Pectin utilization by the methylotrophic yeast *Pichia methanolica*

Tomoyuki Nakagawa, Kaichiro Yamada, Shuki Fujimura, Takashi Itou, Tatsuro Miyaji and Noboru Tomizuka

Department of Food Science and Technology, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri, Hokkaido 099-2493, Japan

The methylotrophic yeast *Pichia methanolica* was able to grow on pectic compounds, pectin and polygalacturonate, as sole carbon sources. Under the growth conditions used, *P. methanolica* exhibited increased levels of pectin methylesterase, and pectin-depolymerizing and methanol-metabolizing enzyme activities. On the other hand, *P. methanolica* has two alcohol oxidase (AOD) genes, MOD1 and MOD2. On growth on pectin, the *P. methanolica* mod1Δ and mod1Δmod2Δ strains showed a severe defect in the growth yield, although the mod2Δ strain could grow on polygalacturonate to the same extent as the wild-type strain. The expression of MOD1 was detected in pectin-grown cells, but the MOD2-gene expression detected by pectin was much lower than that of MOD1. Moreover, pectin could induce peroxisome proliferation in *P. methanolica*, like methanol and oleic acid. These findings showed that *P. methanolica* was able to utilize the methylester moiety of pectin by means of methanol-metabolic enzymes in peroxisomes, and that the functional AOD subunit for pectin utilization was Mod1p in *P. methanolica*.

INTRODUCTION

Methylotrophic yeasts that are able to utilize methanol as sole carbon source have attracted much attention as tools for molecular and cell biology; for example, powerful methanol-inducible gene expression systems have been established for some methylotrophic yeasts (Cregg, 1993; Gellissen, 2000; Hollenberg & Gellissen, 1997; Raymond, 1999; Sakai et al., 1999). However, only a few attempts have been made so far to determine the significance of such yeasts in natural environments.

In natural environments, plants are the major source of methanol; for example, methanol can be detected in leaf extracts and is emitted from leaves into the atmosphere (MacDonald & Fall, 1993; Nemecek-Marshall et al., 1995). In plants, one of the major sources of methanol is pectin, which is hydrolysed to methanol and polygalacturonate by pectin methylesterase (PME) (Sakai et al., 1993). It has been reported that many methylotrophic yeasts are able to grow on pectic compounds as sole carbon sources (Lee & Komagata, 1980). Therefore, we believe that this pectin-growth ability of methylotrophic yeasts may be one of the clues for revealing the physiological and ecological roles of yeasts and the methanol cycle in natural environments. Indeed, the methylotrophic yeast *Candida boidinii* grown on pectin media exhibits the activities of pectin-metabolizing enzymes and methanol-metabolizing enzymes (Nakagawa et al., 2000; Stratilova et al., 1998).

In a methylotrophic yeast, the first reaction in methanol metabolism is the oxidation of methanol to formaldehyde catalysed by alcohol oxidase (AOD) (Tani et al., 1978), which is localized in peroxisomes (Goodman et al., 1984). The methylotrophic yeast *Pichia methanolica* has nine AOD isozymes, AOD being the first enzyme for methanol utilization during methylotrophic growth (Gruzman et al., 1996; Nakagawa et al., 1996). The AOD isozymes of *P. methanolica* are encoded by two genes, MOD1 and MOD2 (Nakagawa et al., 1999, 2001; Raymond et al., 1998). However, little is known about the physiological roles of methanol-metabolizing enzymes and AOD isozymes in pectin utilization by *P. methanolica*, although one of the major sources of methanol is pectin in natural environments.

This study was conducted to reveal the metabolic pathway for pectin degradation in the methylotrophic yeast *P. methanolica*, and to determine the physiological roles of AOD isozymes in pectin metabolism in natural environments.

METHODS

Yeast and bacterial strains, media and cultivation. *P. methanolica* IAM 12901 and PMAD11 (Invitrogen) (Raymond et al., 1998)
were used as the wild-type strains. MOD1 and/or MOD2 disruption strains, that is, the mod1Δ (PMAD12), mod2A (PMAD13) and mod1Δmod2Δ (PMAD14) strains (Raymond et al., 1998), were used for the growth test on pectic compounds. These strains were kindly provided by ZymoGenetics, Inc.

Complex YPD and synthetic MI media were used for cultivation of the P. methanolica strains (Sakai et al., 1995, 1998). The carbon source was one of the following: 1% (w/v) glucose, 1% (v/v) glycerol, 1% (v/v) methanol, 1% (v/v) oleic acid, 1% (w/v) pectin or 1% (w/v) polygalacturonate (Sigma). The degree of esterification (DE) of pectins from citrus fruit (Sigma) was approximately 90%. The initial pH of the medium was adjusted to 4.0 with 6N HCl or 6N NaOH. Growth was monitored by measuring OD660.

Preparation of extracellular and intracellular fractions. Yeast cells grown on pectic compounds were separated by centrifugation at 12000 g for 10 min at 4°C, supernatants of the culture media being used as extracellular fractions. The yeast cells were resuspended in 50 mM sodium phosphate buffer (pH 7.0), and then disrupted with a mini bead-beater (Biospec Products) for periods of 30 s, with intermediate cooling periods of 1 min on ice. The cell debris were removed by centrifugation at 12000 g for 10 min at 4°C, and the supernatants were used as intracellular fractions for enzyme assays.

Enzyme assays. Pectin methylesterase (PME), polygalacturonase (PG), pectin lyase (PNL) and pectate lyase (PAL) activities were determined as described previously (Nakagawa et al., 2000, 2005). One unit of PME was defined as the amount of enzyme that released 1 µmole of carboxylic acid per minute. One unit of PG was defined as the amount of enzyme that released 1 µmole of reducing group per minute, and reducing groups derived from polygalacturonate were measured by the method of Somogyi and Nelson (Nelson, 1944; Somogyi, 1952). One unit of PNL and PAL, respectively, was defined as an increase in A235 of 1.0 of the reaction mixture per minute (Ishi & Yokotsuka, 1972).

Alcohol oxidase (AOO) (Tani et al., 1985), dihydroxyacetone synthase (DHAS) (Nash, 1953; Yanase et al., 1995), glutathione-dependent formaldehyde dehydrogenase (FDL) (Schütte et al., 1976; Nakagawa et al., 2004) and formate dehydrogenase (FDH) (Schütte et al., 1976) activities were determined as described previously, and each activity was determined as given in the respective paper.

Protein was determined by the method of Bradford (1976) with a protein assay kit (Bio-Rad), using BSA as standard.

Electrophoresis of AOD isozymes. For AOD-zyogram analysis of P. methanolica mutant strains, 20 µg aliquots of cell-free extracts of strains grown on pectin were subjected to non-denaturing 5% native PAGE at 4°C according to the method of Laemmli (1970). After electrophoresis, the polyacrylamide gels were stained by oxidation with guaiacol (Lee & Komagata, 1980; Nakagawa et al., 1996).

Northern analysis. P. methanolica cells grown on or induced with several carbon sources were harvested by centrifugation at 6700 g for 10 min at 4°C, and then total RNAs were extracted from the cells by the acid-guanidinium thiocyanate-phenol/chloroform method using ISOGEN (Nippon Gene Co.). Briefly, 10 µg aliquots of RNA samples were electrophoresed on 1.0% agarose gels containing 20 mM MOPS buffer, 1 mM EDTA and 2-2 M formaldehyde. After electrophoresis, capillary transfer to nylon membranes (Hybond-N+; Amersham Pharmacia Biotech) in 20 x SSC (1 x SSC = 0.15 M NaCl plus 0.015 M sodium citrate) was performed. The DNA probe consisted of the entire coding region of MOD1 or MOD2 and was labelled with an AlkPhos DIRECT kit (Amersham Pharmacia Biotech).

Expression of green fluorescent protein (GFP). Expression vector pMETB (Invitrogen) was used for the expression of GFP in P. methanolica. GFP-coding regions were PCR-amplified, using the GFP-ATG primer, 5'-CTCggATggTpaAgCAaggCgAgg-3', and GFP-SKL primer, 5'-TgTCgACCTTTATAAATTTAGACTTcagCTCg-TCCATgC-3'. The obtained PCR fragment was introduced into the Xhol and Sall sites of pMETB under the PmMOD1 promoter, yielding pMET-GFP-SKL. The vector was linearized with PstI and used for the transformation of strain PMAD11. The transformant was termed strain GFP-SKL/wt.

RESULTS AND DISCUSSION

P. methanolica has the ability to utilize the polygalacturonate skeleton of pectin compounds as the sole carbon source

The P. methanolica wild-type strain was able to produce a hydrolysis halo on a polygalacturonate/glycerol plate, like C. boidinii (Fig. 1A), and to grow on polygalacturonate

![Fig. 1. Growth of P. methanolica on pectin and polygalacturonate.](image-url)

(A) Hydrolysis haloes on polygalacturonate/glycerol medium. Pm, P. methanolica; Cb, C. boidinii. The plate was stained with a 0.02% ruthenium red solution. (B) Growth curve. Symbols: ●, polygalacturonate (DE 0%); ○, pectin (DE 90%).
(Fig. 1B). Moreover, an extracellular fraction of *P. methanolica* grown on pectin exhibited the activities of pectin-depolymerizing enzymes, in other words, polygalacturonase (PG), pectin lyase (PNL) and pectate lyase (PAL) (86 mU ml\(^{-1}\), 8 mU ml\(^{-1}\) and 14 mU ml\(^{-1}\), respectively). These results indicate that *P. methanolica* is able to hydrolyse the polygalacturonate skeleton of pectic compounds by means of PG, PNL and PAL, and has the ability to utilize the polygalacturonate skeleton.

**P. methanolica** has the ability to utilize methanol produced from pectin

On the other hand, *P. methanolica* was also able to grow on pectin (Fig. 1B). When the degree of methyl esterification (DE) of pectin used as the carbon source became higher, the cell yield of *P. methanolica* increased, the growth yield on pectin of DE 90\% finally being about twice that on polygalacturonate (DE 0\%) (Fig. 1B). This difference between the growth yield obtained from polygalacturonate and pectin of DE 90\% corresponds to the growth yield on 0-05\% methanol, since ~33\% of the methylester moiety in the pectin of DE 90\% is calculated to be utilized (Nakagawa et al., 2000). Moreover, *P. methanolica* exhibited pectin methylesterase (PME) activity. PME activity was detected in the extracellular fraction, and was remarkably induced in cells grown on polygalacturonate or pectin (73.3 U mg\(^{-1}\) and 194 U mg\(^{-1}\), respectively), although it was not found in the intracellular fraction. These findings indicate that *P. methanolica* is able to hydrolyse pectin at the methylester moiety by means of PME extracellularly, and it is possible that *P. methanolica* utilizes methanol derived from the methylester moiety of pectin.

Since methyl esterification of pectin affected the growth yield of *P. methanolica* on pectin and the yeast exhibited PME activity, it seems that methanol produced from pectin was utilized by the methanol-metabolizing enzymes in *P. methanolica* cells. Next, we studied the regulation of the methanol-metabolizing enzymes of *P. methanolica* by pectic compounds. As shown in Table 1, alcohol oxidase (AOD), dihydroxyacetone synthase (DHAS), glutathione-dependent formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH) activities were detected in pectin-grown cells, although these activities were lower than those in methanol-grown cells. Also, these enzyme activities in pectin-grown cells were ~1.7- to 4.3-fold higher than those in polygalacturonate-grown cells.

These results showed that there are methanol-metabolizing enzymes in *P. methanolica* cells grown on pectic compounds, and that *P. methanolica* is able to utilize methanol produced from pectin through hydrolysis by PME, as well as the polygalacturonate skeleton.

**Mod1p is able to function in pectic metabolism and growth, but Mod2p is not**

*P. methanolica* grown on pectin exhibits AOD activity, but *P. methanolica* has AOD isozymes (Ashin & Trotsenko, 1998; Gruzman et al., 1996; Nakagawa et al., 1996) and two AOD-encoding genes, *MOD1* and *MOD2* (Nakagawa et al., 1999; Raymond et al., 1998). To determine to what extent the AOD genes are directly involved in pectin metabolism, AOD gene knock-out strains were grown on pectin or polygalacturonate as a carbon source, their growth being compared with that of the wild-type strain (Fig. 2).

The *mod1Δ* and *mod1Δmod2Δ* strains showed a severe defect in the growth yield on pectin as a sole carbon source compared with the wild-type strain. However, these knockout strains were still able to grow on pectin, their growth yields on pectin being almost the same as those on polygalacturonate. Also, reduced growth yields such as those

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**Table 1.** Specific activities of enzymes related to methanol metabolism during growth on polygalacturonate (DE 0\%), pectin (DE 90\%), methanol and glucose

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>AOD (U mg(^{-1}))</th>
<th>DHAS (U mg(^{-1}))</th>
<th>FLD (U mg(^{-1}))</th>
<th>FDH (U mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE 0%</td>
<td>1.85 ± 0.11</td>
<td>0.09 ± 0.02</td>
<td>0.49 ± 0.04</td>
<td>0.09 ± 0.16</td>
</tr>
<tr>
<td>DE 90%</td>
<td>3.14 ± 0.21</td>
<td>0.33 ± 0.05</td>
<td>1.20 ± 0.09</td>
<td>0.39 ± 0.16</td>
</tr>
<tr>
<td>Methanol</td>
<td>3.72 ± 0.18</td>
<td>0.47 ± 0.09</td>
<td>2.71 ± 0.12</td>
<td>0.29 ± 0.11</td>
</tr>
<tr>
<td>Glucose</td>
<td>ND</td>
<td>ND</td>
<td>0.02 ± 0.01</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Fig. 2.** Growth of the *P. methanolica* wild-type (○), *mod1Δ* (□), *mod2Δ* (⊙) and *mod1Δmod2Δ* (■) strains on pectin (DE 90\%) medium. The initial pH of the medium was 6.0.
observed with these knock-out strains grown on pectin were not observed for cells grown on polygalacturonate (data not shown). These results indicated that the methanol-metabolizing pathway plays a significant role in pectin assimilation, and that Mod1p can function as an AOD in pectin metabolism and the pectic growth of *P. methanolica*. The mod2Δ strain was still able to grow on pectin to the same extent as the wild-type strain (Fig. 2). On the other hand, the growth on polygalacturonate was not impaired in AOD gene knock-out strains (data not shown).

Next, we attempted to identify the gene encoding the pectin-inducible AOD by zymogram analysis and Northern analysis. A cell-free extract of pectin-grown cells gave a single AOD band on zymogram analysis, the *R*~m~ value corresponding to that of Mod1p (Fig. 3A). The cell-free extracts of the *mod1Δ* and *mod1Δmod2Δ* strains did not give any AOD-active band, although those of the wild-type and *mod2Δ* strains each gave a single band corresponding to Mod1p (Fig. 3A). In addition, *MOD1*- and *MOD2*-gene expression was followed at the mRNA level. The expression of *MOD1* was detected by pectin, but the *MOD2*-gene expression detected by pectin was much lower than that of *MOD1* (Fig. 3B). Moreover, disruption of *MOD1* caused a growth defect on pectin medium. These findings indicated that Mod1p is the functional AOD subunit in pectin utilization, and that the presence of Mod2p alone is not sufficient for growth on pectin. It has been reported that Mod2p exhibits a ~10-fold higher *K*~m~ value toward methanol compared to Mod1p (Gruzman *et al*., 1996; Nakagawa *et al*., 1996, 2002). Therefore, it seems that Mod2p cannot function in pectin medium with a low methanol concentration, although the expression of *MOD2* to utilize methanol derived from pectin, can be detected slightly by pectin.

These findings indicated that *P. methanolica* can utilize methanol derived from pectin as a carbon source, that the pectin-inducible AOD was encoded by the *MOD1* gene, and that the *MOD2* gene was not necessary for pectin metabolism.

### Pectin is an inducer of peroxisome proliferation

Since (i) Mod1p, which is detected in pectin-grown cells, is a peroxisomal enzyme, (ii) methanol derived from pectin is metabolized in peroxisomes, and (iii) peroxisome proliferation in *C. boidinii* was induced by pectin (Nakagawa *et al*., 2000), we examined whether or not pectin could induce peroxisome proliferation in *P. methanolica*. Morphometric analysis of peroxisomes was performed using *P. methanolica* strain GFP-SKL producing GFP-PTS1 (GFP tagged with an SKL sequence at the carboxyl terminus). When strain GFP-SKL was grown on glycerol medium, there were very small peroxisomes (Fig. 4B). On the other hand, cells grown on pectin had large peroxisomes, like cells grown on oleic acid, which is one of the peroxisome inducers (Fig. 4A, D), although their morphology was smaller than that of methanol-grown cells (Fig. 4C). These findings showed that pectin is a peroxisome inducer, like methanol and oleic acid.

### Conclusions

In this study, we showed that *P. methanolica* has the ability to utilize pectic compounds, in other words, polygalacturonate and pectin, as carbon sources, and that *P. methanolica* is able to assimilate the polygalacturonate skeleton and the methanol derived through hydrolysis from the methylester moiety of pectin by extracellular PME, using extracellular pectinases and the methanol-metabolic pathway.

From the pectic-growth data for *P. methanolica* and *C. boidinii* (Nakagawa *et al*., 2000; Stratilova *et al*., 1998), it seems that utilization of both the methylester moiety and the

![Fig. 3. Regulation of *MOD1*- and *MOD2*-gene expression in *P. methanolica*. (A) Zymogram analysis of AOD isozymes from *P. methanolica* strains. Protein (20 μg) was analysed by native PAGE on a 5 % polyacrylamide gel. After electrophoresis, the gel was stained for AOD activity by the improved method of Lee and Komagata (Lee & Komagata, 1980; Nakagawa *et al*., 1996). The cell-free extracts were: lane 1, purified Mod1p; lane 2, purified Mod2p; lane 3, wild-type strain; lane 4, *mod1Δ* strain; lane 5, *mod2Δ* strain; lane 6, *mod1Δmod2Δ* strain. These strains were grown on 1 % (w/v) pectin (lanes 3–6) as carbon source. (B) Northern analysis. Total RNA (20 μg) was loaded on each lane. The carbon sources were: lane 1, 1 % (w/v) methanol; lane 2, 1 % (w/v) pectin; lane 3, 1 % (w/v) polygalacturonate; lane 4, 1 % (w/v) glucose.](image-url)
polygalacturonate skeleton of pectin as carbon sources is a general feature of methylotrophic yeasts, and that methylotrophic yeasts are significantly involved in the ecological carbon cycle of pectin in natural environments.

ACKNOWLEDGEMENTS

We are grateful to ZymoGenetics, Inc. for providing us with the AOD-gene disruption mutants of \textit{P. methanolica}, and to Messrs Kenji Muraoka, Shigeki Masuda, Takuya Nakajima, Toshinori Nagaoka and Shintaro Niwa for their skillful assistance. This research was supported in part by a Grant-in-Aid for Young Scientists (B) 15780061 from the Ministry of Education, Culture, Sports, Science and Technology to T. N.

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\begin{figure}
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\begin{tabular}{ccc}
\includegraphics[width=0.3\textwidth]{A.png} & \includegraphics[width=0.3\textwidth]{B.png} & \includegraphics[width=0.3\textwidth]{C.png} \\
(A) & (B) & (C) \\
\includegraphics[width=0.3\textwidth]{D.png} & \includegraphics[width=0.3\textwidth]{E.png} & \includegraphics[width=0.3\textwidth]{F.png} \\
(D) & (E) & (F) \\
\end{tabular}
\caption{Fluorescence of GFP-PTS1 induced by (A) pectin (DE 90 %), (B) glycerol, (C) methanol, (D) oleic acid. The induced cells were visualized by Nomarski (upper) or fluorescence (lower) microscopy.}
\end{figure}
formaldehyde dehydrogenase gene FLD1 from the methylotrophic yeast* *Pichia methanolica.* Yeast 21, 445–453.


