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Physiological Study and Alcohol Oxidase Gene(s) of Thermotolerant Methylotrophic Yeasts Isolated in Thailand

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ABSTRACT

Two thermotolerant methylotrophic yeasts, *Candida sithepensis* sp. nov. and *Pichia siamensis* sp. nov. isolated in Thailand were studied for their physiology and alcohol oxidase gene(s). Optimum and maximum temperatures for growth on methanol of *C. sithepensis* sp. nov. were 34°C and 37°C whereas those of *P. siamensis* sp. nov. were 37°C and 40°C, respectively. Effect of methanol on the methylotrophic growth of the two yeast strains was investigated within the ranges of 0.25% to 2% (v/v) methanol. At 34°C, *C. sithepensis* sp. nov. exhibited a decline in μ_{max} from 0.16 h^{-1} to 0.13 h^{-1} with respect to increase in methanol concentration. When cultivated at 37°C, the μ_{max} of *C. sithepensis* sp. nov. was found to be drastically decreased from 0.16 h^{-1} to 0.10 h^{-1} within the same ranges of methanol concentration studied. *P. siamensis* sp. nov. showed an insignificant decrease of μ_{max} with respect to an increase in methanol supplement when cultivated at 37°C.

Key enzymes involved in methanol dissimilatory pathway, i.e., alcohol oxidase (AOD), glutathione-dependent formaldehyde dehydrogenase (FLD), formate dehydrogenase (FDH) and catalase (CAT) of the two yeasts were assessed. As for the favorable growth of *C. sithepensis* sp. nov. at its optimum temperature, fairly high level of specific AOD activity was observed but lower level of specific AOD activity was exhibited at maximum temperature. In contrast, specific FLD and FDH activity profiles were found to be higher when the yeast *C. sithepensis* sp. nov. was grown at its maximum temperature compared to the methylotrophic growth at its optimum temperature. Catalase production appeared to be slightly disturbed under all conditions studied. In case of *P. siamensis* sp. nov., stationary phase growth under low methanol supplement at its optimum temperature appeared soon after 24h cultivation.

Nucleotide sequences of *AOD* gene(s) of the two yeasts were investigated. *C. sithepensis* sp. nov. showed two *AOD* genes designated as *AOD1* and *AOD2* whereas *P. siamensis* sp. nov. revealed only one *AOD* gene within the genome.

Key words: methylotrophic yeasts, alcohol oxidase gene, thermotolerant, physiological study

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INTRODUCTION

Methylotrophic yeasts are widely used for heterologous protein production (Cereghino and Cregg, 2000; Gellissen, 2000; Faber *et al*., 1995). Two yeast genera, i.e., *Pichia* spp. and *Candida* spp. have now been recognized as methylotrophic (Houard *et al*., 2002). Several species: *P. pastoris, P. methanolica, P. angusta, C. boidinii* and *C. methanolovescens* are known as representatives of these two genera (Demain *et al*., 1998). Methanol metabolism starts with the oxidation of methanol to formaldehyde and hydrogen peroxide. The reaction is catalyzed by alcohol oxidase and the hydrogen peroxide produced is subsequently removed by the reaction catalyzed by catalase. This beginning part of methanol metabolism is localized in peroxisomes whereas the subsequent reactions in methanol assimilation and dissimilation are localized in the cytosol (Fukui *et al.,* 1975; Douma *et al.,* 1985; Goodman, 1985; Veenhuis and Harder, 1987). Dissimilation of formaldehyde proceeds to formate and further to CO_2, and is catalyzed by formaldehyde dehydrogenase and formate dehydrogenase, respectively, resulting in the generation of two molecules of NADH (Veenhuis and Harder, 1987).

The merits of methylotrophic yeasts applied to various processes are due to the capability to utilize methanol as the sole carbon and energy source. However, industrial applications have mostly been limited to the inability of these yeast strains to grow at high temperature. This affects not only the yeast growth but also the production of yeast proteins or metabolites. It is, therefore of advantage to search for thermotolerant methylotrophic yeast strains to overcome this detrimental effect of high temperature. Thailand is one of the tropical countries having reasonably high ambient temperature throughout the year. As a result of such climate, the thermotolerant microbial resources have therefore been the target of interest. Limtong *et al.* (2004) described three new thermotolerant methylotrophic yeasts isolated in Thailand, i.e., *Candida krabiensis* sp. nov., *C. sithepensis* sp. nov. and *Pichia siamensis* sp. nov.

In this study it was aimed to determine the optimum and maximum temperatures for growth of the two new yeast isolates described by Limtong *et al.* (2004). In addition, the effect of methanol on the methylotrophic growth at optimum and maximum temperatures was evaluated. Methanol utilizing enzymes of these yeast isolates were investigated under different growth conditions. Furthermore, alcohol oxidase gene(s) of the isolates of interest were also studied.

MATERIALS AND METHODS

Yeast strains

All yeast isolates, except *Pichia angusta*, were isolated in Thailand from natural samples (flowers, fruits, leaves, soils and water) by the selective and enrichment isolation technique. *Pichia angusta*, on the other hand, was obtained from Laboratory of Microbial Biotechnology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Japan.

Cultivation and media

Yeast isolates were maintained in YPD slant culture. Methanol synthetic medium (Sakai *et al.*, 1995) was used for the shake flask cultivation. Methanol concentration varied as 1% (v/v) for determination of optimum and maximum temperatures for growth, 0.25-2% (v/v) to study the effect of methanol on methylotrophic yeast growth and 0.75-2% (v/v) for the production of methanol utilizing enzymes. Yeast cultivation (100 ml) to determine temperatures and methanol concentrations critical to yeast growth was conducted aerobically at various temperatures, i.e.,

20-40°C to identify optimum and maximum temperatures for growth; 34°C, 37°C and 40°C, depending on yeast isolate, for the study of yeast growth as well as methanol utilizing enzymes production which might be affected by methanol supplement. All incubations were performed with rotary shaking at 180 rpm in 250 ml Erlenmeyer flasks. However, 400 ml culture in 1 L Erlenmeyer flasks were used to investigate the methanol utilizing enzymes.

Growth determination

Yeast growth was determined by measuring the optical density at 610 nm (OD_{610}).

Preparation of cell-free extracts

The methanol-grown cells were washed in 50mM potassium phosphate buffer (pH 7.5), and then transferred to a 2 ml Eppendorf tube containing an equal volume of 0.5 mm glass beads. The tube was shaken vigorously for 30 s on a mini-beadbeater (Biospec Products, UK) and chilled on ice for 30 s. This procedure was repeated six times. Cell debris was discarded after centrifugation at 16,000g for 5 min at 4°C. The supernatant was then subjected to enzyme activity assay and protein content determination.

Assay of methanol utilizing enzymes and protein content

Alcohol oxidase activity was determined by the ABTS/POD method (Tani *et al.,* 1985) whereas catalase activity was determined as a decrease in H_2O_2 in the reaction mixture (Bergmeyer, 1955). Activities of formaldehyde dehydrogenase and formate dehydrogenase were measured by determining the rate of NADH formation at 340 nm as described by Schutte *et al.* (1976). Protein concentrations of cell-free extracts were determined by the method of Bradford (1976) using a protein assay kit (Bio-Rad Laboratories) having bovine serum albumin as the standard. Methanol in the culture medium was determined by gas chromatography (Shimadzu GC-9A, Japan) and chromatopac recorder (Shimadzu C-R3A, Japan).

DNA methods

Genomic DNA of the yeast strains was prepared using the protocols described by Higgins and Cregg (1998). The primer employed for PCR amplification of *AOD* gene was designed. Two mixed primers, AODFp (5′-AAYCCS TGGGTSTAYYTSCCNGG-3′) and AODRp (5′-GGRTAYTCNTRRAARTGRAACAT-3'), were obtained as a result of the primer design based on the amino acid sequences of highly conserved regions in several AOD encoding genes (NPWVYLPG and NFHFLEY). Using these two primers and genomic DNA from strain *C. sithepensis* sp. nov. or *P. siamensis* sp. nov. as a template, a 1.1 kb fragment was specifically amplified in a BIO-RAD I Cycler thermal cycler. Purified PCR product was subjected to the TA-cloning into the bacterial host, *Escherichia coli* DH5a using pGEM-T Easy Vector System (Promega). Transformed colonies were picked up to prepare the plasmid DNA. The plasmids obtained were checked for the correct insertion of *AOD* gene by digestions with the restriction enzymes EcoRI and NotI. The PCR amplified *AOD* fragment was then labeled with dioxetane chemiluminescence using the Gene ImagesTM AlkPhos DirectTM labelling and detection system from Amersham Pharmacia Biotech. DNA hybridization was performed at 55°C as recommended by the manufacturer. Southern analysis was then carried out to investigate the number(s) of *AOD* gene of both yeast isolates by individual digestion of the genomic DNA with the restriction enzymes BamHI, EcoRI, HindIII, SacI, XbaI and XhoI. DNA sequences of the cloned fragments were determined using dye deoxy method in DSQ2000L DNA sequencer (Shimadzu Co., Ltd) and nucleotide sequences were analyzed.

RESULTS AND DISCUSSION

Screening for thermotolerant methylotrophic yeasts

253 isolates of methylotrophic yeast were obtained from natural samples such as fruits, flowers, leaves, soil and natural water using the selective isolation technique at 30°C. Further screening on 1% methanol agar medium revealed that 124 isolates were able to grow at 37°C and 54 isolates showed good to very good growth. Among these, 10 isolates exhibited higher maximum specific growth rate than that observed in *P. angusta* when cultivated under shake flask cultivation in synthetic broth containing 1% methanol as sole carbon and energy source. In comparison to *P. angusta*, the isolates identified as *P. siamensis* sp. nov. and *C. sithepensis* sp. nov. (Limtong *et al*., 2004) were selected for further studies according to the highest maximum specific growth rate (0.152 h^{-1}) for *P. siamensis* sp. nov. and to the highest maximum cell density determined as OD_{610} (14.100) for *C. sithepensis* sp. nov. Results are summarized in Table 1. In addition, when cultivated at 25°C and 30°C, *C. sithepensis* sp. nov. also exhibited higher maximum cell density than *P. angusta*.

Further screening at 40°C indicated that 17 isolates showed methylotrophic growth on 1% methanol agar medium. However, *P. siamensis* sp. nov. was the only isolate that showed the methylotrophic growth at 40°C under shake flask cultivation in synthetic broth containing 1% methanol. *C. sithepensis* sp. nov. and *P. siamensis* sp. nov. were, therefore, subject to further characterization.

Determination of optimum and maximum temperatures for growth

Optimum and maximum temperatures for growth of the selected yeast isolates were determined from the relationship between m_{max} and growth temperatures as shown in Figure 1. Results indicated that *C. sithepensis* sp. nov. possessed the ranges of optimum temperature of 30-34°C and a maximum temperature of 37°C. Although negligible difference in maximum specific growth rate could be observed, it should be noted that 34°C were preferably used as an optimum temperature for further investigation due to a slightly greater growth rate obtained. Similar pattern was shown between *P. siamensis* sp. nov. and *P. angusta* regarding their optimum and maximum temperatures which appeared to be 37°C and 40°C, respectively.

Table 1 Maximum specific growth rate and cell density of ten yeast isolates cultivated in 1% methanol synthetic broth at 37°C.

isolates	μ_{max} (h^{-1})	OD_{610} max / (time)
Pichia siamensis sp. nov.	0.152	10.100 / (60h)
S051	0.145	9.950 / (84h)
FS30	0.143	7.650 / (48h)
Candida sithepensis sp. nov.	0.143	14.100 / (72h)
M02	0.142	11.350 / (60h)
FS95	0.141	11.400 / (72h)
FS101	0.140	11.950 / (60h)
FS26	0.139	12.450 / (60h)
FS56	0.138	9.950 / (72 h)
PT27	0.128	9.950 / (60 h)
Pichia angusta	0.123	12.100 / (60h)

Effect of methanol on methylotrophic yeast growth

Effect of methanol on yeast growth was individually investigated at optimum and maximum temperatures for growth. The two isolates of interest, *C. sithepensis* sp. nov. and *P. siamensis* sp. nov., were cultivated along with the reference strain *P. angusta* under shake-flask cultivation in synthetic medium supplemented with various methanol concentrations (0.25% - 2%). Results shown in Figure 2 indicated that, at its optimum temperature (34°C), *C. sithepensis* sp. nov. exhibited a decline in μ_{max} from 0.16 h^{-1} to 0.13 h^{-1} (decreased to 81.25% of its original value) when methanol concentration increased up to 2%. When cultivated at its maximum temperature (37° C), the maximum specific growth rates of *C. sithepensis* sp. nov. were found to decrease from 0.16 h^{-1} to 0.10 h^{-1} (decreased to 62.5% of its original value) within the same ranges of methanol concentration studied. *P. siamensis* sp. nov. showed a decline in μ_{max} from 0.14 h^{-1} to 0.12 h^{-1} (decreased to 85.71% of its original value) with respect to an increase in methanol supplement from 0.25% to 2% when cultivated at 37°C. Methylotrophic growth of *P. siamensis* sp. nov. at 40°C appeared to be inferior to that observed at 37°C cultivation as shown by generally lower level of μ_{max} (0.08-0.10 h^{-1}). However, similar profile, i.e., negligible change in μ_{max} was noticed (Figure 3). Effect of methanol concentrations on methylotrophic growth of the reference strain, i.e., *P. angusta* at 34°C, 37°C and 40°C was also investigated (Figure 4). Results obtained in *P. angusta* were found to be comparable to those observed in *P. siamensis* sp. nov. in terms of negligible changes in μ_{max} (0.11-0.14 h^{-1}) at all cultivation temperatures.

Study of growth and methanol utilizing enzymes production

It is interesting to find that, at the maximum temperature for growth of the two isolates of interest, *C. sithepensis* sp. nov. exhibited a drastic response to methanol concentration in the culture medium whereas a decline in m_{max} of *P. siamensis* sp. nov. was observed in a lesser extent. Contrary to the methylotrophic growth at

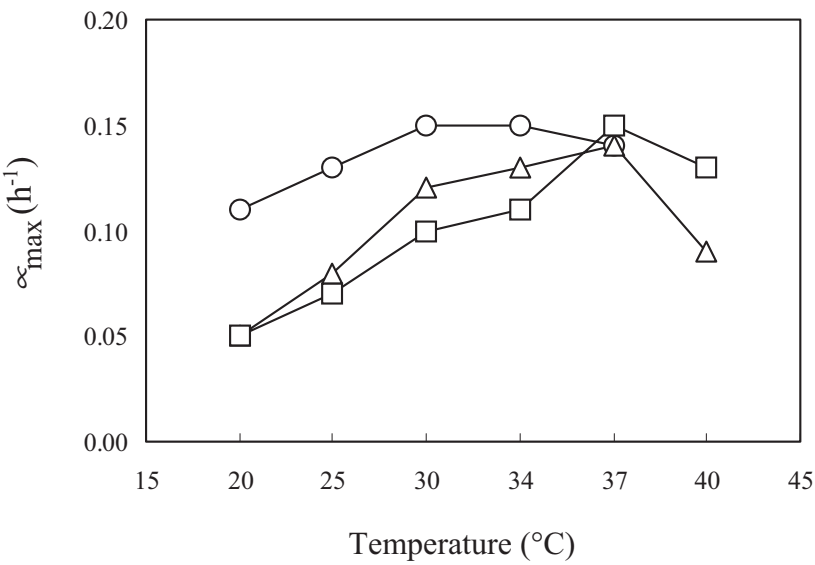

Figure 1 Maximum specific growth rate of the methylotrophic yeast *C. sithepensis* sp. nov. (○), *P. siamensis* sp. nov. (△) and *P. angusta* (□), on 1% methanol synthetic broth under shake flask cultivation at different temperatures for growth.

optimum temperature, insignificant differences between the two isolates could be pointed out. This may indicate some physiological role of *AOD* gene(s) of the strain grown at high temperature as described in *P. methanolica* (Nakagawa *et al*., 2002). This part of experiment was therefore aimed to investigate enzymatic profiles of the methylotrophic yeast *C. sithepensis* sp. nov. and *P. siamensis* sp. nov. at their optimum and maximum temperatures for growth

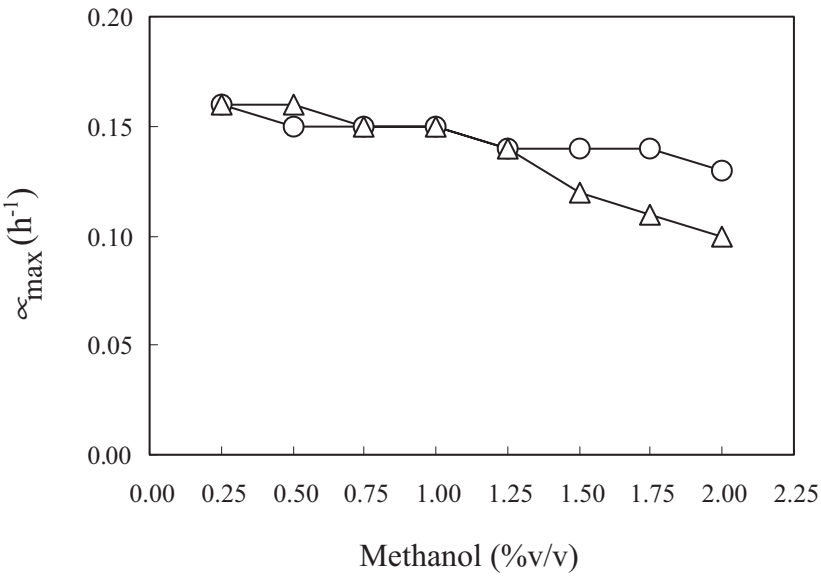

Figure 2 Maximum specific growth rates of *C. sithepensis* sp. nov. cultivated at 34°C (○) and 37°C (△) in the synthetic methanol broth supplemented with various concentrations of methanol.

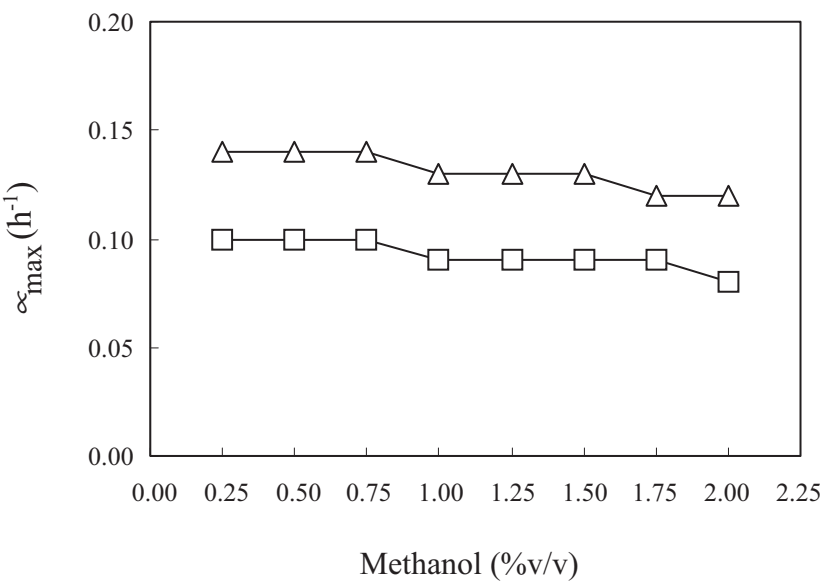

Figure 3 Maximum specific growth rates of *P. siamensis* sp. nov. cultivated at 37°C (△) and 40°C (□) in the synthetic methanol broth supplemented with various concentrations of methanol.

under low and high methanol supplement to the culture.

Depending on yeast isolate and experimental results obtained at optimum and maximum temperatures for growth, the profile of methanol utilizing enzymes produced under low and high methanol concentrations would be individually assessed. Despite the fact that, in some cases, low methanol concentrations, e.g., 0.25% appeared to yield an amenable growth rate, however, the culture possessed low cell density and shortly reached a stationary phase growth due to the depletion of carbon and energy source, i.e., methanol. This obviously resulted in an inadequate amount of harvested cells needed for enzyme activity assay. The culture density was, therefore, taken into account when "low" methanol concentration was assigned. Activities of catalase (CAT) and some key enzymes responsible for dissimilatory pathway of methanol utilization, i.e., alcohol oxidase (AOD), glutathione-dependent formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH) were assessed with growth of the methylotrophic yeast *C. sithepensis* sp. nov. and *P. siamensis* sp. nov. along with those observed in *P. angusta*.

A time-course determination of growth, methanol and specific enzyme activities of the isolate *C. sithepensis* sp. nov. was conducted at various growth conditions (Figure 5). Methanol concentration appeared to introduce insignificant effect to yeast growth at its optimum temperature cultivation whereas a retarded growth pattern was observed as a result of high methanol supplement at its maximum temperature. As for the favorable growth at optimum temperature, fairly high level of specific AOD activity was observed (maximum activity of 7.65 U/mg protein) as indicated in Figure 5C. However, lower level of specific activity was exhibited at maximum temperature for growth. During exponential growth of *C. sithepensis* sp. nov. (12-36 h of cultivation), specific AOD activity appeared to be lower when methanol concentration increased. These results agreed with high AOD activity observed at low methanol concentration in the inflow growth

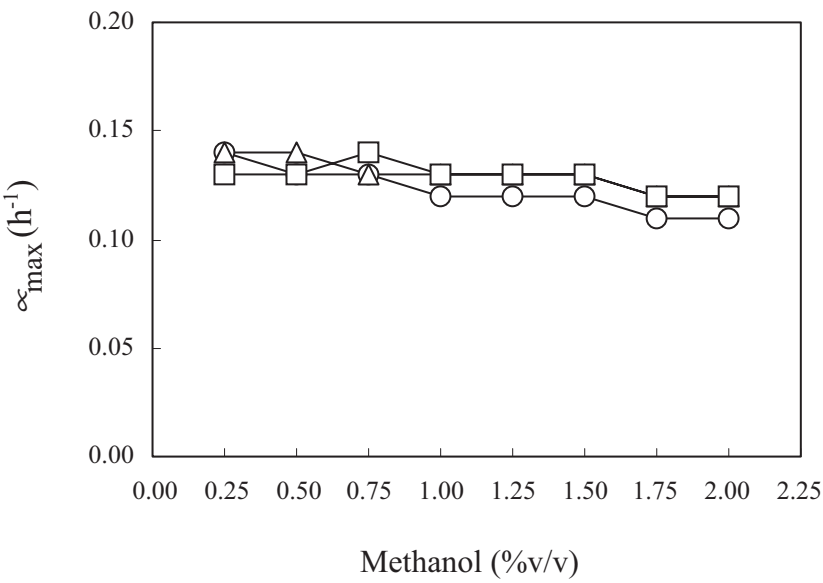

Figure 4 Maximum specific growth rates of the isolate *P. angusta* cultivated at 34°C (○), 37°C (△) and 40°C (□) in the synthetic methanol broth supplemented with various concentrations of methanol.

medium of *C. boidinii* (Volfova *et al*., 1992). However, AOD production profiles appeared to be contrary to those observed for FLD production shown in Figure 5E. Specific FLD activity profiles was found to be higher when the yeast *C. sithepensis* sp. nov. grown at its maximum temperature compared to the methylotrophic growth at its optimum temperature. Similar pattern, although in the lesser extent, was shown for FDH production. Figure 5D shows

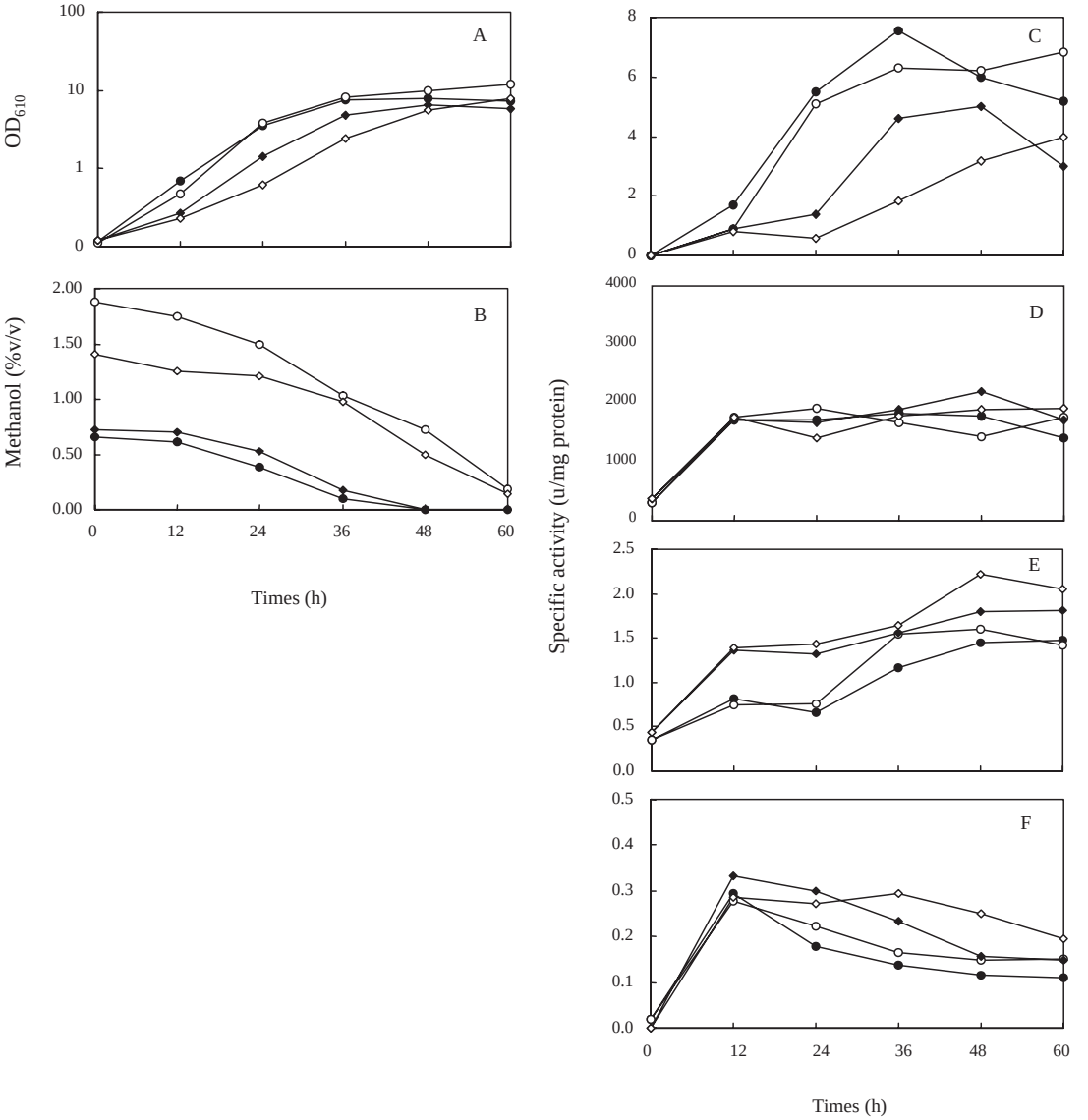

Figure 5 Growth (A), methanol residue (B) and specific activities of alcohol oxidase (C), catalase (D), glutathione-dependent formaldehyde dehydrogenase (E) and formate dehydrogenase (F) of *C. sithepensis* sp. nov. cultivated at various growth conditions. 34 °C and 0.75% (v/v) methanol, (●); 34 °C and 2% (v/v) methanol, (○); 37 °C and 0.75% (v/v) methanol, (◆); 37 °C and 1.5% (v/v) methanol, (◇)

insignificant differences on specific CAT activity observed under all conditions studied. A relatively stable CAT production of *C. sithepensis* sp. nov. was concurrent to those appeared in *C. boidinii* cultures (Volfova *et al*., 1992).

Figure 6 summarizes the results of time-course determination for growth and enzymatic profiles of *P. siamensis* sp. nov. Stationary phase growth of *P. siamensis* sp. nov. under low methanol supplement (0.75%) at its optimum temperature

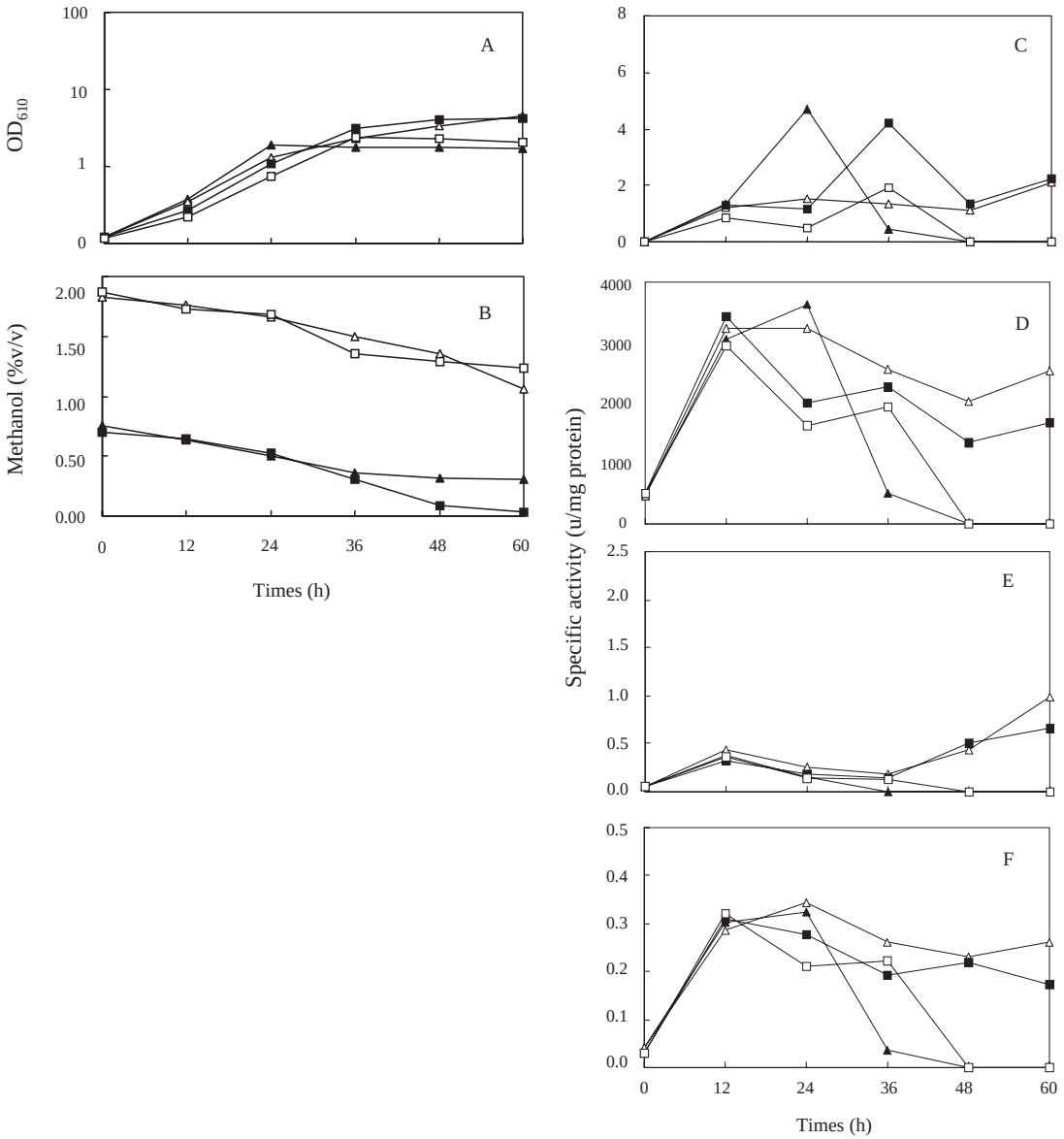

Figure 6 Growth (A), methanol residue (B) and specific activities of alcohol oxidase (C), catalase (D), glutathione-dependent formaldehyde dehydrogenase (E) and formate dehydrogenase (F) of *P. siamensis* sp. nov. cultivated at various growth conditions. 37 °C and 0.75% (v/v) methanol, (▲); 37 °C and 2% (v/v) methanol, (△); 40 °C and 0.75% (v/v) methanol, (■); 40 °C and 2% (v/v) methanol, (□)

was observed soon after 24h cultivation. This obviously resulted in the decrease of methanol utilizing enzyme production and, probably, the incomplete methanol utilization under low methanol supplement. The highest specific AOD activity observed when the yeasts *C. sithepensis* sp. nov. and *P. siamensis* sp. nov. grown under the same growth condition, i.e., low methanol level and optimum temperature for growth were compared. Result obtained from *P. siamensis* sp. nov. (4.5 U/mg protein) was approximately half of that from *C. sithepensis* sp. nov. (7.5 U/mg protein).

Determination of growth, methanol and specific enzyme activities of *P. angusta* cultivated at various growth conditions were also investigated in the same manner and results are summarized in Figure 7. Insignificant differences could be observed under all growth conditions studied.

Study of alcohol oxidase gene(s)

AOD fragment was amplified from yeast genomic DNA using *AOD* degenerated primers as follows:

AODFp
5'-AAYCCSTGGGTSTAYYTSCCNGG-3'
AODRp
5'-GGRTAYTCNTRRAARTGRAACAT-3'

This fragment was then cloned into *E. coli* prior to the partial nucleotide sequencing. Yeast genomic DNA was digested with various restriction enzymes and subjected to Southern analysis using PCR amplified *AOD* fragment as a probe. Results of Southern analysis are shown in Figure 8. DNA band numbers were determined between genomic DNA samples of individual yeast isolates digested with the same set of restriction enzymes. Obviously, *C. sithepensis* sp. nov. contained more than one *AOD* gene because Southern analysis of genomic DNA digested with BamHI, EcoRI, XbaI and XhoI showed two bands whereas *P. siamensis* sp. nov. possessed only one *AOD* gene as seen from a single band when the genomic DNA was digested with the same set of restriction enzymes. These indications were coincident with the results obtained from partial nucleotide sequence indicating that *C. sithepensis* sp. nov. possessed two different *AOD* genes whereas *P. siamensis* sp. nov. had only one *AOD* gene. Alcohol oxidase genes have been isolated and characterized from *P. angusta* (Ledeboer *et al.*, 1985), *P. pastoris* (Koutz *et al.*, 1989), *C. boidinii* (Sakai and Tani, 1992) and *P. methanolica* (Nakagawa *et al.*, 1996). Among these yeast species, *P. pastoris* and *P. methanolica* have been reported to have two methanol oxidase genes. These results were therefore the first evidence of two *AOD* genes existed in the genus *Candida* of methanol-utilizing yeasts. Alignment of deduced amino acid sequences of *AOD* of *C. sithepensis* sp. nov. and *P. siamensis* sp. nov. compared to the database (Figure 9) suggested that the *C. sithepensis* sp. nov.- *AOD1* had 90% homology to *P. methanolica- MOD1* whereas the *C. sithepensis* sp. nov.- *AOD2* had 84% homology to *P. methanolica- MOD2*. The *P. siamensis* sp. nov.- *AOD* was found to be 97% homology to the *P. angusta- MOX*. Similarity of alcohol oxidase of the two methylotrophic yeasts is summarized in Figure 10 using UPGMA analysis.

As a result of two *AOD* genes exist in *C. sithepensis* sp. nov., further studies on the regulation of these two genes at transcriptional level should be performed. In addition, an existence of alcohol oxidase isozymes will also be investigated under different growth conditions.

CONCLUSIONS

Optimum and maximum temperatures for growth on methanol of the two thermotolerant

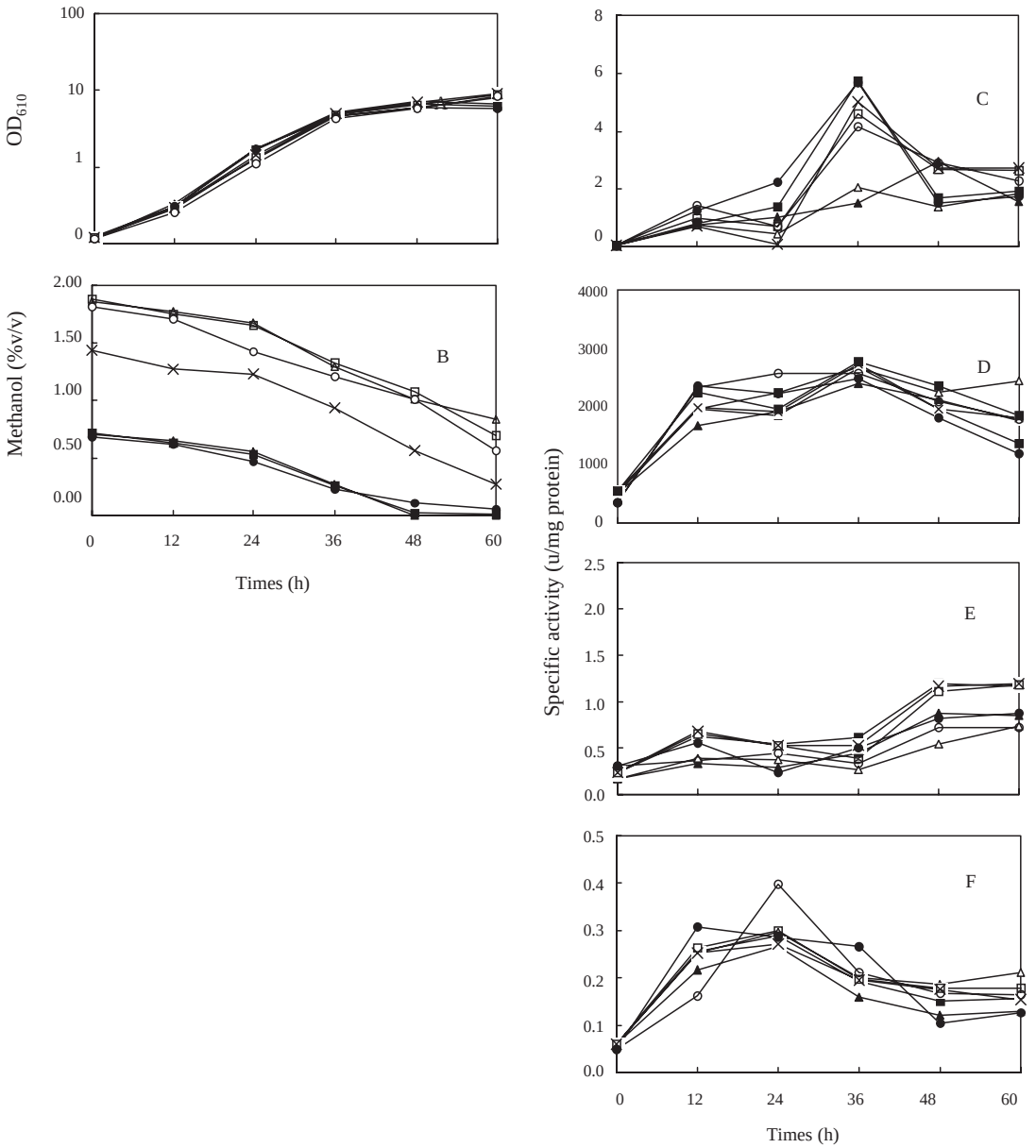

Figure 7 Growth (A), methanol residue (B) and specific activities of alcohol oxidase (C), catalase (D), glutathione-dependent formaldehyde dehydrogenase (E) and formate dehydrogenase (F) of *P. angusta* cultivated at various growth conditions. 34 °C and 0.75% (v/v) methanol, (▲); 34 °C and 0.75% (v/v) methanol, (△); 37 °C and 0.75% (v/v) methanol, (■); 37 °C and 1.5% (v/v)methanol, (×); 37 °C and 2% (v/v) methanol, (□); 40 °C and 0.75% (v/v) methanol, (●); 40 °C and 2% (v/v) methanol, (○)

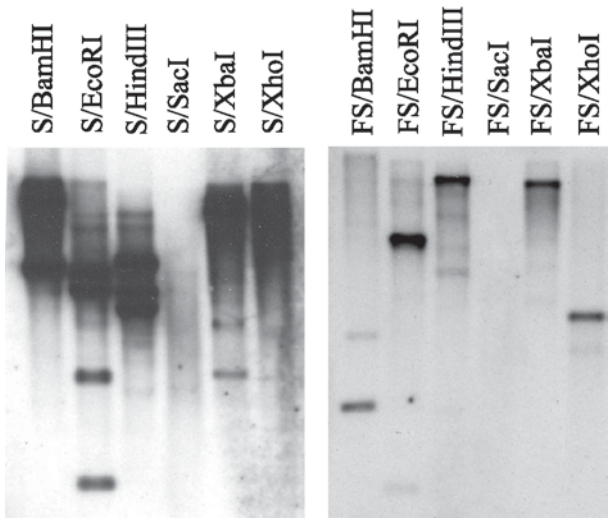

Figure 8 Southern blot analysis of genomic DNA of the methylotrophic yeast *C. sithepensis* sp. nov. (S, left panel) and *P. siamensis* sp. nov. (FS, right panel) with partial AOD gene(s). Genomic DNAs were digested with various restriction enzymes as indicated.

methylotrophic yeasts, *Candida sithepensis* sp. nov. and *Pichia siamensis* sp. nov., isolated from Thai fruits and soil were 34°C and 37°C for *C. sithepensis* sp. nov. and 37°C and 40°C for *P. siamensis* sp. nov., respectively. The μ_{max} of the two yeasts was affected by methanol supplement. At its optimum temperature for growth (34°C), increasing methanol concentration within the range of 0.25 to 2% (v/v) decreased the μ_{max} of *C. sithepensis* sp. nov. from 0.16 h^{-1} to 0.13 h^{-1}. When cultivated at its maximum temperature (37°C), the μ_{max} of *C. sithepensis* sp. nov. drastically decreased within the same ranges of methanol concentration studied. Methanol supplement appeared to have slight effect to *P. siamensis* sp. nov. at its optimum temperature for growth.

Reasonably high level of specific AOD activity was observed during the favorable growth of *C. sithepensis* sp. nov. at its optimum temperature. Lower level of specific AOD activity was shown at its maximum temperature as for an inferior growth appeared. Formaldehyde dehydrogenase production was contrast to AOD production since higher specific FLD activity profile was observed when the yeast *C. sithepensis* sp. nov. grown at its maximum temperature compared to the methylotrophic growth at its optimum temperature. Similar pattern, although in the lesser extent, was shown for FDH production. Insignificant differences on specific CAT activities could be observed. In case of *P. siamensis* sp. nov., stationary phase growth under low methanol supplement at its optimum temperature appeared soon after 24h cultivation.

C. sithepensis sp. nov. showed two *AOD* genes designated as *AOD1* and *AOD2* whereas *P. siamensis* sp. nov. revealed only one *AOD* gene within the genome. *AOD1* of *C. sithepensis* sp. nov. had 90% homology to *MOD1* of *P. methanolica* whereas the *AOD2* of *C. sithepensis* sp. nov. had 84% homology to the *MOD2* of *P. methanolica*. High similarity (97% homology) of *P. siamensis* sp. nov. - *AOD* to the *P. angusta- MOX* was exhibited.

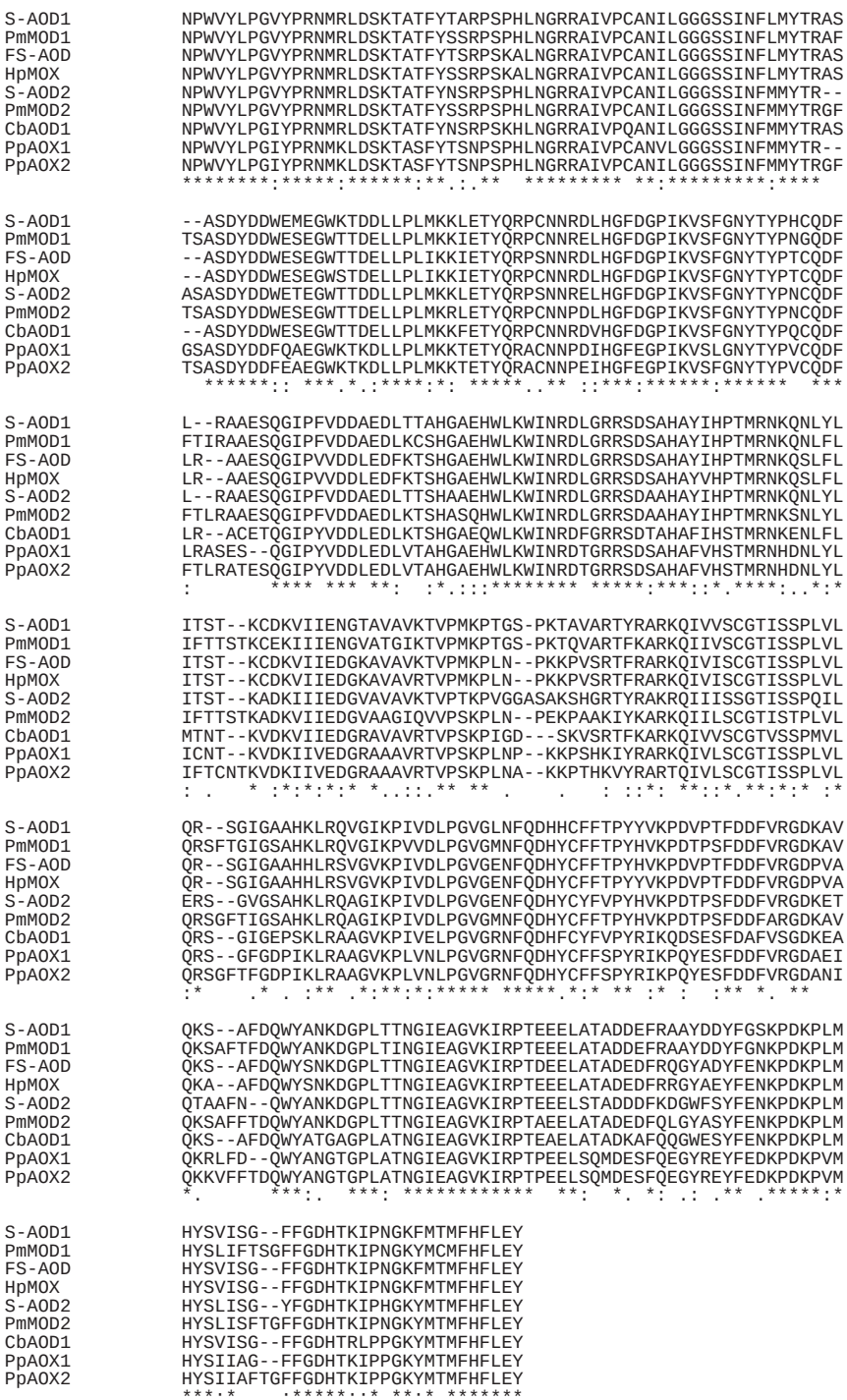

Figure 9 Alignment of deduced amino acid sequences of partial *AOD* gene(s) of the methylotrophic yeast *C. sithepensis* sp. nov. (S-AOD1 and S-AOD2) and *P. siamensis* sp. nov. (FS-AOD) with that of *Pichia methanolica* (PmMOD1, PmMOD2), *P. pastoris* (PpAOX1, PpAOX2), *P. angusta* (HpMOX) and *Candida boidinii* (CbAOD1). Identical and conserved residues are shown as *

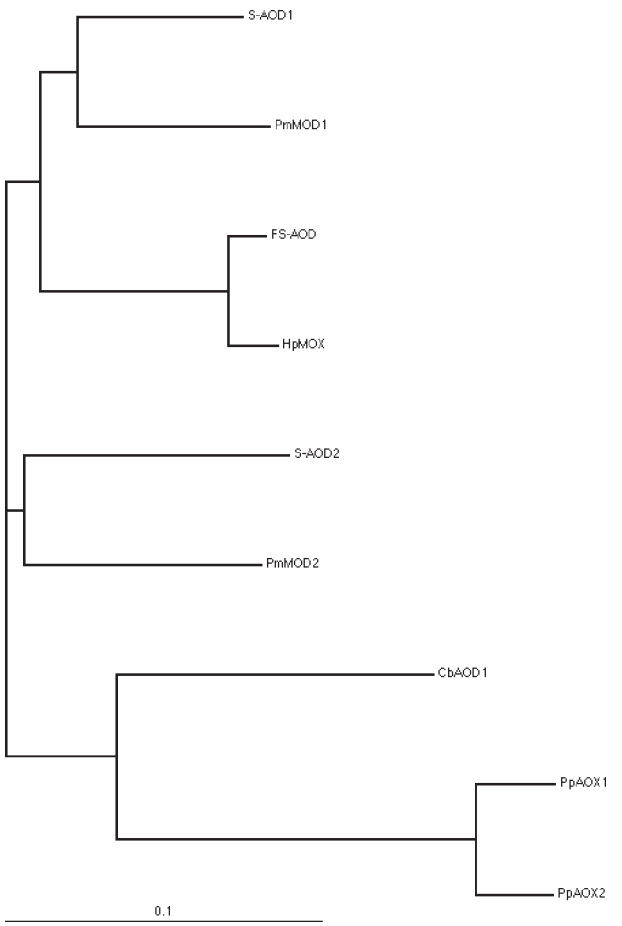

Figure 10 Similarity of the *AOD* gene(s) of the methylotrophic yeast *C. sithepensis* sp. nov. and *P. siamensis* sp. nov. The tree was constructed by UPGMA analysis.

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