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Non-Repressing Carbon Sources for Alcohol Oxidase (*AOX1*) Promoter of *Pichia pastoris*

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The growth of *Pichia pastoris* in a mixture of either glycerol or glucose and methanol follows a diauxic growth, with C_1 utilizing enzymes being repressed. Therefore, these carbon sources can not be used as a mixture with methanol to simultaneously grow *P. pastoris* and induce C_1 utilizing enzymes, especially in a shake flask cultures of AOX-deficient *P. pastoris*. Among the alternative carbon sources tested, alanine, sorbitol, mannitol and trehalose, did not repress β-gal production when methanol was used as an inducer in mut⁻ strain of *P. pastoris*. Our results show that either one of alanine, sorbitol, mannitol or trehalose can be used as a sole carbon and energy source for *P. pastoris*, although the doubling time on trehalose was very long. Mut⁻ strains growing in media containing trehalose, alanine, sorbitol and mannitol with methanol (0.5%) as an inducing agent expressed as much or higher amount of β-gal as compared to the mut⁺ growing in methanol containing media.

[**Key words:** *Pichia pastoris*, carbon catabolite repression, *AOX1* promoter]

Pichia pastoris, a methylotrophic yeast, has proven to be an outstanding host for the production of heterologous proteins for academic and industrial interest. A unique feature of the expression system is the promoter employed to drive heterologous gene expression, which is derived from the methanol regulated alcohol oxidase I gene (*AOX1*) of *P. pastoris*, one of the most efficient and tightly regulated promoters known (1).

The production of heterologous proteins in fermentor cultures of *P. pastoris* involves a three-stage high cell-density fermentation scheme. The first stage is the batch phase in which the culture is grown in a salt medium on a non-fermentable carbon source, such as glycerol. Upon glycerol depletion, the second phase (transition phase) is initiated by adding glycerol at a growth-limiting rate. The second phase is important since by-products *i.e.*, ethanol generated during batch phase is consumed and cells are primed for induction. The third phase is the induction phase, which is initiated by adding limited methanol (2).

Three *m*ethanol-*ut*ilizing phenotypes (mut⁻, mut^s and mut) of *P. pastoris* have been used for the production of heterologous proteins. The genome of the *P. pastoris* contains two copies of the alcohol oxidase (*AOX*) gene. The *AOX1* promoter, which regulating 85% of the alcohol oxidase activity in the cell, is the promoter used to drive heterologous protein expression in *Pichia*. The *AOX1* promoter-Gene X expression cassette is inserted into the *Pichia* genome along with a *HIS4* (histidinol dehydrogenase) gene for selection of transformed cells in *his* host strain, *i.e.* GS115 (*his4*) strain. Insertion of the expression cassette into *HIS4* locus generates a mut⁻ strain (*m*ethanol *ut*ilization plus), a phenotype indistinguishable from wild type *P. pastoris*. When the expression cassette is inserted within the *AOX1* locus, a mut^s strain (*m*ethanol *ut*ilization *s*low) is generated (3). Another way of obtaining mut^s phenotype of host strain is by disruption of *AOX1* gene by gene insertion *i.e.* KM71 (*arg4his4 AOX1Δ::SARG4*) strain (4).

A third host strain used for heterologous protein expression is the mut (*m*ethanol *ut*ilization *n*egative) strain in which both the *AOX1* and *AOX2* genes are disrupted *i.e.* MC100-3 (*arg4 his4 aox1Δ::SARG4 aox2Δ::Phis4*) (5).

Induction of protein expression by the *AOX1* promoter is controlled by growing the cells on methanol as the sole source of carbon and energy for the mut⁻ and mut^s strains. *P. pastoris* mut^s strains grow very slowly in methanol containing media as a sole carbon source because these stains are defective in *AOX1* gene (6).

Advantages of expressing genes of interest in mut strain of *Pichia* are two fold; first, higher yields of protein were observed in mut^s and mut strain (7, 8). Second, storage of a large amount of methanol in explosion-proof facilities is expensive. The amount of methanol can be reduced using mut strain since 35 fold less methanol is used for induction of a mut strain as oppose to a mut⁻ strain. (8).

The alcohol oxidase defective strain, *i.e.* MC100-3, can not utilize methanol as its sole carbon source. This inability to grow on methanol requires the use of an alternate carbon source, such as glycerol, for growth and recombinant protein production. However non-limiting glycerol concentrations in the shake flask culture causes repression of the *AOX1* promoter (8).

In all methylotrophic yeasts, batch growth on a mixture of glucose and methanol results in a classical diauxic growth

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pattern with glucose being utilized first and concomitant repression of C_1 utilization enzymes (9–11). Among the methanol utilizing yeasts, *Hansenula polymorpha* alcohol oxidase (MOX) is derepressed during the exponential growth on carbon sources such as, sorbitol, glycerol, ribose and xylose (12). The extent of derepression by different carbon sources varied considerably, with ethanol showing the highest repression potential. The repression potential of carbon sources as indicated by expression of MOX in *H. polymorpha* is as follows; ethanol > glucose > ribose > sorbitol > glycerol > xylose > xylitol (12). Although the *H. polymorpha MOX* gene and *P. pastoris AOX1* and *AOX2* genes are not regulated identically, they show some common futures of their expression patterns. Both of them are fully repressed in ethanol and unlimited glucose. Although the *P. pastoris AOX1* gene is not fully derepressed in any limited or unlimited carbon sources (>C_1), non-limiting glycerol and carbon starvation cause some degree of the derepression of the promoter (13).

Utilization of D-alanine as a sole source of carbon and energy by *Candida utilis* has been reported (14). D-Amino acid oxidase converts the D-alanine to pyruvate. Pyruvate can be subsequently used for both energy and synthesis of cell material. Ammonia may be assimilated into amino acids via NADP-dependent glutamate dehydrogenase. Growth of *C. utilis* on D-alanine led to excretion of ammonia, due to relatively low C/N ratio of this substrate. In contrast to utilization of alkylated amines, this ammonia excretion did not result in inhibition of growth (14).

Although fed-batch fermentation has been developed for mut strains (8), there is no carbon source or media composition published, which can be used in shake flask experiments to screen transformed cells. Screening each transformant in fermentor conditions could be highly time consuming and expensive for mut strains. Although mannitol (15) sorbitol and alanine (16, 17) have been considered as nonrepressible carbon sources for *P. pastoris*, comparison of these carbon sources and supporting data have not been reported in the literature. Therefore, the aim of this report is to compare different carbon sources in terms of their ability to support growth and expression of an *AOX1-lacZ* fusion in shake flask studies of a *P. pastoris* mut strain.

MATERIALS AND METHODS

Organisms and cultivation *P. pastoris* strains, MC100-3 (*arg4 his4 aox1Δ::SARG4 aox2Δ::Phis4*) (pSAOH5) and GS115 (*his4*) (pSAOH5) were the generous gift of J. M. Cregg, Keck Graduate Institute, Claremont, CA, USA. The mut⁻ MC100-3 (pSAOH5) is defective in *AOX1* and *AOX2* genes. As a control organism GS115 (pSAOH5) was chosen because it is a wild type strain with a single integrated copy of *AOX1-lacZ* containing fusion vector, pSAOH5. The mut⁻ MC100-3 (pSAOH5) is isogenic to GS115 (pSAOH5) except for the defective *AOX1* and *AOX2* genes. Details of the vector construction and transformations are reported elsewhere (4, 5, 13).

The minimal medium used for cultivation to obtain growth curves contains 1.34% yeast nitrogen base w/o amino acids (YNB), 4×10^{-5}% biotin and 1% carbon source, glycerol, methanol, ethanol, D-L alanine, mannitol, sorbitol, trehalose and acetate.

The *P. pastoris* strains were maintained as frozen stocks and transferred to 10 ml-MGY (Minimal Glycerol media) containing test tubes for preparation of inoculum. After growing the cells in a rotary shaker at 30°C for 12–16 h, the culture was used as the inoculum for a shake flask containing 50 ml of the minimal media supplemented with one of the carbon sources. Samples were taken at time intervals to monitor the growth.

The inoculum for induction studies was prepared in minimal glycerol media (MGY) as described above. This culture was used to inoculate 50 ml MGY containing 250-ml shake flasks. The culture was grown to ~5 O.D at the same conditions. The cells were centrifuged at $2000\times g$ for 10 min, dissolved in minimal media containing one of the tested carbon source plus 0.5% methanol as an inducer, and 0.5% methanol without any carbon source. Growth temperatures were maintained at 30°C in rotary shaker at 200-rpm speed. Samples were taken at time intervals to determine methanol concentration and β-gal activity.

As a control organism GS115 (pSAOH5) mut⁺ strain was used. Methanol was the sole carbon source for growth and induction media. The other conditions were same as for the mut⁻ strain.

Determination of methanol and cell concentrations Methanol was determined with a Shimadzu 17A gas chromatography using a Stabilwax (0.53 mm, 2 μm, 15 m) fused silica capillary column (polyethylene glycol stationary phase) Restek Corporation, Bellefonte, PA, USA. Helium was used as the carrier gas at a flow rate of 10 ml/min, and *n*-propanol was used as an internal standard. Flame ionization detector and injector temperatures was be maintained at 250 and 220°C, respectively. Column temperature was held at 40°C for 10 min, and ramped to 100°C at the rate of 10°C/min.

Turbidity of cells in the fermentation broth was measured with a spectrophotometer (DU-70 Beckman Inc., Fullerton, CA, USA) at 600 nm.

Determination of β-galactosidase activity β-Gal activity was assayed by ONPG as reported by Miller (18) and with the modification proposed by Guarante and Ptashne (19). The cells were permeabilized with chloroform and sodium dodecyl sulfate (SDS). Results were reported in Miller Units. β-gal activity was normalized per OD for all samples.

RESULTS AND DISCUSSION

Evaluation of different carbon sources for mut⁻ strain Although the host and vector system and the fed-batch fermentation process have been developed (8, 20), the use of mut strain of *P. pastoris* as an expression host is limited. After transforming the mut strain with appropriate expression vector to screen optimum expression clones, the common carbon sources glycerol, glucose, ethanol, acetate or methanol can not be used since glucose, glycerol and ethanol represses the *AOX1* promoter (21) and methanol can not be utilized due to deficiency of AOX enzyme. We therefore examined the ability of different carbon compounds to support growth and *AOX1-lacZ* expression in this strain.

MC100-3 (pSAOH5) was grown in minimal media containing 1% of the indicated carbon sources together with 0.5% methanol. GS115 (pSAOH5) was used as a control organism. The carbon source for GS115 (pSAOH5) was methanol, and was monitored during growth. Growth curves are shown in Fig. 1–3. The samples were taken during the logarithmic growth phase to determine non-limiting carbon concentrations and β-gal production (Table 1). Because the growth rate of *P. pastoris* changed depending on the carbon source used, time for sampling also changed. From Table 1,

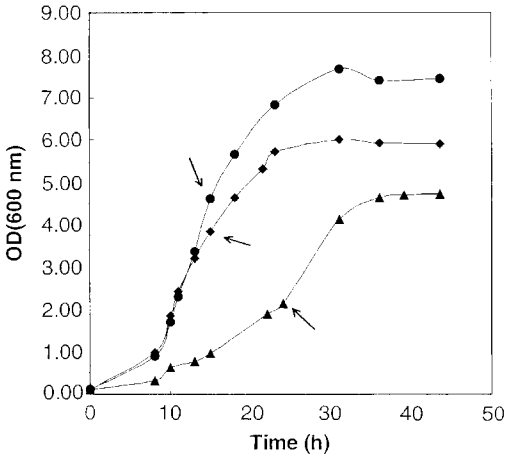

FIG. 1. Growth of *P. pastoris* (mut) in glucose, glycerol and ethanol. MC100-3 (pSAOH5) was grown in minimal media containing 1% of indicated carbon source and 0.5% methanol. Arrows indicate the time at which samples were taken for β-gal assay in Table 1. Solid diamond, glucose; solid circle, glycerol; solid triangle, ethanol.

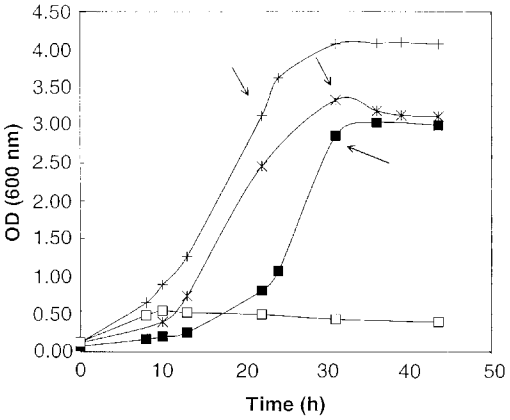

FIG. 2. Growth of *P. pastoris* (mut) in alanine, acetate, methanol and mut in methanol. MC100-3 (pSAOH5) was grown in minimal media containing 1% alanine or acetate and 0.5% methanol. GS115 (pSAOH5) was grown in minimal media containing 0.5% methanol. Arrows indicate the time at which samples were taken for β-gal assay in Table 1. Open square, methanol (mut); plus, alanine; star, acetate; solid box, methanol (mut).

it is very clear that glucose, glycerol, ethanol and acetate supported growth but repressed the expression of β-gal. These are well documented in literature (13, 21). On the contrary, MC100-3 (pSAOH5) growing in media containing alanine, mannitol, sorbitol and trehalose expressed as much or higher amount of β-gal compared to the mut⁻ strain. Methanol was needed, however, to induce the *AOX1* promoter. In the absence of methanol, with the carbon sources tested, no induction or derepression of *AOX1* promoter was observed (data not shown) indicating that the *AOX1* promoter is regulated independently by methanol induction and carbon repression. We have also tested xylose as an alternative carbon source, but *P. pastoris* was not able to utilize it as its sole carbon source.

Chauhan *et al.* (22) have found that the addition of alanine and case amino acids as a supplement improved the HbsAg in shake flask and fermentor conditions. Addition of

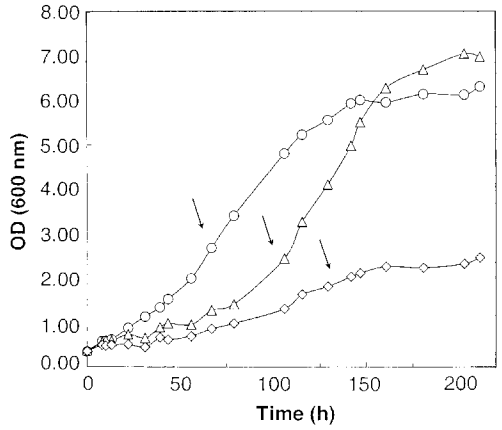

FIG. 3. Growth of *P. pastoris* (mut) in sorbitol, mannitol and trehalose. MC100-3 (pSAOH5) was grown in minimal media containing 1% of indicated carbon source and 0.5% methanol. Arrows indicate the time at which samples were taken for β-gal assay in Table 1. Open circle, sorbitol; open triangle, mannitol; open diamond, trehalose.

TABLE 1. β-Gal production of *P. pastoris* in different carbon sources in presence of methanol as an inducer

Carbon source	Strain phenotype	β-Galactosidase activity (Miller units)	Sampling time[a] (h)
Glucose	Mut	0	15
Glycerol	Mut	0	15
Ethanol	Mut	2±1	24
Acetate	Mut	2±1	31
Trehalose	Mut	781±142	132
Sorbitol	Mut	1116±168	67
Mannitol	Mut	799±125	106
Alanine	Mut	421±48	22
Methanol	Mut	70±5	8
Methanol	Mut	385±35	31

[a] Samples were taken at mid-log range $O.D_{600}$ of 1.5–2.5.

sorbitol decreased the HbsAg expression. In continuous fermentation of *P. pastoris*, the use of sorbitol in mixed feed has been reported (17). Sears *et al.* (15) observed GUS activity under the control of *AOX1* promoter when cells were grown in mannitol as sole carbon source, but we have not observed any β-gal expression in mannitol growing cells unless methanol was added. Difference may be due to expression vector or reporter gene.

In batch culture of *H. polymorpha* with glycerol, sorbitol, ribose and xylose as the carbon source, substantial amount of MOX activity was observed (23). Excess glucose results in complete repression of MOX synthesis, which is partially derepressed under glucose-limiting conditions (24). Ethanol is an even stronger repressor of MOX since total repression observed in batch culture was not relieved by ethanol limitation in continuous culture (9). *P. pastoris* AOX synthesis, however, is fully repressed in glucose, glycerol and ethanol and no derepression occur. Our previous study showed that a very little amount of ethanol and acetate (10 mg/*l*) repressed the *AOX1* promoter in shake flask culture (21).

Expression of β-gal in media containing alanine, mannitol, sorbitol and trehalose The mut strain expressing β-gal was tested in media containing as a carbon source

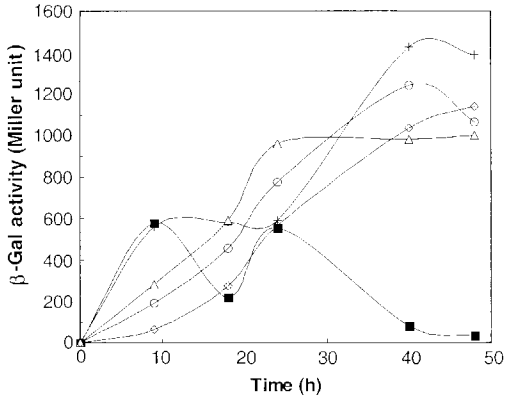

FIG. 4. β-gal production in methanol, alanine, sorbitol, mannitol and trehalose. MC100-3 (pSAOH5) and GS115 (pSAOH5) were grown in MGY to ~5 OD_{600}. The cells were resuspended in minimal media containing 0.5% methanol and the indicated carbon source. Methanol was the sole carbon source for GS115 (pSAOH). Solid box, methanol (mut); plus, alanine; open circle, sorbitol; open triangle, mannitol; open diamond, trehalose.

either alanine, mannitol, sorbitol or trehalose, and 0.5% methanol as an inducer. For a control GS115 (pSAOH5) mut$^-$ strain was grown with methanol as the carbon source. Figure 4 shows the effect of different carbon sources on β-gal expression. Since GS115 (pSAOH5) mut$^-$ strain quickly consumed the methanol in the media, β-gal expression decreased after methanol was depleted. β-gal expression resumed upon the addition of methanol (at 18 h). β-gal expression dropped again at 40 h because no additional methanol was added. Guarna *et al.* (25) also observed fluctuations in protein expression in shake flask experiment of mut$^-$ strains because methanol was consumed quickly and addition of methanol resumed protein production. MC100-3 (pSAOH5) mut strain growing in alanine, mannitol, sorbitol and trehalose continued accumulating β-gal protein throughout the 48-h fermentation process. From these results it is clear that either one of alanine, mannitol, sorbitol or trehalose can be used as sole carbon source for mut strain of *P. pastoris* for shake flask studies with methanol required as an inducer.

In conclusion, the use of alternate carbon source in shake flask studies will promote the use of mut strain as a host, and will reduce the amount of methanol needed, which is required in large amounts for mut$^-$ strain of *P. pastoris*. It will also reduce the time to screen the transformed host cells to choose the best clone in order to scale up individual clone. Furthermore, one of these carbon sources can also be used as a supplemental carbon source for mut$^-$ and mut^s strain of *P. pastoris* employing the *AOX1* promoter driven heterologous protein expression.

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