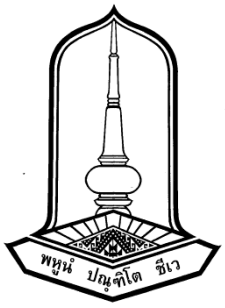

XYLITOL PRODUCTION BY ISOLATED YEAST STRAIN FROM SUGARCANE BAGASSE WASTE

KUSUMAWADEE THANCHAROEN

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology at Mahasarakham University

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The examining committee has unanimously approved this dissertation, submitted by Mrs. Kusumawadee Thancharoen, as a partial fulfillment of the requirements for the Doctor of Philosophy degree in Biotechnology at Mahasarakham University.

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30 April 2018

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Kusumawadee Thancharoen

ชื่อเรื่อง การผลิตไซลิทอลโดยยีสต์ที่คัดแยกได้จากกากชานอ้อย
ผู้วิจัย นางกุสุมาวดี ฐานเจริญ
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บทคัดย่อ

ไซลิทอลเป็นน้ำตาลแอลกอฮอล์ที่มีคุณค่าสูงในการเป็นสารให้ความหวาน จากรายงานที่ผ่านมากระบวนการทางชีววิทยาในการใช้น้ำตาลไซโลสจากวัตถุดิบประเภทลิกโนเซลลูโลสเพื่อเปลี่ยนเป็นไซลิทอลมีความน่าสนใจเพิ่มขึ้น เนื่องจากเป็นวิธีการที่มีประสิทธิภาพ การศึกษาครั้งนี้ใช้ชานอ้อยเป็นวัตถุดิบสำหรับการผลิตไซลิทอล เนื่องจากเป็นสารตั้งต้นที่มีศักยภาพสูง ลดค่าใช้จ่ายในระดับอุตสาหกรรม และมีความเข้มข้นของน้ำตาลไซโลสสูง การปรับสภาพชานอ้อยด้วยกรดซัลฟูริกโดยการแปรผันสภาวะต่างๆ พบว่า สภาวะในการปรับสภาพ คือ กรดซัลฟูริกความเข้มข้น 3.1% (ปริมาตรต่อปริมาตร) ที่อุณหภูมิ 126 องศาเซลเซียส เป็นเวลา 18 นาที สามารถปลดปล่อยน้ำตาลไซโลสเท่ากับ 19 กรัมต่อลิตร จากการคัดแยกยีสต์จากชานอ้อยและคัดเลือกยีสต์ที่มีความสามารถผลิตไซลิทอลสูงสุด พบว่า *Candida tropicalis* KS10-3 (จากยีสต์ทั้งหมด 72 ไอโซเลต) สามารถผลิตไซลิทอลสูงสุดเท่ากับ 0.47 กรัมไซลิทอลต่อกรัมไซโลส โดยมีความเข้มข้นของไซโลสเริ่มต้นเท่ากับ 32.30 กรัมต่อลิตร ใช้เวลาในการเพาะเลี้ยง 96 ชั่วโมง

การหาสภาวะที่เหมาะสมเพื่อผลิตไซลิทอลจากยีสต์ *Candida tropicalis* KS10-3 ด้วยไฮโดรไลเสทชานอ้อยซึ่งใช้เป็นสารตั้งต้น โดยการวางแผนแบบ Central composite design (CCD) เป็นการออกแบบการทดลอง เพื่อตรวจวัดตัวแปรที่มีผลต่อการผลิตไซลิทอล และทำนายข้อมูลที่ได้จากการทดลอง ตัวแปรที่มีผลต่อการผลิตไซลิทอลประกอบด้วยความเข้มข้นของไซโลส ความเร็วในการเขย่า และพีเอช สภาวะของตัวแปรที่มีผลสามารถให้ค่าความน่าเชื่อมั่น (R2) เท่ากับ 0.9365 จากการทดลองพบความสัมพันธ์ของตัวแปรร่วมระหว่างความเร็วในการเขย่าและพีเอชอย่างมีนัยสำคัญ สภาวะที่เหมาะสมที่ได้จากการวิเคราะห์ คือ ความเข้มข้นของไซโลส 60 กรัมต่อลิตร ความเร็วในการเขย่า 248.16 รอบต่อนาที และพีเอชเท่ากับ 5.27 โดยสภาวะดังกล่าวจะให้ผลได้ไซลิทอลจากการคำนวณเท่ากับ 0.67 กรัมต่อกรัมไซโลสแตกต่างจากไซลิทอลที่ได้จากการทดลอง 24.07 เปอร์เซ็นต์

การผลิตไซลิทอลระดับถังหมักแบบกะขนาด 1 ลิตรโดยใช้ไฮโดรไลเสทชานอ้อยเป็นสารตั้งต้น และใช้ยีสต์ *Candida tropicalis* KS10-3 โดยใช้ความเร็วในการเขย่า 150 รอบต่อนาที อัตราการให้อากาศ 1 vvm และความเข้มข้นของเชื้อตั้งต้นเท่ากับ 10 เปอร์เซ็นต์ปริมาตรต่อปริมาตร หลังจากเวลา 96 ชั่วโมงให้ค่าไซลิทอลเท่ากับ 31.04 กรัมต่อลิตร

คำสำคัญ การปรับสภาพ, การหมักแบบกะ, ชานอ้อย, ไซลิทอล, ไซโลส

TITLE Xylitol production by isolated yeast strain from sugarcane bagasse waste.
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ABSTRACT

Xylitol is a high value sugar alcohol used as a sweetener. Recently, the production of xylitol by D-xylose reductase enzymes from lignocellulosic material has gained increasing interest. Sugarcane bagasse was used as the raw material for xylitol production because of its high efficiency, low industrial cost, and high concentration of xylose. Pre-treatment of sugarcane bagasse with sulfuric acid was evaluated under various conditions. Results showed that the optimum condition for xylose production at 19 g/L was exhibited by 3.1% sulfuric acid at 126°C for 18 min. Isolated yeasts from sugarcane bagasse were selected and tested for their xylitol production ability from xylose. Results showed that *Candida tropicalis* KS 10-3 (from 72 isolates) had the highest ability, producing 0.47 g xylitol/ g xylose in 96 hrs of cultivation with 32.30 g/L xylose used as the production medium.

A statistical experimental designs was adopted to optimize the culture medium in xylitol production by *Candida tropicalis* KS10-3 using sugarcane bagasse hemicellulose hydrolysate as the substrate. Central composite design (CCD) was used to determine the optimum level of each of the significant variables. A polynomial was determined by the multiple regression analysis of the experimental data. The influence of various process variables namely xylose concentration, agitation speed and pH on the xylitol production were evaluated. Optimum levels of these variables were used to establishment a significant mathematical model with a co-efficient of determination R^2 = 0.9365. The optimum levels of the process variables were xylose concentration-60 g/L, agitation speed (248.16 rpm) and pH (5.27) giving 0.67 g/g xylitol which differed from the experimental value by 24.07%

Batch production of xylitol from the hydrolysate of sugarcane bagasse hemicellulosic using *Candida tropicalis* KS10-3 was carried out in a stirred tank reactor (agitation speed 150 rpm, aeration rate of 1 vvm and initial cell concentration 10%v/v). After 96 hours, xylitol reached 31.04 g/L.

Keywords Pretreatment, Batch fermentation, Sugarcane bagasse, Xylitol, Xylose

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CHAPTER 1
INTRODUCTION

1.1 Background and rationale

Xylitol, a natural pentahydroxy polyol ($C_5H_{12}O_5$) has a similar sweetening power to sucrose. The global demand xylitol is ever increasing because of its unique functional properties like insulin independent metabolism, low caloric value (2.4 cal/g), and it inhibits the growth of the tooth-decaying bacterium *Streptococcus mutans*, conveying an anticariogenic effect (Sam Ko *et al*., 2011; Rafiqul and Sakinah, 2013; Albuquerque *et al*., 2014; Rafiqul *et al*., 2015). Furthermore, xylitol does not participate in Maillard reactions, cause the formation of browning compounds in food, or reduce protein content. These characteristics confirm its applicability in food production processes, such as candies, caramels, chocolates, ice creams, jellies, marmalades and beverages (Monedero *et al*., 2010). Industrial production of xylitol has been on-going for almost four decades and, since the early years, solutions of purified D-xylose typically undergo a catalytic hydrogenation process, under conditions of high-temperature (80-140^{o}C) and pressure (up to 50 atm) (Chen *et al*., 2010), until polyol formation is achieved (chemical process). Due to the operating conditions and the need for purity in the employed xylose, this traditional process becomes quite expensive. Several studies have sought alternatives to chemical routes, with particular attention paid to biotechnological processes, which led them to become a major focus in scientific studies. Aiming to further reduce processing costs and, in addition, searching for solutions to the recycling of agro-industrial waste, the potential utilization of renewable raw materials is being evaluated, such as sugarcane bagasse, corn cob, corn stover, wheat, rice straw, bamboo, vegetable residues and sunflower stalks. Especially, bagasse, a waste in the process of sugar and ethanol extraction, is one of the most abundant low-cost lignocellulosic material, renewable, and inexpensive lignocellulosic biomass available in MahaSarakham Province, Thailand. An absolute minimum of about 70% of all bagasse is needed to generate heat and power to run the sugar milling process and the remainder can be stockpiled. The stockpiled bagasse is of low economic value and constitutes an environmental problem to sugar mills and surrounding districts, especially if

stockpiled for extended periods, due to the risk of spontaneous combustion occurring within the pile. Thus, several processes and products that utilize bagasse as a raw material have been reported (Candido *et al.*, 2012). Hemicellulose is a major constituent of lignocellulosic biomass and is mainly composed of D-xylose which is the second most abundant sugar found in nature. The conversion of xylose to value-added product xylitol will have a significant role in the economic viability of cellulose bioconversion process. These wastes, in turn, can be hydrolyzed, generally by diluted acids, with further release of D-xylose available for microbial xylitol conversion (Prakash *et al*., 2011; Albuquerque *et al*., 2014).

Xylitol can be produced from D-xylose by many wild-type microorganisms are reported (e.g., bacteria, yeasts, and fungi) in the literature for xylitol production, among which yeasts are considered the best xylitol producers, including *Candida guilliermondii*, *C.parapsilosis*, *C.tropicalis*, *C.athensensis*, *Hansenula polymorpha* and *Debaryomyces hansenii* (Barbosa *et al*., 1988; Kim *et al*., 1997; Kim *et al*., 1998; Zhang *et al*., 2012; Pal *et al*., 2016). Xylose-fermenting yeasts have a metabolic system with NAD(P)H-dependent xylose reductase (XR; EC 1.1.1.21) and $NAD(P)^+$-dependent xylitol dehydrogenase (XDH; EC 1.1.1.9) and these enzymes are induced by xylose. The metabolism of xylose inside the microbial cells occurs mostly in two steps: (i) xylose is reduced to xylitol by XR, and (ii) the produced xylitol is either secreted from the cell or oxidized to xylulose by XDH. These two reactions are considered to be rate-limiting for xylose fermentation and xylitol production. Xylulose is then phosphorylated by xylulokinase (EC 2.7.1.17) to xylulose 5-phosphate, which can be catabolized by pentose phosphate, glycolytic, or by phosphoketolase pathways (Rafiqul *et al*., 2015). Yeast under the genus *Candida* is still regarded as the best source of XR among the microorganisms. In this study, XR was synthesized from *Candida tropicalis* because it is one of the most efficient xylitol producers. This study focused on the XR preparation from *C.tropicalis* cultivated on sugarcane bagasse hydrolysate medium and characterization of the enzyme.

1.2 Purposes of the research

1. To investigate yeast growth characteristics on xylose and its ability to assimilate xylose.

2. To establish the process model for xylitol production and to achieve the optimized condition of the production

3. To use the hemicellulosic fraction of sugarcane bagasse as a feedstock for xylose for xylitol bioconversion by the isolated xylose-fermenting yeast.

4. To produce xylitol in process operated in reactions under controlled conditions, for large-scale xylitol production by biotechnological means.

1.3 Hypothesis of research

Xylose-fermenting yeast strains able to assimilate sugarcane bagasse hydrolysate as sole carbon source and produce xylitol by D-xylose fermentation.

1.4 Scope of research

1. Xylose-fermenting yeast strains with the ability to produce xylitol from high concentrations of xylose were screened from sugarcane bagasse waste in sugar factories by enrichment culture using xylose as a sole carbon source.

2. The hydrolyzed hemicellulose fraction of sugarcane bagasse was subjected to hydrolyze by dilute-acid hydrolysis of H_2SO_4.

3. The fermentation samples were analyzed by high performance liquid chromatography (HPLC)

4. Optimization of xylose concentration, inoculum size, agitation speed, temperature and pH for determination of xylitol yield.

1.5 Benefit of research

1. We report the screening of xylose-fermenting yeast and production of high concentrations of xylitol by the selected strain.

2. Response surface methodology (RSM) using the central composite design (CCD) which is a mathematical and statistical analysis was utilized extensively for optimizing different biotechnological process.

3. Batch fermentation was used for the development of an efficient technology for large scale xylitol production.

1.6 Definition of terms

1. Xylitol is a five-carbon sugar alcohol obtained from xylose reduction. Xylitol production through bioconversion has been proposed as an alternative process utilizing microorganism such as bacteria, filamentous fungi and yeasts.

2. Hemicellulose is a portion of agricultural residues like *Eucalyptus grandis*, rice straw, corn cobs and sugarcane bagasse.

3. Hydrolysis of hemicellulose yields glucose, D-xylose, L-arabinose and other minor sugars.

CHAPTER 2
LITERATURE REVIEW

2.1 Xylitol

Xylitol (CH_2OH-CHOH-CHOH-CHOH-CH_2OH, molecular weight 152.15 g/mol) is a polyol, with a sweetening power similar to sucrose, found in fruits and vegetables.

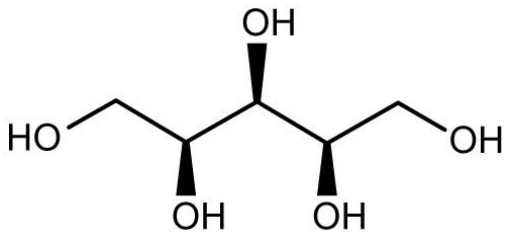


Figure 2.1. Xylitol chemical structure
Reference: http://chemistry.about.com/od/factsstructures/ig/Chemical-Structures---X/Xylitol.htm

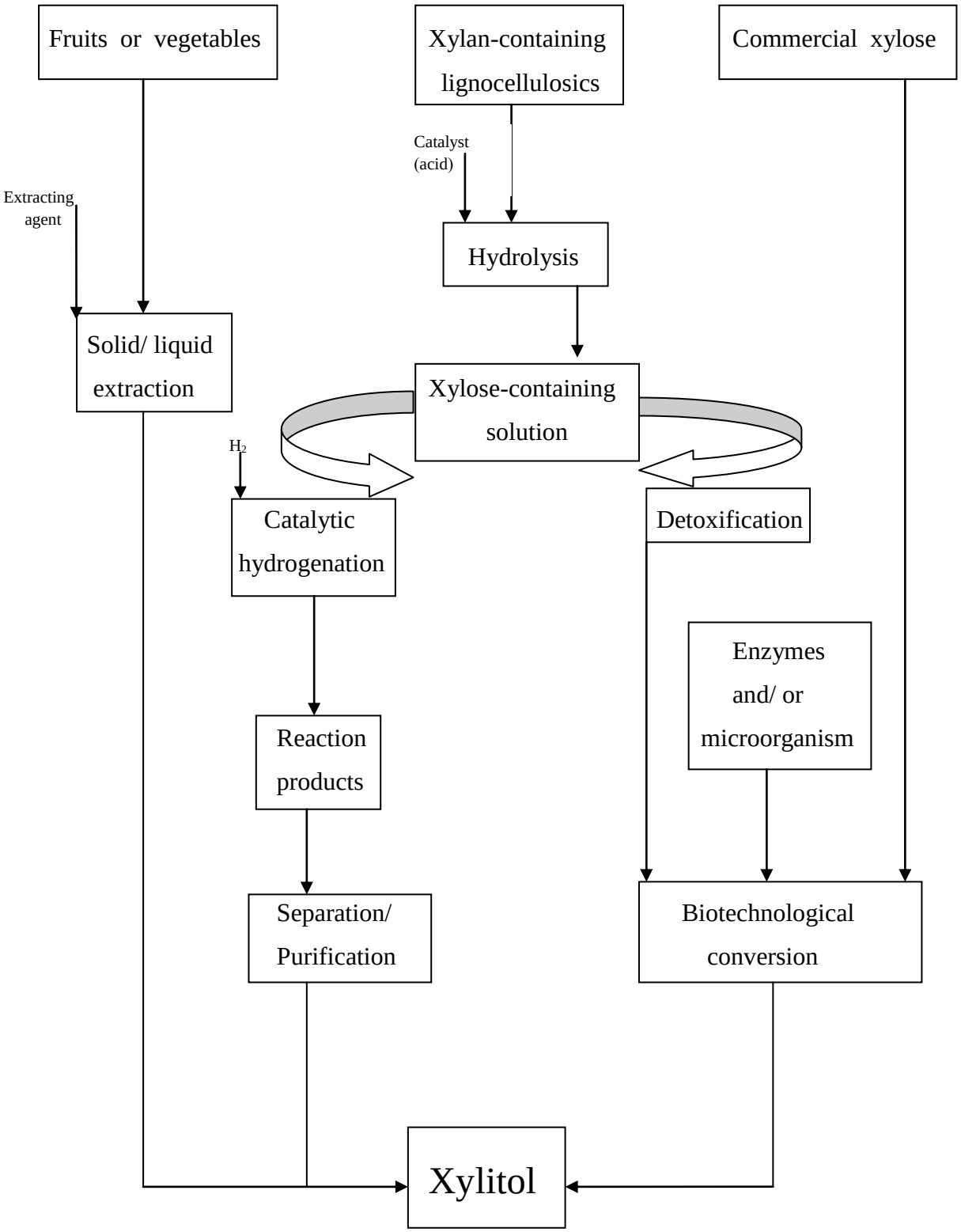
Xylitol has many advantages as a food ingredient. It does not undergo a Maillard reaction, responsible for both darkening and reduction in the nutritional value of proteins; when continuously supplied in the diet, it limits the tendency to obesity, and the incorporation of xylitol in food formulations improves the color and taste of preparations without causing undesired changes in properties during storage.

A number of studies have shown the beneficial effects of xylitol as a sweetener when used alone or formulated in combination with other sugars in yogurts, jams (providing texture, color and taste, stable for longer periods than those of products formulated with conventional sugars such as sucrose) or frozen desserts. With fructose, xylitol is the sugar recommended for diabetic patients. The xylitol tolerance by diabetics lies in the fact that the two different pathways of its human metabolism (direct absorption, mainly in the liver, and indirect metabolism by intestinal bacteria) are not insulin-mediated. Xylitol causes only limited increases in the glucose and insulin blood levels in comparison with the

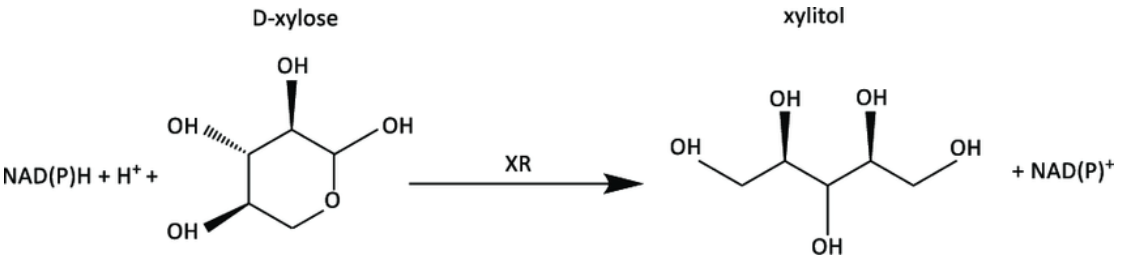
changes caused by sucrose or glucose. These properties make xylitol useful for post-operative or post-traumatic states, when the excessive secretion of stress hormones (cortisol, catecholamines, glucagon, growth hormone, etc.) causes insulin resistance and hinders the efficient utilization of glucose. In the same way, xylitol can be used to correct catabolic disorders (peripheral lipolysis, simulation of gluconeogenesis, degradation of muscle proteins) and contribute to the anabolic effects. Furthermore, xylitol does not react with amino acids, allowing its utilization for parenteral nutrition. The anticariogenic properties of xylitol have important commercial implications related to their ability to inhibit the growth of oral bacteria (so reducing plaque formation). Other effects related to the intake of xylitol include the enhanced remineralization of carious lesions and the stimulation of the flow of saliva without a pH decrease. The above properties can be maintained in mixtures of xylitol and difficult to ferment carbohydrates. As a component in toothpaste, xylitol also possesses an important ability to retain moisture.

Because of its negative heat of dissolution, xylitol produces a feeling of vaporization in the oral and nasal cavities, and is used as a part of the coating of confectionery or pharmaceutical products (such as vitamins or expectorants, usually in combination with mannitol, sorbitol and citric or adipic acids) and in the formulation of dietary complements (such as amino-acids, vitamins, trace elements and non-reducing sugars).

2.2 Processes for xylitol production



A.



B.

Figure 2.2. A.Technologies available for xylitol production (modified from Parajo *et al*., 1998) B. Reduction of D-xylose to xylitol structure (https://www.researchgate.net/figure/Reduction-of-D-xylose-to-xylitol-by-Xylose-Reductase-XR_314163790)

Figure 2.2 summarizes the several technologies available for xylitol production and structure of xylose to xylitol, which are described in the following sections.

2.2.1 Solid-liquid extraction. Xylitol is found naturally in fruits and vegetables (lettuce, cauliflower, yellow plums, raspberry, strawberry, grape, banana), as well as in yeast, lichens, seaweed and mushrooms. Xylitol can be recovered from these sources by solid-liquid extraction, but its small proportion in the raw materials (less than 900 mg/ 100 g) is a major economic problem (Parajo *et al*., 1998).

2.2.2 Chemical synthesis. This process starts with the production of xylose from xylan (a polysaccharide belonging to the hemicellulosic fraction of vegetable biomass) by acid-catalyzed hydrolysis. Xylan accounts for 11-35% (dry weight basis) of lignocellulosic materials such as hardwoods and agricultural residues (including corncob, wheat and rice straw, cotton seeds, sunflower or coconut hulls, sugarcane bagasse and pulp). The open and non-crystalline structure of xylan allows an easy diffusion of the hydronium ions (catalysts of the hydrolysis reaction) in the polymer matrix, favoring the hydrolysis reaction. However, the hemicellulosic fraction of the vegetable biomass contains polymers different from xylan and hydrolysates usually contain a variety of sugars (including glucose, arabinose, mannose and galactose) in proportions that are

dependent on the raw material and operational conditions. When pure xylose is necessary, expensive purification steps are required. After purification and color removal and in the presence of metal catalysts, xylose containing hemicellulose hydrolysates can be employed for xylitol production by hydrogenation of the pentose sugar at 80-140^{o}C and hydrogen pressures up to 50 atm. The solution produced in the hydrogenation step requires further processing (chromatographic fractionation, concentration and crystallization) to obtain pure xylitol. About 50-60% of the initial xylose is converted into xylitol, the purification and separation steps being the most expensive. Although the above process is similar to sorbitol production, the production cost of xylitol is much higher because of the hydrolysis stage (glucose can be obtained from starch in high concentrations at high yields and with high purity under mild hydrolysis concentrations) and the conditioning of hydrolysates and products (Parajo *et al*., 1998).

2.2.3 Biotechnological procedures. Biotechnological methods for xylitol production are based on the utilization of microorganisms and/ or enzymes. Some general ideas on these alternatives are summarized in the following sections (Parajo *et al*., 1998).

2.2.3.1 Production of xylitol by bacteria

Most bacteria possess the enzyme xylose isomerase, able to convert xylose into xylulose, which is further phosphorylated by xylulokinase into D-xylulose-5-phosphate (a common intermediate in the prokariotics and eukariotics metabolism). D-xylulose-5-phosphate can be incorporated into the pentose-phosphate pathway (Figure 2.3) or transformed by xylulose-5-phosphate phosphoketolase into glyceraldehyde-3-phosphate and acetyl-phosphate. This step produces an intermediate of glycolysis without production of NADPH, similar to the metabolism of glucose by yeasts. Since bacterial strains such as *Corynebacterium* and *Enterobacter* strains produce xylitol, they could possess an enzymatic oxido-reductive system (in addition to or in replacement of xylose-isomerase) allowing the reduction of xylose by reduction into xylitol, with a further oxidation to xylulose. In experiments leading to xylitol production, Yoshitake *et al*. (1973), using an *Enterobacter* strain, reached 33.3 g xylitol/ L in a fermentation medium containing 100 g initial xylose/ L with volumetric

productivity Qp = 0.35 g/ L/ h. In contrary to the results observed by the same authors with *Corynebacterium* spp. the addition of gluconate hindered the xylitol production. In Table 2.1, we list three D-xylitol producing bacteria which have been used. *Corynebacterium* spp. produced 69 mg/ ml of D-xylitol after 14 days of incubation. *Enterobacter liquefaciens*, which was isolated from soil, could yield 33.3 mg/ ml of D-xylitol when D-xylose was used as a single carbon source. When grown in anaerobic condition, *Mycobacterium smegmatis* could transform 70% of D-xylose to D-xylitol (Parajo *et al*., 1998).

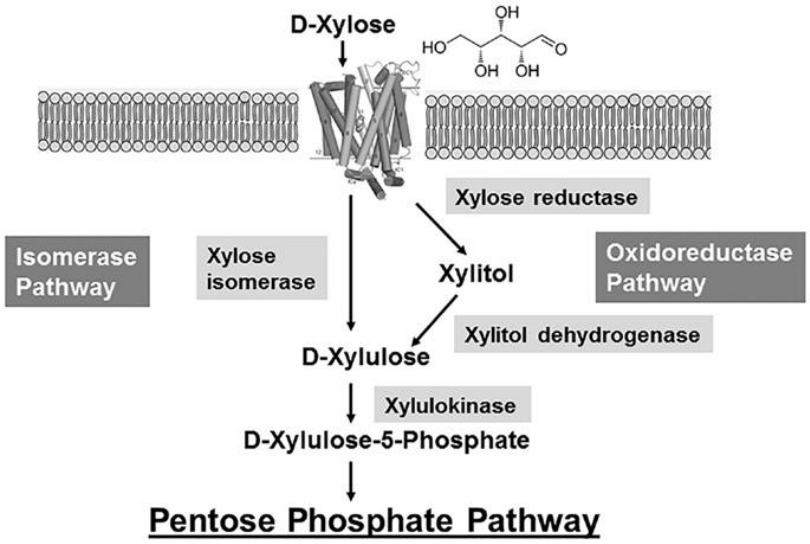


Figure 2.3. Xylose metabolism in bacteria

Reference: Nieves *et al*. (2015).

Table 2.1. Bacteria strains for D-xylitol production.

Strains	Carbon sources	Growth conditions	Yield	Reference
Corynebacterium spp.	D-xylose	Temperature 30°C, 48 h., pH 6.5	69 mg/ ml	Yoshitake *et al*., 1971
Enterobacter liquefaciens	D-xylose	Temperature 30°C, 48 h., pH 7.0	33.3 mg/ ml	Yoshitake *et al*., 1973; Yoshitake *et al*., 1976
Mycobacterium smegmatis	D-xylulose, xylitol or D-mannitol	Anaerobic	0.7 g/ g	Izumori *et al*., 1988

2.2.3.2 Production of xylitol by fungi

Fungi metabolize xylose by oxido-reductive conversion into xylulose. Isomerization has been proposed by several authors, but Suomalainen *et al*. (1989) reported on the inability of *Rhodosporidium toruloides* to grow on xylose in the presence of xylose isomerase. Since this report, there has been a general agreement that xylose is metabolized by fungi following the oxido-reductive pathway (Ojamo, 1994). In experiments leading to xylitol production, small concentrations of this compound were found by Chiang and Knight (1961) when culturing filamentous fungi (*Penicillium*, *Aspergillus*, *Rhizopus*, *Gliocladium*, *Byssochlamys*, *Myrothecium* and *Neurospora* spp.) on xylose-based media. A similar finding was reported by Ueng and Gong (1982) for fermentations of sugarcane bagasse hemicellulose hydrolysates by *Mucor* spp. Suihko (1984) detected xylitol (< 1 g/ L) after two days of aerobic culture of *Fusarium oxysporum* on media containing 50 g initial xylose/ L. Dahiya (1991), in cultures with *Petromyces albertensis*, reached 39.8 g xylitol/ L and 2.8 g xylulose/ L after 10 days in a culture medium containing 100 g initial xylose/ L (Parajo *et al*., 1998).

2.2.3.3 Production of xylitol by yeasts

Some yeast strains are able to transform xylose into D-xylose through an oxido-reductive route consisting of two sequential reactions. In the first, xylose-reductase (XR), in the presence of NADH and/ or NADPH, transforms D-xylose into the intermediate xylitol, although the relative stability of this product asks the question of whether xylitol is an intermediate, a product or both. In a subsequent reaction, xylitol is transformed into D-xylulose by either NAD^+-linked or $NADP^+$-linked xylitol dehydrogenase (XDH). Fig. 2.4 shows a simplified scheme of the yeast xylose metabolism based on the data of Prior *et al*. (1989).

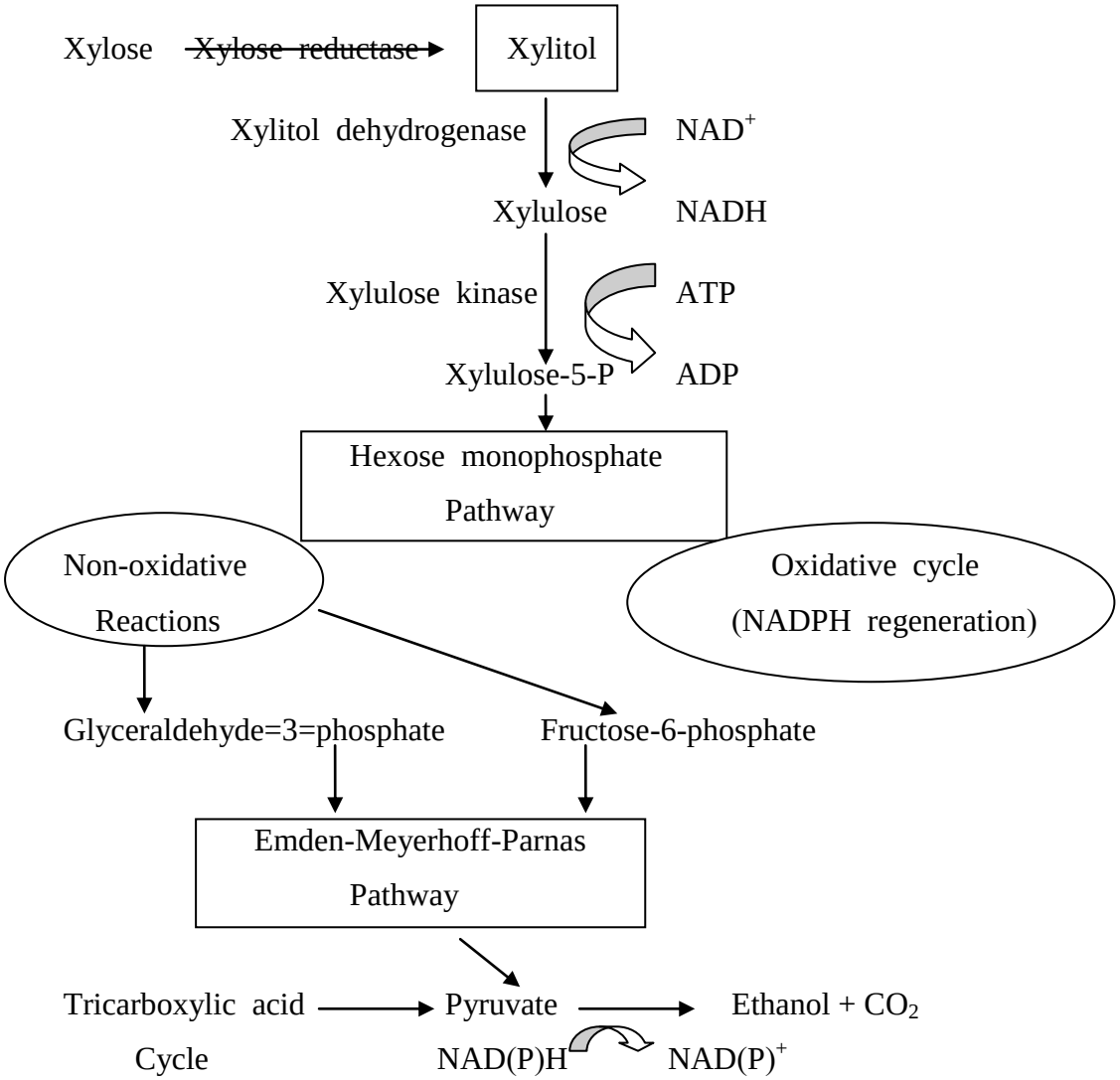


Figure 2.4. Simplified scheme of xylose metabolism by yeasts. Reference: Ghindea *et al*. (2010).

A close relationship between the reduction of xylose into xylitol, the cofactor linked to XR and the products of the metabolism of xylose has been reported. Under both anaerobic and oxygen-limited conditions, the yeasts with XR activity linked to both NADH and NADPH (i.e. *Pichia stipitis*) can regenerate the NAD^+ consumed in the second step of the xylose metabolism (Fig. 2.4). In the case, the major reaction product is ethanol and no xylitol accumulation occurs owing to the redox balance between the cofactors of XR and XDH. In contrast, the yeasts that consume xylose by XR activity dependent only on NADPH (with complete absence of NADH-linked XR) in the first step of the xylose metabolism (i.e. *Debaryomyces hansenii*) accumulate xylitol. In the second step, xylitol is oxidized by XDH, usually in the presence of NAD^+ (Ghindea *et al*., 2010).

2.3 Xylitol production characteristics of the studied *Candida* species

The xylitol production characteristics of three different *Candida* strains.

2.3.1 *Candida milleri*

C. milleri has been used in sourdough rye baking in Finland as part of a mixed culture together with lactic acid bacteria for centuries. It is an acid tolerant food grade organism and thus ideal for production of xylitol type sweeteners. According to Mantynen *et al*. (1999) *C. milleri* is taxonomically closely related to *Saccharomyces cerevisiae*, which could facilitate its genetic modification. *C. milleri* has not been used in xylitol production studies before (Granstrom, 2002).

2.3.2 *Candida guilliermondii*

C. guilliermondii was chosen on the basis of its high xylitol production capacity. Ojamo (1994) demonstrates that *C. guilliermondii* (VTT-C-71006) is an efficient xylitol producer. A xylitol yield of 0.74 g/ g xylose was obtained within 50 h at an initial xylose concentration of 100 g/ L. Using a fed batch cultivation the xylitol yield was increased to 0.78 g/ g and process time was decreased by 40%, In addition the initial xylose concentration could be increased to 250 g/ L (Ojamo, 1994). Roberto *et al*. (1999) studied the effect of k_La on the conversion of

xylose to xylitol by *C. guilliermondii* (FTI 20073) in batch fermentation. With rice straw hydrolysate as a substrate (containing 62 g/ L of xylose) the maximum volumetric productivity was 0.52 g/ L/ h and the highest xylitol concentration (36.8 g/ L) was attained at k_La 15/ h after 70 h cultivation. Dominguez *et al*. (1999) studied xylitol production by Ca^{2+}-alginate entrapped *Debaryomyces hansenii* and *C. guilliermondii*. They reached volumetric productivities of 0.91 and 0.58 g/ L/ h respectively. Barbosa *et al*. (1988) achieved 77.2 g/ L xylitol concentration from 104 g/ L of xylose with *C. guilliermondii* FTI-20037 using shake flasks with high cell density and defined medium. Meyrial *et al*. (1991) achieved 221 g/ L xylitol yield from 300 g/ L of xylose with average specific production rate of 0.19 g/ g/ CDW/ h in the shake flask study (Granstrom, 2002).

2.3.3 *Candida tropicalis*

C. tropicalis is often considered to be an opportunistic pathogen, which limits its use in food processing industry. It has potential industrial importance, because of its high xylose uptake rate, xylitol production capacity and alkane and fatty acid degradation in its peroxisomes. Its morphology changes from mycelium culture to single cell culture according to oxygen availability in the chemostat (data not shown). *C. tropicalis* is one of the most common model organisms in xylitol production studies. Production of xylitol in cell recycle fermentation by *C. tropicalis* ATCC13803 was studied by Choi *et al*. (2000). The cell recycling studies indicated that the feeding of xylose, glucose and yeast extract in the xylitol production phase was most effective in enhancing xylitol productivity. It resulted in 0.82 g xylitol/ g xylose yield and 4.94 g xylitol/ L/ h productivity. Horitsu *et al*. (1992) studied the effect of culture conditions on xylitol production rate with *C. tropicalis*. They achieved a maximum xylitol production rate of 2.67 g/ L/ h, when initial xylose and yeast extract concentration were 172 and 21 g/ L, respectively. Oh and Kim (1998) carried out feeding experiments with different ratios of xylose and glucose and their effect on xylitol production of *C. tropicalis*. The maximum xylitol yield from 300 g/ L of xylose was 91% at a glucose/ xylose feeding ratio of 15%, while the maximum volumetric production rate of xylitol was 3.98 g/ L/ h at a glucose/ xylose feeding ratio of 20%. Yahashi *et al*. (1996) studied the xylitol production with *C. tropicalis* using a fed

batch cultivation and glucose as a cosubstrate. Xylitol was produced with a yield of 0.82 g/ g xylose consumed and with volumetric production rate of 3.26 g/ L/ h. Faster growth occurred when a mixture of glucose and xylose was used as a substrate. Azuma *et al*. (2000) studied the effect of salt on xylitol production by *C. tropicalis*. An addition of 4% NaCl increased the yield of xylitol from 5% D-xylose by 1.3-fold. The addition of salts also increased the xylitol production even in the presence of 2% D-glucose. They suggested that the increase in xylitol production by NaCl may be due in part to the increase in xylose reductase production (Granstrom, 2002).

A summary of the results is presented in Table 2.2. It can be concluded that different growth conditions employed in the studies make comparison difficult between different strains and species. Volumetric productivity (g/ L/ h) is used, even though specific productivity (g/ CDW/ h) would give a better comparison. This is due to the fact that in many articles, biomass concentration is not given.

Table 2.2. Summary of xylitol production studies with *Candida* species.

Strain	Yield g/ g	Initial xylose g/ L	Productivity g/ L/ h	Process strategy	Reference
C. guilliermondii	0.78	250	nr	Fed batch	Ojamo, 1994
C. guilliermondii	0.73	62	0.52	Batch, oxygen limit	Roberto *et al*., 1999
C. guilliermondii	0.2	15.5	0.58	Packed bed reactor	Dominguez *et al*., 1999
C. guilliermondii	0.74	104	nr	Shake flask	Barbosa *et al*., 1988
C. guilliermondii	0.75	300	nr	Shake flask	Meyrial *et al*., 1991
C. tropicalis	0.82	750	4.94	Cell recycling Yeast extract Glu-xyl feed O_2 limit	Choi *et al*., 2000
C. tropicalis	0.64	172	2.67	Yeast extract O_2 limit	Horitsu *et al*., 1992
C. tropicalis	0.91	300	3.98	Glucose-xylose feed O_2 limit	Oh and Kim, 1998
C. tropicalis	0.82	127	3.26	Fed batch glu-xyl feed O_2 limit	Yahashi *et al*., 1996
Saccharomyces cerevisiae (recombinant)	0.95	190	0.40 (calc)	Fed batch glu-xyl feed	Hallborn *et al*., 1991

nr = not reported; calc = calculated from the values given in the article.

2.4 Xylose transport within the yeast cell

Yeasts from genera *Candida*, *Pichia*, *Debaryomyces*, and *Pachysolen* are especially able to produce xylitol from D-xylulose, through successive metabolic reactions, with various yields.

It was recently proved that *Saccharomyces cerevisiae* can grow slowly in the presence of xylose as sole carbon source, in aerobic conditions. Until recently, it was considered that *S. cerevisiae* does not present specific transporters for xylose and that grows poorly in its presence. The initial studies regarding the xylose transport alternatives within the yeast cell involved strains of *P. stipitis*, *P. heedii*, *C. shehatae*, and *C. intermedia* and proved the existence of two transport systems.

A. A facilitated diffusion system, with low affinity-the genes involved here are, for example, *SUT1* (sugar transporter 1) for *P. stipitis*, or *GXF1* (glucose/ xylose facilitator 1) for *C. intermedia*, which are constitutively expressed genes that code the glucose/ xylose transporter proteins.

B. A symport xylose-proton system, with high affinity-*GXS1* (glucose/ xylose symporter) that codes proteins involved in the symport monosaccharides-protons transport in various species of yeasts and fungi.

In the case of *S. cerevisiae* species, the facilitated diffusion of xylose takes place with the aid of transporter proteins coded by *HXT* (hexose transporter) genes (Figure 2.5). The xylose transport in the *S. cerevisiae* cell is, however, less efficient than glucose transport, the transport proteins (code by the genes *XHT2*, *XHT6*, *XHT7*) manifesting higher affinity for glucose.

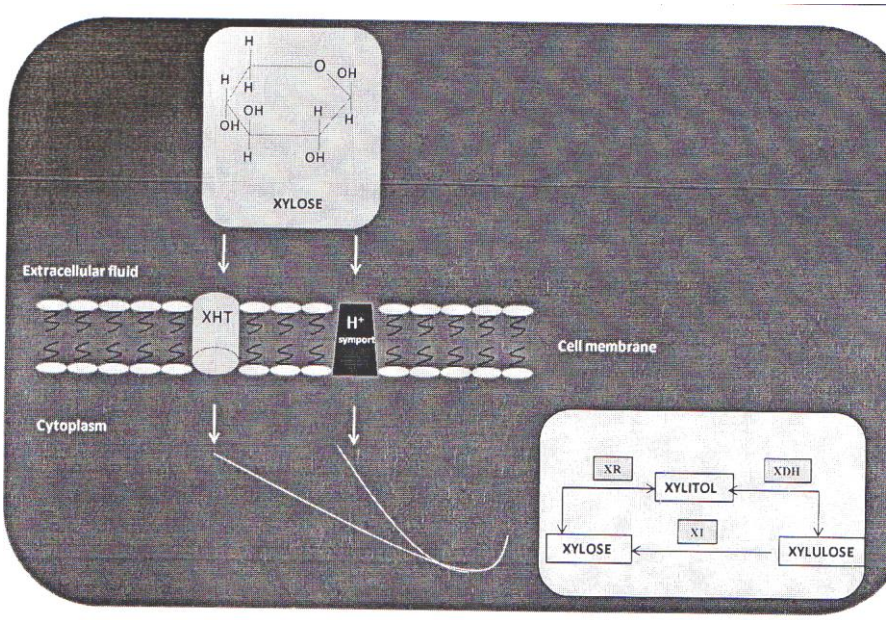


Figure 2.5. Xylose transport within yeast cells, XI = Xylose Isomerase, XR = Xylose Reductase, XDH = Xylose Dehydrogenase.
Reference: Ghindea *et al*. (2010).

2.5 Factors effecting xylitol production

2.5.1 Medium components. Although various media have been used to culture xylitol-producing yeasts, a few generalizations can be made: (i) For some yeasts, yeast extract is an important nutrient for xylitol production. (ii) For other yeasts, sometimes also including yeasts from the first group, but reported by different researchers, yeast extract has no significant effect on xylitol formation. These yeasts prefer urea and Casamono acids. (iii) For kinetic studies, synthetic media are used which provide all the necessary minerals and vitamins. The conversion of xylose to xylitol by *C. guilliermondii* was effected by the nutrient source. Horitsu *et al*. (1992) studied the influence of culture conditions on xylitol formation by *C. tropicalis* and optimized the volumetric xylitol production rate by the Box-Wilson method. In this respect, initial xylose concentration, yeast extract concentration and k_La were chosen as independent factors in 2^3-factorial design. Optimal product formation ($r_{xylitol}$ = 2.67 g/ L/ h, $C_{xylitol}$ = 110 g/ L) was obtained at 172 g/ L xylose, 21 g/ L yeast extract and a k_La of 451.5 L/ h. The culture media for *C. parapsilosis* ATCC28474 (Furlan *et al*., 1994), *C. boidinii* no.2201 (Vongsuvanlert and Tani, 1989), *C. guilliermondii* NRC5578 (Meyrial *et al*., 1991)

and *C. tropicalis* IFO0618 (Horitsu *et al*., 1992) contain yeast extract in concentrations ranging from 10 to 20 g/ L. Yeast extract at a maximum concentration of 1 g/ L was sufficient for *C. tropicalis* DSM7524. Concentrations higher than 15 g/ L, blocked the conversion of D-xylose to xylitol (Silva *et al*., 1994). Increased concentrations of yeast extract of 5 and 10 g/ L increased the biomass production of *C. guilliermondii* FTI20037, but sharply decreased its xylitol productivity (Silva *et al*., 1997). Similarity, the addition of yeast extract and peptone to the defined medium for *C. mogii* ATCC18364 enhanced cell growth markedly but had no significant effect on the yield and specific productivity of xylitol (Sirisansaneeyakul *et al*., 1995).

2.5.2 Xylose concentration. D-xylose concentration has been shown to be critical for yeast growth and fermentation. In the absence of D-xylose, xylitol formation does not occur. Together with aeration, D-xylose concentration affects xylitol formation the most. D-xylose is required for the induction of xylose reductase and xylitol dehydrogenase activities in yeasts. Initial xylose concentration is an important factor to obtain high xylitol production. Meyrial *et al*. (1991) reported that an increase in the initial xylose concentration from 10 g/ L to 300 g/ L led to activation of xylitol production by *C. guilliermondii*. The xylitol yield increased gradually with substrate, the highest xylitol yield (0.75 g/ g xylose) was obtained at a substrate concentration of 300 g/ L. However, the growth of the yeast was gradually inhibited by an increase in initial xylose concentration in the medium. Both the yield and specific rate of cells production declined when xylose concentration initially present in the culture increased. Chen and Gong (1985) reported a xylitol yield of 84.5% of theoretical and a maximum production rate of 0.269 g/ g/ h from 249 g/ L xylose by *Candida* sp. B-22. *C. tropicalis* HXP2 (Gong *et al*., 1981) and *C. boidinii* (Vongsuvanlert and Tani, 1989) produced the highest amounts of xylitol (144 g/ L and 39 g/ L, respectively) at respective values of substrate concentration of 200 g/ L and 100 g/ L. Dahiya (1991) reported maximum xylitol production by *P. albertensis* was 36.8 g/ L at the initial xylose concentration of 100 g/ L. Xylitol production declined when the initial xylose concentration was increase to 150 g/ L. This might be due to an osmotic effect on cells of *P. albertensis* or substrate repression of xylose

metabolizing enzymes. When *C. mogii* was grown under oxygen-limited conditions in synthetic medium containing different concentrations of xylose (5-53 g/ L), the xylitol formation rates showed a hyperbolic dependency on the initial substrate concentration. Vandeska *et al*. (1995) reported that an increase in initial xylose concentration induced xylitol production in *C. boidinii* but simultaneously acted as a growth inhibitory substrate leading to a long fermentation time. To overcome these problems, fed batch cultures were then used in which higher xylitol yields (0.57-0.68 g/ g) and production rates (0.32-0.46 g/ L/ h) were obtained as compared with a batch process. A fed batch process with highest initial xylose concentration (100 g/ L) and lowest level of aeration in the first phase, resulted in the highest yield of xylitol (75% of theoretical). A potentiometric biosensor for xylose to monitor fermentative conversion of xylose to xylitol was devised (Reshetilov *et al*., 1996). High D-xylose concentration induces xylitol formation in yeasts. Increased xylose concentration favors xylitol production at the expense of ethanol production, resulting in an increase in the xylitol/ ethanol production, a decrease in the ethanol yield and without exception, an increase in xylitol yield (42, 48, 77, 80). As the initial D-xylose concentration increases, the specific growth rate decrease, demonstrating substrate inhibition, whereas the overall xylitol productivity depends on the yeast type. However, all yeasts need a relatively long time for the conversion of D-xylose to xylitol (Table 2.3). For most yeasts, the initial D-xylose concentrations resulting in the highest yields are between 100 and 200 g/ L and with *C. guilliermondii* NRC5578 being an exception for witch 300 g/ L is the most suitable concentration. Certain xylose concentrations inhibit xylitol formation, and such inhibitory concentrations differ with yeast species (Table 2.3).

Table 2.3. Influence of initial substrate concentration on the fermentation parameters for some xylitol-producing yeasts.

Yeast strains	D-xylose (g/ L)	S_c (%)	C_x (g/L)	$Y_{x/s}$ (g/g)	$Y_{x/t}$ (%)	Q_x (g/Lh)	C_e (g/L)	$Y_{cm/s}$ (g/g)	μ (L/h)	T (h)	Reference
C. boidinii (*Kloeckera* sp.) no.2201	50	64.0	17.1	0.53	58.4	0.35	n.r.	n.r.	n.r.	48	Vongsuvanlert *et al*., 1989
	100	81.8	36.0	0.44	48.4	0.25	n.r.	n.r.	n.r.	144	
	150	51.0	17.0	0.22	24.2	0.01	n.r.	n.r.	n.r.	144	
C. boidinii NRRL Y-17213	50	77.6	4.8	0.12	13.2	0.05	6.4	0.20	n.r.	96	Vandeska *et al*., 1995
	100	85.6	25.2	0.29	31.9	0.13	11.6	0.11	n.r.	132	
	150	74.7	53.1	0.47	51.7	0.16	15.1	0.07	n.r.	336	
	200	22.2	10.0	0.22	24.2	0.04	9.0	0.09	n.r.	192	
C.guilliermondii NRC5578	10	100	6.2	0.62	68.1	0.13	0.4	0.31	0.110	46	Meyrial *et al*., 1991
	50	98.3	30.9	0.63	69.1	0.19	0.9	0.09	0.110	165	
	150	100	110.3	0.74	81.3	0.46	3.0	0.04	0.030	238	
	300	100	221.0	0.74	81.3	0.54	6.0	0.02	0.01	406	
C.guilliermondii NRC5578	50	100	22.5	0.45	49.5	0.055	n.r.	0.036	0.050	409	Nolleau *et al*., 1993
	100	100	49	0.49	53.8	0.066	n.r.	0.014	0.030	742	
	200	100	116	0.58	63.7	0.099	n.r.	0.002	0.007	1172	
	300	100	207	0.69	75.8	0.164	n.r.	0.004	0.010	1269	
C. mogii ATCC18364	10.1	100	1.7	0.17	18.7	n.r.	n.r.	n.r.	0.005	n.r.	Sirisansa neeyakul *et al*., 1995
	28.9	100	14.5	0.50	54.9	n.r.	n.r.	n.r.	0.004	n.r.	
	53.3	100	37.3	0.70	76.9	n.r.	n.r.	n.r.	0.003	n.r.	
C. parapsilosis ATCC28474	50	100	29.5	0.59	64.8	0.111	n.r.	0.050	0.026	266	Vongsuvanlert *et al*., 1989
	100	100	61.0	0.61	67.0	0.123	n.r.	0.018	0.020	477	
	200	100	116.0	0.58	63.7	0.115	n.r.	0.016	0.020	1009	
	300	100	93.0	0.31	34.1	0.050	n.r.	0.004	0.009	1860	

n.r. = not reported, S_c = substrate concentration, C_x = biomass concentration, $Y_{x/s}$ = biomass yield on substrate, $Y_{x/t}$ = biomass productivity, Q_x = specific biomass rate, μ = specific growth rate, T = time

2.5.3 Presence of other sugars. Yahashi *et al*. (1996) investigated the effect of glucose feeding on the production of xylitol from xylose by *C. tropicalis*. In the bench-scale fermenter (3 L scale) experiment, xylitol was produced up to 104.5 g/ L at 32 h cultivation and a yield of 0.82 (g/ g xylose consumed) which is 1.3 times higher than that without glucose feeding. Meyrial *et al*. (1991) evaluated the

ability of *C. guilliermondii* to ferment non-xylose sugars such as glucose, mannose, galactose and L-arabinose commonly found in hemicellulose hydrolysate. The strain did not convert glucose, mannose and galactose into their corresponding polyalcohol but only to ethanol and cell mass. Arabinose was converted to arabitol. Silva *et al*. (1996) studied batch fermentation of xylose for xylitol production in stirred tank bioreactor. The efficiency of substrate conversion to xylitol was 66% in a medium containing xylose but decreased to 45% in a medium containing xylose and glucose. Vandeska *et al*. (1996) investigated xylitol production by *C. boidinii* in fed batch fermentation with xylose (50, 100 g/ L) and a mixture of glucose (25 g/ L) and xylose (25 g/ L). All fermentations were initially batch processes with high levels of aeration and rapid production of biomass. Faster growth occurred when a mixture of glucose and xylose was used instead of xylose. Glucose was assimilated first and maximum xylitol production was 39-41 g/ L, compared with 46.5 and 59.3 g/ L with xylose alone.

2.5.4 Nitrogen source and organic nutrients. Dahiya (1991) studied the effect of 8 ammonium salts and 4 organic nitrogen sources on the production of xylitol from xylose by *P. albertensis*. Ammonium acetate was most effective for xylitol production. Yeast extract was the most suitable organic nutrient for enhancement of xylitol production. Lu *et al*., (1995) investigated the effect of nitrogen sources (asparagine, casein hydrolysate, glycine, Traders protein, yeast extract, urea, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $NH_4H_2PO_4$) on xylitol production from xylose in shake flaks by an efficient xylitol producing yeast, *Candida* sp. L-102. Different nitrogen sources influenced xylitol production rate, average specific productivity, and xylitol yield. Maximum xylitol production (100 g/ L of xylitol from 114 g/ L of xylose) was obtained with urea (3 g/ L) as the nitrogen source. Silva *et al*. (1994) evaluated the xylose conversion into xylitol by *C. guilliermondii* in semi-synthetic media supplemented with different nitrogen sources (urea, NH_4Cl_2, $(NH_4)_2SO_4$) in a ratio C/N equal 25.6. The type of nitrogen source did not influence this bioconversion and the xylitol yield was around 80%. On the other hand, Barbosa *et al*. (1988) reported that the use of urea led to higher xylitol productivity by *C. guilliermondii* than with ammonium sulfate, and

supplementation of urea with casamino acids improved performance over urea alone only slightly. Yeast extract improved yields, but only slightly.

2.5.5 Magnesium and biotin. Mahker and Guebel (1994) studied the influence of Mg^{2+} concentration on growth, ethanol and xylitol production from xylose by *P. stipitis* NRRL Y-7124. Under constant oxygen uptake rate, biomass/ xylose and biomass/ Mg^{2+} yields increased with Mg^{2+} concentration with a maximum value at 4 mm. Ethanol was the main product formed. At low Mg^{2+} levels (1 mM), 49% of carbon flux to ethanol was redirected to xylitol production, and was correlated with intracellular accumulation of NADH. Lee *et al*. (1988) reported that the relative amount of ethanol and xylitol accumulated in xylose fed aerobic batch cultures of *P.tannophilus* and *C. guilliermondii* depended on the limitation by biotin. In high biotin containing media (2 μg/ L) *P. tannophilus* favored ethanol production over that of xylitol while *C. guilliermondii* favored xylitol formation.

2.5.6 Methanol supplementation. Dahiya (1991) reported that addition of 1% methanol to the medium with 100 g/ L xylose increased the xylitol production from 36.8 g/ L to 39.8 g/ L by *P. albertensis*. No significant difference in fungal biomass and xylulose accumulation was observed and only 0.015% methanol was consumed. This could be due to the oxidation of methanol to yield NADH which would enhance the reduction of xylose and xylulose to xylitol. Vongsuvanlert and Tani (1989) reported about 18 and 26% increase in xylitol production from xylose in presence of 1 and 2% methanol, respectively by *C. boidinii*. This is also the case with the production of sorbitol from glucose and idiotol from L-sorbose by *C. boidinii* (1988).

2.5.7 Initial cell density. Cao *et al*. (1994) investigated the effect of cell density on the production of xylitol from xylose by *Candida* sp. B-22. The rate of xylitol production from xylose increased with increasing yeast cell density. At high initial yeast cell concentration of 26 mg/ ml, 210 g/ L of xylitol was produced from 260 g/ L of xylose after 96 h of incubation with a yield of 81% of the theoretical value. Vandeska *et al*. (1995) reported that high initial cell densities improved xylitol yields and specific production rate of xylitol by *C. boidinii*. The susceptibility of wood hydrolysate to fermentation by *D. hansenii*

NRRL Y-7426 was strongly dependent on the initial cell concentration (Pajaro *et al*., 1996).

2.5.8 Oxygen supply. A variety of yeasts such as *Candida*, *Hansenula*, *Kluyveromyces*, and *Pichia* require oxygen for sugar uptake (Sims and Barnett, 1978) and availability of oxygen has significant influence on xylose fermentation by these yeast (Gong *et al*., 1983). However, oxygen limitation is the main factor stimulation the formation of xylitol (Furlan *et al*., 1994). Roseiro *et al*. (1991) reported that xylitol production by *D. hansenii* required semi-anaerobic conditions. The presence of oxygen enhanced NADH oxidation and a high NAD^+/ NADH ratio led to xylitol oxidation to xylulose, therefore, less xylitol was accumulated. Thus the yield of xylitol depended strongly on the oxygen transfer rate (Nolleau *et al*., 1993). Horitsu *et al*. (1992) reported that higher level of dissolved oxygen is required only at the earlier phase of cultivation and afterwards it should be decreased to the lower level of respiration by the yeast. Barbosa *et al*. (1988) reported that increasing oxygen limitation led to increased xylitol productivity and decreased ethanol production with *C. guilliermondii*. Nolleau *et al*. (1995) evaluated the ability of *C. guilliermondii* and *C. parapsilosis* to ferment xylose to xylitol under different oxygen transfer rates. In *C. guilliermondii*, a maximal xylitol yield of 0.66 g/ g was obtained when oxygen transfer rate was 2.2 mmol/ L/ h. Optimal conditions to produce xylitol by *C. parapsilosis* (0.75 g/ g) arose from cultures at pH 4.75 with 0.4 mmols of oxygen/ L/ h. The oxygen is not only an important factor to optimize the xylitol production but it is also an essential component for xylose assimilation. When aerobic batch cultures of *C. guilliermondii* and *C. parapsilisis* provided with xylose, were shifted to anaerobic conditions, the xylose concentration remained at a constant level and all metabolic activities stopped immediately. *C. mogii* produced xylitol from xylose under aerobic and oxygen-limiting conditions, but not without oxygen (Sirisansaneyakul *et al*., 1995). Xylose conversion into xylitol by *C. guilliermondii* FTI 20037 was investigated in a stirred tank bioreactor at different stirring rates (Silva *et al*., 1996). Maximal xylitol production (22.2 g/ L) was obtained at 30^oC, with an aeration rate of 0.46 vvm using a stirring rate of 300 per min (k_La = 10.6/ h). An increase of k_La caused an increase in the consumption of xylose in

detriment to xylitol formation. Winkelhausen *et al*. (1991) investigated xylitol formation by *C. boidinii* in oxygen limited chemostat culture. The production of xylitol by the yeast occurred under conditions of an oxygen limitation at specific oxygen uptake rates lower than 0.91 mmol/ gh. The effect of aeration on xylitol production from xylose by some yeasts is summarized in Table 2.4.

Table 2.4. Effect of aeration on xylitol production from xylose by some yeasts.

Yeast	**Xylose (g/ L)**	**Aeration**	**Xylitol yield (g/g)**	**Reference**
C. tropicalis	100	100 ml/ min	0.49	Horitsu *et al*., 1992
	100	400 ml/ min	0.57	
	100	500 ml/ min	0.45	
	100	700 ml/ min	0.38	
C. guilliermondii	100	Microaerobiosis	0.50	Nolleau *et al*., 1993
	100	Semiaerobiosis	0.49	
	100	Aerobiosis	0.56	
C. parapsilosis	100	Microaerobiosis	0.74	Nolleau *et al*., 1993
	100	Semiaerobiosis	0.61	
	100	Aerobiosis	0.50	
C. parapsilosis (continuous culture)	10	0.15 vvm	0.31	Furlan *et al*., 1991
	10	0.30 vvm	0.27	
	10	0.60 vvm	0.08	
	10	1.00 vvm	0.04	
	10	1.50 vvm	0.02	
	10	2.00 vvm	0.04	

2.6 Xylose Reductase (Branco *et al*., 2012)

For any enzyme catalyzed bioconversion, basic characteristics and properties of the enzyme (optimum temperature, pH, isoelectric point, kinetic, etc.) are essential for detailed understanding. Xylose reductase (EC 1.1.1.21) belongs to the superfamily of aldo-keto reductase, being a NAD(P)H dependent

oxidoreductase. XR is present in some strains of yeast and fungi. Table 2.5 summarizes some microorganisms capable of synthesizing the XR.

Table 2.5. Some examples of xylose reductase producing microorganisms

Microorganism	**Xylose reductase Specific activity (U mg^{-1} of protein)**
Candida pelliculosa	1.73
C. pelliculosa var. *acetaetherius*	0.09
C. utilis	0.26
C. guilliermondii FTI20037	1.10
Debaryomyces hansenii	0.16
Hansenula anomala Y1	0.09
Hormoacus platypoides AM93	0.43
Pichia nakazawae	0.24

XR plays a key role in microbial metabolism, since it allows using xylose as an energy source for microorganisms. XR initiates the first step of xylose metabolism, catalyzing the reduction reaction of xylose (aldose) to xylitol (alcohol) with the aid of the NAD(P)H coenzyme as an electron donor. The molecular weight of XR varies (30-70 kDa) widely depending on the producing microorganism. According to Cortez (2002), the XR present in Candida guilliermondii FTI20037 is composed of one or two 30-60 kDa subunits. In another study, Kratzer et al. (2004) found that the XR from Candida tenuis has a molecular weight of around 36 kDa. Several studies have demonstrated that different types of XR are possible in regards to their structure, which can be monomeric or dimeric depending on the microorganism.

2.7 Pretreatment of lignocellulosic materials (Rao *et al.*, 2016)

Pretreatment is the prerequisite step which plays a key role in subsequent saccharification of biomass and its conversion to xylitol. The methods of pretreatment can be divided into physical, chemical, physic-chemical and biological

methods as there are many reports available on pretreatment, in the current review we tried to put forth in brief about the recent pretreatment technologies.

2.7.1 Physical pretreatment

Physical pretreatment methods disrupt the structural integrity of lignocellulosic substrates and thereby increase their accessibility to acids or enzymes. Various types of physical pretreatment methods which can improve the efficiency of lignocellulosic saccharification include reducing size through chipping, grinding, or milling and irradiation by gamma rays, electron beam or microwave.

2.7.2 Chemical pretreatment

Chemical pretreatment methods involve the use of alkali, dilute acid, ammonia, organic solvents, oxidative agents and other chemicals. These methods are effortless and give good conversion yields in less span of time. However, the alkali used in the treatment has to be recovered after the process which needs additional energy input. Alkaline pretreatment digests lignin and makes holocellulose accessible for acid or enzymatic degradation. Generally hydroxides of sodium, potassium, calcium and ammonium are employed in this process. Xu *et al*. (2007) studies the influence of equal concentrations of sodium and potassium hydroxides in the recovery of hemicellulose from perennial ryegrass leaves, and found sodium hydroxide was effective for the removal of hemicelluloses whereas use of potassium hydroxide resulted in higher purity of hemicellulose. Manas and Krishnan (2015) studied the pretreatment of rice straw using 15%aqueous ammonia at 120^{o}C, which removed a significant amount of lignin i.e., 65.84% and enhanced subsequent enzymatic digestibility by four fold.

In acid pretreatment method, acids like sulfuric, hydrochloric, hydrofluoric, nitric and phosphoric acid etc. are used as catalysts. Dilute sulfuric acid hydrolysis is a well-known process for depolymerization of hemicelluloses in lignocellulosic biomass into monomeric sugars. In the presence of concentrated acids, hemicellulose and cellulose get hydrolyzed completely and high yield of monomeric sugars are obtained but the major drawback is their corrosive nature and the need to recycle acids to lower the cost of pretreatment. In recent times, organic acids were also recommended as alternatives to inorganic acids to avoid

reactor corrosions and reduce energy requirement for acid recovery. Acids like maleic, succinic, oxalic, fumaric and acetic acid have been extensively studied. Qin *et al*. (2012) observed that, when corn stover was pretreated with sulfuric, oxalic, citric, tartaric and acetic acid, 91.7-96.8% glucan as well as higher xylan and less furfural was obtained using acetic acid. Pretreating lignocellulosic biomass using oxidative reagents involves the reduction of crystallinity in hemicellulose and cellulose and also disrupts the association between carbohydrates and lignin. Nevertheless the cost of oxidative pretreatment is more than conventional alkali and acid pretreatments hence it is generally used as a support for other pretreatments to remove residual lignin from lignocellulosic substrates.

The oxidative methods comprise of alkaline peroxide pretreatment, ozonolysis, and wet oxidation. In wet oxidation, the biomass is treated with water and air or oxygen at high temperatures for comparatively short residence time. Recent studies have stated that hydrogen peroxide is the most frequently used oxidizing agent that disintegrate about 50% of lignin and most of the hemicellulose with 2%H_2O_2 at 30^{o}C. As ozone has the electron deficiency in the terminal oxygen, it is used as the strongest oxidizing agent. It attacks lignin, an electron-rich substrate selectively than carbohydrates. In a study conducted by Garcia Cubero *et al* (2009), O_3 appreciably reduced lignin content with meagre loss in hemicellulose during the oxidative delignification process.

Organic solvent pretreatment is an additional probable method in the extraction of hemicelluloses. As compared to other chemical pretreatments, this process has many advantages such as easy recovery of solvent by distillation, low environmental impact, and recovery of high quality lignin as a by-product. Dimethyl sulfoxide (DMSO) is the most common neutral solvent that has been used to recover hemicelluloses from holocellulose without disturbing the acetyl ester compounds and the glycosidic bonds. Though most of the solvents used in the organosolv pretreatment can be recycled from the reactor to decrease the cost, the high price and potential hazards of handling large volumes of organic solvents limit the utilization of organosolv process. Process using ionic liquids (IL) is another alternative for pretreatment of lignocellulosic materials. Ionic liquids disturb the non-covalent interactions in the lignocellulosic matrix without

any significant degradation. Hemicellulose liberated from IL solutions show enhanced enzymatic digestibility. Although inhibitor formation is minimal, the small amount of ILs remaining in the residual pretreated materials are significantly toxic to fermentative microorganisms. On the other hand, ILs are mostly expensive and thus further research is essential to standardize their solubility for lignocellulosic substrates.

2.7.3 Physico-chemical pretreatment

This category includes the majority of pretreatment technologies such as ultrasonication, microwave irradiation, steam explosion, twin screw extruder and so on. These forms of pretreatment exploit the use of conditions and compounds that affect the physical and chemical properties of biomass. Twin-screw extruder or extrusion reactor is a thermo-mechanic and chemical fractionation system that allows the integration of extrusion, cooking, liquid-solid extraction, and filtration in a single step. The twin-screw reactor extracts up to 90% of the original hemicellulose depending upon the content of xylan. The yield after extraction depends significantly on temperature whereas reduction in screw rotation speed and solid flow rate influenced the average residence time of the liquid phase which increased the extraction yield of hemicellulose.

Hydrothermal pretreatment includes steam explosion and liquid hot water hydrolysis. In the process of steam explosion, biomass is generally treated with high pressure and temperature for a certain amount of time after which the sample is rapidly decompressed resulting in the breakdown of the lignin carbohydrate complex. The principal fraction of the biomass digested in the liquid phase during pretreatment is hemicellulose whereas the lignin is transformed as result of the high temperature. In hot-compressed water or liquid hot water pretreatment, temperature above 200^{o}C at various pressures below the critical point is used. This method can recover comparatively high hemicellulose with low levels of inhibitory compounds. Moretti *et al*. (2014) reported that microwave-assisted extraction can be a novel eco-friendly way for the recovery of hemicellulose. The difficulty in microwave-assisted extraction is to get a good yield of sugars without extensive degradation of the hemicelluloses and

contamination with dissolved lignin. This method is efficient than the traditional chemical pretreatment.

2.7.4 Biological pretreatment

Biological retreatment using either organism directly or its metabolite for pretreatment of biomass is a promising eco-friendly technology as it has various advantages and is an economically viable strategy for enhancing the rate of enzymatic saccharification. Microorganisms like brown, white and soft rot fungi were used for degradation of lignin in biological pretreatment. Biological pretreatment of *Eucalyptus grandis*, saw dust degradation patterns and saccharification kinetics with white rot fungi was reported by Castoldi *et al*. (2014). The treatment produced structural changes in the saw dust fibers and after pretreatment there was a twenty fold increase in reducing sugars. Fungal laccases are the most widely used lignolytic enzymes in biotechnology but pretreatment with a single enzyme cannot completely remove lignin in either wood or herbaceous species.

2.8 Lignocellulose hydrolysates

Lignocellulosic materials are renewable, widespread and cheap. The main component of the hemicellulosic fraction of hardwoods and agricultural residues is xylan, a polymer made from xylose units that can be hydrolyzed to this sugar by mineral acids. Under selected conditions, the solid residue from acid hydrolysis contains both the cellulosic and lignin fractions, that can be separated in a further reaction step and utilized for different product applications. The liquid phase from the hydrolysis reaction (containing xylose, byproducts and compounds derived from other fractions of the raw materials such as extractives or lignin) can be utilized for making fermentation media suitable for xylitol production.

D-xylitol is industrially produced by the chemical reaction of D-xylose derived mainly from photosynthetic biomass hydrolysates. Photosynthetic biomass is the most abundant renewable resources in the world, consisting of cellulose, hemicellulose, lignin and a low quantity of pectin, protein, extractives, and ash (Figure 2.6). Hemicellulose is the second most abundant polysaccharide in nature, representing 19-34% of the photosynthetic biomass, just next to the most

abundant biopolymer; cellulose 34-50%. Hemicellulose, a good resource for producing D-xylitol, is composed of D-glucose, D-galactose, D-mannose, D-xylose, D-arabinose, and D-glucuronic acid with acetyl side chains.

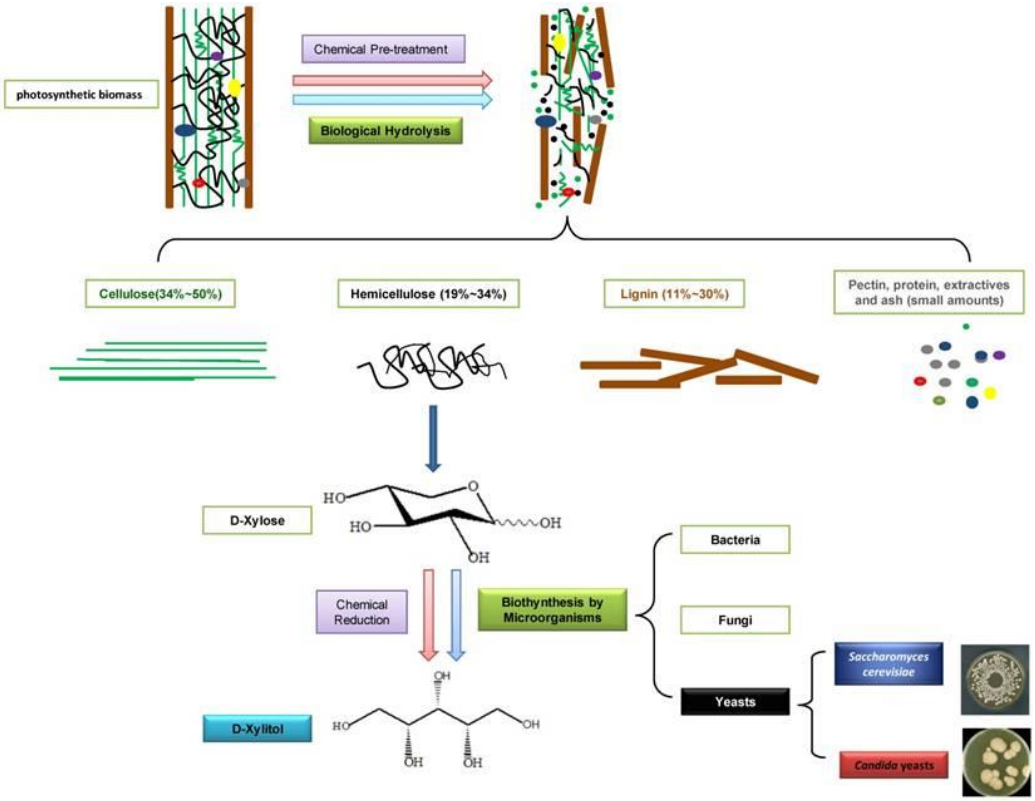


Figure 2.6. Production of D-xylitol from photosynthetic biomass. Reference: Chen *et al.*, (2010).

Leonard and Hajny (1945) considered the following as inhibitors: (i) minerals or metals contained in the lignocellulosic materials or resulting from the corrosion of the hydrolysis equipment; (ii) products derived from the hydrolysis of hemicelluloses, as acetic acid (liberated from the acetyl groups of the raw material) or furfural and hydroxymethyl furfural (generated by degradation of sugars); (iii) products derived from lignin degradation (phenolic compounds, aromatic acids and aldehydes); and (iv) compounds derived from extractives. Vanillic, syringic, caproic, caprylic, pelargonic and palmitic acids have been cited among the organic acids with inhibitory ability present in fermentation media obtained from lignocellulose.

2.9 Detoxification of acid hydrolysate

Several detoxification strategies were used to remove toxic compounds from the hydrolysates as the minute amounts may interfere and prevent the microbial growth. The efficiency of detoxification process depends on the raw material, type of hydrolysis process, and microorganism employed.

2.9.1 Chemical detoxification

Adsorption with activated charcoal is a well-established and low cost detoxification method. The detoxification of sago trunk hydrolysate with 2.5% activated charcoal at the reaction time of 60 min facilitated the reduction of 53% and 78% of furfural and phenolic compounds. It is evident that the maximum removal of phenolic compounds is attained by activated charcoal with a xylitol yield of 0.78 g/g when compared with the untreated hydrolysate (0.307 g/g). This study proposes that detoxification method using activated charcoal has a significant impact on xylitol production.

Arruda *et al*. (2011) elaborated the process involved in using ion-exchange resins. Sugarcane hemicellulosic hydrolysate was treated with the resins at the ratio of 1:2 v/v. The hydrolysate was vacuum evaporated and then subjected to ion-exchange resins like A-860S (macroporous strong base anion exchanger), A-500PS (macroporous type I strong base anion exchanger) and C-150 (macroporous strong acid cation exchanger), which led to the removal of 93% phenolics and 64.9% acetic acid.

Zhu *et al*. (2011) performed trialkylamine extraction to remove the inhibitors from corn stover prehydrolysate. The advantages of using trialkylamine are that it is highly efficient in removing inhibitors, and requires low temperature, and low energy at low cost. The triakylamine contained 50% n-octanol-20% kerosene. It was able to remove 73.3% of acetic acid, 45.7% of 5-HMF and 100% of furfural.

2.9.2 Nanofiltration

Nanofiltration (NF) is a potential and cost-competitive membrane separation technology. The molecular weight cut-off ranges from 150 to 1000 g/mol, thus facilitating the retention of compounds with molecular weight up to 150-250

g/mol as well as charged molecules. NF finds its applications in detoxification, fermentation broth separation, fractionation of sugars and sugar concentration.

Recently Nguyen et al. (2015) worked on NF and reverse osmosis (RO). The low molecular weight cut-off (150-400 g/mol) of the membranes of NF and RO were monitored on a flat-sheet plant for their capability in separating C5 and C6 sugars from acetic acid, furfural, 5-HMF and vanillin in a model solution. RO led to the sugar rejection of > 97% butr with the low transmission in inhibitors. The membranes of NF chiefly NF270, NF- and NF245 (Dow) and DK (GE Osmonics) were suitable with > 94% glucose rejection and > 80% inhibitors transmission.

Bras *et al*. (2014) used nanofiltration under diafiltration mode for the removal of inhibitory compounds from diluted acid hydrolysate of olive pomance substrate to improve its xylose fermentability. They also used the membranes NF270 and NF90 to explore the best membrane suitable for detoxification. NF270 was chosen to be used in the diananofiltration experiment as it showed the lowest rejection for toxic compounds and highest permeate flux and can deplete 99.7% of acetic acid and 100% of formic acid and furfural.

2.9.3 Vacuum membrane distillation

Chen *et al*. (2013) studied the efficiency of vacuum membrane distillation (VMD), a membrane separation technology which is an increasingly popular and cost-effective in recovering inhibitors. It works on the principle of liquid-vapor phase equilibrium that controls the process selectivity, facilitating the removal of volatile components. VMD was used to eliminate two inhibitors acetic acid and furfural from lignocellulosic hydrolysates. More than 98% of furfural could be removed by VMD under optimal conditions but, the removal of acetic acid was considerably lower when compared with furfural.

2.9.4 Electrochemical detoxification

Electrochemical detoxification is used for the removal of phenolic compounds, p-coumaric acid, ferulic acid, syringaldehyde, and vanillin. The phenolic compounds were converted electrochemically to a radical form at oxidation potential and are then removed after radical polymerization as polymeric compounds. Nearly 71% of total phenolics were removed from rice straw hydrolysate without any sugar-loss.

2.9.5 Biological detoxification

There is increasing interest regarding the biological mode of detoxification because the formation of low side reaction products, requires less energy without the use of chemicals. For the proficient microbial detoxification to occur, there should be a focus on the process parameters like optimal nutrient addition, pH 4-6, temperature 25-50^{o}C, incubation time 12-144 h, size of inoculum 1-10% v/v or 0.5-10 g/L (dry weight) and the strain employed in carrying out detoxification. The use of an efficient *Coniochaeta ligniaria* NRRL30616 metabolizes furfural, HMF, and other inhibitory compounds present in the dilute acid hydrolysate.

Zhang *et al.* (2013) performed detoxification of oil palm empty fruit bunch hydrolysate with a newly isolated *Enterobacter* sp. FDS8 and reported the depletion of furfural and HMF at a rate of up to 0.54 g/L/h and 0.12 g/L/h with total sugar loss of < 5%. It is even possible to recover the cells and used again for at least 5 times without losing their ability of detoxification. Biological detoxification is performed in single step without any additional manipulation, and without volume loss. It also offers numerous other benefits like complete reduction of furfural, acetic acid, and guaiacil over chemical detoxification.

The enzyme mediated detoxification involves the enzymes laccases and peroxidases. Lee *et al*. (2012) were the first to report a novel laccase from the yeast, *Yarrowia lipolytica*, which demonstrates a higher efficiency in the reduction of non-phenolic and phenolic components than any previously reported enzyme from bacteria or fungi.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Chemical and reagents

All chemicals and reagents used in this work are listed in Table 3.1.

Table 3.1 List of chemicals used.

Chemical	Structural formula	Company
Acetic acid	-	QReC
Agar	CH_3COOH	LABCHEM
Ammonium sulfate	$(NH_4)_2SO_4$	ACI Labscan
Arabinose	$C_5H_{10}O_5$	SIGMA-ALDRICH
Calcium oxide	CaO	UNIVAR
Charcoal	-	-
Di-potassium hydrogen phosphate	K_2HPO_4	UNIVAR
Di-sodium hydrogen phosphate dodecahydrate	$Na_2HPO_4.12H_2O$	ACI Labscan
Ethanol	C_2H_6O	ACI Labscan
Furfural	$C_5H_4O_2$	SIGMA-ALDRICH
Glucose	$C_6H_{12}O_6$	UNILAB
Hydrochloric acid	HCl	CARLO ERBA
5-hydroxymethyl furfural (HMF)	$C_6H_6O_3$	SIGMA-ALDRICH
Magnesium sulfate hepta-hydrate	$MgSO_4.7H_2O$	Fisher Scientific
Malt extract	-	BD

Table 3.1 (Cont.)

Chemical	Structural formula	Company
Mercaptoethanol	C_2H_6OS	SIGMA-ALDRICH
β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH)	-	SIGMA-ALDRICH
β-nicotinamide adenine dinucleotide,z-phosphate reduced, tetrasodium salt (NADPH)	-	SIGMA-ALDRICH
Peptone	-	HIMDIA
Phenolic	C_6H_6O	QReC
Phosphoric acid	H_3PO_4	QReC
Potassium di-hydrogen phosphate	KH_2PO_4	ACI Labscan
Sodium di-hydrogen phosphate di-hydrate	$NaH_2PO_4.2H_2O$	ACI Labscan
Sodium hydroxide	NaOH	UNIVAR
Sulfuric acid	H_2SO_4	QReC
Tris-hydroxyl methyl aminomethane	$C_4H_{11}NO_3$	UNIVAR
Xylitol	$C_5H_{12}O_5$	SIGMA-ALDRICH
Xylose	$C_5H_{10}O_5$	HIMEDIA
Yeast extract	-	HIMEDIA

3.2 Material and methods

3.2.1 Material

The bagasse was provided by Wang Kanai Sugar Factories, Maha Sarakham Province and Mit Phu Wiang Factories, Khon Kaen Province, Thailand

3.2.1.1 Preparation of sugarcane bagasses hydrolysate, pretreated with different concentrations of sulfuric acid with sugarcane bagasse and sulfuric acid ratio of 1:10, hydrolysis including 1%, 2%, and 3.1% v/v to determine the highest yield of xylose. After pre-treatment the sugarcane bagasse was hydrolyzed under different temperatures and incubation times based on the acid concentration which were 1% (v/v) H_2SO_4 concentration 121°C, 60 min. (Rao *et al*., 2006), 2% (v/v) H_2SO_4 134°C, 60 min. (Jeon *et al*., 2010) and 3.1% (v/v) H_2SO_4 126°C, 18 min. (modified from Paiva *et al*., 2008) in an autoclave. The liquid fraction was then filtered through Whatman no.1 filter paper and the pH was raised to 9 with calcium oxide and then decreased to 5.5 using phosphoric acid. After this, the hydrolysate was mixed with activated charcoal concentration 1, 2.5 and 3% w/v, and agitated 150 rpm, 30°C, 60 min. The hydrolysate was then concentrated under vacuum at 70°C to increase the xylose concentration using an evaporator (Buchi Rotavapor® 215+v-700/ v-855). The sugarcane bagasse hydrolysate was used as a fermentation medium for xylitol production.

3.2.1.2 Sugar quantification by HPLC analysis, The hydrolysate was first filtered and concentrated at 70^{o}C under vacuum to obtain a sixfold increase in the xylose content using an evaporator (Buchi Rotavapor® 215+v-700/ v-855). Xylose, glucose, arabinose, acetic acid, HMF, furfural, phenolic compound and xylitol concentrations were determined using HPLC with an Aminex HPX-87H, (Biorad, USA) carbohydrate column (300 x 7.8 mm) at 45°C, using 5 mM H_2SO_4, as an eluent. A flow rate of 0.5 ml/min and a sample volume of 20 µl were maintained. The eluate was monitored with refractive index (RI) detector. Peaks were identified and quantified by comparison with the retention times of authentic standards (xylose and xylitol purchased from Sigma Company).

3.2.1.3 Toxic agents, acetic acid, HMF, furfural, phenolic compound and xylitol concentrations were determined using HPLC with an Aminex HPX-87H, (Biorad, USA) carbohydrate column (300 x 7.8 mm) at 45°C, using 5 mM H_2SO_4, as an

eluent. A flow rate of 0.5 ml/min and a sample volume of 20 µl were maintained. The eluate was monitored with refractive index (RI) detector. Peaks were identified and quantified by comparison with the retention times of authentic standards (xylose and xylitol purchased from Sigma Company).

3.2.2 Primary screening of xylose fermenting-yeasts and culture conditions

Samples of sugarcane bagasse waste were obtained from 2 sugar factories in Maha Sarakham and Khon Kaen Province, Thailand. For screening, 10 g of each sample was placed into 90 ml of malt extract-xylose-yeast extract-peptone, MXYP, medium containing 30 g/l xylose, 3 g/l yeast extract, 3 g/l malt extract, and 5 g/l peptone, in a 250 ml Erlenmeyer flask and incubated at 30°C for 72 hrs. The enriched cells were spread on MXYP agar plates, 100 g/l xylose. After incubation at 30°C for 48 hrs, the cross streak method was used to obtain pure isolates individual colonies of microorganism. Colonies with different characteristics were picked up and maintained on slant YM agar (containing 4 g/l yeast extract; 10 g/l malt extract, 4 g/l glucose and 20 g/l agar) at 30°C for 48 hrs, maintained at 4°C and subcultured at regular intervals. The assays were carried out in 10 ml MXYP medium (initial xylose 30 g/l) and cultivated on a shaker incubator at 150 rpm/ min and 30°C. Cell growth was measured by optical density at 600 nm. After 48 hrs cultivation, samples of each strain were analyzed.

3.2.3 Secondary screening using xylose fermentation of selected strain

3.2.3.1 Inoculum development

Six isolated xylose fermenting-yeasts from xylose assimilation tests, were used for xylitol production. From the subculture, one loopful of yeast cells was inoculated into 100 ml Erlenmeyer flasks containing 50 ml of MXYP medium, and then cultivated at 30°C for 24 hrs on a rotary shaker at 150 rpm. Inoculated cells were counted using a hemacytometer; final cell concentration was $1x10^8$ cells/ ml and used as an inoculum in xylitol fermentation process.

3.2.3.2 Fermentation conditions

Flask batch fermentations were performed by shaking in 125 ml Erlenmeyer flasks containing 50 ml of sugarcane bagasse hydrolysate (initial xylose concentration

32.30 g/l) into a fermentation medium, containing 2 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4.7H_2O$, 10 g/l yeast extract, 20 g/l peptone, 0.5 g/l KH_2PO_4, and 0.5 g/l K_2HPO_4, and cultivated in a rotary-shaker at 150 rpm, 30°C for 96 hrs. They were inoculated to a final concentration of 10^8 cells/ ml. The samples were collected at regular intervals of 24, 48, 72, and 96 h. of incubation. Aliquots of the cultures were centrifuged at 12,000 x g for 10 min and the supernatant subjected to high performance liquid chromatography (HPLC) analysis for determining the sugar consumption and xylitol concentration.

3.2.4 Optimization of xylitol production using flask batch fermentation

3.2.4.1 Design of experiment (DOE)

The response surface methodology (RSM) has several classes of designs, with its own properties and characteristics. Central composite design (CCD), Box-Behnken design and three-level factorial design are the most popular designs applied by the researchers. Prior knowledge with an understanding of the related bioprocesses is necessary for a realistic modeling approach.

3.2.4.2 Central composite design

The CCD is used to study the effects of the variables towards their responses and subsequently in the optimization studies. This method is suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, as well as to analyze the interaction between the parameters. In order to determine the existence of a relationship between the factors and the response variables, the data collected are analyzed in a statistical manner, using regression. A regression design is normally employed to model a response as a mathematical function (either known or empirical) of a few continuous factors and good model parameter estimates are desired. The coded values of the process parameters are determined by the following equation (1):

$$x_i = \frac{X_i - X_0}{\Delta x} \qquad (1)$$

Where x_i-coded value of the i^{th} variable, X_i-uncoded value of the i^{th} test variable and X_0-uncoded value of the i^{th} test variable at center point.

This method is based on the mathematical model, which can be represented by equation (2):

$$\hat{y} = b_0+b_1x_1+b_2x_2+b_{12}x_1x_2+b_{11}x^2_1+b_{22}x^2_2 \quad (2)$$

in which $\hat{y}$ represents the response variable, b_o is the interception coefficient, b_1 and b_2 the linear terms, b_{11} and b_{22} the quadratic terms and x_1 and x_2 the variables studied.

Table 3.2 Ranges of variables used in RSM

No.	Variables	Code	Levels				
			-2.37	-1	0	1	2.37
1	Xylose concentration	A	36.2159	50	60	70	83.7841
2	Inoculum size	B	1.62159	3	4	5	6.37841
3	Agitation speed	C	115.54	150	175	200	234.46
4	Temperature	D	19.6619	30	37.5	45	55.3381
5	pH	E	2.62159	4.0	5.0	6.0	7.37841

3.2.4.3 Model Fitting and Statistical Analysis

The regression and graphical analysis with statistical significance are carried out using Design-Expert software (version 7.1.5, Stat-Ease, Inc., Minneapolis, USA). In order to visualize the relationship between the experimental variables and responses, the response surface and contour plots are generated from the models. The optimum values of the process variables are obtained from the regression equation.

The adequacy of the models is further justified through analysis of variance (ANOVA). Lack-of-fit is a special diagnostic test for adequacy of a model that compares the pure error, based on the replicate measurements to the

other lack of fit, based on the model performance. *F*-value, calculated as the ratio between the lack-of-fit mean square and the pure error mean square, is the statistic parameter used to determine whether the lack-of-fit is significant or not, at a significance level.

3.2.4.4 Validation of the experimental model

The statistical model was validated with respect to xylitol production under the conditions predicted by the model in shake-flasks level. Samples were drawn at the desired intervals and xylitol production was determined as described above.

3.2.4.5 Sugar quantification by HPLC analysis

Xylose, glucose, arabinose, and xylitol concentrations were determined using HPLC with an Aminex HPX-87H, (Biorad, USA) carbohydrate column (300 x 7.8 mm) at 45°C, using 5 mM H_2SO_4, as an eluent. A flow rate of 0.5 ml/min and a sample volume of 20 µl were maintained. The eluate was monitored with refractive index (RI) detector. Peaks were identified and quantified by comparison with the retention times of authentic standards (xylose and xylitol purchased from Sigma Company).

3.2.5 Xylitol production in 5 L bioreactor

3.2.5.1 Bioreactor condition

Xylitol production was scaled up in a 5 L fermenter using 1 L of optimized and treated sugarcane bagasses hemicellulosic hydrolysate medium containing (g/L): 50 g/l of xylose, along with other medium components (%): yeast extract 10 g/l, Peotone 20 g/l, $(NH_4)_2SO_4$ 2.0 g/l, $MgSO_4 \cdot 7H_2O$ 5 g/l, K_2HPO_4 5 g/l and KH_2PO_4 5 g/l . The appropriate medium was sterilized in situ at 121 ^oC for 15 min and inoculated with 10% of the inoculum. Fermentation was carried out at 30 ^oC and pH 6.0. Agitation and aeration rate were adjusted to 200 rpm with a constant rate of 0.3 vvm (adapted aeration) for rest of the fermentation run. Foaming was controlled by adding silicon antifoam agent (50%, v/v prepared in distilled water). Samples were withdrawn for 96 h at regular time intervals of 12 h, centrifuged and were analyzed for xylitol production, leftover xylose and cell mass.

3.2.5.2 Strain

Candida tropicalis KS 10-3 was grown on the preculture medium containing KH_2PO_4 2 g/l, $(NH4)_2SO_4$ 5 g/l, yeast extract 4 g/l, $MgSO_4.7H_2O$ 0.5 g/l, xylose 20 g/l. The seed cells for the bioreactor were prepared in 500 ml flasks containing 100 ml preculture medium. The flasks were incubated at 30 ^oC and 200 rpm for 24 h and subsequently inoculated into the fermentation medium.

3.2.5.3 Determination of xylose reductase (XR) activity

Ten percent (v/v) of the adapted yeast inoculum was inoculated into batch fermenter containing 1 L of sugarcane bagasse hydrolysate medium, and incubated at 30^oC, 1 vvm and 150 rpm. Cells were harvested at the end by centrifugation at 8000 x g for 20 min at 4^oC. The washed cell pellet was resuspended in potassium phosphate buffer (0.1 M, pH 7.0) at a cell biomass to buffer ratio (w/v) of 1:2 and the resulting cell suspension was disrupted by ultrasonication following the protocol reported elsewhere (Rafiqul and Sakinah, 2014). The homogenate was then centrifuged as before to obtain a supernatant solution. The supernatant was re-centrifuged at 10,000 x g for 30 min. The refined supernatant was stored at -80^oC, and used as crude XR enzyme determination.

Xylose reductase activity was measured at 35^oC by following the xylose-dependent NADPH consumption in a reaction mixture consisting of 100 µl cell-free extract, 100 µl distilled water, 100 µl 2-mercaptoethanol (100 mM), 500 µl potassium buffer (250 mM, pH 7.0), 100 µl NADPH (3.4 mM) and 100 µl D-xylose (0.5M) in a final volume of 1 ml. The rate of dinucleotide oxidation was measured at 340 nm by UV-vis spectrophotometer at 30-s intervals for 5 min against an extract blank lacking xylose. Activity was calculated over the linear portion of the curve from a molar extinction coefficient of 6220 $M^{-1}cm^{-1}$.

XR activities were determined by spectrophotometry at 340 nm at room temperature. An enzyme activity unit (U) was defined as the amount of enzyme catalyzing the reduction/ oxidation of 1 µmol NADPH/ min, at room temperature, by using the molar extinction coefficient for NADPH and NAD^+ co-factors of 6220 M/cm (Silva and Felipe, 2006). The activity of XR and XDH were determined using the extinction coefficient of NADPH and NAD^+ through Eqs. (2) and (3) (Nidetzky *et al*., 1996).

$$\Delta A340/\text{min} = ((A340\text{at 1 min}) - (A340\text{ at 5 min}))/ 4\text{ min} \quad (2)$$

$$\text{Enzyme activity} = (\Delta A340\text{at 1 min(test)} - (\Delta A340\text{at 1 min(control)}) \times V_a \times DF / (E^{mM} \times V_e) \text{ (U/ ml)} \quad (3)$$

Where $\Delta A340/\text{min}$ is the rate of decrease in absorbance at 340 nm per min; V_a is the volume of assay (ml); V_e is the volume of enzyme (ml), *DF* is the dilution factor, and E^{mM} is the millimolar extinction coefficient of NADPH (6.22 $nM^{-1}cm^{-1}$).

3.2.5.4 Sugar quantification by HPLC analysis

Xylose, glucose, arabinose, and xylitol concentrations were determined using HPLC with an Aminex HPX-87H, (Biorad, USA) carbohydrate column (300 x 7.8 mm) at 45°C, using 5 mM H_2SO_4, as an eluent. A flow rate of 0.5 ml/min and a sample volume of 20 µl were maintained. The eluate was monitored with refractive index (RI) detector. Peaks were identified and quantified by comparison with the retention times of authentic standards (xylose and xylitol purchased from Sigma Company).

3.2.6 Strain identification

Partial sequences of the D1/D2 domain approximately 600-800 bp of the LSU rDNA and the SSU rDNA were determined from PCR products using extracted genomic DNA from yeast cells by using a slightly modified version of the method (Lachance *et al*., 2001). A divergent 5' domain of the gene was amplified by a PCR with the forward primer NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and the reverse primer NL-4 (5'-GGTCCGTGTTTCAAGACGG) (O'Donnell,1999); amplification of the SSU rDNA was done with the forward primer P1 sequence and the reverse primer P2 sequence(Sjamsuridzal *et al*., 1997). DNA product was subjected to electrophoresis on 3% agarose gel, recovered using the QIAquick purification kit (Qiagen) and cycle-sequenced using the ABI Big Dye terminator cycle sequencing kit, version 3.1 (Applied Biosystems), with the external primers NL-1 and NL-4 for the D1/D2 domain (Kurtzman and Robnett, 1998) and eight primers, P1–P8, for the SSU rDNA (Yamada *et al*., 1999). The sequences were determined with an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems) according to the instructions of

the manufacturer. Database sequences were compared with the BLASTN homology search (http:// www.ncbi.nlm.nih.gov/BLAST/, NCBI) and generated sequences were aligned with related species retrieved from GenBank using the CLUSTAL X, version 1.81 (Thompson *et al*., 1994).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Sugarcane bagasse hydrolysis

Sugarcane bagasse is a lignocellulosic material waste product from sugar mills consisting of cellulose, hemicellulose, lignin and other types of sugar containing five and six carbon atoms which is commercially used as a carbon source to manufacture a variety of products. Sugars produced from sugarcane bagasse include glucose, galactose, mannose, xylose, and arabinose. Galacturonic acid can be produced from dilute acid or base hydrolysis. The remaining sugars in the bagasse are formed from the breakdown of the monosaccharide in the beta-glucosidic bond. Chandel *et al*. (2011) illustrated auto hydrolysis as a simple method to break down the hemicellulosic backbone into monomeric sugar constituents with fewer by-products. However, a significant fraction of hemicellulosic material may remain with the substrate after the auto hydrolysis. To overcome this problem, dilute acid hydrolysis is used for the maximum degradation of hemicelluloses into monomers. Compared to other pretreatment methods, dilute acid hydrolysis is more useful for the conversion of maximum hemicellulosic fraction into xylose and other sugars, which can be fermented by specialized microorganisms.

The chemical composition of the sugarcane bagasse which is similar to any other type of plant biomass is presented in Table 4.1 and composed of cellulose, hemicelluloses, lignin, and small amounts of extractives and mineral salts.

Table 4.1 Chemical composition of sugarcane bagasse.

Component	Composition (%)
Hemicellulose (xylan)	25.8
Cellulose (glucan)	42.8
Lignin	22.1
Extractives	6.1
Ash	1.4
Total	98.2

Reference : Sarrouh *et al*., 2009

Results showed that maximum xylose concentration of 19.0 g/l was obtained at 3.1% sulfuric acid concentration (126°C, 18 min) (Table 4.2). Increasing sulfuric acid concentration enhanced the decomposition of lignin and released xylose sugars from the sugarcane bagasse during acid hydrolysis. Lignin was broken down into phenolic compound including vanillin, syringaldehyde, 4-hydroxybenzoic acid, and ferulic acid which have a toxic effect on microbial growth and xylose metabolism in yeast cells.

Table 4.2 Sugar composition of bagasse pre-treatment by under different conditions.

Condition	Sugar composition (g/l)		
	Xylose	**Glucose**	**Arabinose**
H_2SO_4 1%Temp. 121^oC, 60 min.	18.51±9.35	2.42±9.35	2.20±9.35
H_2SO_4 2%Temp. 134^oC, 60 min.	12.06±5.43	3.23±5.43	2.17±5.43
H_2SO_4 3.1%Temp. 126^oC, 18 min.	19.00±9.42	2.75±9.42	2.63±9.42

Rao *et al.* (2006) found that using sulfuric acid concentration of 1% v/v under a temperature of 121°C, 60 min produced xylose, glucose and arabinose at 56, 15, and 24%, respectively. Paiva *et al.* (2009) reported that sulfuric acid concentrations of 3.1% v/v at a temperature of 126°C, 18 min gave xylose at 266.73 mg/g dry weight of

sugarcane bagasse. Jeon *et al*. (2010) used sulfuric acid concentrations of 2% v/v under a temperature of 134°C, 60 min. and produced xylose, glucose and arabinose at 21, 5.4, and 3 g/l, respectively. They used dilute acid as a catalyst in the hydrolysis. Hemicellulose and lignin dissolved mostly at low concentrations (0.05-5%) this minimized damage to products and offered the highest sugar yield at the end of the process. Furthermore, dilute acid enhances the economic potential for industrial production, since it is easy to control and can treat large amounts of the biomass in a short time. The mechanism of the reaction of acid hydrolysis is as follows, (1) diffusion of protons through the wet lignocellulosic matrix, (2) heterocyclic bonding of oxygen protons and sugar monomers, (3) breaking of the ether bond, (4) intermediate carbocation, (5) solvation of the carbocation with water, (6) regeneration of protons with the sugar energy monomer, (7) distribution of the reaction in the liquid phase, and (8) resumption of the second stage, as developed from the experimentals model (Aguilar *et al*., 2002).

Table 4.3 Sugarcane bagasse hydrolysate composition from Wang Kanai Factories, Maha Sarakham Province.

Treatment	Sugar composition (g/l)			Toxic compound (g/l)			
	Xylose	Glucose	Arabinose	HMF	Furfural	Acetic acid	Phenolic compound
Original	14.95± 10.55	1.27± 2.03	3.13± 2.04	0.02± 0.02	0.03± 0.02	3.90± 1.66	5.24±3.57
Evaporation	44.58± 10.55	5.50± 2.03	8.00± 2.04	0.07± 2.02	0.09± 0.02	5.54± 1.66	7.78±3.57
H + Charcoal 1%	32.13± 10.55	5.98± 2.03	7.59± 2.04	0.03± 2.02	0.05± 0.02	2.37± 1.66	0.25±3.57
H + Charcoal 2.4%	31.62± 10.55	5.78± 2.03	7.54± 2.04	0.03± 2.02	0.04± 0.02	1.85± 1.66	0.19±3.57
H + Charcoal 3%	32.30± 10.55	5.90± 2.03	7.60± 2.04	0.02± 2.02	0.04± 0.02	1.56± 1.66	0.15±3.57

Original = Hydrolysate not evaporation, H = Hydrolysate, HMF = Hydroxy methyl furfural

Evaporation under vacuum can eliminate volatile compounds such as acetic acid, furfural and vanillin from the lignocellulosic hydrolysate. However, this method retains the concentration of non-volatile toxic compounds (extractives and lignin derivatives) in the hydrolysates. Table 4.3 shows the toxic compounds byproducts

obtained from the acid hydrolysis step which can be removed by charcoal. The main disadvantage of the synthesis of lignocellulosic, material using the diluted acid hydrolysis process is the degradation of the many sugar types during hydrolysis, and the formation of undesirable by-products, which inhibit the fermentation process. The by-products in diluted acid concentration are divided into three main groups: (1) weak acids, e.g. acetic and formic acids, (2) furan derivatives, e.g. furfural and 5-hydroxymethylfurfural, and (3) phenolic compounds. Several detoxification methods have been reported to overcome the inhibitory effect of these compounds during fermentation by yeasts, including adaptation of microorganisms to the medium, treatments with molecular sieves, ion-exchange resins or charcoal, steam stripping and overtitration to remove various inhibitory compounds from lignocellulosic hydrolysates. Charcoal is effective in removing some toxic compounds which attach to its surface. Kamal *et al*. (2011) and Aguilar *et al*. (2002) noted that activated charcoal has been widely used in the removal of toxic compounds in different proportions varying from 1% to 30% in addition, they observed that 1% of charcoal was enough to remove 94% of the phenolic compounds. Mussatto and Roberto (2001) reported an increase in xylitol production by *Candida guilliermondii* using cells previously adapted to the hemicellulosic hydrolysate of rice straw. This study describes detoxification using activated charcoal concentration 3% (w/v) to benefits xylitol production by removing inhibitors. The toxic substance attaches to the surface of the charcoal and maximizes xylose residual sugar from the hydrolysate.

4.2 Primary screening of xylose-fermenting-yeasts and culture conditions

4.2.1 Isolation of xylose fermented-yeasts on solid media

Xylose fermenting-yeasts have the ability to use xylose as a carbon source on solid media. The isolates was screened from sugarcane bagasse waste collected from sugar factories in Kosum Phisai, Maha Sarakham Province and Phu Wiang, Khon Kaen Province, led to 34 and 38 yeast isolates, respectively (Table 4.4). Morphological characteristics shown in Table 4.4 demonstrate xylose-fermenting yeast of sugarcane bagasse waste, from sugar factories, at Kosum Phisai, Maha Sarakham Province, as 34 isolates exhibiting 25 isolates of white colony, 4 isolates of cream colony, 4 isolates of pink colony and 1 isolate of yellow colony. From Phu Wiang, Khon Kaen Province, 38

isolates presented 25 isolates of white colony, 11 isolates of cream colony, 1 isolate of pink colony and 1 isolate of yellow colony.

Table 4.4 Morphological characteristics and growth ability of xylose fermenting yeasts isolated from sugarcane bagasse waste.

Sample source	Isolates	Morphological characteristics					Growth on xylose medium (OD_{600nm})	
		Form	**Elevation**	**Surface**	**Margin**	**Color**	**0 h.**	**48 h.**
Kosum Phisai, MahaSarakham Province	KS1-2	Irregular	Convex	Rough	Undulate	Cream	0.134	0.444
	KS1-3	Circular	Convex	Glistening	Entire	Cream	0.057	0.833
	KS1-4	Circular	Raised	Glistening	Entire	White	0.069	1.181
	KS1-5	Circular	Convex	Glistening	Entire	Pink	0.044	0.319
	KS2-1	Irregular	Raised	Glistening	Undulate	White	0.062	0.351
	KS2-2	Circular	Convex	Glistening	Entire	White	0.056	0.684
	KS2-3	Irregular	Convex	Glistening	Undulate	White	0.031	0.235
	KS2-4	Circular	Convex	Glistening	Entire	White	0.052	0.793
	KS3-1	Circular	Raised	Glistening	Entire	Cream	0.214	0.601
	KS4-1	Irregular	Pulvinate	Glistening	Undulate	White	0.063	0.853
	KS4-2	Circular	Flat	Glistening	Entire	Cream	0.078	0.908
	KS4-4	Circular	Convex	Glistening	Entire	Pink	0.093	0.423
	KS4-5	Filamentous	Flat	Rough	Lobate	White	0.046	0.489
	KS4-6	Circular	Convex	Glistening	Entire	Pink	0.105	0.514

Table 4.4 Continued

Sample source	Isolates	Morphological characteristics					Growth on xylose medium (OD_{600nm})	
		Form	**Elevation**	**Surface**	**Margin**	**Color**	**0 h.**	**48 h.**
Kosum Phisai, MahaSarakham Province	KS5-1	Irregular	Raised	Glistening	Lobate	White	0.109	0.406
	KS6-1	Circular	Flat	Rough	Entire	White	0.066	0.555
	KS6-2	Circular	Convex	Rough	Entire	White	0.090	0.618
	KS7-1	Irregular	Convex	Rough	Undulate	White	0.082	0.467
	KS7-2	Irregular	Flat	Glistening	Undulate	White	0.159	0.300
	KS7-3	Irregular	Effuse	Rough	Undulate	White	0.086	0.680
	KS7-4	Irregular	Convex	Rough	Undulate	White	0.096	0.589
	KS7-5	Irregular	Flat	Rough	Lobate	White	0.094	0.669
	KS7-6	Irregular	Flat	Glistening	Undulate	Yellow	0.057	0.242
	KS7-7	Circular	Convex	Glistening	Entire	Pink	0.058	0.990
	KS7-8	Irregular	Convex	Glistening	Lobate	White	0.059	0.266
	KS7-9	Irregular	Pulvinate	Glistening	Undulate	White	0.083	0.539
	KS8-1	Circular	Flat	Glistening	Undulate	White	0.068	0.197
	KS9-1	Irregular	Convex	Glistening	Undulate	White	0.133	0.861

Table 4.4 Continued

Sample source	Isolates	Morphological characteristics					Growth on xylose medium (OD_{600nm})	
		Form	**Elevation**	**Surface**	**Margin**	**Color**	**0 h.**	**48 h.**
Kosum Phisai, MahaSarakham Province	KS9-2	Filamentous	Raised	Rough	Filamentous	White	0.082	0.755
	KS10-1	Rhizoid	Effuse	Glistening	Filamentous	White	0.068	0.493
	KS10-3	Circular	Convex	Rough	Undulate	White	0.086	1.032
	KS10-4	Circular	Pulvinate	Rough	Entire	White	0.097	0.691
Phu Wiang, KhonKaen Province	PV1-1	Irregular	Convex	Glistening	Undulate	White	0.079	0.508
	PV1-2	Circular	Raised	Glistening	Entire	Cream	0.223	0.864
	PV1-4	Rhizoid	Convex	Rough	Filamentous	White	0.044	0.405
	PV1-6	Circular	Convex	Glistening	Entire	Cream	0.069	0.907
	PV2-1	Circular	Convex	Glistening	Entire	White	0.083	0.488
	PV2-2	Circular	Pulvinate	Glistening	Entire	Cream	0.059	0.454
	PV2-3	Circular	Convex	Glistening	Entire	Cream	0.057	0.180
	PV3-1	Irregular	Pulvinate	Glistening	Undulate	White	0.082	1.280
	PV3-3	Circular	Pulvinate	Glistening	Entire	Pink	0.095	0.414
	PV3-4	Circular	Raised	Glistening	Entire	Cream	0.060	0.117

Table 4.4 Continued

Sample source	Isolates	Morphological characteristics					Growth on xylose medium (OD_{600nm})	
		Form	**Elevation**	**Surface**	**Margin**	**Color**	**0 h.**	**48 h.**
Phu Wiang, KhonKaen Province	KS3-7	Circular	Convex	Glistening	Undulate	White	0.052	0.126
	PV4-2	Circular	Pulvinate	Glistening	Entire	White	0.111	0.526
	PV4-4	Circular	Convex	Glistening	Entire	White	0.087	0.541
	PV4-5	Circular	Convex	Rough	Entire	White	0.097	0.566
	PV5-1	Irregular	Flat	Glistening	Undulate	Cream	0.055	0.720
	PV5-3	Filamentous	Pulvinate	Rough	Filamentous	White	0.120	0.513
	PV5-4	Irregular	Convex	Glistening	Undulate	Cream	0.120	0.241
	PV5-5	Rhizoid	Flat	Rough	Curld	Cream	0.030	0.040
	PV5-7	Rhizoid	Flat	Rough	Curld	Cream	0.027	0.190
	PV6-1	Circular	Convex	Glistening	Entire	White	0.097	0.623
	PV6-2	Circular	Convex	Glistening	Entire	White	0.062	0.485
	PV6-3	Circular	Convex	Rough	Entire	White	0.062	0.368
	PV6-7	Rhizoid	Pulvinate	Rough	Curld	Cream	0.041	0.335
	PV7-1	Circular	Convex	Rough	Entire	White	0.058	0.492

Table 4.4 Continued

Sample source	Isolates	Morphology Characteristics					Growth on xylose medium (OD_{600nm})	
		Form	**Elevation**	**Surface**	**Margin**	**Color**	**0 h.**	**48 h.**
Phu Wiang, KhonKaen Province	PV7-4	Circular	Raised	Rough	Entire	White	0.083	0.319
	PV7-5	Circular	Convex	Rough	Entire	White	0.075	0.374
	PV8-1	Rhizoid	Pulvinate	Glistening	Curld	White	0.046	0.477
	PV8-3	Irregular	Flat	Rough	Undulate	White	0.048	0.362
	PV8-4	Circular	Convex	Rough	Entire	White	0.044	0.518
	PV9-1	Irregular	Raised	Rough	Undulate	White	0.026	0.344
	PV9-2	Circular	Pulvinate	Glistening	Entire	White	0.043	0.480
	PV9-3	Circular	Convex	Glistening	Entire	White	0.127	0.541
	PV9-4	Irregular	Convex	Glistening	Undulate	White	0.055	0.149
	PV9-5	Irregular	Flat	Rough	Undulate	White	0.045	0.328
	PV9-6	Circular	Convex	Glistening	Entire	Yellow	0.115	0.317
	PV10-3	Rhizoid	Convex	Rough	Filamentous	White	0.043	0.085
	PV10-4	Circular	Pulvinate	Rough	Entire	White	0.043	0.288

The sugar xylose contains five-carbon atoms and is converted into xylitol by microbial fermentation using bacteria, fungi and yeast. The best known xylitol producers are yeasts, with special emphasis on the genus *Candida* (Ikeuchi *et al*., 1999; Kang *et al*., 2005; Guo *et al*., 2006; Sampaio *et al*., 2008) and *Debaryomyces* (Altamirano *et al*., 2000; Sampaio *et al*., 2008; Prakash *et al*., 2011). Microbial production of xylitol has the advantage of being a more attractive process, since its downstream processing is simple, and its fermentation process provides high cell density, thus resulting in high xylitol yields. Moreover, it is more economic and can be achieved in the industry without high pressure, temperature or xylose purification (Silva *et al*., 1999; Kang *et al*., 2005). Ideally microorganism xylitol producers are easy to cultivate, highly capable of productivity and have special

resistance to pressure and toxins. According to recent research, the use of a variety of wild type yeast strains mostly genera *Candida* has shown potential production on an industrial scale such as *C. boidinii* (Vandeska *et al*., 1995), *C. guilliermondii* (Zagustina *et al*., 2001; Rodrigues *et al*., 2003), *C. parapsilosis* (Oh *et al*., 1998), *C. peltata* (Saha *et al*., 1999) and *C. tropicalis* (Kim *et al*., 2002; López *et al*., 2004). Sirisansaneeyakul *et al.* (1995) selected a xylitol producer as *C. mogii* ATCC 18364 from 11 strains of D-xylose, utilizing yeasts they had screened in previous research. Their results indicated maximum xylitol yield of Yp/s = 0.62 g/g and a specific rate for product formation that was higher than the other yeasts. Ikeuchi *et al.* (Ikeuchi *et al*., 1999) demonstrated microorganisms with the ability to produced xylitol from high concentrations of xylose screened from 1,018 types of soil from farms and parks in Osaka, Japan by enrichment medium culture. A chemically defined (CD) medium using xylose as a sole carbon source for primary selection was obtained from 102 isolates capable of metabolizing xylose and showed rapid growth on media containing xylose concentrations of 200 g/l. For secondary selection, a semi defined (SD) medium with xylose concentration at 250 g/l was used. The yeast 559-9 strain gave the maximum cell concentration at 204 g/l and this was identified as *Candida* sp. reference from 'The Yeasts' (3rd edition) (Baz *et al*., 2011). The biochemical and morphological characteristics of the strain were similar to *Candida* sp. Altamirano *et al.* (2000) isolated 33 yeast strain from natural resources, including corn silage (ASM strains) and viticulture residues (SJV strains) with growth on a medium with xylose as a

carbon source. These strains were identified as *C. membranifaciens, C. tropicalis, C. guilliermondii, C. shehatae, Pichia capsulate, C. utilis, Candida* sp., and *P. angusta* by C.P. Kurtzman, National Center for Agricultural Utilization Research (U.S.A.) and accessioned into ARS Culture Collection (NRRL) as NRRL Y-27290. The results of selected strains capable of producing xylitol showed that *C. tropicalis* has the highest potential to yield 0.69 g/g, using TLC techniques and HPLC. Kang *et al*. (2005) isolated a novel yeast strain from many kinds of soil from rice farming. The isolated strain was identified as *C. tropicalis* HY200 based on systematic characterization using general approaches of Biolog MicrologTM and 18S rRNA sequence analyses. This strain obtained high xylitol yield at 77% and a productivity amount of 2.57 g/l/h from 200 g/l of xylose concentration. Guo *et al*. (2006) explained the principles of xylitol producer screening for 45 out of 274 isolates grown on solids and broth with xylose as a carbon source. They comprised nine genera. *Saccharomyces, Schizoblastosporion, Candida, Geotrichum, Pichia, Hansenula, Trichosporon, Sporobolomyces,* and *Rhodotorula*. Five species were selected for further experiments, for the high utilization of xylose and D1/D2 of 26S rDNA identified as *C. guiliermondii* Xu280 and *C. maltosa* Xu316 produced the highest xylose consumption and xylitol yield in batch fermentation under microaerobic conditions.

4.2.2 Isolation of xylose fermented-yeasts on liquid media

Seventy-two isolates from sugarcane bagasse were tested for their abilities to assimilate xylose. All strains showed significant growth on liquid medium with xylose as the sole carbon source. Analysis of optical determination (OD_{600nm}) showed that the strains, which grew well on xylose medium consumed xylose rapidly up to 48 h. From these 72 strains, six strains including KS 1-4, KS 7-7, KS 10-3, PV 1-6, PV 3-1 and PV 5-1 (Table 4.4) were chosen for further study because of their high-growth rates on xylose medium. Previously, different methods have been used for selecting the best xylitol producer yeast from several strains because diverse species of yeasts break down xylose at different rates.

Xylitol production using all yeast species was difficult and it was necessary to find a method for selecting the best yeast culture to demonstrate the potential of using xylose as a carbon source. Altamirano *et al*. (2000) studied the isolation and identification of xylitol-producing yeasts from agricultural residues. Thirty-six yeast

strains were screened for their capacities to convert D-xylose into xylitol using a conventional method by TLC adapted for easy determination of xylose and xylitol production. This technique is suitable for the first steps of a screening program to select xylitol-producing yeasts from natural environments. *C. tropicalis* ASM III (NRRLY-27290), isolated from corn silage gave high xylitol yield of 0.88 g xylitol/g of xylose consumed. Guo *et al*. (2006) accurately cultivated 274 strains on both solid and liquid screening media with xylose as the sole carbon source. Five strains were selected based on the significant high growth of assimilated xylose, which showed that the strains with rapid growth rate also consumed xylose rapidly. This method demonstrated that in the early testing it is easy to select varieties that produce high xylitol yield, and further study the conditions that affect xylitol production. Xylitol production using all yeast strains from isolation at flask scale may be difficult, since it is time consuming, inconvenient to harvest and increases the cost of the analysis. Therefore, this study used the primary screening method by Guo *et al*. (2006), which preliminary selected the yeast which used xylose quickly within 48 hours, observed from changes in the growth of yeast in the broth and significant turbidity increase. These strains were selected as inoculum for yeast fermentation of xylose in sugarcane bagasse hydrolysate to compare their ability to produce xylitol production in the secondary screening of xyltitol-yeast producers from the xylitol yields.

4.3 Secondary screening using xylose fermentation of selected strains

Owing to their abilities to assimilate xylose rapidly, six strains were selected for further evaluation. Physiological behavior of these strains on hydrolysate fermentation medium (xylose concentration 32.30 g/l) was investigated. Xylitol production accompanied xylose consumption during yeasts growth. KS 10-3 exhibited the highest xylitol yield as shown in Table 4.2. Results in Table 4.5 show the six yeast strains (KS 1-4, KS 7-7, KS 10-3, PV 1-6, PV 3-1, and PV 5-1) primarily selected using xylitol fermentation of xylose in sugarcane bagasse hydrolysate. In batch fermentation was carried out with flask 250 ml (initial xylose 32.30 g/l) and time variation. Results showed that KS 10-3 strain gave maximum xylitol yield at 0.47 g/g with 96 hours at 30^{o}C.

Table 4.5 Xylose fermentation of selected strains cultivated on a hydrolysate medium under micro-aerobic conditions. Initial xylose concentration was 32.30 g/l.

Strains	Culture time (h)	Sugar consumption (g/l)			$Y_{xl/s}$;xylitol yield (g xylitol/ g xylose consumed)	$Y_{e/s}$;ethanol yield (g ethanol/ g xylose consumed)
		xylose	glucose	arabinose		
KS1-4	24	30.16±1.33	2.36±0.19	9.2±0.17	ND	ND
	48	28.96±1.33	2.18±0.19	9.30±0.17	ND	ND
	72	29.44±1.33	2.30±0.19	9.0±0.17	ND	ND
	96	27.04±1.33	1.94±0.19	8.94±0.17	ND	ND
KS7-7	24	30.70±0.49	2.88±0.15	7.10±0.62	ND	0.02±0.005
	48	31.18±0.49	3.11±0.15	8.40±0.62	ND	0.01±0.005
	72	31.18±0.49	3.06±0.15	7.97±0.62	ND	0.01±0.005
	96	31.90±0.49	3.23±0.15	8.41±0.62	ND	0.01±0.005
KS10-3	24	30.40±13.66	3.06±1.17	2.22±2.02	0.11±0.18	0.01±0.009
	48	2.66±13.66	1.14±1.17	6.90±2.02	0.14±0.18	0.03±0.009
	72	5.80±13.66	1.22±1.17	5.70±2.02	0.42±0.18	0.03±0.009
	96	1.50±13.66	0.28±1.17	4.14±2.02	0.47±0.18	0.02±0.009
PV1-6	24	28.54±0.85	2.08±0.17	9.26±0.25	ND	ND
	48	29.03±0.85	2.16±0.17	9.08±0.25	ND	ND
	72	30.51±0.85	2.46±0.17	9.66±0.25	ND	ND
	96	29.60±0.85	2.33±0.17	9.46±0.25	ND	ND
PV3-1	24	27.96±0.95	2.18±0.19	9.40±0.22	ND	ND
	48	27.06±0.95	2.06±0.19	9.22±0.22	ND	ND
	72	28.06±0.95	2.28±0.19	9.46±0.22	ND	ND
	96	29.36±0.95	2.50±0.19	9.76±0.22	ND	ND
PV5-1	24	29.50±0.52	2.33±0.13	9.57±0.21	ND	ND
	48	29.00±0.52	2.26±0.13	9.21±0.21	ND	ND
	72	29.20±0.52	2.36±0.13	9.52±0.21	ND	ND
	96	30.19±0.52	2.56±0.13	9.72±0.21	ND	ND

ND = Not Detect

Results showed that sugarcane bagasse as waste from the sugar factory is a good local alternative as a low cost substrate, with added value since residual xylose and other sugar especially xylose, are a substrates in xylitol production. These findings agreed with Silva *et al*. (2011) who studied the glucose:xylose ratio effect to *C. guilliermondii* during fermentation of sugarcane bagasse and showed that a ratio of 1:5 had the highest xylitol yield at 0.59 g/g demonstrating that glucose, arabinose and acetic acid all assist in the fermentation. Xylitol production from sugarcane bagasse hydrolysate mostly using *C. guilliermondii*, found to be *C. guilliermondii* FTI 20037, gave high xylitol yields at 0.69, 0.75 and 0.81 g/g from initial xylose concentrations of

30, 48, and 80 g/l, respectively. *C. guilliermondii* gave a xylitol yield of 0.59 g/g from 45 g/l of xylose. *C. langeronii* RLJ Y-019 presented xylitol yield of 0.40 g/g from 47.2 g/l xylose concentration. Several recent reports suggested that *C. guilliermondii* had high potential of xylitol yield from sugarcane bagasse hydrolysate. Rao *et al.*, (2006) showed that *C. tropicalis* as a xylitol yeast produced high xylitol yield similar to *C. guilliermondii*. These findings suggested that the maximum xylitol yield (0.45 g/g) was achieved with sugarcane bagasse hydrolysate but xylitol yields were lower with mixed sugar because of limited yeast growth rate and inhibitor effect on cell adaptation. Results were similar to these observed in xylitol production by Baz *et al*. (2011) using *C. tropicalis* under different condition such as rice bran, ammonium sulfate and xylose resulting in xylose giving a maximum xylitol yield at 57.2% obtained from xylose 20 g/l, rice bran 15 g/l and ammonium sulfate 1 g/l pH 5.5. However, xylitol increased to 72.5% when initial xylose was 50 g/l. Xylitol production on a large scale requires oxygen aeration by semi-synthetic media and hydrolysate. Using *C. tropicalis* AY2007 under a limit of O_2 0.3 vvm gave xylitol 36 g/l in 59 hours of fermentation and increase aeration of 1.5 vvm obtained 30.99 g/l. In comparison, xylitol yield from semisynthetic media and hydrolysate were 0.704 and 0.783 g/g, respectively.

In this study, KS 10-3 gave the maximum xylitol yield at 0.47 g/g obtained at 32.30 g/l of xylose (Table 4.5) lower than some report in Table 4.6 towards initial xylose is low and necessary optimized condition suitable for this xylitol-yeast producer strain of xylitol production particularly xylose concentration and agitation rate influence cell yeast growth and increase xylitol production efficiency.

Table 4.6 Sugarcane bagasse hydrolysate for xylitol production by genus *Candida*.

Yeast strain	Condition	Initial xylose (g/L)	$Y_{p/s}$ (g/g)	Q_p (g/L/h)	Time (hour)	Reference
C. guilliermondii FTI20037	Temperature at 30°C, agitation rate 300 rpm	48	0.75	0.57	22	Felipe *et al*., 1997
C. langeronii RLJ Y-019	Temperature at 42°C, agitation rate 700 rpm	47.2	0.40	0.97	-	Nigam, 2000
C. guilliermondii FTI20037	Temperature at 30°C, agitation rate 300 rpm	30	0.69	0.68	-	Martinez *et al*., 2003
C. guilliermondii	Temperature at 30°C, agitation rate 500 rpm	45	0.59	0.53	48	Silva *et al*., 2007
C. guilliermondii FTI20037	Temperature at 30°C, agitation rate 200 rpm	80	0.81	0.60	48	Arruda *et al*., 2011
C. tropicalis	Temperature at 30°C, agitation rate 200 rpm	56	0.45	-	48	RaO *et al*., 2006
C. tropicalis	Temperature at 30°C, agitation rate 200 rpm	50	36.25	-	96	Baz *et al*., 2011
C. tropicalis AY2007	Temperature at 30°C, agitation rate 200 rpm, aeration 0.3 vvm	29.8 45.5	0.783 0.704	0.239 0.506	94 65	

Q_p = Specific product rate, $Y_{p/s}$ = Xylose-xylitol bioconversion yield

4.4 Optimization of xylitol production

4.4.1 Central composite design

The levels of process variables xylose concentration, agitation speed, and pH and the effect of their interactions on xylitol production were determined by central composite design of response surface methodology (RSM). The design matrix of experimental results by test was planned according to the 17 full factorial designs.

Table 4.7 Ranges of variables used in RSM

No.	Variables	Code	Levels				
			-1.68	**-1**	**0**	**1**	**1.68**
1	Xylose concentration	A	43.18	50	60	70	76.82
2	Agitation speed	B	132.96	150	175	200	217.05
3	pH	C	3.32	4.0	5.0	6.0	6.68

Table 4.8 Central Composite Design (CCD) in coded levels with xylitol production as response

Runs	A	B	C	Xylitol (g/g)
1	0.000	0.000	0.000	0.29
2	1.000	-1.000	-1.000	0.24
3	-1.000	1.000	-1.000	0.50
4	0.000	1.682	0.000	0.40
5	1.000	1.000	-1.000	0.26
6	-1.000	-1.000	1.000	0.54
7	0.000	0.000	-1.682	0.26
8	0.000	0.000	0.000	0.27
9	-1.682	0.000	0.000	0.48
10	-1.000	1.000	1.000	0.46
11	0.000	0.000	0.000	0.29
12	-1.000	-1.000	-1.000	0.38
13	1.000	-1.000	1.000	0.37
14	1.000	1.000	1.000	0.38
15	1.682	0.000	0.000	0.22
16	0.000	-1.682	0.000	0.36
17	0.000	0.000	1.682	0.38

Seventeen experiments were performed at different combinations of the factors shown in Table 4.4 and the central point was repeated three times (1, 8, 11). The observed response along with design matrix are presented in Table 4.8. Results were analyzed by ANOVA (Table 4.9). A second order regression equation provided the levels of xylitol production as a function of xylose concentration, agitation speed and pH, which can be presented in terms of coded factors in the following equation:

$$Y = +3.74224 - 0.053261A - 0.017813B - 0.084115C - 5.00000E\text{-}006AB + 1.62500E\text{-}003AC - 1.05000E\text{-}003BC + 3.18301E\text{-}004A^2 + 6.78988E\text{-}005B^2 + 0.021224C^2$$

where Y is the xylitol yield (g/g) and A, B and C are xylose concentration, agitation speed, and pH respectively. ANOVA for the response surface is shown in (Table 4.10). The *F*-value of 11.46 implied that the model was significant with only a 0.1% chance that a "Model *F*-value" this large could occur due to noise. Value of "prob > *F*" less than 0.05 indicated model terms as significant. Values greater than 0.1 indicated model terms as not significant. The linear terms of A and C and the combination of BC were significant for xylitol production. The co-efficient of determination (R^2) for xylitol production was calculated as 0.9365, which is very close to 1 and can explain up to 93.65% variability of the response.

The model can be used to predict xylitol production within the limits of experiment factors, and actual response values agreed well with predicted response values.

Table 4.9 Analyses of variance (ANOVA) for the response surface quadratic model for production of xylitol.

Source	Sum of squares	df	Mean square value	F-value	p-value Probe > F
Model	0.14	9	0.016	11.46	0.0020
A-xylose concentration	0.083	1	0.083	60.81	0.0001
B- Agitation speed	1.380E-003	1	1.380E-003	1.01	0.3493
C- pH	0.024	1	0.024	17.45	0.0041
AB	1.250E-005	1	1.250E-005	9.113E-003	0.9266
AC	2.112E-003	1	2.112E-003	4.02	0.2546
BC	5.513E-003	1	5.513E-003	1.955E-003	0.0850
$\mathbf{A^2}$	0.011	1	0.011	8.33	0.0235
$\mathbf{B^2}$	0.020	1	0.020	14.80	0.0063
$\mathbf{C^2}$	5.078E-003	1	5.078E-003	3.70	0.0958
Residual	9.602E-003	7	1.372E-003		
Lack of fit	9.335E-003	5	1.867E-003	14.00	0.0680
Pure Error	2.667E-004	2	1.333E-004		
Cor Total	0.15	16			
R-squared	0.9365				

The interaction effects of the variables on xylitol production were studied by plotting 3D surface curves against any two independent variables, while keeping the other variable at its central (0) level. The 3D curves of the calculated response (xylitol yield) and contour plots from the interactions between the variables. Figures 4.1-4.2 shows the dependency of xylitol production on agitation speed and pH. Xylitol production increased with an increase in agitation speed and thereafter with a further increase in pH.

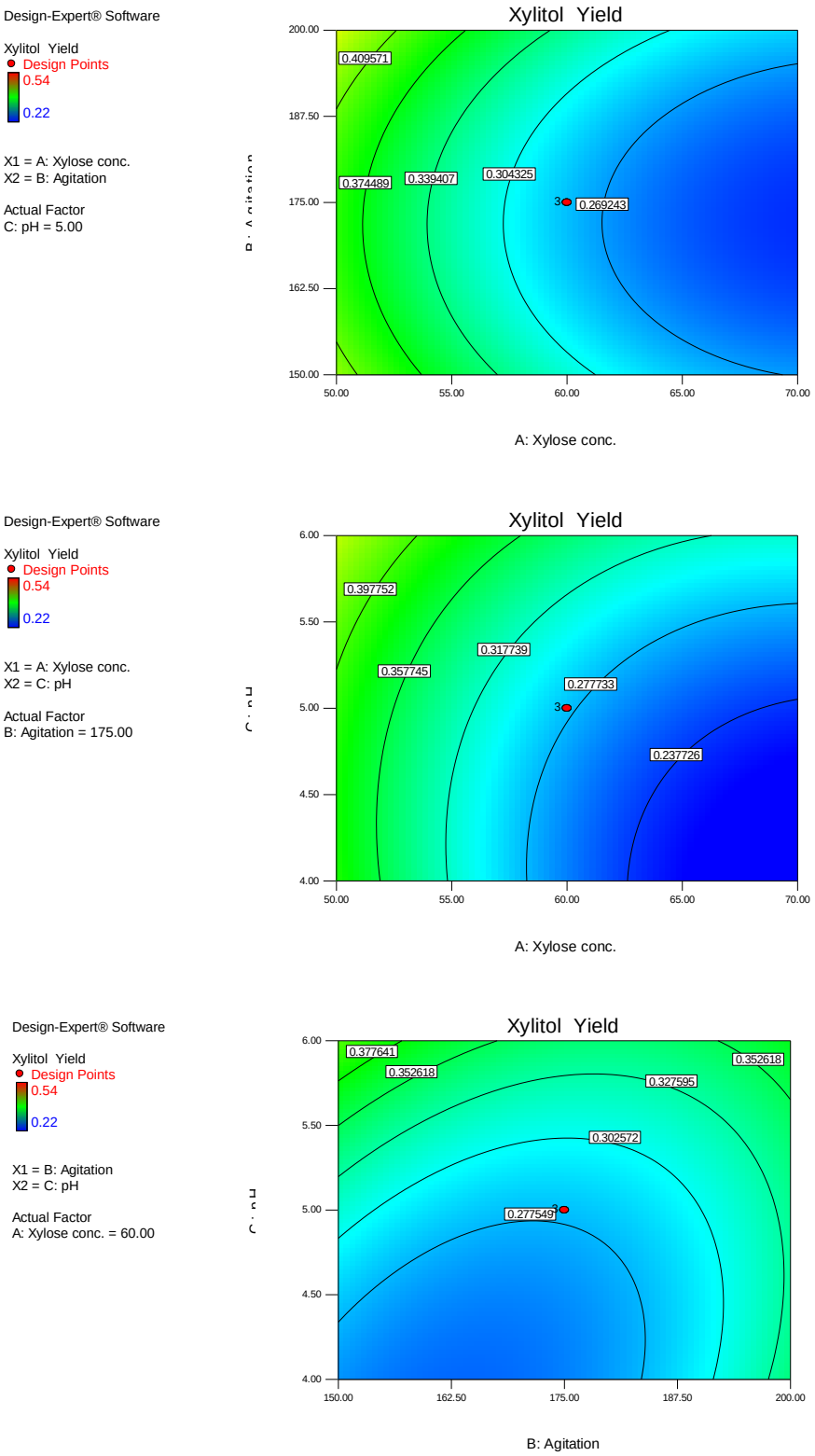


Figure 4.1 contour plot showing the effects of different fermentative conditions (X_1: xylose concentration; X_2: agitation speed; X_3: pH) on the response variable Y.

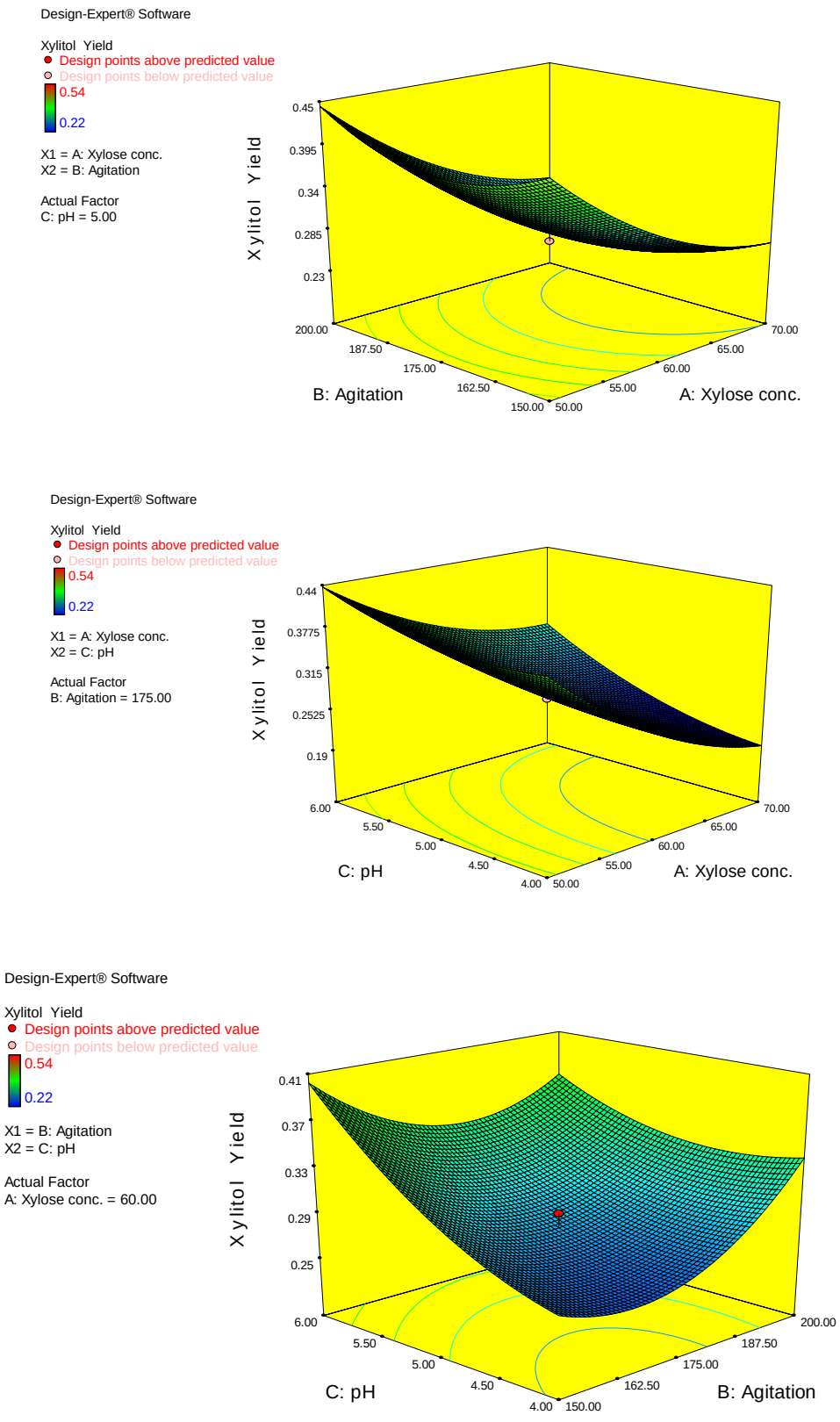


Figure 4.2 3D plot showing the effects of different fermentative conditions (X_1: xylose concentration; X_2: agitation speed; X_3: pH) on the response variable Y.

Table 4.10 Recently published studies on xylitol production using lignocellulosic hydrolysates by *Candida tropicalis*

Strains	Feedstock	Hydrolysate conditioning	Xylitol yield (g/g)	Sugar	Reference
C. tropicalis ATCC96745	Corn fiber	$Ca(OH)_2$ neutralized Activated charcoal overliming	0.4	48% xylose 22% glucose	Buhner and Agblevor (2004)
C. tropicalis IF00618	Bech wood	Enzymatic hydrolysis	0.5	-	Tran *et al*. (2004)
C. tropicalis	Corn fiber Sugarcane bagasse	$Ca(OH)_2$ neutralized Activated charcoal Anion exchange resin	0.43 0.45	30 g/ L xylose	RaO *et al*. (2006)
C. tropicalis BCRC20520	Wood chip	$Ca(OH)_2$ neutralized Activated charcoal Anion exchange resin	0.54	60 g/L xylose 30 g/L glucose	Ko *et al*. (2008)
C. tropicalis W103	Corncob	Overliming Ethyl acetate extraction	0.71	66 g/L xylose 10 g/L glucose	Cheng *et al*. (2009)

Table 4.10 Continued

Strains	Feedstock	Hydrolysate conditioning	Xylitol yield (g/g)	Sugar	Reference
C. tropicalis ATCC750	Prairie grass	Enzymatic hydrolysis	0.46		West (2009)
C. tropicalis ATCC20215			0.38		
C. tropicalis JH030	Rice straw	NaOH-neutralized	0.71	46 g/ L xylose 9 g/ L glucose	Huang *et al*. (2011)
C. tropicalis JH030	Sugarcane bagasse	NaOH-neutralized	0.51	26 g/ L xylose 3.5 g/ L glucose	
C. tropicalis KS10-3	Sugarcane bagasse	$Ca(OH)_2$ neutralized Activated charcoal	0.54	32 g/ L xylose	This study

To date, several strains of *C .tropicalis* have been used to ferment xylose-rich hydrolysate from various lignocellulosic materials for xylitol production. A summary of the results is listed in Table 4.7. The hydrolysates studied were often detoxified either by overliming or a combination of other methods such as active charcoal or ion exchange to improve fermentability. Maximum xylitol yield from other studies varies widely from 0.11-0.71 g/g depending on the efficacy of the detoxification process and the hydrolysate composition. Although many authors reported on xylitol production from lignocellulosic hydrolysate, only a few focused on the fermenting hydrolysate without detoxification. *C. tropicalis* KS10-3 used here, clearly demonstrated potential for xylitol production with a good xylitol yield using lignocellulosic hydrolysate.

Many authors concentrated on the engineering of recombinant *Saccharomyces cerevisiae* for use in xylitol production and these strains showed almost full conversion of xylose-to-xylitol; however the recombinant *S. cerevisiae* requires a substrate such as glucose for cell growth and intracellular reduction-oxidation maintenance. *C. tropicalis* is a neutral xylose-fermenting yeast with a NADH-NADPH dual cofactor dependent xylose reductase. This could partly release the stress from the rate-limiting step of NADPH regeneration, needed for xylitol accumulation in other yeasts.

4.5 Batch bioreactor xylitol production

Batch fermentation was carried out from sugarcane bagasse hydrolysate under aeration 1 vvm, temperature at 30^{o}C, agitation speed 150 rpm, pH 6.0, inoculum size 10% (v/v) and xylose concentration 60 g/L. Fermentation of xylose-rich resulted in a maximum xylitol concentration of 30.62 g/L in 96 h (Table 4.11).

Table 4.11 Sugar consumption and xylitol during batch 1 fermentation

Fermentation time (h)	Xylose	Glucose	Arabinose	Xylitol (g/L)	Ethanol (g/L)	XR activity (U/ 100ml)
0	49.97±3.51	6.74±1.66	11.47±0.06	0±8.81	0±0.11	0±0.53
12	44.97±3.51	6.41±1.66	11.47±0.06	22.97±8.81	0±0.11	0.08±0.53
24	44.55±3.51	6.35±1.66	11.33±0.06	25.21±8.81	0±0.11	0.77±0.53
36	44.13±3.51	6.18±1.66	11.33±0.06	25.23±8.81	0±0.11	1.24±0.53
48	43.96±3.51	6.06±1.66	11.33±0.06	25.45±8.81	0±0.11	0.08±0.53
60	43.29±3.51	5.94±1.66	11.33±0.06	24.79±8.81	0±0.11	0.09±0.53
72	39.71±3.51	5.86±1.66	11.33±0.06	24.99±8.81	0±0.11	1.24±0.53
84	38.93±3.51	2.68±1.66	11.33±0.06	26.35±8.81	0.27±0.11	0.08±0.53
96	39.18±3.51	2.30±1.66	11.33±0.06	30.62±8.81	0.25±0.11	0.032±0.53

These results suggest that batch fermentation is an effective approach to overcome the effect on xylitol production using strain KS10-3 and can be potentially used for high xylitol production, it should be further improved to enhance its productivity for futurebbindustrial applications.

A comparison of fermentation studies on sugarcane bagasse using different saccharification techniques and microorganisms reported by various researchers is shown in Table 4.12.

Table 4.12 Xylose-to-xylitol bioconversion using sugarcane bagasse based on batch fermentation prepared from *Candida* species.

Strains	Operation mode	X_0 (g/L)	S_0 (g/L)	P_F (g/L)	$Y_{p/s}$ (g/g)	Reference
Candida guilliermondii FTI20037	Batch at 30 ^oC, 300 rpm, pH 5.5, 22.5 h	1	69	36.29	0.64	Rodrigues *et al*. (2003)
Candida guilliermondii	Batch at 30 ^oC, 500 rpm, pH 5.5, 72 h	1	45	-	0.56	Silva *et al*. (2005)
Kluyveromyces sp. IIPE453	Batch at 50 ^oC, pH 5.0, 1 vvm	2	30	-	0.61	Kumar *et al*. (2014)
Candida tropicalis KS10-3	Batch at 30 ^oC, 200 rpm, pH 6.0, 96 h	1	50	31.04	0.62	This study (2016)

X_0 = Initial cell concentration, S_0 = Initial xylose concentration, P_F = Final xylitol production, Yp/s = Xylose-xylitol bioconversion yield

4.6 Identification of the 10-3 strain

Taxonomic identification of this 10-3 strain was performed according to the methods described in ``The Yeasts". This strain was classified and designated as *C. tropicalis* according to the molecular characteristics. The conserved 5' large subunit of 26S ribosomal DNA and its variable flanking region can be used to study the taxonomic and phylogenic relationship among closely related yeast strains and species (Kurtzman and Robnett, 1997; Wuczkowski and Prillinger, 2004). BLAST searches indicated that the sequence D1/D2 of 26S rDNA analysis results identified this strain as *C. tropicalis* KS 10-3 with 100% sequence identity to the strain in the database (length 576 nucleotides). 26S ribosomal RNA gene, partial sequence showed

AAACCAACAGGGATTGCCTTAGTAGCGGCGAGTGAAGCGGCAAAAG
CTCAAATTTGAAATCTGGCTCTTTCAGAGTCCGAGTTGTAATTTGAAGAAGG
TATCTTTGGGTCTGGCTCTTGTCTATGTTTCTTGGAACAGAACGTCACAGAG
GGTGAGAATCCCGTGCGATGAGATGATCCAGGCCTATGTAAAGTTCCTTCG
AAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCT
AAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAA
AGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGA
AAGGGAAGGGCTTGAGATCAGACTTGGTATTTTGTATGTTACTTCTTCGGGG
GTGGCCTCTACAGTTTATCGGGCCAGCATCAGTTTGGGCGGTAGGAGAATT
GCGTTGGAATGTGGCACGGCTTCGGTTGTGTGTTATAGCCTTCGTCGATACT
GCCAGCCTAGACTGAGGACTGCGGTTTATACCTAGGATGTTGGCATAATGA
TCTTAAGTCGCCCGTCT.

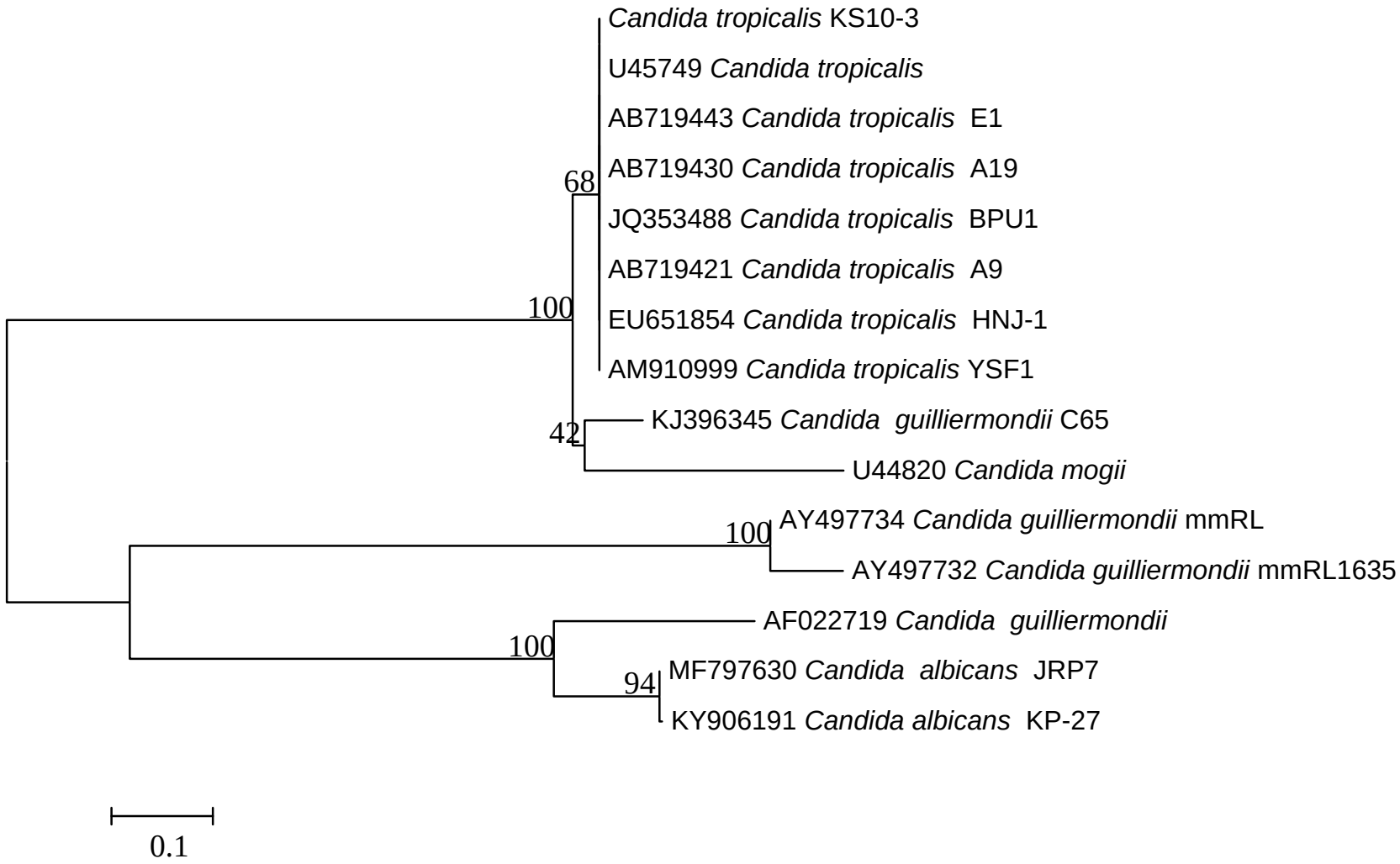


Figure 4.3 Phylogenetic tree showing the positions of KS10-3 and related species based on 26S rDNA sequences.

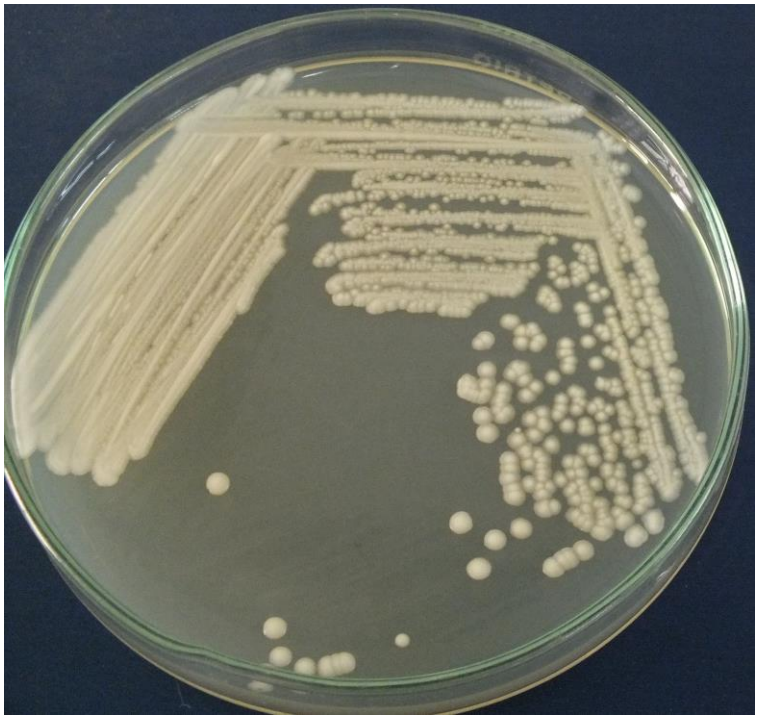


Figure 4.4 Morphological characteristic of KS10-3

CHAPTER 5

CONCLUSION

We reported a xylose-fermenting yeast *Candida tropicalis* KS10-3 that demonstrated efficient xylitol production at high xylose concentration, indicating a possible application for practical use. Strain KS10-3 is expected to play an important role in further research to identify of related enzymes, and complete genome sequences. *C. tropicalis* KS10-3 was characterized as a strong potential candidate for xylitol production from xylose. Compared to other xylose-fermenting strains, KS10-3 has the potential for development of a practical biotechnological application for xylitol production.

Central composite design was shown to be a powerful tool to optimize xylitol production by *Candida tropicalis* KS10-3 using sugarcane bagasse hemicellulose hydrolysate to test the relative importance of process variables. Optimization values of the process variables for xylitol production were identified as xylose concentration at-60 g/L, agitation speed-248.16 and pH-5.27. Sugarcane bagasse is a good source for production of xylitol. Validation experiments verified the availability and accuracy of the model. Under optimized conditions, xylitol production reacheds 0.54 g/g with close concordance between expected and obtained levels.

Concentration of xylose in the hydrolysates was higher than other sugars, showing greater loss of hemicellulose than cellulose during treatment with sulfuric acid. A arabinose, acetic acid and furfural are also generated by breakdown of the hemicellulose chains, this difference was even greater. Fermentation of xylose from hemicellulosic material is important to produce xylitol from yeast in a cost-effective manner. Sugarcane bagasse hemicellulosic hydrolysate has potential as a substrate for xylitol production by *C. tropicalis* KS10-3.

Increasing demand for xylitol in the food and pharmaceutical industries has generated development interest in low cost biotechnological xylitol production an efficient approach with economic advantages compared to chemical

processes. *C. tropicalis* KS10-3 is suitable for the development of a large-scale fermentation process for xylitol production from hemicellulosic hydrolysates.

Suggestion

Future work should focus on xylitol purification and recovery from sugarcane bagasse hydrolysate medium, thus allowing a better evaluation of the economic viability of the biotechnological production process.

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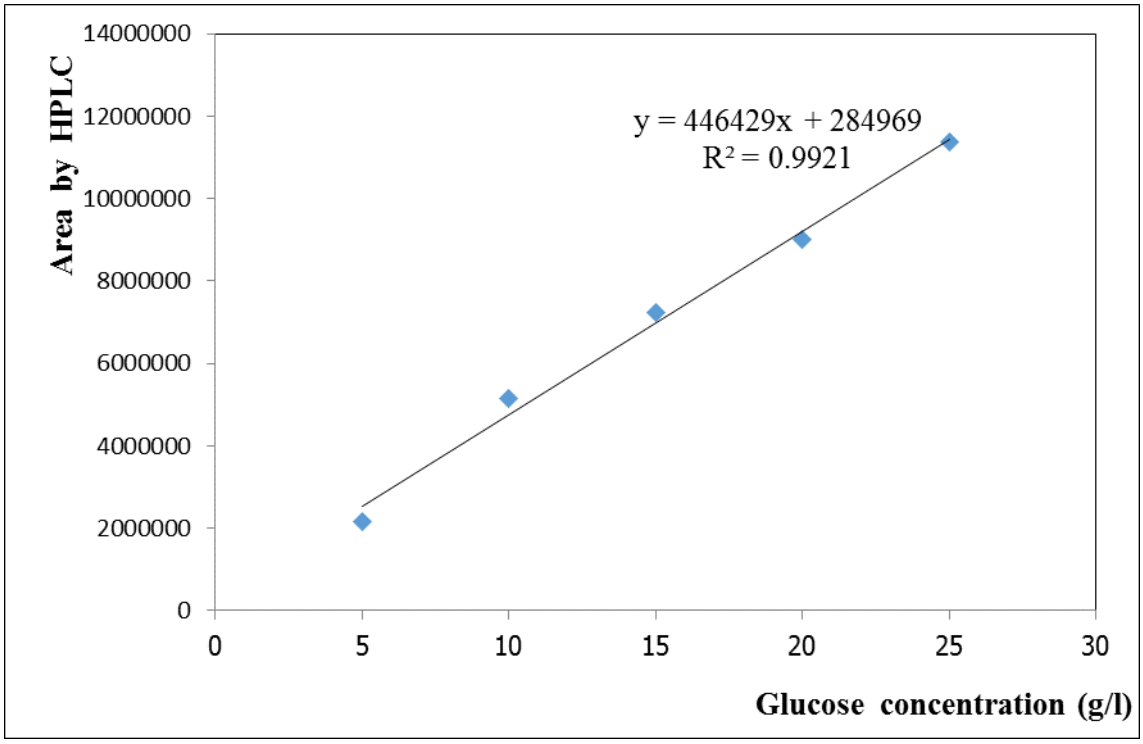
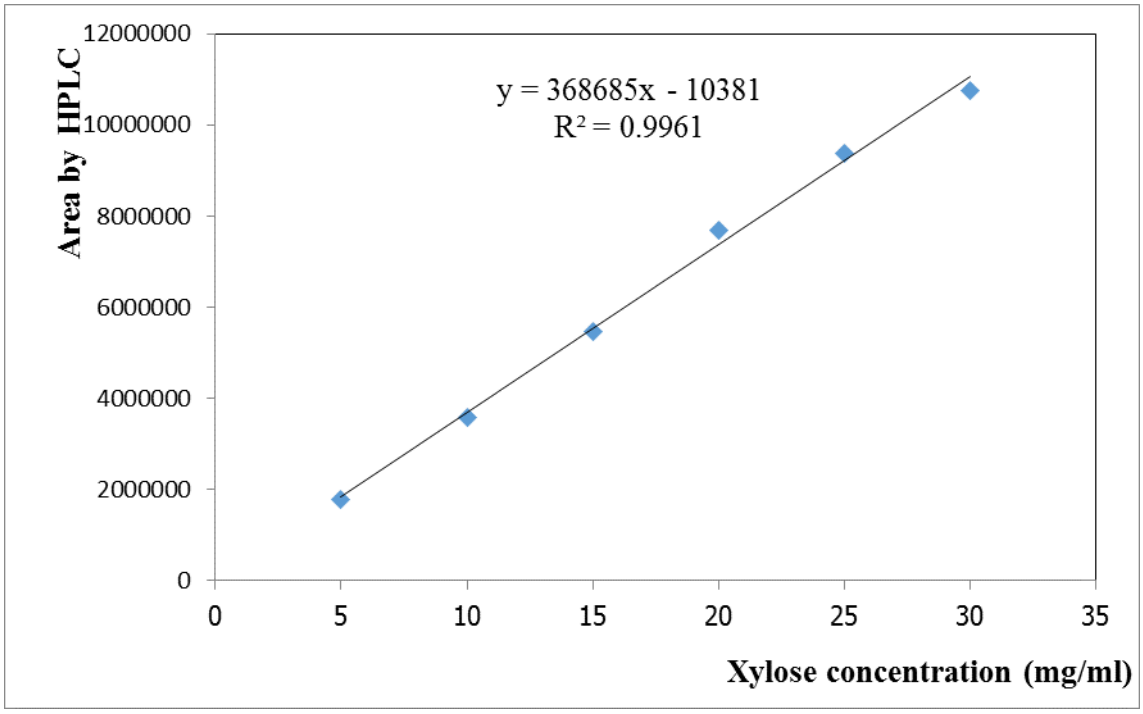
APPENDICES

APPENDIX A

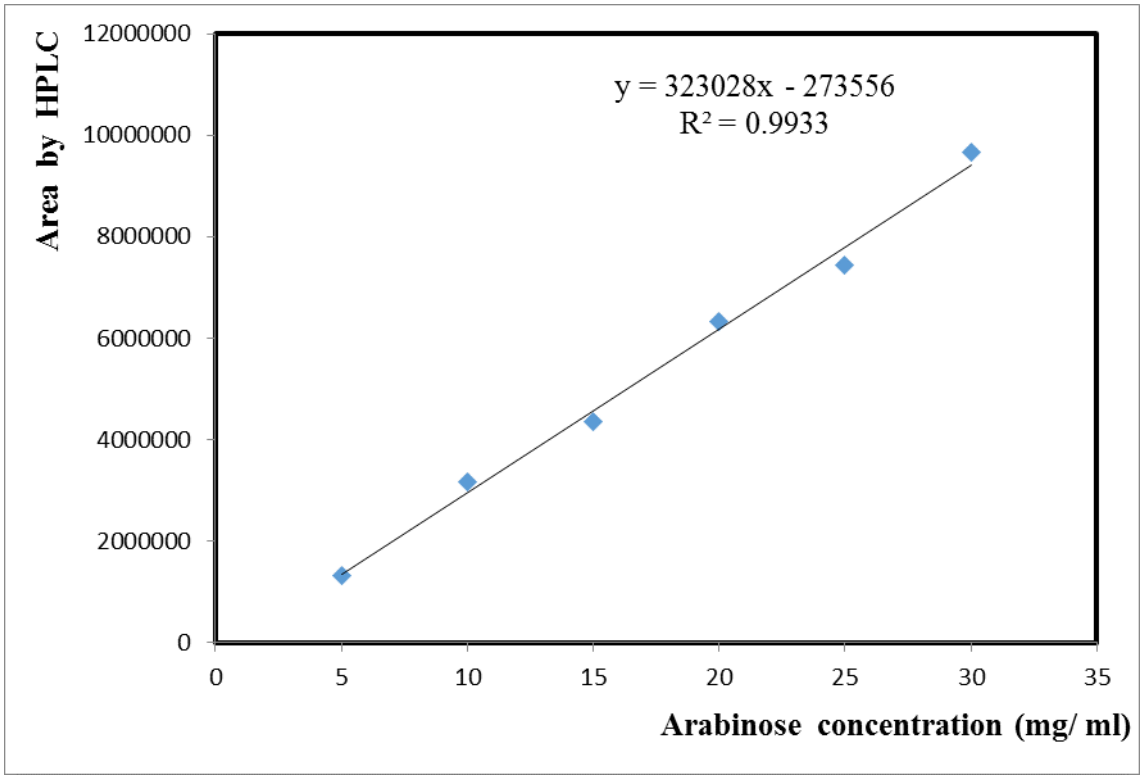
Standard Graphs



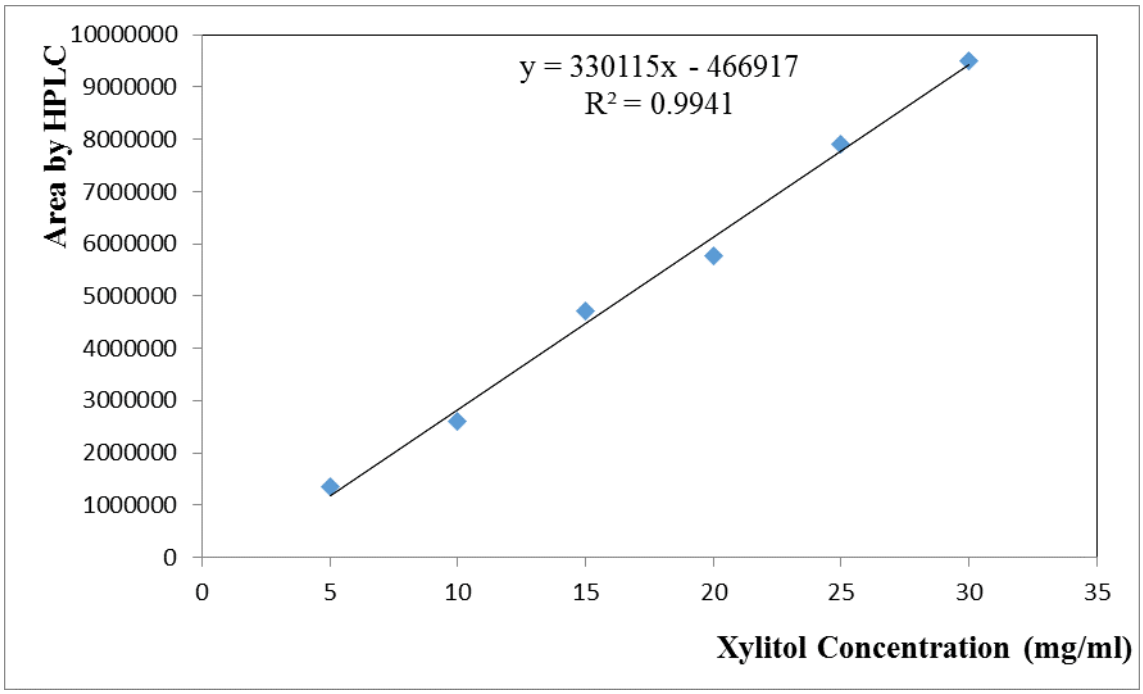
Appendix Figure A1. Standard curve of glucose.



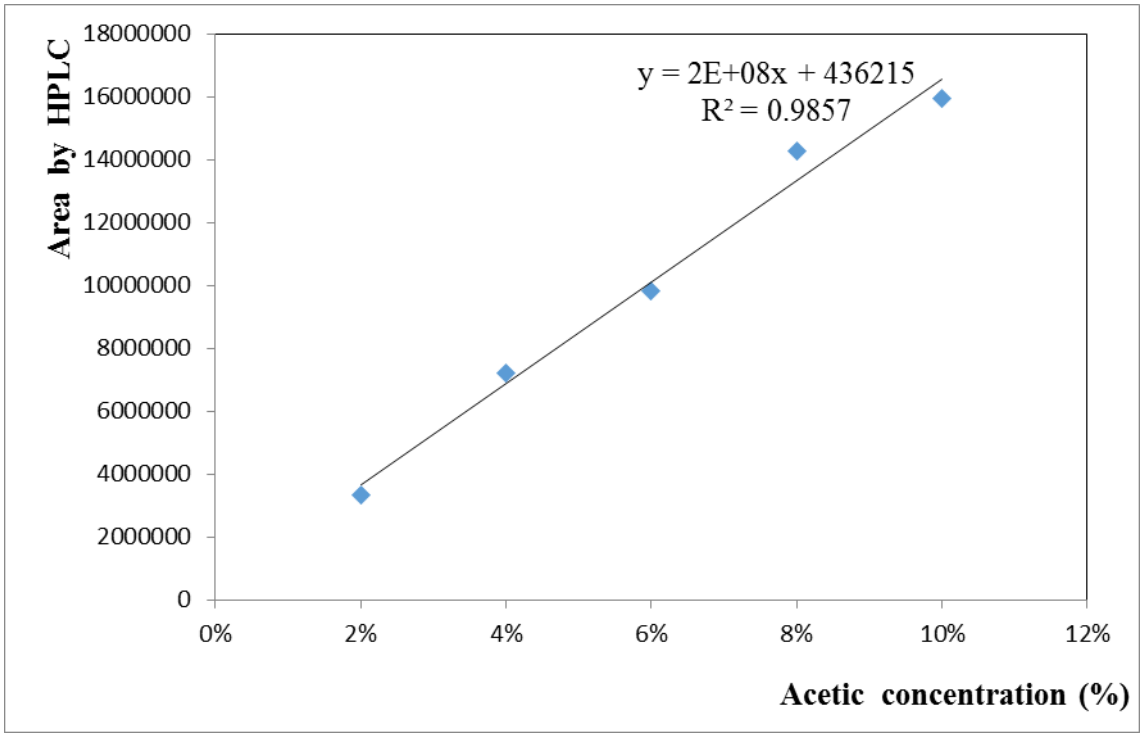
Appendix Figure A2. Standard curve of xylose.



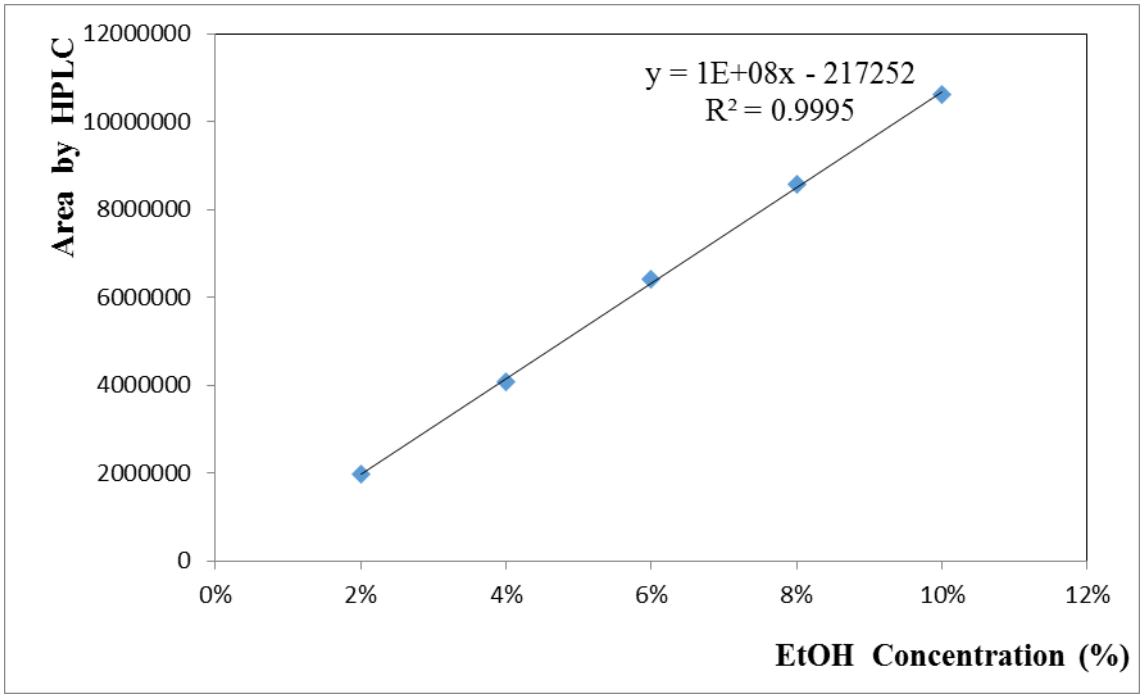
Appendix Figure A3. Standard curve of arabinose.



Appendix Figure A4. Standard curve of xylitol.



Appendix Figure A5. Standard curve of acetic acid.



Appendix Figure A6. Standard curve of ethanol.

APPENDIX B

Standard HPLC Data

==== Shimadzu LCsolution Analysis Report ====

D:\kusumawadee\23-04-56\Acetic2%.lcd

Acquired by	: Admin
Sample Name	: Acetic2%
Sample ID	:
Tray#	: 1
Vail #	: 1
Injection Volume	: 20 uL
Data File Name	: Acetic2%.lcd
Method File Name	: method HPX87-H for sample.lcm
Batch File Name	: BATCH.lcb
Report File Name	: Default.lcr
Data Acquired	: 4/23/2013 2:39:00 PM
Data Processed	: 4/23/2013 3:19:02 PM

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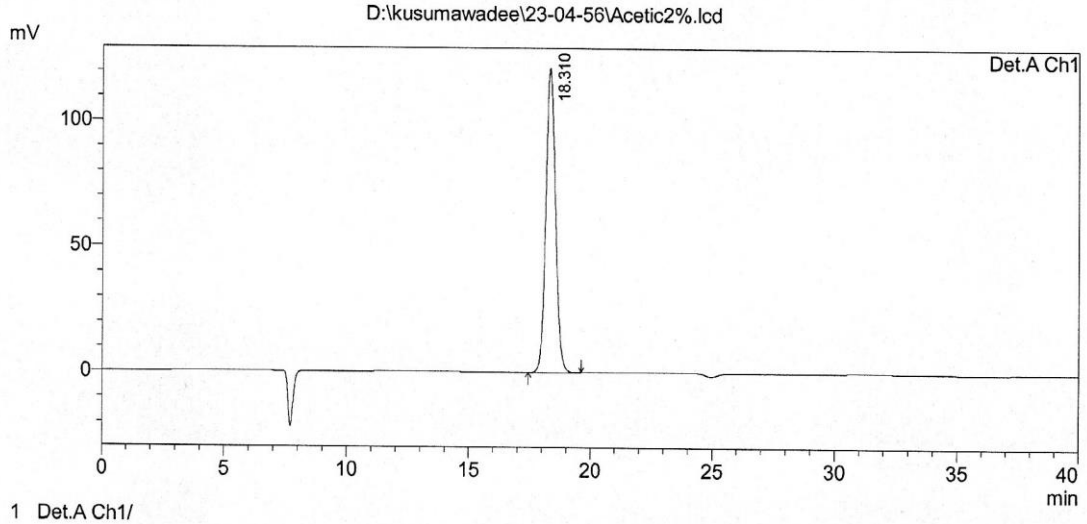

1 Det.A Ch1/

PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	18.310	3341704	121420	100.000	100.000
Total		3341704	121420	100.000	100.000

==== Shimadzu LCsolution Analysis Report ====

D:\kusumawadee\arabinose5.lcd

Acquired by	: Admin
Sample Name	: arabinose5
Sample ID	:
Tray#	: 1
Vail #	: 31
Injection Volume	: 20 uL
Data File Name	: arabinose5.lcd
Method File Name	: method.lcm
Batch File Name	: batch.lcb
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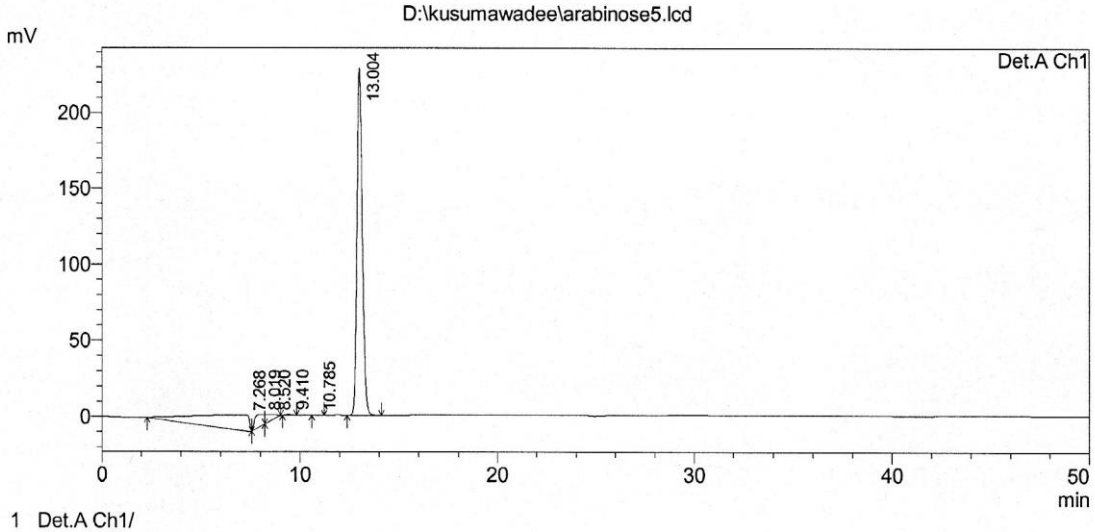

1 Det.A Ch1/

PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	7.268	1667894	10156	26.146	4.043
2	8.019	257904	7660	4.043	3.049
3	8.520	147062	3987	2.305	1.587
4	9.410	8514	534	0.133	0.213
5	10.785	1275	79	0.020	0.031
6	13.004	4296500	228807	67.352	91.077
Total		6379149	251224	100.000	100.000

==== Shimadzu LCsolution Analysis Report ====

D:\kusumawadee\23-04-56\EtOH2%.lcd

Acquired by	: Admin
Sample Name	: EtOH2%
Sample ID	:
Tray#	: 1
Vail #	: 6
Injection Volume	: 20 uL
Data File Name	: EtOH2%.lcd
Method File Name	: method HPX87-H for sample.lcm
Batch File Name	: BATCH.lcb
Report File Name	: Default.lcr
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Data Processed	: 4/23/2013 6:41:18 PM

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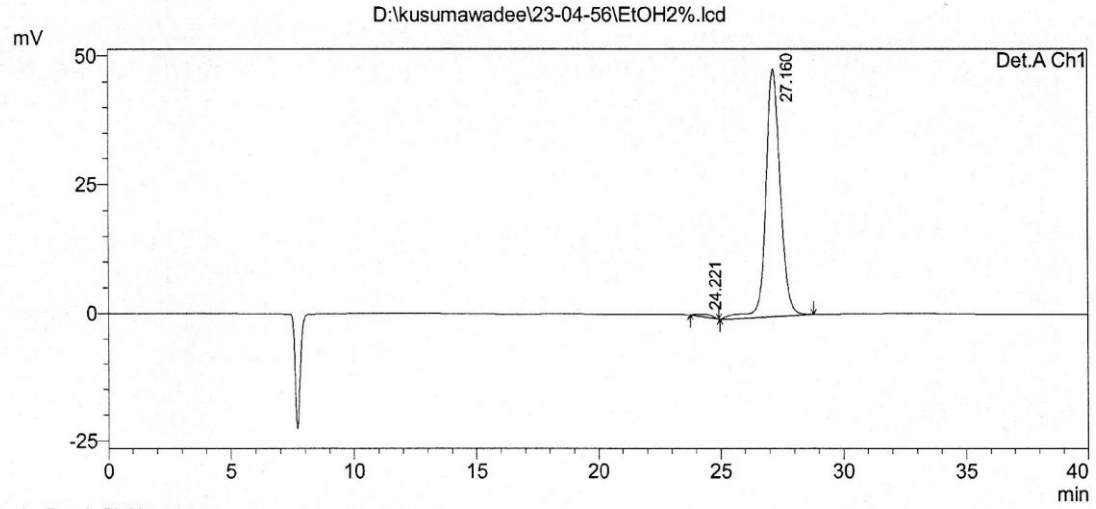

1 Det.A Ch1/

PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	24.221	20421	424	1.040	0.877
2	27.160	1943207	47942	98.960	99.123
Total		1963629	48366	100.000	100.000

==== Shimadzu LCsolution Analysis Report ====

D:\kusumawadee\23-04-56\EtOH2%.lcd

Acquired by	: Admin
Sample Name	: EtOH2%
Sample ID	:
Tray#	: 1
Vail #	: 6
Injection Volume	: 20 uL
Data File Name	: EtOH2%.lcd
Method File Name	: method HPX87-H for sample.lcm
Batch File Name	: BATCH.lcb
Report File Name	: Default.lcr
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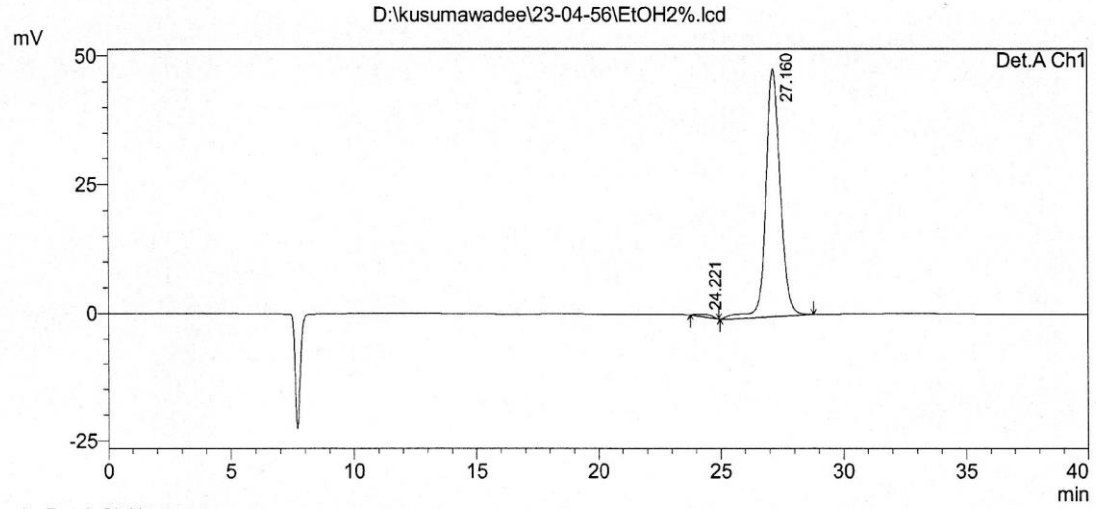

1 Det.A Ch1/

PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	24.221	20421	424	1.040	0.877
2	27.160	1943207	47942	98.960	99.123
Total		1963629	48366	100.000	100.000

==== Shimadzu LCsolution Analysis Report ====

D:\kusumawadee\glucose5.lcd

Acquired by	: Admin
Sample Name	: glucose5
Sample ID	:
Tray#	: 1
Vail #	: 37
Injection Volume	: 20 uL
Data File Name	: glucose5.lcd
Method File Name	: method.lcm
Batch File Name	: batch.lcb
Report File Name	: Default.lcr
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Data Processed	: 1/30/2013 2:34:18 AM

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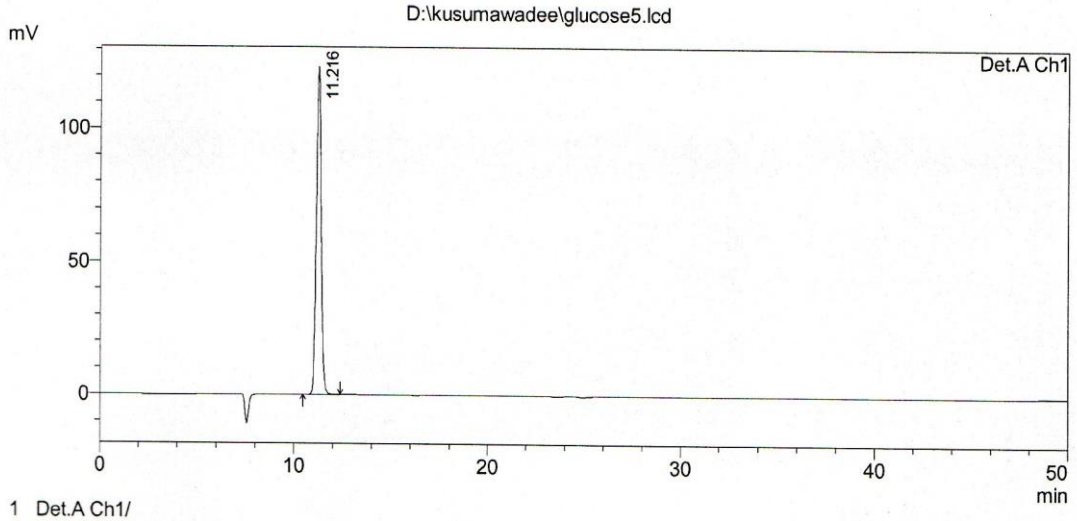

1 Det.A Ch1/

PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	11.216	2153711	123463	100.000	100.000
Total		2153711	123463	100.000	100.000

==== Shimadzu LCsolution Analysis Report ====

D:\kusumawadee\23-04-56\Xylitol 5.lcd

Acquired by	: Admin
Sample Name	: Xylitol 5
Sample ID	:
Tray#	: 1
Vail #	: 11
Injection Volume	: 20 uL
Data File Name	: Xylitol 5.lcd
Method File Name	: method HPX87-H for sample.lcm
Batch File Name	: BATCH.lcb
Report File Name	: Default.lcr
Data Acquired	: 4/23/2013 9:23:30 PM
Data Processed	: 4/23/2013 10:03:32 PM

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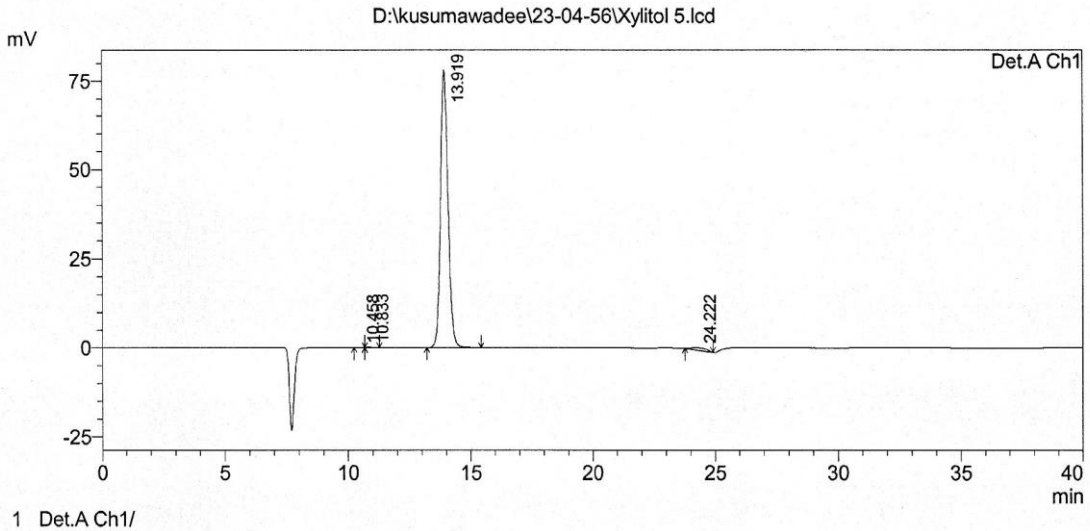


1 Det.A Ch1/

PeakTable

Detector A Ch1

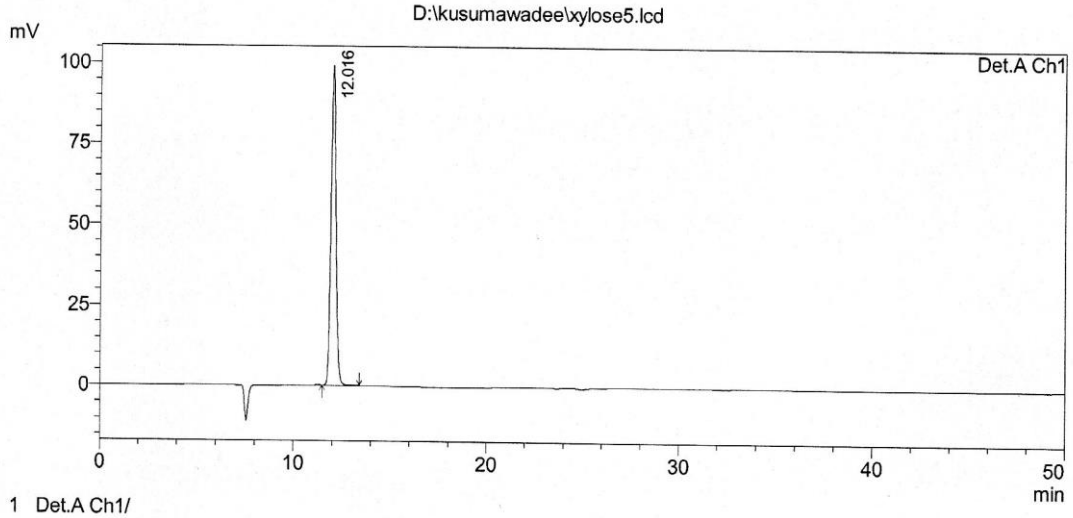
Peak#	Ret. Time	Area	Height	Area %	Height %
1	10.458	1157	61	0.069	0.077
2	10.833	1804	90	0.107	0.115
3	13.919	1653660	77993	98.331	99.137
4	24.222	25111	528	1.493	0.671
Total		1681732	78672	100.000	100.000

==== Shimadzu LCsolution Analysis Report ====

D:\kusumawadee\xylose5.lcd

Acquired by	: Admin
Sample Name	: xylose5
Sample ID	:
Tray#	: 1
Vail #	: 43
Injection Volume	: 20 uL
Data File Name	: xylose5.lcd
Method File Name	: method.lcm
Batch File Name	: batch.lcb
Report File Name	: Default.lcr
Data Acquired	: 1/30/2013 6:47:00 AM
Data Processed	: 2/13/2013 11:18:34 AM

<Chromatogram>



1 Det.A Ch1/

PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	12.016	1776440	99031	100.000	100.000
Total		1776440	99031	100.000	100.000

APPENDIX C

Statistic Data

Descriptives

[DataSet2] E:\Ph.D.Student\งานเขียนเล่มจบ\เล่มวิทยานิพนธ์\ล่าสุด\SPSS.sav

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
Xylose	5	14.95	44.58	31.1160	10.55110
Glucose	5	1.27	5.98	4.8860	2.02958
Arabinose	5	3.13	8.00	6.7720	2.04430
HMF	5	.02	.07	.0340	.02074
Furfural	5	.03	.09	.0500	.02345
Acetic	5	1.56	5.54	3.0440	1.66169
Phenolic	5	.15	7.78	2.7220	3.57284
Valid N (listwise)	5				

Descriptives

[DataSet1] E:\Ph.D.Student\งานเขียนเล่มจบ\เล่มวิทยานิพนธ์\ล่าสุด\SPSS.sav

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
Xylose	9	38.93	49.97	43.1878	3.51445
Glucose	9	2.30	6.74	5.3911	1.66857
Arabinose	9	11.33	11.47	11.3611	.06173
Xylitol	9	.00	30.62	22.8456	8.81119
Ethanol	9	.00	.27	.0578	.11476
XR	9	.00	1.24	.4011	.53013
Valid N (listwise)	9				

APPENDIX D

Batch fermentation Data

Table 1D Sugar consumption and xylitol during batch 1 fermentation

Fermentation time (h)	Xylose	Glucose	Arabinose	Xylitol (g/L)	Ethanol (g/L)	XR activity (U/ 100ml)
0	49.95±3.54	6.46±2.04	11.18±0.13	0±12.36	0±0.11	0±0.53
12	46.18±3.54	6.44±2.04	11.18±0.13	0±12.36	0±0.11	0.08±0.53
24	45.34±3.54	6.40±2.04	10.90±0.13	27.45±12.36	0±0.11	0.77±0.53
36	45.15±3.54	6.16±2.04	10.89±0.13	27.30±12.36	0±0.11	1.24±0.53
48	45.05±3.54	6.06±2.04	10.89±0.13	26.92±12.36	0±0.11	0.08±0.53
60	44.54±3.54	5.95±2.04	10.88±0.13	26.60±12.36	0±0.11	0.09±0.53
72	40.46±3.54	5.92±2.04	10.88±0.13	26.97±12.36	0±0.11	1.24±0.53
84	39.52±3.54	1.82±2.04	10.88±0.13	28.76±12.36	0.27±0.11	0.08±0.53
96	39.18±3.54	1.39±2.04	10.88±0.13	31.04±12.36	0.25±0.11	0.032±0.53

Table 2D Sugar consumption and xylitol during batch 2 fermentation

Fermentation time (h)	Xylose	Glucose	Arabinose	Xylitol (g/L)	Ethanol (g/L)
0	49.98±3.90	7.02±1.98	11.76±0.01	0±7.38	0
12	43.76±3.90	6.38±1.98	11.76±0.01	22.97±7.38	0
24	43.76±3.90	6.29±1.98	11.76±0.01	23.00±7.38	0
36	43.10±3.90	6.19±1.98	11.76±0.01	23.11±7.38	0
48	42.87±3.90	6.05±1.98	11.76±0.01	23.97±7.38	0
60	42.04±3.90	5.92±1.98	11.76±0.01	22.97±7.38	0
72	38.96±3.90	5.80±1.98	11.76±0.01	23.01±7.38	0
84	38.34±3.90	3.54±1.98	11.76±0.01	23.94±7.38	0
96	38.02±3.90	3.20±1.98	11.76±0.01	30.19±7.38	0
108	37.52±3.90	2.19±1.98	11.76±0.01	26.22±7.38	0
120	37.15±3.90	1.89±1.98	11.76±0.01	25.56±7.38	0
132	37.07±3.90	1.87±1.98	11.72±0.01	25.22±7.38	0

Table 3D Sugar consumption and xylitol during batch 3 fermentation

Fermentation time (h)	Xylose	Glucose	Arabinose	Xylitol (g/L)	Ethanol (g/L)
0	49.33±14.38	6.72±1.22	11.44±1.94	0±27.08	0
12	41.84±14.38	4.35±1.22	11.34±1.94	0±27.08	0
24	41.63±14.38	4.25±1.22	11.14±1.94	0±27.08	0
36	38.62±14.38	3.45±1.22	10.96±1.94	45.38±27.08	0
48	37.78±14.38	3.35±1.22	10.73±1.94	45.15±27.08	0
60	34.82±14.38	3.29±1.22	10.54±1.94	51.07±27.08	0
72	32.13±14.38	3.25±1.22	10.36±1.94	54.53±27.08	0
84	27.96±14.38	3.16±1.22	9.75±1.94	57.19±27.08	0
96	21.75±14.38	3.08±1.22	9.74±1.94	62.21±27.08	0
108	15.38±14.38	2.75±1.22	6.79±1.94	65.56±27.08	0
120	8.47±14.38	2.32±1.22	6.51±1.94	67.36±27.08	0
132	3.21±14.38	1.97±1.22	6.36±1.94	68.32±27.08	0

APPENDIX E

Research publication

Research Publication

Kusumawadee Thancharoen, Sirirat Deeseenthum and Kanit Vichitphan. (*2014*). Xylitol production from Xylose-fermented yeast using sugarcane bagasse hydrolysate as carbon source. *International Conference on Beneficial Microbes (ICOBM 2014), 27-29 May 2014., Pinang, Malaysia*. (Oral presentation)

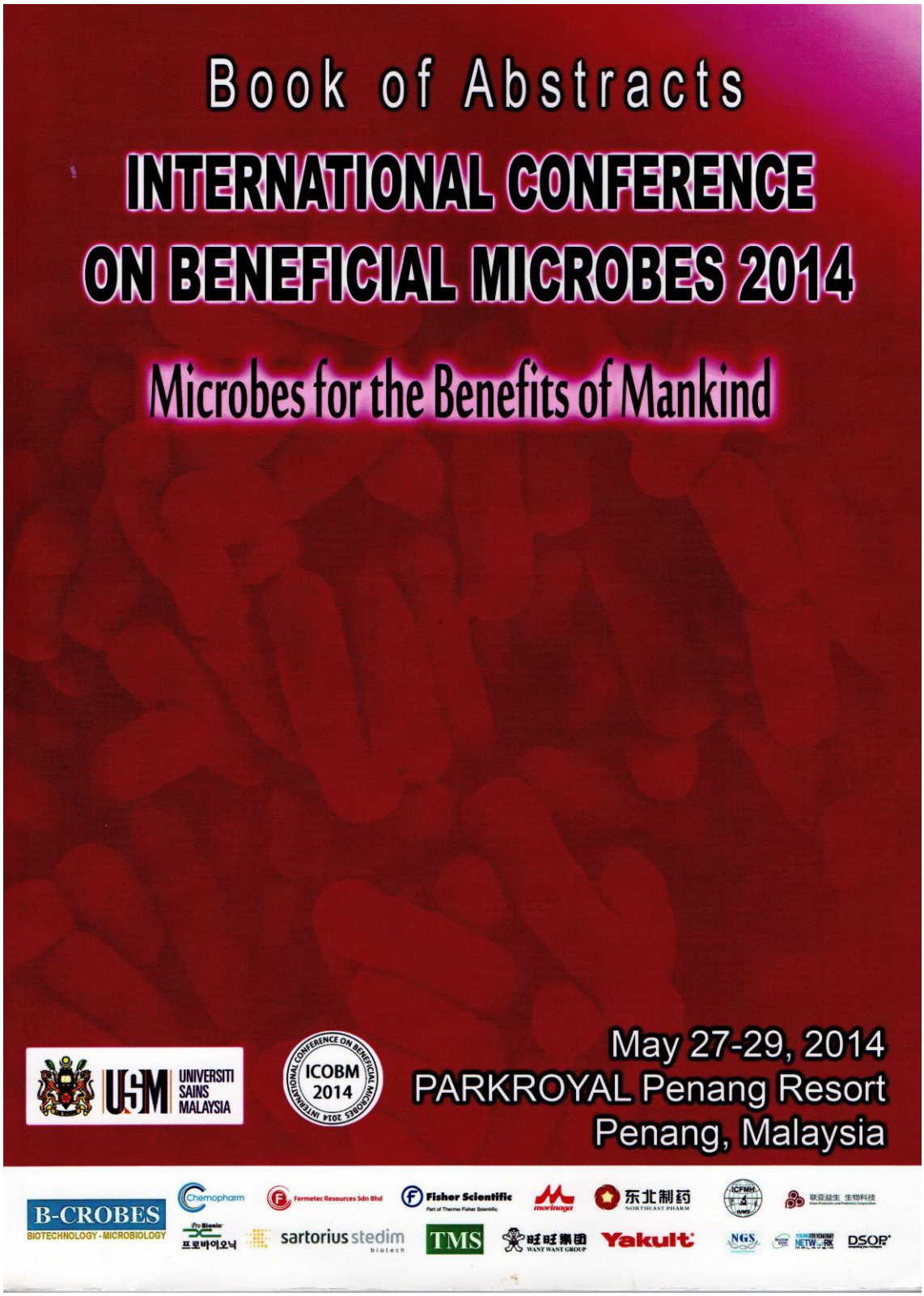
Kusumawadee Thancharoen, Sirirat Deeseenthum and Kanit Vichitphan. (*2014*). Acid hydrolysis of sugarcane bagasses for xylitol production from *Candida guilliermondii*. *International Postgraduate Symposium on Food, Agricultural and Biotechnology, 20-21 August 2014., MahaSarakham, Thailand.* (Poster presentation)

Kusumawadee Thancharoen, Sirirat Deeseenthum, and Kanit Vichitphan. (*2016*). Potential of xylose-fermented yeast isolated from sugarcane bagasse waste for xylitol production using hydrolysate as carbon source. Songklanakarin J. Sci. Technol. 38 (5), 473-483.

27-29 May 2014

International Conference on Beneficial Microbes (ICOBM 2014)

Pinang, Malaysia.


Book of Abstracts
INTERNATIONAL CONFERENCE
ON BENEFICIAL MICROBES 2014
Microbes for the Benefits of Mankind
USM UNIVERSITI SAINS MALAYSIA
ICOBM 2014
May 27-29, 2014
PARKROYAL Penang Resort
Penang, Malaysia
B-CROBES
BIOTECHNOLOGY - MICROBIOLOGY
Chemopharm
Fermetec Resources Sdn Bhd
Fisher Scientific
Part of Thermo Fisher Scientific
morinaga
东北制药
NORTHEAST PHARM
ICFMH
联亚益生 生物科技
프로바이오닉
sartorius stedim
biotech
TMS
旺旺集团
WANT WANT GROUP
Yakult
NGS
DSOP

Concurrent Session 5.3 : Young Scientist Awards (Food Sciences & Technology / Nutrition & Nutraceutical)			
	Chairperson: **Svetoslav Dimitrov TODOROV** Sao Paulo University, Sao Paulo, Brazil	**Serai Room, Level 1**	
1400 - 1415	**Muhammad JUNAID** University of Veterinary and Animal Science, Punjab, Pakistan	**OPC-1**	74
1415 - 1430	**Ming-Zhan TOH** National University of Singapore, Singapore	**OPC-2**	74
1430 - 1445	**S. Anto Jeya DAYALAN** Taylor's University, Selangor, Malaysia	**OPC-3**	75
1445 - 1500	**Yosep JI** Handong Global University, Gyeoungbuk, South Korea	**OPC-4**	75
1500 - 1515	**Shahidah MD. NOR** Universiti Sains Islam Malaysia, Negeri Sembilan, Malaysia	**OPC-5**	76
1515 - 1530	**Li-Oon CHUAH** Universiti Sains Malaysia, Penang, Malaysia	**OPC-6**	76
1530- 1500	**Afternoon Break**		
	Chairperson: **Huey-Shi LYE** Department of Agricultural and Food Science, Faculty of Science, Universiti Tunku Abdul Rahman, Perak, Malaysia	**Serai Room, Level 1**	
1600 - 1615	**Ili Farhana binti ABD HAMID** Universiti Sains Islam Malaysia, Negeri Sembilan, Malaysia	**OPC-7**	77
1615 - 1630	**Yasmi LOUHA SAKUL** Prince of Songkla University, Songkhla, Thailand	**OPC-8**	77
1630 - 1645	**Muhammad Faiz Bin ABDUL KUTHOOSE** Universiti Sains Islam Malaysia, Penang, Malaysia	**OPC-9**	78
1645 - 1700	**Phanwipa PANGSRI** Kasetsart University, Bangkok, Thailand	**OPC-10**	78
1700 - 1715	**Kusumawadee THANCHAROEN** Mahasarakham University, Mahasarakham, Thailand	**OPC-11**	79
1715 - 1730	**Hassan PYAR** Universiti Sains Malaysia, Penang, Malayisa	**OPC-12**	79
1730 - 1800	**Judging of Posters**		
1900	**"Cocktail with the Experts" at the Garden of Parkroyal Hotel (upon invitation only).** Sponsored by Young Scientists Network - Academy of Sciences Malaysia (**YSN-ASM**)		

Young Scientist Award (Oral Presentation) OPC-11

Xylitol production from xylose-fermented yeast using sugarcane bagasse hydrolysate as carbon source

Kusumawadee Thancharoen

Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand

Email:kusumawadee_yeast@hotmail.com

Abstract

Xylitol is a high value sugar alcohol with anticariogenic properties that is used as sweetener for diabetic patients and metabolic disorders. In recent years, the bioconversion of D-xylose from lignocellulosic residues into xylitol gained an increased attention as an alternative procedure. In this study, sugarcane bagasse was used as raw material for xylitol production because of its high efficiency, reduced cost industrial, and contained high concentration of xylose. Pre-treatment of sugarcane bagasse with sulfuric acid was performed with various conditions of temperature, reaction time and acid concentration (1% H_2SO_4 121^{0}C, 60 mins, 2% H_2SO_4 134^{o}C, 60 mins and 3.1% H_2SO_4 126^{o}C, 18 mins). The result showed that the optimum condition of 3.1% sulfuric acid at 126 ^{o}C for 18 mins exhibited 19 g/ L xylose. Isolated yeast from sugarcane bagasse waste of Wangkanai factory, Mahasarakham province and mitrphol factory, Khonkaen province were selected and tested the xylitol production ability from xylose. The results showed that KS 10^{-3} (from 81 isolates) had the highest ability to produce 7.35 g/ L xylitol in 96 hrs of cultivation when sugarcane bagasse hydrolysate contained 32.30 g/ L xylose was used as production medium.

Young Scientist Award (Oral Presentation) OPC-12

Nutritional and medicinal values of pineapple and cabbage wastes to grow beneficial probiotics strain

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*Email: mintze.liong@usm.my

Abstract

There is a growing interest in research to make use of the wastes and byproducts of fruits and vegetable around the world. There are number of techniques that can be employed to reutilize the waste materials for several industrial purposes such as, fermentation, extraction of bioactive components and functional ingredients, etc. This study was focused on to evaluate the nutritional value of two agro-based waste materials, pineapple and cabbage and to make use of these waste products primarily as low-cost raw material to grow lactobacilli species and secondarily to produce bioactive metabolites from the species through fermentation. To study the nutritional value of pineapple and cabbage wastes products, a proximate analysis was conducted and the extent of variation in moisture, protein, lipid, ash and fiber content of both wastes were studied. Probiotics lactobacilli species was cultivated in cabbage and pineapple culture wastes for 36 hours at 37 °C. After incubation, the activity was measured with viable count, pH and also antibacterial activity was tested using supernatant against *E. coli*, *Staphylococcus* spp., *Salmonella* spp., *Pseudomonas* spp. and *Klebsiella* spp. The proximate analyses showed that moisture content, crude protein, lipid, ash and crude fiber in pineapple were 85.51, 0.56, 0.21, 0.61 and 1.81%, respectively and in cabbage were 94.56, 1.22, 0.15, 0.85 and 2.52%, respectively. The pH was found to be decreased from 6 to 4 which indicates the production of organic acid. Viable count was approximately 10^8 CFU/mL. The supernatant from both media showed significant antibacterial activity against all tested pathogenic bacteria. From the results of the present study it can be concluded that, pineapple and cabbage waste products could be the cheapest and valuable bio-resource to cultivate probiotics lactobacilli for the production of bioactive metabolites with high nutritional value and antibacterial properties.

20-21 August 2014

International Postgraduate Symposium on Food, Agricultural and Biotechnology, MahaSarakham, Thailand.

Contents

IPSFAB-2014
International Postgraduate Symposium on Food, Agriculture and Biotechnology 2014

P-09

Acid hydrolysis of sugarcane bagasses for xylitol production from *Candida guilliermondii*

Kusumawadee Thancharoen[1,2*], Sirirat Deeseenthum[2,3] and Kanit Vichitphan[4]

[1] Doctoral candidate of Biotechnology, Faculty of Technology, Mahasarakham University
[2] Unit research of Applied microbiology, Faculty of Technology, Mahasarakham University
[3] Department of Biotechnology, Faculty of Technology, Mahasarakham University
[4] Department of Biotechnology, Faculty of Technology, Khonkaen University

* Corresponding author: kusumawadee_yeast@hotmail.com

Abstract:

Xylitol production from sugarcane bagass hydrolysate by *C. guilliermondii* was experimentally investigated. Acid hydrolysis of sugarcane bagasses using sulfuric acid was performed under different conditions of temperature, reaction time and acid concentration (1% H_2SO_4 121 ^{0}C, 60 mins, 2% H_2SO_4 134^{0}C, 60 min and 3.1% H_2SO_4, 126 ^{0}C, 18 min). Pre-treatment of sugarcane bagasses with 3.1% sulfuric acid at 126^{0}C for 18 min obtained 19 mg/ ml of xylose. Xylitol production in batch fermentation was carried out in shake flasks using various substrate concentrations (2, 4, 6, 8 and 10 g/ 100 ml) under the same conditions (temperature at 30^{o}C, agitation rate was 150 rpm/min, 96 hours). The results showed that initial sugarcane bagass concentrations at 2 g/100 ml provided the highest xylitol yield of 2.206 g/l from 0.925 g/l xylose consumption.

Keywords: *C. guilliermondii*, sugarcane, bagasses, hydrolysate, xylose, xylitol

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38 (5), 473-483.

Songklanakarin Journal of Science and Technology

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Songklanakarin J. Sci. Technol.
38 (5), 473-483, Sep. - Oct. 2016

SJST
http://www.sjst.psu.ac.th

Original Article

Potential of xylose-fermented yeast isolated from sugarcane bagasse waste for xylitol production using hydrolysate as carbon source

Kusumawadee Thancharoen[1]*, Sirirat Deeseenthum[1], and Kanit Vichitphan[2,3]

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Received: 1 July 2015; Accepted: 18 January 2016

Abstract

Xylitol is a high value sugar alcohol that is used as a sweetener. In the past years, the biological process of D-xylose from lignocellulosic material into xylitol has gained increasing interest as an alternative production method. In this study, sugarcane bagasse was used as raw material for xylitol production because of its high efficiency, reduced industrial cost, and high concentration of xylose. Pre-treatment of sugarcane bagasse with sulfuric acid was performed with various conditions. The results showed that the optimum condition was exhibited for 3.1% sulfuric acid at 126°C for 18 min producing 19 g/l xylose. Isolated yeasts from the sugarcane bagasse were selected and tested for xylitol ability from xylose. Results showed that Candida tropicalis KS 10-3 (from 72 isolates) had the highest ability and produced 0.47 g xylitol/ g xylose in 96 hrs of cultivation containing 32.30 g/l xylose was used as the production medium.

Keywords: acid hydrolysate, sugarcane bagasse composition, xylose-fermenting yeast, xylitol

1. Introduction

Xylitol ($C_5H_{12}O_5$) is a natural five-carbon sugar alcohol; recently it has become very interesting because of its use as an industrial food sweetener which can be used by diabetics' patients, as it enters the bloodstream slowly and is a natural insulin stabilizer. Xylitol is anticacinogenic and prevents the formation of acids that attack the tooth enamel. Moreover, xylitol has the ability to inhibit the growth of oral bacterial species, which cause acute otitis medium including *Streptococcus pneumonia* and *Haemophilus influenza*, (Rao *et al*., 2006; Jeevan *et al*., 2011; Rubio *et al*., 2012). Xylitol is found in various fruits and vegetables, but some are not used for xylitol extraction because their low content, less than 9 mg/g, makes manufacturing expensive (Rubio *et al*., 2012). Xylitol is currently produced on an industrial scale by a catalytic reduction (hydrogenation) of xylose obtained from wood sources, such as white birches. There are some disadvantages because a xylose purification step is necessary and makes the process expensive. The hydrogenated solution produced requires further processing (chromatographic fractionation, concentration and crystallization) to attain pure xylitol. About 50-60% of the xylose is converted into xylitol, and the refining and separation steps are more effective. Xylose obtained from hemicellulose hydrolysates can be used for xylitol production by hydrogenation of xylose sugar at 80-140°C and hydrogen pressures of up to 50 atm in the presence of Raney nickel catalyst (Paraj *et al*., 1998). However, the biotechnological process based on the utilization of various microorganisms, such as bacteria, mold, and yeasts

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that can convert xylose into xylitol, a highly specific and economic process since 80% of the sugar is transformed into sugar alcohol. The biotechnological method uses mostly potential agricultural waste as substrate which is low cost and environmental friendly (Rubio *et al.*, 2012). Microbial xylitol production from agricultural wastes containing hemicelluloses could be suitable because this effectively uses renewable resources. In general, among xylose-producing microorganisms, the best xylitol producers are yeasts especially genus *Candida* (Fabio *et al.*, 2008) such as *Candida parapsilosis, C. guilliermondii, C. intermedia, C. boidinii, C. mogii, C. shehatae, C. tenuis, C. tropicalis, C. utilis, Debaryomyces hansenii, Hansenula anomala, Kluyveromyces franilis, K. marxianus, Pachysolen tannophilus, Pichia stipites* and *Schizosaccharomyces pombe* (Winkelhuazen and Kuzmanova, 1998; Fabio *et al.*, 2008; Ghindea, 2010).

Sugarcane bagasse, *Saccharum officinarum* L., is a byproduct of the extraction process in sugar production. For each mill the generated bagasse makes 35-40% of the weight of the milled sugarcane. Bagasse contains mostly lignocelluloses composed of lignin, cellulose, and hemicellulose. Lignocellulose from sugarcane bagasse is a substrate which is available in abundance, widespread, cost effective, and an economical source of biomass. There are many sources of sugarcane bagasse all over MahaSarakham Province in Thailand because of a high number of sugarcane factories for processing sugarcane cultivation. Sugarcane bagasse is composed of hemicellulose, a good resource for producing D-xylitol, D-glucose, D-galactose, D-mannose, D-xylose, D-arabinose and D-glucuronic acid with acetyl side chains (Chen *et al.*, 2010). The objective of this research was to isolate xylose fermenting-yeast from sugarcane bagasse waste and evaluate the xylitol production using the bagasse acid hydrolysate as a carbon source.

2. Materials and Methods

2.1 Preparation of sugarcane bagasses hydrolysate

The sugarcane bagasse was pretreated with different concentrations of sulfuric acid with a sugarcane bagasse and sulfuric acid ratio of 1:10, hydrolysis including 1%, 2%, and 3.1% v/v to determine the highest yield of xylose. After pre-treatment the sugarcane bagasses were hydrolyzed under different temperatures based on the acid concentration by 1% v/v) H_2SO_4 concentration 121°C, 60 min, Rao *et al.*, 2006), 2% v/v) H_2SO_4 134°C, 60 min, Jeon *et al.*, 2010) and 3.1% v/v) H_2SO_4 126°C, 18 min, modified of Paiva *et al.*, 2008) in an autoclave. The liquid fraction was then filtered through Whatman no.1 filter paper and the pH was raised to 9 with calcium oxide and then decreased to 5.5 using phosphoric acid. After this, the hydrolysate was mixed with activated charcoal concentration 1, 2.5 and 3% w/v, and agitated 150 rpm, 30°C, 60 min. The hydrolysate was then concentrated under vacuum at 70°C to increase the xylose concentration using an evaporator (Buchi Rotavapor® 215+v-700/ v-855). The sugarcane bagasses hydrolysate was used as a fermentation medium for xylitol production.

2.2 Isolation of xylose fermenting-yeasts and culture conditions

Samples of sugarcane bagasse waste were obtained from sugar factories in MahaSarakham and Khon Kaen Province, Thailand. For screening, 10 g of each sample was placed into 90 ml of malt extract-xylose-yeast extract-peptone, MXYP, medium containing 30 g/l xylose, 3 g/l yeast extract, 3 g/l malt extract, and 5 g/l peptone, in a 250 ml Erlenmeyer flask and incubated at 30°C for 72 hrs The enriched cells were spread on MXYP agar plates, 100 g/l xylose. After incubation at 30°C for 48 hrs, the cross streak method was used to obtain pure isolates individual colonies of microorganism. The isolated yeast were picked up and maintained on slant YM agar (containing 4 g/l yeast extract; 10 g/l malt extract, 4 g/l glucose and 20 g/l agar) at 30°C for 48 hrs, maintained at 4°C and subcultured at regular intervals. The assays were carried out in MXYP medium, containing 10 ml MXYP medium (initial xylose 30 g/l) and cultivated on a shaker incubator at 150 rpm/ min and 30°C. Cell growth was measured by optical density at 600 nm. After 48 hrs cultivation, samples of each strain were analyzed.

2.3 Inoculum development

Six isolated xylose fermented-yeasts from xylose assimilation tests including *C. guilliermondii* from the Thailand Institute of Scientific and Technological Research, TISTR, were used for xylitol production. From the subculture, one loopful of yeast cells was inoculated into 100 ml Erlenmeyer flasks containing 50 ml of MXYP medium, and then cultivated at 30°C for 24 hrs on a rotary shaker at 150 rpm. Inoculated cells were counted using a haemacytomer; final cell concentration was $1x10^8$ cells/ ml and used as an inoculum in xylitol fermentation process.

2.4 Fermentation

Flask batch fermentations were performed by shaking in 125 ml Erlenmeyer flasks containing 50 ml of sugarcane bagasses hydrolysate (initial xylose concentration 32.30 g/l) into a fermentation medium, containing 2 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4.7H_2O$, 10 g/l yeast extract, 20 g/l peptone, 0.5 g/l KH_2PO_4, and 0.5 g/l K_2HPO_4, and cultivated in a rotary-shaker at 150 rpm, 30°C for 96 hrs. They were inoculated to a final concentration of 10^8 cells/ ml. The samples were collected at regular intervals of 24, 48, 72, and 96 h. of incubation. Aliquots of the cultures were centrifuged at 12,000 xg for 10 min and the supernatant subjected to high performance liquid chromatography (HPLC) analysis for determining the sugar consumption and xylitol concentration.

2.5 Strain identification

Partial sequences of the D1/D2 domain approximately 600-800 bp of the LSU rDNA and the SSU rDNA were determined from PCR products from genomic DNA extracted from yeast cells by using a slightly modified version of the method (Lachance *et al.*, 2001). A divergent 5' domain of the gene was amplified by a PCR with the forward primer NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and the reverse primer NL-4 (5'-GGTCCGTGTTTCAAGACGG) (O'Donnell, 199); amplification of the SSU rDNA was done with the forward primer P1 and the reverse primer P2 (Sjamsuridzal *et al.*, 1997). DNA product was subjected to electrophoresis on 3% agarose gel, recovered using the QIAquick purification kit (Qiagen) and cycle-sequenced using the ABI Big Dye terminator cycle sequencing kit, version 3.1 (Applied Biosystems), with the external primers NL-1 and NL-4 for the D1/D2 domain (Kurtzman and Robnett, 1998) and eight primers, P1–P8, for the SSU rDNA (Yamada *et al.*, 1999). The sequences were determined with an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems) according to the instructions of the manufacturer. Sequences data base was compared with the BLASTN homology search (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI) and generated sequences were aligned with related species retrieved from GenBank using the CLUSTAL X, version 1.81 (Thompson *et al.*, 1994).

2.6 Analytical methods

Xylose, glucose, arabinose, acetic acid, HMF, furfural, phenolic compound and Xylitol concentrations were determined using HPLC with an Aminex HPX-87H, (Biorad, USA) carbohydrate column (300 x 7.8 mm) at 45°C, using 5 mM H_2SO_4, as an eluent. A flow rate of 0.5 ml/min and a sample volume of 20 µl were maintained. The eluate was monitored with refractive index (RI) detector. Peaks were identified and quantified by comparison with the retention times of authentic standards (Xylose and Xylitol purchased from Sigma Company).

3. Results and Discussion

3.1 Sugarcane bagasses hydrolysis

Sugarcane bagasse is a lignocellulosic material waste from sugar mills consisting of cellulose, hemicellulose, lignin and other compounds particularly various types of sugar containing five and six carbon atoms it is commercially used as a carbon source to produce a variety of products. The sugars produced from sugarcane bagasse are glucose, galactose, mannose, xylose, and arabinose. Galacturonic acid can be produced from dilute acid or base hydrolysis. For the remaining sugars in the bagasse there is a breakdown of the beta-glucosidic bond that has a monosaccharide. Chandel *et al.* (2011) illustrate that auto hydrolysis is a simple method to break down the hemicellulosic backbone into monomeric sugar constituents with fewer by-products. However, a significant fraction of hemicellulosic may remain with the substrate after the auto hydrolysis. To overcome this problem, dilute acid hydrolysis is used for maximum degradation of hemicelluloses into monomers. Compared to other pretreatment methods, dilute acid hydrolysis is more useful for the conversion of maximum hemicellulosic fraction into xylose and other sugars, which can be fermented by specialized microorganisms.

The results showed that the maximum xylose concentration of 19.0 g/l was obtained at 3.1% sulfuric acid concentration (126°C, 18 min) (Table 1). The increase in sulfuric acid concentration enhanced the decomposition of lignin and the xylose sugars released from the sugarcane bagasses during acid hydrolysis more other methods. Lignin was broken down into phenolic compounds, such as vanillin, syringaldehyde, 4-hydroxybenzoic acid and ferulic acid which have a toxic effect on microbial growth and xylose metabolism in yeast cells.

According to recent research on the use of dilute sulfuric acid to digest sugarcane bagasse Rao *et al.* (2006) found that using sulfuric acid concentration of 1% v/v under a temperature of 121°C, 60 min produced xylose, glucose and arabinose at 56, 15, and 24%, respectively. Paiva *et al.* (2009) have reported that sulfuric acid concentrations of 3.1% v/v at a temperature of 126°C, 18 min gave xylose at 266.73 mg/g dry weight of sugarcane bagasse. Jeon *et al.* (2010) used sulfuric acid concentrations of 2% v/v under a temperature of 134°C, 60 min. and produced xylose, glucose and arabinose at 21, 5.4, and 3 g/l, respectively. They used dilute acid as a catalyst in the hydrolysis. Hemicellulose and lignin dissolved mostly at low concentrations (0.05-5%) this will minimize damage to products and offer the highest sugar yield at the end of the process. Furthermore, dilute acid enhances the economic potential for industrial production, since it is easy to control and can treat large amounts of the biomass in a short time. The mechanism of the reaction of acid hydrolysis are as follows, (1) diffusion of protons through the matrix of lignocellulosic wet, (2) heterocyclic cyclic bonding of oxygen protons and sugar monomers, (3) the ether bond is broken, (4) intermediate carbocation, (5) the solubility of carbocation with water, (6) the restoration of protons with the sugar energy monomer, (7) the distribution of the reaction in the liquid phase, and (8) the resumption of the second stage, which is developed from the experiments model (Aguilar *et al.*, 2002).

Results in Table 2 show that toxic compounds are byproducts obtained from the acid hydrolysis step and they could be removed by charcoal. A main disadvantage of the synthesis of lignocellulosic, which comes from the diluted acid hydrolysis process, is the degradation of the many sugars type in hydrolysis processes and the formation of undesirable by-products, which inhibit the fermentation process. The by-products in diluted acid concentrations are divided into three main groups: (1) weak acids, e.g. acetic

Table 1. Sugar composition of sugarcane bagasse pre-treatment by different conditions.

Condition	Sugar composition (g/l)		
	Xylose	Glucose	Arabinose
H_2SO_4 1%Temp. 121°C, 60 min.	18.51	2.42	2.20
H_2SO_4 2%Temp. 134°C, 60 min.	12.06	3.23	2.17
H_2SO_4 3.1%Temp. 126°C, 18 min.	19.00	2.75	2.63

Table 2. Sugarcane bagasse hydrolysate composition.

Treatment	Sugar composition (g/l)			Toxic compound (g/l)			
	Xylose	Glucose	Arabinose	HMF	Furfural	Acetic acid	Phenolic compound
Original	14.95	1.27	3.13	0.02	0.03	3.90	5.24
Evaporation	44.58	5.50	8.00	0.07	0.09	5.54	7.78
H+Charcoal 1%	32.13	5.98	7.59	0.03	0.05	2.37	0.25
H+Charcoal 2.4%	31.62	5.78	7.54	0.03	0.04	1.85	0.19
H+Charcoal 3%	32.30	5.90	7.60	0.02	0.04	1.56	0.15

H = Hydrolysate

and formic acids, (2) furan derivatives, e.g. furfural and 5-hydroxymethylfurfural, and (3) phenolic compounds. Several detoxification methods have been reported to overcome the inhibitory effect of these compounds during fermentation by yeasts, such as adaptation of microorganisms to the medium, treatments with molecular sieves, ion-exchange resins or charcoal, steam stripping and overtitration to remove various inhibitory compounds from lignocellulosic hydrolysates, especially charcoal cane effective remove some toxin compounds owing to the toxic substance attaches to the surface of the charcoal. Kamal *et al.* (2011) and Aguilar *et al.* (2002) presented activated charcoal that has been widely used in the removal of carbon compounds hydrolysate concentration; contact time greatly influenced the removal of toxicity compounds. Different proportions varying from 1% to 30% in addition, they observed that 1% of charcoal was enough to remove 94% of the phenolic compounds. Mussatto and Roberto (2001) reported an increase in xylitol production by *Candida guilliermondii* using cells in the past appropriate to hemicellulosic hydrolysate of rice straw. This work, detoxification using activated charcoal concentration 3% (w/v) benefits the xylitol production by removing inhibitors because the toxic substance attaches to the surface of the charcoal and maximizes xylose residual sugar from the hydrolysate.

3.2 Isolation of xylose fermented-yeasts and culture conditions

Isolation xylose fermenting-yeasts have the ability to use xylose as a carbon source. Samples of sugarcane bagasse waste, collected from sugar factories in Kosum Phisai, MahaSarakham Province and Phu Wiang, Khon Kaen Province, led to the isolation of yeasts, 34 and 38 isolates, respectively. Morphological characteristics shown in Table 3 demonstrate xylose-fermenting yeast of sugarcane bagasse waste, from sugar factories, at Kosum Phisai, MahaSarakham Province, 34 isolates exhibit 25 isolates of white colony, 4 isolates of cream colony, 4 isolates of pink colony and 1 isolate of yellow colony. From Phu Wiang, Khon Kaen Province, 38 isolates presented 25 isolates of white colony, 11 isolates of cream colony, 1 isolate of pink colony and 1 isolate of yellow colony.

The sugar xylose contains five-carbon atoms and is converted into xylitol by microbial fermentation using bacteria, fungi and yeast. The best known xylitol producers are yeasts, with special emphasis on the genus *Candida* (Ikeuchi *et al.*, 1999; Kang *et al.*, 2005; Guo *et al.*, 2006; Sampaio *et al.*, 2008) and *Debaryomyces* (Altamirano *et al.*, 2000; Sampaio *et al.*, 2008; Prakash *et al.*, 2011). Microbial

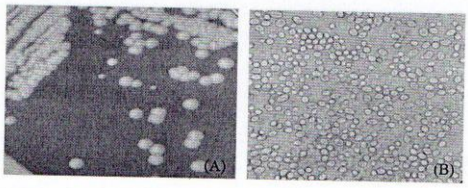

Figure 1. Characteristics of xylose fermented-yeast strain KS 10-3. (A) Colony of KS 10-3 strain on YM agar plate. (B) KS 10-3 from bright microscope (40X).

Table 3. Morphology and growth ability in xylose medium of the xylose fermented-yeasts isolated from sugarcane bagasses waste.

Sample Source	Isolated	Morphology Characteristics					Growth on xylose medium (OD600 nm)	
		Form	Elevation	Surface	Margin	color	0 h.	48 h.
	KS 1-2	Irregular	Convex	Rough	Undulate	Cream	0.134	0.444
	KS 1-3	Circular	Convex	Glistening	Entire	Cream	0.057	0.833
	KS 1-4	Circular	Raised	Glistening	Entire	White	0.069	1.181
	KS 1-5	Circular	Convex	Glistening	Entire	Pink	0.044	0.319
	KS 2-1	Irregular	Raised	Glistening	Undulate	White	0.062	0.351
	KS 2-2	Circular	Convex	Glistening	Entire	White	0.056	0.684
	KS 2-3	Irregular	Convex	Glistening	Undulate	White	0.031	0.235
	KS 2-4	Circular	Convex	Glistening	Entire	White	0.052	0.793
	KS 3-1	Circular	Raised	Glistening	Entire	Cream	0.214	0.601
	KS 4-1	Irregular	Pulvinate	Glistening	Undulate	White	0.063	0.853
	KS 4-2	Circular	Flat	Glistening	Entire	Cream	0.078	0.908
	KS 4-4	Circular	Convex	Glistening	Entire	Pink	0.093	0.423
	KS 4-5	Filamentous	Flat	Rough	Lobate	White	0.046	0.489
	KS 4-6	Circular	Convex	Glistening	Entire	Pink	0.105	0.514
	KS 5-1	Irregular	Raised	Glistening	Lobate	White	0.109	0.406
	KS 6-1	Circular	Flat	Rough	Entire	White	0.066	0.555
	KS 6-2	Circular	Convex	Rough	Entire	White	0.090	0.618
	KS 7-1	Irregular	Convex	Rough	Undulate	White	0.082	0.467
	KS 7-2	Irregular	Flat	Glistening	Undulate	White	0.159	0.300
	KS 7-3	Irregular	Effuse	Rough	Undulate	White	0.086	0.680
	KS 7-4	Irregular	Convex	Rough	Undulate	White	0.096	0.589
	KS 7-5	Irregular	Flat	Rough	Lobate	White	0.094	0.669
	KS 7-6	Irregular	Flat	Glistening	Undulate	Yellow	0.057	0.242
	KS 7-7	Circular	Convex	Glistening	Entire	Pink	0.058	0.990
	KS 7-8	Irregular	Convex	Glistening	Lobate	White	0.059	0.266
	KS 7-9	Irregular	Pulvinate	Glistening	Undulate	White	0.083	0.539
	KS 8-1	Circular	Flat	Glistening	Undulate	White	0.068	0.197
	KS 9-1	Irregular	Convex	Glistening	Undulate	White	0.133	0.861
	KS 9-2	Filamentous	Raised	Rough	Filamentous	White	0.082	0.755
	KS 10-1	Rhizoid	Effuse	Glistening	Filamentous	White	0.068	0.493
	KS 10-3	Circular	Convex	Rough	Undulate	White	0.086	1.032
	KS 10-4	Circular	Pulvinate	Rough	Entire	White	0.097	0.691
Phu Wiang, Khonkaen Province	PV 1-1	Irregular	Convex	Glistening	Undulate	White	0.079	0.508
	PV 1-2	Circular	Raised	Glistening	Entire	Cream	0.223	0.864
	PV 1-4	Rhizoid	Convex	Rough	Filamentous	White	0.044	0.405
	PV 1-6	Circular	Convex	Glistening	Entire	Cream	0.069	0.907
	PV 2-1	Circular	Convex	Glistening	Entire	White	0.083	0.488
	PV 2-2	Circular	Pulvinate	Glistening	Entire	Cream	0.059	0.454
	PV 2-3	Circular	Convex	Glistening	Entire	Cream	0.057	0.180
	PV 3-1	Irregular	Pulvinate	Glistening	Undulate	White	0.082	1.280
	PV 3-3	Circular	Pulvinate	Glistening	Entire	Pink	0.095	0.414
	PV 3-4	Circular	Raised	Glistening	Entire	Cream	0.060	0.117
	PV 3-7	Circular	Convex	Glistening	Undulate	White	0.052	0.126
	PV 4-2	Circular	Pulvinate	Glistening	Entire	White	0.111	0.526
	PV 4-4	Circular	Convex	Glistening	Entire	White	0.087	0.541
	PV 4-5	Circular	Convex	Rough	Entire	White	0.097	0.566
	PV 5-1	Irregular	Flat	Glistening	Undulate	Cream	0.055	0.720

Table 3. Continued

Sample Source	Isolated	Morphology Characteristics					Growth on xylose medium (OD 600 nm)	
		Form	Elevation	Surface	Margin	color	0 h.	48 h.
	PV5-3	Filamentous	Pulvinate	Rough	Filamentous	White	0.120	0.513
	PV5-4	Irregular	Convex	Glistening	Undulate	Cream	0.120	0.241
	PV5-5	Rhizoid	Flat	Rough	Curld	Cream	0.030	0.040
	PV5-7	Rhizoid	Flat	Rough	Curld	Cream	0.027	0.190
	PV6-1	Circular	Convex	Glistening	Entire	White	0.097	0.623
	PV6-2	Circular	Convex	Glistening	Entire	White	0.062	0.485
	PV6-3	Circular	Convex	Rough	Entire	White	0.062	0.368
	PV6-7	Rhizoid	Pulvinate	Rough	Curld	Cream	0.041	0.335
	PV7-1	Circular	Convex	Rough	Entire	White	0.058	0.492
	PV7-4	Circular	Raised	Rough	Entire	White	0.083	0.319
	PV7-5	Circular	Convex	Rough	Entire	White	0.075	0.374
	PV8-1	Rhizoid	Pulvinate	Glistening	Curld	White	0.046	0.477
	PV8-3	Irregular	Flat	Rough	Undulate	White	0.048	0.362
	PV8-4	Circular	Convex	Rough	Entire	White	0.044	0.518
	PV9-1	Irregular	Raised	Rough	Undulate	White	0.026	0.344
	PV9-2	Circular	Pulvinate	Glistening	Entire	White	0.043	0.480
	PV9-3	Circular	Convex	Glistening	Entire	White	0.127	0.541
	PV9-4	Irregular	Convex	Glistening	Undulate	White	0.055	0.149
	PV9-5	Irregular	Flat	Rough	Undulate	White	0.045	0.328
	PV9-6	Circular	Convex	Glistening	Entire	Yellow	0.115	0.317
	PV10-3	Rhizoid	Convex	Rough	Filamentous	White	0.043	0.085
	PV10-4	Circular	Pulvinate	Rough	Entire	White	0.043	0.288

production of xylitol has the advantage of being a more attractive process, since its downstream processing is simple, and its fermentation process provides high cell density, thus resulting in high xylitol yields. Moreover, it is more economic and can be achieved in the industry without high pressure, temperature or xylose purification (Silva *et al.*, 1999; Kang *et al.*, 2005). Ideally microorganism xylitol producers are easy to cultivate, highly capable of productivity and have special resistance to pressure and toxins. According to recent research, the used of a variety of wild type yeast strains mostly genera *Candida* has shown potential production on an industrial scale including *C. boidinii* (Vandeska *et al.*, 1995), *C. guilliermondii* (Zagustina *et al.*, 2001; Rodrigues *et al.*, 2003), *C. parapsilosis* (Oh *et al.*, 1998), *C. peltata* (Saha *et al.*, 1999) and *C. tropicalis* (Kim *et al.*, 2002; López *et al.*, 2004). Sirisansaneeyakul *et al.* (1995) selected a xylitol producer as *C. mogii* ATCC 18364 from 11 strains of D-xylose, utilizing yeasts they have screened in previous research. Their results indicated maximum xylitol yield of Yp/s = 0.62 g/g and a specific rate for product formation that was more than the other yeasts. Ikeuchi *et al.* (Ikeuchi *et al.*, 1999) demonstrate microorganisms with the ability to produced xylitol from high concentrations of xylose were screened from 1,018 types of soil from farms and parks in Osaka, Japan by enrichment culture. A chemically defined (CD) medium using xylose as a sole carbon source for primary selection was obtained from yeast strain capable of metabolizing xylose and showed rapid growth on media containing xylose concentrations of 200 g/l. Results showed that 102 isolates and secondaries were selected using a semi defined (SD) medium with xylose concentration 250 g/l found that yeast 559-9 strain gave the maximum result at 204 g/l and this was identified as *Candida* sp. reference 'The Yeasts' (3rd edition) (Baz *et al.*, 2011). In this strain, the biochemical and morphological characteristics are similar to *Candida* sp. Altamirano *et al.* (2000) isolated thirty-three yeast strain from natural resources, including corn silage (ASM strains) and viticulture residues (SJV strains) with growth on a medium with xylose as a carbon source. These strains were identified as *C. membranifaciens*, *C. tropicalis*, *C. guilliermondii*, *C. shehatae*, *Pichia capsulate*, *C. utilis*, *Candida* sp., *P. angusta* by C.P. Kurtzman, National Center for Agricultural Utilization Research (U.S.A.) and accessioned into ARS Culture Collection (NRRL) as NRRL Y-27290. The results of selected strains capable of producing xylitol showed that *C. tropicalis* has the highest potential to yield 0.69 g/g, using TLC techniques and HPLC. Kang *et al.* (2005) isolated a novel yeast strain from many kinds of soil from rice farming. The isolated strain was identified as *C. tropicalis* HY200 based on systematic characterization using general approaches of Biolog MicrologTM and

18S rRNA sequence analyses. This strain obtained high xylitol yield at 77% and a productivity amount of 2.57 g/l/h from 200 g/l of xylose concentration. Guo *et al.* (2006) explained the principles of xylitol producer screening for forty-five isolates, from 274 isolates grown on solids and broth with xylose as a carbon source. They comprised 9 genera. *Saccharomyces*, *Schizoblastosporion*, *Candida*, *Geotrichum*, *Pichia*, *Hansenula*, *Trichosporon*, *Sporobolomyces*, and *Rhodotorula*. Five species were selected for further experiments, toward the high utilization of xylose and D_1/D_2 of 26S rDNA identified as *C. guiliermondii* Xu280 and *C. maltosa* Xu316 which produced the highest xylose consumption and xylitol yield in batch fermentation under microaerobic conditions.

3.3 Xylose assimilation tests

Seventy-two isolates from sugarcane bagasse were tested for their abilities to assimilate xylose. All strains showed significant growth on liquid medium with xylose as the sole carbon source. Analysis of optical determination (OD600) showed that the strains, which grew well on xylose medium consumed xylose rapidly up to 48 hours. From these 72 strains, six strains including KS 1-4, KS 7-7, KS 10-3, PV 1-6, PV 3-1 and PV 5-1 were chosen for further study because of their high-growth rates on xylose medium. Results are shown in Table 3.

Previously different methods have been used for selecting the best xylitol producer yeasts from several strains, because different yeasts break down xylose at different rates because of species diversity. To ferment xylitol using all yeast species is difficult and it is necessary to find a method of selecting the best yeast culture to demonstrate the potential of using xylose as a carbon source. Altamirano *et al.* (2000) studied the isolation and identification of xylitol-producing yeasts from agricultural residues. Thirty-six yeast strains were primary screened for their capacities to convert D-xylose into xylitol using a conventional method by TLC adapted for easy determination of xylose and xylitol production. This technique is suitable for the first steps of a screening program to select xylitol-producing yeasts from natural environments. *C. tropicalis* ASM III (NRRLY-27290), isolated from corn silage had to high xylitol yield of 0.88 g xylitol/g of xylose consumed. Guo *et al.* (2006) accurately cultivated 274 strains on both solid and liquid screening media with xylose as the sole carbon resource. Five strains were selected based on significant high growth of assimilated xylose, which showed that the strains with rapid growth rate also consumed xylose rapidly. This method demonstrated that in the early testing it is easy to select varieties that produce high xylitol yields, and further study the conditions that affect xylitol production. Xylitol production using all yeast strains from isolation in the flask scale may be difficult, since it is time consuming, inconvenient to harvest and will increase the cost of the analysis. Therefore, this study used the primary screening method by Guo *et al.* (2006), which preliminary selected the yeast which used xylose quickly within 48 hours, observed from changes in the growth of yeast in the broth and significant turbidity increase. These strains were selected as inoculum for yeast fermentation of xylose in sugarcane bagasse hydrolysate to compare their ability to produce xylitol production, in the secondary screening of xyltitol-yeast producers from the xylitol yields.

3.4 Xylitol fermentation

Owing to their abilities to assimilate xylose rapidly, six strains were selected for further evaluation. Physiological behavior of these strains on hydrolysate fermentation medium (xylose concentration 32.30 g/l) was investigated. Xylitol production accompanied xylose consumption during yeasts growth. KS 10-3 exhibited the highest xylitol yield as shown in Table 4. The results in Table 4 show the six yeast strains (KS 1-4, KS 7-7, KS 10-3, PV 1-6, PV 3-1, and PV 5-1) primarily selected using xylitol fermentation of xylose in sugarcane bagasse hydrolysate compared to the reference strain *C. guilliermondii.* In batch fermentation was carried out with flask level (initial xylose 32.30 g/l) and time variation. Results showed that KS 10-3 strain gave maximum xylitol yield of 0.47 g/g with 96 hours at 30°C. Sequence D_1/D_2 of 26S rDNA analysis results identified this strain as *Candida tropicalis* KS 10-3 because 100% sequence identity is strain to that in database (length 576 nucleotides). 26S ribosomal RNA gene, partial sequence showed

AAACCAACAGGGATTGCCTTAGTAGCGGCGAGTGAAG
CGGCAAAAGCTCAAATTTGAAATCTGGCTCTTTCAGAG
TCCGAGTTGTAATTTGAAGAAGGTATCTTTGGGTCTGG
CTCTTGTCTATGTTTCTTGGAACAGAACGTCACAGAGG
GTGAGAATCCCGTGCGATGAGATGATCCAGGCCTATGT
AAAGTTCCTTCGAAGAGTCGAGTTGTTTGGGAATGCA
GCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATA
TTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGG
AAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAA
GTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCA
GACTTGGTATTTTGTATGTTACTTCTTCGGGGGTGGCCT
CTACAGTTTATCGGGCCAGCATCAGTTTGGGCGGTAGG
AGAATTGCGTTGGAATGTGGCACGGCTTCGGTTGTGTG
TTATAGCCTTCGTCGATACTGCCAGCCTAGACTGAGG
ACTGCGGTTTATACCTAGGATGTTGGCATAATGATCTT
AAGTCGCCCGTCT.

Moreover, this result shows that sugarcane bagasse as waste from the sugar factory is a good local alternative to use as a low cost substrate, with added value since residual xylose and other sugar especially xylose, is a substrate in xylitol production. Results agree with the Silva *et al.* (2011) study of glucose:xylose ratio effect to *C. guilliermondii* during fermentation of sugarcane bagasse and show that the glucose:xylose ratio of 1:5 has the highest xylitol yield at 0.59 g/g and demonstrates that glucose, arabinose and acetic acid all assist in the fermentation.

Xylitol production from sugarcane bagasse hydrolysate mostly using *C. guilliermondii* in Table 5 found to be

Table 4. Xylose fermentation of selected strains cultivated on a hydrolysate fermentation medium under micro-aerobic conditions. Initial xylose concentration was 32.30 g/l.

Strains	Culture time (h)	Sugar consumption (g/l)			$Y_{xl/s}$: Xylitol Yield (g xylitol/ g xylose consumed)
		xylose	glucose	arabinose	
KS 1-4	24	30.16	2.36	9.2	ND
	48	28.96	2.18	9.30	ND
	72	29.44	2.30	9.0	ND
	96	27.04	1.94	8.94	ND
KS 7-7	24	30.70	2.88	7.10	ND
	48	31.18	3.11	8.40	ND
	72	31.18	3.06	7.97	ND
	96	31.90	3.23	8.41	ND
KS 10-3	24	30.40	3.06	2.22	0.11
	48	2.66	1.14	6.90	0.14
	72	5.80	1.22	5.70	0.42
	96	1.50	0.28	4.14	0.47
PV 1-6	24	28.54	2.08	9.26	ND
	48	29.03	2.16	9.08	ND
	72	30.51	2.46	9.66	ND
	96	29.60	2.33	9.46	ND
PV 3-1	24	27.96	2.18	9.40	ND
	48	27.06	2.06	9.22	ND
	72	28.06	2.28	9.46	ND
	96	29.36	2.50	9.76	ND
PV 5-1	24	29.50	2.33	9.57	ND
	48	29.00	2.26	9.21	ND
	72	29.20	2.36	9.52	ND
	96	30.19	2.56	9.72	ND
C. guillier mondii	24	32.00	3.30	8.66	0.09
	48	32.36	3.36	7.24	0.11
	72	34.12	3.64	7.52	0.11
	96	32.94	3.48	7.32	0.11

ND = Not Detected

C. guilliermondii FTI 20037 high xylitol yields were 0.69, 0.75 and 0.81 g/g from xylose initial concentrations 30, 48, and 80 g/l, respectively. *C. guilliermondii* has a xylitol yield of 0.59 g/g from 45 g/l of xylose. *C. langeronii* RLJ Y-019 present xylitol yield 0.40 g/g from 47.2 g/l xylose concentration. Reports of recent research suggest that *C. guilliermondii* has a high potential of xylitol yield from sugarcane bagasse hydrolysate. Previous results show that *C. tropicalis* is a xylitol-yeast which produces a high xylitol yield similar to *C. guilliermondii*. Such findings suggest that the maximum xylitol yield of 0.45 g/g was achieved with the sugarcane bagasse hydrolysate but xylitol yields were lower with mixed sugar because of the limited yeast growth rate and inhibitor effect on cell adaptation (Rao *et al.*, 2006). Results are similar to these observed in xylitol production by Baz *et al.* (2011) using *C. tropicalis* under different condition such as rice bran, ammonium sulfate and xylose resulting in xylose giving a maximum xylitol yield of 57.2% obtained from xylose 20 g/l, rice bran 15 g/l and ammonium sulfate 1 g/l pH 5.5. However, xylitol increased to 72.5% when initial xylose was 50 g/l. Xylitol production on a large scale requires oxygen aeration by semi-synthetic media and hydrolysate. Using *C. tropicalis* AY2007 under a limit of O_2 0.3 vvm gave xylitol 36 g/l in 59 hours of fermentation and increase aeration of 1.5 vvm obtained 30.99 g/l. In comparison, xylitol yield from semi-synthetic media and hydrolysate were 0.704 and 0.783 g/g, respectively.

For this work, KS 10-3 gave the maximum xylitol yield at 0.47 g/g obtained at 32.30 g/l of xylose lower than some report in Table 5 towards initial xylose is low and necessary optimized condition suitable for this xylitol-yeast producer strain of xylitol production particularly xylose concentration and agitation rate influence cell yeast growth and increase xylitol production efficiency.

Table 5. Summary using sugarcane bagasse hydrolysate for xylitol production by genus *Candida*.

Yeast strain	Condition	Initial xylose	Yp/s (g/l)	Qp (g/g)	Time (g/l/hr)	Reference (hour)
C. guilliermondii FTI20037	Temperature at 30°C, agitation rate 300 rpm	48	0.75	0.57	22	Felipe *et al.*, 1997
C. langeronii RLJ Y-019	Temperature at 42°C, agitation rate 700 rpm	47.2	0.40	0.97	-	Nigam, 2000
C. guilliermondii FTI20037	Temperature at 30°C, agitation rate 300 rpm	30	0.69	0.68	-	Martinez *et al.*, 2003
C. guilliermondii	Temperature at 30°C, agitation rate 500 rpm	45	0.59	0.53	48	Silva *et al.*, 2007
C. guilliermondii FTI20037	Temperature at 30°C agitation rate 200 rpm	80	0.81	0.60	48	Arruda *et al.*, 2011
C. tropicalis	Temperature at 30°C agitation rate 200 rpm	56%	0.45	-	48	Rao *et al.*, 2006
C. tropicalis	Temperature at 30°C agitation rate 200 rpm	50	36.25 g/l	-	96	Baz *et al.*, 2011
C. tropicalis AY2007	Temperature at 30°C,	29.8	0.783	0.239	94	Baz *et al.*, 2011
	agitation rate 200 rpm, aeration 0.3 vvm	45.5	0.704	0.506	65	
C. tropicalis	Temperature at 30°C agitation rate 200 rpm	32.30	0.47	-	96	This study

4. Conclusions

In summary, we reported a xylose fermented-yeast had the ability to use xylose as a carbon source from sugarcane bagasse, KS 10-3 can grew well on xylose medium consumed xylose rapidly and produced a higher xylitol yield than *C. guilliermondii*. Therefore, a better understanding of the regulation of xylose metabolism will contribute to the design of xylose reductase and xylitol dehydrogenase activity to enhance the xylitol production and optimization that affect xylitol yield. Together with the complete genome sequence available from related strains, detailed genetic studies are expected to provide some clues for this information. This strain is expected to be a good candidate for studying xylitol production in large scale fermentation.

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