CHAPTER 4
RESULTS AND DISCUSSION

4.1 Bagasse pretreatment by acid hydrolysis

The lignocellulose hydrolysates like sugarcane bagasse can be used as fermentation media to obtain xylitol, ethanol and other useful products. During the hydrolysis of sugarcane bagasse, the sugars are released to liquors, mainly xylose and glucose. These sugars can be derived from the cellulosic fraction or from some hetero-polymers of hemicellulosic fraction (Aguilar *et al.*, 2002). It is important to obtain high reducing sugar concentrations since they are the main carbon source of most microorganisms.

The sugarcane bagasse was 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, respectively. The particles of bagasse, then were hydrolysed using sulphuric acid (H_2SO_4). The results in term of reducing sugar (as xylose) are summarised in Table 6-8. The treatments with H_2SO_4 showed no effect of the particle sizes of bagasse in both reducing sugar contents (xylose and glucose). The particle size (< 0.850-4.75 mm) tested during the same reaction time did not result in significant difference ($p \leq 0.05$) in xylose production.

Another important factor in the hydrolysis process is reaction time, as the reaction time increased, the conversion of cellulose and hemicellulose to xylose increased 2-3 folds (Figure 2). However, if the reaction time is longer than 1 hour, xylose concentration decreased due to degradation into furfural and hydroxymethyfurfural (Cruz *et al.*, 2000; Palmqvist & Hahn-Hägerdal, 2000).

The reducing sugar contents were affected by the sulphuric concentration applied (see in Figure 2). The 3% H_2SO_4 pretreated samples yielded higher sugar contents than the samples obtained from 1 and 2% H_2SO_4 pretreated bagasse. The maximum value of xylose was 57.7 g/l treated in the hydrolysate from the 3% H_2SO_4 treatment and the 60 min reaction time.

Table 6 Reducing sugar concentrations (as xylose; g/l) obtained in the 1% (w/v) H_2SO_4 hydrolysis of sugarcane bagasse at 126.7°C (1.5 kg/cm^2 pressure gauge) in the range of 15-60 min reaction time

Time (min)	Particle sizes (mm)	Reducing sugar concentration (as xylose; g/l)
15	< 0.85	10.96[a]
	0.85-1.70	11.03[a]
	1.70-2.38	10.55[a]
	2.38-4.75	12.10[a]
30	< 0.85	13.97[b]
	0.85-1.70	14.08[b]
	1.70-2.38	12.50[b]
	2.38-4.75	13.10[b]
45	< 0.85	24.24[c]
	0.85-1.70	25.58[c]
	1.70-2.38	21.99[c]
	2.38-4.75	21.72[c]
60	< 0.85	32.70[d]
	0.85-1.70	33.34[d]
	1.70-2.38	31.35[d]
	2.38-4.75	30.35[d]

[a,b,c,d]: significant difference ($p \leq 0.05$)

Table 7 Reducing sugar concentrations (as xylose; g/l) obtained in the 2%(w/v H_2SO_4 hydrolysis of sugarcane bagasse at 126.7°C (1.5 kg/cm^2 pressure gauge) in the range of 15-60 min reaction time

Time (min)	Particle sizes (mm)	Reducing sugar concentration (as xylose; g/l)
15	< 0.85	13.19[a]
	0.85-1.70	14.17[a]
	1.70-2.38	13.15[a]
	2.38-4.75	13.73[a]
30	< 0.85	23.32[b]
	0.85-1.70	22.45[b]
	1.70-2.38	21.91[b]
	2.38-4.75	18.60[b]
45	< 0.85	37.36[c]
	0.85-1.70	37.96[c]
	1.70-2.38	35.11[c]
	2.38-4.75	31.51[c]
60	< 0.85	47.38[d]
	0.85-1.70	45.56[d]
	1.70-2.38	45.54[d]
	2.38-4.75	43.58[d]

[a,b,c,d]: significant difference ($p \leq 0.05$)

Table 8 Reducing sugar concentrations (as xylose; g/l) obtained in the 3%(w/v) H_2SO_4 hydrolysis of sugarcane bagasse at 126.7°C (1.5 kg/cm^2 pressure gauge) in the range of 15-60 min reaction time

Time (min)	Particle sizes (mm)	Reducing sugar concentration (as xylose; g/l)
15	< 0.85	20.12[a]
	0.85-1.70	20.10[a]
	1.70-2.38	17.77[a]
	2.38-4.75	18.31[a]
30	< 0.85	31.18[b]
	0.85-1.70	30.63[b]
	1.70-2.38	28.96[b]
	2.38-4.75	30.12[b]
45	< 0.85	45.21[c]
	0.85-1.70	45.76[c]
	1.70-2.38	45.14[c]
	2.38-4.75	39.96[c]
60	< 0.85	57.71[d]
	0.85-1.70	56.64[d]
	1.70-2.38	54.41[d]
	2.38-4.75	53.72[d]

[a,b,c,d]: non significant difference ($p \leq 0.05$)

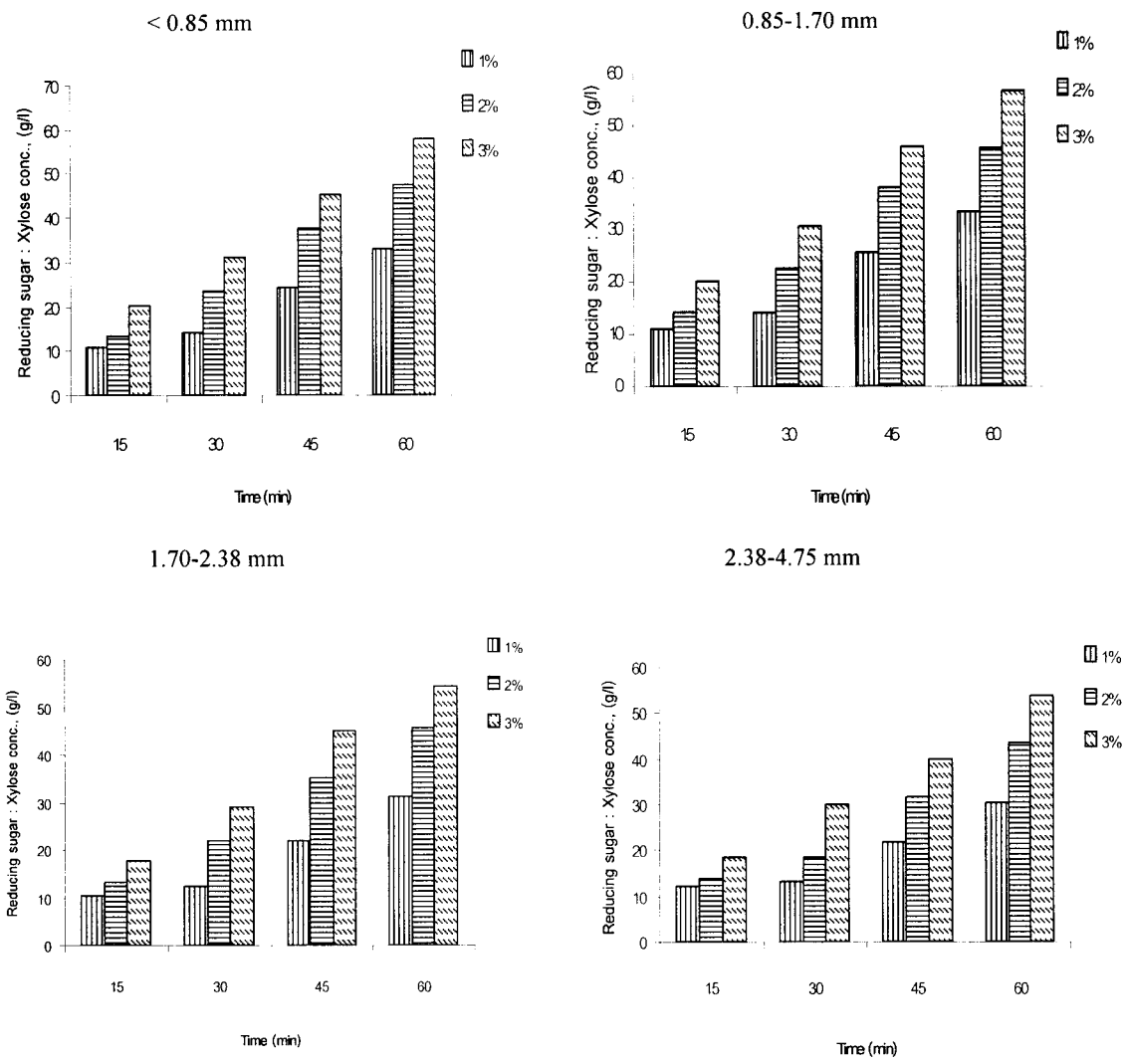

Figure 2 Reducing sugar concentrations (as xylose; g/l) obtained in 1-3%(w/v) H_2SO_4 hydrolysis of sugarcane bagasse at 126.7°C (1.5 kg/cm^2 pressure gauge) in the range of 15, 30, 45 and 60 min reaction times

In conclusion one could observe that, the particles sizes (≤0.85, 0.85-1.70, 1.70-2.38, and 2.38-4.75 mm) of sugar cane bagasse had no effect on hydrolysis, however 3% H_2SO_4 hydrolysis of sugarcane bagasse at 126.7°C (autoclaved at 1.5 kg/cm^2 pressure gauge) for 60 min resulted in the maximum yield (57.7 g/l) of xylose. Furthermore, the lignocellulosic substrates need to be pretreated and neutralised to attain the fermentation pH, thereby becoming more suitable for microorganism

metabolism (Roberto *et al.,* 1991a&b; Winkelhausen & Kuzmanova, 1998 and Mussatto & Roberto, 2004).

Consequently, 3% H_2SO_4 was used for hydrolysis of sugarcane bagasse at 126.7°C (autoclaved at 1.5 kg/cm^2 pressure gauge) for 60 min. After hydrolysis, the sugarcane bagasse hydrolysate was treated and neutralised for selection of yeast strains use.

4.2 Genetic manipulation of yeasts from selected xylitol producing yeasts

4.2.1 Growth of selected yeasts on different carbon source media

The yeasts were grown on YPD agar plate majority containing glucose, on agar medium containing xylose and the medium containing xylitol. Since glucose is the most readily fermentable sugar, most of yeasts; *C. guilliermondii* 5068, *K. marxianus* 5057 and *H. anomala* 5302 which utilise glucose can grow in YPD agar plate (see in Figure 3- 5). Both *C. guilliermondii* 5068 and *H. anomala* 5302 showed the predominant growth on the most of three different carbon source plates. Meanwhile, *K. marxianus* 5057 was clearly grown only on the glucose agar plate.

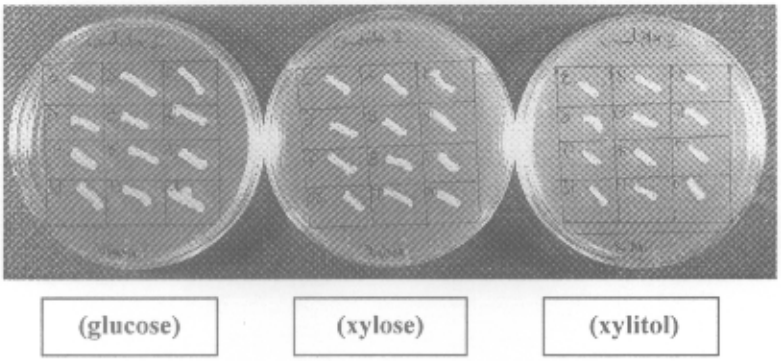

Figure 3 Growth of *C. guilliermondii* 5068 on different carbon sources media.

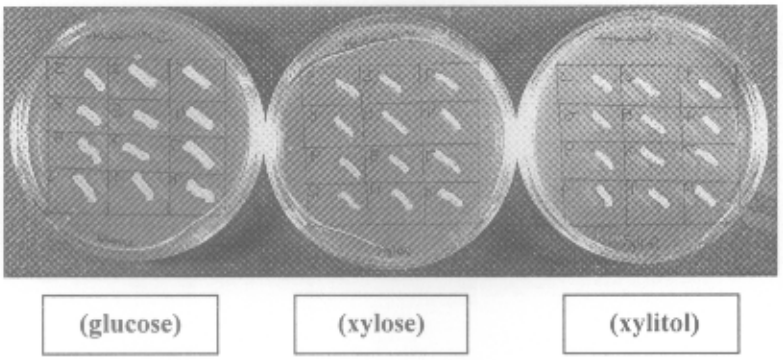

Figure 4 Growth of *H. anomala* 5302 on different carbon sources media.

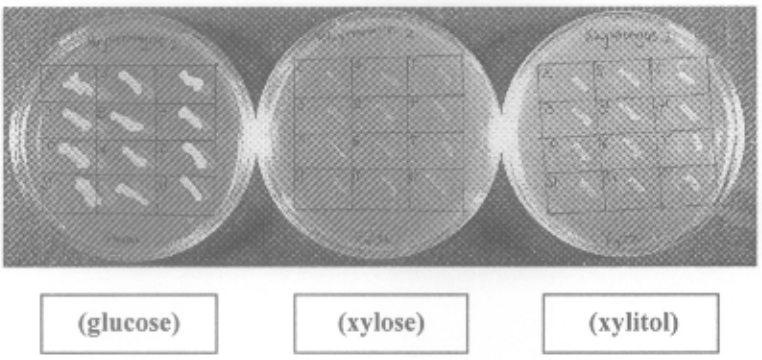

Figure 5 Growth of *K. marxianus* 5057on different carbon sources media.

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

Xylose reductase (XR) is a key enzyme in D - xylose metabolism, catalysing the reduction of D-xylose. After extraction of genomic DNA, the extracted DNA from *C. guilliermondii* 5068, *K. marxianus* 5057 and *H. anomala* 5302 was analysed on agarose gel. The result of DNA gel showed in Figure 6. In order to isolate of xylose reductase gene from *C. guilliermondii* 5068, specific primers for *C. guilliermondii* 5068 (XR) gene, based on the sequence alignment of *C. guilliermondii* which reported in GenBank database was used. 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*, *Pichia stipitis*, *P. guilliermondii* and *K. lactis* which reported in GenBank database.

After genomic DNA of *C. guilliermondii* 5068 and *K. marxianus* 5057 were extracted and amplified, XR fraction appeared in an agarose gel as a single band XR fraction *K. marxianus* 5057 appeared in a gel as a single band with about 900 bases pair in length (see Figure 7 & 8). This *K. marxianus* 5057 PCR product was used to generate DNA probe as the specific xylose reductase (XR) PCR probe labeling with digoxygenin for further experiment.

Figure 6 Genomic DNA extraction from *C. guilliermondii, H. anomala* and *K. marxianus.*

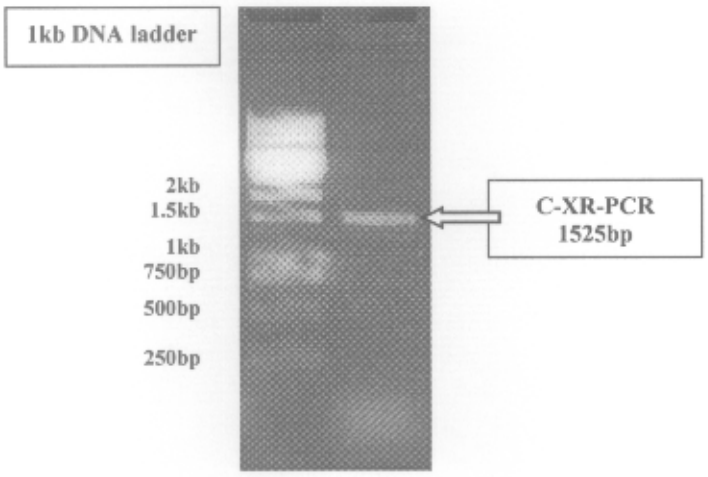

Figure 7 PCR production of xylose reductase (XR) gene from *C. guilliermondii.*

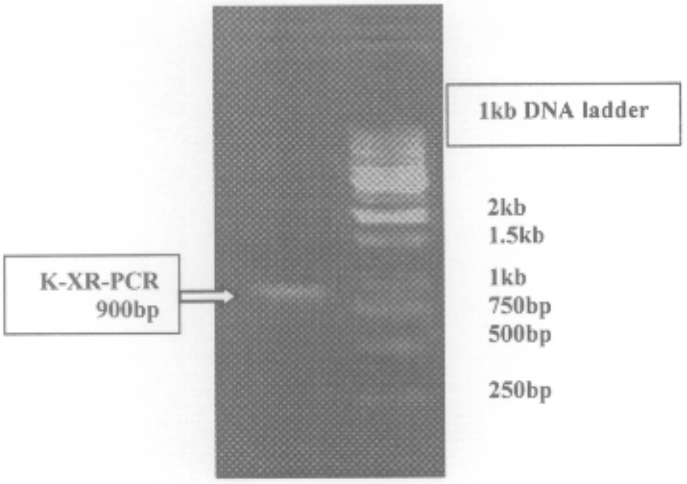

Figure 8 PCR production of xylose reductase (XR) gene from *K. marxianus* 5057.

Since XR gene of *K. marxianus* 5057 was isolated and showed the least ability to grow on xylitol medium, meanwhile, xylitol dehydrogenase, the important enzyme converted xylitol to xylulose, could not isolated from *K. marxianus* 5057 (data not showed). Consequently, *K. marxianus* 5057 was chosen for increasing XR gene to maximise the xylitol production.

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

PCR amplified DNA products of *K. marxianus* 5057 were cloned into pCR® - TOPO vectors and sequenced. The result showed nucleotide alignment between the PCR product and those yeast species are 79 % identity (see in Figure 14-15).

4.2.4 Determination of xylose reductase (XR) expression

K. marxianus 5057 were grown in varies carbon sources growth media; YPD (glucose broth), YPDX (glucose-xylose broth) and YPX (xylose broth) at 30°C in shaking incubator (200 rpm) for 5 days. The 50 ml of medium cultures were collected different time at 2, 3, 4 and 5 days and harvested by centrifugation at 3000 rpm for 10 min at room temperature. Total RNA was extracted from each sample according to Schmitt (1990), run onto a formaldehyde gel and transferred to nylon membrane for Northern - blot hybridization. The positive result of digested RNA hybridization with the specific xylose reductase PCR probe was visualised by colorimetric detection. Colorimetric detection was performed in the presence of nitroblue tetrazulium (NBT) and 5-bromo-4chloro-3-indolyl phosphate (BCIP),

substrates of the alkaline phosphatase enzyme conjugated to the anti-digoxigenin antibodies.

In this experiment, expression of *K. marxianus* 5057 XR gene could not determined. So, DNA blotting was conducted for XR gene expression. However, the result of DNA blotting showed the expression of *K. marxianus* 5057 XR gene (see in Figure 9).

Consequently, hydrolysate from sugarcane bagasse was used as the carbon source. *K. marxianus* 5057 was grown in sugarcane bagasse hydrolysate broth as the same conditions of growing in YPD, YPDX and YPX broth. After total RNA was extracted, run onto a formaldehyde gel and transferred to nylon membrane for Northern - blot hybridization. The positive result of digested RNA hybridization with the specific xylose reductase PCR probe was visualised by colorimetric detection which gave the expression of xylose reductase in *K. marxianus* 5057.

K. marxianus 5057 showed the highest expression of xyloes reductase in sugarcane hydrolysate after 4 day culture time (Figure 10). This condition of culture was used for growing of *K. marxianus* 5057 to produce xylose reductase protein in the next trial.

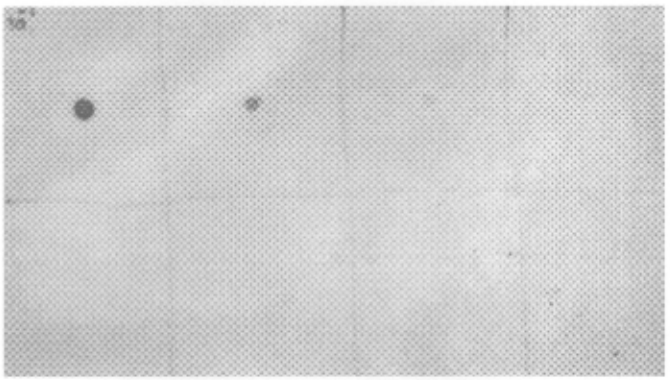

Figure 9 DNA Dot blotting analysis showing xylose reductase (XR) gene in *K. marxianus* 5057.

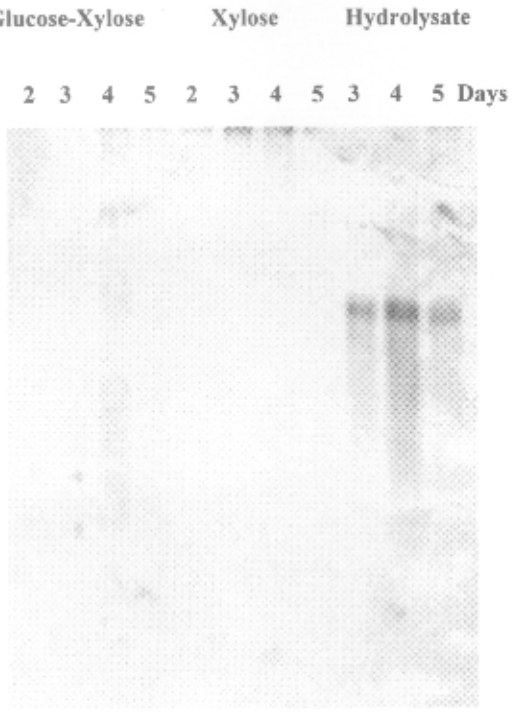

Figure 10 Northern - blot analysis showing xylose reductase (XR) gene in *K. marxianus* 5057 grown on various carbon sources and period of time; (YPDX Glucose-Xylose medium), YPX (Xylose medium) and sugarcane bagasse hydrolysate medium and for 2, 3, 4 and 5 days, respectively.

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

To isolate the full length xylose reductase gene, *K. marxianus* was cultured in YPDX medium at 30°C in shaking incubator (200 rpm) for 24 h, then the genomic DNA was extracted from *K. marxianus* 5057 and digested with *AccI, AsnI, BglII, ClaI, Hind III, PvuI, PvuII, PstI, SacI, SmaI* and *XhoI* restriction enzymes. The digested DNA from each enzyme digest was run onto an agarose gel and transferred to a nylon membrane. After hybridization of the digested DNA on the nylon membrane with the specific xylose reductase PCR probe, the result showed Southern blot hybridization in Figure 11. The size of the hybridization fragment increased to the favoured 4.5 kb as a site of *SacI* digested genomic DNA, the others were to be big size to encode into the yeast cells.

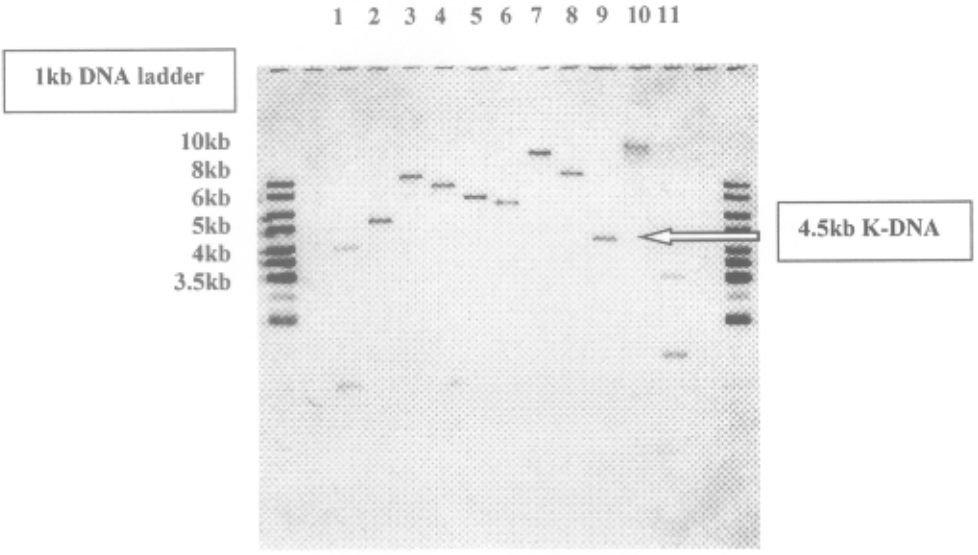

Figure 11 Southern – blot analysis showing the 4.5 kb *SacI* fragment.

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

The positive band at the size of 4.5 kb was gel purified, subcloned in pUC 18 and transformed into *E. coli* following the mention above. The transforming bacteria, *E. coli,* was grown in LB broth at 37°C in shaking incubator at 250 rpm for 24 h. The culture was dropped onto Gridded membranes, the membranes then transferred into YPX agar medium and cultivated for 24 h. The bacterial colonies were used for colony hybridization. Colony hybridization was performed at 42°C using the same specific DNA probe.

As shown in Figure 12, two colonies showed positive hybridisation to the specific probe. Plasmids were then extracted from each colony for sequencing analysis. The nucleotide sequences of *K. marxianus* 5057 XR gene (from 2 colonies) showed in Figure 13 & 14, which were compared with *K. lactis* xylose reductase (XYL1) gene from database GenBank (see in Figure 15 & 16).

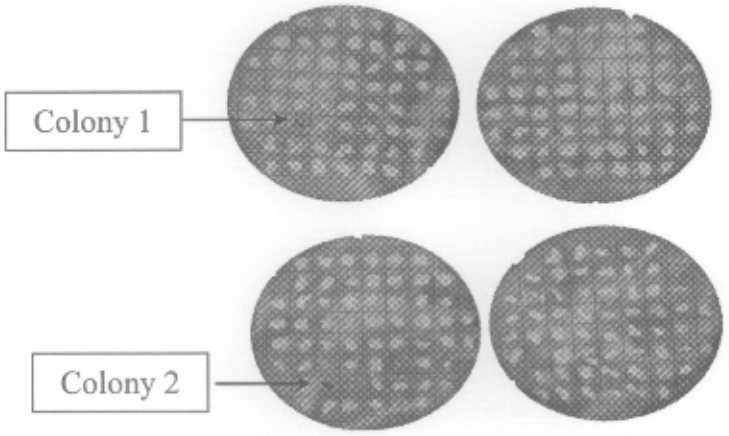

Figure 12 Positive colonies hybridisation with blue colour.

Colony 1 Sequence

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1       GAGCTCATTG TTTCGTCTGG TGCTGTAATT TGCTTGGGTT TTCTTTCGCC TACTGCCTTG
61      TGTTTTTGAC TTTGTCTCCA AGGACGTGCG CACCCACCTG GACTTAAGGG ACAATCTGGC
121     AGAGGCAGAG GCAGAGGCAG AGGCCCAGGC ATTGCTCTGG GCCTGGGCCT GGAACTATGC
181     CTGGGCTGCG CCCAGGCATT GGTTGGCTTA GGCCCTTCCC ACCACCCATA TCAAACCCAT
241     ATCAACCGCA ATTGCTCTCG TGAAGGTTTG TATTTTGCAT TTTTCTGTAT AAATAGGGCT
301     GGCATGGAAT TGTCACAGCA ATCTGATCAG TCAGTCCCCT TACTACTTGC TAGTAGTTGT
361     AACACTGGTT GTAATAGTAT ATGTAGTAAA TAGTCGTGAC AGCAGTGTCA ATTATCTCCA
421     CCAAAACACA GTATAGAAAC ACCATGACAT ACCTCCCACC AACAGTTACC TTGAACAATG
481     GATCCAAGAT GCCGCTAGTC GGCTTGGGAT GCTGGAAAAT CCCAAACGAA GTGTGTGCCG
541     AACAGGTGTA CGAAGCCATC AAGTTGGGCT ACCGCTTGTT CGACGGCGCG CAGGACTACG
601     CCAACGAAAA AGAGGTGGGC CAAGGTATTA ACAGAGCCAT CAAGGAAGGA ATCGTCAAGA
661     GAGAAGACTT GGTCGTCGTT TCTAAGTTGT GGAACAGTTT CCACCACCCA GACAACGTGC
721     GTACCGCAGT CGAAAGAACC TTGAACGACT TGCAATTGGA CTACTTGGAC TTGTTCTACA
781     TCCATTTCCC ATTGGCTTTC AAGTTCGTGC CACTAGACGA AAAGTACCCT CCAGGTTTCT
841     ACACAGGTAA GGACAATTTC GCCAAGGAAA TCATCGAAGA GGAGCCTGTC CCAATCTTGG
901     ACACCTACAG AGCCCTCGAG AAGTTGGTCG ACGAAGGTTT GATCAAATCT TTGGGTATCT
961     CAAACTTTTC GGGTGCATTG ATCCAGGACT TGTTGCGTGG CGTCCGTATC AAGCCAGTCG
1021    CCTTGCAGAT CGAACACCAC CCATACTTGG TCCAGGACCG TTTGATCACG TACGCCCAAA
1081    AGGTGGGCTT GCAAGTCGTC GCCTACTCCA GTTTCGGCCC ACTATCCTTT CTCGAGTTGA
1141    ACAACGAAAA GGCCTTGCAC ACAAAGACTT TGTTCGAAAA CGACACCATC AAGGCCATCG
1201    CTCAAAAACA TAACGTAACC CCATCCCACG TCTTGTTGAA GTGGTCCACC CAACGTGGTA
1261    TCGCCGTCAT TCCAAAGTCC TCCAAGAAGG AACGTCTCCT CGAGAACTTG AAGATCGAAG
1321    AGACCTTTAC CTTGTCCGAC GAAGATATCA AGGAGATCAA CGGCTTGGAC CAGGGATTGA
1381    GATTTAACGA CCCATGGGAC TGGTTGGGCA ACAAATTCCC AACCTTTATC TAAGATACTT
1441    TCTTTCCCCT TCCCATGTCA AATGATGAAA CGAATGCTTA TATACTCTGT ATATTGGATG
1501    GGCGTCCCAC CACCCTTTCC TCCCCTATAT AGATGATGGC TTTCAAAAAT CAAATGTGTT
1561    TCCGTTGTCC CTCTGGACAA CGTTCACTAA CTTCTTCCTT CTTTTGACAC TTTTTTTTGT
1621    CAGATCGAAC GTGCCTAAAC TATATGTCTA GGCTATGCCC CTCACTTTAC TAATATGTCC
1681    GGTGGCTCTA GCTTCTGGCT TCTGGTTTCT GATTGTGTTT TCTTCTTATT CTGACTAAAC
1741    TCGCTTTGTT CTGGCTCATC GCTTTGATTT GTCACACAGG CCTGGAATTT TTCATGAAAA
1801    CTGATATAAA GACCCGACCT AATTGTCAAA AGCAGCCACA CCAAACGAAA GTGAAAACAC
1861    ACTGTTAATA TGTACAATCT AAATTCAATA GAAGTGTATC ACCACTAGTA CATGCGTATA
1921    CCCATACAAC AAATACAACG CAGAATGGTT CATCACAGGG TAACAATTAT TGGGTTCCGG
1981    CCCAGCAGCC CACACCGCCG CCATTTACTT GGCTAGAAGC AGAAATCAAG CCTACCCTAT
2041    ACGANGGTTT CATGGCTAAC GGTATCGCTG CTGGTGGTCA ACTAACNAAC CACCACTGAA
2101    ATCGAAAACT TCCCAAGGTT TCCAGAAAGG TTTGACCGGN ANGTGAAATG GATGGGATAA
2161    NATGAANGGT CCAATCTGTC AAGNTTTGG
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Figure 13 Sequences of *K. marxianus* 5057 XR gene (colony 1).

Colony 2 Sequence

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1       GAGCTCATTG TTTCGTCTGG TGCTATAATT TGCTTGGGTT TTCTTTCGCC TACTGCCTTG
61      TGTTTTTGAC TTTGTCTCCA AGGACGTGCG CACCCACCTG GACTTAAGGG ACAATCTGGC
121     AGAGGCAGAG GCAGAGGCCC AGGCATTGCT CTGGGCCTGG GCCTGGAACT ATGCCTGGGC
181     TGCGCCCAGG CATTGGTTGG TTTAGGCCCT TCCCACCACC CATATCAAAC CCATATCAAC
241     CGCAATTGCT CTCGTGAAGG TTTGTATTTT GCATTTTTCT GTATAAATAG GGCTGGCATG
301     GAATTGTCAC AGCAATCTGA TCAGTCAGTC CCCTTACTAC TTGCTAGTAG TTGTAACACT
361     GGTTGTAATA GTATATGTAG TAAATAGTCG TGACAGCAGT GTCAATTATC TCCACCAAAA
421     CACAGTATAG AAACACCATG ACATACCTCC CACCAACAGT TACCTTGAAC AATGGATCCA
481     AGATGCCGCT AGTCGGCTTG GGATGCTGGA AAATCCCAAA CGAAGTGTGT GCCGAACAGG
541     TGTACGAAGC CATCAAGTTG GGCTACCGCT TGTTCGACGG CGCGCAGGAC TACGCCAACG
601     AAAAAGAGGT GGGCCAAGGT ATTAACAGAG CCATCAAGGA AGGAATCGTC AAGAGAGAAG
661     ACTTGGTCGT CGTTTCTAAG TTGTGGAACA GTTTCCACCA CCCAGACAAC GTGCGTACCG
721     CAGTCGAAAG AACCTTGAAC GACTTGCAAT TGGACTACTT GGACTTGTTC TACATCCATT
781     TCCCATTGGC TTTCAAGTTC GTGCCACTAG ACGAAAAGTA CCCTCCAGGT TTCTACACAG
841     GTAAGGACAA TTTCGCCAAG GAAATCATCG AAGAGGAGCC TGTCCCAATC TTGGACACCT
901     ACAGAGCCCT CGAGAAGTTG GTCGACGAAG GTTTGATCAA ATCTTTGGGT ATCTCAAACT
961     TTTCAGGTGC ATTGATCCAG GACTTGTTGC GTGGCGTCCG TATCAAGCCA GTCGCCTTGC
1021    AGATCGAACA CCACCCATAC TTGGTCCAGG ACCGTTTGAT CACGTACGCC CAAAAGGTGG
1081    GCTTGCAAGT CGTCGCCTAC TCCAGTTTCG GCCCACTATC CTTTGTCGAG TTGAACAACG
1141    AAAAGGCCTT GCACACAAAG ACTTTGTTCG AAAACGACAC CATCAAGGCC ATCGCTCAAA
1201    AACATAACGT AACCCCATCC CACGTCTTGT TGAAGTGGTC CACCCAACGT GGTATCGCCG
1261    TCATTCCAAA GTCCTCCAAG AAGGAACGTC TCCTCGAGAA CTTGAAGATC GAAGAGACCT
1321    TTACCTTGTC CGACGAAGAT ATCAAGGAGA TCAACGGCTT GGACCAGGGA TTGAGATTTA
1381    ACGACCCATG GGACTGGTTG GGCAACGAAT TCCCAACCTT TATCTAAGAT ACTTTCTTTC
1441    CCCTTCCCAT GTCAAATGAT GAAACGAA
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Figure 14 Sequences of *K. marxianus* 5057 XR gene (colony 2).

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gi|559294|gb|L36993.1|YSKXYL  Kluyveromyces lactis xylose reductase (XYL1) gene,
complete cds Length=1752

Score =  137 bits (69), Expect = 2e-28 Identities = 312/393 (79%), Gaps = 0/393 (0%)
Strand=Plus/Plus   K45

Query  461   AACAGTTACCTTGAACAATGGATCCAAGATGCCGCTAGTCGGCTTGGGATGCTGGAAAAT  520
             ||||||||| || ||||||||    ||||||||||||||||||| || |||||||| ||
Sbjct  717   AACAGTTACTTTAAACAATGGCGAAAAGATGCCGCTAGTCGGCTTAGGTTGCTGGAAGAT  776

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Sbjct  897   CAAAGAAGGGCTTGTTAAGAGAGAGGATTTAGTTGTTGTCTCCAAGCTATGGAACAGTTT  956


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              ||||| |||||||||||||||| ||||||||
Sbjct  1077  GAAGTATCCTCCAGGTTTCTACACCGGTAAGGA  1109

Score = 56.0 bits (28), Expect = 5e-04 Identities = 76/92 (82%), Gaps = 0/92 (0%)
Strand=Plus/Plus

Query  927   GTCGACGAAGGTTTGATCAAATCTTTGGGTATCTCAAACTTTTCGGGTGCATTGATCCAG  986
             |||||| ||||| |||||| || |||||||| || |||||||| ||||| ||||||||
Sbjct  1183  GTCGACCAAGGTAAGATCAAGTCCTTGGGTATTTCCAACTTTTCAGGTGCGTTGATCCAA  1242

Query  987   GACTTGTTGCGTGGCGTCCGTATCAAGCCAGT  1018
             || ||| | ||||| |  ||||||||||||||
Sbjct  1243  GATTTGCTACGTGGTGCTCGTATCAAGCCAGT  1274
```

Figure 15 Sequences blast of *K. marxianus* 5057 XR gene (colony 1) with *K. lactis* xylose reductase (XYL1) gene from database GenBank.

gi|559294|gb|L36993.1|YSKXYL *Kluyveromyces lactis* xylose reductase(XYL1)gene,complete cds Length=1752

Score = 137 bits (69), Expect = 1e-28 Identities = 312/393 (79%),Gaps = 0/393 0%)
Strand=Plus/Plus

```
Query  455   AACAGTTACCTTGAACAATGGATCCAAGATGCCGCTAGTCGGCTTGGGATGCTGGAAAAT  514
             ||||||||| || ||||||||    ||||||||||||||||||| || ||||||||  ||
Sbjct  717   AACAGTTACTTTAAACAATGGCGAAAAGATGCCGCTAGTCGGCTTAGGTTGCTGGAAGAT  776

Query  515   CCCAAACGAAGTGTGTGCCGAACAGGTGTACGAAGCCATCAAGTTGGGCTACCGCTTGTT  574
              || ||||| || |||||||| ||  | ||||||||||| ||| | || || || || ||
Sbjct  777   GCCCAACGACGTTTGTGCCGACCAAATTTACGAAGCCATTAAGATCGGATATCGTTTATT  836

Query  575   CGACGGCGCGCAGGACTACGCCAACGAAAAAGAGGTGGGCCAAGGTATTAACAGAGCCAT  634
             ||| || || || || ||||||||||| ||||| || || || ||| | ||||||||||
Sbjct  837   CGATGGTGCCCAAGATTACGCCAACGAGAAAGAAGTTGGACAGGGTGTCAACAGAGCCAT  896

Query  635   CAAGGAAGGAATCGTCAAGAGAGAAGACTTGGTCGTCGTTTCTAAGTTGTGGAACAGTTT  694
             ||| |||||  | || |||||||| || || || || || || ||| | ||||||||||
Sbjct  897   CAAAGAAGGGCTTGTTAAGAGAGAGGATTTAGTTGTTGTCTCCAAGCTATGGAACAGTTT  956

Query  695   CCACCACCCAGACAACGTGCGTACCGCAGTCGAAAGAACCTTGAACGACTTGCAATTGGA  754
             |||||| || |||||||| | |   ||  | ||||||||  |   ||| ||||||||||
Sbjct  957   CCACCATCCGGACAACGTACCTCGTGCTTTGGAAAGAACTCTTTCCGATTTGCAATTGGA  1016

Query  755   CTACTTGGACTTGTTCTACATCCATTTCCCATTGGCTTTCAAGTTCGTGCCACTAGACGA  814
             |||  | ||| | ||||||||||||||||||||||| ||||||  ||||||  | || ||
Sbjct  1017  CTATGTTGACATATTCTACATCCATTTCCCATTGGCCTTCAAGCCTGTGCCATTCGATGA  1076

Query  815   AAAGTACCCTCCAGGTTTCTACACAGGTAAGGA  847
              ||||| ||||||||||||||| ||||||||
Sbjct  1077  GAAGTATCCTCCAGGTTTCTACACCGGTAAGGA  1109
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Score = 63.9 bits (32), Expect = 1e-06 Identities = 77/92 (83%), Gaps = 0/92 (0%)
Strand=Plus/Plus

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Query  921   GTCGACGAAGGTTTGATCAAATCTTTGGGTATCTCAAACTTTTCAGGTGCATTGATCCAG  980
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Sbjct  1183  GTCGACCAAGGTAAGATCAAGTCCTTGGGTATTTCCAACTTTTCAGGTGCGTTGATCCAA  1242

Query  981   GACTTGTTGCGTGGCGTCCGTATCAAGCCAGT  1012
             || ||| | ||||| |  ||||||||||||||
Sbjct  1243  GATTTGCTACGTGGTGCTCGTATCAAGCCAGT  1274
```

Figure 16 Sequences blast of *K. marxianus* 5057 XR gene (colony 2) with *K. lactis* xylose reductase (XYL1) gene from database GenBank.

4.2.7 Expression of the *K. marxianus* 5057 XR gene in *Picha pastoris* GS115

Plasmid DNA of *E. coli* transformant (amplified xylose reductase from DNA of *K. marxianus* 5057 at 4.5 kb) was subcloned in TOPO, and digested with *Sna*B*I* and *Not*I restriction enzymes. The subclone was then ligated in pPIC 9 and transformed into *P. pastoris* using electroporation method according to Becker and Guarente (1991). The transforming *P. pastoris* GS115 was grown on histidine-deficient medium at 30°C for 48-72 h. The colonies of transformants were selected on their ability to grow on histidine-deficient medium. The chosen colonies were then cultivated in Minimmal methanol (MM) medium followed by Minimal dextrose (MD) medium at 30°C. The XR protein was observed on SDS-PAGE from those colonies.

The xylose reductase protein of *K. marxianus* 5057 expressed in *P. pastoris* GS115 was shown in Figure 17. The apparent molecular weight value is 37.5 kD. The protein sequence of XR gene from *Pichia* expression has 329 amino acids (see in Figure 18).

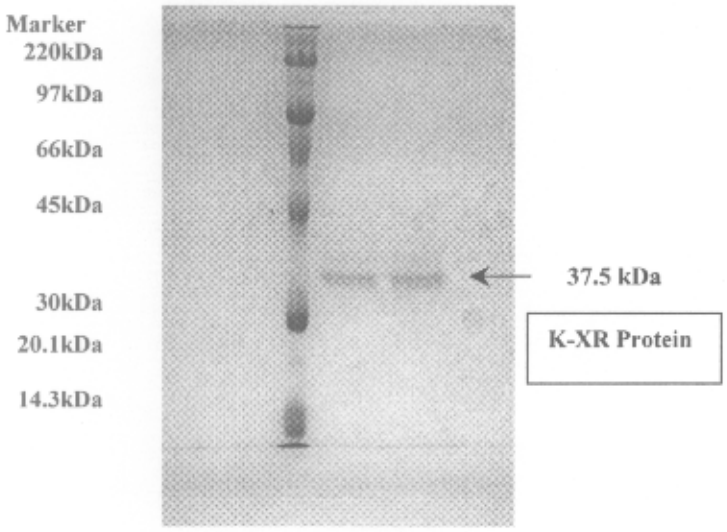

Figure 17 SDS-PAGE showing XR protein and the molecular mass in *P. pastoris* GS115

Colony 1 protein

```
1      MTYLPPTVTL NNGSKMPLVG LGCWKIPNEV CAEQVYEAIK LGYRLFDGAQ DYANEKEVGQ
61     GINRAIKEGI VKREDLVVVS KLWNSFHHPD NVRTAVERTL NDLQLDYLDL FYIHFPLAFK
121    FVPLDEKYPP GFYTGKDNFA KEIIEEEPVP ILDTYRALEK LVDEGLIKSL GISNFSGALI
181    QDLLRGVRIK PVALQIEHHP YLVQDRLITY AQKVGLQVVA YSSFGPLSFL ELNNEKALHT
241    KTLFENDTIK AIAQKHNVTP SHVLLKWSTQ RGIAVIPKSS KKERLLENLK IEETFTLSDE
301    DIKEINGLDQ GLRFNDPWDW LGNKFPTFI*
```

Figure 18 Amino acid sequences of XR gene of *K. marxianus* in *P. pastoris* GS115

4.2.8 Transformation of XR gene into *K. marxianus* 5057

Plasmid DNA of *K. marxianus* 5057 at 4.5 kb from pUC18 was digested with *Sac*I, then ligated in pPICZ A, and transformed into *K. marxianus* using electroporation method. The transformants, *K. marxianus*, were cultivated on YPD agar plates containing 100 μg/ml of zeocin at 30°C for 48-72 h. The selected colonies were then transferred and grown on YPD agar plates containing zeocin; varies in the concentration of 200, 500 and 1,000 μg/ml, at 30°C for 48-72 h. The colonies that grown on YPD agar plates containing 1,000 μg/ml of zeocin were collected.

Consequently, the genomic DNA from cloned *K. marxianus* 5057 was extracted and digested with *Sac*I restriction enzyme. The DNA fragment was probed using the specific xylose reductase PCR probe. In this present work, 6 colonies of recombinant *K. marxianus* 5057 (rKm1, rKm2, rKm3, rKm4, rKm5, rKm6) were chosen for further experiment to maximise xylitol production from sugarcane baggase hydrolysate.

4.3 Growth and xylitol productivity of the isolates and recombinant yeasts

Growth and xylitol production of yeasts both wild type strains (*C. guilliermondii* 5068, *H. anomala* 5302, *K. marxianus* 5057) and clone cultures of *K. marxianus* 5057 (rKm1, rKm2, rKm3, rKm4, rKm5, rKm6) were investigated. It was found that during the first 24 h fermentation, the wild tested cultures appeared to utilise glucose more rapidly in bagasse medium for growth (Figure 19 & 21) and complete depletion, while a slightly changes of xylose utilisation was occurred during fermentation as compared with the cloned cultures, except for *C. guilliermondii* 5068 (see Figure 20).

The concentration profiles of another sugar (arabinose), acetic acid and ethanol as a function of fermentation time are shown in Figure 23 - 25. Like most of xylose - metabolising yeasts, the isolated wild type cultures and the cloned tested yeasts were not able to strongly ferment arabinose (see Figure 18). The results agreed with the reported for *Candida* species (Kim *et al.*, 1999), especially for *C. guilliermondi* (Roberto *et al.*, 1994; Sene *et al.*, 2001).

Approximately 7.0 g/l of acetic acid was found in sugarcane bagasse hydrolysate that was higher amounts than from those corncob and rice straw hemicellulose hydrolysate (Roberto *et al.*, 1994; Kim *et al.*, 1999). During the fermentation process, acetic acid concentration slightly decreased in the yeast cultures, except for the *C. guilliermondii* 5068, acetic acid concentration appeared to decrease sharply reaching nearly zero after 72 h fermentation. In contrast, in the culture media fermented by *H. anomala* 5302 and the recombinant rKm6, acetic acid concentration appeared to increase after 48 h fermentation. No negative influence of acetic acid was clearly identified. However, Lee & Mc Caskey (1983) have suggested that low concentrations of acetic acid in the medium can improve the growth of microrganisms.

The high amounts of ethanol production were found in the yeast culture media during 24 h fermentation and appeared to decrease until the fermentation processes were finished, except for the hydrolysate medium fermented by *C. guilliermondii* 5068. This is because *Candida* species is able to produce both ethanol and xylitol from xylose (Domínguez, 1996)

Among the 3 chosen wide type strains and 6 clone cultures in shaking flask cultures (200 rpm) regarding their ability to produce xylitol from baggase hydrolysate medium, more yield of xylitol was performed in the clone tested cultures than in the wild type cultures. The concentrations of xylitol yield were rapidly increased in clone tested cultures after 48 h fermentation. The highest yield of xylitol (15.64 g/l) with 0.13 g/l/h xylitol productivity was found in rKm6 cloned culture after 120 h fermentation (see Figure 22).

On the conclusion, the xylitol production after 120 hours fermentation of tested cultures, *C. guilliermondii* 5068, *H. anomala* 5302, *K. marxianus* 5057, rKm1, rKm2, rKm3, rKm4, rKm5 and rKm6 were 7.41, 3.41, 2.62, 11.04, 9.67, 6.91, 12.48,

14.83 and 15.64 g/l with the xylitol productivity of 0.06, 0.03, 0.02, 0.09, 0.08, 0.06, 0.10, 0.12 and 0.13 g/l/h, respectively. Since the high potential ability for xylitol production, the clone tested cultures, rKM6 was chosen for the production of xylitol in a reactor. However, the xylose consumption and the xylitol production were strongly affected by the aeration level (Mayerhoff *et. al,* 1997). Consequently, the effect of aeration rate on xylitol production would be continued observed.

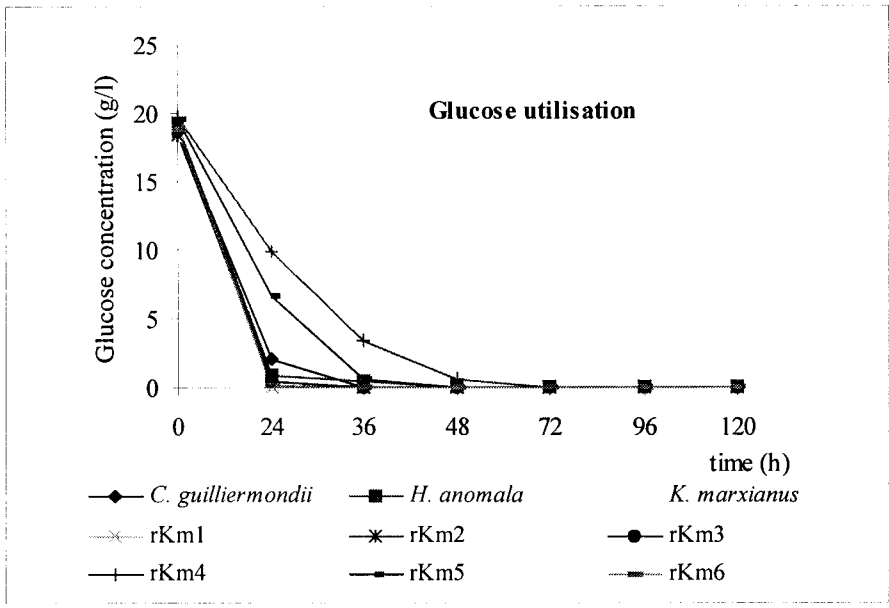

Figure 19 Glucose utilisation (g/l) during batch fermentation in sugarcane bagasse hydrolysate by different yeasts.

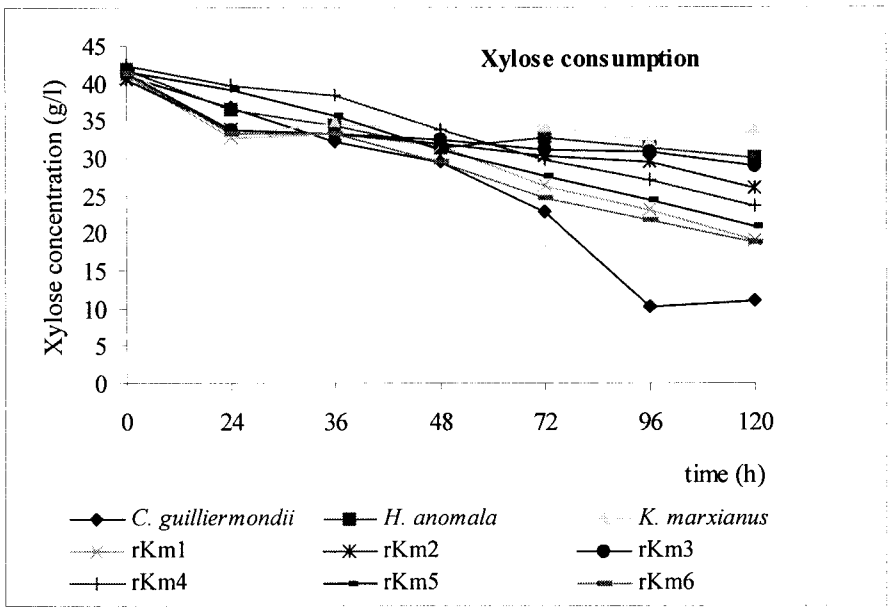

Figure 20 Xylose consumption (g/l) during batch fermentation in sugarcane bagasse hydrolysate by different yeasts.

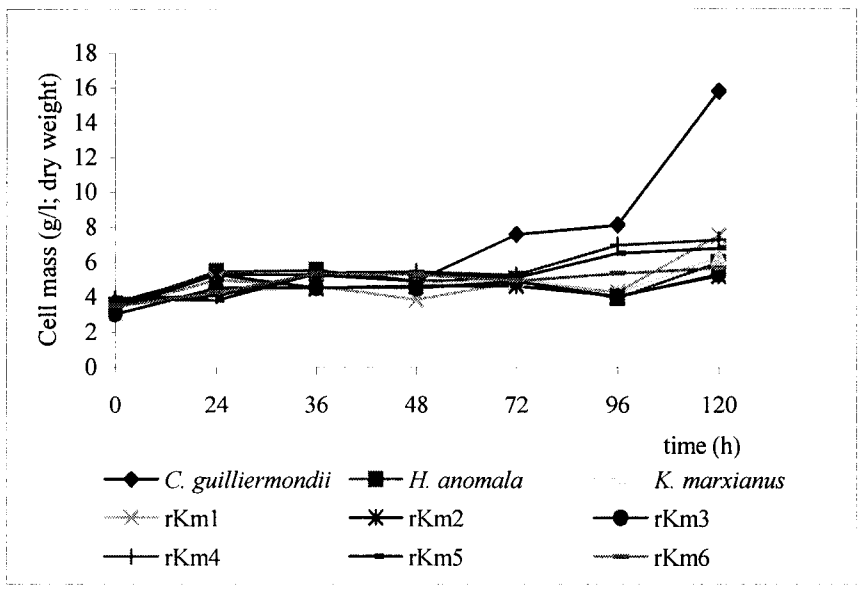

Figure 21 Cell mass (as dry weight; g/l) during batch fermentation in sugarcane bagasse hydrolysate by different yeasts.

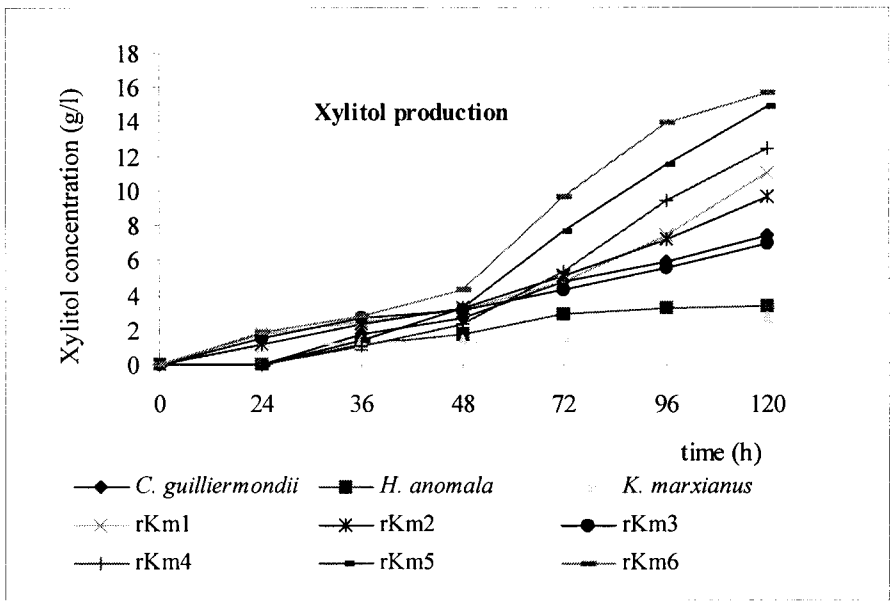

Figure 22 Xylitol production (g/l) during batch fermentation in sugarcane bagasse hydrolysate by different yeasts.

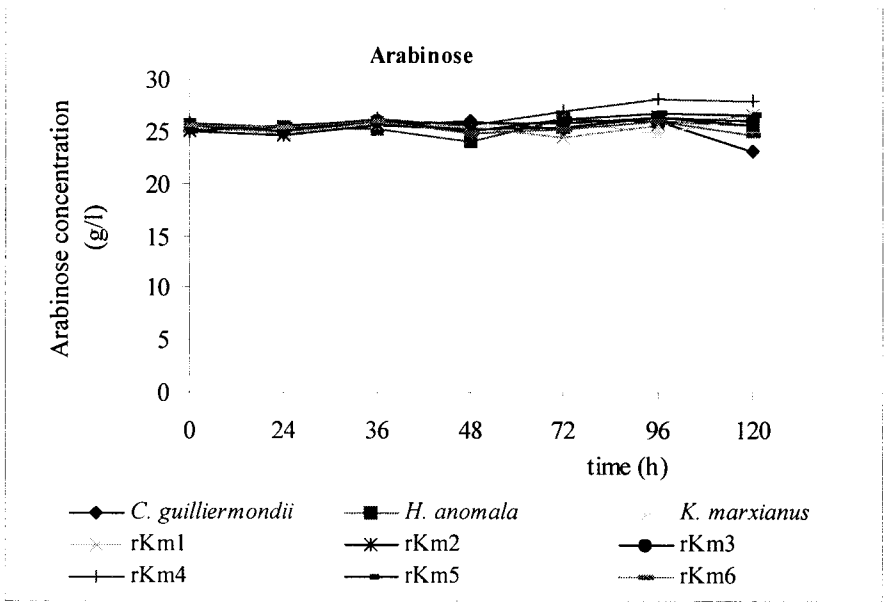

Figure 23 Arabinose concentrations (g/l) during batch fermentation in sugarcane bagasse hydrolysate by different yeasts.

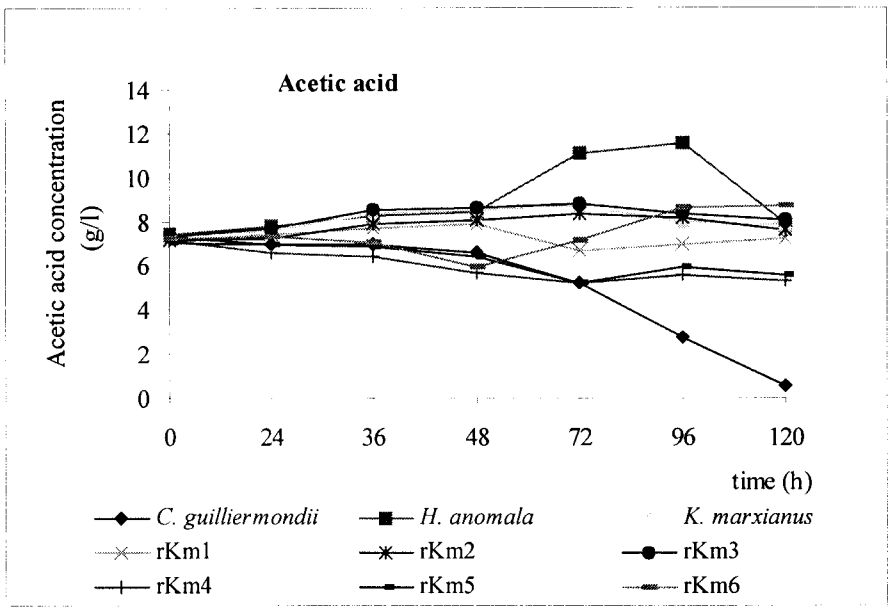

Figure 24 Acetic acid concentrations (g/l) during batch fermentation in sugarcane bagasse hydrolysate by different yeasts.

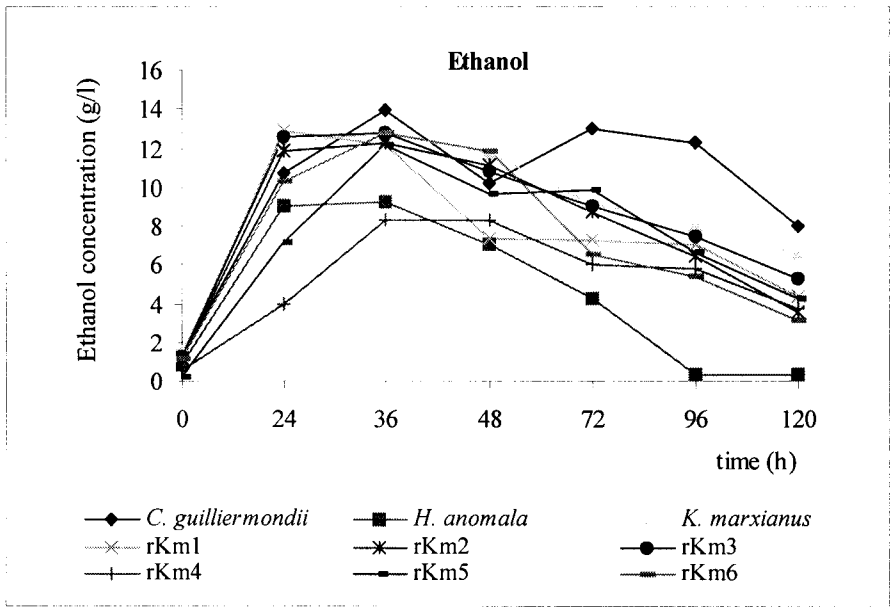

Figure 25 Ethanol concentrations (g/l) during batch fermentation in sugarcane bagasse hydrolysate by different yeasts.

Table 9 Xylitol production from bagasse hydrolysate by different yeasts during batch fermentation.

Microorganisms	Initial xylitol (g/l)	Xylitol concentration (g/l)						Xylitol productivity (g/l/h)
	0 h	24 h	36 h	48 h	72 h	96 h	120 h	
C. guilliermondii 5068	0.00	0.00	1.70	2.69	4.76	5.91	7.41	0.06
H. anomala 5302	0.00	0.00	1.12	1.72	2.88	3.24	3.41	0.03
K. marxianus 5057	0.00	1.07	1.22	1.29	1.54	1.83	2.62	0.02
rKm1	0.00	1.74	2.47	3.19	4.75	7.46	11.04	0.09
rKm2	0.00	1.12	2.31	3.20	5.07	7.23	9.67	0.08
rKm3	0.00	1.49	2.63	3.15	4.29	5.53	6.91	0.06
rKm4	0.00	0.00	1.09	2.35	5.38	9.46	12.48	0.10
rKm5	0.00	0.00	1.36	3.20	7.62	11.54	14.83	0.12
rKm6	0.00	1.83	2.74	4.35	9.69	13.97	15.64	0.13

4.4 Batch culture by recombinant *K. marxianus* (rKm6) for xylitol production

Xylose bioconversion into xylitol occurs as a function of the presence of xylose reductase enzyme in the xylose fermenting yeast (Martinez *et al.*, 2000). Aeration stimulates sugar transport in some yeasts and a wide variety of microorganisms including *Candida, Hanensula, Klyuveromyces* and *Pichia*, require oxygen for sugar uptake (Nigam & Singh, 1995). Aeration of the fermenting medium enhances xylose conversion to xylitol because xylitol production is directly coupled to growth of biomass and is strongly influenced by oxygen consumption. However, some organisms such as *C. guillermondii* and *Debaromyces hansenii* can produce xylitol under micro-aerophilic condition. (Silva *et al.*, 1998)).

Recombinant *K. marxianus* (rKm6) was evaluated in the stirred tank reactor. The batch processes were carried out in bagasse hydrolysate medium under three different aeration rate of 0.5, 1.0 and 1.5 vvm and fixed stirring rate of 200 rpm at 30°C for approximately 168 h. Glucose utilization, xylose consumption and xylitol production are shown in Figure 27- 32. The result showed the cell mass increased when the aeration rate of oxygen supply was increased (see in Figure 26), whereas xylitol production seem to be no difference contents during the first 120 h

fermentation. However, after 120h fermentation, the high yield of xylitol was occurred in the fermentation batch with the increased aeration rate (see in Figure 30). The xylitol yield from the batch process with the oxygen supply of 0.5, 1.0 and 1.5 vvm at 200 rpm stirring rate were 40.48, 43.09 and 45.38 g/l, resulting the xylitol productivity were 0.24, 0.25 and 0.27 g/l/h, respectively.

The shaking / agitation speed is also related to the concentration of dissolved oxygen that plays an important role in the fermentation of xylose into xylitol. In this experiment, when the agitation speed (stirring rate) was increased from 200 to 300 rpm at the aeration rate of 0.5 vvm. The result showed that the biomass was sharply increased (see in Figure 27) leading to increase in xylitol yield during the first 96 h fermentation and trended to be constant until 168 h fermentation. Whereas, the higher xylitol yield after 96 h fermentation were performed in the process with the stirring rate at 200 rpm and the aeration rate of 1.0 and 1.5 vvm, respectively. In this experiment, the xylitol formation was strongly dependent on the oxygen supply. By increasing the aeration rate and stirring rate, the xylitol formation increased until a suitable stirring / aeration rate relationship was reached. Similar results were found by Silva *et al.* (1996) using synthetic medium containing xylose as the major carbon source.

For the concentration profiles of arabinose, acetic acid and ethanol during batch fermentation are shown in Figure 28 – 30. Again, the present arabinose was hardy consumed by the recombinant rKm6 for those conditions of batch processes. In contrast, both of acetic acid and ethanol contents decreased continuously reaching a zero value during the fermentation. The results showed that oxygenated conditions appeared to be effected on the acetic acid concentration. For the fermentation conditions with high aeration rate, the acetic acid were completely utilised in the shortage time.

In agreement with Preez (1994) and Martin *et al.* (2002) who reported that as the extent of oxygen limitation increases, the maximum concentration of ethanol increases. The results of ethanol contents in batch processes showed that ethanol was produced by recombinant yeast during the first 24 h fermentation and sharply decreased depend upon the oxygenated conditions until reaching to the zero value.

On the conclusion, XR gene from *K. marxianus* 5057 expressed into the recombinant *K. marxianus* 5057 was able to convert xylose to xylitol very efficiently in sugaecane baggase hydrolysate, compared of those wild types (C. *guillermondii* 5068, *H. anomala* 5302 *K. marxianus* 5057 (see in Table 9 & 10). The maximum xylitol production (45.38 g/l) with 0.27 g/l/h xylitol productivity from hydrolysate was attained under stirring rate set at 200 rpm and aeration rate of 1.5 vvm which is about two times that of shake flask culture.

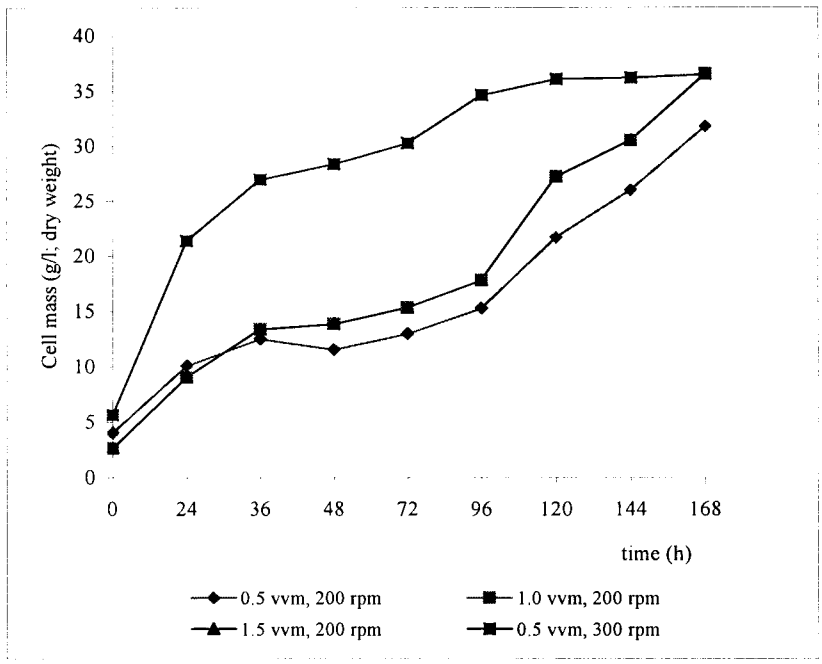

Figure 26 Cell mass (as dry weight; g/l) during batch fermentation in sugarcane bagasse hydrolysate by recombinant *K. marxianus* (rKm6) under different aeration rate of 0.5, 1.0 and 1.5 vvm and stirring rate of 200 rpm at 30°C for approximately 168 h.

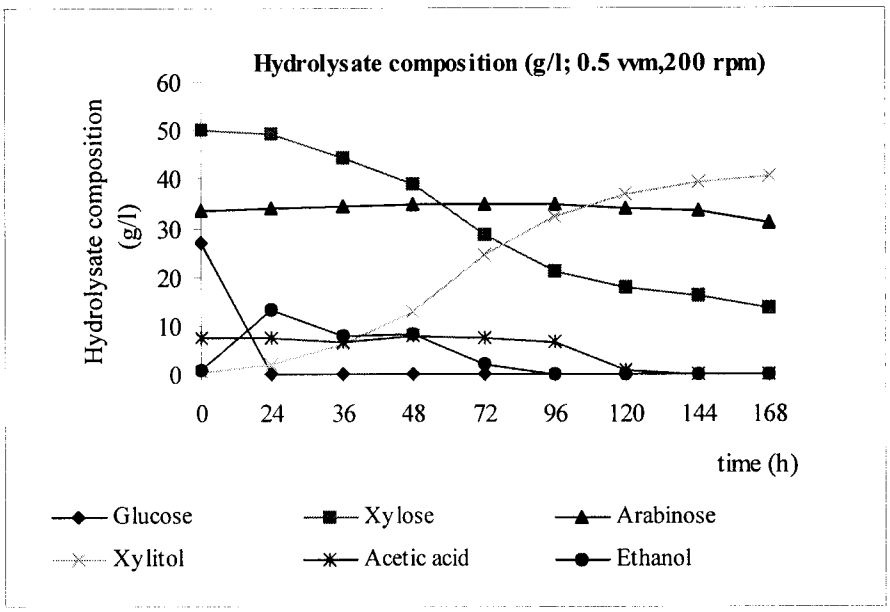

Figure 27 Composition of sugarcane bagasse hydrolysate (g/l) during batch fermentation by recombinant *K. marxianus* (rKm6) under aeration rate of 0.5 vvm and stirring rate of 200 rpm at 30°C for approximately 168 h.

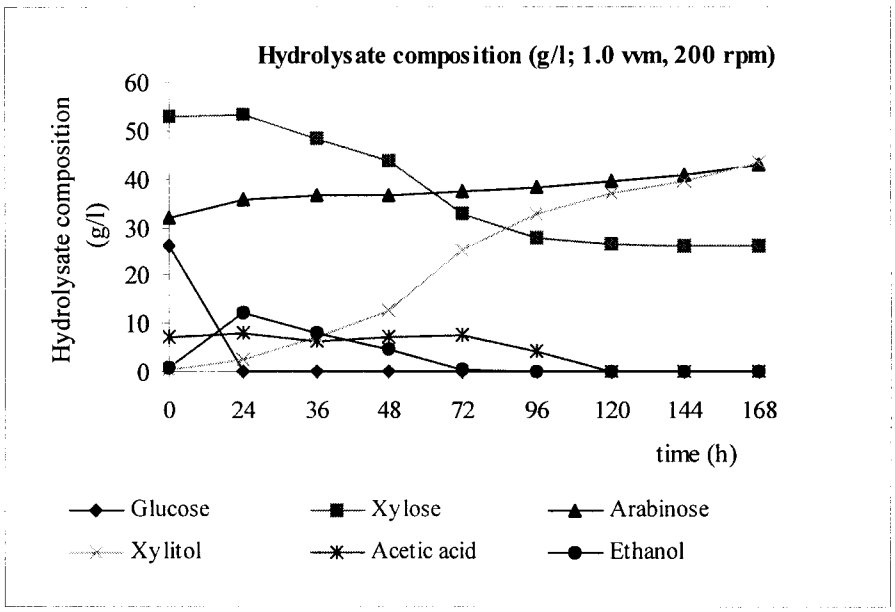

Figure 28 Composition of sugarcane bagasse hydrolysate (g/l) during batch fermentation by recombinant *K. marxianus* (rKm6) under aeration rate of 1.0 vvm and stirring rate of 200 rpm at 30°C for approximately 168 h.

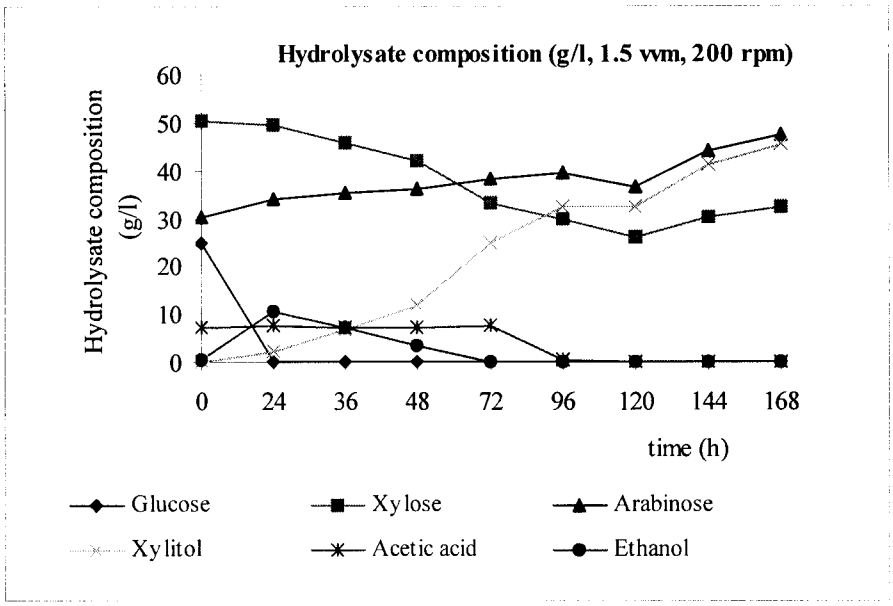

Figure 29 Composition of sugarcane bagasse hydrolysate (g/l) during batch fermentation by recombinant *K. marxianus* (rKm6) under aeration rate of 1.5 vvm and stirring rate of 200 rpm at 30°C for approximately 168 h.

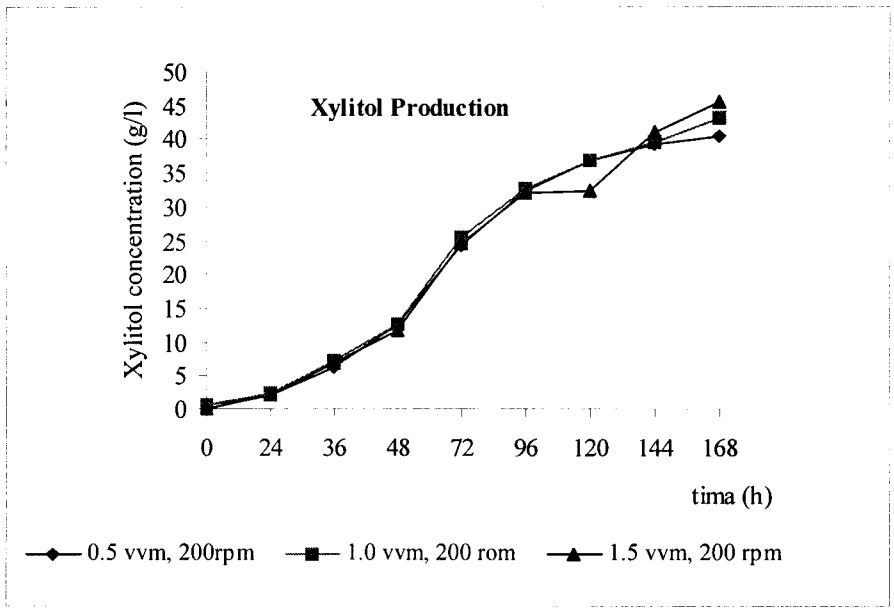

Figure 30 Xylitol production (g/l) during batch fermentation in sugarcane bagasse hydrolysate by recombinant *K. marxianus* (rKm6) under three different aeration rate of 0.5, 1.0 and 1.5 vvm and stirring rate of 200 rpm at 30°C for approximately 168 h.

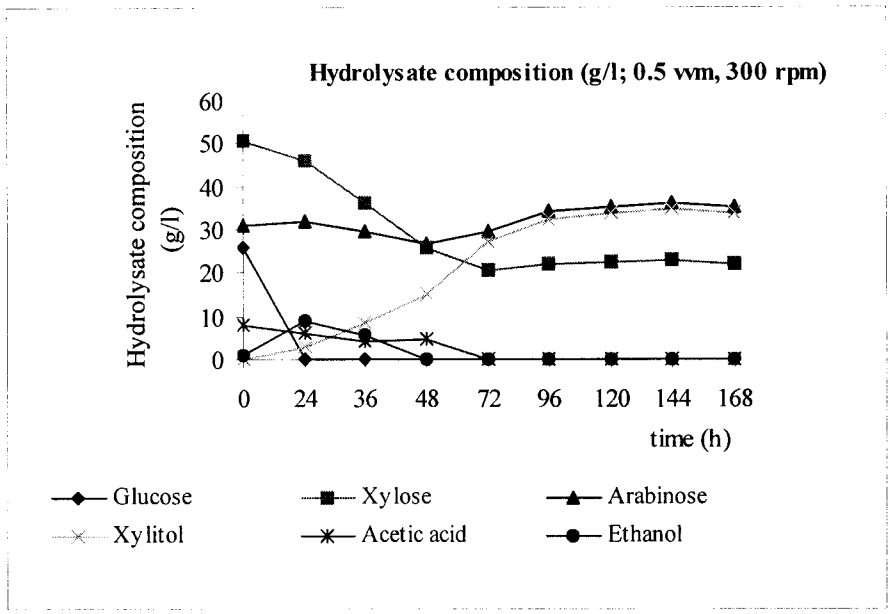

Figure 31 Composition of sugarcane bagasse hydrolysate (g/l) during batch fermentation by recombinant *K. marxianus* (rKm6) under aeration rate of 0.5 vvm and stirring rate of 300 rpm at 30°C for approximately 168 h.

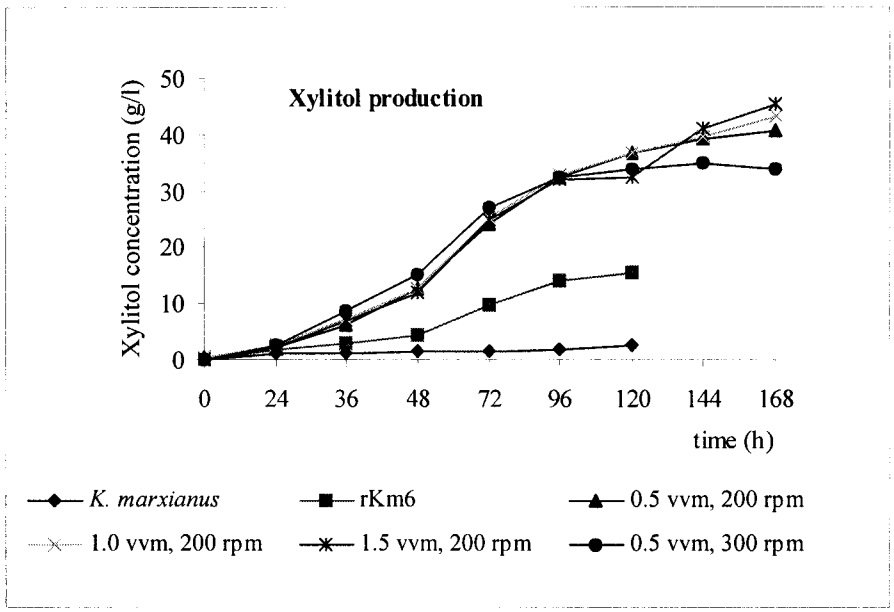

Figure 32 Xylitol production (g/l) during batch fermentation in sugarcane bagasse hydrolysate by recombinant different yeasts under different aeration rate of 0.5, 1.0 and 1.5 vvm and stirring rate of 200 rpm at 30°C for approximately 168 h.

Table 10 Xylitol production during batch fermentation in sugarcane bagasse hydrolysate by recombinant K. marxianus (rKm6) under three different aeration rate of 0.5, 1.0 and 1.5 vvm and stirring rate of 200 and 300 rpm at 30°C for approximately 168 h.

Conditions	Initial xylitol (g/l)	Xylitol concentration (g/l)								Xylitol productivity (g/l/h)
	0 h	24 h	36 h	48 h	72 h	96 h	120 h	144 h	168 h	
0.5 vvm / 200 rpm	0.50	2.01	6.19	12.64	24.21	32.36	36.71	39.34	40.48	0.24
1.0 vvm / 200 rpm	0.47	2.52	7.12	12.62	25.30	32.73	36.83	39.60	43.09	0.25
1.5 vvm / 200 rpm	0.00	2.10	6.78	11.74	24.66	32.18	32.45	41.04	45.38	0.27
0.5 vvm / 300 rpm	0.00	2.64	8.62	15.16	27.14	32.41	33.66	34.78	33.95	0.20