Barnettozyma siamensis f.a., sp. nov., a lipid-accumulating ascomycete yeast species

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Two strains, DMKU-UbN24(1)T and DMKU-CPN24(1), of a novel yeast species were obtained from soil and palm oil fruit, respectively, collected in Thailand by an enrichment isolation technique using a nitrogen-limited medium containing glycerol as the sole source of carbon. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, the two strains were found to represent a novel species of the genus Barnettozyma although the formation of ascospores was not observed. The novel species was related most closely to the type strain of Candida montana but differed by 5.4 % nucleotide substitutions in the D1/D2 region of the LSU rRNA gene and by 10.3–10.5 % nucleotide substitutions in the ITS region. The name Barnettozyma siamensis f.a., sp. nov. is proposed. The type strain is DMKU-UbN24(1)T (=BCC 61189T=NBRC 109701T=CBS 13392T).

The genus Barnettozyma was proposed based on phylogenetic analysis of nucleotide sequence divergence in the genes coding for large subunit (LSU) rRNA, small subunit (SSU) rRNA and translation elongation factor-1α of the six ascosporic species placed in the genus Barnettozyma, Barnettozyma hawaiensis, Barnettozyma populi, Barnettozyma salicaria and Barnettozyma wickerhamii had been previously assigned to the genus Pichia, whereas Barnettozyma californica and Barnettozyma pratensis were previously members of the genus Williopsis (Kurtzman et al., 2008). From this analysis two species of the genus Candida, Candida montana and Candida norvegica, were found to be members of the Barnettozyma clade. Subsequently, Barnettozyma vistinii (Yurkov et al., 2009) and Barnettozyma sucrosica (Imanishi et al. 2010) have been proposed. In addition, Candida qinlingensis and Candida sanyiensis have been included as members of the Barnettozyma clade (Lachance et al., 2011).

Some bacteria, yeasts, moulds and algae are able to accumulate high levels of cellular lipid, with those that accumulate lipid to a level higher than 20 % of their biomass being called oleaginous microorganisms (Ratledge & Cohen, 2008). Microbial lipid is believed to be a potential feedstock for biodiesel production due to it having similar fatty acid composition to that of vegetable oils (Liu et al., 2011). Among known yeast species only a minor proportion (5 %) have been reported to accumulate lipid levels higher than 20 % of their dry biomass (Meng et al., 2009), with examples being Candida curvata, Cryptococcus albidus, Kodamaea ohmeri, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis, Rhodotorula graminis, Trichosporon cacaoliposimilis, Trichosporon oleaginosus, Trichosporonoides spathulata and Yarrowia lipolytica (Ageitos et al., 2011; Galafassi et al., 2012; Gujjari et al., 2011; Kitcha & Cheirsilp, 2011; Meng et al., 2009; Ratledge & Cohen, 2008).

Glycerol is generated as a by-product of the biodiesel production process. An exponential increase in the demand for biodiesel has generated increased amounts of glycerol and resulted in a lowering of its price. The low price of glycerol makes it very competitive with sugars for the production of various products by microbial fermentation. Among these, lipid production by oleaginous yeasts is considered to be a potential use for glycerol rather than simple disposal. Therefore, we carried out a screening to obtain yeast strains suitable for lipid production from glycerol. During the screening of yeasts that accumulated high lipid levels when glycerol was used as the carbon source, two yeast strains were found to represent a novel species of the genus Barnettozyma belonging to the order Saccharomycetales based on the analysis of the D1/D2

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 region of the LSU rRNA genes of strains DMKU-UbN24(1)T and DMKU-CPN24(1) are AB741519 and AB741520, respectively. Those for the internal transcribed spacer region are KJ413944 and KJ413945, respectively. The Mycobank number for Barnettozyma siamensis f.a., sp. nov. is MB807942.
domain of the LSU rRNA gene sequence. In this article, two strains, DMKU-UbN24(1) and DMKU-CPN24(1), are described as representing *Barnettozyma siamensis* f.a., sp. nov.

In total, 323 yeast strains were isolated from 142 samples of soil and other materials such as palm oil fruits and the female flowers of palm, collected from natural habitats around Thailand by an enrichment technique using two media, i.e. a nitrogen-free medium (0.117 g Difco yeast carbon base l−1) supplemented with 250 mg sodium proprioionate l−1 and 200 mg chloramphenicol l−1, pH 3.3 and a nitrogen-limited medium containing glycerol as the sole source of carbon (30 g glycerol l−1, 1.5 g yeast extract l−1, 0.5 g NH₄Cl l−1, 7.0 g KH₂PO₄ l−1, 5.0 g Na₂HPO₄·12H₂O l−1, 1.5 g MgSO₄·7H₂O l−1, 0.08 g FeCl₃·6H₂O l−1, 0.01 g ZnSO₄·7H₂O l−1, 0.1 g CaCl₂·2H₂O l−1, 0.1 mg MnSO₄·5H₂O l−1 and 0.1 mg CuSO₄·5H₂O l−1) (Kimura et al., 2004) supplemented with 250 mg sodium proprioionate l−1 and 200 mg chloramphenicol l−1 at pH 5.3. Approximately 2 g of sample was added to 30 ml of each enrichment medium and incubated on a rotary shaker at 150 r.p.m. and 28 °C for 3 days. The enrichment culture was then spread on the agar medium with the same composition as the enrichment broth and incubated at 28 °C until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by cross streaking on YM agar (10 g glucose l−1, 5 g peptone l−1, 3 g yeast extract l−1, 3 g malt extract l−1, 20 g agar l−1). Purified yeast cultures were maintained in YM broth supplemented with 10 % glycerol at −80 °C.

All yeast strains were two-step screened for strains accumulating high amounts of lipid when cultivated in the nitrogen-limited medium containing glycerol as the sole carbon source. In the secondary screening, yeast strains were cultivated in 50 ml nitrogen-limited medium II containing glycerol (70 g glycerol l−1, 0.75 g yeast extract l−1, 0.55 g (NH₄)₂SO₄ l−1, 0.40 g KH₂PO₄ l−1 and 2.00 g MgSO₄·7H₂O l−1) in a 250 ml Erlenmeyer flask on a rotary shaker at 150 r.p.m. and 28 °C for 120 h. The lipid was extracted according to the modified method of Bligh & Dyer (1959), the methyl esters from fatty acids were obtained by the method of Holub & Skeaff (1987) and the fatty acid methyl esters were analysed by GC (GC14-A; Shimazu) using a capillary column containing a silica megabore column (30 mm × 0.52 mm × 1 μm, Durabond 225; J and W Scientific) and a flame ionization detector. The results showed that 34 yeast strains accumulated lipid in the range 15.3–71.02 % of biomass whereas two references oleaginous yeast strains, *Rhodosporidium toruloides* CBS 14 and *Cryptococcus curvatus* CBS 570, accumulated lipid amounting to 37.7 and 18.2 % of biomass, respectively. These 34 strains were identified on the basis of molecular taxonomy by analysis of the sequence similarity of the D1/D2 domain of the LSU rRNA gene. The sequences of strains, DMKU-UbN24(1)T and DMKU-CPN24(1), represented a single novel species. Strains DMKU-UbN24(1)T and DMKU-CPN24(1) accumulated lipid levels of 18.0 % of biomass (2.6 g biomass l−1 and 0.47 g lipid l−1) and 15.3 % of biomass (9.85 g biomass l−1 and 1.51 g lipid l−1), respectively. Strain DMKU-UbN24(1)T was isolated from soil collected in Na Chaluai district, Ubon Ratchathani province, and strain DMKU-CPN24(1) was obtained from palm oil fruit collected in Lang Suan district, Chumphon province.

The strains of the novel species were characterized morphologically, biochemically and physiologically by standard methods (Kurtzman et al., 2011). Ascospore formation was investigated for individual strains or strain pairs grown on 5 % malt extract agar, Gorodkovka agar, Fowell’s acetate agar and corn meal agar at 25 and 15 °C for up to 4 weeks. Carbon assimilation tests were conducted using liquid medium assimilation and nitrogen compounds were examined in liquid media with starved inocula as described by Kurtzman et al. (2011). Growth at various temperatures was determined by cultivation of the strains in YM broth. Ubiquinones were extracted from cells cultivated in a 500 ml Erlenmeyer flask containing 250 ml yeast extract peptone glucose (YPG) broth (1 % yeast extract, 2 % peptone and 2 % glucose) on a rotary shaker at 150 r.p.m. and 28 °C for 72 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC as described previously (Limtong et al., 2007).

**Novel species delineation and identification**

Analysis of the D1/D2 region of the LSU rRNA gene sequences revealed that the sequences of the two strains, DMKU-UbN24(1)T and DMKU-CPN24(1), were identical. The closest relative in terms of pairwise sequence similarity was *Candida montana* CBS 8057T (EF550275) but with...
5.4% nucleotide substitution (29 nt substitutions with two gaps of 2 nt out of 541 nt). The sequences of the ITS regions of the two strains differed from each other by only one gap (1 nt) but differed by 10.3–10.5% nucleotide substitutions (52–53 nt substitutions with 14–15 gaps of 1 nt) but differed by 10.3–10.5% nucleotide substitution (29 nt substitutions with 1 gap (2 nt) out of 541 nt). The sequences of the ITS region, we conclude that the two strains, DMKU-UbN24(1)T and DMKU-CPN24(1), represent a novel species. Although formation of ascospores was not observed, according to the nomenclatural rules for fungi of the International Code of Nomenclature for algae, fungi and plants (Melbourne Code) the most important requirement is the adoption of 'one fungus, one name' (McNeill et al., 2012). Consequently, the novel species was assigned to the genus Barnettozyma, and the designation forma asexualis (f.a.) was included following the recommendation of Lachance (2012). The name Barnettozyma siamensis f.a., sp. nov. (MB807942) is proposed.

**Description of Barnettozyma siamensis Polburee & Limtong sp. nov. (MB807942)**

*Barnettozyma siamensis* (siam.en’s N.L. fem. adj. siamensis of or belonging to Siam, the old name of Thailand, where the type strain was isolated).

Growth in YM broth: after 3 days at 25 °C, cells are globose to subglobose (2–4 × 3–4 μm) and occur singly or in pairs (Fig. 2). Budding is multilateral. Ascospores are not produced from individual strains or strain pairs on 5% malt extract agar, Fowell’s acetate agar, Gorodkowa agar or corn meal agar at 15 and 25 °C after up to 6 weeks. Fermentation is absent. D-Glucose, raffinose (weakly), salicin, L-rhamnose, ethanol, glycerol, ethylene HCl, D-mannitol, D-lactate, succinate and citrate are assimilated, but inulin, sucrose, melibiose, D-galactose, lactose, z-x-trehalose, maltose, melezitose, methyl α-D-glucoside, soluble starch, erythritol, celllobiose, L-sorbitose, D-xyllose, L-arabinose, D-arabinose, D-ribose, methanol, ribitol, galactitol, D-glucitol, myo-inositol, D-glucuronate, N-acetylglucosamine, xylitol, 2-ketoglucuronic acid, 5-ketoglucuronic acid, D-glucuronate, D-galacturonic acid and D-glucono-1,5-lactone are not. Ammonium sulfate, L-lysine and cadaverine are absent.

**Fig. 1.** Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of *Barnettozyma siamensis* f.a., sp. nov. [strains DMKU-UbN24(1)T and DMKU-CPN24(1)], with respect to closely related species. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbour-joining method with MEGA software version 5.0. Numbers indicate percentages of bootstrap values, derived from 1000 samples. The numbers in parentheses are GenBank accession numbers. *Candida melibiosica* CBS 5814T was the outgroup in the analysis. Bar, 0.02 Kńuc.
assimilated but potassium nitrate is not. Growth in vitamin-free medium is absent. Grows on medium containing 50 % glucose but not on 60 % glucose, 10 % NaCl/5 % glucose and 16 % NaCl/5 % glucose. No growth with 0.01 or 0.1 % cycloheximide. Grows at 30, 37, 40 and 42 °C. Formation of acid is positive. Starch-like compounds are not produced. Diazonium blue B colour and urease reactions are negative. The major ubiquinone is Q-7.

The holotype is DMKU-UbN24(1)T (=BCC 61189T=NBRC 109701T=CBS 13392T), isolated from soil collected in Na Chaluai district, Ubon Ratchathani province, Thailand. The Mycobank registration number is MB 807942.

In practice, B. siamensis sp. nov. can be distinguished from the most closely related species, Candida montana, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and ITS region but also by some phenotypic characteristics. B. siamensis sp. nov. assimilates DL-lactate and raffinose (weakly), while the type strain of Candida montana does not. The novel species does not assimilate cellubiose or 2-ketogluconic acid but the type strain of Candida montana does. Formation of acid is positive for the novel species but negative for the most closely related species. Growth on medium containing 50 % glucose and at 37, 40 and 42 °C is present for B. siamensis sp. nov. but absent for the type strain of Candida montana.

Species of the Barnettozyma clade have been found from a wide variety of substrates. B. californica was isolated from soil, water, tree fluxes and animal dung. Candida norvegica has been recovered from clinical specimens, food and beverages (Kurtzman, 2011). Some species of this genus were isolated from substrates associated with plants, namely B. hawaiensis isolated from the bark of native trees in Hawaii, B. populi from the fluxes of cottonwood trees in western Canada and Alaska, and B. salicaria are from the slime fluxes of willow trees (Salix sp.) from the USA. B. wickerhamii is recognized only from the frass of moth larvae (Pyralidae) found in a species of cycad (Encephalartos sp.) from South Africa (van der Walt, 1956) and Candida montana is known only from wild grapes (Goto & Oguri, 1983). All known isolates of B. pratensis, Candida sanyiensis and Candida qinlingensis were obtained from soil in Russia, Taiwan and China, respectively. Therefore, it is not unusual that the strains of the novel species B. siamensis sp. nov. were found from soil and palm oil fruit collected in Thailand as noted above. All species of the genus Barnettozyma and most species of the genus Candida in this clade can assimilate glycerol (Kurtzman et al., 2008; Lachance et al., 2011). This is in agreement with the fact that the two strains of the novel species in this study were isolated by the enrichment isolation technique using a nitrogen-limited medium containing glycerol as the sole source of carbon. However, information in relation to lipid accumulation in yeast strains of this genus has not been reported. Only the lipase-producing strain of Barnettozyma (Williopsis) californica has been reported by Ciafardini et al. (2006).

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