CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw material (Bagasse)

The raw material, sugarcane bagasse, used in this experiment was collected from a local sugar industry (Mitt-phu-wieng Industry, Khon Kaen, Thailand). The sugarcane bagasse was air dried, milled and cut to different fraction of particles with a size lower than 0.85 mm, 0.85-1.70 mm, 1.70-2.38 mm and 2.38-4.75 mm, then homogenized in each single lot and stored at room temperature until used.

3.1.2 Microorganisms

C. guilliermondii 5068, *Kluyveromyces marxianus* 5057 and *Hansenula anomala* 5302 were purchased from MIRCEN, Thailand. These yeasts were used to screen yeast for xylitol production.

3.1.3 Media for yeasts growth

3.1.3.1 YPD broth and agar

This medium was prepared by suspending 20 g of glucose, 20 g of peptone, 10 g of yeast extract in 1 litre of distilled water for broth and 20 g of agar was added for YPD agar.

3.1.3.2 YPX broth and agar

This medium was prepared by suspending 50 g of xylose, 20 g of peptone, 10 g of yeast extract in 1 litre of distilled water for broth and 20 g of agar was added for YPX agar

3.1.3.3 YPDX broth and agar

This medium was prepared by suspending 10 g of glucose, 40 g of xylose, 20 g of peptone, 10 g of yeast extract in 1 liter of distilled water for broth and 20 g of agar was added for YPDX agar.

3.1.3.4 Sugarcane bagasse hydrolysate medium

(1) Preparation of sugarcane bagasse hydrolysate

Acid hydrolysis

Sugarcane bagasse was hydrolysed with 3% (w/v) H_2SO_4 (solid:liquid ratio of 1:5) under the condition of autoclaving, at steam gauge pressure of 1.5 Kg/cm^2 for 60 min. The hydrolysate was concentrated to three-fold of solid content under vacuum at 70°C, then neutralised and reduced the inhibitors.

Neutralisation of acid hydrolysate

The hydrolysate was first treated with CaO pellets to adjust the pH to 7.0 and subsequently treated with H_3PO_4 to lower the pH to 5.5. The $CaSO_4$ was removed by filtration, and the neutralised hydrolysate liquors were subjected to charcoal adsorption.

Treatment with activated charcoal

Activated charcoal at 2.4% (w/v) was added into neutralised hydrolysate and shaken at 30°C and 200 rpm for 1 h. The precipitate was removed by vacuum filtration. This bagasse hydrolysate was stored at 4°C until used.

(2) Sugarcane bagasse hydrolysate medium

This medium was prepared by adding the following nutrients: 2.0 g/l $(NH_4)_2SO_4$, 0.1 g/l $CaCl_2 \cdot 2H_2O$ and 20.0 g/l rice bran extract into the prepared sugarcane bagasse hydrolysate that containing approximately 27.4 g/l xylose and 7.0 g/l glucose for broth and 20 g of agar was added for agar medium.

3.1.4 Strains, vectors, enzyme, media and chemicals used in genetic manipulation

K. marxianus 5057, purchased from MIRCEN Thailand was used to produce recombinant xylose reductase enzyme with the expression vector pPICZ A.

Escherichia coli TOP 10F′ (Invitrogen) was used as the bacterial host for DNA manipulation with the pCR®2.1-TOPO and pUC 18 cloning vector (both TOPO TA Cloning® kit and pUC 18 cloning vector from Invitrogen).

Pichia pastoris strian GS115 (his4) was used to produce recombinant enzymes with the expression vector pPIC9 (Invitrogen).

Media components were purchased from Oxoid (Basingstoke, UK). Antibiotics and chemicals were purchased from Sigma and BDH (Poole, UK).

Oligonucleotide primers were purchased from Sigma Genosys UK.

Molecular biology enzyme and regents were purchased from Life Technologies Inc. (Paisley, UK), Promega, Boehringer Mannheim and Stratagene UK.

PCR reactions were performed using deoxynucleoside triphosphate perchased from Promega, Taq DNA polymerase perchased from *Ab*gene with homemade 10X PCR buffer 2 (500 mM Tris-HCl pH 9.2, 140 mM $(NH_4)_2SO_4$, 22.5 mM $MgCl_2$), *Vent* DNA polymerase with 10X ThermoPol buffer (10 mM KCl, 20 mM Tris-HCl pH8.8, 10 mM $(NH_4)_2SO_4$, 22.5 mM $MgCl_2$, 0.1% Titon X-100) purchased from Biolabs or *Pfu* DNA polymerase with 10X Cloned *Pfu* buffer (200 mM Tris-HCl pH 8.8, 20 mM $MgSO_4$, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1 mg/ml nuclease-free BSA) purchased from Stratagene.

Long template PCR reactions were carried out using the Expand Long Template PCR System with 10X PCR buffer 3 (500 mM Tris-HCl pH 9.2, 140 mM $(NH_4)_2SO_4$, 22.5 mM $MgCl_2$, 20% (v/v) DMSO, 1% (v/v) tween® 20) purchased from Boehringer, Mannheim.

Restriction enzymes and buffer were purchased from Boehringer, Mannheim. *P. pastoris* expression system was supplied by Invitrogen; Living Science (The Netherlands).

3.2 Analytical Methods

3.2.1 Determination of reducing sugar

Reducing sugars (glucose and xylose) were determined according to Somogyi –Nelson method.

3.2.2 Determination of monosaccharides, acetic acid and ethanol in sugarcane bagasse

Xylose, glucose, arabinose, xylitol, acetic acid and ethanol were analysed in a Shimadzu high performance liquid chromatograph (HPLC), using a Bio-Rad

Aminex HPX-87 H column at 45°C and 0.005 M H_2SO_4 was used as the eluent at a flow rate of 0.6 ml/min.

3.2.3 Determination of cell growth

Cell growth was monitored by measuring the culture turbidity at 600 nm. The cell mass was estimated by dry weight measurement.

3.3 Methods

3.3.1 Pretreatment of bagasse

Sugarcane bagasse prepared as described above (in 3.1.1) were hydrolysed in 250 ml Erlenmeyer flasks using 100 ml of sulphuric acid (H_2SO_4). Treatments were performed in the solutions containing 1, 2 and 3% H_2SO_4 liquor. All experiments were carried out using a mass ratio of solid to liquid (1:5). The mixtures were autoclaved at 126.7°C (1.5 kg/cm^2 pressure gauge) for several reaction times in the range of 15-60 min. The acid hydrolysis consisted of 48 experimental sets. There were 4 major treatments with regard to hydrolysis time, viz. 15, 30, 45 and 60 min; for each duration three levels of H_2SO_4 concentrations, 1, 2, and 3% (w/v) were applied to the four different bagasse particles sizes, <0.850, 0.850-1.70, 1.70-2.83 and 2.83-4.75 mm, respectively.

At the end of the reaction time period, the sample was taken and filtered. Samples of the filtrate were analysed for reducing sugars (glucose and xylose) according to Somogyi –Nelson method.

3.3.2 Genetic manipulation of yeasts from selected xylitol producing yeasts

3.3.2.1 Growth of selected yeasts on different carbon source media

C. guilliermondii 5068, *Kluyveromyces marxianus* 5057 and *Hansenula anomala* 5302 purchased from MIRCEN, Thailand, were selected for growth and xylitol production based on the previously reported for efficiently xylitol production in sugarcane bagasse hydrolysate. The yeasts were grown using sterile techniques on YPD agar plate and the agar medium containing; 20/l xylose, 20g/l peptone and 10 g/l yeast extract. The colonies forming of the yeasts were observed for growth after 24 h incubation at 30°C. Also, the growth of those yeasts on agar medium containing; 20/l xylitol, 20g/l peptone and 10 g/l yeast extract at the same condition was conducted.

3.3.2.2 Isolation of xylose reductase (XR) gene from selected yeasts

(1) Extraction of genomic DNA from yeasts

Genomic DNA was extracted from *C. guilliermondii* 5068, *K. marxianus* 5057 and *H. anomala* 5302. After growing the yeasts in YPD broth for overnight, the cell cultures were then harvested. The culture broth (2 ml) was transferred into eppendorf tubes and centrifuged using micro-centrifugation. After decant off the supernatant, breaking cells was made by adding 0.3 g acid glass beads, 0.2 ml Smash and Grab Solution (1% SDS, 2% Trition X-100, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 4 M NH_4OAc) and 0.2 ml of mixed solution (phenol:chloroform:isoamyl alcohol; 25:24:1 ratio) and mixed for 2 min by hand or 5 min using a multi-tube vortex. After centrifugation at 13,000 rpm for 5 min, the upper phase was transferred into a fresh eppendorf tubes. The DNA was precipitated with 1 ml of absolute cold - ethanol and incubated on ice for a further 30 min. After centrifugation at 13,000 rpm for 20 min at 4°C, the DNA pellet was resuspended in 10-50 µl of water.

(2) Polymerase chain reaction (PCR reaction)

PCR reaction was performed by adding genomic DNA (10 ng) as a template in 50 µl of reaction mixtures containing; 50 pmol of primers (forward and reverse primers), 500 mM of each deoxynucleoside triphosphate (A, T, C, G bases), 0.1 volume of 10X PCR buffer 3 and 2.5 U *Taq* DNA polymerase. After the initial denaturing step at 95°C for 5 min, PCR amplifications were carried out using 30 sec denaturation at 95°C, 45 sec annealing at 50°C, 120 sec primer extension at 72°C for 35 cycles and the final extension step at 72°C for 10 min. The PCR products were analysed using agarose gel electrophoresis.

Specific primers for *C. guilliermondii* 5068 (XR) gene, based on the sequence alignment of *C. guilliermondii* which reported in GenBank database are;

Forward primer: AAGACCTAGTCGGCAACAC
Reverse primer: GACAACTGGAGATGTTTCC

For *K. marxianus* 5057 and *H. anomala* 5302, degenerated primers for amplification of XR gene were designed based on the conserved sequence alignment from the other yeast species: *Candida shehatae*, *C. tenuis*, *C. tropicalis*,

Pichia stipitis, *P. guilliermondii* and *K. lactis* which reported in GenBank database. In this experiment, the degenerated primers are;

Forward primer: 5′ TSGGYTTNGGYTGYTGGAAG 3′

Reverse primer: 5′CAKTCCCAWGGRTYRTTGAATC3′

(3) Agarose gel electrophoresis

A suspension of agarose (typically 0.8%; w/v) in TAE buffer (40 mM Tris Acetate, 2 mM EDTA, pH 7.9) was melted using a microwave oven and added with ethidium bromide at a concentration of 1 μg/ml. The agarose suspension was poured into a casting tray and allowed to set. Once set, the gel was immersed in a buffer tank containing TAE buffer. Samples to be loaded, were mixed with 0.2 volumes of loading buffer (0.025 M EDTA pH 8.0, 0.01% (w/v) bromophenol blue powder and 50% (v/v) glycerol). Samples were loaded with a constant voltage (45-150 V) applied across the gel.

At neutral pH, nucleic acids have a negative charge and so migrated towards the anode. The DNA loading buffer contains bromophenol blue dye, so the migration of the DNA could be followed. Separation by agarose gel electrophoresis could be used to size DNA fragments by comparing their migration with that of fragments of known sizes. The ethidium bromide in the gel intercalates into nucleic acids causing it to fluoresce under UV irradiation, so that band position could be visualised.

3.3.2.3 Sequencing of *K. marxianus* 5057 (XR) gene

(1) Growth and storage of bacteria *E. coli*

E. coli was grown using sterile techniques in either Luria-Bertani Broth (LB broth) at 37°C in shaking incubator at 250 rpm for overnight or on LB agar plate in an inverted position at 37°C for overnight, in the presence of the adequate antibiotics as a selection factor. Liquid culture was inoculated from single colonies taken from LB agar plate or 1 night - old culture taken from LB broth at the dilution of 10^{-2}. Long term storage of bacteria was done by addition 50% (v/v) glycerol into aliquot cultures, followed by brief vortex-mixing, flash freezing in liquid nitrogen and storage at -70°C. Frozen stocks were used for all transformed bacterial

strains from which DNA was isolated and analysed by restriction digest or DNA sequenced.

(2) Extraction of genomic DNA from *K. marxianus* 5057 and PCR reaction

Extraction of genomic DNA of *K. marxianus* 5057 and PCR reaction was conducted as described above in 3.3.2.2 (1) and 3.3.2.2 (2). After PCR amplifications, the resulting PCR products were gel purified before cloning into the pCR® 2.1-TOPO vector and sequenced.

(3) TOPO TA Cloning ® of PCR amplified DNA products

The PCR product was mixed with pCR®-TOPO® vector (see in appendix C) in an eppendrof tube; the mixture contained: 4 µl of PCR product, 1 µl of salt solution and 1 µl of TOPO® vector. The mixed reaction was incubated at room temperature for 20 min and then placed on ice cubes for 5 min. A 2 ml aliquot of the mixed reaction was used to transform TOP10F′ into *E. coli* competent cells, which were plated onto LB agar plates containing ampicillin and X-gal.

(4) Transformation of chemically competent *E. coli* cell

Competent cells were thawed on ice cubes. Plasmid DNA (10-100 ng) or ligation reaction in a maximum volume of 10 µl was added into the 200 µl of thawed competent cells and incubated on ice cube for 30 min. The cells were heat shocked for 45 sec at 42°C, incubated on ice cube for 2 min, followed by addition of 0.25 ml of LB broth (containing no antibiotic). The cells were incubated 37°C for 1 h with gently agitation to initiate expression and replication of the plasmid DNA and allow expression of the antibiotic resistance gene. Aliquots of 50-100 µl were spread onto LB agar plates (containing require antibiotics) and incubated for overnight at 37°C. For blue/white screening, plates also contained X-gal and IPTG.

(5) Blue/white screening

To produce an enzymatically active β-galactosidase protein, two domains are required: the α-peptide coded by a plasmid vector, and the truncated from of the enzyme produced by a bacterial host that has the ΔM15 deletion mutation of β-galactosidase gene (*lacZ*). These two domains fold to form a functional enzyme, the α-region complementing the missing amino acids resulting from the ΔM15

mutation. IPTG, a chemical analogue of lactose, is necessary for specific induction of *lacZ* transcription. When functional β-galactosidase is produced, it convert colourless X-gal into a blue product. As the multiple cloning site (MCS) of plasmid vectors is flanked by DNA coding for the N-terminus of β-galactosidase (α-peptide), when cDNA is presented in the polylinker, expression from the *lacZ* gene is disrupted and white plaques are produced. In contrast, without insert in the polylinker, the amino terminus of β-galactosidase is expressed and non- recombinant strains can be scored visually by presence of blue plaques.

(6) Alkaline Lysis Fast "mini - prep" method for preparation of plasmid DNA

A single colony of bacteria was picked from LB agar plate containing appropiated antibiotics for plasmid DNA selection, and cultivated for overnight in 10 ml of LB broth also containing antibiotic for selection. Approximately 1.5-3.0 ml of the overnight culture was pelleted by centrifugation at 12,000 g for 1 min in an eppendorf tube and discarded the supernatant. The pellet was completely resuspended in 100 μl of Solution I and incubated at room temperature for 5 min. Then, 200 μl of Solution II was added, mixed gently and incubated on ice cubes for 5 min, followed by addition 150 μl of Solution III and incubated on ice cubes for a further 15 min. After centrifugation at 12,000 g for 15 min at 4°C, the supernatant was transferred into a fresh eppendorf tube and the DNA was precipitated with 1 ml of absolute cold ethanol. After centrifugation at 12,000 g for 10 min at 4°C, the DNA pellet was resuspended in 300 μl of sterile distillate water, followed by addition of 1 U. of Rnase-A plus 150 μl of 7.5 mM ammonium acetate and then incubated at -20°C for 15 min. After centrifugation at 12,000 g for 15 min at 4°C, the supernatant was transferred into a fresh eppendorf tube and the DNA was precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol (-20°C). After centrifugation at 12,000 g for 15 min at 4°C, the pellet was washed with 500 μl of 70% ethanol and again, centrifugation at 12,000 g for 5 min at room temperature.

The resulting pellet was resuspended in 30-60 μl of sterile distilled water and analysed using restriction digest or sequenced. In the experiments, the composition of Solution used contained the following;

Solution I: 50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0,

Solution II: 0.2 NaOH and 1% SDS

SolutionIII: 60 ml of 5M KOAc, 11.5 ml glacial acetic acid and 28.5 ml H_2O

3.3.2.4 Determination of xylose reductase (XR) expression

(1) Growth and storage of yeast

Yeast was grown using sterile techniques in YPD, YPDX, YPX and sugarcane bagasse hydrolysate broth at 30°C in shaking incubator (200 rpm) for overnight or on YPD, YPDX and YPX agar plate in an inverted position at 30°C.

Long-term storage of yeast was done by addition 50% (v/v) of glycerol to aliquot cultures, followed by brief vortex-mixing, flash freezing in liquid nitrogen and storage at -70°C. Frozen stocks were used for Genomic DNA extraction and RNA isolation.

(2) Isolation of total RNA from yeast *K. marxianus* 5057

RNA was extracted according to Schmitt *et al.* (1990). *K. marxianus* 5057 was grown in 200 ml of YPD, YPDX, YPX and sugarcane hydrolysate culture medium. The 50 ml of culture medium was taken at the time points of 2, 3, 4 and 5 days and harvested from each time point by centrifugation at 3,000 rpm for 10 min at room temperature. The cell pellet was resuspended in 400 µl of AE buffer (50 mM Sodium acetate pH 5.3, 1 mM EDTA) and transferred into a micro centrifuge tube. The cell was mixed with 40 µl of 10% SDS using vortex for 20 sec. An equal volume (450-500 µl) of saturated phenol buffer was added and mixed for about 20 sec, incubated at 65°C for 4 min, then incubated in a dry ice/ethanol bath until crystallisation was occurred (~1 min). After centrifugation at the speed of 3,000 rpm for 2 min at 4°C, the supernatant was transferred into a new eppendorf tube and added an equal volume of the solution of phenol and chloroform, mixed again for 20 sec. After centrifugation at the speed of 3,000 rpm for 2 min at 4°C, the upper phase was removed into a new eppendorf tube. The RNA was precipitated with 40 µl of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol (-20°C) by centrifugation at the speed of 3,000 rpm for 15 min at 4°C. The RNA pellet was washed with 500 µl

of 80% ethanol (-20°C). After centrifugation at the speed of 3,000 rpm for 5 min at room temperature and air dry briefly, the total RNA was resuspended in 20 µl of DEPC-treated water and stored at -80°C.

(3) Dnase treate of RNA

To remove genomic DNA contaminating, the RNA samples were treated with Rnase-free DnaseI supplied by Promega (RQ1 Rnase-free Dnase). One unit of enzyme (1U/µl) was used to treat 1 µg of RNA. The digestion was performed according to the manufacturer's procedure, at 37°C for 30 min. Extraction by Phenol:chloroform (v/v) solutoin and ethanol precipitation were performed after the digestion. Before using the RNA for Northern-blot hybridisation, the RNA integrity was determined and checked using agarose gel electrophoresis (integrity of the rRNA bands).

(4) Spectrophotometric determination of nucleic acid concentration and purity

Both DNA and RNA can absorb at wavelengths of 260 and 280 nm and their concentrations can be calculated by measuring absorbency at wavelengths of 260. The value 1 of an optical density (OD_{260}) corresponds to a concentration of 50 µg/ml for double strands DNA, 40 µg/ml for RNA and 33 µg/ml for single strand DNA. The extinction coefficient varies with the environment of the bases such that pure dsDNA has a ratio of absorbency at 260 and 280 nm (OD_{260}/OD_{280}) values of 1.8 and pure RNA one of around 2.0. If there is the contamination with protein or phenol, this ratio will be less and accurate quantification is not possible.

(5) RNA separation in formaldehyde agarose gel

In all cases, the gel tank, former and comb were treated with 1M NaOH and 1% of SDS for 30 min before rinsing with freshly autoclaved sterile distill water. Gloves, eye mouth protection were employed and the experiments were carried out in a fume hood.

The (1.2%; v/v) formaldehyde (6.6%; w/v) agarose gel was prepared as follows: 0.6 g of agarose was melted in 36 ml of distilled water and allowed to cool to approximately 55°C. Five ml of 10X running buffer (0.2 M MOPS,

10 mM EDTA and 10 mM sodium acetate pH 7.0) were mixed with 9 ml of 37% (w/v) formaldeyde and added to the cooled agarose. The gel was cast and allowed to solidify for 1 h at room temperature in a fume hood. Total RNA (~5 μg) was precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volume of ethanol. The pellet was resuspended in 2 μl of sterile distilled water. Formamide (5 μ l), formaldehyde (2 μl), 10X running buffer (1 μl) and 400 μg/ml ethidium bromide (1 μl) were added to each sample. The samples were mixed thoroughly and heated for 10 min at 65°C. After heating, the samples were placed on ice cubes. Following a brief centrifugation to collect any condensate, 1 μl of 10X loading buffer (0.2%; w/v) bromophenol blue, 10 mM EDTA, 50%; w/v glycerol) was added. The gel was electrophoresed for overnight at 1.5 V/cm in 1X running buffer.

(6) Northern-blotting

Northern-blotting was performed according to The DIG System User's Guide for Filter Hybridisation (1995). RNA was electrophoresed in formaldehyde denaturing agarose gels with 1X running buffer. The electrophoresed RNA was then blotted by capillary transfer with 20X SSC buffer (3 M NaCl, 0.3 M sodium citrate pH 7.0) onto Hybong-N^+ nylon membrane (Amersham Pharmacia biotect), at room temperature for overnight. The RNA was UV cross-linked to the menbrane and probed in DIG Easy Hyb formamide based buffer (Boehringer, Mannheim) at 42°C with PCR fragment from XR-F primer and XR-R primer, which was labeled using the DIG-labeling (Digoxygenin-11-dUTP) system (Boehringer, Mannheim). Digoxygenin-11-dUTP (DIG dTUP) can be incorporated by *Taq* polymerase during the polymerase chain reaction. The resulting probes are very sensitive, and the yield from the labeling reaction is high.

Colorimetric detection was performed in the presence of NBT (Nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate), substrates of the alkaline phosphatase enzyme conjugated to the anti-digoxigenin antibiodies.

3.3.2.5 Isolation of xylose reductase full length gene by Southern-blotting

(1) Extraction of genomic DNA from *K. marxianus* 5057

Genomic DNA from *K. marxianus* 5057 was extracted as described above in 3.3.2.2 (1). After extraction, DNA was digested with restriction enzymed then analysed.

(2) Restriction digests of DNA

Digestion of DNA by restriction enzymes was carried out using the condition recommended by the enzyme supplier. Typically, digestions were performed in a total volume of 10-50 µl with approximately 0.5-2.0 units of enzymes for 0.2-1.5 µg of DNA, at appropriate temperature for 2 h. The appropriate restriction buffer supplied with the enzyme. If the DNA preparation being digested contained RNA, Rnase (1 U/µg DNA) was also included in the digestion reaction.

(3) Southern-blotting

Southern - blotting was performed according to The DIG System User's Guide for Filter Hybridisation (1995). Genomic DNA was digested with varies restriction enzymes; *Acc*I, *Asn*I, *Bgl*II, *Cla*I, *Hind*III, *Pvu*I, *Pvu*II, *Pst*I, *Sac*I, *Sma*I and *Xho*I. DNA was then electrophoresed in agarose gels with 1X TAE running buffer (40 mM Tris Acetate, 2 mM EDTA pH 7.9) and blotted by capillary transfer with 10X SSC buffer onto Hybond-N^+ nylon membrane, at room temperature for overnight. DNA was UV cross-linked to the membrane and probed in DIG Easy *hyb* formamide based buffer (Boehringer, Mannheim) at 42°C with PCR fragment from XR-F primer and XR-R primer, which was labeled using the DIG-labeling system (Boehringer, Mannheim). The colorimetric detection was performed in the presence of NBT and BCIP substrates.

3.3.2.6 Nucleotide sequence of the XR full length gene in *E. coli* transformant

XR full length gene of *K. marxianus* 5057 was subcloned into pUC 18 vector, transformed into *E. coli* cell and sequenced.

(1) Ligation reaction

T4 DNA ligase (Stratagene, UK) was used to join DNA strands between 5′-phosphate and 3′-hydroxyl groups of adjacent nucleotides with

cohesive or blunt ends. Generally a molar ratio of insert to vector (pUC 18) of 3:1 was used when cloning a fragment into a plasmid vector. For the ligation, total DNA about 0.01-1 μg, 0.5 μl of T4 DNA ligase (4 U/μl), 1 μl of 10 mM ATP, 1 μl of 10X ligase buffer, and sterile distill water for final volume of 10 μl was prepared. The reaction mixture was incubated at room temperature for 3-4 h and following at 4°C for overnight or 15°C for 4-18 h.

The optimal temperature for a ligation reaction is a balance between the optimal temperature for T4 DNA ligase enzyme activity (25°C) and the temperature necessary to ensure annealing of fragment ends. Blunt ends ligations are generally efficient at temperature between 15-20°C for 16-24 h, which cohesive ends are ligated efficiently at room temperature (22°C) for 3 h or at 4-8°C for overnight.

(2) Transformation of chemically competent *E. coli* cell

Transformation of chemically competent *E. coli* cells were performed as described above in 3.3.2.3 (4).

(3) Colony hybridisation

Colony hybridisation was performed according to The DIG System User's Guide for Filter Hybridisation (1995). The bacterial colonies were transferred onto Hybond™-C Gridded membrane (Amersham Life Science). The membrane was treated with alkaline treatment (0.5 N NaOH, 1.5 m NaCl) serves to lyse the colonies. The denatured DNA was then immobilised and UV cross-linked on the membrane. A digoxigenin - labled DNA probe was used for hybridisation at 42°C. Detection was carried out with colorimetric method.

(4) DNA Dot Blotting

DNA dilution series of *K. marxianus* in 10^{-1}-10^{-8} were prepared. DNA target in the dilution was denatured for 10 min at 95°C and chilled immediately on ice. The membrane was marked lightly with a pencil to identify each dilution before spotting on positively charged Nylon Membranes (Boehringer Mannheim). Each dilution (1 μl) was dispensed onto the membrane and mixed well before dotting on membrane. The DNA was UV cross-linking to the membrane by for 5 min. A digoxigenin - labled DNA probe was used for hybridisation at 42°C. Detection was carried out with colorimetric method

(5) Colorimetric Detection with NBT and BCIP

After hybridisation and post-hybridisation were washed, equilibrated the membrane in washing buffer for 1 min. A freshly washed dish or bag, was blocked the membrane by gently agitation in blocking solution for 30-60 min. Near the end of the blocking period, the antibody solution was prepared. Anti-Digoxigenin-AP was dilluted 1:5,000 (after centrifugation) in blocking buffer for a working concentration of 150 mU/ml, mixed gently and added 6 µl of anti-Digoxigenin-AP into 30 ml blocking solution, and mixed well. This working antibody solution is stable for about 12 h at 4°C. The blocking solution was poured off, incubated the membrane for 30 min in the prepared antibody solution and discarded the antibody solution. The membrane was then washed twice in 100 ml of washing buffer to remove unbound antibody, mixed 45 µl NBT solution and 35 µl BCIP solution in 10 ml of detection buffer. This freshly prepared color substrate solution will be used next step. The membrane was equilibrated in 20 ml detection buffer for 2 min, poured off the detection buffer and add approximately 10 ml color substrate solution to the membrane. The membrane was incubated in a sealed plastic bag in dark. The membrane can be exposed to light for short periods to monitor the color development. The color precipitate starts to form within a few minute, and the reaction is usually complete after 12 h. Once the desired bands are detected, washed the membrane with H_2O to prevent over-development. If the membrane is to be reused, use sterile H_2O to stop the development.

3.3.2.7 Expression of the *K. marxianus* 5057 (XR) gene in *Pichai pastoris* GS115

(1) Protein expression in *P. pastoris* GS115

The procedure for protein expression in *P. pastoris* GS115 was examined according to Guthrie and Fink (1994). The *P. pastoris* strain GS115 possesses a mutation in the gene for histidinol dehydrogenase gene (*his4*) that prevents synthesis of histidine, which must therefore be added to growth media. The *HIS4* gene presents in *Pichia* expression vectors and allows to select transformants (phenotype His^+) by their ability to grow on histidine-deficient medium. Induction of expression is driven specifically by methanol, acting at the *AOX1* promoter (P_{AOX1}) presented in *Pichia* expression vectors (see in appendix C).

(2) Preparation of electrocompetent *P. pastoris* GS115 cell

P. pastoris GS115 was grown in 5 ml YPD broth in a 50 ml conical tube at 30°C for overnight. On the following day, 0.1-0.5 ml of the overnight culture was transferred into a 2 l flask with 500 ml of fresh YPD medium, incubated at 30°C for overnight with vigorous shaking (250-300 rpm) until the OD_{600} value of 1.3-1.5 was reached. Cells were centrifuged at 1,500 g for 5 min at 4°C and the pellet was resuspended in 500 ml of ice-cold sterile water. Cells were then centrifuged and, again resuspended in 250 ml of ice-cold sterile water. Centrifugation was repeated once again and cells were resuspended in 20 ml of 1 M ice-cold sorbitol, followed by a new centrifugation and resuspension in 1 ml of 1 M ice-cold sorbitol. Cells were kept on ice cubes and used the same day.

(3) Transformation of electrocompetent *P. pastoris* GS115 cell

Transformation of *P. pastoris* GS115 was performed using the electroporation method. For transformation according to Backer & Guarente (1991), 1-5 μg of vector was linearised with *Dra*I restriction enzyme to allow the gene replacement at *P. pastoris AOX1* gene. *Pichai* cells (80 μl) prepared as described above in 3.4.5.2 were mixed with 1-5 μg of linearised DNA (in 5 μl sterile water) and then incubated on ice for 5 min. Cells were transferred into a 0.2 cm ice-cold electroporation cuvette and pulsed according to the parameters for yeast (suggested by the manufacturer; 1.5 kV, 200 Ω, 25 μF, using a BioRad gene pulse with a pulse controller). Immediately 1 ml of 1 M ice-cold sorbitol was added into the cuvette. Transformed cells were directly plated out by spreading of 100 μl cell culture on RDB plates (Regeneration Dextrose Base). Plates were incubated for 2 to 3 days at 30°C. The transformants were selected by their ability to grow at 30°C on histidine-deficient medium.

(4) Screening of transformants

To access His^+ transformants for Methanol Utilisation phenotype (Mut), colonies from above (3.4.5.3) were picked up using sterile toothpick and patched onto a MM (Minimal Methanol) plate and then onto a MD (Minimal

Dextrose) plate in a regular pattern. Normal growth on dextrose and slow growth on methanol is indicated as the Mut^S phenotype.

(5) Small-scale expression

Single colonies on MD plate were used to inoculate into 10 ml BMGY medium (Buffer Complex Glycerol Medium). Covered by loose caps, the cultures were incubated at 30°C with vigorous shaking (>200 rpm) until saturation grown (~2 days), with an OD_{600} of 10-20. Cells were harvested by centrifugation at 4,000 g for 10 min at room temperature and resuspended in 2 ml BMMY medium (Buffer Complex Methanol Medium). The cultures were covered with loose caps and incubated at 30°C with vigorous shaking for 3 days. Absolute methanol was added to a final concentration of 0.5% methanol every 24 h to maintain induction. Cells were then pelleted by centrifugation at 4,000 g and supernatants was analysed by SDS-PAGE (12% bis-acrylamide) to secrete expressed product.

(6) SDS - Polyacrylamide Gel Electrophoresis (SDS - PAGE)

Protein samples were mixed with 0.2 volumes of 5X loading buffer (0.0625 M Tris pH 6.8, 20% (v/v) glycerol, 0.2% (w/v) SDS, 20% (v/v) β-mercaptoethanol, 0.00125% bromophenol blue). The reaction mixture was then heated for 2 min and centrifuged for 5 min. If the samples contained particulate material, samples were separated on a 12% separating gel with a 5% stacking gel.

A 12% separating gel was prepared by mixing 1.5 M Tris-HCl (pH 8.8) and 0.4% SDS solution with 30% acrylamide, TEMED, 10% APS and water, so that the final concentrations were 0.375 M Tris-HCl (pH 8.8), 0.1% SDS solution with 12% acrylamide, 0.5% TEMED and 0.5% APS. The TEMED and APS were added just before pouring the gel. Approximately 5 ml of separating gel was made for each gel to be poured. Before the separating gel was polymerised, it was overlaid by water saturated with butanol. When the gel had set, the butanol was removed by thoroughly rinse with water, and the top of the gel blotted was dried using filter paper.

A 5% stacking gel was prepared by mixing 0.25 M Tris-HCl (pH 6.8) and 0.2% SDS solution with 30% acrylamide, TEMED, 10% APS and water, so that the final concentration were 0.125 M Tris-HCl (pH 6.8), 0.1% SDS solution with 3% acrylamide, 0.1% TEMED and 0.1% APS. Approximately 1 ml of stacking

gel was poured on the top of the separating gel. The comb was positioned in the stacking gel as soon as possible after pouring. When the stacking gel was polymerised, gels were arranged into the pairs in a gel tank electrophoresis. The tank was filled with 1X running buffer (1l of 5X running buffer containing: 7.5 g glycine, 15 g Tris, 5 g SDS in distilled water), the comb was carefully removed and samples were loaded into the wells. Protein samples were separated under 200 V for 45 min. Then the separating gel was cut away from the stacking gel, stained in Coomassie Blue Gel Stain solution(0.25% Coomassie Blue Brillant Blue R, 10% glacial acetic acid, 25% methanol and water) for about 1-2 h and de-stained for about 1 h in the mixture of 10% glacial acetic acid and 25% of methanol and water. The gel was used for protein electro-blotting onto PVDF membrane for protein sequencing.

3.3.2.8 Transformation of *K. marxianus* 5057 (XR) gene into *K. marxianus* 5057 cells

(1) Preparation of electrocompetent *K. marxianus* 5057 cell

Electrocompetent *K. marxianus* 5057 cells were prepared following the same method for preparation of the Electrocompetent *P. pastoris* as described above in 3.3.2.7 (2).

(2) Transformation of electrocompetent *K. marxianus* 5057 cell

Transformation of *K. marxianus* 5057 was performed using the electroporation method. For transformation, 1-5 μg of vector was linearised with *Hind*III restriction enzyme to allow the gene integration. *Kluyveromyces* cells (80 μl) prepared as described above in 3.4.6.1 were mixed with 1-5 μg of linearised DNA (in 5 μl sterile water) and then incubated on ice cubes for 5 min. Cells were transferred into a 0.2 cm ice-cold electroporation cuvette and pulsed according to the parameters for yeast (suggested by the manufacturer; 1.5 kV, 200 Ω, 25 μF, using a BioRad gene pulse with a pulse controller). Immediately 1 ml of 1 M ice-cold sorbitol was added into the cuvette. Transformed cells in the cuvette were then transferred into 15 ml sterile tube. The tube was let to incubate at 30°C without shaking for 1-2 h. After finish incubating, transformed cells were plated out by spreading of 50-200 μl on separated, labeled YPD plates containing the appropriate concentrations of Zeocin™.

Plates were incubated for 2-3 days at 30°C. The transformants were selected by their ability to grow at 30°C on YPD plates containing 200 μg/ml of Zeocin™.

(3) Screening of transformants

Colonies (from 3.3.2.8 (2)) were taken using sterile toothpick and patched onto YPD plates containing the various concentrations of Zeocin™ of 200, 500 and 1,000 μg/ml. Plates were incubated for 2-3 days at 30°C. The transformants were selected by their ability to grow at 30°C on YPD plates containing 1,000 μg/ml of Zeocin™.

3.3.3 Growth and xylitol productivity by isolates and recombinant yeasts

3.3.3.1 Preparation of inocula and inoculum media

A loopful of cells, each chosen isolate (*K. marxianus* 5057, *C. guilliermondii* 5068 and *Hansenula anomala* 5302) and recombinant yeasts (rKm1, rKm2, rKm3, rKm4, rKm5, rKm6) was transferred into 50 ml Erlenmeyer flasks with 10 ml of medium containing; 1% glucose, 1% xylose, 3 g/l yeast extract, 3 g/l malt extract and 5 g/l peptone, and cultivated on the rotary shaker at 200 rpm for 24 h at 30°C.

The inocula (10%; v/v), then were grown in 125 ml Erlenmeyer flasks with 25 ml of non-concentrate bagasse hydrolysate containing; approximately 38.5 g/l xylose, 17.0 g/l glucose and the following nutrients: 2.0 g/l $(NH_4)_2SO_4$, 0.1 g/l $CaCl_2 \cdot 2H_2O$ and 20.0 g/l rice bran extract, at 30°C on the rotary shaker (200 rpm) for 24 h. These were used as fermentation inocula.

3.3.3.2 Fermentation medium and fermentation conditions

For all assays, 10% (v/v) inocula culture broth (24 h – old) were incubated in 250 ml Erlenmeyer flasks with 100 ml of bagasse hydrolysate medium containing approximately 42.8 g/l xylose, 19.0 g/l glucose and the following nutrients: 2.0 g/l $(NH_4)_2SO_4$, 0.1 g/l $CaCl_2 \cdot 2H_2O$ and 20.0 g/l rice bran extract, on the rotary shaker (200 rpm) at 30°C for 120 h.

The fermentation tests were monitored by periodic sampling to determine the growth, sugar consumption and xylitol formation using the same condition of the mentioned methods. Samples of appropriate dilutions were prepared by filtration through a 0.45 micron filter.

3.3.4 Batch culture for xylitol production by recombinant *K. marxianus* 5057

3.3.4.1 Microorganisms and inoculum preparation

A loopful of cells, recombinant *K. marxianus* 5057 (rKm6), was grown in 50 ml Erlenmeyer flasks with 10 ml of medium containing; 1% glucose, 1% xylose, 3 g/l yeast extract, 3 g/l malt extract and 5 g/l peptone and cultivated on the rotary incubator at 200 rpm for 24 h at 30°C.

The inoculum (10%; v/v), then was transferred into 125 ml Erlenmeyer flasks with 25 ml of non-concentrate bagasse hydrolysate containing; approximately 37.0 g/l xylose, 17.0 g/l glucose and the following nutrients: 2.0 g/l $(NH_4)_2SO_4$, 0.1 g/l $CaCl_2 \cdot 2H_2O$ and 20.0 g/l rice bran extract, and cultivated for 24 h at 30°C on the rotary shaker (200 rpm). This was used as inoculum for batch fermentation.

3.3.4.2 Fermentation conditions

Aerobic fermentations were operated as batch process in the sugarcane bagasse hydrolysate fermentation medium of the mentioned composition in approximately 50 g/l xylose, 17.0 g/l glucose and the following nutrients: 2.0 g/l $(NH_4)_2SO_4$, 0.1 g/l $CaCl_2 \cdot 2H_2O$ and 20.0 g/l rice bran extract, and cultivated for up to 168 h. The temperature (30°C) was controlled during the process.

Fermentation experiments were performed in 2.5 l bench top fermentor (Eyela M -100, type MOB-2, RIKAKIKAI Co., LTD, JAPAN) with a working volume of 1.2 l. The 10% (v/v) of inoculum culture broth was transferred into a reactor. The agitation speeds using a flat-blade turbine were set at 200, 300 rpm and aeration were varied in the range of 0.5, 1.0 and 1.5 vvm (volume of air per volume of medium per min.) during fermentation.

The fermentation tests were monitored by periodic sampling to determine the growth, sugar consumption and xylitol formation. Samples of appropriate dilutions were prepared by filtration through a 0.45 micron filter. Glucose utilisation, xylose consumption, xylitol production and cell growth were determined using the same condition of the mentioned methods.