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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 C1 utilizing enzymes being repressed. Therefore, these carbon sources can not be used as a mixture with methanol to simultaneously grow *P. pastoris* and induce C1 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 excellent 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 (*AOXI*) 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 methanol-utilizing phenotypes (mut-, mut+ 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 *AOXI* promoter, which regulating 85% of the alcohol oxidase activity in the cell, is the promoter used to drive heterologous protein expression in *Pichia*. The *AOXI* 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 (methanol utilization plus), a phenotype indistinguishable from wild type *P. pastoris*. When the expression cassette is inserted within the AOX1 locus, a mut+ strain (methanol utilization slow) is generated (3). Another way of obtaining mut- phenotype of host strain is by disruption of AOX1 gene by gene insertion i.e. KM71 (arg4his4 AOX1A::SARG4) strain (4).

A third host strain used for heterologous protein expression is the mut (methanol utilization negative) strain in which both the AOX1 and AOX2 genes are disrupted i.e. MC100-3 (arg4 his4 aox1A::SARG4 aox2A::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+ strains. *P. pastoris* mut- strains grow very slowly in methanol containing media as a sole carbon source because these strains are defective in AOX1 gene (6).

Advantages of expressing genes of interest in mut strains of *Pichia* are two fold; first, higher yields of protein were observed in mut- 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
pattern with glucose being utilized first and concomitant repression of C\textsubscript{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 \textgreater glycerol \textgreater ribose \textgreater sorbitol \textgreater glycerol \textgreater xylose \textgreater 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\textsubscript{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 non-repressible 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.

**RESULTS AND DISCUSSION**

Evaluation of different carbon sources for mut\textsuperscript{−} 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 repress 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 \(\beta\)-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,
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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.

Time (h)

**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 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

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

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

* Samples were taken at mid-log range O.D. of 1.5-2.5.
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' strain of P. pastoris employing the AOX1 promoter driven heterologous protein expression.

REFERENCES


