Candida xylanilytica sp. nov., a xylan-degrading yeast species isolated from Thailand

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Xylan is a major component of hemicellulose, which constitutes about 40 % of plant biomass. Hydrolysis of xylan into simple sugars is one of the important steps in the conversion of lignocellulosic material to value-added products. During an investigation of cellulose- and xylan-degrading yeasts, two yeast strains that were able to use cellulose and xylan as sole carbon source were found to represent a phylogenetically distinct species in the *Spathaspora* clade. The closest species in terms of pairwise sequence similarity in the D1/D2 domain of the LSU rRNA gene was *Candida subhashii*. The novel species can be distinguished from the other species in the *Spathaspora* clade based on the ability to assimilate methanol and raffinose, growth in medium containing 60 % glucose, and growth at 42 °C. It ferments glucose but not other carbohydrates. The name *Candida xylanilytica* sp. nov. is proposed for this species. The type strain is KU-Xn11^T (=NBRC 106499^T =BCC 34694^T =CBS 11761^T).

Xylan is the second most abundant polysaccharide in nature and is one of the principal components of hemicellulose in plant cell walls. Most xylans occur as heteropolysaccharides containing β -xylopyranose residues as the backbone with substituent acetyl, arabinofuranose and glucuronosyl residues in the side chains. In plants, hemicelluloses link between lignin and cellulose microfibrils. The xylan layer, which forms a covalent linkage with lignin and a non-covalent linkage with cellulose, is considered to be an important key to maintain cellulose integrity and protect the fibrils from degradation by cellulases (Beg *et al.*, 2001; Gilbert *et al.*, 2008). Therefore, hydrolysis of xylan into simple sugars is also one of the important steps to utilize and convert lignocellulosic materials to value-added products such as ethanol and biodiesel. Several yeasts, such as *Candida ergatensis*, *Cryptococcus albidus*, *Pichia stipitis*, *Trichosporon cutaneum* and a yeast-like fungus, *Aureobasidium pullulans*, have been reported to have xylan-utilizing ability (Beg *et al.*, 2001; Bhadra *et al.*, 2008).

The *Spathaspora* clade was recognized by Nguyen *et al.* (2006) and comprised *Spathaspora arborariae*, *Spathaspora passalidarum* and five anamorphic species in the genus

Candida. The xylose-fermenting species, *Candida lyxophilila* and *S. arborariae*, were isolated from surface woodland soil in South Africa and rotting wood in Brazil, respectively, while *Candida jeffriesii* and *S. passalidarum* were found to associate with the gut of wood-boring beetles, *Phrenapates bennettii* in Panama and *Odontotaenius disjunctus* in Louisiana, USA (Cadete *et al.*, 2009; Nguyen *et al.*, 2006; van der Walt *et al.*, 1987). The other three species of the *Spathaspora* clade do not ferment xylose. *Candida insectamans* and *Candida materiae* were found in frass of buprestid beetle larvae and rotting wood, respectively, while *Candida subhashii* was isolated from a fungal peritonitis patient in 2006 (Adam *et al.*, 2009; Barbosa *et al.*, 2009; van der Walt *et al.*, 1972).

In the course of an investigation of cellulose- and xylan-degrading yeast in Thailand, two yeast strains which were able to use cellulose and xylan as sole carbon source were found to represent a novel species in the *Spathaspora* clade, based on analysis of the D1/D2 domains of the large-subunit (LSU) rRNA gene sequence. In the present study, a novel species in the genus *Candida* is described.

Strains KU-Xn11 and KU-Xn20 were isolated from decayed corn cobs and decayed grasses, respectively, at the National Corn and Sorghum Research Center, Nakhon Ratchasima, Thailand, by an enrichment technique using

The GenBank/EMBL/DDBJ accession number for the D1/D2 domain sequence of the LSU rRNA gene of strain KU-Xn11^T is AB523752.

xylan broth (1 % oat spelt xylan, 0.67 % yeast nitrogen base). The cultures were incubated on a rotary shaker at 150 r.p.m. at 25 °C for 5 days. The culture broth was spread on xylan agar (1 % oat spelt xylan, 0.67 % yeast nitrogen base, 2 % agar) and incubated at 25 °C until yeast colonies developed. The different morphotypic colonies were purified by streaking on yeast extract/malt extract (YM) agar. Xylan and cellulose degradation was determined on xylan YM agar (1 % oat spelt xylan, 0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 2 % agar) and carboxymethyl cellulose YM agar (1 % carboxymethyl cellulose, 0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 2 % agar). After incubation at 25 °C for 5 days, the culture plates were stained with 0.5 % (w/v) Congo red for 10 min and destained with 1N NaCl (Zhang *et al.*, 2006). Strains KU-Xn11 and KU-Xn20 showed positive hydrolysis zones in xylan and cellulose degradation tests.

The strains were characterized morphologically, biochemically and physiologically according to the standard methods described by Yarrow (1998). Cultures grown on 5 % malt extract agar, cornmeal agar, Fowell's acetate agar, diluted vegetable juice agar (Kagome Co., Japan) and YM agar were examined for ascospore formation. Individual strains and mixed cultures were incubated at 15 °C and 25 °C for up to 1 month. Fermentation of D-xylose was determined in Durham tubes containing 2 % (w/v) xylose and 0.67 % yeast nitrogen base solution. After incubation at 25 °C for up to 28 days, the results were scored according to standard methods of fermentation of carbohydrates described by Yarrow (1998). Ubiquinones were extracted from intact cells cultivated in YM broth on a rotary shaker at 25 °C for 3 days. Isolation, purification and identification of ubiquinone homologues were performed according to the method described by Mikata & Yamada (1999). Genomic DNA was prepared according to Holm *et al.* (1986), as modified by Kaneko & Banno (1991). The DNA base composition was determined by HPLC as described by Tamaoka & Komagata (1984) using the DNA-GC kit (Yamasa Shoyu Co., Japan).

Sequences of the D1/D2 domain of the LSU rRNA gene were determined as described by Limtong *et al.* (2008). The sequences were compared pairwise by BLASTN search (Altschul *et al.*, 1997) and were aligned with sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL X version 1.83 (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987) and by the maximum-parsimony method using the max-mini branch-and-bound algorithm of MEGA version 4 (Nei & Kumar, 2000; Tamura *et al.*, 2007). Confidences for the phylogenetic tree were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

The D1/D2 domains of the LSU rRNA gene of strains KU-Xn11 and KU-Xn20 have identical sequences which are

present as two distinct copies in the genome. One copy has seven consecutive T nucleotides (copy 1, 569 nt), the other has eight T nucleotides (copy 2, 570 nt) in the same region. This may result from variability in tandem repeats of the rRNA gene. The closest species in terms of pairwise sequence similarity was *Candida subhashii*, with 5.6 % nucleotide substitution (32 bp) and 17 indels in copy 1, and 5.8 % nucleotide substitution (33 bp) and 16 indels in copy 2. The two strains showed greater than 1 % nucleotide substitution in the D1/D2 domain of the LSU rRNA gene and were presumed to represent a distinct species from *Candida subhashii*, according to the guidelines of Kurtzman & Robnett (1998). Phylogenetic analysis based on the D1/D2 domain of the LSU rRNA gene demonstrated that the two strains formed a cluster with *Candida insectamans* with high bootstrap support and were related to the other known species of the *Spathaspora* clade (Fig. 1).

The two strains proliferated by multilateral budding and pseudohyphae were abundant (Fig. 2). The strains were negative for Diazonium blue B and urease reactions and had Q-9 as the major ubiquinone. Lipid accumulated in cells grown in 5 % malt extract agar, cornmeal agar or YM agar after 5 days at 25 °C. Ascospores were not produced from individual strains or mixed culture on 5 % malt extract agar, cornmeal agar, Fowell's acetate agar, diluted vegetable juice agar or YM agar after 5 weeks at 15 °C. Xylose fermentation was absent, similar to *Candida insectamans*, *Candida materiae* and *Candida subhashii*. The novel strains could be distinguished from the other species of the *Spathaspora* clade based on the ability to assimilate methanol and raffinose, growth in medium containing 60 % glucose and growth at 42 °C. Based on the data above, we concluded that the two strains represent a single novel species of the genus *Candida* in the *Spathaspora* clade, for which the name *Candida xylanilytica* sp. nov. is proposed.

Latin diagnosis of *Candida xylanilytica* Boonmak, Limtong, Jindamorakot, Am-In, Yongmanitchai, Suzuki, Nakase et Kawasaki sp. nov.

In liquido 'YM', post dies 3 ad 25 °C, cellulae spheroidae, ovoidae, ellipsoideae vel elongatae, 3.2–9.5 × 3.2–23.2 µm, singulae, binae vel conglobatae, per germinationem multipolarem reproducentes. Pseudomycelium formatur. In agaro 'YM', post unum mensem ad 25 °C, cultura butyrosa vel rugosus, cremea, umbonatus vel pulvinatus, margine fimbriata cum mycelio. In agaro farinae Zea mays post die 7 ad 25 °C pseudohyphae formantur. Ascosporae non formantur.

Glucosum (exiguum) fermentur at non galactosum, sucrosum, maltosum, lactosum, raffinotum, trehalosum, cellobiosum nec D-xylosum. Glucosum, sucrosum, raffinotum (exiguum vel nullum), trehalosum, maltosum, melezitotum, α-methyl D-glucosidum, amyllum solubile, cellobiosum, salicinum, D-xylosum, L-arabinosum, D-ribosum, methanolum (fortasse tardum), ethanolum, glycerolum, ribitolum,

Fig. 1. Phylogenetic tree based on sequences of the D1/D2 domain of the LSU rRNA gene, showing the positions of the two strains of *Candida xylanilytica* sp. nov., with respect to closely related species. The neighbour-joining (NJ) phylogenetic tree was reconstructed from evolutionary distance data corrected by the two-parameter transformation of Kimura (1980). The maximum-parsimony (MP) phylogenetic tree was reconstructed using the max-mini branch-and-bound algorithm (Nei & Kumar, 2000). Numbers indicate percentages of bootstrap sampling, derived from 1000 samples. Bars, 0.02 substitutions per nucleotide position (NJ) or 5 steps (MP).

D-mannitolum, D-glucitolum, acidum DL-lacticum, acidum succinicum (lente vel nullum), acidum D-gluconicum (lente vel nullum), D-glucosaminum, N-acetyl-D-glucosaminum, 2-ketogluconicum, propanum-1,2-diolum (lente), xylanum et arbutinum assimilantur at non inulinum, melibiosum, galactosum, lactosum, L-sorboseum, L-rhamnosum, D-arabinosum, erythritolum, galactitolum, inositolum, acidum citricum, hexadecanum, xylitolum, 5-ketogluconicum, acidum D-glucuronicum, arabinitolum, D-glucono-1,5-lactonum, acidum D-galacturonicum nec butanum-2,3-diolum. Ethylaminum, L-lysinum et cadaverinum assimilantur at non

nitricum nec nitroculum. Crescit sine vitaminis (exiguum). Crescit in 50 % glucosum et 60 % glucosum (exiguum). Non crescit in 0.01 % cycloheximido, 0.1 % cycloheximido nec 10 % natrii chloridum/5 % glucosum. Amylum non formatur. Diazonium caeruleum B non respondens. Ureum non hydrolysatur. Maxima temperatura crescentiae: 42 °C. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 38.3, 39.2 mol% (per HPLC). Ubiquinonum majus: Q-9. Typus strips KU-Xn11^T (=NBRC 106499^T =BCC 34694^T =CBS 11761^T) isolatus ex putrefacio Zea mays axis, Nakhon Ratchasima Provincia, Thailandia, conservatur in

Fig. 2. Morphology of vegetative cells of strain KU-Xn11^T. (a) Vegetative cell grown in YM broth for 3 days at 25 °C. (b) Pseudomycelia produced on slide culture with cornmeal agar after 7 days at 25 °C. (c) Pseudomycelia produced on Dalmau plate culture with cornmeal agar after 14 days at 25 °C. Bars, 10 μm.

collectione culturarum in 'NITE Biological Resource Center (NBRC)', Kisarazu, Chiba, Japania ut NBRC 106499^T, et collectione culturarum in 'BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC)', Pathumthani, Thailandia ut BCC 34694^T, et 'Centraalbureau voor Schimmelcultures (CBS)', Utrecht, the Netherlands ut CBS 11761^T deposita est.

Description of *Candida xylanilytica* Boonmak, Limtong, Jindamorakot, Am-In, Yongmanitchai, Suzuki, Nakase & Kawasaki sp. nov.

Candida xylanilytica (xy.lan.i.ly'ti.ca. N.L. n. *xylanum* xylan, a plant polysaccharide; Gr. adj. *lutikos* -ê -on able to loose, able to dissolve; N.L. fem. adj. *xylanilytica* hydrolysing xylan).

After growth in YM broth for 3 days at 25 °C, loose floating islets and heavy sediment are present. Cells proliferate by multilateral budding and are spheroidal, ovoidal, ellipsoidal or elongate, 3.2–9.5 × 3.2–23.2 µm and occur singly, in pairs or in small clusters. Pseudomycelia are produced. After growth in YM agar for 1 month at 25 °C, streak culture is smooth, butyrous, cream-coloured and umbonate with ciliate margin. Sometimes pulvinate wrinkled colonies are obtained from smooth colonies. In Dalmau plate culture on cornmeal agar after 5 days at 25 °C, pseudomycelia are produced abundantly. Blastoconidia are formed in clusters. Ascospores are not produced from individual strains or mixed culture on 5 % malt extract agar, cornmeal agar, Fowell's acetate agar, diluted vegetable juice agar or YM agar after 5 weeks at 15 °C. Fermentation of glucose is positive but fermentation of galactose, sucrose, maltose, lactose, raffinose, trehalose, cellobiose and xylose is negative. Glucose, sucrose, raffinose (weakly positive or negative), trehalose, maltose, melezitose, α-methyl D-glucoside, soluble starch, cellobiose, salicin, D-xylose, L-arabinose, D-ribose, methanol (slow), ethanol, glycerol, ribitol, D-mannitol, D-glucitol, DL-lactic acid, succinic acid (latent or negative), D-gluconic acid (latent or negative), D-glucosamine, N-acetyl-D-glucosamine, 2-ketogluconic acid, propane-1,2-diol (latent), xylan, ethylamine, L-lysine and cadaverine are assimilated, but inulin, melibiose, galactose, lactose, L-sorbose, L-rhamnose, D-arabinose, erythritol, galactitol, inositol, citric acid, hexadecane, xylitol, 5-ketogluconic acid, D-glucuronic acid, arabinitol, D-glucono-1,5-lactone, D-galacturonic acid, butane-2,3-diol, nitrate and nitrite are not assimilated. Growth in vitamin-free medium is weak. Growth at 42 °C but not at 45 °C. Growth on medium containing 0.01 % cycloheximide and medium containing 10 % NaCl and 5 % glucose is negative. Growth on medium with 50 % glucose is positive but weakly positive with 60 % glucose. Starch formation and gelatin liquefaction are negative. Tween 80 hydrolysis is positive. Diazonium blue B and urease reactions are negative. DNA G+C content is 38.3–39.2 mol%. Major ubiquinone is Q-9.

The type strain, KU-Xn11^T (=NBRC 106499^T =BCC 34694^T =CBS 11761^T), was isolated from decayed corn cobs at the National Corn and Sorghum Research Center, Nakhon Ratchasima, Thailand. A living culture of the type is deposited at the NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation (NITE), Chiba, Japan, as NBRC 106499^T, the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 34694^T, and the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as CBS 11761^T.

Acknowledgements

This work was supported in part by the Institute for Fermentation, Osaka (IFO) fund. Special thanks go to Mr Shinya Ninomiya and Mr Atsushi Yamasaki for kindly support and suggestion. Many thanks are due to the students of the laboratory of Professor Savitree Limtong for their assistance. This work was facilitated by the Joint Research Project between BIOTEC and NITE.

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