การผลิตไบโอเอทานอลและไซลิทอลของยีสต์หมักไซโลสที่คัดเลือกได้

นางสาววัลลภา หล่อเหลี่ยม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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BIOETHANOL AND XYLITOL PRODUCTION OF SELECTED XYLOSE
FERMENTING YEAST

Miss Wanlapa Lorliam

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ยีสต์ที่หมักไซโลสได้จำนวน 129 ไอโซเลท คัดแยกได้จากมูลสัตว์กินพืชในประเทศไทยจำนวน 60 ตัวอย่าง
และจากดินในประเทศญี่ปุ่นจำนวน 3 ตัวอย่าง จากการศึกษาลักษณะทางฟีโนไทป์และจีโนไทป์โดยการวิเคราะห์
ลำดับเบสนิวคลิโอไทด์บริเวณ D1/D2 ของ 26S rDNA สามารถพิสูจน์เอกลักษณ์ยีสต์ จำนวน 93 ไอโซเลทได้เป็น
แอสโคไมซีตัสยีสต์ ในสกุล *Candida* 62 ไอโซเลท, *Sporopachydermia* 9 ไอโซเลท, *Kluyveromyces* 3 ไอโซเลท,
Meyerozyma 3 ไอโซเลท, *Zygoascus* 1 ไอโซเลท, *Barnettozyma* 3 ไอโซเลท, *Pichia* 4 ไอโซเลท, *Issatchenkia*
2 ไอโซเลท *Geotrichum* 14 ไอโซเลท, *Cyberlindnera* 2 ไอโซเลท และพิสูจน์เอกลักษณ์ยีสต์จำนวน 26 ไอโซเลทได้
เป็นแบสิดิโอไมซีตัสยีสต์ในสกุล *Trichosporon* ในการศึกษานี้พบยีสต์สปีชีส์ใหม่ได้แก่ *Candida* sp. ELP19 และ
Geotrichum sp. จำนวน 14 ไอโซเลท โดยอาศัยผลของลำดับเบสนิวคลิโอไทด์บริเวณ D1/D2 ของ 26S rDNA ซึ่งมี
ความเหมือนกับสายพันธุ์มาตรฐานเพียง 94-97 เปอร์เซนต์ และมีผลของฟีโนไทป์ที่แตกต่างไป

จากการศึกษาความสามารถในการหมักไซโลสที่ระยะเวลา 48 ชั่วโมง พบว่ายีสต์จำนวน 117 ไอโซเลท
สามารถหมัก 4% ไซโลสเป็นเอทานอลได้ โดยมีปริมาณเอทานอลอยู่ในช่วง 0.01-2.388 กรัมต่อลิตร และ ยีสต์จำนวน
95 ไอโซเลท สามารถหมักไซโลส (4%) ได้เป็นไซลิทอล 0.03-29.47 กรัมต่อลิตร และพบว่า *Zygoascus meyerae* E23
สามารถผลิตเอทานอลได้สูงถึง 3.61 กรัมต่อลิตร ที่ระยะเวลา 72 ชั่วโมง ส่วน *Candida tropicalis* A26 สามารถผลิต
ไซลิทอลได้สูง 21.30 กรัมต่อลิตร เท่ากับ 0.53 กรัมของไซลิทอลต่อกรัมของไซโลส ภายหลังการหมักที่ 24 ชั่วโมง
ดังนั้นจึงคัดเลือก *C. tropicalis* A26 เพื่อศึกษาสภาวะการหมักที่เหมาะสมต่อไป โดยพบว่าสามารถผลิตไซลิทอลได้สูง
ถึง 42.517 กรัมต่อลิตร เมื่อหมักในอาหารที่มีส่วนผสมของไซโลส 80 กรัม และเปบโตน 40 กรัม พบว่าใกล้เคียงกับ
ค่าที่ได้จากการออกแบบทางสถิติ ที่สามารถประมาณค่าไซลิทอลได้ 45.83 กรัมต่อลิตร และจากการนำสภาวะที่
เหมาะสมเพื่อใช้หมักในถังหมัก 5 ลิตร พบว่าสามารถผลิตไซลิทอลได้ 71.59 กรัมต่อลิตร คิดเป็นค่า yield เท่ากับ 0.89
กรัมต่อกรัมไซโลส ซึ่งแสดงว่า *C. tropicalis* A26 มีประสิทธิภาพในการหมักไซโลสได้ดีมาก และจากการศึกษายีน
ไซโลสรีดักเทสของ *Z. meyerae* E23 และ *C. tropicalis* A26 พบว่าลำดับเบสของกรดอะมิโนของไซโลสรีดักเทสของ
Z. meyerae E23 มี % identity 58.96 และ % similarity 73.88 เปรียบเทียบกับลำดับเบสของยีนไซโลสรีดักเทสของ
Ogataea siamensis (ACN78427) ส่วน *C. tropicalis* A26 นี้มี % identity 98.42 และ % similarity 98.95 กับยีนไซโลส
รีดักเทสของ *C. tropicalis* (ABX60132C) จากลำดับเบสของยีนทั้ง 2 จะพบบริเวณโมทีฟแสดงให้เห็นว่า เอนไซม์นี้
เป็นประเภท NADPH-dependent xylose reductase.

สาขาวิชา   เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ   ลายมือชื่อนิสิต_____
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KEYWORDS: XYLOSE FERMENTING YEAST / ETHANOL / XYLITOL / XYLOSE REDUCTASE.

WANLAPA LORLIAM: BIOETHANOL AND XYLITOL PRODUCTION OF SELECTED XYLOSE FERMENTING YEAST. ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., CO-ADVISOR: ASSOC. PROF. ANCHARIDA AKARACHARANYA, D. Eng., 160 pp.

One hundred and twenty-nine xylose-utilization yeasts were isolated from 60 herbivore animal fecal samples in Thailand and 3 soils sample in Japan. These yeast isolates were divided into ten groups based on their phenotype and genotype of D1/D2 region of large-subunit ribosomal RNA gene. Ninety-three ascomycetous yeasts were identified as *Candida* (62 isolates), *Sporopachydermia* (9 isolates), *Kluyveromyces* (3 isolates), *Meyerozyma* (3 isolates), *Zygoascus* (1 isolate), *Barnettozyma* (3 isolates), *Pichia* (4 isolates), *Issatchenkia* (2 isolates), *Geotrichum* (14 isolates) and as *Cyberlindnera* (2 isolates). Twenty-six basidiomycetous yeasts isolates were identified as *Trichosporon*. *Candida* sp. ELP19 and *Geotrichum* sp. (14 isolates) were identified as the novel specie based on their differential phenotype and D1/D2 region of large subunit ribosomal RNA gene showing the similarity 94 to 97%.

In the xylose fermentation, 117 isolates could ferment xylose (4%) to ethanol (0.01-2.388 g/l after 24 h.) while 95 isolates could ferment xylose to xylitol (0.03-29.47 g/l after 24 h). *Zygoascus meyerae* E23 produced the highest ethanol (3.61 g/l after 72h). *C. tropicalis* A26 produced the highest xylitol (21.30 g/l) which corresponded to 0.53 g xylitol/g xylose after 24 h. Thus, *C. tropicalis* A26 was selected to optimize for xylitol production. This strain produced highest xylitol production in shake flask with 42.517g/l when xylose and peptone were 80 g/l and 40 g/l, respectively. Statistical regression model predicted the maximum xylitol production at 45.83 g/l. In 5L stirred fermenter under the optimized condition, the highest xylitol production and xylitol yield were 71.59 g/l and 0.89 g/g xylose, respectively. Then characterization of xylose reductase gene (*xyl1*) of both strains E23 and A26 was carried out. Deduced amino acids sequence of the cloned xylose reductase gene of *Z. meyerae* E23 showed 58.96% identity and 73.88% similarity to xylose reductase of *Ogataea siamensis* (ACN78427) while *C. tropicalis* A26 xylose reductase gene showed 98.42% identity and 98.95% similarity to *C. tropicalis* (ABX60132C). Moreover, deduce amino acids of both strains showed tetra-amino acid motif (Ile-Pro-Lys-Ser) which is conserved among NADPH-dependent xylose reductase.

Field of Study: <u>Pharmaceutical Chemistry</u>   Student's Signature _____

<u>and Natural Products</u>   Advisor's Signature _____

Academic Year: <u>2012</u> _____   Co-advisor's Signature _____

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LIST OF ABBREVATIONS

α	=	Alpha
cm	=	Centimeter
CCD	=	Central composite design
°C	=	Degree Celsius
Df	=	Degree of freedom
DDBJ	=	DNA Data Bank of Japan
dNTP	=	Deoxyribonucleotide triphosphate
DNA	=	Deoxyribonucleic acid
rDNA	=	Ribosomal deoxynucleic acid
EDTA	=	Disodiumethylenediaminetetraacetate
EMBL	=	European Molecular Biology Laboratory
Fe^{2+}	=	Iron ion
g	=	Gram
g/l	=	Gram per liter
μg	=	Microgram
mg	=	Milligram
μg	=	microgram
g	=	Gravity
G+C	=	Guanine-plus-cytosine
GenBank	=	National Institute of Health genetic sequence database
h	=	Hour
HCl	=	Hydrochloric acid
l	=	latant
μl	=	microliter
ml	=	milliliter
μm	=	micrometer
mm	=	millimeter
min	=	Minute
MS	=	mean square
MW	=	Molecular weight

nt	=	nucleotide
Na^+	=	Sodium ion
NaCl	=	Sodium chloride
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NADH	=	Nicotinamide adenine dinucleotide
NaOH	=	Sodium hydroxide
NBRC	=	NITE Biological Resource Center
nm	=	Nanometer
ng	=	Nanogram
nov.	=	Novel
nd	=	not determine
OD	=	Optical density
%	=	Percent
PBD	=	Placket-Burman design
PCR	=	Polymerase chain reaction
ppm	=	part per million
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
s	=	slow
sec	=	Second
sp.	=	Species
SS	=	sum of square
TAE	=	Tris-acetate EDTA
TBE	=	Tris-borate EDTA
Tm	=	Melting temperature
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
v	=	Variable
v/v	=	volume / volume
w/v	=	weight / volume
w	=	Weak
XR	=	Xylose reductase

XDH	=	Xylitol dehydrogenase
*xyl*1	=	xylose reductase gene
Ye/s	=	ethanol yield
Yx/s	=	xylitol yield
YP	=	Yeast peptone medium
YPD	=	Yeast peptone glucose medium
YPX	=	Yeast peptone xylose medium
YM	=	Yeast extract-malt extract medium
YX	=	Yeast nitrogen base xylose medium

CHAPTER I

INTRODUCTION

Lignocellulose is an interesting non-food fermentable sugar resource for industrial production of ethanol, which it is a world most abundant biomass, low price and environmental benefits (Roberto *et al*., 1990). Lignocellulosic consists of 30-40% (w/w) cellulose and 15-25% (w/w) hemicellulose (Lee, 1997; Jeffries and Alexander, 1990). Complete hydrolysis of the cellulose and the hemicellulose results in glucose and xylose, respectively which several microorganisms can ferment to ethanol (Dien *et al*., 2003). *Saccharomyces cerevisiae* is the most popular yeast for fermenting glucose to ethanol due to its high ethanol production yield and comparatively high ethanol tolerance (Byron and Hung, 2007; Kutzman and Robnett, 1998), however *S. cerevisiae* strains can not ferment xylose to ethanol.

Several yeasts such as *Candida shehatae*, *Pachysolen tannophilus*, *Brettanomyces naardenensis*, *C. tenui*s, *Picihia segobiensis, C. lyxosophila*, *C. intermedia*, *C. jeffriesii*, *Spathaspora passalidarum*, *Spathaspora arboraria, C. prachuapepsis* and *Scheffersomyces stipitis* have been reported as xylose fermenting yeasts (Barnett *et al*., 2000; Nitiyon *et al*., 2011; Cadete *et al*., 2009; Jefferies and Kurtzman,1994; Nguyen *et al*., 2010). *S. stipitis* strain produces significant amounts of ethanol from xylose and it has been studied extensively (Jefferies and Kurtzman, 1994).

To increase an ethanol production yield from lignocellulose, both glucose and xylose liberated should be fermented to ethanol. Though *S. stipitis* strain can ferment both glucose and xylose to ethanol. It's ethanol yield from glucose and ethanol tolerance are much lower than those of *S. cerevisiae* (Jefferies and Kurtzman, 1994; Jeffries, 2006). Co-cultivating of *S. cerevisiae* and *S. stipitis* strains to co-ferment glucose and xylose is not satisfactory, due to their difference in fermenting condition and ethanol tolerance (Taniguchi *et al*., 1997; Agbogbo *et al*., 2006). *S. stipitis* strain prefers to ferment glucose more than xylose while it has lower ethanol tolerance than *S. cerevisiae* strain (Watanabe *et al*., 2007). So xylose was not fermented. Therefore, there has been an attempt to construct recombinant *S. cerevisiae* strain capable of

fermenting xylose by overexpressing of genes encoding enzymes in xylose fermentation pathway of *S. stipitis* strain (Byron and Hung, 2007; Jefferies and Jin, 2004; Watanabe *et al*., 2007). 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 co-factor (Verduyn *et al*., 1985; Zhan *et al*., 2011). 2) xylitol dehydrogenase (EC.1.1.1.9), which converts xylitol to xylulose using NAD as cofactor (Rizzi *et al*., 1989). 3) xylulose kinase (EC 2.7.1.17), which converts xylulose to xylulose-5-phosphate (Jeppsson *et al*., 1996). Because under oxygen-limit condition which *S. cerevisiae* ferments glucose to ethanol, NAD becomes its limiting factor (Jefferies and Jin, 2004). So ethanol produced 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 for construction of recombinant *S. cerevisiae* capable of fermenting xylose (Bengtsson *et al*., 2009; . Van Vleet and Jeffries, 2009; Watanabe *et al*., 2007).

Furthermore, xylitol is side product from xylose metabolic pathway to ethanol production, which is an attractive sugar substitute due to its high sweetening power and unique pharmacological properties. Xylitol is produced by chemical reduction of D-xylose in a presence of nickel catalyst at high temperature and pressure. By this method, separation and product recovery is expensive (Chen, 2010; Sampaio *et al*., 2006). Microbial xylitol production is an alternative process which is produced from renewable resource of agricultural residues such as hemicelluloses. Bacteria, filamentous fungi and yeasts were known to produce xylitol. Various yeast species such as *C. boidinii, C. guilliermindii, C. shehatae, C. parasilosis, C. peltata, C. mogii, C. maltosa C. tropicalis, Pichia stiptis, Pachysolen tannophilus, Debaryomyces hansenii* and *D. nepatensis* were reported as high xylitol producers (Sampaio *et al*., 2006; Furlan *et al*, 2001; Silva *et al*., 2006; Sirisansaneeyakul *et al*., 1995; Guo *et al*., 2006; Sreenivas Rao *et al*, 2007; Kumdam *et al*., 2012). However, the xylitol production yields reported required improvement through an optimization for economically viable.

Conventional optimization procedure, one variable at a time, is effective in some situation but fails to consider the combined effects of the entire factors involved. In addition, it is time consuming and can not provide an interaction of parameter on

the desired outcome (Sreenivas Rao, 2004). Statistical optimization procedure has an advantage over the conventional procedure for rapidity, reliability, and understanding of interaction among parameters at various concentrations (Ayse *et al*., 2007). Plackett-Burman design is statistics design, which was two levels of high and low variables to identify the critical parameter in fermentation process (Naveena *et al*., 2005). Central composite design (CCD) is one of the popular mathematical and statistical methodology, response surface methodology (RSM) used for optimization process (Anderson and Whitcomb, 2005).

This work deals with the isolation, screening and identification of the xylose-fermenting yeasts based on their phenotypic and chemotaxonomic characteristics including DNA sequencing analysis. Screening of ethanol and xylitol production, and optimization of xylitol production by selected xylose-fermenting isolate will be performed according to experimental design using the response surface method in shake flasks and upscale in 5L fermentor. The characterization of *xyl1* gene encoding xylose reductase will be also investigated.

OBJECTIVE

1. To isolate, screen xylose-fermenting yeasts and to identify based on the phenotypic, chemotaxonomic and genotypic characteristics.
2. To screen ethanol and xylitol production
3. To optimize xylitol production from xylose by selected isolate.
4. To characterize xylose reductase encoding gene (*xyl1*).

CHAPTER II

LITERATURE REVIEW

2.1 Yeast taxonomy

Yeasts are eukaryotic unicellular microfungi that are widely distributed in the natural environments. The ascomycetous and basidiomycetous fungi reproduce vegetative by budding or fission, with or without pseudohyphae and hyphae, and forming sexual states that are not enclosed in fruiting bodies (Boekhout and Kuetzman, 1990). Some yeasts may reproduce sexually, resulting in an alternation of generation with the formation of characteristic cells in which reduction division (meiosis) take place. In ascomycetous yeast, the cell is ascus, in which ascospores are formed. In basidiomycetous yeasts the site of meiosis is called a basidium, on which basidiospores are exogenously formed.

Basidiomycetous yeasts can be differentiated from the ascomycetous yeasts based on urease test which is positive in the former group (Choudhary and Johri, 2009). The dominated polysaccharide composition in Basidiomycete yeast was chitin and in Ascomycete yeast was β-glucan (Suh *et al.,* 2006). The differential characteristics of Basidiomycete yeast and Ascomycete yeast was summarized by Suh *et al* (2006) as in Table 2.1.

Asexual reproduction yeasts are referred to as imperfect, mitosporic or anamorphic yeasts (e.g. *Cryptococcus neoformans*, *Candida utilis*), and sexually reproducing yeasts are called perfect, meiosporic or teleomorphic yeasts (e.g. *Filobasidiella neoformans* and *Pichia jadinii*).

Table 2.1 Differential characteristics of Basidiomycete yeast and Ascomycete yeast.

Characteristic	Ascomycete yeast	Basidiomycete yeast
Spore forming	Ascospore	Basidiospore
Cell wall polysaccharide	β-glucan	Chitin
Nuclear DNA G+C content	Higher than 50%	Lower than 50%
Bud formation	Holoblastic	Enteroblastic
Diazonium Blue B reaction	negative	positive
Urease test	negative	positive

2.1.1 Classification

The Yeasts, a Taxonomic Study 4th edition (Kurtzman and Fell, 1998) was published in 1998. This book composed of 689 species in 94 genera and the yeasts were classified into the Phylum Ascomycota and Phylum Basidiomycota. The ascomycetous yeasts (Phylum ascomycota) are divided into 3 classes, Archiascomycetes, Euascomycetes and Hemiascomycetes. The basidiomycetous yeast (Phylum Basidiomycota) are divided into 3 classes, Hymenomycetes, Urediniomycetes and Ustilaginomycetes.

In the present edition, the Yeasts, a Taxonomic Study 5th edition (Kurtzman *et al*., 2011a) was published in 2011. There are 149 genera and nearly 1500 yeast species. The application of gene sequence analysis is largely responsible for the increasing in the number of taxa presented in this edition. Ascomycetes are mainly classified into the Saccharomycetes and Schizosaccharomycetes, while Basidiomycetes are divided into 3 classes, Hymenomycetes, Urediniomycetes and Ustilaginomycetes.

The classification of ascomycetous yeasts and basidiomycetous yeasts are shown in Table 2.2 and Figure 2.1, respectively.

Table 2.2 Class, order and families of yeast and yeast-like genera of Ascomycota.

Neolectomycetes
      Neolectales Landvik, O.E. Eriksson, Gargas & P. Gustafsson
            Neolectaceae Redhead
                Neolecta Spegazzini (T)
Pneumocystidomycetes
      Pneumocystidales O.E. Eriksson
            Pneumocystidaceae O.E. Eriksson
                Pneumocystis P. Delanöe & Delanöe (T)
Schizosaccharomycetes
      Schizosaccharomycetales Prillinger, Dörfler, Laaser, Eckerlein & Lehle ex Kurtzman
            Schizosaccharomycetaceae Beijerinck ex Klöcker
                Schizosaccharomyces Lindner (T)
Taphrinomycetes
      Taphrinales Gäumann & C.W. Dodge
            Protomycetaceae Gray
                Burenia M.S. Reddy & C.L. Kramer (T)
                Protomyces Unger (T)
                Protomycopsis Magnus (T)
                Saitoella S. Goto, Sugiyama, Hamamoto & Komagata (A)
                Taphridium Lagerheim & Juel ex Juel (T)
                Volkartia Maire (T)
            Taphrinaceae Gäumann & C.W. Dodge
                Lalaria R.T. Moore (A)
                Taphrina Fries (T)
Saccharomycetes
      Saccharomycetales Kudryavtsev
            Ascoideaceae J. Schröter
                Ascoidea Brefeld & Lindau (T)
            Cephaloascaceae L.R. Batra
                Cephaloascus Hanawa (T)
            Debaryomycetaceae Kurtzman & M. Suzuki
                Debaryomyces Lodder & Kreger-van Rij (T)
                Kurtzmaniella Lachance & Starmer (T)
                Lodderomyces van der Walt (T)
                Meyerozyma Kurtzman & M. Suzuki (T)
                Millerozyma Kurtzman & M. Suzuki (T)
                Priceomyces M. Suzuki & Kurtzman (T)
                Scheffersomyces Kurtzman & M. Suzuki (T)
                Schwanniomyces Klöcker emend. M. Suzuki & Kurtzman (T)
                Spathaspora Nguyen, S.-O. Suh & M. Blackwell (T)
                Wickerhamia Soneda (T)
                Yamadazyma Billon-Grand (T)
            Dipodascaceae Engler & E. Gilg
                Dipodascus Lagerheim (T)
                Galactomyces Redhead & Malloch (T)
                Geotrichum Link:Fries (A)
            Endomycetaceae J. Schröter
                Endomyces Reess (T)
                Helicogonium W.L. White (T)
                Phialoascus Redhead & Malloch (T)

Table 2.2 (Continued).

Lipomycetaceae E.K. Novak & Zsolt
    Lipomyces Lodder & Kreger van Rij (T)
    Myxozyma van der Walt, Weijman & von Arx (A)
Metschnikowiaceae T. Kamienski
    Aciculoconidium King & Jong (A)
    Clavispora Rodrigues de Miranda (T)
    Hyphopichia von Arx & van der Walt (T)
    Kodamaea Y. Yamada, T. Suzuki, Matsuda & Mikata emend.
            Rosa, Lachance, Starmer, Barker, Bowles & Schlag-Edler (T)
    Metschnikowia T. Kamienski (T)
Phaffomycetaceae Y. Yamada, Kawasaki, Nagatsuka, Mikata & Seki
    Komagataella Y. Yamada, Matsuda, Maeda & Mikata (T)
    Phaffomyces Y. Yamada, Higashi, S. Ando & Mikata (T)
Pichiaceae Zender
    Brettanomyces Kufferath & van Laer (A)
    Dekkera van der Walt (T)
    Kregervanrija Kurtzman (T)
    Pichia E.C. Hansen (T)
    Saturnispora Liu & Kurtzman (T)
Saccharomycetaceae G. Winter
    Cyniclomyces van der Walt & D.B. Scott (T)
    Eremothecium Borzi emend. Kurtzman (T)
    Kazachstania Zubkova (T)
    Kluyveromyces Kurtzman, Lachance, Nguyen & Prillinger (T)
    Lachancea Kurtzman (T)
    Nakaseomyces Kurtzman (T)
    Saccharomycetaceae G. Winter
    Naumovia Kurtzman (T)
    Saccharomyces Meyen ex Reess (T)
    Tetrapisispora Ueda-Nishimura & Mikata (T)
    Torulaspora Lindner (T)
    Vanderwaltozyma Kurtzman (T)
    Zygosaccharomyces Barker (T)
    Zygotorulaspora Kurtzman (T)
Saccharomycodaceae Kudryavtsev
    Hanseniaspora Zikes (T)
    Kloeckera Janke (A)
    Saccharomycodes Hansen (T)
Saccharomycopsidaceae von Arx & van der Walt
    Saccharomycopsis Schiönning (T)
Trichomonascaceae Kurtzman & Robnett
    Blastobotrys von Klopotek (A)
    Sugiyamaella Kurtzman & Robnett (T)
    Trichomonascus H.S. Jackson emend. Kurtzman & Robnett (T)
    Wickerhamiella van der Walt (T)
    Zygoascus M.Th. Smith (T)

Table 2.2 (Continued).

Wickerhamomycetaceae Kurtzman, Robnett & Basehoar-Powers
  Barnettozyma Kurtzman, Robnett & Basehoar-Powers (T)
  Lindnera Kurtzman, Robnett & Basehoar-Powers (T)
  Starmera Y. Yamada, Higashi, S. Ando & Mikata (T)
  Wickerhamomyces Kurtzman, Robnett & Basehoar-Powers (T)

Saccharomycetales incertae sedis
  Ambrosiozyma van der Walt (T)
  Ascobotryozyma J. Kerrigan, M.Th. Smith & J.D. Rogers (T)
  Babjeviella Kurtzman & M. Suzuki (T)
  Blastobotrys von Klopotek (A)
  Candida Berkhout (A)
  Citeromyces Santa María (T)
  Coccidiascus Chatton emend. Lushbaugh, Rowton & McGhee (T)
  Kuraishia Y. Yamada, Maeda & Mikata (T)
  Macrorhabdus Tomaszewski, Logan, Snowden, Kurtzman & Phalen (A)
  Magnusiomyces Zender (T)
  Nadsonia Sydow (T)
  Nakazawaea Y. Yamada, Maeda & Mikata (T)
  Ogataea Y. Yamada, Maeda & Mikata (T)
  Pachysolen Boidin & Adzet (T)
  Peterozyma Kurtzman & Robnett (T)
  Saprochaete Coker & Shanor ex D.T.S. Wagner & Dawes (A)
  Schizoblastosporion Ciferri (A)
  Sporopachydermia Rodrigues de Miranda (T)
  Trigonopsis Schachner (A)
  Wickerhamia Soneda (T)
  Yarrowia van der Walt & von Arx (T)

(A), Anamorphic genus; (T), Telemorphic genus.
Anamorphic and teleomorphic genera are placed together in the same family when relationships areknown. For many anamorphic and teleomorphic genera, phylogenetic relationships are unclear and the genera are placed in *Saccharomycetales* incertae sedis until family relationships become known.
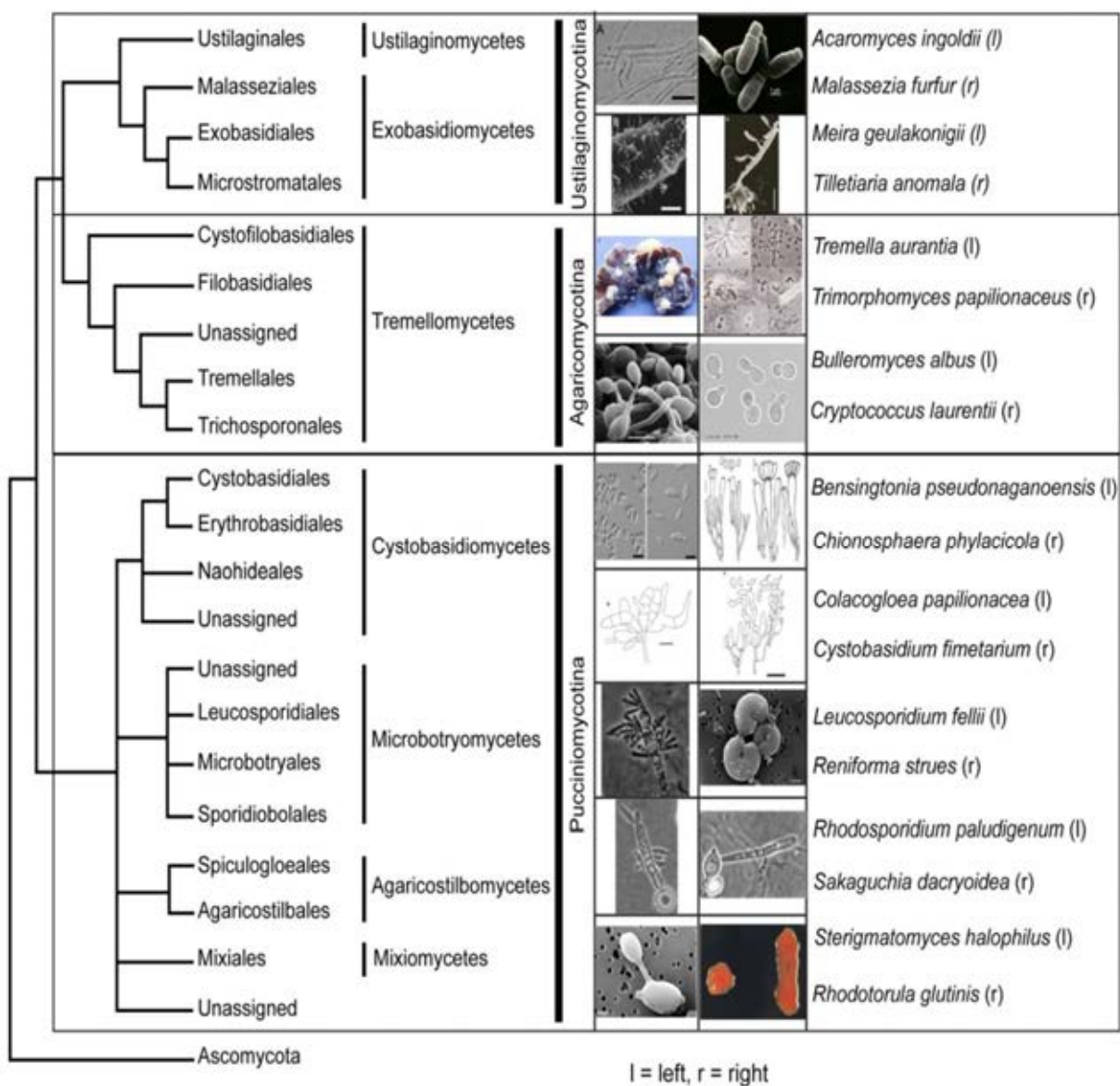
Figure 2.1 Phylogenetic relationships between three subphyla of the Basidiomycota that contained yeast states. r, right picture; l, left picture.

2.1.2 Identification

The first classification of yeasts at the lower taxonomic levels were based on the presence or absence of sexual state, cell division, hyphae and pseudohyphae, fermentation simple sugar and growth on various carbon and nitrogen compounds. The transition from phenotypic identification of yeast to molecular identification began with the determination of mol (%) G+C ratio of nuclear DNA (Suh *et al*., 2006). The identification method was improved to accuracy and rapid (Querol *et al*., 2003).

The criteria used for yeast identification are based on the conventional phenotypic (Yarrow, 1998), chemotaxonomic (Phaff, 1998; Yamazaki *et al*., 1998) and molecular taxonomic characteristics (Kurtzman, 1998a; Kurtzman and Blanz, 1998; Kurtzman *et al*., 2011a) as in Table 2.3.

Table 2.3 Criteria used in yeast classification and identification (Walker, 1998).

Morphological characteristics	Physiological characteristics	Immunological characteristics	Molecular characteristics
Giant colony	Fermentation of	Serology:	rRNA and rDNA
Morphology cell	sole C sources	agglutination	phylogeny
Morphology in liquid media	Assimilation of	Immunoelectro-	RFLP of mitDNA
Mode of vegetative and/or	sole C sources	phresis	DNA base composition
sexual reproduction	Assimilation of	Immunofluore-	(mol % G + C)
Spore characteristics	sole N sources	scence microscopy	Karyotpe analysis
Presence/absence of hyphae	Pigment		DNA hybridization
or pseudohyphae	production		Random amplification
Pellicle formation at liquid	Acid production		of Polymorphic DNA
surfaces	Osmophilia		(RAPD)
Flocculation in liquid media			

2.1.2.1 Phenotypic characteristics

Identification of yeast genera can often be achieved by the morphology supplemented with a few physiology tests (Walker, 1998). Morphological and physiological tests still need to support identification although molecular technique (DNA associated, gene sequencing) was normally determined now. Some laboratories have no sequencing facilities, so the identification was depended on the morphological and physiological tests. Moreover, new species descriptions, morphological and physiological properties should also be reported as such information describes the general biological properties of the species. Metabolic properties may help to understand the ecology rely on physiological data to understand ecological interactions and to selectively isolate species groups; and biotechnologists utilize the data to predict which species or strains may have novel or improved applications (Kurtzman *et al*., 2011b).

Morphology Characteristics

Study of morphological characteristics are included feature of colonies (e.g. suface texture or colour), vegetative reproduction, and characteristics of sexual reproduction such as ascospore formation and basidiospore formation (Walker, 1998). For example, *Saccharomyces cerevisiae*, cells divide by multilateral budding, *Saccharomycodes ludwigii* by bipolar budding and *Schizosaccharomyces pombe* by fission (Figure 2.2 (2-4)).

Pseudohyphae or true hyphae formations the criterion for the separation of many species, *Candida* (pseudohyphae present) is separated from *Turulopsis* (pseudohyphae absent). Yarrow and Meyer (1978) transferred *Torulopsis* species to *Candida*, the genus of taxonomic priority. The present of true hyphae and the structure of hyphal septa have been considerd as taxonomic characters. For example, Ambrosiozyma is unique among of the Saccharomycetales because most species form dolipore-like, exceptions are found in *A. ambrosiae* and *A. angophora* (Smith and de Hoog, 1998; Kurtzman, 2011e).

The shape of ascospore has been considered to be a defining character for many genera. The genus *Metschnikowia* are all characterized by needle-shaped ascospores and all species of *Eremothecium* have elongated ascospore (Figure 2.2 (5)) (Kurtzman, 2011e). In *Pachysolen tannophilus*, ascus forms on the tip of an elongated refractile tube. The ascus wall becomes deliquescent and releases four hat-shaped ascospores (Figure 2.2 (6)). *Lodderomyces elongisporus* forms persistent ascus with a single ellipsoidal ascospore. This is the only species of the clade, which includes *Candida albicans* and *C. tropicalis*, that is known to form ascospores (Figure 2.2 (7)). Asci with 1–2 spherical ascospores and form an elongated extension that may function as a bud conjugant of *Torulaspora delbrueckii* (Figure 2.2 (8)). Asci with spherical ascospores and often conjugant with ascus then giving rise to the term ''dumbbell-shaped'' asci. This species is one of the most aggressive food spoilage yeasts known of *Zygosaccharomyces bailii* (Figure 2.2 (9)). For *Pichia bispora* is hat-shaped ascospore and released from the asci at maturity (Figure 2.2 (10)). Hat-shaped ascospores are produced by species in a variety of different genera.

Physiology and biochemical characteristics

Physiological and biochemical characteristics are based on the fermentation of carbohydrates, assimilation of carbon compounds, assimilation of nitrogen compounds, growth in vitamin-free medium and vitamin requirements, growth in media of high osmotic pressure, growth at 37°C and at other temperatures, acid formation from glucose, formation of extracellular amyloid compounds (starch formation), hydrolysis of urea and cycloheximide resistance (Kurtzman *et al*., 2011b).
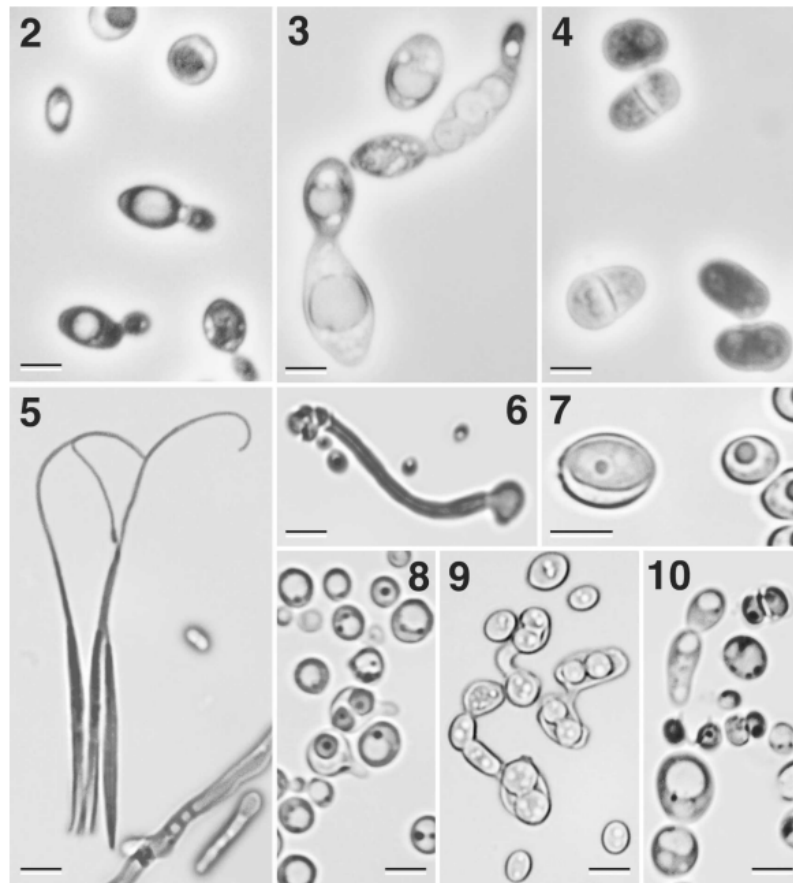
Figure 2.2 Ascomycete yeasts in pure culture (2) *Saccharomyces cerevisiae*. Cells dividing by multilateral budding (3) *Saccharomycodes ludwigii*. Cell division by bipolar budding (4) *Schizosaccharomyces pombe*. Cell division by fission (5) *Eremothecium* (*Nematospora*) *coryli*. Free, needle-shaped ascospores with whip-like tails of extended wall material (6) *Pachysolen tannophilus*. A single ascus forms on the tip of an elongated refractile tube (7). *Lodderomyces elongisporus*. Persistent ascus with a single ellipsoidal ascospore (8) *Torulaspora delbrueckii*. Asci with 1–2 spherical ascospores. Asci often form an elongated extension that may function as a bud conjugant(9) *Zygosaccharomyces bailii*. Asci with spherical ascospores and form ''dumbbell-shaped'' asci. (10) *Pichia bispora*. Ascospores are hat-shaped.

For example, *Kluyveromyces nonfermentans* was absence fermentation of sugar but *K. aestuarii* was strong fermentation of several sugar (Lachance, 2011). So some biochemical characteristics were separated yeasts in speciec level. The *Scheffersomyces* clade most species ferment D-xylose such as *Scheffersomyces stipitis* and *Candida shehatae* (Kurtzman, 2011e)

Growth on *myo*-inositol is a property that correlates fairly well with certain basidiomycete genera, but relatively few ascomycetous yeasts utilize this compound. Taxa that do utilize *myo*-inositol include some species of *Ascoidea, Babjeviella, Blastobotrys, Candida, Dipodascopsis, Lipomyces, Myxozyma, Saccharomycopsis* and *Zygoascus.* However, there are not all species of these genera utilize *myo*-inositol (Kurtzman, 2011e).

Assimilation of nitrate as a sole source of nitrogen is a criterion for circumscription of genera. *Pichia* and *Hansenula* were separated on the basis of this reaction, which was negative for *Pichia*, and positive for *Hansenula* (Kurtzman, 1984).

2.1.2.2 Chemotaxonomic characteristics

Chemotaxonomic characteristics, such as carbohydrate composition of cell walls and capsules (Weijman and Miranda, 1983; Suzuki and Nakase, 1998; Prillinger *et al*., 1993, Van der Klei *et al*., 2011), ubiquinone system (Yamada and Kendo, 1973; Yamada, 1998), fatty acid composition (Cottrel *et al*.,1986; Viljoen *et al*. 1986) were extensively used for taxonomic distinctions and nucleic acid base composition (mol% G+C) can differentiate phenotypically of similar strains to different species (Kurtzman and Phaff, 1987).

The range of nucleic acid base compositions differs for ascomycetous and basidiomycetous yeasts. Most ascomycetous yeasts have a mol% G+C about 27-50%, while that of basidiomycetous yeasts is approximately 50-70% (Kurtzman, 1998a)

Ubiquinone (Q) or coenzyme Q can be characterized from the number of isoprene units. For ascomycetous yeasts were varies ubiquinone-5 to 10. Species of the families *Pichiaceae* and Wickerhamomycetaceae form ubiquinone-7, whereas members of the *Debaryomycetaceae* and *Trichomonascaceae* form Q-9. Most species

of the genus *Lipomyces* produce Q-9, although *L. lipofer* (Q-10), *L. oligophaga* (Q-8) and *L. suomiensis* (CoQ-8) are exceptions (Kurtzman, 2011c)

2.1.2.3 Molecular characteristics

Molecular characteristics are important to the identification of yeast genera and species. The D1/D2 domain of 26S rDNA, internal transcribed spacers (ITS), and 18 S small subunit are important regions of the ribosomal RNA, the great impact on the taxonomy of yeasts.

D1/D2 is variable domains of the 26S rDNA or large subunit ribosomal RNA, the variable D2 domain (ca. 300 nucleotides) near the 5' end of large subunit (26S) rRNA (Peterson & Kurtzman, 1991). These regions are considered to be chronometers because of their universal occurrence, functional constraints and the presences of both variable and conserved regions (Worse, 1987). The length of the sequenced of D1/D2 region was about 600 nucleotides (63-642 bp of *Saccharomyces cerevisiae*). This region is useful for analysis at the species level (Kurtzman and Robnett, 1998) and a large database is now available.

Fell *et al*. (2000) published the D1/D2 sequences of known basidiomycetous yeasts, thus completing the database for basidiomycetous yeast. D1/D2 domain was estimated from comparisons of taxa determined to be closely related from DNA associations (Kurtzman and Robnett, 1998). The detection of new species that showed six or more noncontiguous substitution (1%) sequenced of D1/D2 region. If nucleotide sequencing no more than zero or three nucleotide difference (0-0.5%), they are same species(Kurtzman and Robnett, 1998, Kurtzman *et al*., 2007; Kurtzman and Fell, 2006)

However, the D1/D2 sequence might not distinguish varieties species or sister species so a phylogenetic analysis of the dataset provides an overview of close species relationships.

The internal transcribed spacer region ITS1 and ITS2, which are separated by the 5.8S gene of rDNA, are located between the small subunit (SSU) and large subunit (LSU) rRNA gene (Figure 2.3). Although, ITS sequences give no greater resolution than that obtained from D1/D2 LSU rRNA gene, ITS sequence was resolved species that unclear in the D1/D2 LSU rRNA gene (Jame *et al*., 1996;

Kurtzman and Robnett, 2003). Scorzetti *et al.*(2002) reported ITS sequences to provide greater resolution among many basidiomycetous yeast species than was found for D1/D2 domain.

The intergenic spacer (IGS) region of rDNA, one of rDNA region used for species identification, appears the most substituted and offers the greatest resolution of closely related species. *Cryptococcus, Xanthophyllomyces, Mrakia* and *Saccharomyces* were successed to separate from closely related species (Diaz *et al.*, 2000; Diaz and Fell, 2000; Bovers *et al.*, 2006). Because of the occurrence of repetitive sequences and homopolymeric regions, the IGS region tend to difficult to sequence for some species (Kurtzman and Fell, 2006). Small subunit (18S) rDNA is generally too conserved to allow the separation of individal species and its important in broad-base phylogenetic analysis (Kurtzman and Robnett, 2003)

In addition, gene sequence other than those of the rDNA repeat have been used to separation of species. Belloch *et al.* (2000) demonstrated the utility of cytochrome oxidase II for resolution of *Kluveromyces* species. Daniel *et al.* (2001) successful used actin-1 for species of *Candida,* and Kurtzman and Robnett (2003) showed the usefulness of elongation factor 1-α and RNA polymerase II for resolution of *Saccharomyces* species.

From single gene analysis such as the D1/D2 domain, it is apparent that many of ascomycetous yeast genera are not well circumscribed and not clear (Kurtzman and Robnett, 1998). Multigene sequence analyses have been applied to identification. Multigene sequence analysis of the *Kazachstania* (*Arxiozyma*) *telluris* species complex. D1/D2 LSU rRNA gene sequence analysis resolved the complex into five species, as did analysis of mitochondrial SSU rRNA gene sequences. However, analysis of RNA polymerase II detected four species because *K. pintolopesii* and *K. heterogenica* had nearly identical sequences for this third gene (Kurtzman *et al.* 2005). From the preceding analyses, it appears that *K. heterogenica* is a hybrid between *K. pintolopesii* and an undescribed species of *Kazachstania.*

Consequently, multigen sequence analysis will be required to resolve relationship between the preceding genera as well as for determining relationships within the genera (Kurtzman and Fell, 2006). Tsui *et al.* (2008) re-examining the

phylogeny of clinically relevant *Candida* species because single gene analysis was not sufficient information and misleading. Multigene analysis has been conducted to clarify the phylogenetic relationships of genus *Candida* and its allied genera (Kurtzman and Robnett, 2003; Diezman *et al*., 2004), these investigated multiple protein code gene (actin, RNA polymerase largest subunit (RPB1), RNA polymerase second largest subunit (RPB2) and second subunit of mitochondrial cytochrome oxidase gene (COX2) and D1/D2 LSU rRNA gene.

Presently, combined data analysis from these ribosomal gene (e.g. ITS-D1/D2) was largely been applied in number of studies involving delineation and description of new species (Pagnocca *et al*., 2010; Peter *et al*., 2010)
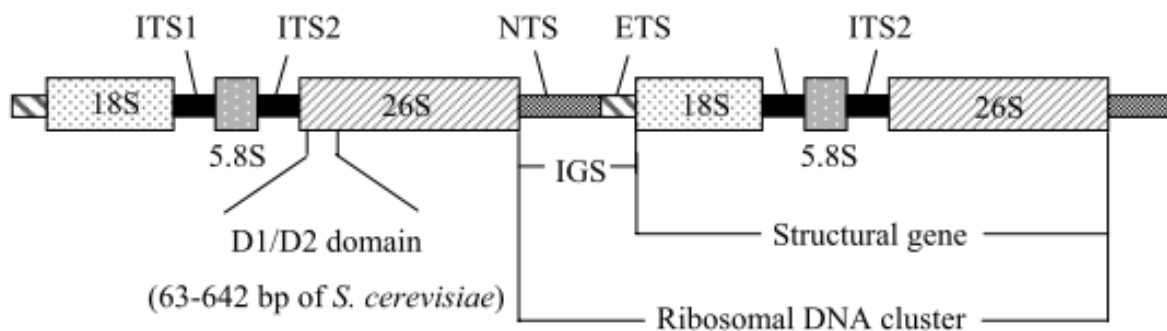
Figure 2.3 Structure of the ribosomal RNA gene cluster of yeasts. The cluster is split into coding (18S, 5.8S and 26S genes) and non-coding (Internal Transcribed Spacer or ITS and Inter-Genic Spacer or IGS. IGS consist of Non-Transcribed Spacer (NTS) and External Transcribed Spacer (ETS) regions (Jindamorakot, 2006).

2.2 Xylose fermentation

The applications of yeasts are important sectors including food, beverages, however, the most important biotechnology application is the production of bioethanol (Deak, 2009). In Brazil and Canada, considerable amount of ethanol was fermented from can-juice or other sugar-rich agricultural raw materials (Wheals *et al*., 1999). The improvement of fermentation technology and the utilization of cheap ago-industrial or wastes are studied (Girio *et al*., 2010).

2.2.1 Lignocellulose

Lignocellulose are abundant source of carbohydrate polymers. The major components of lignocellulose which is composed of mainly 40-50% cellulose, 25-30% hemicelluloses and 15-20% lignin (Figure 2.4) (Menon and Rao, 2012). Cellulose is a long-chain of D –glucose molecule linked by β-1,4 glycosidic bonds. Hemicellulose is a branched polymer composed primarily of xylose molecules (simple 5-carbon sugars) and other sugars, hexoses (glucose, galactose, and mannose) and pentoses (xylose and arabinose). Lignin is not a carbohydrate but a mainly phenolic compounds and their derivatives (Ortiz *et al*., 2011). It is a main component in plant cell walls and structure of each component in Figure 2.5.

The hemicellulose fraction contains varying levels of xylose-based hemicelluloses. The relative proportion of the individual sugars depends on the raw material (Table 2.4); the hemicellulose fraction of hardwoods and agricultural raw materials is rich in pentose sugars, while softwood hemicellulose only contains minor fractions of the pentose sugars D-xylose and L-arabinose (Hanh-Hägerdal *et al*., 2007). Therefore, agricultural residues and hardwood are most commonly considered for fuel ethanol and xylitol production.

Lignocellulose hydrolysate contains not only glucose, but also various monosaccharides, such as xylose, microorganisms should be required to efficiently ferment these sugars for the successful industrial production of ethanol.
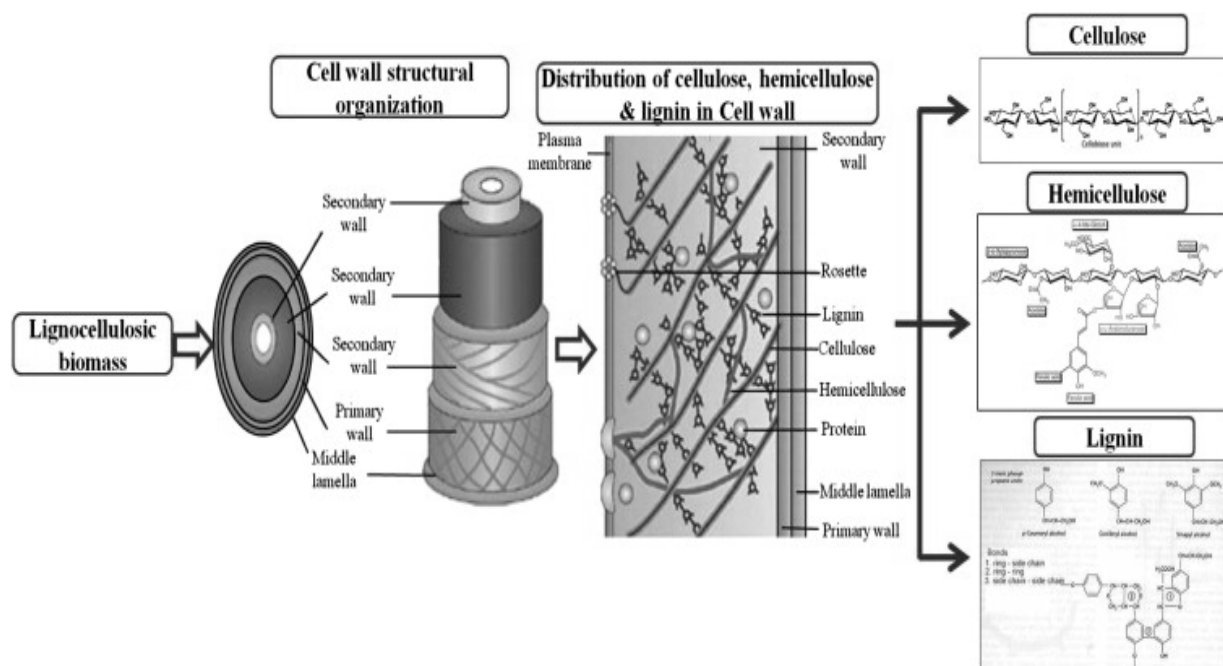
Figure 2.4 Diagrammatic illustration of the framework of lignocellulose (Menon and Rao, 2012).
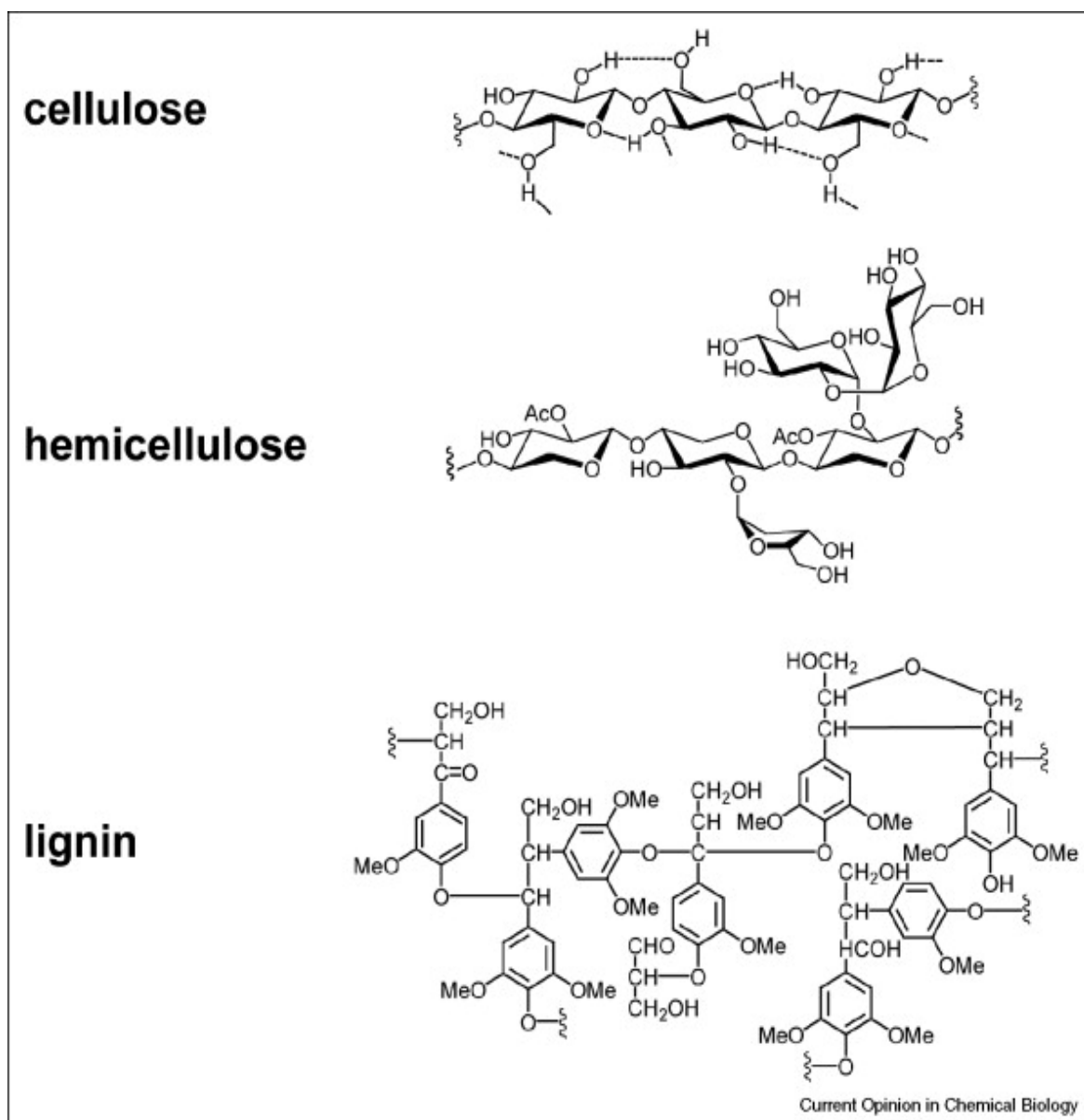
Figure 2.5 Biopolymers found in lignocelluloses (Chang, 2007).

Table 2.4 Hemicelluloses composition of various lignocellulosic materials (Gírio *et al*., 2010).

Raw material	Xyl	Ara	Man	Gal	Rha	UA	AcG
Softwoods							
Douglas fir	6.0	3.0	–	3.7	–	–	–
Pine	5.3–10.6	2.0–4.2	5.6–13.3	1.9–3.8	–	2.5–6.0	1.2–1.9
Spruce	5.3–10.2	1.0–1.2	9.4–15.0	1.9–4.3	0.3	1.8–5.8	1.2–2.4
Hardwoods							
Aspen	18–27.3	0.7–4.0	0.9–2.4	0.6–1.5	0.5	4.8–5.9	4.3
Birch	18.5–24.9	0.3–0.5	1.8–3.2	0.7–1.3	0.6	3.6–6.3	3.7–3.9
Black locust	16.7–18.4	0.4–0.5	1.1–2.2	0.8	–	4.7	2.7–3.8
Eucalypt	14–19.1	0.6–1	1–2.0	1–1.9	0.3–1	2	3–3.6
Maple	18.1–19.4	0.8–1	1.3–3.3	1	–	4.9	3.6–3.9
Oak	21.7	1	2.3	1.9	–	3	3.5
Poplar	17.7–21.2	0.9–1.4	3.3–3.5	1.1	–	2.3–3.7	0.5–3.9
Sweet gum	19.9	0.5	0.4	0.3	–	2.6	2.3
Sycamore	18.5	0.7	1	–	–	–	3.6
Willow	11.7–17.0	2.1	1.8–3.3	1.6–2.3	–	–	–
Agricultural and agro-industrial materials							
Almond shells	34.3	2.5	1.9	0.6	–	–	–
Barley straw	15	4	–	–	–	–	–
Brewery's spent grain	15	8	0	1	0	2	0.8
Cardoon	26	2.5	3.7	1.4	0.9	–	–
Corn cobs	28–35.3	3.2–5.0	–	1–1.2	1	3	1.9–3.8
Corn fibre	21.6	11.4	–	4.4	–	–	–
Corn stalks	25.7	4.1	<3.0	<2.5	–	–	–
Corn stover	14.8–25.2	2–3.6	0.3–0.4	0.8–2.2	–	–	1.7–1.9
Olive stones	2.0–3.7	1.1–1.2	0.2–0.3	0.5–0.7	0.3–0.5	1.2–2.2	–
Rice husks	17.7	1.9	–	–	–	–	1.62
Rice straw	14.8–23	2.7–4.5	1.8	0.4	–	–	–
Sugar cane bagasse	20.5–25.6	2.3–6.3	0.5–0.6	1.6	–	–	–
Wheat bran	16	9	0	1	0	2	0.4
Wheat straw	19.2–21.0	2.4–3.8	0–0.8	1.7–2.4	–	–	–

Expressed as g/100 g of dry material.

The percentages of hemicelluloses composition were, in some cases, calculated from the corresponding "polymers". Xyl, xylose; Ara, arabinose; Man, mannose; Gal, galactose; Rha, rhamnose; UA, uronic acids; AcG, acetyl groups.

2.2.2 Xylose-fermenting microorganisms

The D-xylose-fermenting microorganisms are reported so far and are belonged to different species of yeasts, fungi and bacteria (Hahn-Hägerdal *et al*., 2006).

2.2.2.1 Bacteria

Bacteria which have been reported to have good potential for fermenting D-xylose to ethanol (Fong *et al*., 2006; Sommer *et al*., 2004; Chandel *et al*., 2011) are mainly the facultative anaerobes and obligate anaerobic, included *Clostridium thermosaccharolyticum*, *Clostridium acetobutylicum*, *Bacillus macerans*, *Aeromonas hydrophila*, *Lactobacillus* sp., *Thermomonobacter ethanolicus*, *Thermoanaerobacter ethanolicus* and members of the family *Enterobacteriaceae* (Table 2.4). For fermenting D-xylose to xylitol are a few bacteria as *Corynebacterium* sp., *Enterobacter liquefaciens*, and *Microbacterium smegmatis* (Parajó *et al*., 1998). Moreover, the facultative anaerobic bacteria, *Serratia, Cellulomonas* and *Corynebacterium*, were screened for the production of xylitol (Rangaswamy and Agblevor, 2002).

However, bacteria have the major problem in using D-xylose because bacteria produce a wide mixture of metabolic products which is unwanted metabolic by-products such as organic acids (acetic, lactic, and butyric acids) and butanediol (Banerjee, 1989). Anaerobic bacteria are inhibited already at low sugar and ethanol concentrations (Hahn-Hägerdal *et al*., 2007). Moreover, bacteria provide product recovery more difficult than yeasts. Yeast has larger cells and thicker cell walls, which makes cell harvest and recycle easier. Perhaps most importantly, yeast fermentations are not as susceptible to contamination by bacteria or viruses. For these reasons many industrial ethanol processors retain an interest in xylose-fermenting yeasts.

2.2.2.2 Yeasts

A large number of yeast species metabolize xylose but this only 1% display fermentative capacity. The fermentation of xylose to ethanol was first reported by Karczewska in 1959. Even though many yeasts were known to assimilate xylose, this discovery did not enter the review literature and was not cited for over 20 years. The ability of yeasts to ferment xylose has been recognize only since the early 1980s, that the discovery of the ability of yeasts to ferment xylose to ethanol (Chandrakant and Bisaria, 1998; Jeffries and Kurtzman, 1994).

The naturally xylose-fermenting yeast was to know as *Candida shehatae, Candida tenuis, Candida tropicalis, Kluyveromyces maxianus, Pichia segobiensis, Pachysolen tannophilus, Scheffersomyces stipitis* (Slininger *et al*.,1982; Jeffries and Kurtzman, 1994, Silva and Afschar, 1994)

Morikawa *et al.*(1985) screened 213 species of yeasts for their abilities to ferment both xylose and cellobiose and identified *Kluyveromyces cellobiovorus*, a single strain belonging to a new species as capable of fermenting both sugars. Then Suh *et al*. (2004) screened xylose fermenting yeasts from gut of beetles and they were identified to be *Enteroramus dimorbhus*, a novel xylose-fermenting yeast that belonging to same clade as *S. stipitis*. Many more new species were isolated and characterized from beetles, wood-boring beetle and rootting wood such as *Spathaspora passalidarum, Spathaspora arborariae, Candida jeffriesii* and *Candida lyxosophila* were novel D-xylose fermenting yeast (Suh *et al*., 2003; Nguyen *et al*., 2006; Cadete *et al*., 2009). *Candida laoshanensis* and *Candida qingdaonensis* utilized D-xylose as sole carbon source, which isolated from decayed wood (Wang *et al*., 2010).

Table 2.5 Growth characteristics of natural pentose-fermenting microorganisms (Chandel *et al*., 2011).

Microorganisms	Glu	Xyl	Ara	Man	Cel	Temperature range (°C)	pH range
Filamentous fungi							
Fusarium oxysporum	+	+	+	+	+	28 - 32	5 - 6
Neurospora crassa	+	+	-	-	+	28 - 37	5 - 6
Monilia sp.	+	+	-	-	-	26	5
Mucor sp.	+	+	-	-	-	30	5.4
Yeast							
Saccharomyces cerevisiae	+	-	-	+	-	30 - 35	3-7
Klyuvermyces marxians	+	+	+	+	-	30 - 35	3 - 7
Pachysolen tannophilus	+	+	+	-	-	28 - 32	2.5 - 7
Candida shehatae	+	+	+	+	-	28 - 32	3 - 7
Pichia stiptis	+	+	+	+	-	28 - 32	3 - 7
Mesophilic bacteria							
Bacillus polymyxa	+	+	+	+	-	35 - 37	5.5 - 8
Aerobacter hydrophila	+	+	+	+	-	35 - 37	5.5 - 9
Klebsiella pneumonia	+	+	+	+	-	36 - 37	5 - 6
Clostridium acetobutylicum	+	+	+	+	+	37 - 37	4 - 8
Thermophilic bacteria							
Clostridium thermocellum	+	+	+	-	+	65	4 - 8
C. thermohydrosulfuricum	+	+	+	-	-	65	4.7 - 8
C. thermosaccharolyticum	+	+	+	+	-	60	5 - 8
C. thermosulfurogenes	+	+	+	+	-	60	4.5 - 7.5
Thermoanerobacter ethanolicus	+	+	+	+	-	69	4.4 - 9.5

+, positive; -, negative. Glu, glucose; Xyl, xylose; Ara, arabinose; Man, mannose; Cel, cellulose.

Nitiyon *et al*. (2011) isolated xylose-utilizing yeast strains from samples of decaying agricultural residues and soils collected in various areas of Thailand. Seventy-eight strains were assigned to Ascomycota: *Barnettozyma californica, Candida blankii, Candida coipomoensis, Candida maltosa, Candida membranifaciens, Candida pseudointermedia, Candida pyralidae, Candida solani, Candida tropicalis, Debaryomyces hansenii var. fabryi, D. nepalensis, Geotrichum silvicola, Lindnera rhodanensis, Pichia caribbica, Pichia kudriavzevii, Saturnispora saitoi, Sporopachydermia lactativora, Zygoascus hellenicus Candida prachuapensis* and *Candida saraburiensis.* Forty-two strains were assigned to Basidiomycota: *Cryptococcus* cf. *podzolicus, Cryptococcus heveanensis, Cryptococcus humicola, Cryptococcus laurentii, Cryptococcus terrestris, Trichosporon asahii, T. moniliiforme, T. terricola* and *T. mycotoxinivorans*. Although, many species belong to xylose-utilizing yeast, only a few species were fermented D-xylose to ethanol. Such as *Zygoascus hellenicus, Candida blankii* and *Candida saraburiensis* produced ethanol at 3.1–3.6 g/l at 72 h. Until today, *S. stipitis* and *C. shehatae* still to be the best ethanol producing (Jeffries and Kurtzman, 1994).

In addition, the other substitute for ethanol production can be the formation of xylitol from lignocellulosic hydrolyzates by the yeasts such as *Candida tropicalis* and *Candida guilliermondii* (Barbosa *et al*., 1988; Horitsu *et al*., 1992; Rafiqul and Sakinah, 2012). Species acceptable for biotechnology production of xylitol were *C. boidinii, C. guilliermondii, C. maltosa, C. parasilosis, C.tropicalis, C. pelliculosa, Debaromyces hansenii, Pachysolen tannophilus, Pichia caribica, Pichia farinose, P. miso, Issatchenkia* sp*., Trichosporon* sp., *Rhodotorula* sp., and *Clavispora* sp. (Sirisansaneeyakul *et al*., 1995; Sreenivas *et al*., 2007; Praksham *et al*., 2009; Ghindea *et al*., 2010)

2.**2.2.3 Filamentous fungi**

The ability of filamentous fungi to ferment pentose sugar has been known about 70 years. Several fungal species belonging to genera *Fusarium*, *Rhizopus*, *Monilia*, *Neurospora* and *Paecilomyces* were found to have potential for fermenting glucose as well as xylose. Among the ethanol producing fungi, *Fusarium oxysporum* has shown more ethanol production than *Neurospora crassa* and *Mucor* sp. (Chandel *et al*., 2011)

Skory *et al.* (1997) screened the ethanol-producing filamentous fungi such as *Aspergills* sp. and *Rhizopus* sp. They showed ability to ferment simple sugars (glucose, xylose, and arabinose). *Rhizopus* sp. produce ethanol more than 31 g ethanol/l under anaerobic stress at 72 h. Thermophilic fungal species such as *Sporotrichum thermophile, Thermoascus aurantiacus*, *Thielavia terrestris* and *T. emersonii* have been proposed as good candidates for bioconversion of lignocellulosic residues to sugars and offer the great potential to be used at industrial scales (Dashtban *et al*., 2009; Fernades *et al*., 2008).

Aerobic filamentous fungi could ferment pentose sugar and tolerate industrial substrates. Some species of aerobic filamentous fungi produce ethanol (Hahn-Hägerdal *et al*., 2007) and xylitol. *F. oxysporum* VTT-D-80134 was the best ethanol production from xylose, 0.42 g ethanol per g xylose (Suihoko and Enari, 1981; Schneider *et al*., 1989), *Petromyces* sp NF1 was 0.4 g ethanol per g xylose (Wu *et al*., 1986; Schneider *et al*., 1989). *Petromyces albertensis* was produced xylitol (39.8 g xylitol/l and 2.8 g xylulose/l) in medium containing 100 g xylose (Dahiya, 1991; Parajo *et al*., 1998).

However, the rate of production of ethanol by fungi is usually considered to be lower than yeasts and poor ethanol tolerance (Hahn-Hägerdal *et al*., 2007).

2.2.3 Xylose utilization pathway

In 1960, Chiang and Knight found that the filamentous fungus *Penicillium chrysogenum* convert D-xylose to D-xylulose through a two-step reduction and oxidation. Enzymes of xylose utilization pathway in yeast were different from xylose isomerase in bacteria. Figure 2.6 schematically illustrates the initial metabolic pathways for xylose utilization in fungi and bacteria. In most fungi and xylose-fermenting yeasts (*e.g., Pichia stipitis, Pachysolen tannophilus, and Candida shehatae*), D-xylose is converted to D-xylulose by two oxidoreductases involving cofactors NAD(P)H and NAD(P)$^+$ through the type I pathway. That is, D-xylose is initially reduced to xylitol by NAD(P)H-dependent xylose reductase (XR; EC 1.1.1.21; Bruinenberg *et al*. 1984), and then xylitol is oxidized to D-xylulose by NAD$^+$-dependent xylitol dehydrogenase (XDH; EC 1.1.1.9; Wang and Jeffries 1990). Finally, xylulokinase XK; EC 2.7.1.17) phosphorylates D-xylulose into D-xylulose 5-phosphate (X5P; Rodriguez-Pena *et al*. 1998), which is further metabolized through the pentose phosphate pathway (PPP). These intermediates are converted to pyruvate in the Embden–Meyerhof Parnas pathway (Hahn-Hägerdal *et al*., 1994). Under anaerobic conditions, pyruvate is decarboxylated by pyruvate decarboxylase to acetaldehyde which is then reduced to ethanol by alcohol dehydrogenase.

On the other hand, in most bacteria (e.g., *Escherichia coli and Streptomyces* sp.), D-xylose is directly isomerized to D-xylulose by xylose isomerase (XI; EC 5.3.1.5) through the type II pathway. As in yeast and fungi, D-xylulose is phosphorylated to D-xylulose 5-phosphate by XK. (Matsushika *et al*., 2009)

Ethanol productivity involved with xylose reductase (XR) and xylitol dehydrogenase. It has been interesting that the conversion of D-xylose to ethanol by yeast require an NADH-dependent xylose reductase activity because reduce redox imbalance. Thus reduce xylitol accumulation and high ethanol production (Verduyn *et al*, 1985). Otherwise, serious imbalance in the cellular redox system are occur with NADPH-dependent xylose reductase activity (du Pressz *et al*, 1989). These results the xylitol formation from D-xylose, but the ethanol amounts produced were low and anaerobic D-xylose utilization was also slow. It was proposed to be due to insufficient capacity of the pentose phosphate pathway and to cofactor imbalance

caused by the different cofactors preferred by the xylose reductase (NADPH) and xylitol dehydrogenase (NAD +) (Kotter and Ciriacy 1993, Walfridsson *et al.* 1995).

In general, the xylose reductase is specific for NADPH coenzyme. Neuhauser *et al.,* (1997) reported that *Candida tenuis* produced xylose reductase was with both NADH and NADPH as coenzyme, the same as *P. stipilis* which reported previously (Verduyn *et al*, 1985)

For xylitol producing yeasts, xylose is reduced to xylitol either by NADH- or NADPH-dependent xylose reductase (aldose reductase EC 1.1.1.21). The produced xylitol is either secreted from the cell or oxidized to xylulose by NAD- or NADP-dependent xylitol dehydrogenase (EC 1.1.1.9). These two reactions are considered to be limiting for D-Xylose fermentation and xylitol production. The ratio of xylose reductase and xylitol dehydrogenase in addition to cofactor regenerating system is the major metabolic regulator for xylitol production. (Hahn-Hagerdal *et al*., 2007).
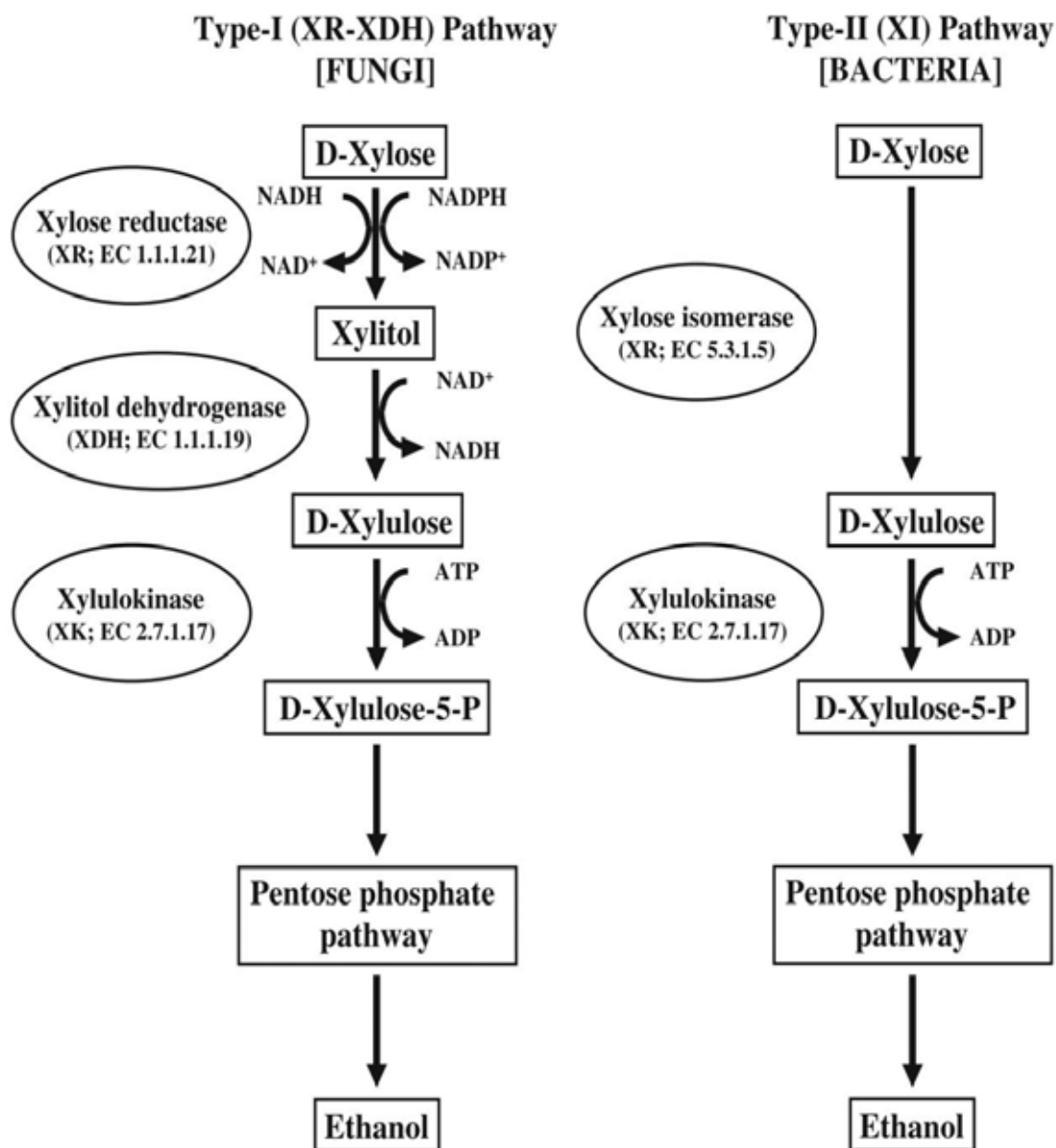
Figure 2.6 Outline of D-xylose metabolic pathway in yeast, fungi and bacteria.

2.2.4 Characterization of the gene encoding XR and XDH

Early as 1983, *Pachystolon tannophilus* was first native xylose-utilizing yeast, and then researchers found aldose (xylose) reductase (XR) that could accept either NADH or NADPH as a cofactor which key to anaerobic assimilation of xylose (Jeffries and Jin, 2004). Similar with *S. stipitis* and *C. shehatae*, at least one XR can use either NADH or NADPH (Verduyn *et al*., 1985). The XR of *C.utilis* used NADH as cofactor. Because the assimilation of xylose requires two oxidoreductase steps and all oxidoreductase reaction following these are balanced (Jeffries and Jin, 2004). Isolate of the first two gene for xylose assimilation led to the initial development xylose fermentation to high production.

From xylose metabolic pathway of xylose-utilising yeasts and filamentous fungi, D-xylose is reduced to xylitol by xylose reductase (XR) after entering the cell and subsequently oxidized to D-xylulose by xylitol dehydrogenase (XDH). (Winkelhausen and Kuzmanova, 1998; Hahn-Hagerdal *et al*., 2007; Mishra and Singh, 1993). The genes encoding the activities of the first two reactions, are namely *XYL1* for xylose reductase and *XYL2* for xylitol dehydrogenase (Jeffries, 2006).

XRs and XDH have gained interest because of their importance both in the fermentation of plant biomass to ethanol and in the production of xylitol, The DNA sequences of many XR gene have been determined and several have been cloned and expressed in a variety of hosts (Amore *et al*., 1991; Ho *et al*., 1990; Kang *et al*., 2003; Lee *et al*., 2003; Mayr *et al*., 2000; Neuhauser *et al*., 1997; Chu and Lee, 2007; Ko *et al.*, 2006). Now, engineering yeast for xylose metabolism attempted to developing yeasts for ethanol production such as cloning and expression gene, mutation gene (Jeffries, 2006).

P. stipitis was the first strain which was isolated and characterized gene encoding XR and XDH. *P. stipitis* have been clone and characterizated gene encoding (*Ps*XR and *Ps*XDH) (Watanabe *et al*., 2007; Amore *et al*., 1991)

Amore *et al*., 1991 cloning and expression in *Saccharomyces cerivisiae* of NAD(P)H-dependent xylose reductase encoding gene (*xyl1*) from *P. stipitis* The gene encodes a NAD(P)H-linked aldose reductase that could be a key enzyme for

anaerobic xylose fermentation in *S. cerevisiae* transformants bearing broth *xyl1* and *xyl2* genes.

Kotter *et al*. (1990) isolated and characterized xylitol dehydrogenase gene (*xyl2*) of *P. stipitis*. The genomic *xyl2* gene was isolated and the nucleotide sequence of the 1089 bp of the structural gene. The *xyl2* open-reading frame codes for a protein of 363 amino acids with a predicted molecular mass of 38.5 kDa. The *xyl2* gene is actively expressed in *S. cerevisiae* transformants. *S. cerevisiae* cells transformed with a plasmid, pRD/, containing both the xylose reductase gene (*xyl1*) and the xylitol dehydrogenase gene (*xyl2*), were able to grow on xylose as a sole carbon source.

Billard *et al*. (1995) described a xylose reductase (XR)-encoding gene (*xyl1*) from the xylose-assimilating yeast *Kluyveromyces lactis* (Kl). *xyl1* was isolated as a highly expressed fusion clone from a 'lacZ translational fusion library. DNA sequence analysis revealed an open reading frame (ORF) of 987 bp capable of encoding a polypeptide of 329 amino acids. Amino acid sequence displayed a 62% overall identity to that of XR from *Pichia stipitis*.

Kang et al. (2003) characterized the aldose reductase (*Cb xyl1*) from *Candida boidinii* and expressed it in *S. cerevisiae*. *Cbxyl1* had an open reading frame of 966 bp encoding 321 amino acids. The *C. boidinii xyl1* is highly similar to other known yeast aldo- reductase and most closely related to the NAD(P)H-linked *XYL1* of *Kluyveromyces lactis*.

Wang *et al*. (2007) cloned XR coding gene (*xyl1*) of *Candida shehatae* and expressed in *Escherichia coil*. The recombinant XR had Km values for NADH more than NADPH of 150 µM and 20 µM, respectively. Optimal pH 6.5 and optimal temperature 35°C. Its high activity, may prove useful in the in vitro production of xylitol.

Zhang *et al*. (2009) cloned *xyl1* gene of *Candida tropicalis* SCTCC 300249 and expressed in *Escherichia coil* BL12 studied the characteristic of XR from *Candida tropicalis* SCTCC 300249, its XR gene (*xyl1*) was cloned and expressed in *Escherichia coil* BL12. The recombinant XR had Km values of NADPH and NADH were 45.5 µM and 161.9 µM, respectively.

In addition, industrial of xylose-fermenting strains and fermentation of lignocellulosic hydrolysates, *S. cerevisiae* strains that limited to ferment xylose (Hahn-Hagerdal *et al*. 2007). Using cell surface display technology, Kondo and collaborators generated the xylose- and cellobiose-assimilating recombinant *S.cerevisiae* strain (Katahira *et al.* 2006) and demonstrated an effective cofermentation process (Nakamura *et al*. 2008).

Recently, Hasunuma and Kondo (2012) reported recombinant *S. cerevisiae* strain displaying cellulolytic and hemicellulolytic enzymes. *Katahira et al*. (2004) reported that the combination of a cell surface display system and additional metabolic functions is effective in the construction of yeast cells capable of multistep bioconversion.

They attempted to generated display of both *T. reesei* XYNII and *A. oryzae* β-xylosidase on the cell surface of *S. cerevisiae*, which made it possible to hydrolyze birchwood xylan to xylose. They also introduced XR, XDH and XK into the xylan-degrading yeast strain, which enabled simultaneous saccharification and fermentation of xylan (Figure 2.7). After 62 h of fermentation, 7.1 g/l ethanol was directly produced from birchwood xylan, and the yield was 0.30 g ethanol per gram carbohydrate.
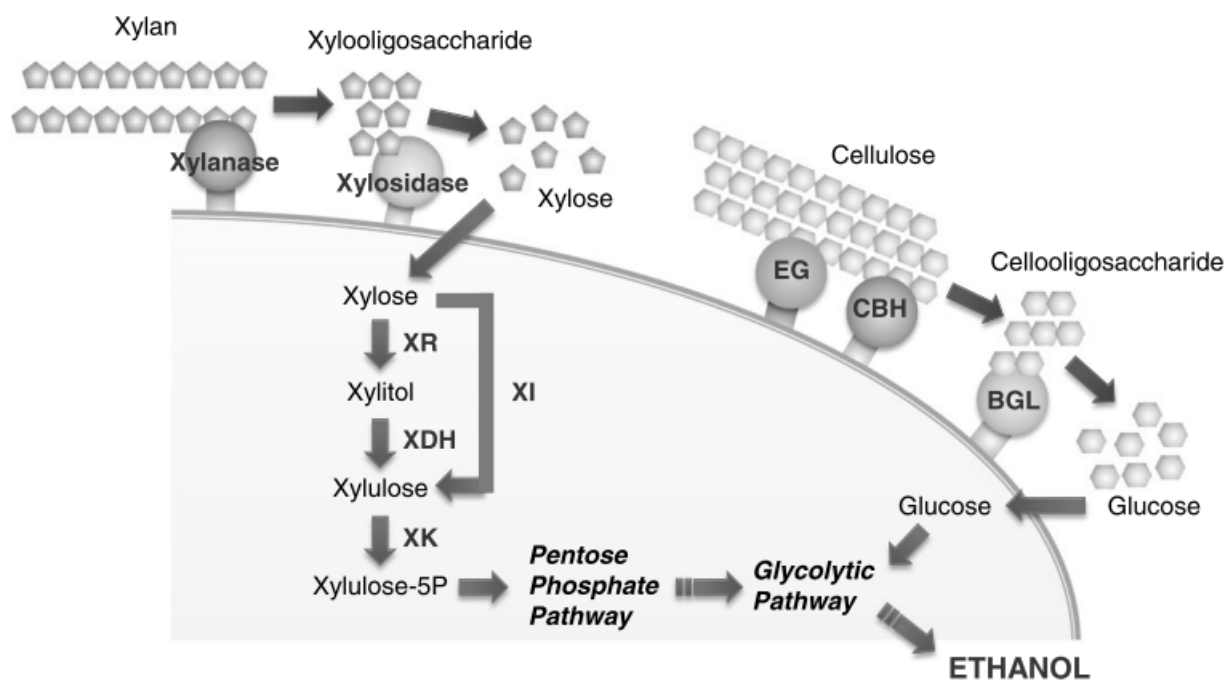
Figure 2.7 Ethanol production pathway from cellulose and xylan using a recombinant *S. cerevisiae* strain displaying cellulolytic and hemicellulolytic enzymes such as endoglucanase (EG), cellobiohydrolase (CBH), β-glucosidase (BGL), xylanase and xylosidase on the cell surface and expressing xylose assimilating enzymes such as xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) in the cell. Xylulose-5P, xylulose-5-phosphate (Hasunuma and Kondo, 2012).

2.2.5 Bio-ethanol production from xylose fermenting yeasts

A number of excellent general reviews on xylose fermenting yeast have appeared in Chapter 2.2.2.2. Only a few strains of *S. stipitis*, *C. shehatae* and *P. tannophilus* produced high ethanol (Jeffries and Kurtzman, 1994).

Pentose-fermenting yeast for ethanol production from D-xylose is restricted mainly by their low ethanol and slow rate of fermentation (Lin and Tanaka, 2006). In this way, review on the environmental factor affecting xylose fermentation. Carbon and nitrogen sources, aeration, pH, and temperature are important for cell growth and product formation.

Aeration plays a critical role such as oxygen limitation induces fermentation in *P. stipitis* and *C. shehatae* (Alexander *et al*., 1988; Passoth *et al.,* 1998). At the same time, these yeasts required oxygen for growth and maximal ethanol production (du Preez *et al.,* 1989). Oxygen plays an important role in cell growth, redox balance, functioning of the mitochondria and generation of energy for xylose transport in *P. stipitis* (Skoog *et al*., 1990). Fermentation in *P. stipitis* is not induced by high sugar concentrations, but inactivated by aerobic conditions (Passoth *et al*. 1996). The investigation by Passoth *et al*. (1996) revealed that the branching point between respirative and fermentative metabolism which includes enzymes such as pyruvate decarboxylase, alcohol dehydrogenase and aldehyde dehydrogenase were induced by a reduction in oxygen tension (Passoth *et al*. 1996).

du Preez *et al.* (1989) found that aeration was one of the factors limiting ethanol production.With *P. stipitis* at ethanol concentrations in excess of 28 g/liter, ethanol assimilation exceeds production, even when the dissolved oxygen tension is kept to 0.2% of saturation. In the absence of aeration, ethanol accumulation continues, but at a much lower rate, and xylitol production increases.

Jeffries and Alexander (1990) attained 56 g/liter ethanol within 38 hr (1.53 g /liter/ hr) with *C. shehatae* in fed-batch fermentation after the cell inoculum had been cultivated at a high dilution rate and shifted to oxygen limited conditions. *Candida shehatae* requires oxygen to maintain viability.

Kastner *et al.* (1999) showed that oxygen starvation induces cell death in *C. shehatae* when it is grown on D-xylose, but not when it is cultivated on D-glucose. Growth of *C. shehatae* was limited to one division or less when cells cultivated aerobically on either glucose or xylose are shifted from aerobic to anaerobic conditions. Cell viability rapidly declined with cells cultivated on xylose, but cells cultivated on glucose remained viable nine times longer.

The initial xylose concentration has an effect on the fermentation parameters of *P. stipitis* and *C. shehatae* with maximum ethanol productivities occurring at a xylose concentration of 50 g/l and highest values at about 50 g/l, respectively. Both yeasts utilized all of the xylose supplied at concentrations of 90 g/l or less. Even at an initial substrate concentration of 100 g/l *C. shehatae* utilized 100% of xylose within 68 h, but *P. stipitis* consumed only 89% after a fermentation time of 193 h. Thus, *P. stipitis* was more susceptible to inhibition by high xylose concentration than *C. shehatae*. But *C. shehatae* was inversely related to xylose concentation and the decrease in ethanol yield was accompanied by an increase in xylitol production (du Preez *et al.*1985).

Roberto *et al.* (1990) studied sugar inhibition of *P. stipitis* CBS 5773 show that ethanol productivity is inhibited at initial xylose concentrations between 76 and 99 g/l and ethanol yield decreased when xylose concentration was above 145 g/l.

Chamy *et al.* (1994) study substrate inhibition effects in the fermentation of xylose by immobilized *Pichia stipitis* found no evidence of xylose inhibition when xylose concentration was 200 g/l . High sugar concentrations increase osmotic stress on organisms and therefore reduces growth and fermentation rate. Therefore, the observation by Chamy and coworkers on xylose inhibition may be due to experimental conditions which reduce the sugar stress.

The pH and temperature optimum for biomass accumulation by *P.tannophilus* on xylose were 3.7 and 31.5, respectively, at an initial xylose concentration of 50 g/liter (Roebuck *et al.,* 1995). As in the case for *P. stipitis* and *C. shehatae,* maximum ethanol productivity and maximum specific growth rate were observed at pH 4 (du Preez *et al.*1985). The detoxified hemicellulose hydrolysate at pH between 6.0 and

7.5, *P. tannophilus* converted 90% of the available xylose into xylitol. At pH values outside this range, cells respired up to 30% of the xylose (Converti *et al*., 1999).

Nutrients in fermentation media play an important part in the growth and ethanol production. Studies on *P. stiptitis* NRRL Y-7124 using a defined medium provied with nitrogen, vitamin, amino acid, purine and pyrimidine show that some of these components could enhance growth and ethanol production in *P. stipitis* (Slininger *et al*., 2006). Ethanol production increased with addition of amino acids and nitrogen was required for non-growth associated ethanol production (Slininger *et al*. 2006). Ethanol production increased with addition of amino acids and nitrogen was required for non-growth associated ethanol production (Slininger *et al*. 2006). Ammonium salts increased the ethanol productivity and the ethanol to biomass yield in *P. stipitis* (Guebel *et al*. 1992;). Magnesium has also been shown to play an important role in redox balance and therefore has an effect on xylitol production (Mahler and Nudel, 2000). Low levels of Mg resulted in xylitol accumulation and a high intracellular NADH content (Mahler and Nudel, 2000). Corn steep liquor is a viable nutrient source for *P. stipitis* fermentation when used as a sole nitrogen source compared to amino acids, vitamins and other nutrients (Amartey and Jeffries 1994). A summary of the effect of various nutrients on growth and ethanol production is shown in Table 2.6

2.2.6 Xylitol fermentation

The development of an economic fermentative process for xylitol production involves the selection of yeast strains with high productivity, establishment of conditions that maximize the conversion of xylose into xylitol and scaling up process (Silva *et al*., 1998).

Recently, a metabolically engineered *Saccharomyces cerevisiae* containing the xylose reductase gene, *xyl1*, was developed to produce xylitol with a yield close to 100% (Roca *et al*., 2003; Lee *et al*., 2000). However, the recombinant strains showed relatively lower production rates and volumetric productivity than the wild - type yeasts. (Kim *et al*., 2002). So need to optimization of xylitol production by the environmental factor affecting.

The initial concentration of xylose should be as high as possible. Aeration also plays an important role in the bioconversion of xylose to xylitol by yeasts (Walther *et al*., 2001) whereas high degree of aeration promotes cell growth, while being detrimental to xylitol accumulation.

Optimum pH for xylitol production by *Candida s*p. was generally ranged from 2.5 to 5.8 (Silva *et al*., 1998). The pH alteration probably affects the growth by influencing the activity of the permeases present in the cytoplasmic membrane or of the enzymes associated to the cellular wall, which tend to coagulate and to precipitate under their isoelectric points (El-Baz *et al*., 2011). As the gap between the extracellular and the intracellular pH values widens, greater stress is placed on the cells and more energy is expected to maintain the intracellular pH within the range that permits growth and survival of the yeast (Thomas *et al*., 2002).

To increase xylitol yield and productivity a two- substrate fermentation was designed: a cell growth step using glucose followed by a bioconversion step from xylose to xylitol without cell growth achieved, by controlling the oxygen supply. In particular, an initial glucose concentration was optimized by using kinetic equations related to effects of ethanol, a major by-product of glucose metabolism, on cell growth and xylose conversion in batch cultures (Kim *et al*., 1999). High concentrations of xylitol, as a polyhydroxyl compound, reduce water activity or increase osmotic pressure, which could inhibit cell metabolism and interfere with the membrane transport system. Kim *et al*., (2002) study fed - batch fermentations were undertaken to extend the bioconversion period for xylitol production and hence to increase the final xylitol concentration. In particular, the manner of feeding the xylose solution in the fed - batch period was determined based on the model equations for cell growth and xylitol production in an attempt to maintain the optimum xylose concentration at 100 g /l.

For the economical production of xylitol, conventional optimization procedure, one variable at a time, is effective in some situation but fails to consider the combined effects of the entire factors involved. In additional, it is time consuming and can not provide an interaction of parameter on the desired outcome (Sreenivas Rao *et al*., 2004). Statistical optimization procedure has an advantage over the

conventional procedure for rapidity, reliability, and understanding of interaction among parameters at various concentrations (Ayse *et al*., 2007). Plackett-Burman design is statistics design, which was two levels of high and low variables to identify the critical parameter in fermentation process (Naveena *et al*., 2005). Central composite design (CCD) is one of the popular mathematical and statistical methodology, response surface methodology (RSM) used for optimization process (Anderson *et al*., 2005). RSM is useful for modeling and analyzing problems where the response of interest is influenced by many variables and successfully to optimize of various parameters related to the bioprocess industry, in producing significant yield of various metabolites.

Table 2.6 Effect of different nutrients on growth and ethanol production in *P. stipitis.*

Nutrient	Strain	Effect on cell growth	Effect on ethanol concentration
Ammonium	NRRL Y-7124 CBS 6054	Slightly decreased	Increase
Amino acids	NRRL Y-7124	Arg, Hist, Isoleu and Prol increased cell growth, Ala, Glutamic acid, Leucine and Tyrosine decreased cell growth	Ala, Arg, Asp, Glu, Gly, Hist, Leu, and Tyr increased ethanol concentration. Isoleucine reduced ethanol oncentration
Calcium	NRRL Y-7124 CBS 6054	Increased growth at low concentration (0.34 mM). Decreased growth at high concentrations (1 mM)	Slightly lower ethanol concentration and ethanol to biomass yield.
Carbon dioxide	CBS 5773 CBS 5776	Decrease in growth rate	Decrease in fermentation rate and final ethanol concentration
Corn steep liquor	CBS 6054	Increase in cell growth	Increase in ethanol fermentation rate and final ethanol concentration
Magnesium (1–4 mM)	NRRL Y-7124	Increase in cell growth	Increase in ethanol concentration
Malt extract, yeast extract and ammonium sulfate	CBS 6054	Decrease in cell growth	Decrease in ethanol fermentation rate and final ethanol concentration
Peptone, yeast extract, $MgSO_4$, and KH_2PO_4	CBS 6054	Decrease in cell growth	Increase in ethanol fermentation rate and final ethanol concentration
Yeast nitrogen base with supplements	CBS 6054	Decrease in cell growth	Decrease in ethanol fermentation rate and but an increase in final ethanol concentration
Vitamins (thiamine, riboflavin, calcium panthotenate, niacin, pyridoxamine, thioctic acid, folic acid, biotin, B_{12})	NRRL Y-7124	Generally improved cell growth	No effect on ethanol production

CHAPTER III

EXPERIMENTAL

3.1 Isolation and screening of xylose-fermenting yeasts

Yeasts were isolated from herbivore feces such as elephants, goat, giraffes, macaques, cows, buffalos, kangaroos, zebras, hog deer, antelope, orlik and barking deers. Sample 0.5 g was placed in tube with 10 ml sterile YX medium (yeast nitrogen base 0.67%, D-xylose 2%, chloramphenicol 0.02%, sodium propionate 0.2%, pH 5.5) at 30°C for 3-10 days (Van der Walt, 1970). When growth was detected, one loopful of culture was streaked on YX agar medium. The plate was incubated at 30°C until yeast colonies developed then the colony was picked up and purified by the conventional streaking technique on YM agar. The pure cultures were suspended in YM broth supplemented with 10% glycerol as a cryoprotectant and maintained in deep freezer (-80°C).

3.2 Identification methods

The morphological, cultural, physiological, biochemical and chemotaxonomic including molecular taxonomic characteristics were used for yeast identification by comparison with standard descriptions in The Yeasts, a Taxonomic Study 5th ed. (Kurtzman *et al*., 2011b).

3.2.1 Morphological and cultural characteristics

Vegetative cells, pseudomycelium and true mycelium formation, morphology of ascospores and colony characteristic on YM agar were observed as described in The Yeasts, a Taxonomic Study 4th ed and 5th ed. (Kurtzman and Fell, 1998; Kurtzman *et al*., 2011b).

3.2.1.1 Growth on malt/yeast extract agar

Cell were grown in malt extract, YM broth or YM agar and incubated for 2-3 days at 25°C. But some strains were incubated at temperatures and times ranging

from 4 to 42 °C and one day to a few weeks, respectively. The cultures were examined for cell shape, cell arrangement (single, pairs or aggregated in clusters), reproduction mode etc. under microscope.

3.2.1.2 Dalmau plate

The Dalmau plate technique is performed on medium which can promote formation of hyphae. Commonly used media cornmeal agar for this technique. Agar is poured into petri plates, which are then put aside for a day or two to allow the surface to dry. The yeast is inoculated as a single streak near one side of the plate (for example from the ten to the two o'clock positions), and as two points near the other side of the plate (for example at the four and eight o'clock positions). A sterile coverglass is placed over the center of the streak and another over one of the point inoculations. The cultures are incubated and examined microscopically in the same way as slide cultures.

3.2.2 Physiological and biochemical characteristics

Physiological characteristic were studied and compared with the standard descriptions in The Yeasts, a Taxonomic Study 4th ed and 5th ed. (Kurtzman and Fell, 1998; Kurtzman *et al*., 2011a).

3.2.2.1 Fermentation of carbohydrate

Yeasts vary in their ability to ferment sugar (D-glucose, D-galactose, sucrose, maltose, lactose, raffinose and trehalose) as measured by the production carbon dioxide. (Van der Walt, 1970).

The fermentation basal medium contained 4.5 g of yeast extract and 7.5 g of peptone in 1 liter of demineralization water. Add 4 ml of stock bromothymol blue (stock 50 mg/75 ml) per 100 ml of fermentation basal medium to give a dark green color. Put 2 ml aliquots of basal medium in to tube 12x150 mm in size. Sterilize at 121ºC for 15 min. Then add 1 ml of 2% (w/v) final sugar concentration which filter-sterilized sugar to sterilized basal medium. Cell suspension was inoculums to fermentation medium and incubated at 25-28ºC for up to 28 days. The results are

indicated as follows, depending on the time taken to fill the insert with gas and change color from green to yellow. The score as follows:

+, strongly positive, insert filled within 7 days.

l, delayed positive (latent), insert rapidly filled, but only after more than 7 days

s, slowly positive, insert slowly filled after more than 7 days.

w, weakly positive, the insert is not fully filled with gas.

-, negative, no accumulation of gas in the insert.

v, variable, some strains are positive, others are negative.

3.2.2.2 Assimilation of carbon compounds

Assimilation of carbon compounds was investigated in liquid media according to the method described by van der Walt and Yarrow (1984) and Yarrow (1998). In the present study 42 carbon compounds were employed in the description of each species (Table 3.1).

Table 3.1 Carbon compounds use in the description of each species.

Group	Carbon compound
Hexoses	Glucose, galactose and sorbose
Disaccharides	Cellobiose, lactose, maltose, melibiose, sucrose and trehalose
Trisacchrides	Melizitose and raffinose
Polysaccharides	Inulin and soluble starch
Pentose	D-arabinose, L-arabinose, D-ribose, L-rhamnose and Dxylose
Alcohols	Galactitol, meso-erythritol, D-glucitol, glycerol, myoinositol, D-mannitol, ribitol, ethanol and methanol
Organic acid	Citric acid, DL-lactic acid, succinic acid and D-gluconic acid
Glycosides	α-methyl-D-glucoside and salicin
Other compound	D-glucosamine hydrochloride, *N*-acetyl-D-glucosamine and hexadecane
Addition compounds	2-keto-D-gluconate, 5-keto-D-gluconate, saccharate, xylitol, D-glucuronate and L-arabinitol

Ten folds medium stock solution (Appendix A) was prepared by filter sterilization and stored in a freezer at -20°C until use. Amount of 0.2 ml of thawed stock solution was added to 1.8 ml of sterilized water in a cotton plugged test tube (13x100 mm). Media for inulin, soluble starch, ethanol, galactitol, 2-ketogluconic acid and 5-ketogluconic acid were prepared at every experiment. In the case of ethanol and 5-ketogluconic acid, 3% and 0.3% solution were employed, respectively. The young culture grown on YM agar was used as the inoculums. A very light suspension in Yeast Nitrogen Base (YNB) medium was prepared and incubated at 25°C for 1 week to exhaust the carbohydrate pool in the cells. A drop of inoculums was inoculated to the carbon assimilation test medium by sterilized Pasteur pipette and incubated at 25°C for 4 weeks. Basal medium without carbon source was employed as a negative control. The growth was observed and recorded every week up to 4 weeks. After dispersed the cells by shaking, the degree of growth was assessed by eye by placing tubes against a white card on which lines of 0.75 mm thick were drown 5 mm apart with Indian Ink. The result was scored as 3+ if the lines are completely obscured; as 2+ if the lines appears as diffuse bands; as 1+ if the lines are distinguishable as such but have blurred edges; as – if the lines were distinct and sharp edged. Results are presented in the descriptions as follows:

+, positive, either a 2+ or a 3+ reading after 1 week, or 2 weeks in some laboratories.

l, delayed positive (latent), either a 2+ or 3+ reading develops rapidly, but after 2 weeks or longer.

s, slow positive, a 2+ or 3+ reading develops slowly over a period exceeding 2 weeks.

w, weakly positive, a 1+ reading.

-, negative.

v, variable, some strains are positive, others are negative.

3.2.2.3 Assimialtion of nitrogen

Assimialtion of nitrogen was investigated on solid media using starved inoculum according to the method of Nakase and Suzuki (1986). Six kinds of nitrogen compounds including amonium sulfate ($(NH_4)_2SO_4$), potassium nitrate (KNO_3), sodium nitrite ($NaNO_2$), ethylamine, L-lysine and cadaverine were employed. The young culture grown on YM agar was used as the inoculums. A very light suspension in YCB medium (Appendix A) was prepared and incubated at 25°C for 1 week to exhaust the carbohydrate pool in the cell. A half-drop of starved culture was inoculated on nitrogen agar plate with sterilized Pasteur pipette and incubated at 25°C. The YCB agar without nitrogen source was used as a negative control. The growth of yeast was observed every 2-4 days up to 14 days.

3.2.2.4 Production of starch-like substances

Production of starch-like substances was determined according to the mothod described by Wicherham (1951). After assimilation test was finished, Lugol's solution was added to glucose assimilation medium and ammonium sulfate assimilation medium. A positive result was indicated by the development in the culture of a color varying from dark blue to green.

3.2.2.5 Other Growth Tests

Other Growth Tests: Growth in vitamin-free medium was determined according to Komagata and Nakase (1967) in The Yeasts, a Taxonomic Study 4th ed and 5th ed. (Kurtzman and Fell, 1998; Kurtzman et al., 2011a). Cycloheximide resistant was determined according to Whiffen (1948) in The Yeasts, a Taxonomic Study 5th ed. (Kurtzman et al., 2011a). This experiment was examined in liquid Bacto Yeast Nitrogen Base with D-glucose (basal medium), with cycloheximide added to give a final concentration of either 100 ppm or 1000 ppm. The inoculation and detection was done in the same way as for the carbon assimilation test.

The ability to growth at high sugar concentration was examined according to the method described by Wickerham (1951). Young culture of yeasts was inoculated on a slant of 50% glucose agar medium. The growth of yeast was observed after 7 days.

The maximum growth temperature was examined according to the method described by Wickerham (1951). Young culture of yeasts was inoculated into YM broth medium. The growth of yeast was observed every week until 3 weeks.

3.2.3 Chemotaxonomic characteristics

Ubiquinone analysis: The analysis of ubiquinone system was carried out following the method of Nakase and Suzuki (1986). Cells grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose) at 25°C for 16-30 hr with shaking were harvested by centrifugation at 6,000 rpm for 5 min. Harvested cells from 400 ml of medium were washed with distilled water and freeze-dried. Dried cells are suspended in 50 ml chloroform/methanol (2:1) and kept at room temperature for one night. Cells were removed by paper filtration. Filtrate was evaporated to dryness using a rotary evaporator and then residues were dissolved with 0.5 ml of acetone. Ubiquinone was purified by using the preparative thin-layer chromatography (0.5 mm silica gel, 60F254 layers on 20 x 20 cm glass plate, Merck, with hexane:diethyl ether (85:15) as developer). A band of ubiquinone detected under short wave UV light was scrapped off. Yeast having respective ubiquinones, Q-6 (*Saccharomyces cerevisiae*, NBRC 10515), Q-7 (*Pichia anomala* NBRC 10213), Q-8 (*Saccharomycopsis vini* NBRC 1749), Q-9 (*Debaryomyces hansenii* var. *hansenii* NBRC 1751) and Q-10 (*Schizosaccharomyces pombe* NBRC 1608) were used as references. Scrapped powder was transferred to a tube and extracted with 1 ml of acetone. The solution was filtered with a 0.2 μm membrane filter and concentrated by the blow of N2 gas. Ubiquinone homologues were identified by HPLC (Cosmosil column (Waters, 5C18,4.6 mm x 250 mm), using methanol:isopropyl alcohol (2:1) as mobile phase at flow rate of 1 ml/min, detected at 275 nm, and identified by comparing with known ubiquinones as standards).

3.2.4 Nucleotide sequencing and phylogenetic analysis

Isolation of DNA was carried out by boiling of cells with lysis buffer. A loopful of yeast cells was transferred to 1.5 ml Eppendorf tube. The 100 μl of lysis buffer was added. Cell suspensions were boiled in water bath or metal block bath for 15 min. After boiling, 100 μl of 2.5 M potassium acetate (pH 7.5) was added and

placed on ice for 1 hr, and centrifuged at 14,000 rpm for 5 min. Supernatant was extracted twice with 100 μl of chloroform:isoamyl alcohol (24:1 v/v). DNA was precipitated with isopropanol, placed at 20°C for 10 min and centrifuged at 15,000 rpm for 15 min. DNA pellet was rinsed with 70% and 90% ethanol and then dried up (15-30 min at room temperature). The dried DNA was dissolved in 30 μl milli Q water.

Polymerase Chain Reaction (PCR) for D1/D2 domain of 26S rDNA. The divergent D1/D2 domain of 26S rDNA and ITS region were amplified with primers NL-1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'); ITS-4 (5'-TCC CTC CGC TTA TTG ATA TG-3') and ITS-5 (5'-GGA AGT AAA GTC GTA ACA AGG-3') respectively (White et al., 1990; Kurtzman and Robnett, 1998). Amplification was carried out in 100 μl reaction mixture conditioning 100 ng of genomic DNA, 2.5 U of Taq polymerase, 20 mM of each dNTP, 40 mM of each primer, 10 mM Tris-HCl and 1.5 mM $MgCl_2$. The reaction was pre-denatured at 94°C for 5 min, then repeated for 30 PCR cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2.5 min and then followed by the final extension at 72°C for 10 min. The amplified DNA was purified with QIAquick PCR Purification Kit according to the manufacturer's instruction. Visualization of the purified amplified DNA was performed by electrophoresis using 0.8% agarose gel in 1X TBE buffer and stained with ethidium bromide (8x10-5 μg/ml) and observed under UV illuminator.

Cycle sequencing of the D1/D2 domain and ITS were obtained with ABI PrismTM BigDyeTM Terminator Cycle sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) according to themanufacturer's instruction. The sequences of D1/D2 domain of 26S rDNA were compared by BLASTn Homology Search (http://www.ncbi.nlm.nih.gov/blast). Generated sequences were aligned with related species by using the CLUSTAL X ver. 1.8 computer programs (Thompson *et al*., 1997). The phylogenetic trees were constructed from the evolutionary distance data according to Kimura (1980) by the neighbor-joining method (Saitou and Nei, 1987). Sites where gaps existed in any sequences were excluded. Bootstrap analysis (Felsenstein, 1985; Felsenstein, 1988) is performed from 1,000 random re-samplings.

3.3 Determination of ethanol and xylitol production

Single colony of yeast isolates grown on YX medium at 30°C, 48 h was inoculated into Yeast-Peptone-Xylose (YPX medium containing 1% yeast extract and 2% peptone, 4% xylose (w/v) and pH 5.0) at 30°C, 200 rpm for 24 h. The culture transferred at 1% (v/v) into fresh YPX 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, 9,793 x g (10 min), resultant supernatants was analysed for ethanol by gas chromatography and for xylitol by high-performance liquid chromatography (HPLC). *S. stipitis* JCM 10742[T] (Japan Collection of Microorganisms, RIKEN BioResource Center, Japan) was used as control, a high efficient ethanolic xylose fermenting yeast.

Zygoascus meyerae ELP23 was selected yeast from the maximum ethanol production to study dynamic growth and ethanol production.

Zygoascus meyerae ELP23 was grown in YPX medium containing 1% yeast extract and 2% peptone, 4% xylose (w/v), pH 5.0 at 30°C for 24h. 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 72 h. The culture was 12 h interval sampled, measure growth cells. After that centrifuged and obtained supernatant was analyzed for ethanol and xylitol.

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. (Akaracharanya *et al*., 2011)

Xylitol was quantified by HPLC (Varian, Prostar, USA) using Lichrospher®100 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.

3.4 Experimental design of xylitol production

      C. tropicalis A26 was selected yeast from the maximum xylitol production to optimize to xylitol production.

3.4.1 Inoculum and cultivation

      Single colony of *C. tropicalis* A26 was transferred to 250 ml Erlenmeyer flasks containing 50 ml of YPX medium (1% yeast extract, 2% peptone and 4% D-xylose, w/v). The flasks were maintained under agitation (200 rpm) at 30˚C for 24-48h and the culture (1% v/v) was subsequently transferred into fresh YPX broth (50 ml in 250 ml Erlenmeryer flask) (200 rpm) at 30˚C for 24h. After 24 h of incubation, the cells were collected by centrifugation at 8000 rpm for 10 min, washed twice with steriled water and used for inoculum.

      The fermentation medium which consisted of xylose, peptone and yeast extract, pH and inoculum volume were varied according to the Plackett-Burman design and central compositie design. All fermentation run was carried out in 250 Erlenmeyer flask containing 50 ml medium at 30˚C and 200 rpm for 24 h. The culture was collected by centrifugation which obtained supernatant and analyzed for the residual xylose and xylitol production.

3.4.2 Plackett-Burman experimental design

      The Plackett-Burman design was used to screen the important parameter with respect to their main effects and not the interaction effects (Plackett and Burman, 1946). This present work was studied on the screening factors affecting xylitol production by *C. tropicalis* A26. The five factors including the concentration of xylose, yeast-extract and peptone, inoculum volume and pH value were selected as key factors affecting the xylitol production. Plackett-Burman factorial design in 8 run and 5 independent variables were used (Table 3.2). Based on Plackett-Burman factorial design, each factor was examined at two levels: -1 for low level and +1 for high level. All experiments were performed in triplicates and the average values of observation were used (Table 3.3). The effect of each variable was determined by following equation:

$$E_{Xi} = 2(\Sigma M_{i+} - \Sigma M_{i-})/N \tag{1}$$

Where E_{Xi} is the concentration effect of the tested variable. M_{i+} and M_{i-} represent xylitol production from trials where the variable (Xi) measured was present at high and low level, respectively. N is the total number of trials.

A statistical procedure was used to calculate the limit to which the effects of important independent variables were assigned. The significant level (*P*-value) of each main effect was determined using *F*-test.

Table 3.2 Variable for screening using Plackett-Burman design.

Variables	Parameter	Units	Low level (-1)	High level(+1)
X1	Xylose	g/l	20	80
X2	Yeast extract	g/l	5	20
X3	Peptone	g/l	10	40
X4	Inoculum value	%	5	20
X5	pH	-	4	6

Table 3.3 Experimental design and results of the Plackett-Burman design.

Run no.	X1	X2	X3	X4	X5	xylitol (g/l)
1	1	1	1	-1	1	20.01
2	-1	1	1	1	-1	6.96
3	-1	-1	1	1	1	8.25
4	-1	-1	-1	1	1	30.12
5	1	-1	-1	-1	1	10.84
6	-1	1	-1	-1	-1	22.82
7	1	-1	1	-1	-1	25.74
8	-1	-1	-1	-1	-1	9.36

X1, xylose (g/l); X2, yeast extract (g/l); X3, peptone (g/l); X4, inoculum (%); X5, pH.

3.4. 3 Central composite design

Response surface methodology was used to optimize the factors for enhancing xylitol production. In this study, a central composite design (CCD) with two factors (xylose and peptone) and five level, including three replicates at the center point, was used for fitting a secondary-order response surface (17). A CCD always contains twice as many star points as factors in the design. The star points represent low and high value for each factor in this design. To maintain rotatability, the value of α depends on the number of experimental runs in the factorial portion of the CCD. In this study, k=2 factors (xylose and peptone) could be written as

$$a=[2^2]^{1/4}=1.414 \tag{2}$$

Table 3.4 and Table 4.13 show the factors, their values, and the experimental design. The variables were xylose and peptone. The responses were of the xylitol production. A second-degree quadratic model was estabilished as Eq. (3) by using the method of least squares as follows:

$$Y=a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 + a_{11}X_{12} + a_{22}X_{22} \tag{3}$$

Where Y is the predicted response (xylitol production, g/l); X_1 and X_2 are the codes forms of the input variables (xylose and peptone); a_0 is constant; a_1 and a_2 are the linear coefficients; a_{12} is cross-product coefficient; a_{11} and a_{22} are the quadratic coefficients. The relation between the coded forms of the input variable and actual value of xylitol production is described by Eq. (4):

$$Xi = (Xi-X0)/ \Delta X \qquad\qquad (4)$$

Where Xi is the dimensionless coded value of the variable X1, X0 is the value of Xi at the center point, and ΔX is the step change.

The data from the experimental design were subjected to second-order multiple regression analysis using the least squares regression method to obtain the parameter estimators of the mathematics model. SPSS Statistics 17.0 and Statistica 5.0 software (Statsoft, USA) were used for regression analysis and graphical analysis of the data, respectively.

Table 3.4 Experimental variable, parameter, ranges and levels of independent variable in the central composite design.

Variables	Parameter (g/l)	Range and levels				
		-1.414	-1	0	1	1.414
X_1	Xylose	51.72	60	80	100	108.2
X_2	Peptone	0.2	1	3	5	5.8

3.5 Batch-fermentation of xylitol production

The batch cultivation was studied by using 5L stirred-vessal bioreactor (model MDL-8C, B.M. Marubishi, Japan) with a 2.5L working volume. The bioreactor was surrounded by water jacket for temperature control at 30˚C. The fermentation condition was under agitation speed at 200 rpm and 1 vvm of aeration. A 20% (v/v) of the cell suspension was inoculated and the fermentation was performed this condition. The culture was 24 h interval sampled for PLA-degrading enzyme activity assay.

3.6 Cloning of xylose reductase gene

Xylose reductase gene (*xyl*) was amplified directly from genomic DNA by PCR method. Primers specific for *xyl* was 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^{TM} DNA Polymerese (Fermentas, California). The PCR reaction mixture containing 20 ng of genomic DNA, 10 pmol of each primers, 2 µl of 10x PCR buffer (contained 15 mM MgCl2), 0.5U of Taq DNA polymerase, 0.02 mM of each dNTP and nuclease free water in the total volume of 20 µl was set up. The reaction was carried out at 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 45-60°C for 1 min, 72°C for 1 min and the last cycle with 72°C for 7 min. PCR product was determined by electrophoresis and purified by GeneJET^{TM} Gel Extraction Kit (Fermentas, California) then ligated to pTZ57R/T vector (InsTAclone^{TM} PCR cloning kit, Fermentas, California).

The ligation reaction was transformed into *Escherichia coil* DH5α competent cells using method described by Sambrook and Russell (2001). Colonies grown on LB agar containing 100 µg/ml ampicillin, 20 mg/ml X-Gal and 100 mM IPTG after incubation at 37°C for 24 h were collected. Clone harbouring the *xyl* was determined by colony PCR. Confirmation of the *xyl* gene was performed by DNA sequence analysis.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation and source of samples

One hundred and twenty-nine yeasts were isolated from 60 herbivore animal feces including the fecal samples of 35 elephants, 1 giraffe, 3 cows, 12 buffalo, 2 kangaroos, 4 zebras, 1 languor and 2 barking deer which were collected from various areas of Thailand; three soil samples were collected in Japan (Table 4.1).

Table 4.1 Location, sample, isolate number, identity and identification of isolates.

Location	Sample	Isolate no.	% Similarity	Nearest Species
	Feces from:			
Bangkok	Macaque	A5	99.8	*Candida tropicalis*
	Zebra	I1, A28	99.8	*Candida tropicalis*
	Baring deer	E1, F1	100	*Candida tropicalis*
	Languor	H1	99	*Issatchenkia terricola*
	Elephant	ELP7	100	*Candida tropicalis*
Nakhonratchasima	Giraffe	A21	100	*Candida tropicalis*
	Kangaroo	A18	100	*Candida tropicalis*
	Kangaroo	L1	99.8	*Candida albican*
	Zebra	A20, M1	99.9	*Candida tropicalis*
Khonkaen	Cow	A26	100	*Candida tropicalis*
Kalasin	Buffalo	A8, A16	100	*Candida tropicalis*
	Cow	A14, A15, A12	100	*Candida tropicalis*
Udonthani	Cow	A9	100	*Candida tropicalis*
Prachinburi	Elephant	A1, G1	100	*Candida tropicalis*
Ayutthaya	Elephant	ELP9	100	*Candida tropicalis*
		ELP21, ELP24	100	*Trichosporon mycotoxinivorans*
		ELP22	100	*Kluyveromyces marxianus*
		E23	99.8	*Zygoascus meyerae*
		ELP20	99.4	*Sporopachydermia lactativora*

Table 4.1 (Continued).

Location	Sample	Isolate no.	% Similarity	Nearest Species
	Feces from:			
Chiangmai	Elephant	ELP16	99.8	*Candida tropicalis*
		ELP19	94.0	*Wickerhamomyces onychis*
Nakonpathom	Elephant	ELP25	99.6	*Sporopachydermia lactativora*
Kanchanaburi	Elephant	ELP26, ELP35	100	*Candida tropicalis*
		ELP34	99.8	*Meyerozyma caribbica*
Surin	Elephant	ELP27	100	*Kluyveromyces marxinus*
		ELP29	100	*Meyerozyma caribbica*
Prachuapkhirikhan	Elephant	ELP30	100	*Candida tropicalis*
		ELP32	99.4	*Sporopachydermia lactativora*
Chonburi	Elephant	ELP37, ELP40	99.8-100	*Trichosporon mycotoxinivorans*
		ELP38	100	*Meyerozyma caribbica*
		ELP39, ELP48, ELP49	100	*Candida tropicalis*
		ELP43, ELP46,	99.4	*Sporopachydermia lactativora*
		ELP45	100	*Kluyveromyces marxinus*
		ELP44	100	*Candida tartarivorans*
Chachoengsao	Buffalo	BUF5-3, BUF4-2	100	*Candida tropicalis*
		BUF4-3, BUF12-1	100	*Candida tropicalis*
		BUF4-4, BUF7-1	100	*Candida tropicalis*
		BUF8-1, BUF8-2	100	*Candida tropicalis*
		BUF9-2, BUF9-4	100	*Candida tropicalis*
		BUF9-5	100	*Candida tropicalis*
		BUF3-6, BUF3-16	99.8	*Candida mengyniae*
		BUF3-18, BUF3-19	100	*Candida parasilopsis*
		BUF1-1, BUF1-2	99.4	*Sporopachydermia lactativora*
		BUF3-5, BUF4-1	99.4	*Sporopachydermia lactativora*

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Table 4.1 (Continued).

Location	Sample	Isolate no.	% Similarity	Nearest Species
	Feces from:			
Chachoengsao	Buffalo	BUF8, BUF9	100	*Trichosporon asahii*
		BUF10, BUF11	100	*Trichosporon asahii*
		BUF2-1, BUF2-2,	100	*Trichosporon asahii*
		BUF2-4, BUF3-3,	100	*Trichosporon asahii*
		BUF3-8, BUF3-9,	100	*Trichosporon asahii*
		BUF3-10	100	*Trichosporon asahii*
Phetchaburi	Wild elephant	7-4, 9-6	100	*Candida tropicalis*
		4-11, 4-12, 4-13	100	*Candida stellimalicola*
		2-1, 3-1, 5-1, 5-3,	99.1	*Candida rugosa*
		7-6, 9-3, 9-4,	99.1	*Candida rugosa*
		10-1, 10-2,	99.1	*Candida rugosa*
		10-3, 10-8	99.1	*Candida rugosa*
		4-7	99.8	*Pichia occidentalis*
		10-7	99.7	*Issatchenkia orientalis*
		4-6, 4-9, 4-10	99.7	*Pichia kluyveri*
		4-1	99.1	*Geotrichum silvicola*
		4-2, 4-3, 4-4, 4-5	96.0	*Galactomyces geotrichum*
		4-14	99.7	*Cyberlindnera mrakii*
		6-2,6-3, 6-4, 6-5, 6-6,	100	*Trichosporon asahii*
		6-8, 6-9, 6-10,	100	*Trichosporon asahii*
		3-5,3-6, 3-7	100	*Trichosporon asahii*
Hiroo park, Japan	Soil	11-1	99.8	*Candida pseudolambica*
		11-2	99.5	*Cyberlindnera saturnus*
		11-4	100	*Geotrichum vulgare*
		12-2, 12-3, 12-4,12-5	99.1	*Geotrichum silvicola*
		15-1, 15-2, 15-3	100	*Barnettozyma californica*
		11-3, 11-5,11-6,11-7	100	*Trichosporon akiyoshidainum*
	Total	129 isolates		

4.2 Identification and characterization of isolates

One hundred and twenty-nine isolates were divided into ten groups and were identified based on their phenotypic characteristics and the D1/D2 domain of large subunit ribosomal RNA gene (LSU rRNA gene) sequence analyses of the representative isolate in each group (Figure 4.1). One hundred and twenty-nine isolates were assigned to ascomycetous (103 isolates), and basidiomycetous (26 isolates) yeasts.

Sixty-two isolates were divided into Group I (a) to I (i) based on the phenotypic properties (Table 4.2). The representative isolates were identified based on D1/D2 domain of LSU rRNA gene sequence and phylogenetic analyses as members of genus *Candida* (Figure 4.1). Yeast strains in the genus *Candida* are widely distributed in nature and have been isolated from various sources both in terrestrial and aquatic habitats. Therefore their phenotypic characteristics have been diverse and not specified to differentiate the species of the genus. (Urubschurov *et al*., 2008; Urubschurov *et al*., 2011; Butinar *et al*., 2005)

Group I (a) contained 40 isolates. The cells are globose in shape. They formed pseudohyphae, consisting of cylindrical cells and branched chains. All isolates fermented glucose, galactose, sucrose, maltose and trehalose. They assimilated galactose, glucose, cellobiose, d-manitol, Maltose, melezitose, α-methyl-d-glucoside, sucrose, trehalose and d-xylose. They did not assimilated arabinose, erythritol, glycerol, inositol, dl-lactate, lactose, melibiose, raffinose, l-rhamnose and d-ribose. They did not grow on 50% glucose which was different from *C. tropicalis* NRRL Y-12968^{T} (Table 4.2). Their D1/D2 LSU rRNA gene sequences were 100% similarity to *C. tropicalis* NRRL Y-12968^{T} (Figure 4.1). Therefore, they were identified as *C. tropicalis*.
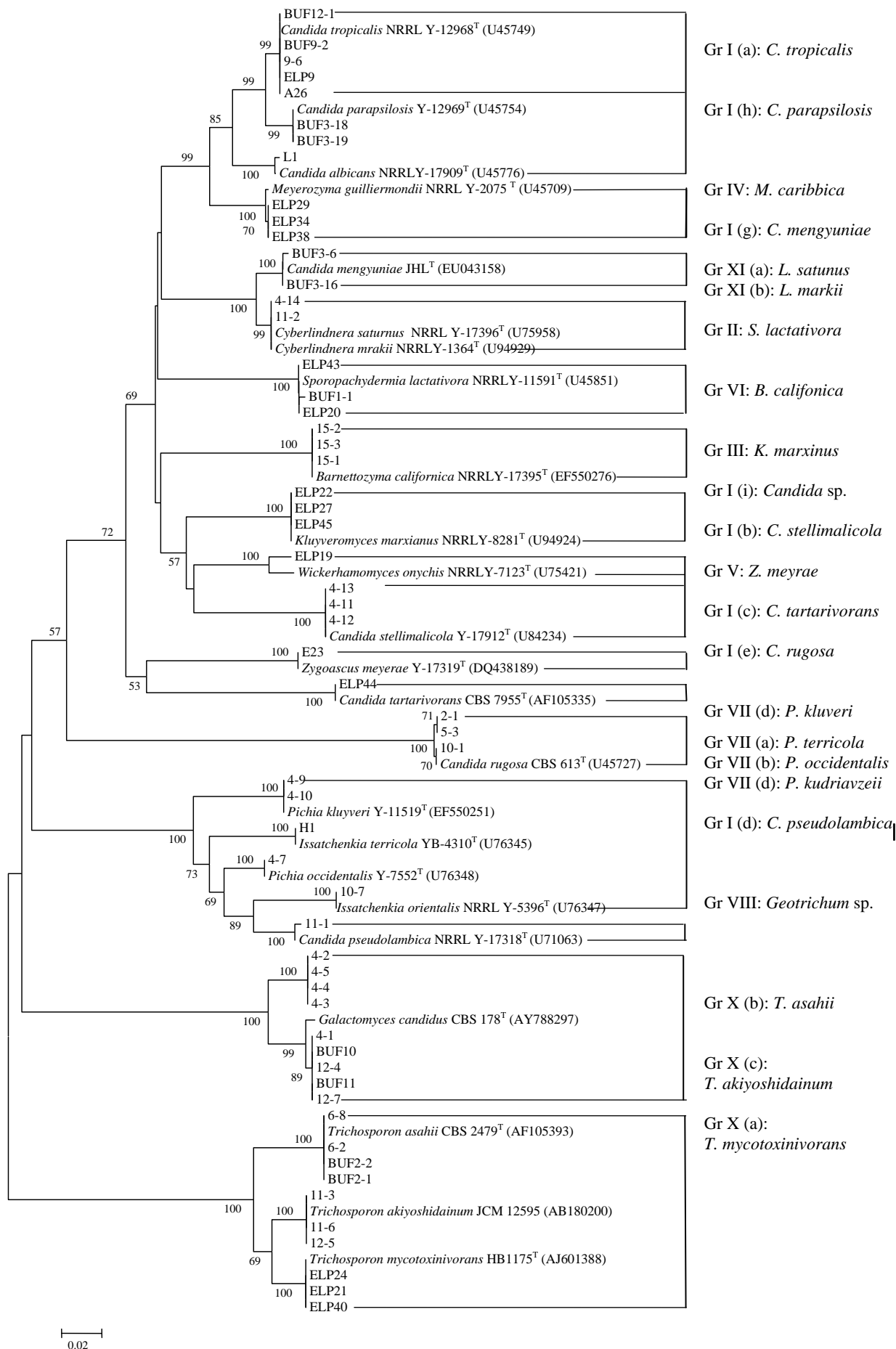
Figure 4.1 Phylogenetic tree of the representative isolates in Group I to X constructed by the neightbor-joining method based on D1/D2 domain of LSU rRNA gene sequences.

This group was the dominant species which was almost found in all the samples of animal feces (Table 4.1). As reported by Urubschurov *et al*. (2011), this species was found in many hosts such as gut of pig, insect, horse and the ruminants cow and sheep. *Candida tropicalis* is one of the most frequently encountered clinical yeast species after *C. albicans* (Moran *et al*. 2002). The species was also known for the ability to degrade hydrocarbons and perform lipid biotransformation. Among the recent reports, their applications were in the fermentation of cocoa beans (Ardhana and Fleet, 2003) and the degradation of polyphenols in waste water (Ettayebi *et al*. 2003).

Group I (b) contained 3 isolates, 4-11, 4-12, 4-13. These strains could ferment in glucose only and assimilated d-mannitol (slow), d-xylose (positive) and glycerol (latent), which were different characteristic from *C. stellimalicola* NRRL Y-17219^{T}. Their D1/D2 LSU rRNA gene sequences were 99-100% similar to *C. stellimalicola* NRRL Y-17219^{T} (Figure 4.1). Suzuki *et al*. (1994) first reported that the *C. stellimalicola* strain was isolated from Ma-Fueng (star fruit) in Thailand; however they were found in traditional fermented buffalo milk of West Sumatera (Dadih) (Jatmiko *et al*., 2012). This species most frequently was exposed in tropical zones such as Thailand and Indonesia.

Group I (c) contained one isolate. Isolate ELP45 could ferment glucose, galactose, sucrose (latent), lactose (latent), raffinose (slow), and trehalose (weak). Differential characteristics of this strain grew on maltose (latent), trehalose (latent), d-xylose (slow), l-arabinose (slow), d-ribose (latent), dl-lactate (slow), 50% glucose and 10% NaCl with 5% glucose. Their D1/D2 LSU rRNA gene sequences were 100% similarity to *Candida tartarivorans* CBS 7955^{T}(Figure 4.1). Therefore, they were identified as *Candida tartarivorans*.

This species was described by Fonseca *et al*., 2000, which were isolated from red wine lees piled in Portugal. *Candida tartarivorans* utilized L-tartaric acid, major organic acids in grapes and wines (Fonseca *et al*., 2000). There was one isolate of elephant feces, maybe contaminated from fruit on which elephant fed.

Group I(d) contained one isolate. Isolate 11-1 utilized ethanol and some organic acids such as dl-lactic acid and succinic acid. The growth on d-xylose was slow and lacked growth at 37°C. These phenotypic properties were similar to *C*.

pseudolambica NRRL Y-17318[T] (Table 4.2). On the basis of D1/D2 LSU rRNA gene sequence analysis, the isolate showed 99.8% similarity with *C. pseudolambica* NRRL Y-17318[T]. Nakase *et al.* (1976) described *C. pseudointermedia* as accommodating three strains recovered from Kamaboko, a popular traditional food made of fish paste in Japan. In this study, the isolate 11-1 was isolated from soil in Hiroo park, Japan.

Group I(e) contained 11 isolates, All isolates were absent the ability to ferment carbon source, but utilized glucose, galactose, dl-lactate, d-mannitol, xylose and glycerol. They could grow in 50% glucose while type strain of *C. rugosa* CBS 613[T]can slowly grow in 50% glucose. From the physiological aspect of this group is found an almost similarity with type strain. In contrast, the morphology of this group was a bit different from *C. rugosa* CBS 613[T] in mycocandida pseudohyphae type (Figure 4.3).

Isolate 2-1 was a representative isolate for this group. After growth on YM medium for 4 days at 25°C, the cells of isolate 2-1 were ovoid and elongate, (1.5-2.5µm) x (4.0-11.0µm), single or in chain (Figure 4.3), flocculent in bottom and thin pellicles. Colonies were cream colored, smooth when it first isolated and became rough when it was kept on agar. Pseudohyphae was formed by Dalmau plate culture on corn meal agar after 7 days at 25°C, pseudohyphae consist of chains of elongate cells (Candida pseudohyphae type) (Figure 4.3). The identification was evaluated by D1/D2 sequencing, and a difference of five substitutions was found, indicating that the group would be differentiated from *C. rugosa* CBS 613[T] (Figure 4.2). Nevertheless, this data was not enough to support a new species; the nucleotide sequence of other genes is required to confirm for this group.

Group I (f) contained one isolate. Isolate L1 had globose cell-shape and formed branched pseudohyphae. It could ferment galactose, sucrose, lactose and raffinose, assimilated sucrose, trehalose, melezitose (slow) and α-methyl-D-glucoside, but did not assimilate melibiose, l-arabnose, and d-ribose, grew on vitamin-free medium, 50% glucose, 10% NaCl with 5% glucose (Table 4.2). *C. albicans* has variable characteristics. Their D1/D2 LSU rRNA gene sequences were 99.8% similar with *C. albicans* NRRLY-17909[T], 2 nucleotides difference. Therefore, it was identified as *C. albicans.* This isolate was concerning to the health of the animal and the kangaroo in the zoo might be infected.
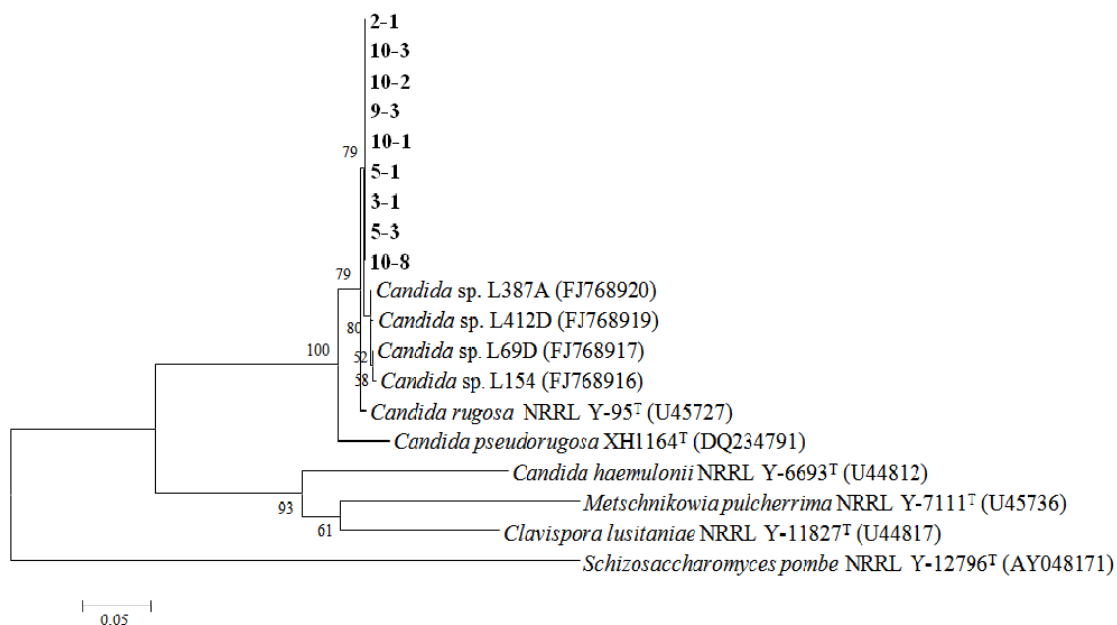
Figure 4.2 Phylogenetic tree of group I (e) and other strains were constructed by the neighbor-joining method based on D1/D2 domain of LSU rRNA gene sequences.
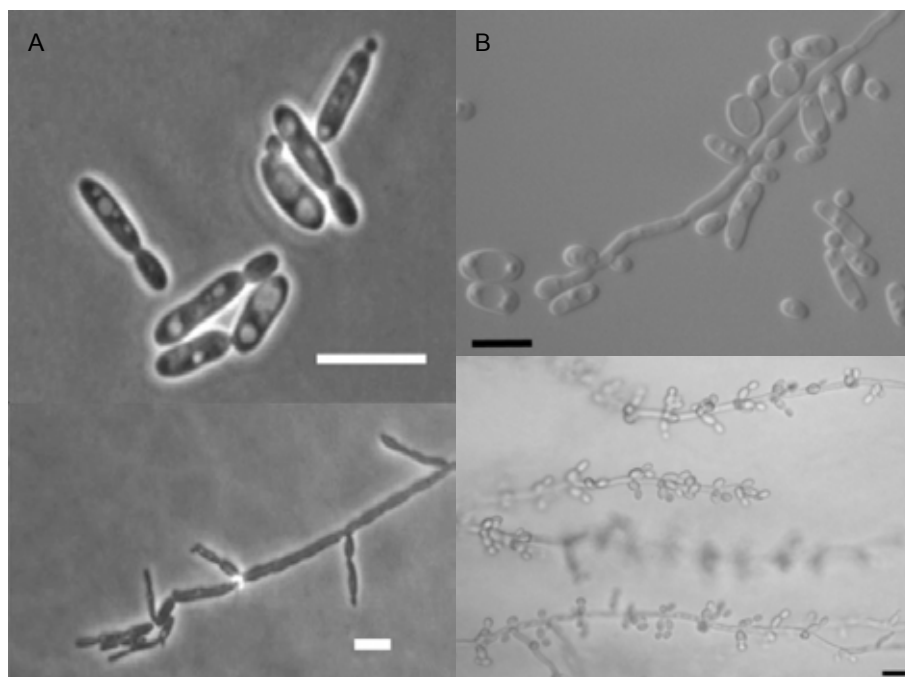
Figure 4.3 Morphology of *C*. rugosa CBS 613[1] and *Candida* sp.2-1 as determined by light microscopy. *C. rugosa* CBS 613[1], budding cells after 3 days, 25°C , YM agar (top) and pseudohypha with blastoconidia after 14 days, 18°C, YCBAS agar (bottom) (A). *Candida* sp.2-1: Vegetative cell grown in YM medium on 4 days at 25°C (top) and pseudohypha with blastoconidia after 7 days in corn meal at 25°C (bottom) (B). [1]Data from The Yeasts, a Taxonomic Study 5th (Lachance *et al*.,2011).

Group I (g) contained 2 isolates. BUF3-6 and BUF3-16 were isolated from the 2-month-old Murrah buffalo. Differential characteristics with type strain, could assimilate maltose, melezitose and dl-lactate (positive) but could not assimilate melibiose (negative) (Table 4.2). Their D1/D2 LSU rRNA gene sequences were 99.8% similarity with *Candida mengyuniae* in *Lindnera* clade (Figure 4.1).

Candida memgyuniae, sulfonylurea herbicide-resistant yeast, was isolated from fields, which were often soaked with discharges from metsulfuron-methyl manufacturing facilities (Xing-Huang *et al*., 2009). The occurrence of *C. mengyuniae* in Murrah buffalo with 2-month age may show that this buffalo could be getting food with contaminated herbicide.

Group I (h) contained 2 isolates, BUF3-18 and BUF3-19. Two isolates were assimilated of xylose, galactose, sucrose, L-arabinose, maltose, trehalose (latent), α-methyl-d-glucoside, mannitol, glycerol, and melezitose but not assimilated ribose (Table 4.2). The phenotypic characteristics were similar to type strain of *C. parasilosis*. Their D1/D2 LSU rRNA gene sequences were 99.8-100% similarity *Candida parasilosis* NRRL Y-12969[T] (Figure 4.1). Therefore, they were identified as *C. parasilosis*.

Group I (i) contained one isolate. Isolate ELP19 showed its phenotypic characteristics similar to *W. onychis*, except it did not ferment raffinose and not produce ascospore. Differential characteristic of this strain from *Candida* species were the growth on trehalose (latent), raffinose (weak), d-xylose (latent), l-arabinose (latent), mannitol (latent), d-glucitol (latent), α-Methyl-D-glucoside (latent), salicin (latent). Their D1/D2 LSU rRNA gene sequences were 94% similar with *Wickerhamomyces onychis* NRRLY-7123[T] (Figure 4.1), 24 nucleotides substituent. The D1/D2 domains among 500-600 nucleotides, so the different nucleotide 6 or more nucleotide (1%) indicated that the strains were different species (Kurtzman *et al*., 2011d; Kurtzman and Robnett, 1998). Therefore, it will be proposed as a new species. In 2008, Liu and staff described four new species of *Candida* on the basis of D1/D2 LSU rRNA gene sequence analysis and found that the two species, *C. yuanshanica* and *C. dajiaensis*, are closely related to *W. onychis*. (Liu *et al*., 2008). The similarity of *Candida* sp. ELP19 compared with *W. onychis, C. yuanshanica* and *C. dajiaensis* are shown in Table 4.3. and Figure 4.5.

Table 4.2 Differential characteristics of the isolates in Group I (a) to I (g) and related type strains.

Characteristics	Gr. I(a)	C. tropicalis [a]	Gr. I(b)	C. stellimalicala [a]	Gr. I(c)	C. tartarivorans [a]	Gr. I(d)	C. pseudolambica [a]	Gr. I(e)	C. rugosa [a]	Gr. I(f)	C. albicans [a]	Gr. I(g)	C. mengyuniae [b]	Gr. I(h)	C. parasilosis [c]
Fermentation of																
Glucose	+	+	s	-	+	+	+	+	-	-	+	+	+	+	+	+
Galactose	+	+	-	-	+	+	-	-	-	-	+	v	n	-	n	v
Sucrose	+	v	-	-	l	l	-	-	-	-	+	v	n	+	n	-/s
Maltose	+	+	-	-	-	l	-	-	-	-	+	+	n	-	n	-/s
Raffinose	-	-	-	-	s	l	-	-	-	-	+	-	n	w	n	-
Trehalose	+	+	-	-	w	l	-	-	-	-	n	v	n	-	n	-/s
Assimilation of																
L-Arabinose	-	-	-	-	s	+	-	-	-	-	v	-	-	-	+	+
Cellobiose	+	v	-	-	+	s	-	-	-	-	-	-	-	v	-	-
Erythritol	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Ethanol	+	+	+	+	-	w	+	+	+	+	s	+	n	n	+	+
Galactose	+	+	-	-	+	+	-	-	+	+	+	+	+	-	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	v	l	+	+	+	-	-	+/l	+	v	-	+	+	+	+
Inositol	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
DL-Lactate	-	v	+	+	s	-	+	+	+	v	+	+	n	+	-	-
Lactose	-	-	-	-	+	+	-	-	-	-	-	-	n	-	-	-
D-Mannitol	+	+	s	+	+	+	-	-	+	v	+	+	+	+	+	+
Maltose	+	+	-	-	+	+	-	-	-	-	+	+	-	+	+	+
Melezitose	+	v	-	-	l	s	-	-	-	-	-	s	-	+	+	+
Melibiose	-	-	-	-	l	+	-	-	-	-	v	-	+	-	-	-
α-Methyl-D-Glucoside	+	v	-	-	+	s	-	-	-	-	v	+	+	v	+	+
Raffinose	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Ribose	-	v	-	-	l	s	-	-	-	-	v	-	-	-	v	-
Sucrose	+	v	-	-	+	+	-	-	-	-	v	+	+	+	+	+
Succinate	+	+	+	+	s	l	+	+	+	v	+	+	-	-	+	+
Trehalose	+	+	-	-	+	+	-	-	-	-	v	+	+	+	+	l
D-Xylose	+	+	+	w	s	+	s	v	+/l	v	+	+	+	+	+	+

Table 4.2 (Continued).

Characteristics	Gr. I(a)	C. tropicalis [a]	Gr. I(b)	C. stellimalicala [a]	Gr. I(c)	C. tartarivorans [a]	Gr. I(d)	C. pseudolambica [a]	Gr. I(e)	C. rugosa [a]	Gr. I(f)	C. albicans [a]	Gr. I(g)	C. mengyuniae [b]	Gr. I(h)	C. parasilosis [c]
Growth in																
Vitamin-free	l	v	-	-	-	-	+	+	-/w	-	+	v	n	+	n	-
50%Glucose	+	+/l	-	n	+	-	-	-	+	l	+	v	n	+	n	+
10% NaCl/ 5% Glucose	l	l	-	-	+	-	-	-	l	n	-	v	n	+	n	+
Growth at 37°C	+	+	+	+	+	n	+	-	+	+	+	v	n	+	n	+

+, positive; -, negative; l, latent (longer than 7 days), s, slow; w, weak; v, variable; n, no data.

[a] Data from Lachance *et al*. (2011);

[b] Data from Xing-Huang *et al*. (2009);

[c] Data from Bourcier et al. (2003).

Presently, D1/D2 LSU rRNA gene sequencing is not enough to identify new species; a study is needed in multiple genes. *W. onychis* is closely related to *W. bovis, W. rabaulensis* and *C. odintsovae* by D1/D2 LSU rRNA gene sequencing, to separate by multigene sequence analysis. The internal transcribed spacers 1 and 2 of the rDNA (ITS) was studied to identify new species (Figure 4.6)

Characterization of *Candida* sp. ELP19 sp.

Growth in 5% malt extract agar after 3 days at 25 ºC, cells were ovoid to cylindrical (2.0-4.0µm x 4.0-10.0µm) and occured singly, in pairs or in short chains (Figure 4.4). Vegetative reproduction proceeds by multilateral budding.

Growth in 5% malt extract agar after 3 days at 25 ºC, the streak culture was cream-coloured, butyrous, glistening and smooth. The margin is irregular.

Dalmau plate on cornmeal agar after 7 days at 25°C, plentifully formed under the coverglass, pseudohyphae consisted of permitative pseudohyphae. Septate hyphae were not formed.

Formation of ascospore. Ascospores were not produced on 5% malt extract agar, Fowell's acetate agar, Gorodkowa agar or YM agar after 8 week at 25°C.

Physiological and growth characteristics. The results of the physiological and growth characteristics tested were shown in Table 4.4 Thin pellicles were formed on the surface of YM medium. The type strain ELP19 was isolated from elephant feces collected in Chiangmai province, Thailand. ELP19 was deposited at Japan Collection of Microorganisms (JCM), Wako, Saitama, Japan.
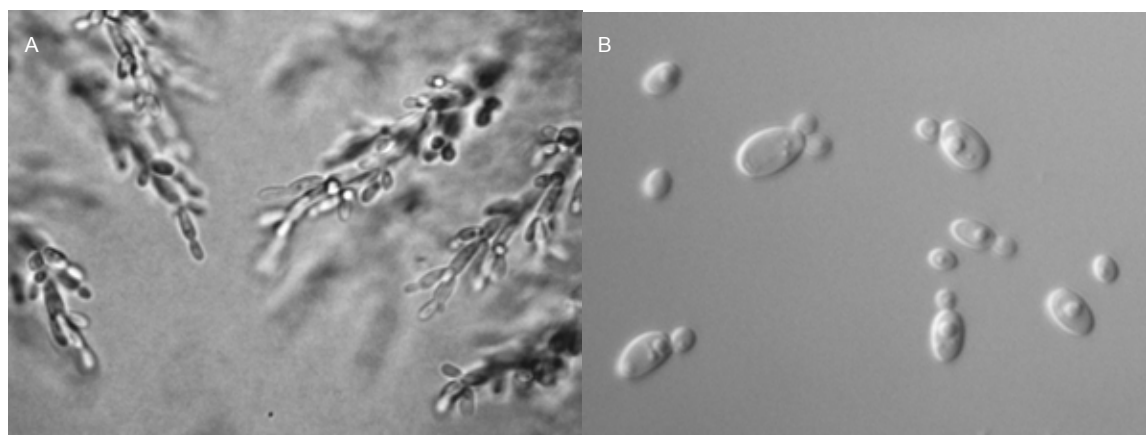
Figure 4.4 Morphology of *Candida* ELP19 as determined by light microscopy. Pseudomycelia formed on slide culture with corn meal agar after 5 days at 25°C (A). Vegetative cell grown in 5% malt extract agar 3 days at 25 °C (B).
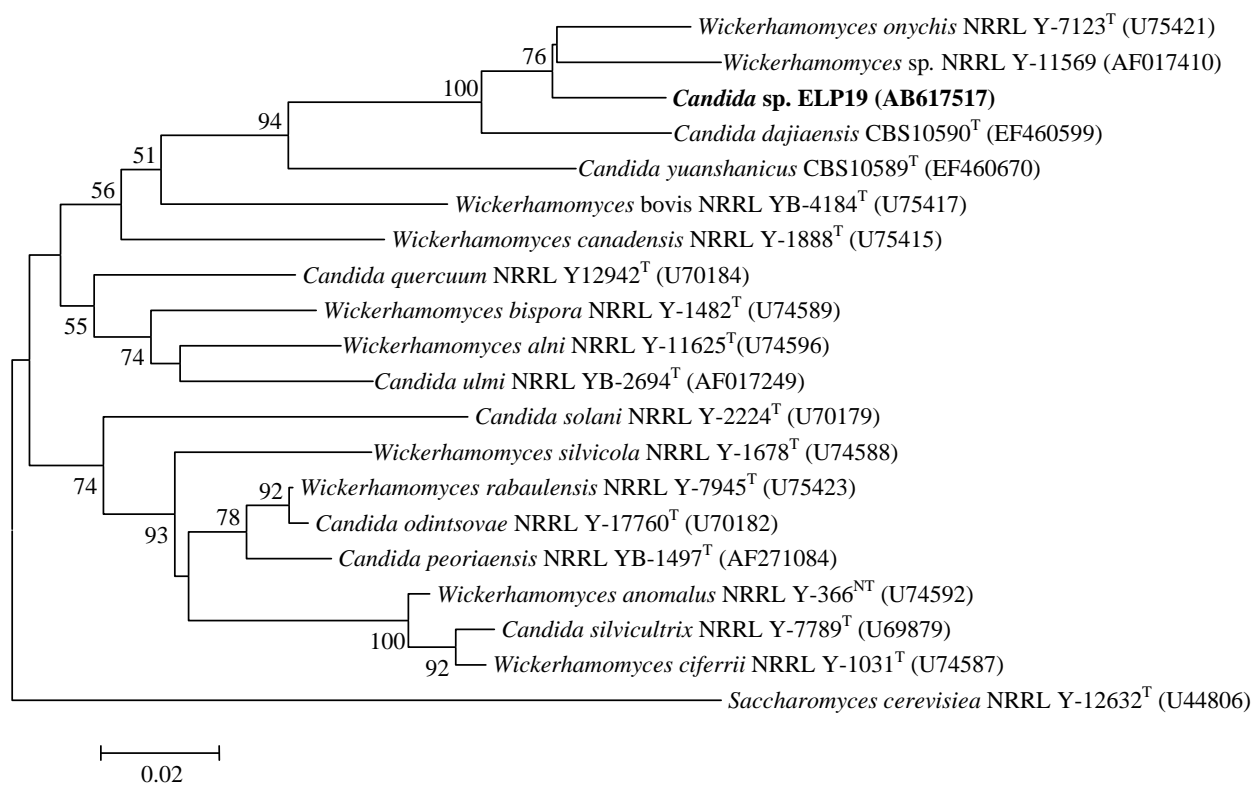
Figure 4.5 Neighbor-joining phylogenetic tree based on the D1/D2 domains of the large subunit (26S) rRNA gene showing the relationship of *Candida* species and related *Wickerhamomyces* and *Candida* species.
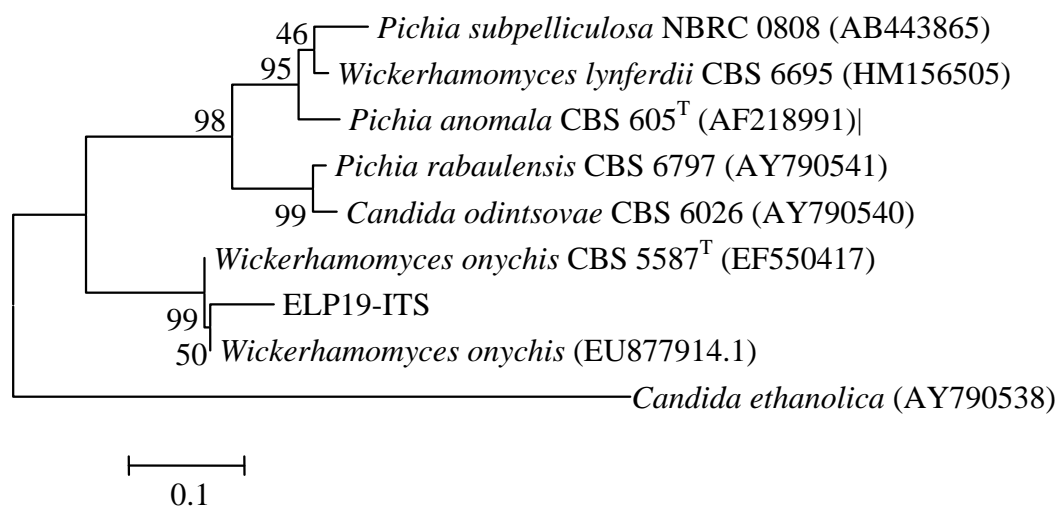
Figure 4.6 Neighbour-joining phylogenetic tree base on the ITS region showing the relationship of *Candida* species and related *Wickerhamomyces* and *Candida* species.

Table 4.3 Physiology and biochemical characteristics of *Candida* sp. ELP19 and other type strains.

Characteristics	ELP19	*W. onychis*[a]	*C. dajiaensis*[b]	*C. yuanshanicus*[b]
Fermentation of				
Glucose	+	+	+	+
Galactose	-	-	-	-
Sucrose	+	+	l	-
Maltose	-	-	-	-
Lactose	-	-	-	-
Raffinose	-	w	-	-
Trehalose	-	-	-	-
C- assimilation of				
D-Arabinose	-	v	-	-
L-Arabinose	l	v	+	+
Cellobiose	+	+	+	+
Citrate	+	+	-	l
Erythritol	-	-	-	-
Ethanol	+	+	-	-
Galactose	-	-	-	-
Galactitol	-	-	-	-
D-Glucitol	l	+	+	-
D-Gluconate	l	+	-	-
D-Glucose	+	+	+	+
D-Glucosamine	-	-	n	n
Glycerol	+	+	+	+
Hexadecane	-	-	n	n
Inositol	-	-	-	-
Inulin	-	v	-	-
2-keto-D-Gluconate	-	-	-	-
5-keto-D-Gluconate	-	-	-	-
Lactose	-	-	-	-
DL-Lactate	+	+	+	-
Maltose	+	+	+	+
D-Mannitol	l	+	+	w
Melezitose	+	+	+	+
Melibiose	-	-	-	-
Methanol	-	-	-	-
α-Methyl-D-glucoside	l	+	+	+
N-acetyl-D-Glucosamine	-	-	-	-
Raffinose	vw	+	-	-
L-Rhamnose	-	-	-	-
D-Ribose	-	-	-	-

Table 4.3 (Continued).

Characteristics	ELP19[T]	W.onychis[a]	C.dajiaensis[b]	C. yuanshanicus[b]
C- assimilation of				
Ribitol	-	-	w/+	-
Salicin	l	+	+	+
Soluble starch	-	-	-	-
L-Sorbose	-	-	-	-
Succinate	+	+	+	-
Sucrose	+	+	+	+
Trehalose	l	+	w	l
D-Xylose	l	+	+	+
N- assimilation of				
Cadavarine	+	n	-	-
Ethylamine	+	n	-	-
L-Lysine	+	n	+	+
Nitrate	-	-	-	-
Nitrite	-	n	-	-
Saccharate	-	-	-	-
Growth in				
Vitamin-free	-	-	n	n
50%Glucose	-	n	-	-
10%NaCl/5%Glucose	-	v	-	-
starch formation	-	-	-	-
Urease	-	n	-	-
0.01% Cycloheximide	-	n	-	-
0.1% Cycloheximide	-	n	-	-
Growth at 35°C	+	n	-	+
Growth at 37°C	+	+	-	+
Growth at 40°C	-	n	-	-
Ubiquinone	7	7	n	n

+, positive; -, negative; l, latent (longer than 7 days), s, slow; w, weak; v, variable;

n, no data.

[a]Data from Kurtzman *et al*. (2011b);

[b]Data from Liu *et al*. (2008);

Group II contained 11 isolates. They assimilated glucose (latent), l-sorbose (latent), inulin (weak), d-xylose (latent), ethanol (latent), ribitol, mannitol (latent) and grew on vitamin free medium (weak). They neither fermented sugars nor assimilated cadavarine (Table 4.4). Their D1/D2 LSU rRNA gene sequences were 99.6-99.4% similarity with *Sporopachydermia lactativora* NRRL Y-11591[T] (Figure 4.1). They were identified as *S. lactativora*.

S. lactativora was appears to be cosmopolitan in distribution – such as soil, fermented food, decaying agriculture residue and bird feces in Antarctica (Nitiyon *et al*., 2011; Lim, *et al.,* 2011; Fell and Phaff, 1967) and this strain grows on high temperature (37°C or more). (Rodrigues de Miranda, 1984).

Group III contained 3 isolates, ELP22, ELP27 and ELP45. These isolates could ferment glucose, galactose, sucrose and raffinose. Differential characteristics of these group were the assimilation of galactose, cellobiose (latent), lactose, d-xylose, l-arabinose, ethanol (latent) glycerol, ribitol (latent), D-mannitol (latent), D-glucitol (latent), salicin (latent), and DL-lactate(weak), but negative assimilation on l-sorbose, D-ribose and citrate. These strains could grow on 50% glucose which *K. marxianus* NRRLY-8281[T] could not (Table 4.4). The nucleotide sequence of D1/D2 LSU rRNA gene showed 100% similarity with *K. marxianus* NRRLY-8281[T]; they were clustered within their nearest phylogenetic neighbors (Figure 4.1). Therefore they were identified as *K. marxianus*.

Group IV contained 3 isolates, ELP29, ELP34 and ELP38. The differential characteristics of these strains could ferment glucose, galactose (latent), sucrose, maltose (weak), raffinose (weak), trehalose (latent) and assimilation on l-sorbose, Inulin(weak), soluble starch(weak), L-arabinose, galactitol, citrate, vitamin free medium (latent) (Table 4.4), when compared with *Meyerozyma caribbica* NRRL Y-27274[T] . Kurtzman and Suzuki (2010) reported that *Meyerozyma caribbica* and *M. guilliermondii* showed no differences in standard fermentation and growth tests. *M. caribbica* forms Saturn-shaped ascospores, in contrast to the hat-shaped ascospores of *M. guilliermondii*, but both species showed sporulate poorly, and the species-separating criterion was uncertain. Consequently, it is recommended that the two species could be separated by the differences in D1/D2 LSU rRNA gene sequences.

Their D1/D2 LSU rRNA gene sequences were 100% similar with *Meyerozyma caribbica* NRRLY-27274[T] (Figure 4.1). Therefore they were identified as *Meyerozyma caribbica.*

Group V contained one isolate. Isolate E23 assimilated galactose (latent), L-sorbose (latent), sucrose (latent), maltose (latent), cellobiose (latent), raffinose (latent), meleziose (latent), soluble starch (latent), ribitol (latent) and 0.1% cycloheximide (latent) after 7 days. It neither fermented raffinose nor assimilated ribose (Table 4.4). Their D1/D2 LSU rRNA gene sequences were 100% similar with *Zygoascus meyerae* Y-17913[T] (Figure 4.1). Therefore, the isolate was identified as *Z. meyerae. Zygoascus meyerae* was distributed in grape must and rotten fruits (Smith *et al*. 2005). So this isolated from elephant feces; that fruit is their normal feed.

Group VI contained 3 isolates, 15-1, 15-2 and 15-3. The morphology of this group showed conjugation between a cell and its bud (Figure 4.7). Isolates fermented glucose, some utilized sucrose (latent), sorbose, maltose (latent), trehalose (latent), rhamnose (slow) and cirate (latent) as carbon sources and utilized nitrate as nitrogen source (Table 4.4). Their D1/D2 LSU rRNA gene sequences were 100% similarity with *Barnettozyma californica* NRRLY-17395[T]. Therefore, they were identified as *Barnettozyma californica.*

Barnettozyma californica appears to have worldwide distribution, as soil, water, tree fluxes and animal dung, and the acidic soils of the Iberian Pyrite Belt (Gadanho *et al*, 2006; Kurtzman *et al*., 2008). Three isolate were isolated from soil of Hiroo park in Japan.

Table 4.4 Characteristics of the isolate in GroupII, III, IV, V, VI and related species.

Characteristics	Gr. II	*S. lactativora* [a]	Gr. III	*K. marxianus* [b]	Gr. IV	*M. caribbica* [c]	Gr.V	*Z. meyerae* [d]	Gr.VI	*B. californica* [e]
Fermentation of										
Glucose	-	-	+	+	+	+	+	+	+	+
Galactose	-	-	+	s	l	+	w	w	-	-
Sucrose	-	-	+	+	+	+	w	w	-	-
Maltose	-	-	-	-	-/w	v	-	-	-	-
Lactose	-	-	-	v	-	-	-	-	-	-
Raffinose	-	-	+	+	w	+/w	-	w/l	-	-
Trehalose	-	-	-	-	l	+	s	w/l	-	-
C-assimilation of										
D-Arabinose	-	-	-	-	+	+	-	-	-	-
L-Arabinose	-	-	+	v	+	v	+	+	-	-
Cellobiose	-	-	l	v	+	+	l	+	+	+
Citrate	-	-	-	v	+	v	s	+	l	v
Erythritol	-	-	-	-	-	-	-	-	-	-
Ethanol	l	+	l	+	+	+	+	+	+	+
Galactose	-	-	+	s	+	+	l	+	-	-
Galactitol	-	-	-	-	+	v	+	+	-	-
D-Glucitol	l	v	l	v	+	+	s	+	+	v
Glucose	l	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	s	+	+	+	+	+	+
2-keto-D-Gluconate	-	-	-	-	+	+	-	-	-	v
5-keto-D-Gluconate	-	n	-	n	v	-	s		-	v
Lactose	-	-	+	v	-	-	-	-	-	-
DL-Lactate	l	+	w	+	v	v	w/-	-	l	+
D-Mannitol	l	v	l	v	+	+	+	+	+	+
Maltose	-	-	-	-	+	+	l	+	l	v
Melezitose	-	-	-	-	+	+	l	v	-	-
Melibiose	-	-	-	-	+	+	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	+	+	-	-	+	v
Inositol	l	+	-	-	-	-	+	+	-	-
Inulin	w	-	+	+	w	+	-	-	-	-
Raffinose	-	-	+	+	+	+	l	+	-	-
L-Rhamnose	-	-	-	-	v	v	+	+	s	v
Ribitol	+	+	l	s	+	+	l	+	-	-
D-Ribose	-	v	-	v	+	+	-	v	-	-
Salicin	-	-	l	v	+	+	+	+	+	+/w
Soluble starch	-	-	-	-	w	-	l	+	-	-

Table 4.4 (Continued).

Characteristics	Gr. II	*S. lactativora* [a]	Gr. III	*K. marxianus* [b]	Gr. IV	*M. caribbica* [c]	Gr.V	*Z. meyerae* [d]	Gr.VI	*B. californica* [e]
C-assimilation of										
L-Sorbose	l	+	-	v	+	v	l	+	+	+
Succinate	l	+	+	+	+	+	+	+	+	+
Sucrose	-	-	+	+	+	+	l	+	l	v
Trehalose	-	-	-	w/-	+	+	+	+	l	v
D-Xylose	l	+	+	s	+	+	+	+	+	+
N-assimilation of										
Cadavarine	-	-	+	+	+		+	+	-	
Ethylamine	+	+	+	+	+	n	+	+	+	
L-Lysine	+	+	+	+	+	n	+	+	+	
Nitrate	-	-	-	-	-	-	-	-	+	+
Nitrite	-				-		-	-	+	
Growth in										
Vitamin-free	w	-	-	-	l	-	-	-	w	-
50%Glucose	-	-	+	-	-	n	+	+	-	
10%NaCl/5%Glucose		-	n	w	+	+		+	-	-
0.01% Cycloheximide	l	+	+	+	+		l	+	-	
0.1% Cycloheximide	+	l	+	+		+	+	l		-
Growth at 37°C	l	+	+	+	+	+	+	+	-	-

+, positive; -, negative; l, latent (longer than 7 days), w, weak;; n, no data.

[a] Data from Rodrigues *et al.* (1984)

[b] Data from Lachance (2011)

[c] Data from Kurtzman (2011a)

[d] Data from Smith *et al.* (2005)

[e] Data from Kurtzman (2011b)
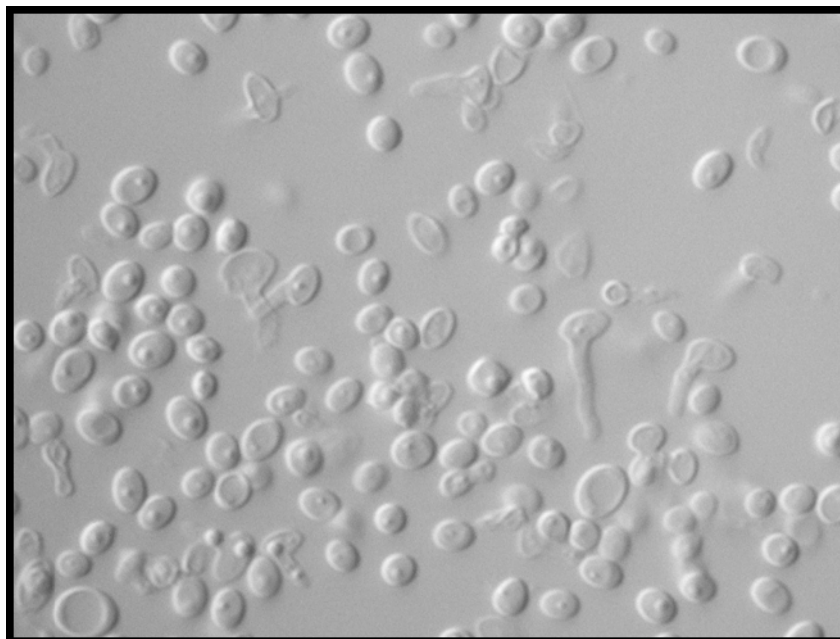
Figure 4.7 *Barnettozyma californica* 15-2. Vegetative cells and conjugation tube after 2 months on YM medium, 25°C.

Group VII contained 5 isolates. These were identified to genus *Pichia*, which were organized into 4 groups by the nucleotide sequence of D1/D2 domain of LSU rRNA gene.

Group VII (a) contained one isolate. Isolate H1 had different characteristics, which was not assimilated xylose and not grown on vitamin-free and 10% NaCl/5% Glucose. Differential from Group VII (b) contained one isolate. Isolated 10-7 was grown on vitamin-free and 10% NaCl/5% Glucose. Group VII (c) contained one isolated, Isolate 10-7 was grown on vitamin-free but not grown on 10% NaCl/5% Glucose. Group VII (d) contained 3 isolates, 4-6, 4-9 and 4-10, were not grown on vitamin-free but grew on 10% NaCl/5% Glucose. Their D1/D2 LSU rRNA gene sequences were 99.8-100% similar with the type strain of species of the genus *Pichia* (Figure 4.1). Therefore, Group VII (a) to Group VII (d) isolates were identified as *I. terricola, P. occidentalis, P.kudrivzevii* and *P.kluyveri,* respectively.

Table 4.5 Differential characteristics of species assigned to the genus *Pichia and Issatchenkia.*

Characteristics	Gr. VII(a)	*I.terricola* [a]	Gr.VII (b)	*I. orientalis* [a]	Gr.VII(c)	*P. occidentalis* [a]	Gr.VII(d)	*P.kluyveri* [a]
Fermentation of								
Glucose	+	+/w	+	+	+	+	+	+
Assimilation of								
D-Arabinose	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-
Citrate	+	w/-	+	+	-	-	l	w/-
DL-Lactate	w	-	+	+	l	+/w	+/s	+/w
L-Sorbose	-	-	-	-	-	v	-	-
D-Xylose	-	-	-	-	-	-	w/-	v
Growth in								
Vitamin-free	-	-	+	+	+	+	-	-
10%NaCl/5%Glucose	-	-	+	+	-	-	+	+
Growth at 37°C	+	v	+	+	+	+	+	v

+, positive; -, negative; l, latent (longer than 7 days), s, slow; w, weak; v, variable; n, no data.

[a]Data from Kurtzman (2011c)

Group VIII contained 14 isolates. These were identified to genus *Geotrichum*. *Geotrichum* represented the teleomorphic states *Dipodascus* and *Galactomyces* (Sybren de Hoog and Smith, 1832). This group was organized into 3 groups by the nucleotide sequence of D1/D2 domain of LSU rRNA gene.

VIII (a) contained 9 isolates, 4-1, 12-2, 12-3, 12-4, BUF7, BUF8, BUF9, BUF10 and BUF11. Different characteristics of this group with *G. bryndzae* and *G. silvicola* (Table 4.6), theirs were weak fermented glucose and assimilated D-mannitol, D-Glucitol, L-lysine, grown on vitamin-free, 0.01% cycloheximide and high temperature at 37°C. They did not assimilate D-glucosamine. Sequence comparison with the GenBank database revealed 5 nucleotide differences in 554 bp sequence from *G. silvicola* UFMG-354-2[T] and 4 nucleotide difference in 543 bp sequence from *G. bryndzae* CCY16-2-1[T] (Figure 4.8). So this group is closest and is related with *G. silvicola.*

VIII (b) contained 4 isolates, 4-2, 4-3, 4-4 and 4-5. A few different characteristics of this group compared with *Ga. geotrichum* (Table 4.6), this group was latent or slow in assimilating galactose, DL-lactate and citrate. This was latent grown on vitamin free and not assimilated D-manitol. Sequence comparison on D1/D2 LSU rRNA gene with the GenBank database revealed 12 nucleotide difference in 546 bp sequence from *Galactomyces geoticum* NRRL Y-17569[T]. Therefore, it will be proposed as a new species.

VIII (c) contained one isolate. Isolated 11-4 different characteristic of slowly assimilated of d-glucitol, citrate and grown well on 0.1% cycloheximide. In contrast, it could not assimilate 2-keto-gluconate. Their D1/D2 LSU rRNA gene sequences were 99% similar with *G. vulgare* HA1379[T]. Therefore, they were identified as *G. vulgare.*

Group VIII (a) and VIII (b) may be proposed as the two new species in the genus *Geotrichum.* However*,* these Groups should to be confirmed again according to The Book of the Yeast: A Taxonomic Study, 5th edition (2011), because many yeast species and genera were reclassified by molecular taxonomic methodology using, for instance, sequence divergence of internal transcription spacer, actin-1gene, RNA polymerase II (RPB1 and RPB2) gene, translation elongation factor-1α (TEF1α) and mitochondrial regions (Kurtzman *et al*., 2011a).
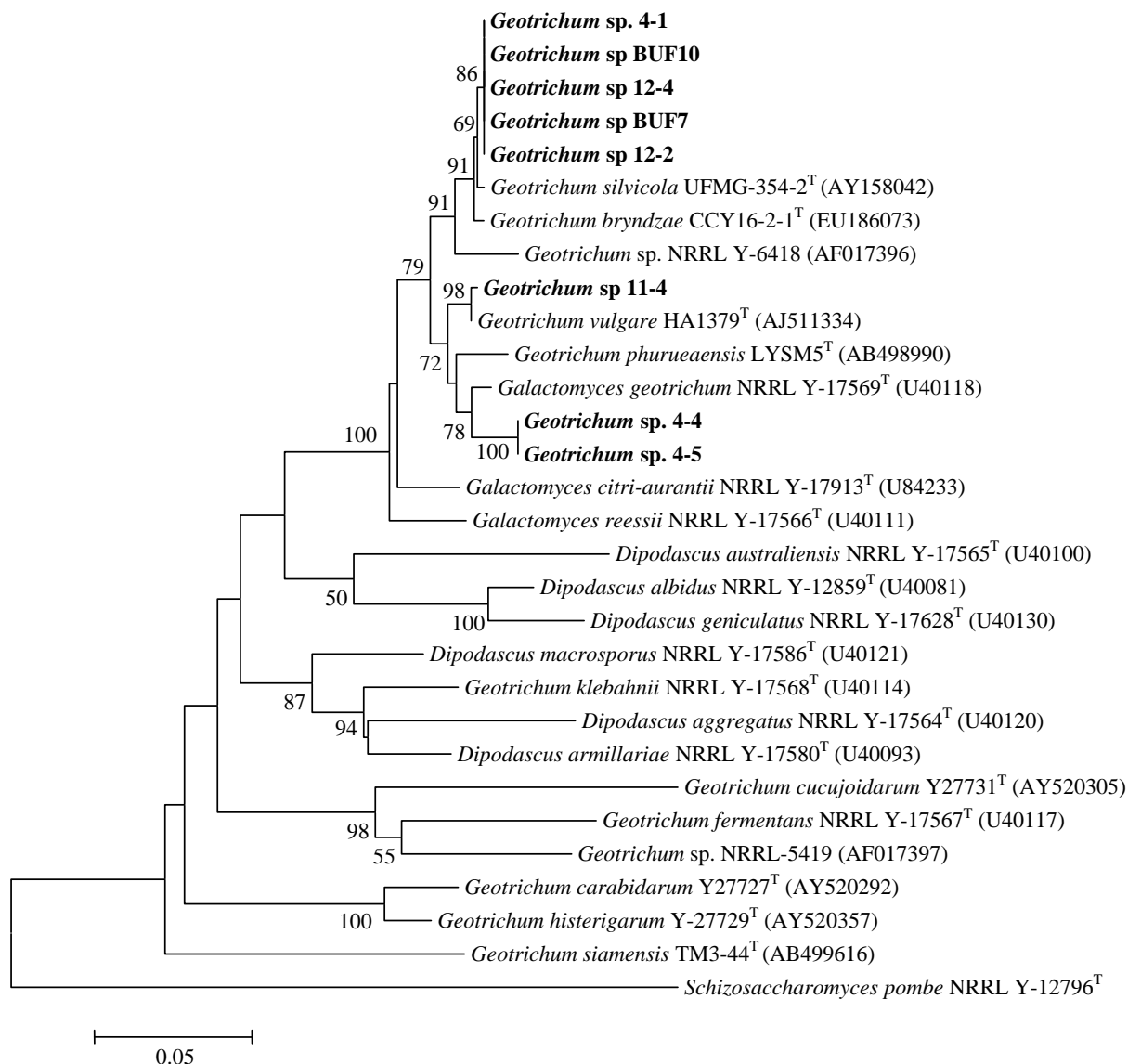
Figure 4.8 Phylogenetic tree of *Galactomyces* sp. and other strain were constructed by the neightbor-joining method based on D1/D2 domain of LSU rRNA gene sequences. The numerals represent the percentages from 1,000 replicate bootstrap resamplings (frequencies less than 50% are not shown).

Table 4.6 Characteristics of the isolates in Group IX and related species.

Characterlistics	Gr. VIII (a)	*G. bryndzae*[a]	*G. silvicola*[a]	Gr. VIII (b)	*G. geotrichum*[a]	Gr. VIII (c)	*G. vulgare*[a]
Fermentation of							
Glucose	-/w	-	-	-	v	-	-
Galactose	-	n	n	-	v	-	n
sucrose	-	n	n	-	-	-	n
Maltose	-	n	n	-	-	-	n
Lactose	-	n	n	-	-	-	n
Raffinose	-	n	n	-	-	-	n
Trehalose	-	n	n	-	n	-	n
C-assimilation of							
D-Arabinose	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-
L-Arabinitol	-	n	n	-	n	-	-
Arbutin	-	n	n	-	n	-	-
Butane 2,3 diol	+	+	n	+	n	+	+
Cellobiose	-	-	-	-	-	-	-
Citrate	+	w	+	l/s	v	l/s	-
Erythritol	-	-	-	-	-	-	-
Ethanol	+	+	+	+	+	+	+
Galactose	+	+	+	l/s	+	l/s	+
Galactitol	-	-	-	-	-	-	-
D-Glucitol	+	-	+	-	+	-	+
D-Gluconate	-	-	v	-	-	-	-
D-Glucose	+	+	n	+	+	+	+
D-Glucosamine	-	w	-	-	-	-	-
D-Glucuronate	-	-	n	-	n	-	-
Glycerol	+	+	+	+	+	+	+
Hexadecane	-	n	-	-	n	-	n
Inositol	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-
2-keto-D-Gluconate	-	w	n	-	-	-	w
5-keto-D-Gluconate	-	n	n	-	n	-	-
Lactose	-	-	-	-	-	-	-
DL-Lactate	+	+	w	l/s	v	l/s	w
Maltose	-	-	-	-	-	-	-
D-Mannitol	+/l	-	+	-	v	-	-
Melibiose	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-
α-Methyl-D-Glucoside	-	-	-	-	-	-	-
Propane 1,2 diol	+	+	n	+	n	+	+
Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Ribose	-	-	-	-	v	-	-

Table 4.6 (Continued).

Characterlistics	Gr VIII (a)	*G. bryndzae*[a]	*G. silvicola*[a]	Gr VIII (b)	*G. Geotrichum*[a]	Gr VIII (c)	*G. vulgare*[a]
C-assimilation of							
Ribitol	-	-	-	-	v	-	-
Salicin	-	-	-	-	-	-	-
L-Sorbose	+	w	+	-	+	-	+
Soluble starch	-	-	-	-	-	-	-
Succinate	+	+	+	+	v	+	+
Sucrose	-	-	-	-	-	-	v
Trehalose	-	-	-	-	-	-	-
D-Xylose	+	+	+	+	+	+	+
Xylitol	-	-	-	-	n	-	-
N-assimilation of							
Cadavarine	+	+	+	+	n	+	+
Ethylamine	w/+	+	+	+	n	+	+
L-Lysine	+	-	+	+	n	+	-
Nitrate	-	-	-	-	-	-	-
Nitrite	-	-	-	-	n	-	-
Growth in							
Acid production 0.01%	-	n	n	-	n	-	n
Cycloheximide	+	-	+	+	n	+	+
0.1% Cycloheximide	+/l	v	+	+	n	+	v
60% Glucose	-	n	n	-	n	-	n
50% Glucose	-	-	-	-	n	-	-
10%NaCl/ 5% Glucose	-	n	-	-	n	-	-
Starch formation	-	-	-	-	n	-	-
Urease	-	-	-	-	n	-	-
Vitamin-free	l	+	+	l	+	+	+
Growth at 30°C	+	+	+	+	n	+	+
Growth at 37°C	+	v	-	+	v	-	-

+, positive; -, negative; l, latent (longer than 7 days), s, slow; w, weak; v, variable; n, no data. [a]Data from Sybren de Hoog *et al*. (2011);

Characterization of Group VIII (a)

After the growth on YM agar for 7 days at 25°C, colonies were white, flat, dry and powdery to finely hairy. Arthoconidia are abundantly present (Figure 4.9). Abundant true mycelium and arthoconidia are formed on Dalmau plate culture with corn meal agar after 7 days. Arthoconidia are 4-5 µm wide, 6-10 µm long. The main branching is 7-10 µm wide (Figure 4.9). It could ferment glucose (variable) and galactose (variable). Assimilation of carbon compounds: glucose, galactose, l-sorbose, xylose, ethanol, glycerol, D-mannitol, D-glucitol, DL-lactate, succinate, citrate, glucono-δ-lactone (latant), propane 1,2-diol, butane 2,3 diol. No growth occurs on sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, Inulin, soluble starch, l-arabinose, d-arabinose, d-ribose, l-rhamnose, d-glucosamine, n-acetlyl-D-glucosamine, methanol, erythritol, ribitol, galactitol, α-methyl-D-glucoside, salicin, D-gluconate, inositol, hexadecane, 2-keto-D-Gluconate, 5-keto-D-Gluconate, saccharate, D-glucoronate, xylitol, or l-arabinitol. Assimilation of nitrogen compounds were found positive for lysine, cadavarine and ethylamine/HCl; assimilation of nitrogen was negative for nitrate and nitrite. Growth in vitamin-free medium is positive. Growth at 30°C is positive and 37°C is variable. Growth on 5% glucose agar with 10% sodium chloride was negative. Growth in 60% glucose and 50% glucose was negative. Starch-like compounds were not produced. Growth on 1% acetic acid medium was negative. In 100 and 1000 µg cycloheximide ml^{-1}, growth was positive. Urease activity is negative (Table 4.6).

The type strain 12-2^T (=JCM 18104^T), was deposited at Japan Collection of Microorganisms (JCM), Wako, Saitama, Japan.
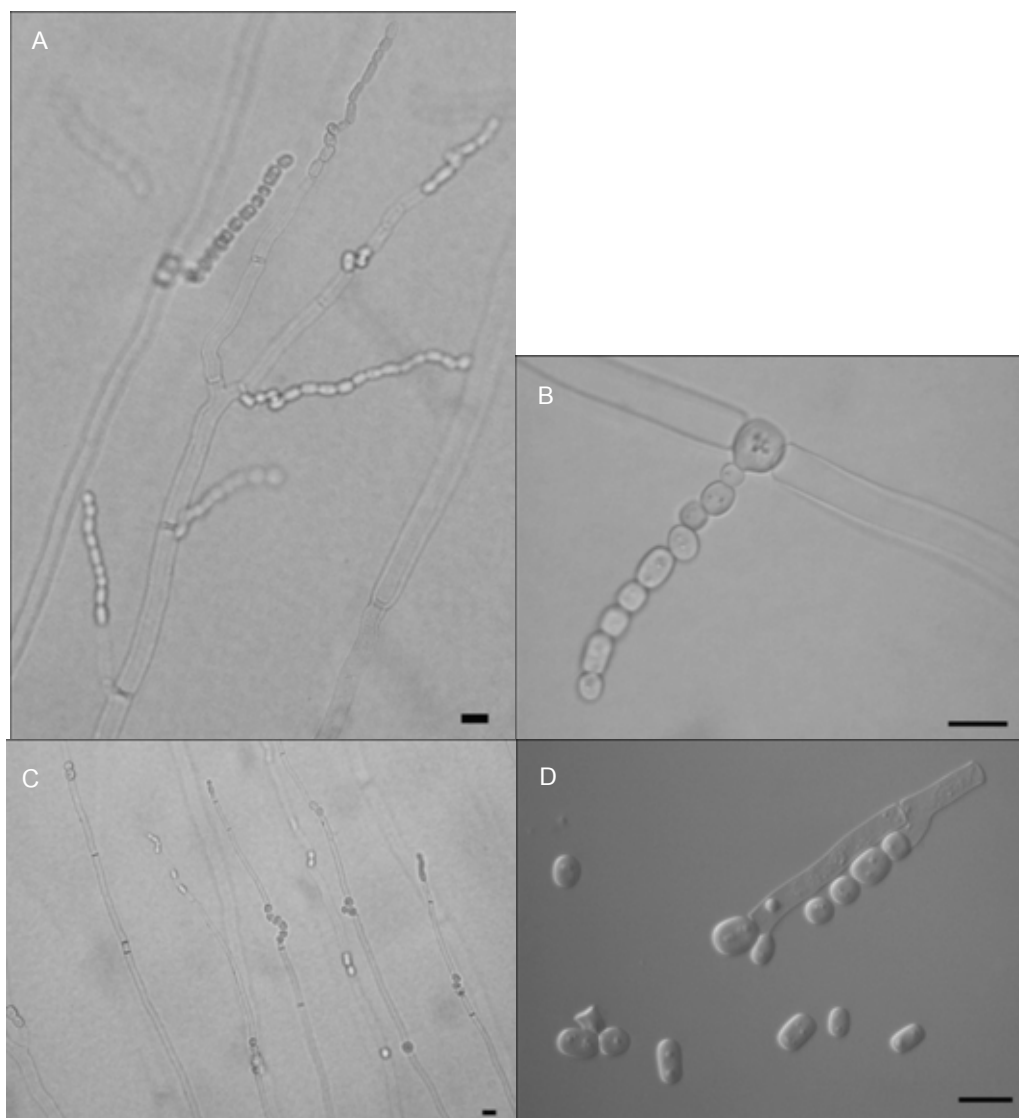
Figure 4.9 Morphology of *Geotrichum* sp. 12-2^{T} as determined by light microscopy. Hyphae formed on Dalmau plate cultivated on corn meal agar after 3 days at 25°C (A, B, C). Vegetative cells and arthrospore formation after 5 days on Fowell's acetate agar at 25°C (D).

Characterization of Group VIII (b)

  After the growth on YM agar for 7 days at 25°C 7, colonies were white, flat and powder to long hairy. Hypha were 7-8 µm wide, with frequent dichotomous branching at the apex, with early disarticulation into cubic arthroconidia. Arthroconidia were 4-5 µm wide, 4.7-17 µm long (Figure 4.10). Abundant true mycelium and arthroconidia were formed on Dalmau plate culture with corn meal agar after 7 days at 25°C. (Figure 4.10). Fermentation was absent. Assimilation of carbon compounds included: glucose, galactose (slow), xylose, ethanol, glycerol, DL-lactate (slow), succinate, citrate (slow), propane-1,2 diol, and butane 2,3 diol. No growth occurred on l-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, l-arabinose, d-arabinose, d-ribose, l-rhamnose, d-glucosamine, n-acetlyl-d-glucosamine, methanol, erythritol, ribitol, galactitol, d-mannitol, d-glucitol, α-methyl-d-glucoside, salicin, d-gluconate, inositol, hexadecane, 2-keto-D-Gluconate, 5-keto-D-Gluconate, glucono-δ-lactone, saccharate, d-glucoronate, xylitol, or l-arabinitol. Assimilation of nitrogen compounds was: positive for lysine, cadavarine and ethylamine/HCl, negative for nitrate and nitrite. Growth in vitamin-free medium was positive (latent). Growth at 30°C and 37°C was positive. Growth on 5% glucose agar with 10% sodium chloride was negative. Growth in 60% glucose and 50% glucose was negative. Starch-like compounds were not produced. Growth on 1% acetic acid medium was negative. In 100 and 1000 µg cycloheximide ml^{-1}, growth was positive. Urease activity was negative.

  The type strain 4-5^T (=JCM 18103 ^{T}), was deposited at Japan Collection of Microorganisms (JCM), Wako, Saitama, Japan.
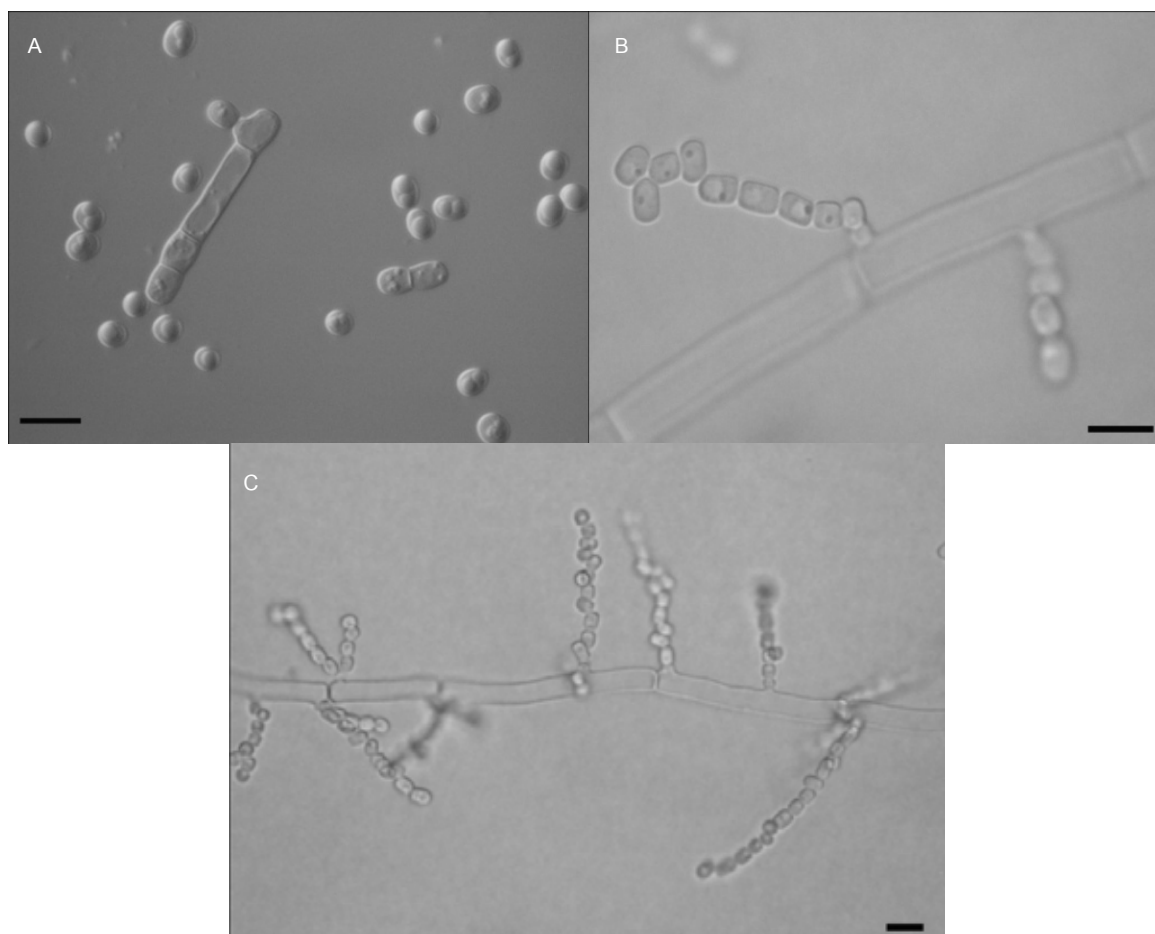
Figure 4.10 Morphology of *Geotrichum* sp. 4-5^T as determined by light microscopy. Vegetative cells and arthrospore formation after 11 days on Fowell's acetate agar at 25°C (A). Hyphae formed on Dalmau plate cultivated on corn meal agar after 7 days at 25°C (B, C).

Group XI contained 11-2 and 4-14. The different characteristics of both isolates were that 11-2 could ferment and assimilate sucrose but in 4-14 that was absent. The identification was evaluated by D1/D2 sequencing, 11-2 showed a 99% similarity with *Cy. saturnus* NRRL Y-17396[T] ,and 4-14 showed a 99% similarity with *Cy. mrakii* NRRLY-1364[T]. The two species were closely related for D1/D2 sequencing analysis. Note the phenotype of differential characteristic species of genus *Lindnera,* for assembling supportive data. (Table 4.7). Therefore, the isolates 11-2 and 4-14 were identified as *Cy. saturnus* and *Cy. mrakii,* respectively.

Table 4.7 Differential characteristics of isolates and *Cyberlindnera* species.

Characteristics	11-2	*Cy. saturnus[a]*	4-14	*Cy. mrakii[a]*
Fermentation of				
Sucrose	+	+	-	-
Assimilation of			-	-
D-Arabinose	-	-		
L-Arabinose	-	-	-	-
Maltose	-	v	-	v
Melezitose	-	v	-	v
Nitrate	+	+	+	+
Raffinose	s	+	s	+
L-Rhamnose	s	v	s	v
Soluble starch	-	-	-	-
Sucrose	+	+	-	-
D-Xylose	+	+	+	+
Vitamin-free	+	+	+	+

+, positive; -, negative; l, latent (longer than 7 days), s, slow; w, weak;
 v, variable; n, no data.
[a]Data from Kurtzman (2011c)

Group X, contained 26 isolates, These were identified to genus *Trichosporon*, which were organize into 3 groups by the nucleotide sequence of D1/D2 domain of LSU rRNA gene.

Group X (a) were composed of 4 isolates. They could assimilate L-sorbose (slow), melibiose (latent), raffinose (latent), melezitose (weak), rhamnose (latent), ribose (slow), and inositol (latent), but could not assimilate inulin, erythritol and galacitol grew on vitamin-free medium (weak), 50% glucose and 10% NaCl with 5% glucose (Table 4.9). They were related to *Trichosporon mycotoxinivorans* HB1175[T] based on 100% D1/D2 LSU rRNA gene sequence similarity (Figure 4.1 and Table 4.8). Therefore, they were identified as *T. mycotoxinivorans.*

Group X (b) contained 17 isolates. The different characteristics of this group were that salicin, inositol, ribitol and sorbose were not assimilated, but sucrose, melezitose, glycerol and DL-lactate (Table 4.8) were assimilated; compare with type strain. Their D1/D2 LSU rDNA gene sequencing were 100% similar with *Trichosporon asahii* CBS 2479[T]. Therefore, they were identified as *T. asahii*.

Group X(c) contained 5 isolates. They had phenotypes similar with *T.akiyoshidainum* HP-2023 except L-sorbose was not assimilated (Table 4.8). Due to type strain of *T.akiyoshidainum* JCM 12595[T] was not formally described of phenotype, so compare phenotype with *T.akiyoshidainum* HP-2023. Hipólito *et al*., 2008, studied phenotypical and genetic characterization of *T. akiyoshidainum* HP-2023, which data was supported describing *T.akiyoshidainum.* Their D1/D2 LSU rDNA gene sequencing were 100% similar with *T.akiyoshidainum* JCM 12595[T]. Therefore, they were identified as *T. akiyoshidainum.*

Table 4.8 Differential characters of species assigned to the genus *Trichosporon.*

Characteristics	Gr. X(a)	*T. mycoxinvora*[a]	Gr. X(b)	*T. asahii*[b]	Gr. X(c)	*T.akiyoshidainum*[C]
C-assimilation of						
D-Arabinose	-	+	l	+	-	v
L-Arabinose	+	+	+	v	+	+
Cellobiose	+	+	+	+	s	+
Citrate	+	+	+	+	+	+
Erythritol	-	w	+	+	-	w
Ethanol	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Galactitol	-	+	-	-	+	+
Glycerol	+	+	s	v	l	+
DL-Lactate	+	+	+	v	+	+
Lactose	+	+	+	+	+	+
Inositol	l	+	-	v	s	+
Inulin	-	+	-	-	-	-
Melibiose	l	+	-	-	+	+
Melezitose	w	+	s	v	w	w/-
Raffinose	l	+	-	-	+	+
L-Rhamnose	l	+	s	+	+	+
Ribitol	s	-	-	v	-	-
Salicin	l	+	-	+	w	+
L-Sorbose	s	+	-	v	-	+
Soluble starch	+	+	l	v	+	+
Succinate	+	+	+	+	+	+

Table 4.8 (Continued).

Characteristics	Gr. X(a)	*T. mycoxinvora*[a]	Gr. X(b)	*T. asahii*[b]	Gr. X(c)	*T.akiyoshidainum*[C]
C-assimilation of						
Sucrose	+	+	+	v	s	+
D-xylose	+	+	+	v	+	+
N-assimilation of						
Ethylamine	+	w	+	+	+	n
L-Lysine	+	+	+	+	+	+
Nitrite	-	-	+	v	-	-
Growth in						
50%Glucose	+	-	+	+	-	-
10%NaCl/ 5% Glucose	+	-	-	+	-	+
Vitamin-free	W	-	-	-	-	-
Growth at 30°C	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	-	-
Growth at 40°C	+	-	+	n	-	n

+, positive; -, negative; l, latent (longer than 7 days), s, slow; w, weak; v, variable; n, no data.
[a] Data from Molnar *et al*. (2004)
[b] Data from Sugita (1994)
[c] Data from Pajot *et al*.(2008)

4.3 Screening and quantitative ethanol and xylitol production

One hundred and twenty-nine isolates in this study utilized xylose as a sole carbon source. 117 isolates could ferment xylose to ethanol (0.01-2.388 g/l) and 95 isolates could ferment to xylitol (0.03-29.47 g/l) using YP medium supplement with 4% xylose 48 h in 50 ml flask (Table 4.9).

As a result, top strains were maximum ethanol production as E23 (2.40 g/l), ELP22 (1.53 g/l), ELP27 (1.63 g/l), 15-1(1.63 g/l), 15-2(1.52 g/l) and 15-3(1.60 g/l), which are compose of *Z. meyerae, K.marxianus* and *B.californica*, respectively. Margaritis and Bajpai (1982) studied direct fermentation of D-xylose to ethanol by *K.marxianus* UB-80-S, which gave a maximum ethanol concentration 5.6 g/l and ethanol yield 0.28 g of ethanol per g of D-xylose in anaerobic condition at 35°C. D-xylose utilization was completed in 48 h. Wilkins *et al*., 2008 reported that *K.marxianus* was thermotolerant yeast strains, which were able to fermente xylose to ethanol and xylitol and were 0.02 and 0.08 g/l, respectively at 40°C. For *B.californica* a few reported about fermentation of xylose to ethanol. Nitiyon *et al*., 2010 reported

that *Barnettozyma californica* was xylose fermenting yeast but did not ferment D-xylose to ethanol. Presently, there is no report of fermenting D-xylose to ethanol by *Z. meyerae*, which maximum ethanol production in this report. In consequence, *Z. meyerae* E23 was selected for further study.

For xylitol production, *Candida tropicalis* (group I(a)) were maximum xylitol production, then *Meyerozyma caribbica* (group IV) were approximately around 20 g/l. The maximum xylitol was 29.47 g/l of *C. tropicalis* A26 and 29.19 g/l of *C. tropicalis* ELP9. Because *C. tropicalis* A26 was yielding 77.2 % xylitol higher than ELP 9 (73%), *C. tropicalis* A26 was selected to optimize xylitol production.

Table 4.9 xylose fermentation parameters ethanol production and xylitol production of the isolates.

Yeast strain	D-xylose consumption (%)	Ethanol concentration (g/l)	%Ye/s	Xylitol concentration (g/l)	%Yxy/s
Group I (a):					
A1	82.1	0.32	0.97	11.55	35.2
A5	100.0	0.26	0.65	20.66	51.7
A8	100.0	0.14	0.35	22.46	56.2
A9	100.0	0.06	0.15	19.31	48.3
A12	100.0	0.35	0.88	25.55	63.9
A14	100.0	0.07	0.18	24.79	62.0
A15	98.5	0.25	0.63	23.73	60.2
A16	96.4	0.58	1.50	20.47	53.1
A18	84.5	0.30	0.89	15.10	44.7
A20	94.3	0.09	0.24	13.85	36.7
A21	97.8	0.13	0.33	21.83	55.8
A26	95.4	0.17	0.45	29.47	77.2
A28	99.5	0.23	0.58	17.09	42.9
E1	98.4	0.08	0.20	20.79	52.8
F1	50.0	0.28	1.40	3.29	16.5
I1	98.5	0.08	0.20	22.55	57.2
M1	100.0	0.70	1.70	26.39	66.0
G1	99.7	0.11	0.28	19.69	49.4
ELP7	99.6	0.17	0.43	22.50	56.5
ELP9	100.0	0.06	0.15	29.19	73.0
ELP16	98.1	0.05	0.13	22.19	56.6
ELP26	96.6	0.12	0.31	14.43	37.3
ELP30	83.4	0.70	2.10	10.99	32.9
ELP35	100.0	0.02	0.05	15.60	39.0
ELP39	100.0	0.06	0.15	17.82	44.6
ELP48	85.9	0.48	1.40	15.93	46.4
ELP49	96.2	0.57	1.48	17.59	45.7
BUF12-1	100.0	0.11	0.11	21.62	36.0
BUF5-3	97.8	0.33	0.34	22.34	38.1
BUF4-2	98.2	0.17	0.17	22.84	38.8

Table 4.9 (Continued).

Yeast strain	D-xylose consumption (%)	Ethanol concentration (g/l)	%Ye/s	Xylitol concentration (g/l)	%Yxy/s
BUF4-3	65.5	0.23	0.34	21.19	53.9
BUF4-4	98.6	0.18	0.18	20.15	34.1
BUF7-1	98.6	0.30	0.30	21.35	36.1
BUF8-1	53.2	0.33	0.63	15.06	47.2
BUF8-2	96.9	0.60	0.62	17.04	29.3
BUF9-2	95.8	0.48	0.49	19.64	34.3
BUF9-4	44.6	0.45	1.01	20.02	74.8
BUF9-5	99.7	0.13	0.13	20.12	33.6
7-4	97.4	0.70	1.80	14.71	37.8
9-6	100.0	0.08	0.20	17.20	43.0
Group I (b):					
4-11	39.52	0.00	0.00	0.10	0.6
4-12	51.97	0.05	0.24	0.27	1.3
4-13	48.07	0.05	0.26	0.35	1.8
Group I (c):					
ELP44	43.88	0.02	0.11	5.17	29.5
Group I (d):					
11-1	20.20	0.00	0	0.00	0
Group I (e):					
10-1	17.52	0.25	3.57	0.00	0.0
2-1	55.20	0.03	0.14	0.00	0.0
5-3	36.17	0.20	1.38	0.00	0.0
3-1	20.50	0.11	1.34	0.00	0.0
10-3	72.08	0.09	0.31	0.45	1.6
10-2	31.57	0.08	0.63	0.15	1.2
10-8	45.27	0.00	0.00	0.10	0.6
5-1	45.27	0.22	1.22	0.00	0.0
7-6	24.25	0.05	0.52	0.00	0.0
9-3	78.60	0.00	0.00	1.43	4.5
9-4	27.43	0.00	0.00	0.16	1.5
Group I (f):					
L1	21.00	0.00	0.00	0.00	0.0
Group I (g):					
BUF3-6	68.59	0.06	0.09	2.59	6.3
BUF3-16	61.60	0.16	0.26	0.90	2.4
Group I (h):					
BUF3-18	58.02	0.01	0.01	0.00	0.0
BUF3-19	58.00	0.05	0.09	0.00	0.0
Group I (i)					
ELP19	45.00	0.29	1.61	0.03	0.2
Group II					
ELP20	6.22	0.21	3.22	0.30	12.1
ELP25	43.79	0.05	0.29	0.39	2.2
ELP32	64.68	0.12	0.46	0.27	1.0
ELP43	7.50	0.08	2.67	0.15	5.0

Table 4.9 (Continued).

Yeast strain	D-xylose consumption (%)	Ethanol concentration (g/l)	%Ye/s	Xylitol concentration (g/l)	%Yxy/s
ELP46	30.62	0.07	0.57	0.12	1.0
BUF1-1	51.57	0.07	0.14	0.00	12.1
BUF1-2	95.05	0.08	0.21	0.00	2.2
BUF3-5	80.22	0.00	0.00	3.68	1.0
BUF4-1	97.78	0.17	0.43	1.51	5.0
Group III					
ELP22	98.52	1.53	7.11	5.49	13.9
ELP27	86.68	1.63	8.74	5.49	15.8
ELP45	44.74	0.60	3.35	3.00	16.8
Group IV					
ELP29	100.00	0.05	0.13	22.95	57.4
ELP34	100.00	0.05	0.13	19.70	49.3
ELP38	99.36	0.11	0.28	17.82	44.8
Group V					
E23	20.00	2.388	5.25	5.26	65.8
Group VI					
15-1	83.33	1.63	4.89	0.00	0.0
15-2	85.00	1.52	4.47	0.00	0.0
15-3	85.20	1.60	4.70	0.00	0.0
Group VII					
H1	19.80	0.00	0.00	0.00	0.0
4-7	14.36	0.00	0.00	0.00	0.0
10-7	12.00	0.00	0.00	0.00	0.0
4-6	19.72	0.06	0.30	0.00	0.0
4-9	19.21	0.06	0.31	0.00	0.0
4-10	18.60	0.03	0.16	0.00	0.0
Group VIII					
4-2	57.38	0.04	0.17	0.08	0.3
4-3	52.83	0.10	0.47	0.00	0.0
4-4	57.53	0.22	0.96	2.47	10.7
4-5	56.98	0.37	1.62	0.00	0.0
4-1	69.41	0.64	2.31	0.00	0.0
11-4	69.27	0.66	2.38	0.00	0.0
12-2	69.17	1.31	4.73	0.00	0.0
12-3	70.23	0.50	1.78	0.00	0.0
12-4	68.67	0.70	2.55	0.00	0.0
BUF7	75.30	0.05	0.06	0.19	0.4
BUF8	65.71	0.07	0.11	0.40	1.0
BUF9	68.04	0.06	0.09	0.00	0.0
BUF10	61.20	0.11	0.18	0.00	0.0
BUF11	70.85	0.04	0.06	0.00	0.0
Group IX					
4-14	48.26	0.06	0.31	2.18	11.3
11-2	37.50	0.00	0.00	1.00	6.7

Table 4.9 (Continued).

Yeast strain	D-xylose consumption (%)	Ethanol concentration (g/l)	%Ye/s	Xylitol concentration (g/l)	%Yxy/s
Group X					
ELP21	81.65	0.03	0.09	1.39	4.3
ELP24	93.41	0.04	0.11	0.96	2.6
ELP37	86.68	0.09	0.26	0.69	2.0
ELP40	63.97	0.02	0.08	2.82	11.0
BUF2-1	67.29	0.04	0.06	0.00	0.0
BUF2-2	80.88	0.13	1.16	0.81	1.7
BUF2-4	65.78	0.03	0.04	1.12	2.8
BUF3-3	58.90	0.04	0.07	0.26	0.7
BUF3-8	89.19	0.01	0.01	0.88	2.2
BUF3-9	95.55	0.03	0.08	0.00	0.0
BUF3-10	8.63	0.03	0.32	0.34	6.5
6-3	40.69	0.00	0.00	0.43	2.6
6-4	40.75	0.06	0.37	0.31	1.9
6-8	41.91	0.03	0.18	0.54	3.2
6-9	82.83	0.06	0.18	0.78	2.4
3-5	10.30	0.03	0.73	0.24	5.8
3-6	36.72	0.03	0.20	0.32	2.2
3-7	34.15	0.13	0.95	0.31	2.3
6-5	68.76	0.06	0.22	0.00	0.0
6-6	28.73	0.00	0.00	0.33	2.9
6-10	46.81	0.00	0.00	0.41	2.2
6-2	50.82	0.22	1.08	3.59	17.7
11-3	28.30	0.00	0.00	0.58	5.1
11-5	28.24	0.18	1.59	0.61	5.4
11-6	40.09	0.03	0.19	0.27	1.7
11-7	39.52	0.00	0.00	0.43	2.7
12-5	49.78	0.00	0.00	0.42	2.1

4.4 Ethanol production of *Z. meyerae* E23

      Z. meyerae E23 determined ethanol production in YPX medium (4% xylose) at 30°C for 72 h with collection every 12 hours. The dynamics of xylose utilization for growth and ethanol production of *Z. meyerae* E23 is shown in Figure 4.11. Maximum ethanol (3.631 g/l) was produced at 72 hours. At this time, no residual xylose was detected in the culture broth. For the first 36 hours *Z. meyerae* ELP32 was in the log phase of growth; it had high xylose utilization but low ethanol production. The *Z. meyerae* E23 entered the late log phase and the stationary phase of growth during 36 to 48 h and 48 to 72 h of cultivation, respectively, when xylose utilization decreased but ethanol production increased.

      These results indicated that *Z. meyerae* E23 first utilized xylose for growth and produced ethanol at a late growth phase. Since *Z. meyerae* E23 had very slow growth, its ethanol production was low. Ethanol production of *Z. meyerae* E23 may increase, if growth phase was shortened by optimization of growth conditions. *S. stipitis* JCM 10742^{T} which is commonly used for producing ethanol from xylose because of its comparatively high ethanol production yield (Jeffries and Jin, 2004), produced maximum ethanol (14.479 g l^{-1}) from xylose at 24 h of incubation. At this time, only 0.5 g/l of residual xylose was detected (Appendix).

      Many attempts were tried to determine ethanol production from D-xylose with xylose fermenting yeasts such as *Candida shehatae*, *Pachysolen tannophilus*, *C. tenui*s and *S. stipitis*. However they produced 6.6 g/l, 2.1 g/l, 6.4 g/l and 5.9 g/l of ethanol from xylose, respectively (Jeffries and Kurtzman, 1994).

      Currently, *C. jeffriesii*, *Spathaspora passalidarum*, *Spathaspora arboraria,* and *C. prachuapepsis* strains are also screened and their ethanol production from xylose ranged from 3.1–3.6 g/l (Nguyen *et al.,* 2006; Cadete *et al.,* 2009; Nitiyon *et al.,* 2011). Unfortunately, the ethanol production from them was still unsatisfactory. In addition, *Rhodotorula graminis* and *R. mucilaginosa* strains isolated from within the stems of poplar (*Populus*) trees could grow in xylose (3%) medium but their ethanol production was not studied (Xu *et al.,* 2011). That study was the first study on ethanol production of *Z. meyerae* E23.

Time (h)
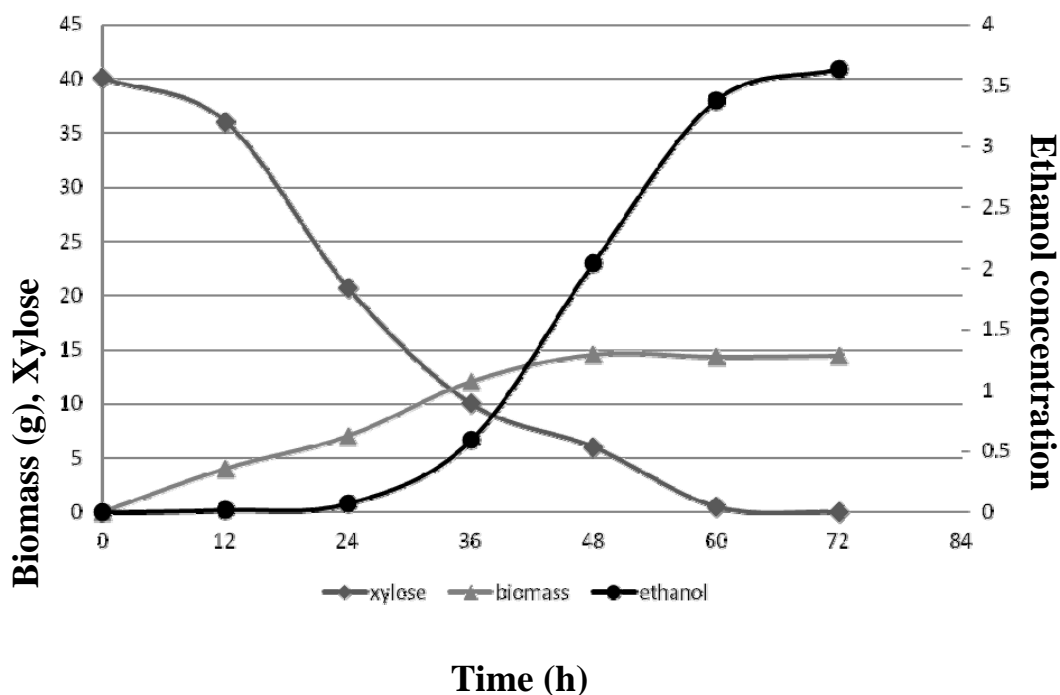
Figure 4.11 Ethanol fermentation profiles of *Zygoascus meyerae* E23 in Yeast-Peptone-Xylose (YPX) medium.

Symbols: ◆, xylose; ● , ethanol; ▲, biomass

4.5 Optimization of xylitol production

The previous result showed that isolate performed a maximal xylitol production at 29.47 g/l at 48 h. Therefore, *C. tropicalis* A26 was selected for further study. Optimization of xylitol production of *C. tropicalis* A26 was carried out in the YPX medium.

The aim of this study was used the Plackett-Burman design and the central composite design to screen for critical parameters and for optimized conditions for xylitol production of *C. tropicalis* A26 screened to Placket-Burman design (PBD) and followed by central composite design (CCD).

4.5.1 Screening experiment

A total of 5 parameters were screened for their effects on xylitol production by *C. tropicalis* A26 using Plackett-Burman Design. Table 3.2 represented the independent variables and their respective high and low levels used in the optimization study. The Plackett- Burman Design, with 8 runs and 5 variables under investigation, and two levels of each factor for optimization of xylitol value (Table 3.3).

Based on the obtained data (Table 4.10), the effect of each variable was calculated and the significance of each variable was determined by the *F*-test. The *P*-values for 2 variables, xylose and peptone, were found to have higher significant values and represent a confidence level of $\geq 90\%$.

It means that xylose and peptone significantly affected xylitol production. It can be seen that the effect E_{xi} of yeast extract, inoculum volume and pH value from low level to high level was insignificant and confidence level was less than 90%. It means that at these levels of yeast extract, inoculum volume and pH value did not improve xylitol production. The low level of them was used for further optimization studies. Initial xylose concentration had the most important effects on xylitol production; xylose is a main substrate to change the xylitol product.

Silva and Roberto (2006) studied the optimization of xylitol production of *C. guilliermondii* FTI 20037 and found that high initial xylose concentration increases the final xylitol production. Maximum xylitol production was 52 g/l from rice straw hemicellulose treated hydrolysate adjust to the desired initial xylose concentration to 80 g/l and inoculum level to 3 g/l. *C. boidinii* and *D. hansenii* had high xylitol yield 0.47 g/g and 0.77 g/g when the initial xylose was increased to 150 g/l and 156 g/l, respectively (Sampaio *et al*., 2006, Vandeska *et al*., 1995). A high concentration of xylose (279 g/l) enhanced xylitol production by *D. hansenii* and xylitol production reached 221 g/l (Dominguez *et al*., 1997). *D. nepalensis* produced xylitol increase from 27 g/l to 36 g/l when using 100 g/l xylose concentration, 10.6 g/l K_2HPO_4 and 8.9 mg/l $ZnSo_4$ (Kumdam *et al*., 2012).

However, high initial concentration of xylose inhibited the growth and xylitol yield. Vongsuvanlert and Tani (Vongsuvanlert *et al*., 1989) reported that xylitol concentration significantly reduced from 36 to 18 g/l when increase xylose from 100

g/l to 150 g/l in a culture of *C. boidinii*. In parallel, the xylitol production by *C.tropicalis* was decreased when increasing the xylose concentration from 100 g/l up to 200 g/l (EI-Baz AF *et al.,* 2011).

Peptone and yeast extract were a good source of organic nitrogen. In contrast, increasing the concentration of peptone and yeast extract effected a decrease in xylitol production yield. Furthermore, the conversion of D-xylose to xylitol by *C.tropicalis* DMS 7524 was blocked at a high concentration of yeast extract (15 g/l) (Silva *et al.,* 1997). To increase the concentration of yeast extract from 5 g/l to 10 g/l, biomass was increased but it decreased xylitol production by *C. guillermondii* FTI20037 (Silva *et al.,* 1997). Similarly the addition of yeast extract and peptone to the defined medium for *C. mogii* ATCC 1834 enhanced biomass but had no significant effect on the yield and specific productivity of xylitol (Sirisansaneeyakul *et al*., 1995). Maximum xylitol yield (0.9007 g/g) of *C. tropicalis* OMV5 was obtained by using 1.32% peptone and 0.48% yeast extract (Sreenivas *et al*., 2008). However, peptone was only one of organic nitrogen sources that significantly improved xylitol production by *C. tropicalis* because peptone was a pool of carbon, nitrogen and growth factors for microorganisms. Increase yield of xylitol was possible at lower levels of peptone (Sreenivas *et al*., 2008).

According to the Plackett-Burman design, 2 variables (xylose and peptone) affected xylitol production. These were selected for further optimization using Central Composite Design (CCD).

Table 4.10 Effect estimated for xylitol production from the results of Plackett-Burman design.

Variables	Parameter	Effect	*F*-value	*P*-value
X_1	Xylose	-15.825	143.257	0.007
X_2	Yeast extract	1.755	1.753	0.317
X_3	Peptone	4.500	11.617	0.076
X_4	Inoculum value	-2.005	2.313	0.268
X_5	pH	-1.080	0.678	0.498

4.5.2. Optimization of xylitol production using Central Composite Design

The variables used for CCD optimization were xylose (X_1) and peptone (X_2). The correspondence between the coded levels and the real levels of the factors are shown in Table 3.4.

The structure of the experiment, the result observed and the results predicted according to the second-order model obtained, are shown in Table 4.11. The xylitol production was selected as the response due to the different cycle of the runs. Eleven experiments were performed in triplicate and the central point was repeated three times (Run 9-11). The repetition at the center point allows the determination of standard error. The experimental results of central composite design (CCD), regression analysis was done following secondary-order polynomial equation. The xylitol production was an empirical function of test variables in a coded unit as shown in the following equation:

$$Y= 45.487+ 0.551X_1 + 2.38 X_2 - 7.494 X_1X_2 -3.399 X_1^2 + 0.748 X_2^2 \quad (5)$$

Where Y is the predicted response (xylitol production); and X_1, X_2 are code values of xylose and peptone. The statistical significance of Eq.(5) was evaluated by the Fisher's *F*-test analysis of variance (ANOVA) for the response surface quadratic model as shown in Table 4.12.

It is evident that the model was significant ($P<0.0001$) at 99% confidence level. The *P*-value of the model was used as the tool to check the significance of each coefficient, and this indicated that the model was suitable to use in the experiment (Myers *et al*., 2002).

The model did not show lack of fit and presented a determination coefficient (r=0.851). The closer the value of R (correlation coefficient) to 1, the better is the correlation between the experimental and predicted values (Stephen *et al.,* 2009).

According to R^2 value (0.724), the model could be explained with the actual value at about 72.4%. The Student t-distribution and the corresponding *P*-value, along with the parameter estimate, are given in Table 4.13. It could be concluded that X_2 had the significant effect at the 95% confidence level for linear effect on the response. The quadric effect of X_1^2 was significant at 99% and the interaction effect of X_1X_2 was significant at a 99% confidence level. It showed that X_1 (xylose) and interaction of X_1X_2 had a significant effect on xylitol production.

Table 4.11 Experimental design with real values and predicted values of xylitol production.

Run no.	X_1	X_2	xylitol production	
			Experimental	Predicted
1	1	1	32.21	38.27
2	1	-1	27.05	32.02
3	-1	1	33.99	35.68
4	-1	-1	31.83	32.41
5	1.414	0	37.81	31.37
6	0	1.414	46.20	42.09
7	-1.414	0	30.05	29.81
8	0	-1.414	37.94	35.37
9	0	0	44.87	45.49
10	0	0	45.82	45.49
11	0	0	45.69	45.49

X_1 xylose (g/l); X_2 peptone (g/l)
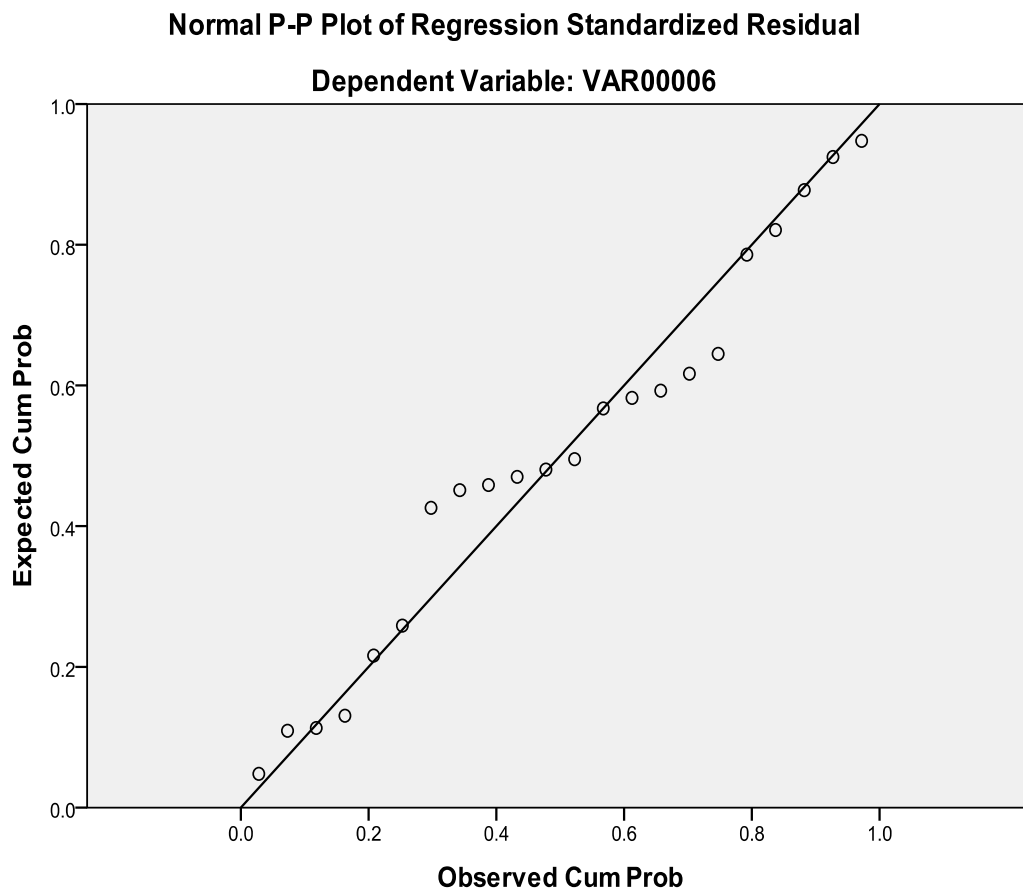
Figure 4.12 Observed xylitol production v.s. predicted xylitol production under optimum fermentation conditions.

Normal P-P Plot of regression standardized residual (Figure 4.12) demonstrated actual xylitol production from experimental design versus the predicted production from the empirical model Eq(5). The predicted data of response from the experimental model are in agreement with observed data in the range of the operating variable.

Table 4.12 Analysis of variance(ANOVA) for the regression model representing xylitol production.

Model	SS	df	MS	F-value	P-value
Model	747.004	5	149.401	8.409	$.000^b$
Residual	284.257	16	17.766		
Total	1031.261	21			

R^2 =0.724; R=0.851; SS, sum of squares; df, degrees of freedom; MS, mean square; Significance level =95%

Table 4.13 Results of regression analysis of the central composite design.

Term	Coefficients	t-statistic	Sig.
(Constant)	45.487	26.434	0.000**
X_1	.551	.522	0.609
X_2	2.380	2.255	0.039*
X_1X_2	-7.494	-5.951	0.000**
X_1^2	-3.399	-2.700	0.016*
X_2^2	.748	.502	0.623

*Significant at 95% level.
** Significant at 99% level.

The contour plot and Response surface plot in Figure 4.13 present the effect of xylose and peptone on xylitol production, while the other two variables were held at a constant level.

(A)

Peptone

1.8
1.2
0.6
0.0
-0.6
-1.2
-1.8

0.5, 0.0

-1.8   -1.2   -0.6   0.0   0.6   1.2   1.8

Xylose

(B)

Xylitol (g/l)

52
48
44
40
36
32
28

Peptone

-1.8
-1.2
-0.6
0.0
0.6
1.2
1.8

1.8 1.2 0.6 0.0 -0.6 -1.2 -1.8
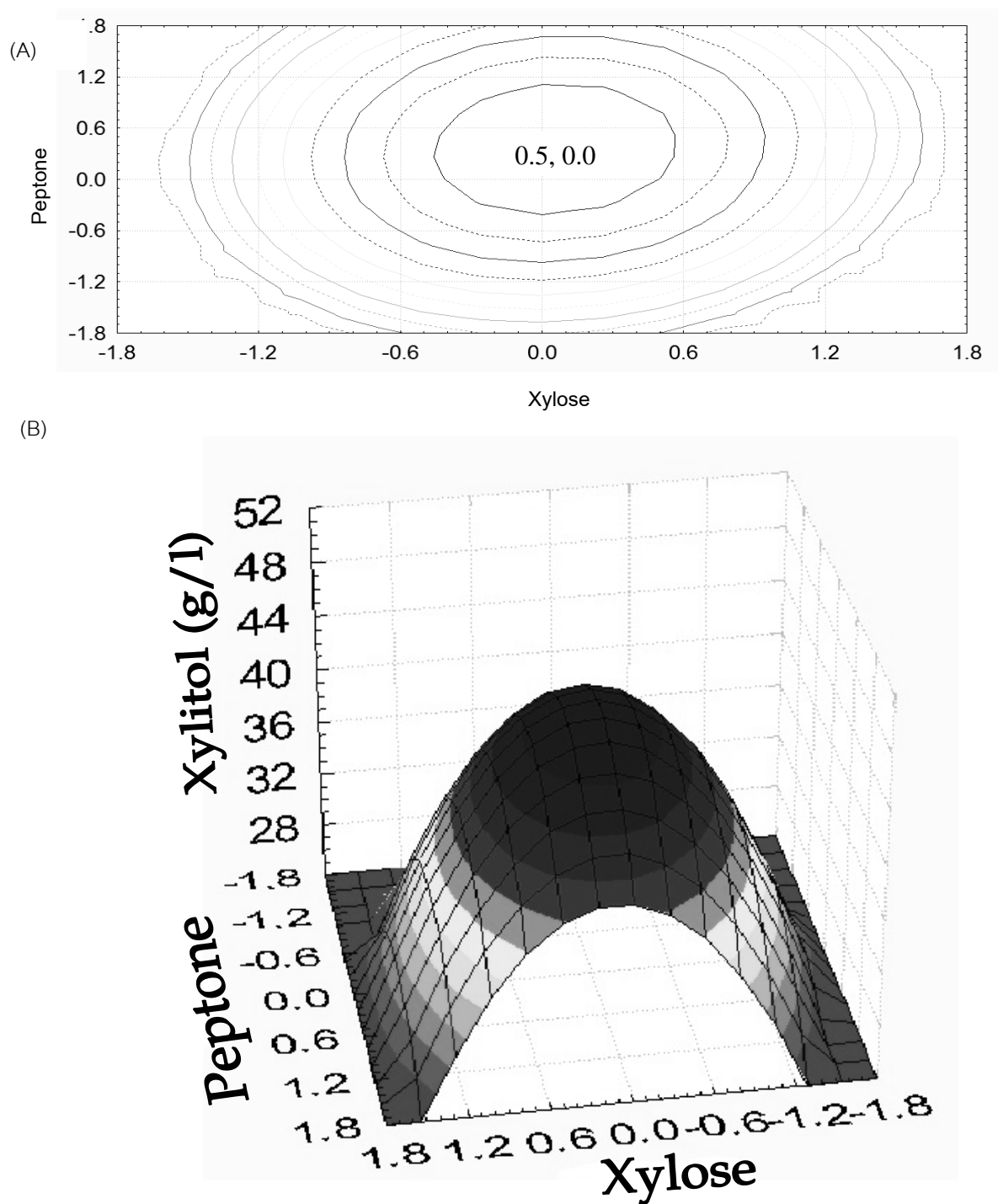
Xylose

Figure 4.13 Contour plot (A) and response surface (B) described by the model, representing xylitol production as function of xylose and peptone.

4.5.3 Statistical verification

The statistical optimal value of variables was investigated when considering carefully that the major and minor axis of response and contour plots at the center point yielded xylitol production. The optimum values of xylose and peptone for xylitol production were 80 g/l and 4 g/l, respectively. The maximum predicted xylitol production of 45.83 g/l was obtained. Verification of calculated optimum conditions for xylitol production was done by performing the experiment under optimized conditions. Under this conditions, *C. tropicalis* produced the highest concentration of 42.517 g/l xylitol at 24 h (Table 4.14) which is in agreement with the predicted values from the statistical model. This statistical design could an improved xylitol production yield from 0.54 g/g xylose of the un-optimization to 0.77 g/g xylose of optimization using CCD (Table 4.14). Moreover, the higher xylitol production yield produced by *C. tropicalis* A26 was obtained when compared to a previous report which showed 29.47 g/l of xylitol production and 0.77 g/g xylose (77.2%) of yielded xylitol production.

Validation of the model in batch experiment performed in 5L fermenter improved xylitol production. (Figure 4.14 and Table 4.15) At the optimized conditions, maximum xylitol production and yield were 71.59 g/l and 0.89 g/g xylose, respectively.
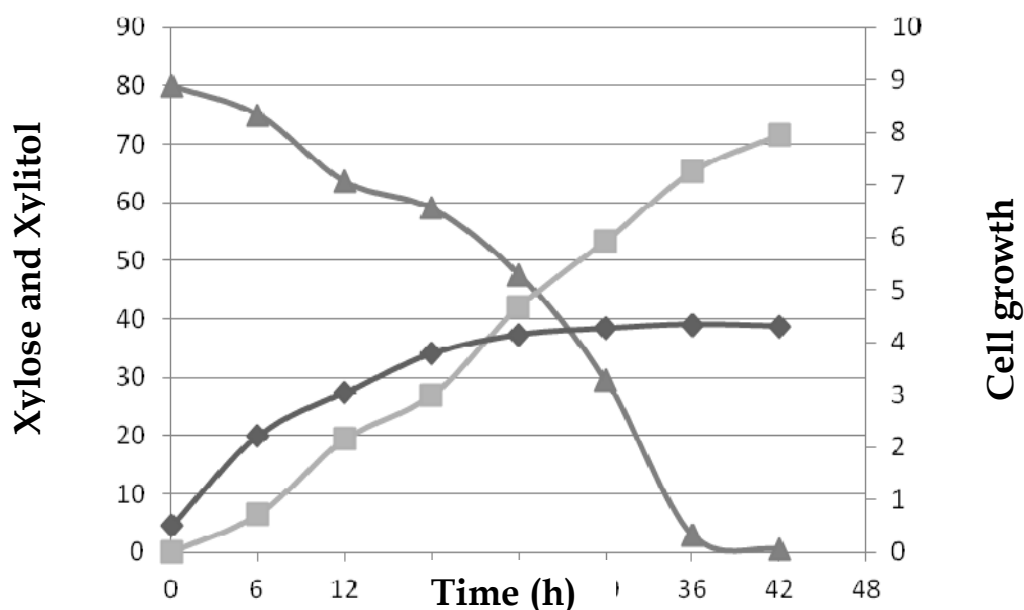
Figure 4.14 Time course of xylitol batch fermentation by *Candida tropicalis* at optimum condition. **Symbols: ▲, xylose; ■ , xylitol; ◆, Cell growth (OD$_{600}$).**

Table 4.14 Summary of improvement of the fermentation process for xylitol production by *C. tropicalis A26.*

Step	Method	Condition	Xylitol production (g/l)	Xylitol yield (g/g xylose)
1.	Un-optimized medium/ condition	40 g/l xylose, peptone 10 g/l, yeast extract 20 g/l.	21.30	0.53
2.	Optimization of medium composition using CCD in skake flasks	80 g/l xylose, peptone 4 g/l, yeast extract 5 g/l.	42.154	0.77
3.	Validation of the model in 5L stirred-vessel fermenter	80 g/l xylose, peptone 4 g/l, yeast extract 5 g/l, air ration rate of 1 vvm, initial pH 5.5 (uncontrol), temperature at 30C	71.59	0.89

4.6 Cloning of *C. tropicalis* A26 and *Z. meyerae* E23 xylose reductase gene

This study deals with characterization of xylose reductase gene of *Z. meyerae* E23 and *C. tropicalis* A26. Partial sequence of *xyl1* cloned of *Z. meyerae* E23 consisted of 807 nucleotides coding for 269 amino acids (Figure 4.16). The GenBank/EMBL/DDBJ accession number for its sequence is KC514137. Comparison of the partial amino acid sequences of *Z. meyerae* E23 to those of other yeast strains in GenBank database revealed that it was 59.16%, 57.59% and 57.06% identical and 67.53%, 67.01%, and 63.35% similar to those of Mg-XR of *Meyerozyma guilliermondii* (AAD09330), Os-XR of *Ogataea siamensis* (ACN78427) and Ctr-XR of *C. tropicalis* (ABX60132C), respectively. (Table 4.15).

Partial sequence of *xyl1* clone of *C. tropicalis* A26 which consisted of 558 nucleotides coding for 186 amino acids, is shown in Figure 4.15. Comparison to obtained partial amino acid sequences of *C. tropicalis* A26 with those of other yeast strains in the GenBank database revealed that *C. tropicalis* A26 xylose reductase possessed 98.42%, 78.53% and 77.48% identical and 98.42%, 78.53% and 77.48% similarity to xylose reductase of CtrXR of *C. tropicalis* (ABX60132C), CtXR of *C.*

tenuis (AAC25601) and CsXR of *C. shehatae* (ABK35120), respectively (Table 4.15). The *xyl1* gene from *C. tropicalis, O. siamensis, M. guilliermondii, C. paraspsilosis, C. shehatae, S. stipitis*, and *K. marxianus* have been extensively studied and showed some different from our strain, *Z. meyerae* E23 and *C. tropicalis* A26 as shown in Figure 4.17

Amino acid sequence alignment of the partial xylose reductase revealed a tetra amino acid motif (Ile-Pro-Lys-Ser), and an NAD(P)H species motif (Fulan and de Castro, 2001; Jefferies and Jin, 2004; Zhan *et al*., 2011) (Figure 4.17). The motif was well-conserved among xylose reductase of several yeast strains except for CpXR of *C. parasilopsis* in which Lys residue was substituted with Arg and resulted in preferentially utilized NADH as a coenzyme. The CtrXR that showed the highest sequence homology to those of A26XR was previously reported as NADPH-specific XR (Jefferies and Jin, 2004; Zhan *et al*., 2011).

A similar result was found in *Z. meyerae* E23, as NADPH-specific XR. Previously, experimatal *Z. meyerae* E23 produced ethanol but not higher than *S. stipitis*. This result suggested that xylose reductase of *Z. meyerae* E23 might be specific for NAD(P)H coenzyme, and redox imbalance might be a possible cause of its low ethanol production. Difference in coenzyme preference between xylose reductase (NADPH) and xylitol dehydrogenase (NAD+) was a major limiting factor for ethanol production from xylose (Zeng *et al*., 2009).

However, this is the first study on cloning and characterization of xylose reductase gene of *Z. meyerae* E23 which was isolated in Thailand.

```
1      CCG CCG GGC TTT TAT TGC GGC GAT GGC GAT AAC TTT CAT TAT GAA     45
1       P   P   G   F   Y   C   G   D   G   D   N   F   H   Y   E      15

46     GAT GTG CCG CTG CTG GAT ACC TGG AAA GCG CTG GAA AAA CTG GTG     90
16      D   V   P   L   L   D   T   W   K   A   L   E   K   L   V      30

91     GAA GCG GGC AAA ATT AAA AGC ATT GGC ATT AGC AAC TTT ACC GGC    135
31      E   A   G   K   I   K   S   I   G   I   S   N   F   T   G      45

136    GCG CTG ATT TAT GAT CTG ATT CGT GGC GCG ACC ATT AAA CCG GCG    180
46      A   L   I   Y   D   L   I   R   G   A   T   I   K   P   A      60

181    GTG CTG CAG ATT GAA CAT CAT CCG TAT CTG CAG CAG CCG AAA CTG    225
61      V   L   Q   I   E   H   H   P   Y   L   Q   Q   P   K   L      75

226    ATT GAA TAT GTG CAG AAA GCG GGC ATT GCG ATT ACC GGC TAT AGC    270
76      I   E   Y   V   Q   K   A   G   I   A   I   T   G   Y   S      90

271    AGC TTT GGC CCG CAG AGC TTT CTG GAA CTG GAA AGC AAA CGT GCG    315
91      S   F   G   P   Q   S   F   L   E   L   E   S   K   R   A     105

316    CTG AAC ACC CCG ACC CTG TTT GAA CAT GAA ACC ATT AAA AGC ATT    360
106     L   N   T   P   T   L   F   E   H   E   T   I   K   S   I     120

361    GCG GAT AAA CAT GGC AAA AGC CCG GCG CAG GTG CTG CTG CGT TGG    405
121     A   D   K   H   G   K   S   P   A   Q   V   L   L   R   W     135

406    GCG ACC CAG CGT AAC ATT GCG GTG ATT CCG AAA AGC AAC AGC CCG    450
136     A   T   Q   R   N   I   A   V   I   P   K   S   N   S   P     150

451    GAA CGT CTG GCG CAG AAC CTG AGC GTG GTG GAT TTT GAT CTG ACC    495
151     E   R   L   A   Q   N   L   S   V   V   D   F   D   L   T     165

496    CAG GAA GAT CTG GAT AAC ATT GCG AAA CTG GAT ATT GGC CTG CGT    540
166     Q   E   D   L   D   N   I   A   K   L   D   I   G   L   R     180

541    TTT AAC GAT CCG TGG GAT    558
181     F   N   D   P   W   D
```

Figure 4.15 Nucleotide and deduced amino acid sequences of the partial *xyl1* gene of *C. tropicalis* A26.

```
1     ATT TTT GAT GGC GCG CAG GAT TAT GGC AAC GAA AAA GAA TGC GGC    45
1      I   F   D   G   A   Q   D   Y   G   N   E   K   E   C   G     15

46    GAA GGC GTG GCG CGT GCG ATT AAA GAT GGC CTG GTG AAA CGT GAA    90
16     E   G   V   A   R   A   I   K   D   G   L   V   K   R   E     30

91    GAT GTG TTT ATT ACC AGC AAA CTG TGG AAC ACC TTT CAT GCG AAA    135
31     D   V   F   I   T   S   K   L   W   N   T   F   H   A   K     45

136   GAA CAT GTG AAA CCG CTG CTG AAA CGT AGC CTG GCG GAT TGG GGC    180
46     E   H   V   K   P   L   L   K   R   S   L   A   D   W   G     60

181   CTG GAA TAT TTT GAT CTG TTT CTG ATT CAT TTT CCG ATT GCG CAG    225
61     L   E   Y   F   D   L   F   L   I   H   F   P   I   A   Q     75

226   AAA TAT GTG GAT CCG GCG GAA CGT TAT CCG CCG GGC ATT GTG AAC    270
76     K   Y   V   D   P   A   E   R   Y   P   P   G   I   V   N     90

271   GAT GTG GAA AAC AAA GTG GCG TAT TTT CAG AAC ACC CCG ATT AGC    315
91     D   V   E   N   K   V   A   Y   F   Q   N   T   P   I   S     105

316   GAA ACC TGG GCG GCG CTG GAA GAA TGC GTG AAA GAA GGC CTG GTG    360
106    E   T   W   A   A   L   E   E   C   V   K   E   G   L   V     120

361   AAA AAC ATT GGC ATT AGC AAC TTT AAC GCG GGC CTG ATT CGT GAT    405
121    K   N   I   G   I   S   N   F   N   A   G   L   I   R   D     135

406   CTG CTG AGC TAT GCG AAA ATT CCG CCG GCG GTG CTG CAG ATT GAA    450
136    L   L   S   Y   A   K   I   P   P   A   V   L   Q   I   E     150

451   CAT CAT CCG TAT CTG ACC CAG GAA CCG CTG GTG AAA TAT GTG CAG    495
151    H   H   P   Y   L   T   Q   E   P   L   V   K   Y   V   Q     165

496   AGC CAG GGC ATT GCG ATT ACC GGC TAT AGC AGC TTT GGC CCG CAG    540
166    S   Q   G   I   A   I   T   G   Y   S   S   F   G   P   Q     180

541   AGC TAT ATT GAA CTG GGC AAC CCG CGT GTG AAA GCG AAC GGC CCG    585
181    S   Y   I   E   L   G   N   P   R   V   K   A   N   G   P     195

586   CTG CTG ACC AAC GAT ATT ATT GAA AAA ATT GCG AAA AGC CAT GGC    630
196    L   L   T   N   D   I   I   E   K   I   A   K   S   H   G     210

631   AAA AGC ACC GCG CAG GTG CTG CTG CGT TGG GCG ACC CAG CGT AAC    675
211    K   S   T   A   Q   V   L   L   R   W   A   T   Q   R   N     225

676   ATT GCG GTG ATT CCG AAA AGC AAC AAC CAG GAA CGT CTG GTG GCG    720
226    I   A   V   I   P   K   S   N   N   Q   E   R   L   V   A     240

721   AAC CTG AAA AGC GAT GAT TTT AAC CTG ACC GAT GCG GAA ATT AAA    765
241    N   L   K   S   D   D   F   N   L   T   D   A   E   I   K     255

766   GAA ATT AGC AGC CTG GAT ATT GGC TTT CGT TTT AAC AAC CCG GGC    810
256    E   I   S   S   L   D   I   G   F   R   F   N   N   P   G     270

811   ACC GGC ATT GGC AGC CGT GCG CGT CGT CTG CAG CGT CCG GCG TGC    855
271    T   G   I   G   S   R   A   R   R   L   Q   R   P   A   C     285

856   AAA CTG AGC CTG    867
286    K   L   S   L
```

Figure 4.16 Nucleotide and deduced amino acid sequences of the partial *xyl1* gene of *Z. meyerae* E23.

Figure 4.17 Alignment of partial amino acid sequences of xylose reductase from *Z. meyerae* E23 and *C.tropicalis* A26 with other yeast strains.

OsXR, *Ogataea siamensis* (ACN78427); KmXR, *Kluyveromyces marxianus* (ADV91498); E23XR, *Z. meyerae* E23 (KC514137); SpXR, *Spathaspora passalidarum*, (EGW32258); SsXR, *Scheffersomyces stipitis* (CAA42072); CsXR, *Candida shehatae* (ABK35120); CtXR, *Candida tenuis* (AAC25601); MgXR from *Meyerozyma guilliermondii* (AAD09330); CpXR, *Candida parapsilosis* (AAO91803); CtrXR, *Candida tropicalis* (ABX60132); A26XR, *C. tropicalis* A26; The underlined conserved sequences were used to design degenerated primers for the *xyl* cloning. Rectangle box indicates NAD(P)H coenzyme utilization

Table 4.15 Comparison of amino acid sequence similarity and identity between *Z. meyerae* E23 xylose reductase, *C.tropicalis* A26 xylose reductase, and those of others.

Code	OsXR	KmXR	E23XR	CsXR	CtXR	SsXR	SpXR	MgXR	CtrXR	CpXR	A26XR
OsXR	■	59.16	57.59	57.59	56.54	58.63	59.16	66.49	64.39	59.68	64.39
KmXR	70.68	■	49.21	61.25	61.25	60.20	55.49	59.16	59.16	54.45	59.68
E23XR	67.01	60.73	■	59.68	60.20	62.30	62.82	67.53	58.11	63.35	62.82
CsXR	68.58	71.20	51.30	■	91.09	81.15	74.86	70.68	77.48	63.87	77.48
CtXR	69.1	71.72	51.30	94.76	■	79.05	74.34	69.10	78.53	61.78	78.53
SsXR	69.63	72.25	50.78	87.43	86.91	■	75.39	69.63	74.34	60.73	74.34
SpXR	71.20	70.68	48.69	84.81	82.19	83.76	■	67.53	72.25	56.54	72.77
MgXR	74.34	70.15	59.16	81.15	80.10	80.62	80.62	■	74.86	65.96	75.39
CtrXR	73.29	71.72	51.83	86.38	86.91	82.72	83.76	82.76	■	75.39	75.19
CpXR	69.10	67.53	57.06	74.86	73.82	73.82	73.82	75.39	82.72	■	98.42
A26XR	72.77	71.72	56.54	85.86	86.38	83.24	84.29	83.76	83.76	98.95	■

% Identity (right side, downward arrow)

% Similarity (bottom, rightward arrow)

Abbreviations: OsXR, *Ogataea siamensis*; KmXR, *Kluyveromyces marxianus*; E23XR, *Z. meyerae* E23; CsXR, *Candida shehatae*; CtXR, *Candida tenuis*; SsXR, *Scheffersomyces stipitis*; SpXR, *Spathaspora passalidarum*; MgXR from *Meyerozyma guilliermondii*; CtrXR, *Candida tropicalis*; CpXR, *Candida parapsilosis*; A26XR, *C.tropicalis* A26.

CHAPTER V

CONCLUSION

In this study, 129 xylose-utilizing yeasts were isolated from 60 samples of animal feces in Thailand and 3 soil samples in Japan. Ninety-three isolates were assigned to 19 recognized species in 8 genera of the phylum Ascomycota and they were identified as *C. tropicalis* (40 isolates), *C. stellimlicola* (3 isolates), *C. tartarivorans* (1 isolate), *C. pseudolambica* (1isolates), *C. rugosa* (11 isolates), *C. albican* (1 isolate), *C. mengyuniae* (2 isolates), *C. parapsilosis* (2 isolates), *S. lactativara* (9 isolates), *K. marxianus* (3 isolates), *M. caribbica* (3 isolates), *Z. meyerae* (1 isolate), *B. californica* (3 isolates), *I. terricola* (1 isolates), *P. occidentalis* (1 isolate), *I. orientalis* (1 isolates), *P. kluyveri* (3 isolates), *Cy. saturnus* (1 isolate) and *Cy. mrakii* (1 isolate). Twenty-six strains were assigned to three recognized species in one genus of the phylum Basidiomycota and identified as *T. mycotoxinivorans* (4 isolates), *T. asahii* (17 isolates) and *T. akiyoshidainum* (5 isolates). An isolate in the genus as *Candida* sp. ELP19 and in *Geotrichum* sp. (14 isolates) were non-described species. However, they were identified as novel species based on their differential phenotypic and the D1/D2 region of the large subunit ribosomal RNA gene. *Candida* sp. ELP19 was closely related to *Wickerhamomyces onychis* (94% identical). The isolates of *Geotrichum* sp. were divided into two Groups, VIII (a) and were closely related to *Geotrichum silvicola* UFMG-354-2[T] and *Geotrichum silvicola* UFMG-354-2[T] with 4-5 different D1/D2 nucleotide sequences (554-543 bp), respectively. The isolates in Group VIII (b) were closely related to *Galactomyces geoticum* NRRL Y-17569[T] with 12 different D1/D2 nucleotide sequences (546 bp).

The ascomycete isolates of *C. tropicalis* were distributed in many animal feces such as macaques, zebras, baring deer, giraffes, kangaroos, cows, buffalo, elephants and wild elephants. *C. stellimlicola, C. rugosa, P. occidentalis, I. orientalis, P. kluyveri* and *Cy. saturnus* strains were isolated from wild elephants feces while *C. tartarivorans, K. marxianus, M. caribbica* and *Z. meyerae* were from non-wild

elephants' feces. *C. pseudolambica, B. californica* and *Cy. mrakii* strains were found in soils from Hiroo park, Japan and *C. albicans* from kangaroo*; while *C. mengyuniae* and *C. parapsilosis* were from buffalo feces*, and *S. lactativara* from elephant and buffalo feces and *I. terricola* from languors. The basidiomycete isolates, *T. mycotoxinivorans* were found in elephant feces*, T. asahii* in buffalo and wild elephant feces and *T. akiyoshidainum* in soils of Hiroo park, Japan.

All 129 strains utilized xylose as the carbon source, 117 strains could ferment D-xylose to ethanol (0.01-2.388 g/l) while 95 isolates could ferment D-xylose to xylitol (0.03-29.47 g/l). *Z. meyerae* E23 produced the maximum ethanol (2.40 g/l). It was selected to study the dynamics of xylose utilization for growth and ethanol production. *Z. meyerae* E23 could produce ethanol production to 3.631 g/l at 72h, which had very slow growth. Therefore, its ethanol production was low. This study was the first determination of ethanol production by the *Z. meyerae* strain.

In the xylitol fermentation, *C. tropicalis* A26 was selected to optimize its xylitol production, and it produced maximum xylitol with 29.47 g/l and 0.77 g/g xylose (77%) yield. Subsequently, the strain was optimized for xylitol production by using Plackett-Burman and Central Composite Design (CCD) model. The critical parameters screened by the Plackett-Burman design experiments were xylose and peptone. An interaction between these two factors were further screened by CCD.

The optimal conditions for xylitol production by *C. tropicalis* A26 were: xylose (80 g/l), peptone (4 g/l) and yeast extract (5 g/l), inoculum (20% v/v) and pH 5.5. The xylitol production and yield at the optimized condition were 2 and 1.45 folds higher than the un-optimized condition, respectively. Batch fermentation at the optimized condition obtained highest xylitol; yield was 0.89 g/g xylose which was 1.67 folds higher than at an un-optimized condition. The results indicated that the statistical design provided useful information for the optimization of xylitol fermentation of *C. tropicalis* A26.

The xylose reductase gene (*xyl1*) was cloned and characterized for *Z. meyerae* ELP23 and *C. tropicalis* A26. A partial xylose reductase gene (*xyl1*) of *Z. meyerae* ELP23 consisted of 807 nucleotides encoding for 269 amino acids, 59.70% identical with *C. tropicalis* (CtXR) and 73.88% similar to *Ogataea siamensis* (OsXR). A part

of xylose reductase gene (*xyl1*) of *C. tropicalis* A26 consisted of 558 nucleotides coding for 186 amino acids, 98.42% identical and 98.95% similar to *C. tropicalis* (CtXR). The xylose reductase gene of both strains was detected as NAD(P)H-dependent xylose reductase. However, the expression of the *xyl*1 clone including characterization of the xylose reductase is required for further study.

Xylose fermenting yeasts isolated from animal feces in Thailand was firstly reported in this study, particularly from wild animals. There are many species and novel species and some strains produced ethanol but some showed potential to produce xylitol from xylose fermentation. Especially, Z. *meyerae* E23 was first reported to produce ethanol but only in small amounts. In the future, if the co-factor preference for xylose reductase enzyme was established by site-directed mutagenesis studies or the change of co-factor specificity to high ethanol yield, the strain could be improved regarding ethanol production. In addition, xylitol production from *C. tropicalis* A26 was improved by statistical design and could be applied for use in industrial scales in the future.

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APPENDICES

APPENDIX A
CULTURE MEDIA

All media were dispended and sterilized in autoclave for 15 min at 15 pounds pressure (121°C) for media except for carbon utilization test which was sterilized at 110 pounds for 110°C for 10 min. All media were prepared in 100 mL of distilled water.

1. Yeast nitrogen base xylose (YX)

Xylose	2	g
Yeast nitrogen base	0.67	g
Agar	1.5	g

2. Yeast nitrogen base xylose (YX) agar plates supplemented with chloramphinical (100 µg/l) and sodium propionate (0.2%)

Xylose	2	g
Yeast nitrogen base	0.67	g
Chloramphenical	10	µg
Sodium propionate	0.2	g
Agar	1.5	g

3. Yeast peptone dextrose (YPD)

Glucose	2	g
Bacto yeast extract (Difco)	1	g
Peptone	2	g

4. Yeast peptone xylose (YPX)

Xylose	4	g
Bacto yeast extract (Difco)	1	g
Peptone	2	g

5. Yeast extract-malt extract agar (YM)

      Bacto yeast extract (Difco)   0.4   g

      Bacto malt extract (Difco)   1.0   g

      Glucose   0.4   g

      Agar   1.5   g

6. 5% malt extract agar

      Malt extract   5   g

      Agar   1.5   g

7. Stock carbon solution (10X)

      Yeast Nitrogen Base (Difco)   6.7   g

      Carbon compound   5.0   g

      Sterilized by filtration and stored in a freezer at -20°C until use

8. Stock nitrogen solution (10X)

      Bacto Yeast Carbon Base (Difco)   11.7   g

      Nitrogen compound*   x   g

      Sterilized by filtration and stored in a freezer at -20°C until use

      * Nitrogen compound: $(NH_4)_2SO_4$ (0.5 g) or KNO_3 (0.78 g) or $NaNO_2$ (0.26 g) or ethylamine-HCl (0.64 g) or L-lysine-HCl (0.56 g) or cadaverine (0.68 g)

9. Yeast Carbon Base (YCB) medium:

9.1 Stock YCB solution (10X)

      Bacto Yeast Carbon Base (Difco)   11.7   g

      Sterilized by filtration and stored in a freezer at -20°C until use

9.2 Ten fold of stock YCB solution (0.2 ml) was added to 13x100 mm test tube containing 1.8 ml sterilized distilled water

10. Yeast Nitrogen Base (YNB) medium

    10.1 Stock YNB solution (10X)

        Bacto Yeast Nitrogen Base (Difco)   6.7   g

        Sterilized by filtration and stored in a freezer at -20°C until use

    10.2 Ten fold of stock YNB solution (0.2 ml) was added into 13 x 100 mm test tube containing 1.8 ml sterilized distilled water.

11. Carbon assimilation medium

Ten fold of stock carbon solution (0.2 ml) was added to 13 x 100 mm test tube containing 1.8 ml sterilized distilled water.

12. Nitrogen agar plate:

Ninety ml of 1.67 % agar solution was sterilized at 121°C for 15 min and left for cool down to 50-55°C, then 10 ml of stock nitrogen solution (10X) was added, mixed well and poured into the petri dishes

13. Fermentation test medium

    13.1 Basal medium

        Yeast extract   4.5   g

        Peptone   7.5   g

        Bromothymol blue (stock 50 mg/75 ml)   small amount

Fermentation basal medium (2 ml) was distributed into the cotton plugged test tubes (13x100 mm), insert Durham tube and sterilize at 121°C for 15 min. After cool down, add concentrate sugar, which was sterilized by membrane filtration to make the final concentration at 2% sugar (except 4% raffinose).

14. Vitamin requirement basal medium

        Vitamin-free cassamino acids (Difco)   5.0   g

        KH_2PO_4 .$7H_2O$ 1   g

        $MgSO_4$ 0.5   g

        NaCl   0.1   g

        $CaCl_2$ 0.1   g

        Adjust pH = 5.5

APPENDIX B

REAGENTS AND BUFFERS

1. Phenol : Chloroform (1 : 1 v/v)

Crystalline phenol was liquidified in water bath at 65 $^{\circ}$C and mixed with chloroform in the ratio of 1 : 1 (v/v). the solution was stored in a light tight bottle.

2. 0.5M EDTA (pH 8.0)

800 mL of distilled water, 186.1 g of disodium ethylenediaminetetraacetate.$2H_2O$ was added and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH (20 g of NaOH pellets). The volume was adjusted to 1 litre. The solution was dispensed into aliquots and sterilized by autoclaving for 15 minutes at 15 lb/in^2.

3. 5M NaCl

292.2 g of sodium chloride was added 800 ml of distilled water and adjusted the volume to 1 litre with distilled water. The solution was sterilized by autoclaving for 15 minutes at 15 lb/in^2

4. 10% Sodium dodecyl sulphate (SDS)

The stock solution of 10% SDS was prepared by dissolved 10 g of sodium dodecyl sulphate in 100 mL sterilized distilled water. Sterilization is not required for the preparation of this stock solution.

5. 1M Tris-HCl pH 8.0

The 1M Tris was prepared by dissolving 121.1 g of Tris base in 800 mL of distilled water. The pH was adjusted to the desired value by adding conc. HCl (pH 8.0, 42 mL of HCl). The solution was cooled to room temperature before making final adjustment to the desired pH. The volume of the solution was adjusted to 1 litter with distilled water and sterilized by autoclving.

6. 0.1 M Tris-HCl buffer, pH 9

Tris	1.21	mg
Distilled water	100	mL

   Adjust the pH to 9 with HCl

7. Lysis buffer

   100 mM Tris (pH 8.0)

   30 mM EDTA (pH 8.0)

   0.5% SDS

8. 2.5 M Potassium acetate (pH7.5)

   245.53 g of Potassium acetate was add 800 ml of distilled water and adjusted the volume to 1 litre with distilled water. The solution was sterilized by autoclaving for 15 minutes at 15 lb/in^2

9. TE buffer

   10 mM Tris HCl (pH 8.0)

   1 m M Na$_2$-EDTA (pH 8.0)

10. Ethidium bromide solution (10 mg/mL)

   The ethidium bromide solution was prepared by dissolved 1 g of ethidium bromide in 100 mL of distilled water. The solution was stored in light-tight container at room temperature.

11. Gel loading buffer

   0.25 of bromophenol blue was dissolved in 20 mL of 15% glycerol.

12. Tris-acetate EDTA (TAE) buffer

1xTBE buffer was used an electrophoresis buffer throughout the study. The working solution of 1xTBE buffer was prepared from stock solution of 5xTAE buffer, as followed.

Tris-base	5.4	g
Boric acid	2.75	g
Na_2-EDTA	0.47	g
Distilled water	100	mL

13. Agarose gel

Agarose	1.6	g
1xTBE buffer	200	mL

APPENDIX C

INFORMATION OF STRAINS

Table 1 Source, location, isolated number and date of isolation.

Source of sample	Isolation No.	Location of sample	Date of isolation
Feces of			
Elephant	A1	Prachinburi	2008
Elephant	A2	Dusit zoo, Bankok	2008
Goat	A3	Dusit zoo, Bankok	2008
Giraffe	A4	Dusit zoo, Bankok	2008
Macaque	A5	Dusit zoo, Bankok	2008
Buffalo	A6	Khonkaen	2008
Buffalo	A7, A8	Kalasin	2008
Cow	A9,A10, A11	Udonthani	2008
Cow	A12,A13,A14,A15	Kalasin	2008
Buffalo	A16,A17	Kalasin	2008
Kangaroo	A18	Korat zoo, Nakhonratchasima	2008
Elephant	A19	Korat zoo, Nakhonratchasima	2008
Zebra	A20	Korat zoo, Nakhonratchasima	2008

Table1 (Continued).

Source of sample	Isolation No.	Location of sample	Date of isolation
Feces of Giraffe	A21	Korat zoo, Nakhonratchasima	2008
Hog deer	A22	Korat zoo, Nakhonratchasima	2008
Antelope	A23	Korat zoo, Nakhonratchasima	2008
Orlik	A24	Korat zoo, Nakhonratchasima	2008
Horse	A25	Korat zoo, Nakhonratchasima	2008
Cow	A26	Khonkaen province	2008
Barking deer	A27	Dusit zoo, Bankok	2008
Zebra	A28	Dusit zoo, Bankok	2008
Elephant	B1	Prachinburi	2008
Barking deer	D1,E1,F1	Dusit zoo, Bankok	2008
Langur	H1	Dusit zoo, Bankok	2008
Zebra	I1	Dusit zoo, Bankok	2008
Kangaroo	L1	Korat zoo, Nakhonratchasima	2008
Zebra	M1	Korat zoo, Nakhonratchasima	2008
Buffalo	BUF1-1, BUF1-2, BUF2-1, BUF2-2, BUF2-2(2), BUF2-3, BUF2-4	Murrah 1 month, Murrah Farm, Chachoengsao	2008

Table1 (Continued).

Source of sample	Isolation No.	Location of sample	Date of isolation
Feces of Buffalo	BUF3-10, BUF3-13, BUF3-17, BUF3-18, BUF3-19, BUF3-3, BUF3-5, BUF3-6, BUF3-8, BUF3-9	Murrah 2 month, Murrah Farm, Chachoengsao	2008
Buffalo	BUF8, BUF9-2, BUF9-3, BUF9-5	Murrah 2.52 years, Murrah Farm, Chachoengsao	2008
Buffalo	BUF8-2, BUF7, BUF7(2)	Murrah 2.58 years, Murrah Farm, Chachoengsao	2008
Buffalo	BUF9, BUF9(2)	Murrah 2.65 years, Murrah Farm, Chachoengsao	2008
Buffalo	BUF7-1, BUF7-3	Thai buffalo 2 month, Murrah Farm, Chachoengsao	2008
Buffalo	BUF4-2, BUF4-3, BUF4-1, BUF4-4, BUF5-1, BUF5-3, BUF5-4	Thai buffalo 3 month, Murrah Farm, Chachoengsao	2008
Buffalo	BUF10, BUF10(2)	Thai buffalo 4.33 years, Murrah Farm, Chachoengsao	2008
Buffalo	BUF11, BUF11(2), BUF12-1, BUF12-2	Thai buffalo 5 years, Murrah Farm, Chachoengsao	2008
Elephant	ELP1, ELP2, ELP3, ELP4	Bankok	2009
Elephant	ELP5, ELP6, ELP7	Bankok	2009
Elephant	ELP11, ELP12	Dusit zoo, Bankok	2009
Elephant	ELP8, ELP9, ELP10	Ayuthya Elephant Camp, Ayuthaya	2009
Elephant	ELP20, ELP21, ELP22, ELP23	Royal Elephant Kraal & Village, Ayuthya	2009
Elephant	ELP13	HomeStay Elephant Thai , Lampang	2009

Table1 (Continued).

Source of sample	Isolation No.	Location of sample	Date of isolation
Feces of			
Elephant	ELP15, ELP18	Thai Elephant conservation , Lampang	2009
Elephant	ELP16	Elephant Camp Mae-sa , Chaimai	2009
Elephant	ELP19	Elephant Camp Mae-ta-moun , Chaimai	2009
Elephant	ELP44, ELP45,ELP46	Elephant Camp, Chonburi	2009
Elephant	ELP48, ELP49, ELP47	The Million Years Stone Park & Pattaya Crocodile Farm, Chonburi	2009
Elephant	ELP39,ELP40	Camp thai thai , Chonburi	2009
Elephant	ELP37	Elephant Village , Chonburi	2009
Elephant	ELP38	Khao Kheow Open Zoo, Chonburi	2009
Elephant	ELP42, ELP43	Secview, Chonburi	2009
Elephant	ELP41	Siam Elephant, Chonburi	2009
Elephant	ELP36	Camp tin-thai , Chonburi	2009
Elephant	ELP33	Camp Kaew Elephan, Kanchanaburi province	2009
Elephant	ELP34, ELP35	Camp Elephant Wang-po, Kanchanaburi province	2009
Elephant	ELP26	Safari zoo, Kanchanaburi	2009
Elephant	ELP25,ELP17	Samphran Elephant Ground & Zoo, Nakhon Pathom	2009
Elephant	ELP32	Asia elephant foundation in Thailand, Prachuapkhirikhan	2009

Table 1 (Continued).

Source of sample	Isolation No.	Location of sample	Date of isolation
Feces of			
Elephant	ELP30	Safari zoo, Prachuapkhirikhan	2009
Elephant	ELP27, ELP28, ELP29, ELP50	Elephant Village, Surin	2009
Wild elephant	2-1, 2-2, 2-3	Krachan National Park, Phetchaburi	2010
Elephant	3-1, 3-2, 3-3, 3-4, 3-5, 3-6, 3-7	Krachan National Park, Phetchaburi	2010
Elephant	4-1, 4-2, 4-3, 4-4, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 4-11, 4-12, 4-13, 4-14	Krachan National Park, Phetchaburi	2010
Elephant	5-1, 5-2, 5-3, 5-4, 5-5, 5-6, 5-7	Krachan National Park, Phetchaburi	2010
Elephant	6-1, 6-2, 6-3, 6-4, 6-5, 6-6, 6-7, 6-8, 6-9, 6-10, 6-11	Krachan National Park, Phetchaburi	2010
Elephant	7-1, 7-2, 7-3, 7-4, 7-5, 7-6, 7-7, 7-8	Krachan National Park, Phetchaburi	2010
Elephant	9-1, 9-2, 9-3, 9-4, 9-5, 9-6, 9-7, 9-8, 9-9	Krachan National Park, Phetchaburi	2010
Elephant	10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7, 10-8, 10-9, 10-10, 10-11	Krachan National Park, Phetchaburi	2010
Soil	11-1, 11-2, 11-3, 11-4, 11-5, 11-6, 11-7	Hiroo park, Tokyo	2011
Soil	12-1, 12-2, 12-3, 12-4, 12-5, 12-6, 12-7	Hiroo park, Tokyo	2011
Soil	15-1, 15-2, 15-3	Hiroo park, Tokyo	2011

Table 2 List of yeasts species isolated from animal feces in Thailand and soil in Japan**.**

Species	No. of strains
Group I :	
Candida tropicalis	40
Candida stellimalicala	3
Candida tartarivorans	1
Candida pseudolambica	1
Candida rugosa	11
Candida albican	1
Candida mengyuniae	2
Candida parasilosis	2
Candida sp. ELP19	1
Group II :	
Sporopachydermia lactativora	9
Group III :	
Kluyveromyces marxianus	3
Group IV :	
Meyerozyma caribbica	3
Group VIII:	
Zygoascus meyerae	1
Group IX:	
Barnettozyma californica	3
Group V :	
Issatchenkia terricola	1
Pichia occidentalis	1
Issatchenkia orentalis	1
Pichia kluyveri	3
Group VI :	
Geotrichum candidum	4
Geotrichum sp.	10
Group VII:	
Cyberlindnera saturnus	1
Cyberlindnera mrakii	1
Group X:	
Trichosporon mycotoxinivorans	4
Trichosporon asahii	17
Trichosporon laibachii	5
TOTAL	129

Table 3. Differential characteristics of the isolates in Group I (a) to I (g) and related type strains.

Characteristics	Gr. I(a)	C. tropicalis [a]	Gr. I(b)	C. stellimalicala [a]	Gr. I(c)	C. tartarivorans [a]	Gr. I(d)	C. pseudolambica [a]	Gr. I(e)	C. rugosa [a]	Gr. I(f)	C. albicans [a]	Gr. I(g)	C. mengyuniae [b]	Gr. I(h)	C. parasilosis [a]
Fermentation of																
Glucose	+	+	s	-	+	+	+	+	-	-	+	+	+	+	+	+
Galactose	+	+	-	-	+	+	-	-	-	-	+	v	n	-	n	v
Sucrose	+	v	-	-	l	l	-	-	-	-	+	v	n	+	n	-/s
Maltose	+	+	-	-	-	l	-	-	-	-	+	+	n	-	n	-/s
Raffinose	-	-	-	-	s	l	-	-	-	-	+	-	n	w	n	-
Trehalose	+	+	-	-	w	l	-	-	-	-	n	v	n	-	n	-/s
C-Assimilation of																
D-Arabinose	-	-	l	-	l	l	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	s	+	-	-	-	-	v	-	-	-	+	+
Cellobiose	+	v	-	-	+	s	-	-	-	-	-	-	-	v	-	-
Citrate	+	+/l	-	-	s	w/l	-	-	-	-	-	-	n	n	+	+
Erythritol	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Ethanol	+	+	+	+	-	w	+	+	+	+	s	+	n	n	+	+
Galactose	+	+	-	-	+	+	-	-	+	+	+	+	+	-	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	v	l	+	+	+	-	-	+/l	+	v	-	+	+	+	+
Inositol	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
2-keto-D-Gluconate	+	+	-	-	w	n	-	-	-	-	+	+	n	n	+	+
5-keto-D-Gluconate	+	+	-	-	s	n	-	n	-	-	+	+	n	n	n	n
DL-Lactate	-	v	+	+	s	-	+	+	+	v	+	+	n	+	-	-
Lactose	-	-	-	-	+	+	-	-	-	-	-	-	n	-	-	-
D-Mannitol	+	+	s	+	+	+	-	-	+	v	+	+	+	+	+	+
Maltose	+	+	-	-	+	+	-	-	-	-	+	+	-	+	+	+
Melezitose	+	v	-	-	l	s	-	-	-	-	-	s	-	+	+	+
Melibiose	-	-	-	-	l	+	-	-	-	-	v	-	+	-	-	-

Table 3. (Continued).

Characteristics	Gr. I(a)	C. tropicalis[a]	Gr. I(b)	C. stellimalicala[a]	Gr. I(c)	C. tartarivorans[a]	Gr. I(d)	C. pseudolambica[a]	Gr. I(e)	C. rugosa[a]	Gr. I(f)	C. albicans[a]	Gr. I(g)	C. mengyuniae[b]	Gr. I(h)	C. parasilosis[a]
α-Methyl-D-Glucoside	+	v	-	-	+	s	-	-	-	-	v	+	+	v	+	+
Raffinose	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Ribose	-	v	-	-	l	s	-	-	-	-	v	-	-	-	v	-
Sucrose	+	v	-	-	+	+	-	-	-	-	v	+	+	+	+	+
Succinate	+	+	+	+	s	l	+	+	+	v	+	+	-	-	+	+
Trehalose	+	+	-	-	+	+	-	-	-	-	v	+	+	+	+	l
D-Xylose	+	+	+	w	s	+	s	v	+/l	v	+	+	+	+	+	+

N-assimilation of

Characteristics	Gr. I(a)	C. tropicalis[a]	Gr. I(b)	C. stellimalicala[a]	Gr. I(c)	C. tartarivorans[a]	Gr. I(d)	C. pseudolambica[a]	Gr. I(e)	C. rugosa[a]	Gr. I(f)	C. albicans[a]	Gr. I(g)	C. mengyuniae[b]	Gr. I(h)	C. parasilosis[a]
Cadavarine	+	+	+	+	+	+	+	+	+	+	+	+	n	n	n	+
Ethylamine	+	+	+	+	+	+	+	+	+	+	+	+	n	n	n	+
L-Lysine	+	+	+	+	l	+	+	+	+	+	+	+	n	+	n	+
Nitrate	-	-	-	-	-	-	-	-	-	-	-	-	n	+	n	-
Nitrite	-	-	-	-	-	-	-	-	-	-	-	-	n	+	n	-

Growth in

Characteristics	Gr. I(a)	C. tropicalis[a]	Gr. I(b)	C. stellimalicala[a]	Gr. I(c)	C. tartarivorans[a]	Gr. I(d)	C. pseudolambica[a]	Gr. I(e)	C. rugosa[a]	Gr. I(f)	C. albicans[a]	Gr. I(g)	C. mengyuniae[b]	Gr. I(h)	C. parasilosis[a]
Vitamin-free	l	v	-	-	-	-	+	+	-/w	-	+	v	n	+	n	-
50%Glucose	+	+/l	-	n	+	-	-	-	+	l	+	v	n	+	n	+
10% NaCl/ 5% Glucose	l	l	-	-	+	-	-	-	l	n	-	v	n	+	n	+
Starch formation	-	-	-	-	-	-	-	-	-	-	-	-	n	n	n	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	n	n	n	N
0.01% Cycloheximide	+	+	-	n	+	+	-	-	-	-	+	+	n	n	n	V
0.1% Cycloheximide	+	+	-	n	+	n	-	n	-	n	+	+	n	n	n	-
Growth at 37°C	+	+	+	+	+	n	+	-	+	+	+	v	n	+	n	+

Table 4. Characteristics of the isolated in genus *Pichia* and *Issatchenkia*.

Characteristics	Gr. VII(a)	*I.terricola* [a]	Gr.VII (b)	*I. orientalis* [a]	Gr.VII(c)	*P. occidentalis* [a]	Gr.VII(d)	*P.kluyveri* [a]
Fermentation of								
Glucose	+	+/w	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-
sucrose	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-
xylose	-	-	-	-	-	-	-	n
Arabinose	-	-	-	-	-	-	-	n
Assimilation of		+						
Glucose	+		+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-
L-sorbose	-	-	-	-	-	V	-	-
sucrose	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-
Soluble starch	-	-	-	-	-	-	-	-
D-xylose	-	-	-	-	-	-	w/-	v
L-Arabinose	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-
D-Ribose	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-
Ethanol	+	+	+	+	+	+	+	+
Glycerol	+	+	l	+	+	+/W	+/l	+
Erythritol	-	-	-	-	-	-	-	-
Ribitol	-	-	-	-	-	-	-	-
Galactitol	-	-	-	-	-	-	-	-
D-mannitol	-	-	-	-	-	-	-	-
D-Glucitol	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-		-	-	-	-
Salicin	-	-	-		-	-	-	-
DL-Lactate	l	-	+	+	l	+/W	+/s	+/w

Table 4. (Continued).

Characteristics	Gr. VII(a)	*I.terricola* [a]	Gr.VII (b)	*I. orientalis*[a]	Gr.VII(c)	*P. occidentalis* [a]	Gr.VII(d)	*P.kluyveri* [a]
Succinate	+	+	+	+	+	+	+/s	+/w
Citrate	+	W/-	+	+/W	+	-	l	w/-
Inositol	-	-	-	-	-	-	-	-
Nitrate	-	-	-	-	-	-	-	-
2-keto-D-Gluconate	-	-	-	-	-	-		-
5-keto-D-Gluconate	-	-	-	-	-	-		-
Growth in		-						
Vitamin-free	-		+	+	-	+	-	-
10%NaCl/5%glucose	-	-	+		-	-		+
starch formation	-	-		-	-	-		-
Growth at 37°C	+	V	+	+	+	+		v
Growth at 40°C	-	-	+	+		-		

Table 5. Physiological and biochemical characteristics of *Cyberlindnera* spp. and type strains.

Characteristics	*Cy. saturnus*	11-2	*Cy. mrakii*	4-14
Fermentation :				
Glucose	+	+	+	+
Galactose	-	-	-	-
sucrose	+	+	-	-
Maltose	-	-	-	-
Lactose	-	-	-	-
Raffinose	+/W	+	+/W	+
Trehalose	-	-	-	-
Assimilation:				
Glucose	+	+	+	+
Galactose	-	-	-	-
L-sorbose	-	-	-	-
sucrose	+	+	-	-
Maltose	v	-	v	-
Cellobiose	+	+	+	+
Trehalose	v	-	v	-
Lactose	-	-	-	-
Melibiose	-	-	-	-
Raffinose	+	S	+	s
Melezitose	v	-	v	-
Inulin	v	-	v	-
Soluble starch	-	-	-	-
D-xylose	+	+	+	+
L-Arabinose	-	-	-	-
D-Arabinose	-	-	-	-
D-Ribose	-	-	-	-
L-Rhamnose	v	S	v	S
Ethanol	+	+	+	+
Glycerol	+	+	+	+
Erythritol	-	-	-	-
Ribitol	-	-	-	-
Galactitol	-	-	-	-

Table 5. (Continued).

Characteristics	*L. saturnus*	11-2	*L. mrakii*	4-14
D-mannitol	v	w	v	w
D-Glucitol	v	w	v	w
α-Methyl-D-glucoside	v	-	v	-
Salicin	v	+	v	+
DL-lactate	+	+	+	+
Succinate	+	+	+	+
Citrate	v	+	v	+
Inositol	-	-	-	-
Nitrate	+	+	+	+
2-keto-D-Gluconate	-	-	-	-
5-keto-D-Gluconate	-	-	-	-
Growth in				
10%NaCl/5%glucose	-	-	-	-
Vitamin-free	+	+	+	+
starch formation	-		-	
Growth at 30°C		+		+
Growth at 35°C		w		+
Growth at 37°C	V	-	v	+

Table 6. Physiological and biochemical characteristics of *Trichosporon* spp. and type strains.

Characteristics	Gr. X(c)	*T. Laibachii*	Gr. X(b)	*T. asahii*	Gr. X(a)	*T. Mycoxinvora*
Fermentation of						
Glucose	-	-	-	-	-	-
Galactose	-	-	-	-	-	-
sucrose	-	-	-	-	-	-
Maltose	-	-	-	-	-	-
Lactose	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-
xylose	-	n	-	-	-	-
Arabinose	-	n	-	-	-	-
Assimilation of						
Glucose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
L-sorbose	-	+	-	v	s	+
sucrose	s	+	'+	v	+	+
Maltose	+	+	'+	+	+	+
Cellobiose	s	+	'+	+	+	+
Trehalose	s	+	l	v	+	+
Lactose	+	+	+	+	+	+
Melibiose	+	+	-	-	l	+
Raffinose	+	+	-	-	l	+
Melezitose	w	v	s	v	w	+
Inulin	-	-	-	-	-	+
Soluble starch	+	+	l	v	+	+
D-xylose	+	+	+	v	+	+
L-Arabinose	+	+	+	v	+	+
D-Arabinose	-	v	l	+	-	+
D-Ribose	s	+	+	+	+	+
L-Rhamnose	+	+	s	+	l	+
Methanol	n	-	n	-		-
Ethanol	+	+	+	+	+	+
Glycerol	l	+	s	v	+	+
Erythritol	-	-	+	+	-	w
Ribitol	-	v	-	v	s	
Galactitol	+	+	-	-	-	+
D-mannitol	-	v	w	v	l	+
D-Glucitol	-	v	-	v	l	+
α-Methyl-D-glucoside	w/-	+	+	+	l	+
Salicin	w	+	-	+	l	+
DL-Lactate	+	+	+	v	+	
Succinate	+	+	+	+	+	+
Citrate	+	+	+	+	+	+

Table 6. (Continued).

Characteristics	Gr. X(c)	T. Laibachii	Gr. X(b)	T. asahii	Gr. X(a)	T. Mycoxinvora
Inositol	s	+	-	v	l	+
Nitrate	-	-	-	-	-	-
2-keto-D-Gluconate	+	+	+	+	+	+
5-keto-D-Gluconate	+	+	+	+	+	+
Glucono-δ-lactone	-	n	-	v	+	
Nitrite	-	v	+	v	-	-
Cadavarine	+	+	+	+	+	w
Creatinine		+		-		w
L-lysine	+	+	+	+	+	+
Ethylamine	+	+	+	+	+	w
Growth in						
Vitamin-free	w	-		-	w	-
60% Glucose	-	n	+	n	+	-
50%Glucose	-	+	+	+	+	-
10%NaCl/5%glucose	-	+	-	+	+	-
Urease	+	+	+	+	+	+
0.01%						
Cycloheximide	+	+	+	+	+	+
0.1% Cycloheximide	+	+	s	v	+	+
Growth at 30°C	+	+	+	+	+	+
Growth at 37°C	-	n	+	+	+	+
Growth at 40°C	-	n	+	n	+	-

ETHANOL AND XYLITOL PRODUCTION OF *S. stipitis*

Table 7. Ethanol and xylitol production of *S. stiptis.*

Time	Xylose	Xylitol	Ethanol	Biomass
12	40.0	0.00	2.96	0.00
24	0.48	0.96	18.66	7.50
36	0.00	1.10	15.84	7.30
48	0.00	1.30	11.68	8.50
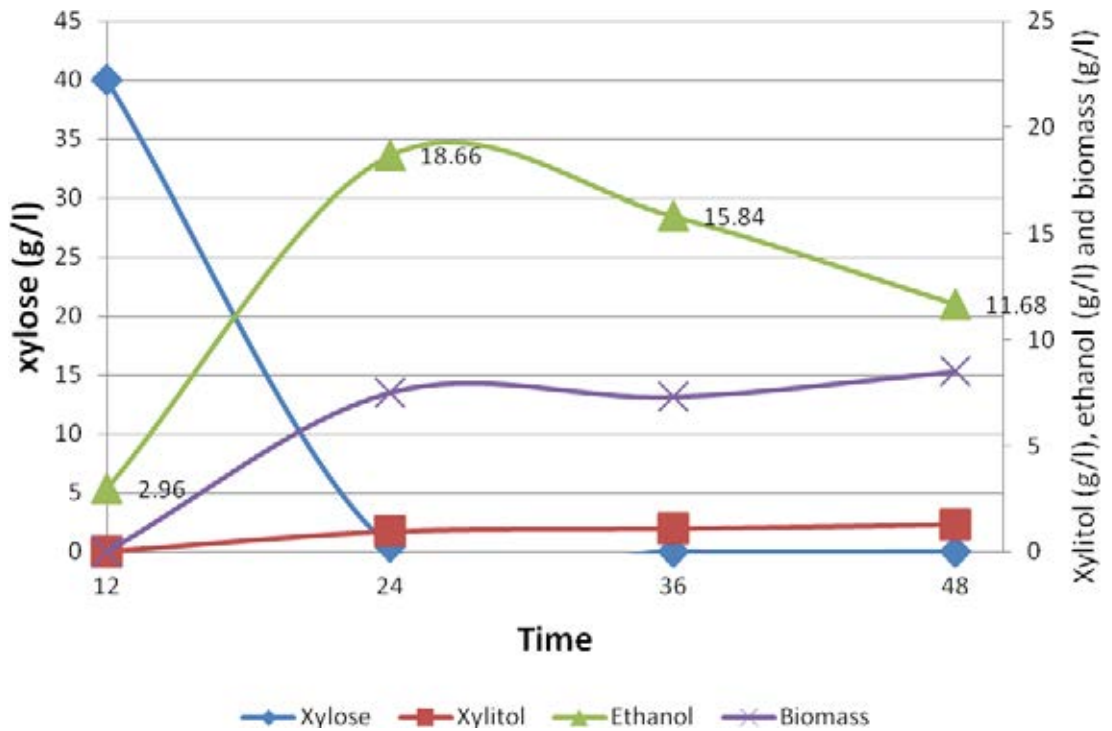
Figure 1. Time course of ethanol and xylitol production by *S. stiptis.*

APPENDIX E

PRIMERS, SEQUENCES OF XYLOSE REDUCTASE GENE AND ACCESION NUMBER OF D1/D2 LSU rRNA, ITS, RPB I GENE SEQUENCES

1. Primers

NL4:   5'-GGT CCG TGT TTC AAG ACG G-3'

NL1:   5'-GCA TAT CAA TAA GCG GAG GAA AAG-3'

ITS5:   5'-GGA AGT AAA AGT CGT AAC AAG G-3'

ITS4:   5'-TCC TCC GCT TAT TGA TAT GC-3'

XR_F: 5'GA(G/A)A(A/G)(A/G)TA(T/C)CC(A/T)GG(A/T) TTCTAC 3'

XR_R: 5'CCT(G/A)TCCCA(T/G)GG(A/G)T(T/C)(G/A)TT(G/A) AATCT-3'

2. Xylose reductase gene nucleotide and amino acid sequence

>A26_XR gene
TACATCCGGATATTCAATCATTAGTTGAGAAAAAATAAATGAAAAAGATTATTTTGACTAT
TGGCTGTCCTGGTTCTGGTAAGAGTACTTGGGCTCGTGAATTTATTGCTAAGAATCCCGGG
TTTTATAATATCAATCGTGATGACTATCGCCAATCTATTATGGCGCATGAAGAACGCGATG
AGTACAAGTATACCAAAAAGAAAGGAGGTATCGTAACTGGTATGCAGTTTGATACAGCTA
AAAGTATTCTGTACGGTGGCGATTCTGTTAAGGGAGTAATCATTTCAGATACTAACCTGAA
TCCTGAACGTCGCCTAGCATGGGAAACTTTTGCCAAAGAATACGGCTGGAAAGTTGAACA
TAAAGTGTTTGATGTTCCTTGGACTGAATTGGTTAAACGTAACTCAAAACGCGGAACTAA
AGCAGTACCAATTGATGTTTTACGTTCAATGTATAAAAGCATGCGAGAGTATCTCGGTCTT
CCAGTATATAATGGGACTCCTGGTAAACCAAAAGCAGTTATTTTTGATGTTGATGGTACAC
TAGCTAAAATGAATGGTCGTGGTCCTTATGACCTTGAAAAATGCGATACCGATGTTATCAA
TCCTATGGTTGTTGAACTGTCTAAGATGTATGCTCTTATGGGTTATCAAATCGTAGTCGTTT
CAGGTCGTGAAAGTGGAACTAAAGAAGACCCAACGAAATATTATCGTATGACCCGTAAAT
GGGTTGAGGACATTGCTGGCGTTCCATTAGTTATGCAATGTCAGCGCGAACAAGGCGATA
CCCGTAAAGACGATGTAGTTAAAGAAGAAATTTTCTGGAAACACATTGCACCGCATTTTG
ACGTGAAATTAGCTATTGATGACCGAACTCAAGTAGTTGAAATGTGGCGTCGTATCGGTG
TTGAATGCTGGCAAGTCGCTTCGGGAGATTTTTAAGGATCCAGA

>A26_XR amino acid
IRGPPGFYCGDGDNFHYEDVPLLDTWKALEKLVEAGKIKSIGISNFTGALIYDLIRGATIKPAVL
QIEHHPYLQQPKLIEYVQKAGIAITGYSSFGPQSFLELESKRALNTPTLFEHETIKSIADKHGKSP
AQVLLRWATQRNIAVIPKSNSPERLAQNLSVVDFDLTQEDLDNIAKLDIGLRFNDPWDRNRIP
GPSTAEACMQAFPIVSRIRAWRNHGHSCFLCEIVIR

>ELP23_XR
ATTTTTGACGGTGCTCAGGATTACGGCAATGAAAAGGAATGTGGTGAAGGTGTTGCCAGA
GCCATCAAGGATGGTCTTGTCAAGCGAGAAGACGTCTTTATTACCTCCAAGCTCTGGAATA
CCTTCCATGCTAAAGAGCATGTCAAGCCTTTGCTCAAGCGATCTCTTGCCGACTGGGGTCT
TGAGTATTTTGACTTGTTTTTGATCCATTTCCCCATTGCTCAGAAGTATGTTGATCCCGCGG
AAAGATACCCCCCTGGTATTGTCAATGACGTTGAGAACAAGGTTGCCTATTTCCAGAACA
CCCCTATTTCTGAGACCTGGGCTGCTCTGGAAGAATGCGTCAAGGAAGGACTTGTTAAGA
ACATTGGTATTTCCAACTTCAATGCCGGCTTGATCCGAGATTTGCTTTCCTATGCTAAGATT
CCTCCTGCCGTCTTGCAGATCGAGCACCACCCTTACTTGACCCAGGAGCCTTTGGTCAAGT
ATGTCCAGTCTCAGGGAATTGCCATCACAGGATACTCCTCATTTGGTCCTCAATCCTACAT
TGAACTTGGAAACCCTCGAGTCAAGGCCAACGGACCCTTTGCTCACCAATGACATCATCGA
GAAGATTGCAAAGTCTCATGGAAAGAGCACTGCTCAGGTTCTGTTGAGATGGGCTACCCA
ACGAAATATTGCTGTTATTCCCAAATCCAACAATCAGGAGAGACTTGTTGCCAACTTGAA
GAGCGACGATTTCAACCTGACTGATGCCGAGATCAAGGAGATCAGTAGCTTGGATATTGG
ATTCAGATTCAACAACCCTGGGACAGGAATCGGATCCCGGGCCCGTCGACTGCAGAGGCC
TGCATGCAAGCTTTCCCTA

> ELP23_XR amino acid
IFDGAQDYGNEKECGEGVARAIKDGLVKREDVFITSKLWNTFHAKEHVKPLLKRSLADWGLE
YFDLFLIHFPIAQKYVDPAERYPPGIVNDVENKVAYFQNTPISETWAALEECVKEGLVKNIGIS
NFNAGLIRDLLSYAKIPPAVLQIEHHPYLTQEPLVKYVQSQGIAITGYSSFGPQSYIELGNPRVK
ANGPLLTNDIIEKIAKSHGKSTAQVLLRWATQRNIAVIPKSNNQERLVANLKSDDFNLTDAEIK
EISSLDIGFRFNNPGTGIGSRARRLQRPACKLSL

Table 8. Accession number of D1/D2 LSU rRNA, ITS and RBP I gene.

Isolate no.	Nearest Species	Accession number		
		D1/D2	ITS	RPB I
A1	*C. tropicalis*	AB719413	-	-
A2	*C. tropicalis*	AB719414	-	-
A3	*C. tropicalis*	AB719415	-	-
A4	*C. tropicalis*	AB719416	-	-
A5	*C. tropicalis*	AB719417	-	-
A6	*C. tropicalis*	AB719418	-	-
A7	*C. tropicalis*	AB719419	-	-
A8	*C. tropicalis*	AB719420	-	-
A9	*C. tropicalis*	AB719421	-	-
A10	*C. tropicalis*	AB719422	-	-
A11	*C. tropicalis*	AB719423	-	-
A12	*C. tropicalis*	AB719424	-	-
A13	*C. tropicalis*	AB719425	-	-
A14	*C. tropicalis*	AB719426	-	-
A15	*C. tropicalis*	AB719427	-	-
A16	*C. tropicalis*	AB719428	-	-
A17	*C. tropicalis*	AB719429	-	-
A18	*C. tropicalis*	AB719430	-	-
A19	*C. tropicalis*	AB719431	-	-
A20	*C. tropicalis*	AB719432	-	-
A21	*C. tropicalis*	AB719433	-	-
A22	*C. tropicalis*	AB719434	-	-
A23	*C. tropicalis*	AB719435	-	-
A24	*C. tropicalis*	AB719436	-	-
A25	*C. tropicalis*	AB719437	-	-
A26	*C. tropicalis*	AB719438	-	-
A27	*C. tropicalis*	AB719439	-	-
A28	*C. tropicalis*	AB719440	-	-
B1	*C. tropicalis*	AB719441	-	-
D1	*C. tropicalis*	AB719442	-	-
E1	*C. tropicalis*	AB719443	-	-
F1	*C. tropicalis*	AB719444	-	-
I1	*C. tropicalis*	AB719445	-	-
M1	*C. tropicalis*	AB719446	-	-
L1	*C. albicans*	AB719447	-	-
H1	*I. terricola*	AB719448	-	-
ELP19	*W. onychis*	**AB617517**	**AB617518**	-
ELP21	*T. mycotoxinivorans*	AB719449	-	-

Table 8. (Continued).

Isolate no.	Nearest Species	Accession number		
		D1/D2	ITS	RPB I
ELP20	*S. lactativora*	AB719450	-	-
ELP25	*S. lactativora*	AB719451	-	-
E23	*Z. meyerae*	AB719452	-	-
ELP24	*T.mycotoxinivorans*	AB719461	-	-
BUF2-1	*T. asahii*	AB741064	-	-
BUF2-2	*T. asahii*	AB741065	-	-
BUF2-4	*T. asahii*	AB741066	-	-
BUF3-10	*T. asahii*	AB741069	-	-
BUF3-16	*C. mengyuniae*	AB741072	-	-
BUF3-18	*C. parapsilosis*	AB741061	-	-
BUF3-19	*C. parapsilosis*	AB741062	-	-
BUF3-3	*T. asahii*	AB741067	-	-
BUF3-5	*S. lactativora*	AB741063	-	-
BUF3-6	*C. mengyuniae*	AB741071	-	-
BUF3-8	*T. asahii*	AB741068	-	-
BUF1-1	*S. lactativora*	AB741078	-	-
BUF9	*Geotrichum* sp.	AB741075	-	-
BUF9-4	*C. tropicalis*	AB741058	-	-
BUF9-2	*C. tropicalis*	AB741056	-	-
BUF9-5	*C. tropicalis*	AB741057	-	-
BUF8	*Geotrichum* sp.	AB741074	-	-
BUF8-1	*C. tropicalis*	AB741054	-	-
BUF8-2	*C. tropicalis*	AB741055	-	-
BUF10	*Geotrichum* sp.	AB741076	-	-
BUF7	*Geotrichum* sp.	AB741073	-	-
BUF7-1	*C. tropicalis*	AB741053	-	-
BUF5-3	*C. tropicalis*	AB741052	-	-
BUF4-2	*C. tropicalis*	AB741060	-	-
BUF4-3	*C. tropicalis*	AB741050	-	-
BUF4-4	*C. tropicalis*	AB741051	-	-
BUF11	*Geotrichum* sp.	AB741077	-	-
BUF12-1	*C. tropicalis*	AB741059	-	-
2-1	*C. rugosa*	-	AB727593	AB727627
3-1	*C. rugosa*	AB727611	AB727596	-
3-2	*C. rugosa*	AB727612	AB727594	-
5-1	*C. rugosa*	AB727613	AB727595	-
5-2	*C. rugosa*	AB727614	AB727597	-
5-3	*C. rugosa*	AB727615	AB727598	AB727628
7-1	*C. tropicalis*	AB727616	AB727599	-

Table 8. (Continued).

Isolate no.	Nearest Species	Accession number		
		D1/D2	ITS	RPB I
7-8	*C. tropicalis*	AB727617	AB727600	-
9-1	*C. rugosa*	AB727618	AB727601	-
9-2	*C. rugosa*	AB727619	AB727602	-
9-3	*C. rugosa*	AB727620	AB727603	AB727629
9-4	*C. rugosa*	AB727621	AB727604	-
10-1	*C. rugosa*	AB727622	AB727605	-
10-2	*C. rugosa*	AB727623	AB727606	-
10-3	*C. rugosa*	AB727624	AB727608	AB727630
10-4	*C. rugosa*	AB727625	AB727609	-
10-8	*C. rugosa*	AB727626	AB727610	-
4-1	Uncultutred fungus	AB727632	AB727642	-
4-2	*Ga. geotrichum*	AB727633	AB727643	-
4-3	*Ga. geotrichum*	AB727634	AB727644	-
4-4	*Ga. geotrichum*	AB727635	AB727645	-
4-5	*Ga. geotrichum*	AB727636	-	-
12-1	Uncultutred fungus	AB727637	AB727646	-
12-2	Uncultutred fungus	AB727638	AB727647	-
12-3	Uncultutred fungus	AB727639	AB727648	-
12-4	Uncultutred fungus	AB727640	AB727649	-
12-7	Uncultutred fungus	AB727641	AB727650	-

Table 9. Nucleotide identity and nucleotide difference in D1/D2 domain compare with closest species.

Strain	Closest species	Nucleotide identity in D1/D2 domain	Nucleotide difference in D1/D2 domain	
			No of nucleotide substitutions	No. of Gap
11-1	*Candida pseudolambica* NRRL Y-17318[T]	575/578 (99%)	3	0
2-1	*Candida rugosa* ATCC 10571[T]	522/528 (99%)	5	1
5-3	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
3-1	*Candida rugosa* ATCC 10571[T]	524/529 (99%)	4	1
10-3	*Candida rugosa* ATCC 10571[T]	513/517 (99%)	5	0
5-2	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
10-5	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
10-2	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
10-4	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
10-8	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
3-2	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0

Table 9. (Continued).

Strain	Closest species	Nucleotide identity in D1/D2 domain	Nucleotide difference in D1/D2 domain	
			No of nucleotide substitutions	No. of Gap
5-1	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
7-6	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
7-8	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
9-1	*Candida rugosa* ATCC 10571[T]	523/528 (99%)	5	1
9-2	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
9-3	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
9-4	*Candida rugosa* ATCC 10571[T]	523/527 (99%)	4	0
4-3	*Galactomyces geotrichum* ATCC 34614	564/589 (96%)	24	1
4-5	*Galactomyces geotrichum* ATCC 34614	564/589 (96%)	24	1
4-2	*Galactomyces geotrichum* ATCC 34614	564/589 (96%)	25	0
4-4	*Galactomyces geotrichum* ATCC 34614	564/589 (96%)	25	0
4-7	*Issatchenkia occidentalis* NRRL Y-7552	576/578 (99%)	2	1
10-7	*Issatchenkia orientalis* NRRL Y-5396	582/584 (99%)	6	1

Table.9 (Continued).

Strain	Closest species	Nucleotide identity in D1/D2 domain	Nucleotide difference in D1/D2 domain	
			No of nucleotide substitutions	No. of Gap
4-1	Uncultured fungus clone	588/589 (99%)	1	0
12-1	Uncultured fungus clone YC21	587/588 (99%)	0	1
12-2	Uncultured fungus clone YC21	591/593 (99%)	1	1
12-3	Uncultured fungus clone YC21	587/588 (99%)	1	0
12-4	Uncultured fungus clone YC21	587/588 (99%)	1	0
12-7	Uncultured fungus clone YC21	587/588 (99%)	1	0
ELP19	*Wickerhamomyces onychis* NRRL Y-7123T	584/620 (94%)	24	11

BIOGRAPHY

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Lorliam,W., Akaracharanya, A., Jindamorakot, S., Suwannarangsee, S., and Tanasupawat, S. 2013. Characterization of xylose-utilizing yeasts isolated from herbivore feces in Thailand. *Sci Asia*. 39: 26-35.

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