.2.1.11)	*P. minioluteum*		(García et al., 2000)
RNA Polymerase (E.C. 2.7.7.6)	Influenza Virus		(Hwang et al., 2000)
Cystatin-C	*H. sapiens*		(Files et al., 2001)
G2 Protein	Hantaan (HTN) Virus		(Ha et al., 2001)
Cinnamoyl Esterase (E.C. 3.1.1.42)	*A. niger*		(Juge et al., 2001)
Surface Antigen I	*T. gondii*		(Letourneur et al., 2001)
Lipase (E.C. 3.1.1.3)	*R. oryzae*		(Minning et al., 2001)
Hexose Oxidase (E.C. 1.1.3.5)	*C. crispus*		(Wolf et al., 2001)
Cellobiose Dehydrogenase (E.C. 1.1.99.18)	*P. chrysosporium*		(Yoshida et al., 2001)
μ-Opioid Receptor	*H. sapiens*		(Sarramegna et al., 2002)
Glucocerebrosidase (E.C. 3.2.1.45)	*H. sapiens*		(Sinclair and Choy, 2002)
Endotoxin Neutralizing Protein	*Limulus*		(Paus et al., 2002)
Penicillin G Amidase (E.C. 3.5.1.11)	*P. rettgeri*		(Sevo et al., 2002)
Polymerases	Hepatitis B Virus	*P. methanolica*	(Choi et al., 2002)
Glycoprotein D (type 1 and type 2)	Herpes Simplex Virus	*P. pastoris*	(van Kooij et al., 2002)
Isopullulanase (E.C. 3.2.1.57)	*Aspergillus* sp.		(Akeboshi et al., 2003)
Interferon-β1	*H. sapiens*		(Skoko et al., 2003)
Xylanase (E.C. 3.1.1.)	*T. lanuginosus*		(Triches et al., 2003)
Nitrate Reductase (E.C. 1.7.1.3)	*R. communis* L		(Tsai et al., 2003)
Granulocyte-macrophage colony-stimulating factor	*H. sapiens*		(Wu et al., 2003)
Alkaline Phosphatase (E.C. 3.1.3.1)	*P. abyssi*		(Zappa et al., 2003)
Defensin 1	*P. sativum*		(Larentis et al., 2004)
Tryptophan Hydroxylase (E.C. 1.14.16.4)	*H. sapiens*		(McKinney et al., 2004)
Lignin peroxidase (E.C. 1.11.1.14)	*P. chrysosporium*	*P. methanolica*	(Wang et al., 2004)
Larval Excretory-Secretory Antigen	*T. canis*	*P. pastoris*	(Fong and Y., 2004)
Laccase (E.C. 1.10.3.2)	*T. versicolor*		(Guo et al., 2005)
Iduronate 2 -sulfate sulfatase (E.C. 3.1.6.13)	*H. sapiens*		(Cordoba-Ruiz et al., 2009)
α-Fetoprotein	*H. sapiens*		(Mashayekhi et al., 2006)
Laccase (E.C. 1.10.3.2)	*T. versicolor*	*P. methanolica*	(Guo et al., 2006)
Lactoferricin	Bovine		(Wang et al., 2007)
	Porcine		(Shan et al., 2007)
Xylanase (E.C. 3.1.1.)	*N. frontalis*		(Tsai and Huang, 2008)
Lignin peroxidase H2 (E.C. 1.11.1.14)	*P. chrysosporium*	*P. pastoris*	(Wei and Xianghua, 2009)

have the tendency to hyper-glycosylated as *S. cerevisiae*; although both strains are able to make mannose-rich N-glycosylation. This glycoside chain length is shorter in *P. pastoris* than in *S. cerevisiae* (Montesino et al., 1998; Lundblad, 1999; Cereghino and Cregg, 2000; Pratap et al., 2000; Medzihradszky et al., 2004). Nevertheless, it has been shown that the glycoside chain in *S. cerevisiae* leads to hyper-antigenicity of the protein expressed in this microorganism, limiting the utilization of this yeast for therapeutic protein expression (Jenkins et al., 1996). For this reason, humanize N-glycosylation strains of *P. pastoris*, have been developed to solve this problem (Bretthauer, 2003; Choi et al., 2003; Jacobs et al., 2009).

On the other hand, the post-translational modifications

in *P. methanolica* have not been well-characterized (Invitrogen, 2009), including the N-glycosylation pattern. In both *P. pastoris* and *P. methanolica*, the α-factor signal peptide from *S. cerevisiae* was correctly processed (Barragan et al., 2005; Invitrogen, 2009).

STRATEGIES FOR CARBON SOURCE UTILIZATION

Many heterologous proteins has been produced using recombinant methylotrophic yeasts such as *P. pastoris* or *P. methanolica* (Table 1) and several carbon source addition strategies has been used to increase the yield products. The carbon source addition strategy depends on the Mut strain type, resulting in the transformation process which depends of the regulation of the AO genes.

In general, the fermentation strategies for the production of recombinant proteins in *P. pastoris* involved two or three stages. The first stage, known as biomass production is designed to get high biomass yield from a non-fermentable carbon source like glycerol. In this stage, a batch culture is carried out, taking care of avoiding oxygen limitation to prevent ethanol or acetate accumulation in the culture media, because glycerol, ethanol and acetate repress the *AOX1* promoter (Cordoba et al., 2003). The D-glucose exerts a transcription repression over the *AOX1* gene (Poutou et al., 2005b).

Mut strains can not use methanol as sole carbon and energy source. In this case, it is necessary that they utilize other carbon source such as trehalose, alanine, sorbitol, or mannitol, which do not inhibit the enzymes that metabolizes methanol and also, the expression of the cloned protein that depends on methanol addition (induction). The behavior of Mut^- strains at this condition is similar to a Mut^+ strain (Zhang et al., 2000). Mut^s strains can use methanol as sole carbon source at lower concentration than a Mut^+ but, at higher concentrations than a Mut^- strains.

Some authors waits for the depletion of glycerol or D-glucose to start before the induction stage (methanol addition). This practice promotes ethanol accumulation and the transient accumulation of acetate. The cell then uses ethanol > acetate as carbon sources and methanol accumulates until the acetate is finished (diauxic growth), (Hardy et al., 2000; Lange et al., 2001; Poutou et al., 2005b; Tsai and Huang, 2008).

In Table 2, it was observed that in some productions, a pre-induction stage (feed-batch culture) is included at which glycerol is feed into the process, at lower concentration to promote the glycerol-limiting growth. Later, the glycerol rate is reduced to start the methanol addition (Lim et al., 2000, 2003; Files et al., 2001; Minning et al., 2001). The addition of methanol before total glycerol depletion means that ethanol will be produced and preferred as carbon source before methanol. But the acetate accumulation should be around 10 fold less with shorter time for diauxic growth and less methanol accumulation (Inan and Meagher, 2001). In this sense, the glycerol-methanol mixture does not promote a complete diauxic behavior in spite of the consumption hierarchy (glycerol > ethanol > acetate (accumulated from ethanol utilization) > methanol, (Poutou et al., 2005b). This indicates that glycerol does not repress the synthesis of the enzymes of the methanol metabolism to the same extent as ethanol, since the enzymes for methanol metabolism appear before the glycerol is completely exhausted (Zhang et al., 2000; Inan and Meagher, 2001). Some authors replace the pre-induction stage by a centrifugation and cell wash, followed by the addition of fresh-culture-media (Poutou et al., 2005a) with the main purpose of eliminating the ethanol and acetate produced from the glycerol metabolism. This is a practice that is unfeasible industrially.

The strategy for *P. methanolica* differs from *P. pastoris* principally because of the regulation that exerts the carbon source over *MOD1* and *MOD2* genes as previously described. For example some authors use D-glucose for batch culture and after carbon source depletion, a methanol feeding strategy is implemented (feed-batch culture) (Tsai and Huang, 2008). In both cases, the best culture conditions (biomass production carbon source, temperature, pH, O_2, methanol feeding strategy) and the results vary according to the strain type of Mut mutant and according to the intrinsic nature of the protein to be expressed (Cos et al., 2006) (Table 2).

Studies made for Inan and Meagher (2001) showed that both ethanol and acetate are consumed by the cell before recombinant β-gal activity is detectable (controlled by *AOX1* promoter) in *P. pastoris* (Inan and Meagher, 2001). These authors showed that the addition of 10 mgl^{-1} of ethanol at the beginning of induction stage delayed the expression of β-gal and the methanol use for at least 4 h repress the *AOX1* promoter, but support the cell growth. In contrast, the addition of 50 mgl^{-1} of acetate delayed the expression of β-gal for 2 h. These results agreed with the Sibirny´s findings that proved the inactivation of AO and CAT caused by ethanol and acetate (Poutou et al., 2005b) (Figure 1).

In relation to the pre-induction stage, all the strategies (Table 2) are very similar for the Mut^S or Mut^+ phenotype, being directed to decrease methanol accumulation through a fast consumption of ethanol and acetate. This is the reason for the use of a pre-induction stage at which a low rate of glycerol feed-batch culture is implemented. During the first stage (Figure 1-IIa) the high glycerol concentration (~40 gl^{-1}) promote the formation of acetate and ethanol (Figure 1-I). These compounds were accumulated and hierarchy consumed after glycerol depletion (Poutou et al., 2005b). The accumulation of ethanol and acetate caused the inhibition of AO during a period of time necessary for the use of both compounds (Sibirny, 1990; Poutou et al., 2005b), which implied that the recombinant protein expression is affected by the accumulation of methanol when the concentration was

Table 2. Recombinant protein production depending on the culture scale and carbon source feeding strategy used.

	Protein	**Human Liver Monoamine Oxidase B (E.C. 1.4.3.4)**	**Human angiostatin**	**Hepatitis B surface antigen**	***Rhizopus oryzae* Lipase (E.C. 3.1.1.3)**	**Human cystatin-C**		**Atrazine-specific Fab fragment K411B**	**Elastase inhibiting peptide**	**Guamerin**	**Iduronate 2-sulfate sulfatase (E.C. 3.1.6.13)**	***Neocallimastix frontalis* Xylanase (E.C. 3.1.1.)**	
	Mut phenotype	**Mut^S**	**Mut^S**		**Mut^S**	**Mut^S**	**Mut^S**	**Mut$^+$**	**Mut$^+$**		**Mut$^+$**	**Mut^S**	**Mut$^+$**
	Microorganism	***P. pastoris***											***P. methanolica***
Stage 1 (biomass production)	Culture volume (L)	2	10	~2000	3.5			4	1.5	1	1		
	Time (h)		7			22	22			15	~25	~25	~26
	pH	4.5–5.0	5.5	5	5.5	5	5	7	5	5	5 - 6	5	6
	DO (%)	30	30		20	30	30	20	20	30	20		
	Batch Culture Media												
	$(NH_4)_2SO_4$			+									
	Biotin		0.4mM										
	$CaSO_4\ 2H_2O$	0.93 gL^{-1}			0.93 gL^{-1}	0.93 gL^{-1}	0.93 gL^{-1}		0.9 gL^{-1}	0.9 gL^{-1}	0.15 gL^{-1}		
	D-Glucose												*BMY + 40 gL^{-1}
	Glycerol (100%)	40.0 gL^{-1}	30 mlL^{-1}	+	40 gL^{-1}	40 gL^{-1}	40 gL^{-1}		40 gL^{-1}		25 gL^{-1}	40 gL^{-1}	
	H_3PO_4 (85% v/v)	27 mlL^{-1}			26.7 mlL^{-1}	26.7 mlL^{-1}	26.7 mlL^{-1}		27 mlL^{-1}	27 mlL^{-1}			
	K_2SO_4	18.2 gL^{-1}			18.2 gL^{-1}	18.2 gL^{-1}	18.2 gL^{-1}		18 gL^{-1}	18.0 gL^{-1}	2.40 gL^{-1}		
	KH_2PO_4		13.6 gL^{-1}	+									
	KOH	4.13 gL^{-1}			4.13 gL^{-1}	4.13 gL^{-1}	4.13 gL^{-1}		4.13 gL^{-1}	4.13 gL^{-1}	0.65 gL^{-1}		
	Methanol (100%)							0.5% (v/v)					
	$MgSO_4$									10.24 gL^{-2}			
	$MgSO_4\ 7H_2O$	14.9 gL^{-1}		+	14.9 gL^{-1}	14.9 gL^{-1}	14.9 gL^{-1}		14.9 gL^{-1}		1.95 gL^{-1}		
	NH_4OH									30 mlL^{-1}			
	Peptone		20 gL^{-1}					20 gL^{-1}					
	PTM1	12 mlL^{-1}	X	+	+	4.35 mlL^{-1}	4.35 mlL^{-1}		6.6 mlL^{-1}	+	4 mlL^{-1}		
	Vitamins			+									
	Yeast extract		10 gL^{-1}					10 gL^{-1}					
	YNB		13.4 gL^{-1}										

Table 2. Contd.

Pre-induction stage (Feed-batch culture)	Time (h)	5-8	1		5	4	4			13		48	24
	OD_{600nm}		~150-200										
	Wet cell mass	180 gL-1								140 g L-1			
	Glycerol feeding rate												
	Glycerol	50% (w/v)	50% (w/v)		50% (w/v)	50% (w/v)	50% (w/v)		80% (w/v)	80% (w/v)			
	PTM1	12 ml L^{-1}			12 ml L^{-1}	12 ml L^{-1}	12 ml L^{-1}		12 ml L^{-1}	12 ml L^{-1}			
	Feeding rate	18 ml h^{-1} L^{-1}	18 ml h^{-1} L^{-1}		2h, 300 ml min^{-1}; 1h, 160 ml min^{-1}; 1h, 100 ml min^{-1}; 1h, 65 ml min^{-1}	15 ml h^{-1} L^{-1}	15 ml h^{-1} L^{-1}		1.6 ml min^{-1}				
Stage 2 (Fedd-batch culture, Induction)	Process time (h)	90	65		120	96		96		65	~120	240	
	Biomas		5kg WW	75-88 g L^{-1} DW	~35 g L^{-1}				average 79.5g L^{-1}		~20g L^{-1} DW		
	DO_2 (%)							20-30%					
	Methanol feeding rate												
	Methanol	100% (w/v)	100% (w/v)	100% (w/v)	100% (w/v)	100% (w/v)	100% (w/v)	40% (w/v)	100% (w/v)	100% (w/v)	100% (w/v)	100% (w/v)	100% (w/v)
	Glycerol		50% (w/v)										
	PTM1	12 ml L^{-1}			12 ml L^{-1}	12 ml L^{-1}	12 ml L^{-1}		12 ml L^{-1}				12 ml L^{-1}
	Peptone							2% (w/v)					
	Yeast extract							1% (w/v)					
	Glycerol feeding rate		2 ml h^{-1} L^{-1}				2.1g h^{-1} L^{-1}						
	Methanol feeding rate	1h, 1 ml h^{-1} L^{-1}; 24h, .9 ml h^{-1} L^{-1}	3h, 1g L^{-1}; 2g L^{-1}	2.9–10.9 g h^{-1} L^{-1}	100 ml min^{-1}	2.25 ml h^{-1} L^{-1}	2.25 ml h^{-1} L^{-1}	~2.7 to 22.5 ml h^{-1}		1.63 to 15.38 g h^{-1}	0.5% (v/v) Methanol each 24h	0.5% (v/v) Methanol in *BMY	1.8ml h^{-1} L^{-1}

over 0.5 gl^{-1} (Poutou et al., 2005a).

The utility of a pre-induction stage strategy (with a glycerol limiting-rate) (Table 2), support the cell growth, allowing the increasing of biomass concentration (high density ~100 gl^{-1} DW). This implied that ethanol and acetate accumulation will not be significantly higher. It is possible to observe that at the end of the pre-induction stage (Figure 1-IIb red circle), the biomass is higher when compared with the biomass concentration at the end of batch culture (Figure 1-IIa). The fact that the ethanol and acetate concentration has a non significant increment; suggest that the time necessary for the consumption of both ethanol and acetate will be lower taking into account the cell concentration. At this moment, it is possible to start the methanol feeding rate, although certain methanol accumulation will occur (Figure 1-IIc),

Table 2. Contd.

	Production information												
Cell lisate or crude extract	Product concentration		20 ± 5 mg L^{-1}	0.097 g L^{-1}		0.778 g L^{-1}		~40 mg L^{-1}	average 166 mg L^{-1}	473 mg L^{-1}			
	Productivity						0.96g h^{-1} L^{-1}						
	Biological activity	1670 U			120 U ml^{-1}						~29 mmol h^{-1} mg^{-1} of total prot.	5400 U ml^{-1}	6200 U ml^{-1}
	Specific activity	0.037 U mg^{-1}											
	References	(Newton, et al., 2000)	(Lin, et al., 2000)	(Hardy, et al., 2000)	(Minning, et al., 2001)	(Files, et al., 2001)		(Lange, et al., 2001)	(Lee, et al., 2003)	(Lim, et al., 2003)	(Córdoba-Ruiz, et al., 2009)	(Tsai and Huang, 2008)	

*: (Invitrogen, 1996b, 2009), DO = dissolve oxygen; DW = dry weight; WW = wet weight; + = authors did not specify concentration.

the toxic levels will be not reached; favoring the expression and yield of the recombinant protein. The glycerol addition during the pre-induction stage does not guaranty the fast consumption of either ethanol or acetate. However, lower glycerol concentrations acts by de-repressing the AO synthesis which increase the cell concentration (as was mentioned before) and prepare it for the methanol induction stage.

That methanol accumulation does not reach toxic levels depends on the methanol feeding rate (at induction stage). It was found that a discrete modification of methanol rate at the beginning of the induction stage followed by a constant flow until the end of process, favors the recombinant protein expression when a gly-cerol feed rate has been included as a pre-induction stage. Bearing this in mind, it was detected that ethanol and acetate at first and pre-induction stages evidence the metabolism process represented in Figure 1-IIab (data proximally to be published). The relation of ethanol/acetate excretion still remains unknown, but the detection in the culture media suggests a carbon source and energy reserve mechanism caused by the depletion of the initial carbon source.

For *P. methanolica*, the analysis was different because glycerol does not limit AO but probably, the production of pyruvate from D-glucose rises to ethanol and acetate, suggesting that a similar treatment as in *P. pastoris* will be necessary.

Summarizing the studies on the carbon source addition strategies for the production of recom-binant protein in two very similar expression systems, *P. pastoris* and *P methanolica,* it was clear that a key fact is related with the pre-induction stage at which it is very important to increase the biomass concentration with the main object of accelerating the consumption of both ethanol and acetate at the beginning of the methanol induction stage. It is urgent to make further studies of this culture step taking into account the importance of understanding the nature of the expressed protein

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