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Original Article

Yeasts in mixed deciduous forest areas of Phujong Nayoy National Park and their ability to produce xylanase and carboxymethyl cellulase

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Abstract

A total of 61 yeast strains were obtained from 132 samples collected from various sources such as soil, mushroom, flowers, fruits, tree barks and insect frass in the mixed deciduous forest areas of Phujong Nayoy National Park, Thailand. Based on D1/D2 region at the 5′ end of the large subunit ribosomal RNA gene (rRNA gene region D1/D2) analysis, 39 strains were identified as ascomycetous yeasts and distributed to 7 genera i.e. *Blastobotrys, Candida*, *Debaryomyces, Dipodascus*, *Kodamaea*, *Pichia* and *Torulaspora*. Twenty strains were identified as basidiomycetous yeasts which belonged to the genera *Asterotremella*, *Cryptococcus*, *Sporidiobolus* and *Trichosporon*. Another two strains of yeast-like fungi were belonged to genus *Aureobasidium*. The predominant genus was *Candida* with a 31.14% contribution. For testing of xylanase and carboxymethyl cellulase production of the 61 strains of yeasts and yeast-like fungi, *Candida glabrata* and *Aureobasidium pullulans* showed xylanase activity of 0.91 and 0.52 UmL^{-1}, respectively, and carboxymethyl cellulase activity of 0.38 and 0.44 UmL^{-1}, respectively.

Keywords: yeast diversity, identification, rRNA gene region D1/D2, xylanase, carboxymethyl cellulase

1. Introduction

Microbial diversity from various habitats such as soil, river water, hypersaline lakes, and insects has been vast exploration as these habitats are the source of useful biomolecules which are a great relevance to the biotechnology industry, and include enzymes, fatty acids, pigments, antibiotics etc. (Butinar *et al*., 2005; Slavikova & Vadkertiova, 2003).

For this reason, the exploration and isolation of the biologically important yeasts from various habitats were investigated. Moreover, the development of biotechnology has raised much interest in using xylanase- and cellulose-producing microorganisms to convert lignocellulosic biomass from agro-industrial wastes to xylose and glucose, respectively, that can be used in applications such as production of bioethanol (Amita *et al*., 2006). Furthermore, xylanase and cellulase could be used in waste water treatment, food industry, pulp and paper industry, textile and laundry and in animal feed (Enari, 1983; Suchita & Ramesh, 2006).

Traditionally, yeasts have been identified and classified by cellular morphology and their distinctive reactions on a standardized set of fermentation and assimilation tests. These methods are laborious and time-consuming. Moreover, these characteristics are influenced by culture conditions and can provide uncertain results because of strain variability. Given these difficulties and the impracticality of identification most species from genetic crosses, molecular techniques, including the sequencing of rRNA genes and/or their flanking ITS regions are increasingly used for identification because the sequencing of rRNA genes and/or their flanking ITS regions have shown that many of these genptypic characteristics are strain specific for recognition of either species or genera. Moreover, these techniques are fast, easy and

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reasonably precise, and thus suitable for the rapid screening of isolates.

The aims of this study were to investigate yeasts in mixed deciduous forest areas of Phujong Nayoy National Park and determine their ability to produce xylanase and carboxymethyl cellulase.

2. Materials and Methods

2.1 Yeast isolation

Two grams of each sample from natural sources such as soil, mushroom, flowers, fruits (one wound per fruit), tree barks and insect frass was incubated in 5 ml of Yeast extract-Malt extract (YM) broth (3 gL^{-1} yeast extract, 3 gL^{-1} malt extract, 5 gL^{-1} peptone and 10 gL^{-1} glucose) containing 0.1 gL^{-1} chloramphenicol and 2 gL^{-1} sodium propionate to suppress bacterial and mold growth, respectively at 30ºC for 2-3 days. An aliquot of 0.1 ml of each yeast sample suspension was spread on YM agar supplemented with 0.1 gL^{-1} chloramphenicol and 2 gL^{-1} sodium propionate. Plates were incubated at 30ºC for 2-3 days. Colonies of yeasts were purified by repeated streaking three times on YM agar plates without the chloramphenicol and sodium propionate.

2.2 Genomic DNA extraction

Pure isolated yeast cells from 3 mL of each 24-h-culture were harvested by centrifugation. Cells were resuspended in 0.2 mL of 100 mM Tris-HCl, pH 8.0, 30 mM EDTA and 0.5% sodium dodecyl sulfate and boiled in a water bath at 100ºC for 15 min. Then 0.2 mL of 2.5M potassium acetate, pH 7.5, was added, thoroughly mixed and incubated on ice for 1 h with subsequent centrifugation at 15,000 rpm for 15 min. Protein contamination was removed by extraction with 0.2 mL of chloroform: isoamyl alcohol (24:1 v/v). DNA was precipitated by adding 0.6 volumes of cold isopropanol. The pellet was washed with 70% ethanol and left for the ethanol to evaporate. Finally, the dry precipitate was resuspended in 30-50 ml of double-distilled water.

2.3 DNA amplification by PCR and purification of PCR products

Cycle sequencing of the 600–650 base pair region D1/D2 at the 5' end of the LSU rRNA domain employed forward primer NL1 (5'GCATATCAATAAGCGGAGGAAAAG) and reverse primer NL4 (5'-GGTCCGTGTTTCAAGACGG). The standard procedure of PCR reaction was performed in 50 mL mixture containing 1.25 units of Taq DNA polymerase, 20 mM Tris-HCl, 10 mM KCl, 2mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1 mg/ml nuclease-free BSA, 1% Triton X-100, 200 mM of each dNTP, 0.1-1 mM of each primer and 20 ng of yeast genomic DNA as template. Denaturation, annealing and polymerization were carried out for 1 min at 94ºC, 1.30 min at 52ºC, and 2 min at 72ºC, respectively, for 30 cycles. Amplification reaction was performed in GeneAmp PCR System 9700 (Applied Biosystems, USA). After amplification, the PCR product was purified by using QIAquick PCR Purification Kit and analyzed with 0.8% agarose gel electrophoresis.

2.4 DNA sequencing and yeast identification

The PCR products were sequenced using the NL-1 and NL-4 primers using either Amersham Pharmacia ALF Express II or ABI 310 (capillary) automated DNA sequencer, following the manufacturer's instructions. For identification, the obtained sequences were compared with those of all known yeast species, available at the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) (Yarrow, 1998).

2.5 Determination of xylanase and carboxymethyl cellulase production

For qualitative analysis of xylanase production; all strains of yeast were incubated at 30°C for 2 days on xylan-agar (1% yeast extract, 0.5% oat–spelt xylan, and 1.8% agar). After incubation, xylanase-producing strains were identified based on the formation of a clear halo around the colonies, which were made visible by Congo red staining.

The xylanase-positive strains were evaluated for xylanase activity by growing the culture in xylan-yeast extract medium (1% yeast extract containing 0.5% oat–spelt xylan) at 30ºC for 7 days. Subsequently, the cells were pelleted by centrifugation, and the cell-free supernatant was used as the source of the enzyme and oat spelt–xylan (0.5% w/v in potassium phosphate buffer, pH 7.0) as the substrate. Xylanase activity was determined by measuring the release of reducing sugars from oat–spelt xylan using the Somogyi–Nelson method (Somogyi, 1992). Reaction mixtures contained 0.45 ml of 0.5% oat spelt–xylan in 50 mM potassium phosphate buffer, pH 7 and 0.05 ml of each enzyme fraction. Control lacked the enzyme fraction. After incubation at 50ºC for 15 min, the reaction was terminated by adding 0.5 ml of Somogyi reagent. The mixture was vortexed, placed in a boiling-water bath for 10 min, and cooled to room temperature. A 0.5 ml of Nelson reagent was added. After being vortexed, the mixture was centrifuged to remove any precipitate, and the absorbance of the supernatant was measured at 660 nm. One international unit (IU) of enzyme activity was defined as the amount of enzyme required to release 1 micromol of xylose from oat–spelt xylan in 1 minute under the assay condition.

Qualitative analysis of carboxymethyl cellulase production was performed in the same way as the qualitative analysis of xylanase production. However, carboxymethyl cellulose sodium salt (CMC-Na salt) agar (1%yeast extract, 0.5% CMC-Na salt and 1.8% agar) was used instead of xylan agar.

The carboxymethyl cellulase-positive isolates were evaluated for carboxymethyl cellulase activity by growing the culture in carboxymethyl cellulose-yeast extract medium (1% yeast extract containing 0.5% carboxymethyl cellulose

sodium salt) at 30ºC for 7 days. Subsequently, the cells were pelleted by centrifugation, and the cell-free supernatant was used as the source of the enzyme. Carboxymethyl cellulase activity was determined by measuring the release of reducing sugars from CMC-Na salt using the Somogyi–Nelson method (Somogyi, 1992). Reaction mixtures contained 0.45 ml of 0.5% carboxymethyl-cellulose sodium salt in 50 mM potassium phosphate buffer, pH 7 and 0.05 ml of each enzyme fraction. Control lacked the enzyme fraction. After incubation at 50ºC for 15 min, the reaction was terminated by adding 0.5 ml of Somogyi reagent. The mixture was vortexed, placed in a boiling-water bath for 10 min, and cooled to room temperature. A 0.5 ml of Nelson reagent was added. After being vortex, the mixture was centrifuged to remove any precipitate, and the absorbance of the supernatant was measured at 660 nm. One international unit (IU) of enzyme activity was defined as the amount of enzyme required to release 1 micromol of glucose from CMC-Na salt in 1 minute under the assay condition.

3. Results and Discussion

3.1 Yeast isolation and identification

A total of 61 yeast strains were obtained from 132 samples collected from natural sources such as soil, mushroom, flowers, fruits, tree barks and insect frass. Based on the sequence analysis of rRNA gene D1/D2 region, 39 strains were identified to be 18 species in Phylum Ascomycota and 20 strains were identified to be 5 species in Phylum Basidiomycota. The predominant genus in the ascomycetous species was *Candida* (48.7%). The remaining 51.3% included species belonging to the genera *Blastobotrys*, *Debaryomyces*, *Dipodascus*, *Kodamaea*, *Pichia* and *Torulaspora* (Figure 1A and Table 1). *Asterotremella* (30%) and *Trichosporon* (45%) were the predominant genera in the basidiomycetous yeasts whereas species of the genera *Cryptococcus* and *Sporidiobolus* together constituted approximately 10 and 15%, respectively (Figure 1B and Table 1). Another 2 strains showed 99.8% similarity with the yeast-like fungi, *Aureobasidium pullulans* (Table 1).

In this report, methods based on DNA/RNA gene sequence analysis have been shown to be the most appropriate tools for rapid yeast identification. Amplified fragments of the 600-nucleotide divergence in the 5'-end of the large subunit (LSU) ribosomal RNA gene (rRNA gene region D1/D2) have been demonstrated to discriminate between different yeast species. On the basis of rRNA gene region D1/D2 analysis, difference yeast species show grater than 1% nucleotide substitution and strains showing 0-3 nucleotide substitution in this region may be nonspecific or sister species (Kurtzman & Robnett, 1998).

Some yeast species isolated from mixed deciduous forest areas of Phujong Nayoy National Park, Thailand, in the present study were reported to be earlier detected in natural habitats in Thailand, i.e. one strain (PJF-1a) obtained from flower was named as *Candida sekii*, which was a new species discovered and proposed from insect frass and forest soil in the Saturnispola clade (Limtong *et al*., 2010) and three strains (PJF-4a, PJF-9 and PJI-1a) were named as *Candida* sp. ST-449 or *Candida uthaithanina* sp. nov, which was a new species discovered from moss in Nakaseomyces clade isolated in Thailand (Limtong *et al*., 2011). Moreover, some species were detected from habitats other than soil, mushroom, flowers, fruits, tree barks and insect frass. For instance, *Trichosporon mycotoxinivorans* was isolated from the hindgut of the lower termite (Molnar *et al*., 2004). *Pichia guilliermondii* was reported to have been isolated from beetle (Suh & Blackwell, 2004). The remaining yeasts were also commonly found in earlier isolates from mosses, mangrove forest (*Torulaspora* sp.), animal, clinical sources (*Aureobasidium pullulans*), goat dung (*Sporidiobolus ruineniae*), insect gut and tree exudates, etc (Kurtzman & Robnett, 1997-1998; Kurtzman & Fell, 1998; Yarrow, 1998; Butinar *et al*., 2007; Bhadra, 2008; Limtong *et al*., 2008).

3.2 Determination of xylanase production by the isolated yeast strains

Of the 61 yeast strains tested for xylanase, 36 strains produced a clear halo around the colonies on xylan-agar. Yeast strains exhibited xylanase activity belonging to spe-

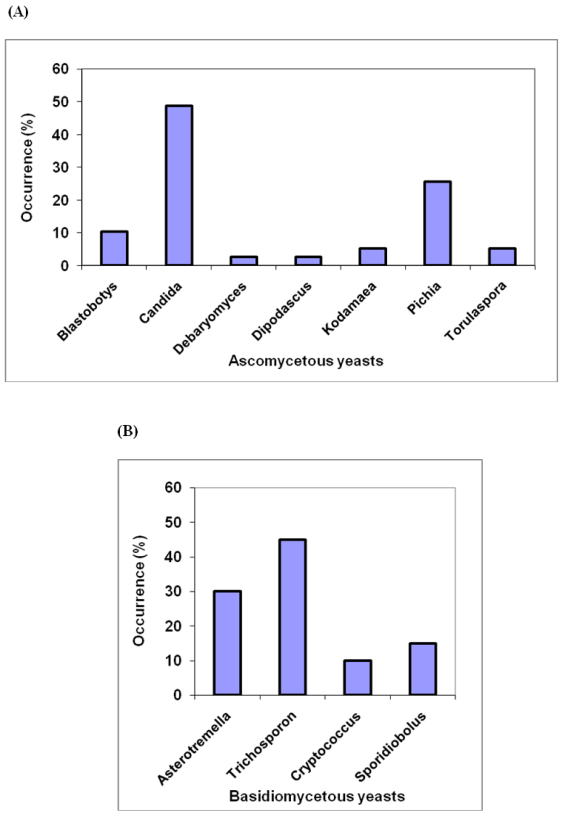

Figure 1. Occurrence of ascomycetous (A) and basidiomycetous (B) yeast genera in the mixed deciduous forest areas of Phujong Nayoy National Park, Thailand.

Table 1. Phylogenetic affiliation of yeasts and yeast-like fungi based on the similarity of D1/D2 domain sequence of LSU rRNA gene.

Result of identification	No. of strains	Strain no.	Accession no. and similarity with D1/D2 sequence of nearest phylogenetic neighbor (%)
Kodamaea ohmeri	2	PJS-3a, PJM-5a	FM180533 (100)
Pichia pijperi	2	PJS-6a, PJM-2a	AB449694 (99.5)
Debaryomyces vanrijiae var. yarrowii	1	PJS-6b	AB281295 (99.8)
Pichia fabianii	3	PJB-1a, PJB-4, PJF-16	EF 550321 (99.0)
Candida orthopsilosis	1	PJB-2a	FJ432622 (100)
Candida sekii	1	PJF-1a	AB495288 (100)
Candida cf. *glabrata*	4	PJF-1b, PJFr-2b, PJS-2a, PJS-5a	AF313362 (99.5)
Candida sp. ST-449	3	PJF-4a, PJF-9,PJI-1a	DQ404525 (99.3)
Pichia guilliermondii	4	PJM-5b, PJF-8b, PJF-15, PJB-2b	FJ515260 (99.8)
Candida parapsilosis	2	PJFr-1a, PJFr-3a	AY894827 (99.7)
Blastobotrys mokoenaii	4	PJF-10, PJS-4b,PJFr-3b, PJF-11	DQ442696 (99.5)
Pichia kudriavzevii	1	PJFr-1b	AB550116 (99.6)
Torulaspora globosa	2	PJI-3a, PJI-4	AB499988 (99.5)
Dipodascus magnusii	1	PJFr-3c	U40097 (99.5)
Candida zeylanoides	1	PJI-2	EU879958 (99.2)
Candida glabrata	2	PJM-3b, PJS-4a	EU373468 (100)
Candida tropicalis	4	PJS-6c, PJM-3a, PJS-1a, PJS-3b	EU293428 (99.7)
Candida litsaeae	1	PJB-3	DQ438184 (99.2)
Asterotremella humicola	6	PJM-5c, PJM-11, PJS-5b, PJM-8, PJM-10, PJM-2b	FJ515231 (99.6)
Cryptococcus humicola	2	PJM-4, PJM-9	DQ645514 (99.5)
Sporidiobolus ruineniae	3	PJM-1a, PJF-6, PJF-13b	AF070438 (100)
Trichosporon asahii	3	PJF-12, PJF-13a, PJFr-2a	GU299480 (99.2)
Trichosporon mycotoxinivorans	6	PJS-2b, PJI-1b, PJM-2c, PJM-6, PJM-12, PJFr-2c	FJ416596 (99.5)
Aureobasidium pullulans	2	PJF-4b, PJF-8a	GQ911487 (99.8)

Note: PJ = Phujong Nayoy National Park, S = strain from soil, M = strain from mushroom, F= strain from flower, Fr = strain from fruit wound, B = strain from tree bark, I = strain from insect frass.

cies; *Asterotremella humicola*, *Aureobasidium pullulans, Candida glabrata, Candida litsaeae, Cryptococcus humicula*, *Kodamaea ohmeri*, *Pichia pijperi*, *Pichia fabianii*, *Pichia guilliermondii*, *Sporidiobolus ruineniae* and *Trichosporon mycotoxinivoran*, while previous reports indicated that xylanase-producing yeasts in the species of *Aureobasidium pullulans*, *Blastobotrys mokoenaii*, *Cryptococcus albidus Cryptococcus adeliensis* sp. nov, *Cryptococcus* sp. S-2, *Pichia stipitis* and *Trichosporon cutaneum* SL409 (Biely *et al*., 1980; Iefuji *et al*., 1996; Liu *et al*., 1998; Scorzetti *et al*., 2000; Basaran *et al*., 2001; Ohta *et al*., 2001; Tanaka *et al*., 2004; Bhadra *et al*., 2008; Preez *et al*., 2009).

Among 36 isolated yeasts, two strains (PJF-4b and PJF-8a) belonging to *Aureobasidium pullulans* and two strains (PJM-3b and PJS-4a) belonging to *Candida glabrata* showed high levels of xylanase activity; the clear halo diameter for these strains ranged from 6.3 to 8.5 mm whereas the lowest clear halo diameter was 1.7 mm (Table 2). Two strains (PJF-4b and PJS-4a) were selected for further study for their ability to produce xylanase. Cells of *Candida glabrata* (PJS-4a) showed xylanase activity (0.91 UmL^{-1}) that was higher than that of the cells of *Aureobasidium pullulans* (PJF-4b) (0.52 UmL^{-1}) when cultivated in xylan-yeast extract medium.

3.3 Determination of carboxymethyl cellulase production by the isolated yeast strains

Of the 61 yeast strains tested for carboxymethyl cellulase, 38 strains produced clear halo around the colonies on carboxymethyl cellulose-agar. Yeast strains exhibiting carboxymethyl cellulase activity belonged to the species *Aureobasidium pullulans*, *Blastobotrys mokoenaii*, *Candida* cf. *glabrata*, *Candida glabrata*, *Candida litsaea*, *Candida sekii*, *Candida* sp. ST-449, *Candida zeylanoides*, *Candida tropicalis*, *Pichia fabianii*, *Pichia guilliermondii*, *Trichosporon asahii* and *Trichosporon mycotoxinivorans*, while

Table 2. Xylanase and carboxymethyl cellulase production of yeast strains isolated from the mixed deciduous forest areas of Phujong Nayoy National Park.

Ascomycetous yeast, Basidiomycetous yeast and Yeast-like fungi	No. of Strains	Xylanaseproduction/ halo diameter (mm)	Carboxymethyl cellulase production/halo diameter (mm)
Ascomycetous			
Kodamaea ohmeri	2	2.8 - 3.2	-
Pichia pijperi	2	2.7 - 2.9	-
Debaryomyces vanrijiae var. yarrowii	1	-	-
Pichia fabianii	3	1.7 - 2.0	4.9 - 5.1
Candida orthopsilosis	1	-	-
Candida sekii	1	-	5.2
Candida cf. glabrata	4	-	7.1 - 7.5
Candida sp. ST-449	3	-	6.1 - 6.3
Pichia guilliermondii	4	2.9 - 3.2	7.3 – 7.5
Candida parapsilosis	2	-	-
Blastobotrys mokoenaii	4	-	6.9 - 7.2
Issatchenkia orientalis	1	-	-
Torulaspora globosa	2	-	-
Dipodascus magnusii	1	-	-
Candida zeylanoides	1	-	7.1
Candida glabrata	2	8.2 - 8.5	12.3 - 12.5
Candida tropicalis	4	-	6.2 - 6.5
Candida litsaeae	1	2.0	10.0
Basidiomycetous			
Asterotremella humicola	6	2.6 - 3.0	-
Cryptococcus humicola	2	2.7 - 3.0	-
Sporidiobolus ruineniae	3	1.8 - 2.0	-
Trichosporon asahii	3	1.6- 2.0	5.3 - 5.5
Trichosporon mycotoxinivorans	6	2.1- 2.5	6.6 - 7.2
Yeast-like fungi			
Aureobasidium pullulans	2	6.3 - 6.5	12.8 - 13.0

Note: (-) = no clear halo

previous reports showed that the species of *Aureobasidium pullulans, Candida albicans, Pichia etchellsii, Pichia guilliermondii, Cryptococcus flavus* and *Cryptococcus* sp. S-2 produced carboxymethyl cellulase (Hatano *et al*., 1991; Cutfield *et al*., 1999; Oikawa *et al*., 1998; Wallecha & Mishra, 2003; Thongekkaew *et al*., 2008; Leite *et al*., 2008; Zhang *et al*., 2011).

Among 38 isolated yeasts, two strains ((PJF-4b and PJF-8a) belonging to species *Aureobasidium pullulans*, two strains (PJM-3b and PJS-4a) belonging to species *Candida glabrata* and one strain (PJB-3) belonging to species *Candida litsaeae* showed high levels of carboxymethyl cellulase activity; the clear halo diameter for these isolates ranged from 10 to 13 mm and the lowest clear halo diameter was 4.9 mm (Table 2). Three strains (PJF-4b, PJS-4a and PJB-3) which produced wide clear halo diameter were selected for further study to assess their ability to produce carboxymethyl cellulase. Cells of *Aureobasidium* pullulans (PJF-4b), *Candida glabrata* (PJS-4a) and *Candida litsaeae* (PJB-3) showed activity of 0.44, 0.38 and 0.36 UmL^{-1}, respectively, when cultivated in carboxymethyl cellulose-yeast extract medium.

When comparing xylanase and cellulase production of our study to different earlier yeast strains, the activities of *A. pullulan* and *C. grabata* xylanase were lower than those of *Cryptococcus adeliae* (24 Uml^{-1}) (Gomes *et al*., 2000) and *Candida stellata* (30.6 Uml^{-1}) (Strauss1, 2001). However, the yield of xylanases and cellulase from our yeasts isolates were three to four-fold higher than those of the most active *Trichosporon* spp. (0.12 and 0.14 Uml^{-1}) (Steven & Payne, 1977).

4. Conclusion

The 61 strains from 132 samples collected from the mixed deciduous forest areas of Phujong Nayoy National Park, Thailand, were identified and tested for xylanase and carboxymethyl cellulase production. The predominant genus in

this work was *Candida* (31.14%). Yeast strain of *Candida glabrata* and yeast-like fungi strain of *Aureobasidium pullulans* produced high activity both of xylanase and carboxymethyl cellulase on xylan-yeast extract medium and on carboxymethyl cellulose-yeast extract medium, respectively. Our study describes yeast diversity, and characterization of this resource can contribute to the development of a microbial bank and provide data on enzyme characteristics for potential industrial applications.

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