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Characterization of xylose-utilizing yeasts isolated from herbivore faeces in Thailand

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ABSTRACT: A total of 39 xylose-utilizing yeast strains were isolated from herbivore faeces in Thailand. They were identified as *Candida tropicalis* (32 isolates), *Candida albicans* (1 isolate), *Pichia terricola* (1 isolate), *Trichosporon mycotoxinivorans* (2 isolates), *Sporopachydermia lactativora* (2 isolates) and *Zygoascus meyerae* (1 isolate) based on their morphological, cultural, physiological and biochemical characteristics including the sequence analysis of the D1/D2 region of the large-subunit ribosomal DNA. Thirty seven isolates could ferment xylose to ethanol. *Zygoascus meyerae* E23 isolated from elephant faeces produced the highest ethanol concentration (3.61 g/l after 72 h). *C. tropicalis* A26 isolated from cow faeces produced the highest xylitol concentration (43.79 g/l) which corresponded to 0.71 g xylitol/g xylose after 24 h. *C. tropicalis* A26 xylose reductase showed 98.4% identity and 99.0% similarity to *C. tropicalis* (ABX60132C) xylose reductase, and showed the tetra-amino acid motif (Ile-Pro-Lys-Ser) which is conserved among NADPH-dependent xylose reductase.

KEYWORDS: *Candida*, *Pichia*, *Sporopachydermia*, *Trichosporon*, xylose fermentation, xylose reductase gene, *Zygoascus*

INTRODUCTION

Lignocellulose is an interesting non-food fermentable sugar resource for ethanol fuel production[1]. It consists of 30–40% (w/w) cellulose and 15–25% (w/w) hemicellulose[1,2] and it is the world most abundant biomass. Completely hydrolysis of the cellulose and the hemicellulose results in glucose and xylose, respectively, which several microorganisms can ferment to ethanol[3]. *Saccharomyces cerevisiae* is the most popular yeast for fermenting glucose to ethanol due to its high ethanol production yield and comparatively high ethanol tolerance[4,5], however *S. cerevisiae* strains cannot ferment xylose to ethanol. Several yeasts such as *Candida shehatae*, *Pachysolen tannophilus*, *Brettanomyces naardenensis*, *C. tenuis*, *Pichia segobiensis*, *C. lyxosophila*, *C. intermedia*, *C. jeffriesii*, *Spathaspora passalidarum*, *Spathaspora arborariae*, *C. prachuapensis*, and *Scheffersomyces stipitis* have been reported as xylose fermenting yeasts[6–10]. The *S. stipitis* strain produces significant amounts of ethanol from xylose and it has been studied extensively[9].

To increase an ethanol production yield from lignocellulose, both glucose and xylose liberated should be fermented to ethanol. Although *S. stipitis* strain can ferment both glucose and xylose to ethanol, its ethanol yield from glucose and ethanol tolerance are much lower than those of *S. cerevisiae*[9,11]. Co-cultivating of *S. cerevisiae* and *S. stipitis* strains to co-ferment glucose and xylose remains unsatisfactory, due to their difference in fermenting condition and ethanol tolerance[12,13]. *S. stipitis* strain prefers to ferment glucose more than xylose while it has lower ethanol tolerance than *S. cerevisiae* strain[14]. So xylose was not fermented. Therefore, there has been an

attempt to construct recombinants *S. cerevisiae* strain capable of fermenting xylose by overexpressing the genes encoding enzymes in the xylose fermentation pathway of *S. stipitis* strain[4,14,15]. Three key step enzymes in *S. stipitis* xylose fermentation pathway are (1) xylose reductase (EC.1.1.1.21), which converts xylose to xylitol using NAD(P)H as a cofactor[16,17] (2) xylitol dehydrogenase (EC.1.1.1.9), which converts xylitol to xylulose using NAD as a cofactor[18], and (3) xylulose kinase (EC 2.7.1.17), which converts xylulose to xylulose-5-phosphate[19]. Because under oxygen-limit condition which *S. cerevisiae* ferments glucose to ethanol, NAD becomes its limiting factor[15]. So ethanol production from xylose of the recombinant *S. cerevisiae* was low. Screening for NADH dependent xylose reductase which has high activity to replace the NAD(P)H dependent xylose reductase of *S. stipitis* will be useful in constructing a recombinant *S. cerevisiae* capable of fermenting xylose[14,20,21]. Digestive tract of herbivores contains microorganisms which can hydrolyse only cellulose in the lignocelluloses consumed to glucose[22]. So the existence of such xylose-utilizing yeasts in their faeces is expected. There is no previous report on the diversity of xylose-utilizing yeasts in digestive tract or faeces of herbivores in Thailand. The objective of this work is to isolate xylose-utilizing yeasts from herbivore faeces to screen for high efficient xylose fermenting and xylitol producing yeasts. Co-enzyme specificity of high activity xylose reductase enzymes of selected yeast was also determined.

MATERIALS AND METHODS

Isolation of xylose utilizing yeasts

Twenty-one herbivore faeces samples from 5 elephants, 1 goat, 2 giraffes, 1 macaque, 3 cows, 1 buffalo, 1 kangaroo, 2 zebras, 1 hog deer, 1 orlik, 1 antelope, 1 horse, and 1 barking deer were collected from various areas in Thailand (Table 1). The sample 0.5 g was enriched in 10 ml of Yeast Nitrogen Base-Xylose (YX) medium (0.67% yeast nitrogen base and 5% D-xylose, w/v) supplemented with 0.02% chloramphenicol and 0.25% sodium propionate in 25×150 mm test tube. The enriched samples were incubated at 30 °C for 3–10 days, then spread on YX agar for isolation. Representative yeast colonies were selected, purified, kept on Yeast extract-Malt extract (YM) slant (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 1.5% agar, w/v) at 4 °C and stored for a long time in YM broth containing 10% (w/v) glycerol at −80 °C[23].

IDENTIFICATION METHODS

Phenotypic characterization

Morphological, physiological and biochemical characteristics of isolated yeasts were examined according to Yarrow[29]. Cells grown on 5% malt extract agar, cornmeal agar or YM agar for 1 or 5 days were examined for ascospore formation under microscope. Sugar fermentation tests of D-galactose, D-glucose, lactose, maltose, D-raffinose, sucrose, trehalose and D-xylose were determined using 0.67% yeast nitrogen base solution containing 2% sugar with Durham tube[30]. Assimilation of carbon and nitrogen compounds were determined in liquid and solid medium, respectively. Nitrogen source tested were KNO_3, $NaNO_2$, ethylamine hydrochloride, cadaverine dihydrochloride, and lysine (Table 2). Capability to grow in 50% glucose and at 37 °C were determined by using YM broth and incubated by using a heat block. Glucose/mineral medium without all vitamins was used to test for an ability to grow in the absence of vitamins. Sensitivity to cycloheximide was performed by using Bacto Yeast Nitrogen Base containing D-glucose and supplemented with 0.1% or 0.01% cycloheximide.

RNA gene sequence and phylogenetic analysis

The D1/D2 domain of large subunit ribosomal RNA gene (*LSU* rRNA gene) was amplified by PCR using the primers NL1 (5′-GCATATCAATAAGCGGAG GAA-3′) and NL4 (5′-GGTCCGTGTTTCAAGAC GG-3′)[5,31]. PCR product was purified by QIAquick purification kit (Qiagen K.K., Japan). The purified PCR product was sequenced by using ABI BigDye Terminator V3.1 Cycle Sequencing RR-100 kit, and ABI Model 3130xl DNA Analyser (Applied Biosystems, Foster City, California, USA). DNA Sequencing reaction was performed using the primers NL1 and NL4. Sequencing data was aligned by Chromas Pro software program (Technelysium Pty Ltd., Australia). The sequences were compared pairwise using a BLASTN search[32] and were aligned with sequences of related species retrieved from GenBank using multiple alignment program CLUSTAL_X version 1.8[33]. The D1/D2 domain of *LSU* rRNA gene sequence similarity of 99–100% to those of related species was identified as the same species[5]. Phylogenetic tree was constructed from an evolutionary distance data with Kimura's two-parameter correction[34] using neighbour-joining method[35]. Topology of the phylogenetic tree was tested by performing 1000 replicates bootstrap resampling[36].

Table 1 Isolation and identification of xylose-utilizing yeasts based on D1/D2 domain of *LSU* rRNA gene sequence.

Animal faeces/Province	Isolate no.	Similarity (%)	Identification
Elephant/Bangkok	A1, A2	100	*C. tropicalis*
Elephant/Nakhonratchasima	A19	100	*C. tropicalis*
Elephant/Prachinburi	B1	100	*C. tropicalis*
Goat/Bangkok	A3	100	*C. tropicalis*
Giraffe/Bangkok	A4	100	*C. tropicalis*
Giraffe/Nakhonratchasima	A21	100	*C. tropicalis*
Cow/Khonkaen	A6, A26	100	*C. tropicalis*
Cow/Udonthani	A9, A10, A11	100	*C. tropicalis*
Cow/Kalasin	A12, A13, A15	100	*C. tropicalis*
Buffalo/Kalasin	A7, A8, A16, A17	100	*C. tropicalis*
Kangaroo/Nakhonratchasima	A18	100	*C. tropicalis*
Hog deer/Nakhonratchasima	A22	100	*C. tropicalis*
Antelope/Nakhonratchasima	A23	100	*C. tropicalis*
Oryx/Nakhonratchasima	A24	100	*C. tropicalis*
Horse/Nakhonratchasima	A25	100	*C. tropicalis*
Barking deer/Bangkok	A27, D1, E1	100	*C. tropicalis*
Zebra/Bangkok	A28, I1	100	*C. tropicalis*
Zebra/Nakhonratchasima	A20, M1	100	*C. tropicalis*
Macaque/Bangkok	A5	100	*C. tropicalis*
Kangaroo/Bangkok	L1	99	*C. albicans*
Macaque/Bangkok	H1	100	*P. terricola*
Elephant/Ayutthaya	E21, E24	100	*T. mycotoxinivorans*
Elephant/Ayutthaya	E20	99	*S. lactativora*
Elephant/Nakhon Pathom	E25	99	*S. lactativora*
Elephant/Ayutthaya	E23	100	*Z. meyerae*

Determination of ethanol and xylitol production

Single colony of yeast isolates grown on YX agar, pH 5.0 at 30 °C, 48 h was inoculated into YX broth (50 ml in 250 ml Erlenmeyer flask), incubated at 30 °C, 200 rpm for 24 h. The culture was transferred at 1% (v/v) into fresh YX broth (50 ml in 250 ml Erlenmeyer flask) and incubated at 30 °C, 200 rpm for 24 h. The inoculum was inoculated at 10% (v/v) into the same medium and incubated at 30 °C, 200 rpm for 24 h. After centrifugation at 4 °C, 9793*g* (10 min), resultant supernatants were analysed for ethanol by gas chromatography[37] and for xylitol by high performance liquid chromatography (HPLC)[38]. *S. stipitis* JCM 10742^{T} (Japan Collection of Microorganisms, RIKEN BioResource Centre, Japan), which is a high efficient ethanolic xylose fermenting yeast, was used as a control.

Single colony of top seven high xylitol producing yeast selected was grown in modified media of Kurtzman and Dien[39] (Yeast-Peptone-Xylose, YPX medium containing 1% yeast extract and 2% peptone, 6.6% xylose (w/v) and pH 5.0) at 30 °C for 24 h. Optical density at 660 nm (OD 660 nm) of the resultant culture was adjusted to 2.0, inoculated into fresh YPX medium (50 ml in 250 ml Erlenmeyer flask) at 10% (v/v) and incubated at 30 °C, 200 rpm for 24–48 h. The culture was centrifuged and obtained supernatant was analysed for ethanol and xylitol.

Cloning of xylose reductase gene

Xylose reductase gene (*xyl*) was amplified directly from genomic DNA of *C. tropicalis* A26 by PCR method. Primers specific for *xyl* were designed based on conserved amino acid sequences of xylose reductase of various yeast species. The primers were designated as XR_F [5′-GA(G/A)A(A/G)(A/G)TA(T/C)CC(A/T)GG(A/T)TTCTAC-3′] and XR_R [5′-CCT(G/A)TCCCA(T/G)GG(A/G)T(T/C)(G/A)TT(G/A)AATCT-3′]. The amplification was performed by gradient PCR using *Tag* DNA Polymerase (Fermentas, California). The amplified product was purified by GeneJET Gel Extraction Kit (Fermentas, California) then ligated to pTZ57R/T vector (InsTAclone PCR cloning kit, Fermentas, California). The ligation reaction was transformed into *Escherichia coli* DH5α competent cells using method described by Sambrook and Russell[40]. Colonies grown on LB agar containing 100 μg/ml ampicillin, 20 mg/ml X-Gal and 100 mM

Table 2 Differential characteristics of yeasts isolated and their related type strains.

Characteristics	1	2[a]	3	4[a]	5	6[b]	7	8[c]	9	10[d]	11	12[e]
Fermentation of:												
Glucose	+	+	+	+	+	+/w	-	-	-	-	+	+
Galactose	+	+	+	v	-	-	-	-	-	-	w	w
Sucrose	+	v	+	v	-	-	-	-	-	-	w	w
Maltose	+	+	+	+	-	-	-	-	-	-	-	-
Lactose	-	-	+	-	-	-	-	-	-	-	-	-
Raffinose	-	-	+	-	-	-	-	-	-	-	-	w/l
Trehalose	+	+/s	+	v	-	-	-	-	-	-	s	w/l
Assimilation of:												
Glucose	+	+	+	+	+	+	+	+	l	+	+	+
Galactose	+	+	+	+	-	-	+	+	-	-	l	+
L-Sorbose	s	v	s	v	-	-	s	+	l	+	l	+
Sucrose	+	v	+	v	-	-	+	+	-	-	l	+
Maltose	+	+	+	+	-	-	+	+	-	-	l	+
Cellobiose	w	+/l	-	-	-	-	+	+	-	-	l	+
Trehalose	+	+	+	v	-	-	+	+	-	-	+	+
Lactose	-	-	-	-	-	-	+	+	-	-	-	-
Melibiose	-	-	-	v	-	-	l	+	-	-	-	-
Raffinose	-	-	-	-	-	-	l	+	-	-	l	+
Melezitose	+	v	s	-	-	-	w	+	-	-	l	v
Inulin	w	-	s	-	-	-	-	+	w	-	-	-
Soluble starch	+	+	l	+	-	-	+	+	-	-	l	+
D-Xylose	+	+	+	+	-	-	+	+	l	+	+	+
L-Arabinose	-	-	-	v	-	-	+	+	-	-	+	+
D-Arabinose	-	-	-	v	-	-	-	+	-	-	-	-
D-Ribose	-	-/l	-	v	-	-	+	+	-	v	-	v
L-Rhamnose	-	-	-	-	-	-	l	+	-	-	+	+
Ethanol	+	+	+	+	+	+	+	+	l	+	+	+
Glycerol	-	v	-	v	+	+	+	+	+	+	+	+
Erythritol	-	-	-	-	-	-	-	w	-	-	-	-
Ribitol	+	+/l	+	+	-	-	s	-	+	+	l	+
Galactitol	-	-	-	-	-	-	-	+	-	-	+	+
D-Mannitol	+	+	+	+	-	-	l	+	l	v	+	+
D-Glucitol	+	+	+	v	-	-	l	+	l	v	s	+
α-Methyl-D-glucoside	+	v	+	v	-	-	l	+	-	-	-	-
Salicin	v	v	s	-	-	-	l	+	-	-	+	+
DL-Lactate	+	v	+	+	l	-	+		l	+	w/-	-
Succinate	+	+	+	+	+	+	+	+	l	+	+	+
Citrate	+	+/l	l	+	+	w/-	+	+	-	-	s	+
Inositol	-	-	-	-	-	-	l	+	l	+	+	+
2-Keto-D-gluconate	+	+	+	+	-	-	+	+	-	-	-	-
5-Keto-D-gluconate	+	+	l	n	-	-	+	+	-	n	s	n
Nitrite	-	-	-	-	-	n	-	-	-	n	-	-
Cadaverine	+	+	+	+	+	n	+	w	-	-	+	+
L-Lysine	+	+	+	+	+	n	+	+	+	+		-
Ethylamine	+	+	+	+	+	n	+	w	+	+	+	+
Growth in:												
Vitamin-free	l	v	+	v	-	-	w	-	w	-	-	-
50%Glucose	-	+/l	+	v	-	n	+	-	-	-	+	-
10%NaCl/5% Glucose	+	l	+	v	-	-	+	-	-	n	+	n
0.01% Cycloheximide	+	+	+	+	-	n	+	+	l	+	l	+
0.1% Cycloheximide	+	+	+	+	-	n	+	+	l	n	l	+
Growth at 37 °C	+	+	+	+	+	v	+	+	l	+	+	+
Starch formation	-	-	-	-	-	-	-	-	-	n	-	-
Urease	-	-	-	n	-	n	+	+	-	n	-	-

1, *C. tropicalis* (32 isolates); 2, *C. tropicalis* NRRL Y-12968^T; 3, *C. albicans* (1 isolate, L1); 4, *C. albicans* NRRL Y-17909^T; 5, *P. terricola* (1 isolate, H1); 6, *P. terricola* YB-4310^T; 7, *T. mycotoxinivorans* (2 isolates); 8, *T. mycotoxinivorans* HB1175^T; 9, *S. lactativora* (2 isolates); 10, *S. lactativora* NRRL Y-11591^T; 11, *Z. meyerae* (1 isolate, ELP23); 12, *Z. meyerae* Y-17319^T. +, positive; -, negative; l, latent (longer than 7 days), s, slow; w, weak; v, variable; n, no data.

[a] Data from Lachance et al[24].

[b] Data from Kurtzman[25].

[c] Data from Molnar et al[26].

[d] Data from Rodrigues et al[27].

[e] Data from Smith et al[28].

IPTG after incubation at 37 °C for 24 h were collected. Clones harbouring the *xyl* were determined by colony PCR. Confirmation of the *xyl* gene was performed by DNA sequence analysis.

Analytical procedures

Ethanol was analysed by gas chromatography (Hewlett-Packard, HP 5890 Series; USA) using Parapak QS (Carbowax 20 M) column and flame ionization detector (FID, 150 °C). Oven temperature was 175 °C and using helium at 35 ml/min flow rate as carrier gas, Xylitol was quantified by HPLC (Varian, Prostar, USA) using Lichrospher100 NH_2 (4–250 mm) column (Merck, Germany) and evaporative light scattering detector. (Alltech, USA). Mobile phase was acetonitide:water (91:9) at 1.5 ml/min flow rate.

RESULTS AND DISCUSSION

Identification of yeast isolates

Thirty-nine xylose-utilizing yeasts were assigned to ascomycetous (37 isolates), and basidiomycetous (2 isolate) yeasts. The 37 yeast isolates were identified as *C. tropicalis* (32 isolates), *C. albicans* (1 isolate), *Pichia terricola* (1 isolate), *Sporopachydermia lactativora* (2 isolates) and *Zygoascus meyerae* (1 isolate), and the 2 basidiomycetous yeasts were identified as *Trichosporon mycotoxinivorans* based on their phenotypic characteristics and D1/D2 domain of *LSU* rRNA gene sequence analysis (Fig. 1 and Table 2).

The majority of the yeast isolates (32 isolates) had the following characteristics: globose cell shape, formed pseudohyphae, consisted of cylindrical cells shape, and branched chains. All isolates fermented glucose, galactose, sucrose, maltose, and trehalose. They assimilated sucrose, L-sorbose (slow), cellobiose (weak), melezitose, inulin (weak), ribitol, α-methyl-D-glucoside, citrate (latent), grew on vitamin-free medium, 10% NaCl with 5% glucose, and grew on YM medium at 37 °C. They did not grow on 50% glucose which was different from *C. tropicalis* NRRL Y-12968^T (Table 2). They were clustered within *C. tropicalis* NRRL Y-12968^T (Fig. 1). Their D1/D2 *LSU* rRNA gene sequences were 100% similarity to *C. tropicalis* NRRL Y-12968^T (Table 1). Therefore, they were identified as *C. tropicalis*[24].

Isolate L1 had globose cell shape and formed branched pseudohyphae. It fermented galactose, sucrose, lactose, and raffinose, assimilated L-sorbose (slow), sucrose, trehalose, melezitose (slow), inulin (slow), glucitol, α-methyl-D-glucoside, salicin (slow), and citrate (latent), but did not assimilate melibiose, L-arabinose, D-arabinose and D-ribose, grew on vitamin free medium, 50% glucose, 10% NaCl with 5% glucose (Table 2). *C. albicans* has variable characteristic[4]. The isolate was related to *C. albicans* (Fig. 1) and was clustered in *Lodderomyces-Spathaspora* clade. It showed 99% D1/D2 *LSU* rRNA gene sequence similarity to *C. albicans* NRRLY-17909^T with 2 nucleotides difference (Table 1). Therefore, it was identified as *C. albicans*[24].

Isolate H1 fermented glucose, grew on YM medium at 37 °C, assimilated DL-lactate, citrate, cadaverine, L-lysine, and ethylamine. It showed almost the same phenotypic characteristics as *P. terricola* YB-4310^T (Table 2). The isolate was nearest with *P. terricola* YB-4310^T based on 100% D1/D2 *LSU* rRNA gene sequence similarity (Fig. 1, Table 1). It belonged to *P. membranifaciens* clade which was transferred from *Issatchenkia* and moved back to *Pichia*[41]. Therefore it was identified as *P. terricola*[25].

Isolates E21 and E24 showed various cell morphology (i.e., unicellular, yeast-like, pseudohyphal, and hyphal). They assimilated L-sorbose (slow), melibiose (latent), raffinose (latent), melezitose (weak), rhamnose (latent), ribose (slow), mannitol (latent), glucitol (latent), α-methyl-D-glucoside (latent), salicin (latent), and inositol (latent), but did not assimilate inulin, D-arabitol, erythritol, or galactitol. They grew on vitamin free medium (weak), 50% glucose, and 10% NaCl with 5% glucose (Table 2). They were related to *T. mycotoxinivorans* HB1175^T based on 100% D1/D2 *LSU* rRNA gene sequence similarity (Fig. 1 and Table 1). Therefore, they were identified as *T. mycotoxinivorans*[26].

Isolates, E20 and E25, assimilated glucose (latent), L-sorbose (latent), inulin (weak), D-xylose (latent), ethanol (latent), ribitol, mannitol (latent), salicin (latent), and grew on vitamin free medium (weak). They neither fermented sugars nor assimilated cadaverine (Table 2). They were related to *Sporopachydermia lactativora* NRRL Y-11591^T based on 99% D1/D2 *LSU* rRNA gene sequence similarity (Fig. 1, Table 1). They were identified as *S. lactativora*[27].

Isolate E23 assimilated galactose, L-sorbose, sucrose, maltose, cellobiose, raffinose, melezitose, soluble starch, ribitol, and 0.1% cycloheximide after 7 days (latent). It neither fermented raffinose nor assimilated ribose (Table 2). The isolate was nearest to *Z. meyerae* Y-17913^T (Fig. 1). The D1/D2 *LSU* rRNA gene sequence of this isolate was 100% similarity to *Z. meyerae* Y-17913^T with only one base substitution (Table 1). Therefore, the isolate was identified as *Z. meyerae*[28].

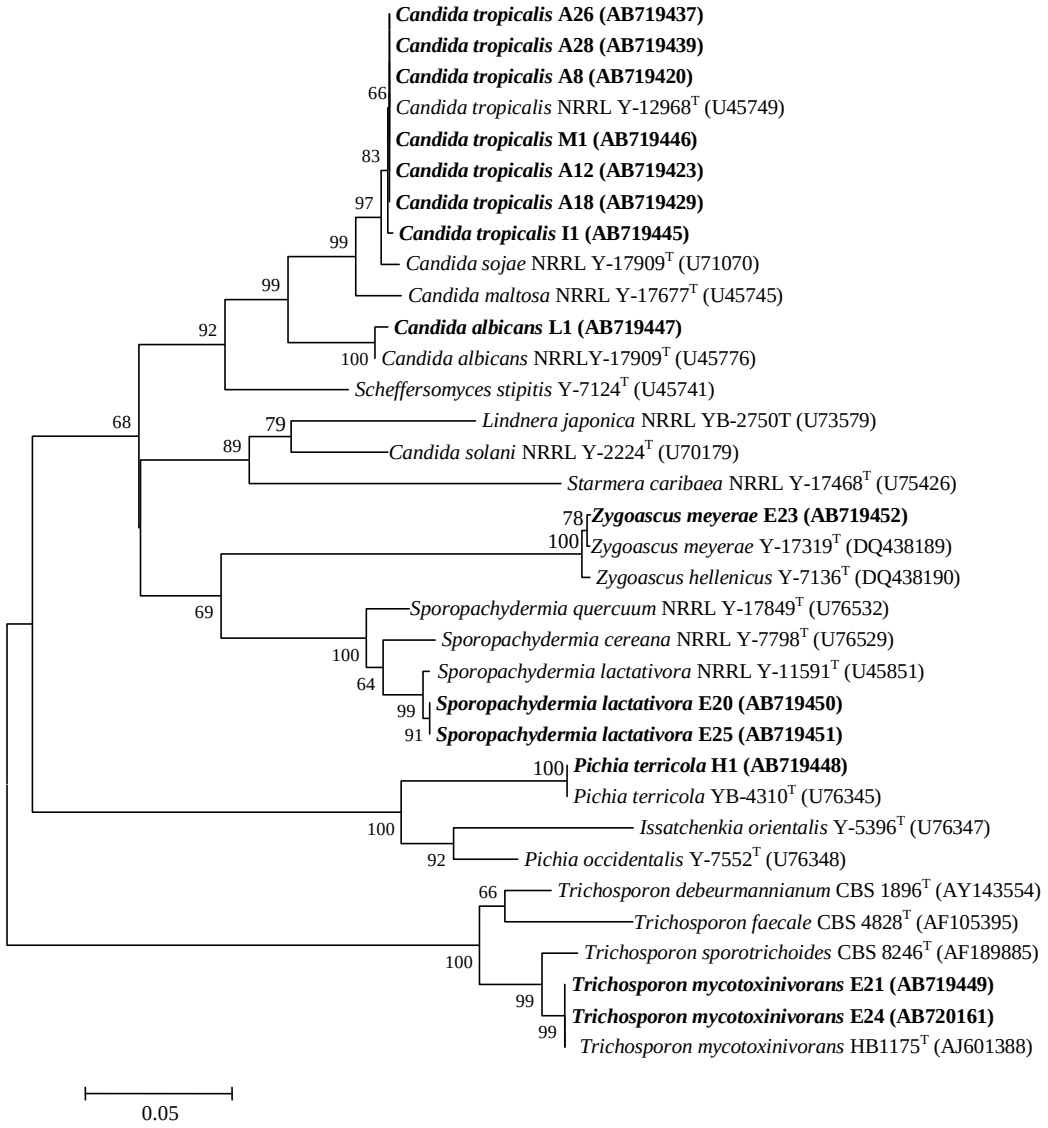

Fig. 1 Phylogenetic tree of xylose assimilation yeasts constructed by the neighbour-joining method based on D1/D2 domain of *LSU* rRNA gene sequences. The numbers represent the percentages from 1000 replicates bootstrap resampling (frequencies less than 50% are not shown).

Ethanol and xylitol production

The 39 xylose-utilizing yeasts isolated were cultured in YX medium. It was found that 37 isolates produced ethanol (0.0016–0.0895 g/l) after 24 h (data not shown), 2 isolates (*P. terricola* H1 and *C. albicans* L1) could not produce ethanol. *Z. meyerae* E23 produced the highest ethanol at 2.04 and 3.61 g/l after 48 and 72 h (data not shown), respectively. For xylitol production, 36 out of 39 xylose-utilizing yeasts isolated produced xylitol in the range of 0.03–43 g/l (data not shown). Seven *C. tropicalis* isolates, A8, A12, A18, A26, A28, I1, and M1 which produced xylitol more than 30 g/l and the *Z. meyerae* E23 which produced the highest ethanol were selected for further studied, since their xylose reductase was expected to have high activity. In YPX medium, the eight selected isolates were found to produce ethanol (0.012–0.017 g/g xylose). Prolongation of the incubation period from 24–48 h only increased ethanol production by *Z. meyerae* E23 from 0.017–0.08 g/g xylose. Xylose consumption rate of *Z. meyerae* E23 was comparatively low during the first 24 h (Table 3). In this study, our isolate produced ethanol from xylose

Table 3 Growth and xylose fermentation parameters of selected isolates.

Strains	$Y_{cm/s}$ (g/g)	Q_s (g l^{-1} h^{-1})	C_{xl} (g/l)	C_e (g/l)	Q_{xl} (g l^{-1} h^{-1})	Q_e (g l^{-1} h^{-1})	$Y_{xl/s}$ (g/g)	$Y_{e/s}$ (g/g)
A8	0.11	2.07	33.69	0.87	1.40	0.04	0.68	0.017
A26	0.08	2.56	43.79	0.73	1.82	0.03	0.71	0.012
M1	0.08	2.43	39.59	0.68	1.65	0.03	0.68	0.012
A12	0.09	2.25	38.33	0.81	1.60	0.03	0.71	0.015
I1	0.11	2.03	33.83	0.72	1.41	0.03	0.69	0.015
A28	0.09	2.44	40.66	0.87	1.69	0.04	0.69	0.015
A18	0.11	2.23	27.36	0.83	1.14	0.03	0.51	0.016
E23	0.28	1.05	7.89	0.42	0.33	0.02	0.31	0.017
JCM 10742^T	0.11	2.73	0.96	18.66	0.04	0.78	0.01	0.285

The values presented are averages of duplicate experiments.
$Y_{cm/s}$, biomass yield (g dry mass/g D-xylose consumed); Q_s, volumetric xylose consumption rate (g l^{-1} h^{-1}); C_{xl}, maximum volumetric xylitol concentration observed (g/l); C_e, maximum volumetric ethanol concentration observed (g/l); Q_{xl}, volumetric xylitol production rate (g l^{-1} h^{-1}); Q_e, volumetric ethanol production rate (g l^{-1} h^{-1}); $Y_{xl/s}$, xylitol yield (g xylitol/g D-xylose consumed); $Y_{e/s}$, ethanol yield (g ethanol/g D-xylose consumed).

with a small amount compared to *S. stipitis* JCM 10742^T (0.285 g/g xylose), *Kluyveromyces marxianus*[42] (0.28 g/g xylose), or *Spathaspora passalidarum*[43] (0.4 g/g xylose) which have been improved for ethanol production. However, *Z. meyerae* E23 was the first report to produce ethanol and further study is required. For xylitol production it was found that maximum xylitol (0.71 g/g xylose) was produced by *C. tropicalis* A26 and A12. Since *C. tropicalis* A26 produced higher xylitol (43.79 g/l) than *C. tropicalis* A12 (38.33 g/l). Therefore, *C. tropicalis* A26 was selected for further studies. *P. miso*, *C. guilliermondii*, *C. tropicalis*, *C. mogii*, *C. maltosa*, and *H. polymorpha* were reported on xylitol production in the range of 0.50–0.65 g/g[44–48]. Guo et al[49] reported that *C. guilliermondii* Xu280 and *C. maltosa* Xu316 produced xylitol 0.73 g/g and 0.70 g/g, respectively, when they were grown in YPX medium supplemented with 50 g/l xylose. Sreenivas et al[50] reported that YS54 was the best xylitol producer (0.58 g/g) among 35 yeasts isolated from gut of coleoptera insects. *C. tropicalis* produced xylitol by cell-recycle fermentation in a cross flow membrane bioreactor[23,38]. Xylitol yields of 0.82 g/g xylose[38] and 0.85 g/g xylose[23] were reported.

Cloning of *C. tropicalis* A26 xylose reductase gene

Several yeasts such as *Candida*, *Pachysolen* and *Debaryomyces* reduced xylose to xylitol by NAD(P)H dependent xylose reductase (XR)[46,51,52]. The xylose reductase is encoded from the xylose reductase gene (*xyl*). Xylose reductase gene (*xy1*) of *C. tropicalis* A26 was amplified from genomic DNA by gradient PCR using designed primers; XR-F and XR-R. Desired amplicon of 540 bp was obtained at annealing temperature range of 45–55 °C. The obtained PCR product was then purified and ligated to pTZ57R/T vector. After transformation into *E. coil* DH5α

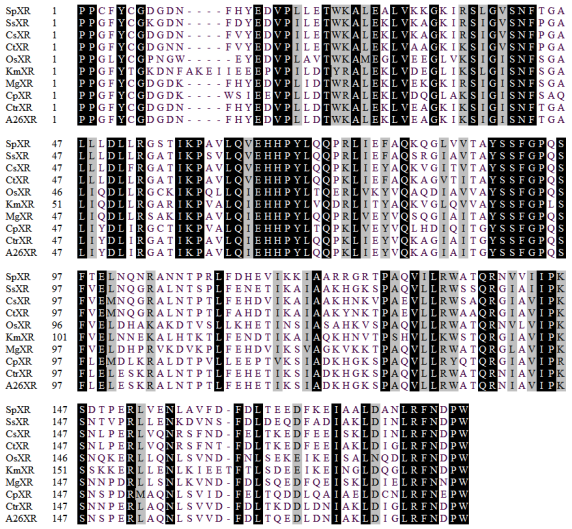

Fig. 2 Alignment of partial amino acid sequences of xylose reductase from *C. tropicalis* A26 and other yeast strains. A26XR, *C. tropicalis* A26; SpXR, *Spathaspora passalidarum*, (EGW32258); SsXR, *Scheffersomyces stipitis* (CAA42072); CsXR, *C. shehatae* (ABK35120); CtXR, *C. tenuis* (AAC25601); OsXR, *Ogataea siamensis* (ACN78427); KmXR, *Kluyveromyces marxianus* (ADV91498); MgXR from *Meyerozyma guilliermondii* (AAD09330); CpXR, *C. parapsilosis* (AAO91803); CtrXR, *C. tropicalis* (ABX60132); The underlined conserved sequences were used to design degenerated primers for the *xyl* cloning. Rectangle box indicates NAD(P)H coenzyme use[43,45,47].

Table 4 Comparison of amino acid sequence similarity and identity between *C. tropicalis* A26 xylose reductase and those of others.

Code	CtrXR	SpXR	SsXR	CsXR	CtXR	OsXR	KmXR	MgXR	CpXR	A26XR
CtrXR		72.3	74.3	77.5	78.5	64.4	59.2	74.9	75.4	98.4
SpXR	83.8		75.4	74.9	74.3	59.2	55.5	67.5	56.5	72.8
SsXR	82.7	83.8		81.2	79.1	58.6	60.2	69.6	60.7	74.4
CsXR	86.4	84.8	87.4		91.1	57.6	61.3	70.7	63.9	77.5
CtXR	86.9	82.2	86.9	94.8		56.5	61.3	69.1	61.8	78.5
OsXR	73.3	71.2	69.6	68.6	69.1		59.2	66.5	59.7	64.4
KmXR	71.7	70.7	72.3	71.2	71.7	70.7		59.2	54.5	59.7
MgXR	83.8	80.6	80.6	81.2	80.1	74.3	70.2		66.0	75.4
CpXR	82.7	73.8	73.8	74.9	73.8	69.1	67.5	75.4		75.9
A26XR	99.0	84.3	84.2	85.2	86.4	72.8	71.7	83.8	83.8	

Numbers on the lower-left half are Similarity (%). Numbers on the upper-right half are Identity (%). Similarity Codes are the same as in Fig. 2. Identity means the same amino acid; Similarity means amino acid in the same groups.

host, the putative positive clones were determined by colony PCR. Nucleotide sequence of an insert DNA extracted from a putative positive clone was analysed. Partial sequence of *xyl* clone which consisted of 558 nucleotides coding for 186 amino acids is shown in Fig. 2. Comparison the obtained partial amino acid sequences of *C. tropicalis* A26 to those of other yeast strains in Gene Bank database revealed that *C. tropicalis* A26 xylose reductase possessed 98.4%, 78.5%, and 77.5% identity and 99.0%, 86.4%, and 85.9% similarity to xylose reductase of CtrXR of *C. tropicalis* (ABX60132C), CtXR of *C. tenuis* (AAC25601), and CsXR of *C. shehatae* (ABK35120), respectively (Table 4). Amino acid sequence alignment of the partial xylose reductase revealed tetra amino acid motif (Ile-Pro-Lys-Ser), an NAD(P)H species motif[14,17,53] (Fig. 2). The motif was well-conserved among xylose reductase of several yeast strains except for CpXR of *C. parapsilosis* which Lys residue was substituted with Arg and resulting in preferentially used NADH as coenzyme[54]. The CtrXR that showed the highest sequence homology to those of A26XR was previously reported as NADPH-specific XR[14,17]. This result suggested that xylose reductase of *C. tropicalis* A26 might specify for an NADPH coenzyme, however, the expression of the *xyl* clone including the characterization of xylose reductase expressed is required.

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