CHAPTER 5
CONCLUSIONS

Xylitol is a five-carbon sugar alcohol, a natural carbohydrate that occurs freely in certain plant parts (for example, in fruits, and also in products made of them) and in the metabolism of humans. It has a sweetness equivalent to sucrose with an extreme cooling effect and has been used as an alternative natural sweetener in food products. It is produced commercially and is used in some foods because of a number of advantageous natural properties. Xylitol can also be produced microbiolocally using xylose-utilising yeast. In xylose-fermenting yeast, D-xylose is metabolised though an inducible pathway in which it is first reduced by an NAD(P)H-dependent xylose reductase (XR) to xylitol. Xylitol is then oxidized by an NAD-dependent xylitol dehydrogenase to xylulose, which is phosphorylated to xylulose-5-phosphate and then channeled through the pentose phosephate pathway and can be converted into ethanol via glycolysis or further metabolized via the tricarboxylic acid cycle and respiration pathway.

In Thailand, sugarcane baggase is an extremely abundant waste product from sugar production, estimated at 14 million tons per annum (Agricultural Statistic Center of Thailand, 1999). Bagasse consists of approximately 50% cellulose and 25% each of hemicellulose and lignin. Consequently, pretreatment of bagasse has often been found useful to improve its digestibility and easy access for microbial use (Alani and Smith, 1988; Doran *et al.,* 1994). Several physical and chemical methods are employed for the pretreatment, which include steam explosion, gamma radiation, treatment with alkali, hydrogen peroxide, solvents, *etc.* Among these, chemical pretreatment (e.g., treatment with alkali or acid solution) have been found effective and economical. The hydrolysate from acid hydrolysis can be used as a fermentation medium for xylitol production.

In general, among microorganisms, the yeasts are considered to be the best xylitol producers and therefore, the majority of publications deal with them. Biological production of xylitol by yeasts has been reported, especially *Candida* spp. such as *C. pelliculosa*, *C. boidinii*, *C. guilliermondii* and *C. tropicalis*, and by

Pachysolen tannophilus. It is obvious concluded that the best xylitol producers belong to the genus *Candida* (Winkelhausen & Kuzmanova, 1998).

In this investigation, an attempt has been made to compare the growth and productivity of xylitol from treated sugarcane baggase by xylitol producing recombinant and wide type yeasts. The whole project was divided into four sections as follow: i) pretreatment of sugarcane baggase using H_2SO_4 solution, ii) genetic manipulation of yeast from selected xylitol producing yeasts, iii) comparison growth and xylitol production both in wild types and clone yeasts in sugarcane bagasse hydrolysate and iv) batch culture for xylitol production by recombinant yeast in bioreactor.

As the starting point, the hydrolysis of sugarcane bagasse of the different particle size with three levels of H_2SO_4 concentrations (1, 2, 3%) for several reaction time were examined. The result showed that the particles sizes (≤0.85, 0.85-1.70, 1.70-2.38, and 2.38-4.75 mm) of sugarcane 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. The bagasse hydrolysate was then used as the fermentation medium.

The xylose–fermenting yeasts; *C. guilliermondii* 5068, *Kluyveromyces marxianus* 5057 and *Hansenula anomala* 5302 were chosen to examine growth and xylitol production in bagasse hydrolysate. On the basis of growth and the ability on conversion xylose to xylitol, the yeast, *K. marxianus* 5057 was chosen to improve the productivity of xylitol using genetic manipulating technique.

After genomic DNA of *K. marxianus* 5057 was extracted and amplified using PCR technique, XR fraction appeared in a agarose gel as a single band with about 900 bases pair in length. This PCR product was used to generate the specific DNA probe. Xylose reductase (XR) of *K. marxianus* 5057 expression was determined by Northern - blotting. *K. marxianus* 5057 showed the highest expression of xyloes reductase in hydrolysate from sugarcane at day 4 of culture time. Consequently, isolation of xylose reductase full length gene of *K. marxianus* 5057 was examined by Southern - blotting. After hybridisation of digested DNA with the specific probe, the result showed the size of the hybridisation fragment increased to 4.5 kb as a site of Sac*I* digested genomic DNA. The positive band at the size of 4.5 kb was then gel purified,

subcloned in pUC 18 and transformed into *Escherichia coli* to determine nucleotide sequence of the XR full length gene.

XR full length gene of *K. marxianus* 5057 was then transformed into *K. marxianus* 5057 cell using electroporation method. In this work, 6 colonies of recombinant *K. marxianus* 5057 (rKm1, rKm2, rKm3, rKm4, rKm5, rKm6) were chosen to maximise xylitol production from sugarcane baggase hydrolysate. Meanwhile, expression of the *K. marxianus* 5057 XR gene in *Pichai pastoris* GS115 was also investigated.

Consequently, comparisons of growth and xylitol production of yeasts both wild type strains (*C. guilliermondii* 5068, *H. anomala* 5302, *K. marxianus* 5057) and cloned cultures of *K. marxianus* 5057 were examined. It was found that 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 improve the production of xylitol in a reactor.

The recombinant *K. marxianus* (rKm6) was processed in the 2.5 l stirred tank reactor with 1.2 l working volume. The batch processes were carried out in bagasse hydrolysate medium under three different aeration rates of 0.5, 1.0 and 1.5 vvm and the stirring rates were set at 200 and 300 rpm at 30°C. The highest yield of xylitol was 45.38 g/l with 0.27 g/l/h xylitol productivity evaluated in bagasse hydrolysate medium under aeration rate of 1.5 vvm and agitation rate of 200 rpm after 168 h fermentation which is about two times that of shake flask culture.