CHAPTER 2
LITERATURE REVIEW

2.1 Bagasse and Pretreatment

Bagasse consists of, on a dry weight basis, approximately 50% cellulose and 25% each of hemicellulose and lignin (Pandey *et al., 2000*). Chemically, bagasse contains about 50% α-cellulose, 30% pentosans and 2.4% ash. Because of its low ash content, bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0%, respectively.

Pretreatment of bagasse has often been found useful to improve its digestibility and easy access for microbial use (Alani & Smith, 1988; Doran *et al.*, 1994; Domínguez *et al.*, 1996; Mussatto & Roberto, 2001). The pretreatment results in enlargement of the inner surface area of substrate particles, accomplished by partial solubilisation and/or degradation of hemicellulose and lignin. Bravo *et al.* (1994) have treated bagasse with water or alkali at three liquid/solid ratios before using it as substrate for microbial protein production. The treatment significantly enhanced fungal growth compared to nontreated bagasse. du-Toit *et al.* (1984) compared pretreatments of bagasse with dilute alkali and acid for the determination of the monosaccharides present in bagasse hemicellulose. The pentosan fraction of the bagasse was successfully hydrolysed and extracted with 5% (w/v) HCl. Treatment with dilute alkali resulted in 39.8% solubilisation of bagasse, but only about 72% of the available hemicellulose could be extracted in this way. Alkaline hydrogen peroxide treatment of bagasse was also found effective in improving its digestibility (Azzam, 1989; Amjed *et al.*, 1992). Furthermore, Kling *et al.* (1987) studied the possibilities of a steam explosion pretreatment of bagasse in terms of hemicellulose solubilisation and enhancement of enzymatic hydrolysis. The pretreatment led to a significant improvement of sugar yield through enzymatic saccharification.

Bagasse hemicellulose hydrolysate has been used for the production of enzymes, single cell protein (SCP), ethanol, xylitol, etc. (Chin *et al.*, 1991; Meyer *et al.*, 1992; Roberto *et al.*, 1991a&b; Katzen & Fowler, 1994; Fontana *et al.*, 1995; Purchase, 1995; Pessoa *et al.*, 1996; Domínguez *et al.*, 1996; Felipe *et al.*, 1997; Sene

et al., 1998). The data on xylitol production from hemicellulosic hydrolysates with yeasts are summarised in Table 1.

2.2 Acid hydrolysis processes

There are basically two types of acid processes. One uses concentrated acid to catalyse saccharification, and the other, dilute acid. Processes using concentrated sulphuric acid for hydrolysis generally give higher yields (Ladisch, 1979). However, separation of the acid from the sugar product is a problem because the sugar product is not fermentable in concentrated acid, and the acid, being relatively expensive, must be recycled if the process is to be economically viable. Various acid hydrolysis processes, primarily for the saccharification of wood, are summarised in Tables 2 and 3

Although the hydrolysis can be performed enzymatically, most fermentation studies have focused on hydrolysates derived from acid hydrolysis. However, for the decomposition of hemicellulose, mild hydrolysis is the most suitable (Lavarack *et al.,* 2000; Aguilar *et al.,* 2002; Kim & Lee, 2002; Mussatto & Roberto, 2004; Rodríguez-Chong *et al.,* 2004). Its advantages are that it prevents the formation of some degradation products, enhances the susceptibility of cellulose to subsequent enzymatic or acid hydrolysis, reduces the requirement for expensive corrosion-proof equipment, and avoids the environmental problems incurred through the use of strong chemical treatments (Magee & Kosaric, 1985). According to Olsson & Hahn-Hägerdal (1996), during the acid hydrolysis of the lignocellulosics, different types of sugars (D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose), and degraded products (furfural, 5-hydromethyl furfural, acetic acid, syringic acid, *p*-hydroxybenzoic acid, vanillin etc.) are formed.

Table 1 Xylitol production by yeasts from hemicellulose hydrolysate

Yeast	Sub strate	Hydrolysis condition	Hydrolysis treatment	Released compounds[a] (g/l)				C_x (g/l)	S_c[b] (%)	Q_x (g/lh)	$Y_{x/}$ (g/g)	$Y_{cm/x}$ (g/g)	t (h)
				D-Xylose	D-Glucose	L-Arabinose	Acetic acid						
C. guiliermondii FTI 20037	sugar cane bagasse	35 mM H_2SO_4, 190°C, 5 min, steam explosion solid/liquid ratio 1/6	$Ca(OH)_2$, pH 6.5	61	15	n.r.[c]	n.r.	0	20	0	0	n.r.	125
			$Ca(OH)_2$, pH 10, H_2SO_4, pH 6.5	68	19	n.r.	n.r.	30	95	0.240	0.48	n.r.	125
			CaO, pH 10 H_2SO_4, pH 6.5	65	19	n.r.	n.r.	24	98	0.192	0.36	n.r.	125
			KOH, pH 6.5	68	18	n.r.	n.r.	0	0	0	0	0	125
			KOH, pH 6.5 H_2SO_4, pH 6.5	63	16	n.r.	n.r.	16	56	0.128	0.48	n.r.	125
C. guiliermondii FTI 20037	rice straw	0.07 g conc. H_2SO_4 , per g rice straw, 145°C, 20 min, solid/liquid ratio 1/10	vaccum concentration at 70°C NaOH,pH 10 H_2SO_4, pH 5.3	45	14	7	8.5	27	86	0.560	0.69	0.14	48
C. mogii ATCC 18364	wheat straw	15% H_2SO_4, 121°C, 30 min,		28	3	6.5	n.r.	6	70	0.130	0.31	0.71	46
Candida sp. 11-2	sugar cane bagasse	2-3%H_2SO_4, 100°C	$CaCO_3$, pH 4.5-6	58	n.r.	n.r.	n.r.	2.6	5.1	0.053	0.88	n.r.	49
			activated charcoal, CaO, $CaCO_3$, pH 4.5-6	43	n.r.	n.r.	n.r.	10.5	94.8	0.205	0.26	n.r.	51
			cation-exchange resins, CaO, $CaCO_3$, pH 4.5-6	46	n.r.	n.r.	n.r.	10.1	83.9	0.195	0.26	n.r.	51

Taken from: Winkelhausen and Kuzmanova,1998;[a]: initial concentrations; [b]: substrate consumed refers to D-xylose only; [c]: n.r., not reported

Table 2 HCl acid processes

Process	Conditions			Maximum Yield	Maximum Sugar Concentration*
	Prehydrolysis	Hydrolysis	Post-Hydrolysis		
Rheinau	None	Cold, fuming HCl	None	-	4% mixed sugars
Rheinau-Bergius	None	41% HCl at 3:1 Acid : Wood ratio	None	70% based on hemi-and α-cellulose	Dilute
Modified Rheinau	1% HCl to hydrolyse hemicellulose to pentose at 130°C	41% HCl	Dilute acid	-	-
Udic-Rheinau	32% HCl at 20°C	41% HCl countercurrent, continuous contacting	Dilute acid	-	12% mixed sugars
Prodor	-	Countercurrent contact w/HCl gas over 8 hours	-	-	-
Hereng	Moist wood chips impregnated with 30% HCl	Recontact w/30% HCl followed by countercurrent gaseous HCl contacting	Conditions not given	-	25% pentoses Hexose not given
Noguchi-Chisso	5% HCl, 100°C, 3 hr	Stagewise, countercurrent contact w/HCl gas at temperatures ranging from –5 to 125°C	-	23% on hemi-cellulose 95% on cellulose	-
Mechanical -chemical	-	Grinding in the presence of 5% HCl	0.2% HCl at 150°C for 80 minutes	90%on hemi-cellulose and α-cellulose	20% mixed sugars

* Prior to concentration by evaporation.

Taken from: Ladisch, 1979

Table 3 H_2SO_4 acid processes

Process	Conditions			Maximum Yield	Maximum Sugar Concentration*
	Prehydrolysis	Hydrolysis	Post-Hydrolysis		
Hokkaido	Water at 180°C to form furfural from pentosans 1.2 to 1.5% H_2SO_4 at 150°C to form xylose	80% H_2SO_4 at room conditions	5 to 15% H_2SO_4 at 100°C for 100 minutes	40% based on hemi-cellulose 90% on α-cellulose	pentose not give 10% glucose
Scholler-Tornesch	1 to 2.5% H_2SO_4 at 140°C	0.4% H_2SO_4 at 170°C and 8 atm	-	55% based on hemi- and α-cellulose	4% mixed sugars
Madison (continuous process)	Impregnation of wood chips with acid	0.5% H_2SO_4 at 185°C	-	72% based on hemi- and α-cellulose	15% pentose 5% mixed sugars
TVA	Similar to Madison process	0.5% H_2SO_4 at 180°C	-	-	6% mixed sugars
Russian Percolation Process	Percolation of dilute acid through vessels of various inclinations	-	-	-	-
Mechanical-chemical process	1.5 to 5% H_2SO_4 impregnated into wood. Wood then dried	Passage through vibratory mill at 4 to 80% H_2SO_4, various times and temperatures	-	60% based on hemi- and α-cellulose	-

* Prior to concentration by evaporation.

Taken from: Ladisch, 1979

2.3 Xylitol and conventional production of xylitol

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 (Aminoff *et al.,* 1978). It is produced commercially and is used in some foods because of a number of advantageous natural properties. Xylitol has been known to organic chemistry at least from the 1890's. German and French researchers were obviously the first ones who made xylitol chemistry more than 100 years ago (Mäkinen, 2000). This reaction was accomplished by means of sodium amalgam reduction of D-xylose (wood sugar).

Primary interest in xylitol therefore centers on its properties and potential uses as an alternative sweetener. In contrast to other alternative noncaloric sweeteners, such as saccharine, xylitol has many properties similar to those of sucrose. It dissolves readily in water, it is as sweet as sucrose and hence approximately twice as sweet as sorbitol and nearly three times as sweet as mannitol (Mäkinen, 1992 & 2000). Its caloric content is the same as that of sucrose, 17 kJ/kg. In addition, it gives a pleasant cool and fresh sensation due to its high negative heat of solution.

The most significant property of xylitol, however, is that it is an anticariogenic sweetener, and can be promoted for oral health and caries prevention. Therefore the bulk of production is consumed in various food products such as chewing gums, sweets, soft drinks and ice cream (Mäkinen, 1992). Recent increase in public awareness of health issues has ensured that substances like xylitol are attracting growing attention.

Various forest and agricultural materials rich in hemicellulose have been used as a raw material in xylitol manufacturing. Hemicelluose is chemically a xylan (pentosan), along polysaccharide molecule consisting of D-xylose units. Xylans are typically present in certain hardwoods such as brich and beech, rice, oak, wheat and cotton seed hulls, various nut shells, straw, corn cob and stalks, sugar cane bagasse, etc. (Mussatto & Roberto, 2004). In the manufacturing process of xylitol, the xylan molecules are first hydrolysed into D-xylose. The later is chemically reduced to xylitol which can be separated by large-scale column chromatography. Xylitol can also be made by means of bacterial fermentation which utilise D-xylose, D-glucose, or other suitable raw materials as substrates.

Although xylitol occurs in many fruits and vegetables, it would be very uneconomical to extract it from such sources due to their high cost and relatively low xylitol content. On a large-scale, xylitol is currently produced by chemical reduction of xylose derived mainly from wood hydrolysates. The conventional process of xylitol production includes four main steps: acid hydrolysis of plant material, purification of the hydrolysate to either a pure xylose solution or a pure crystalline xylose, hydrogenation of the xylose to xylitol and crystallization of the xylitol (Aminoff, *et al.*, 1978).

The critical step in this process is the purification of the xylose from the acid hydrolysate. Ion exchange chromatography is employed to remove salts and charged degradation products and activated carbon is used to remove color. Ion exchange chromatography, however, does not remove or separate the various hemicellulose sugars. This is a problem because acid hydrolysis releases appreciable amounts of D-galactose, D-mannose and L-arabinose in addition to D-xylose. The exact proportions of the various sugars depend on the nature of the feedstock and the manner in which it is hydrolysed. These contaminating sugars can complicate crystallisation and purification of xylose. The yield of xylitol from the xylan fraction is about 50-60% or 8-15% of the raw material employed (Nigam & Singh, 1995).

The existing drawbacks of conventional xylitol production methods motivated researchers to seek alternative ways for its production. One of the most attractive procedures, today, is microbial production.

2.4 Xylitol-producing microorganisms

Microorganisms more readily assimilate and ferment glucose than xylose. However, although in small numbers, there are bacterias, yeasts and fungi capable of assimilating and fermenting xylose to xylitol, ethanol and other compounds (Jaffries, 1983; Barnett, *et al.*, 1990). A few bacteria such as *Corynebacterium* sp., *Enterobacter liquefaciens* and *Mycobacterium smegmatis* (Izumori & Tuzuki, 1988) have been reported to produce xylitol. For the first two bacteria, D-xylose was mainly used as a substrate while for the last one, the substrate was D-xylulose or D-xylose isomerised by commercially immobilised D-xylose isomerase. Regarding the fungi, there is only one significant report regarding *Petromyces albertensis* (Dahiya, 1991).

This fungus accumulated 39.8 g/l of xylitol when cultured for 10 days on 100 g/l D-xylose.

In general, among microorganisms, the yeasts are considered to be the best xylitol producers and therefore, the majority of publications deal with them. Some of the yeasts screened for xylitol production are presented in Table 4. It is obvious that the best xylitol producers belong to the genus *Candida* (Winkelhausen & Kuzmanova, 1998).

Table 4 Screening of yeasts for xylitol production from D-xylose

Yeast	Xylitol (g/l)	Ethanol (g/l)
Candida boidinii NRRL Y-17213	2.9	3.9
C. guilliermondii FTI-20037	16.0	n.d.[a]
C. intermedia RJ-248	5.7	3.6
C. mogii ATCC 18364	31.0	n.r.[b]
C. parapsilosis ATCC 34078	20.0	n.r.
C. pseudotropicalis IZ-431	4.3	3.0
C. tropicalis	2.1	n.r.
C. tropicalis HXP 2	4.8	n.r.
C. tropicalis 1004	17.0	n.d.
C. tropicalis ATCC 7349	20.0	n.r.
C. tropicalis ATCC 20240	5.5	n.r.
C. utilis ATCC 22023	1.8	n.r.
C. utilis C-40	3.0	n.r.
Debaryomyces hansenii C-98 M-21	0.8	n.d.
Hansenula anomala IZ-1420	6.1	n.d.
Kluyveromyces fragilis FTI-20066	4.6	3.5
K. marxianus IZ-1821	6.1	0.6
Pichia (Hansenula) anomala NRRL Y-366	2.0	n.d.
Pachysolen tannophilus NRRL Y-2460	2.2	5.2
Saccharomyces SC-13	0.7	n.r.
Saccharomyces SC-37	2.3	n.r.
Schizosaccharomyces pombe 16979	0.2	n.r.

[a] n.d., Not detected.; [b] n.r., Not reported.

Taken from: Winkelhausen & Kuzmanova, 1998

The development of an economic fermentative process for xylitol production involves the selection of microbial yeast strains with high productivity, the establishment of conditions that maximise the conversion of xylose into xylitol and the optimisation of these parameters for process scale-up (Sirisansaneeyakul *et al.*, 1995; Winkelhausen & Kuzmanova, 1998. and Silva *et al.*(1998) found that the best xylitol producers were *C. polymorpha, C. tropicalis, C. guilliermondii, Pichia miso,* and *Hensenula anomala.* Gong *et al.* (1981) also found that xylitol was the main metabolite formed during the xylose fermentation by yeasts. *C. tropicalis* and *C. guilliermondii* yeasts were proved to be suitable for xylitol production, presenting a high fermentation yield after 48 h cultivation and an insignificant formation of by-products (Barbosa *et al.*, 1988). The yeast *Debaryomyces hansenii* was able to produce xylitol with an efficiency conversion in only 28 h cultivation (Gírio *et al.*, 1989). Although this microorganism is very promising, *C. guilliermondii* has been considered as an outstanding xylitol producer (Winkelhausen & Kuzmanova, 1998).

2.5 Conversion of D–xylose to xylitol by yeasts

The first step in the metabolism of D-xylose is the transport of the sugar across the cell membrane. A number of investigations have shown that under aerobic and oxygen limited conditions the rate of transport can limit the utilisation of D-xylose in *P. stipitis* CBS 7126 and *C. sheatae* ATCC 22984 (Maleszka & Schneider, 1982; Alexander *et al.*, 1988; Kilian & Uden, 1988; Ligthelm *et al.*, 1988; Prior *et al.*, 1989). Under anaerobic conditions, D-xylose metabolism has not appeared to be transport-limited either in *C. sheatae* or in *P. stipitis*. Instead, the limitation was in the two initial steps of D-xylose metabolism, reduction of D-xylose and subsequent oxidation of xylitol. Studying the oxygen requirement for D-xylose uptake, Skoog & Hahn-Hägerdal (1988) found that oxygen induces or activates a transport system in *P. stipitis* CBS 6054.

A typical xylitol-producing yeast, the transport system of which has been studied, is *C. mogii* ATCC 18364 (Sirisansaneeyakul *et al.*, 1995). The D-xylose uptake rate in the yeast followed Michaelis-Menten kinetics, which suggested a carrier-mediated facilitated diffusion transport system. A kinetic analysis of ^{14}C-xylose transport in intact cells of *C. mogii* supported this hypothesis. Chiang &

Knight (1960) found that the filamentous fungus *Penicillium chrysogenum* carried out the conversion of D-xylose to D-xylulose through a two-step reduction and oxidation. It possessed enzymes that differed from xylose isomerase in bacteria. This finding, as well as some further investigations (Chakravorty, 1962) led to the conclusion that the two-step conversion of D-xylose to D-xylulose is specific for yeasts and fungi, whereas in bacteria the same conversion is catalyzed by xylose isomerase in a single step.

Once inside the yeast cell, D-xylose is reduced to xylitol by either NADH- or NADPH-dependent xylose reductase (aldose reductase EC 1.1.1.21; see in Figure 1). Xylitol is either secreted from the cell or oxidised to xylulose by NAD- or NADP-dependent xylitol dehydrogenase (EC 1.1.1.9). The first two reactions are considered to be limiting in D-xylose fermentation. The phosphorylation of xylulose to xylulose-5-phosphate is catalysed by xylulokinase (EC 2.7.1.17) (Smiley & Bolen, 1982; Lache & Jeffries, 1986).

Xylose metabolism in yeasts yields a variety of carbon-containing products, which include carbon dioxide, ethanol, acetic acid and polysaccharides. Product yields are dependent upon the regulation of carbon flow through available metabolic routes (Slininger *et al.,* 1987; Schneider, 1989). D-xylose conversion to xylitol in yeasts can not be separated from the conversion of D-xylose to these products. The process of xylitol formation can not be stopped after the first step, when D-xylose is converted to xylitol. Cell growth depends on some of the above metabolic products and it is also necessary that the cofactors be regenerated through different steps in the metabolic pathway. Therefore, for obtaining good yields of xylitol, the amount of xylose being converted to xylitol and the amount of xylitol, which is available for further metabolism, have to be well balanced.

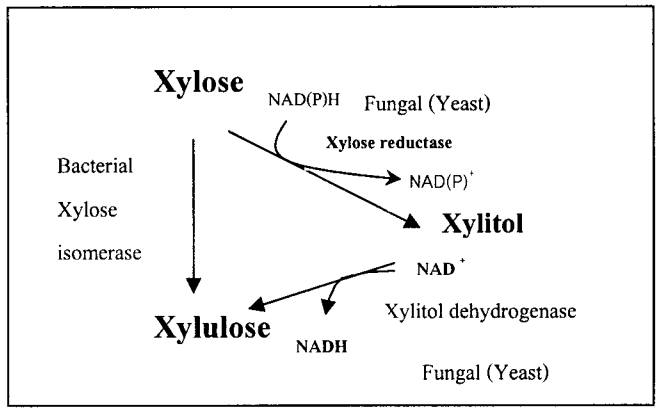

Figure 1 Metabolism of xylose via a xylose reductase and a xylitol dehydrogenase

2.6 Process conditions affecting biological production of xylitol

2.6.1 Effect of aeration rate

Aeration stimulates sugar transport in some yeasts and wide variety of organisms including *Candida, Hansenula, Klyuveromyces* and *Pichia* require oxygen for sugar uptake. Aeration of the fermenting medium enhances xylose conversion to xylitol (Gong *et al.*, 1981; Silva *et al.*, 1996 & 1997; Santos *et al.*, 2005) because xylitol production is directly coupled to growth of biomass and is strongly influenced by oxygen consumption. Some organisms can produce xylitol under micro-aerophilic conditions; Meyrial *et al.* (1991) studied the ability of *C. guilliermondii* to produce xylitol from xylose and non-hemicellulose derived sugars in micro-aerophilic conditions. They achieved a yield of 0.63 g/l xylitol with a negligible amount of ethanol produced from the xylose. The non-xylose sugars were converted to ethanol and biomass. Production of xylitol by *Debaryomyces hansenii* also requires semi-aerobic conditions, since under aerobic conditions the accumulated reduced-adenine-dinucleotide-coenzyme is fully reoxidised, leading to conversion of xylitol into xylulose (Roseiro *et al.*, 1991).

Horitsu *et al.* (1992) reported that for effective xylitol production, the first step to consider is the rapid accumulation of microbial cells in the culture medium. This may be achieved by maintaining an increased level of dissolved oxygen in the culture. *C. tropicalis* accumulated xylitol under oxygen limiting conditions because of at low dissolved oxygen concentrations there are relatively high levels of NADPH and

NADH in the cells, leading to the intensive reduction of D-xylose and the accumulation of xylitol in the medium.

2.6.2 Effect of xylose concentration

A higher initial concentration of substrate favours the production of xylitol by osmophilic microorganisms. Generally, in a batch process, an increase of initial sugar concentration leads to an increase of production rates and yields, if the microorganism is able to tolerate a higher concentration of sugar and higher osmotic pressure. The sufficient cell growth and the xylitol production was obtained at higher aeration rate when increased xylose concentration in cultures of *C. guilliermondii and C. tropicalis* (Meyrial *et al.*, 1991; Horitsu *et al.*, 1992). In contrast to *Candida* sp. B22, xylitol production and growth was gradually inhibited by an increase in xylose concentration (Chen & Gong, 1985).

2.6.3 Effect of presence of other sugars in the medium

Hsiao *et al.* (1982) reported that glucose inhibits D-xylose utilistion in *Candida* and *Schizosaccharomyces*. A short period of time was needed for a maximal inhibitory effect by glucose on D-xylose consumption and the ability to convert xylose was quickly restored once glucose reached a low concentration. The short transition period and fast recovery of the ability to absorb xylose indicated that catabolite repression is not the regulatory mechanism. This evidence supports the idea that inhibition, rather than inactivation and repression, is the main control of xylose transport in the presence of glucose. A short transition period also suggests that inhibition is imposed by intracellular concentration of glucose or its catabolite (Yahashi *et al.*, 1996).

Meyrial *et al.* (1991) evaluated the ability of *C. guilliermondii* to ferment non-xylose sugars such as glucose, mannose, galactose and arabinose, commonly found in hemicellulose hydrolysates. These sugars were rapidly fermented and utilised only for growth and ethanol production; their polyols were not detected in the culture medium. *C. guilliermondii* is characterised by a high potential for the production of xylitol from xylose-rich materials since xylitol production is obligatively achieved by reduction of non isolated xylose in hemicellulosic hydrolysates.

2.6.4 Effect of pH and temperature

Gong *et al.* (1981) have reported that maximal amounts of xylose were converted into xylitol at pH 8.0 and there was a significant reduction in xylitol production when the pH was shifted from alkaline to acidic range. The maximum amount of xylitol was obtained at an initial pH of 6.0-7.0 by *P. albertensis* (Dahaiya, 1991), at pH 6.0 by *C. guilliermondii* (Myrial *et al.*, 1991) and at pH 4.0 by *C. tropicalis*. The optimum temperature for xylitol production by *Candida* and *Schizosaccharomyces* yeasts has been reported to be 30°C (Alexander *et al.*, 1988).

2.7 DNA cloning / recombinant DNA

DNA cloning facilitates the isolation and manipulation of fragments of an organism's genome by replicating them independently as part of an autonomous vector, also called recombinant DNA molecules. Most of the routine manipulations involved in gene cloning are hosts and vectors, subcloning, DNA libraries, screening libraries and analysis of clone (Turner *et al.*, 2001).

Once a clone containing a target gene is identified, the structure of the cloned fragment may be investigated further using restriction mapping, the analysis of the fragmentation of the DNA with restriction enzyme, or ultimately by the analyzed by complete sequence of the protein product determined. The sequence is then available for manipulation in any of the applications.

Many enzymes are used in vitro in DNA cloning and analysis. The properties of the common enzymes are given in Table 5.

2.8 *Pichia pastoris* expression system

P. pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. According to the DIG system user's guide for filter hybridisation (1995), *P. pastoris* has many of the advantage of higher eukaryotic expression system such as protein processing, protein folding and post-translational modification, while being as easy to manipulate as *Escherichia. coli* or *Saccharomyces cerevisiae*. It is faster, easier and generally gives higher expression levels. As yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, and has the

added advantage of 10- to 100-fold higher heterologous protein expression levels. These features make *Pichia* very useful as a protein expression system.

Heterologous expression in *Pichia* can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. While several different secretion signal sequences have been used successfully, including the native secretion signal present on some heterologous proteins. The major advantage of expressing heterologous proteins as secreted proteins is that *P. pastoris* secretes very low levels of native proteins. That, combined with the very low amount of protein in the minimal *Pichia* growth medium, means that the secreted heterologous protein comprise the vast majority of the total protein in the medium and serves as the first step in purification of the protein.

In comparison to *S. cerevisiae*, *Pichia* may have an advantage in the glycosylation of secreted proteins because it may not hyperglycosylate. Both *Saccharomyces cerevisiae* and *Pichia pastoris* have a majority of N-linked glycosylation of the high mannose type; however, the length of the oligosaccharide chains added post-translationally to proteins in *Pichia* (average 8-14 mannose residue per side chain) is much shorter than those in *S. cerevisiae* (50-150 mannose residues). Very little O-linked glycosylation has been observed in *Pichia*. In addition *S. cerevisiae* core oligosaccharides have terminal α1,3 glycan linkages whereas *P. pastoris* does not. It is believed that the α1,3 glycan linkages in glycosylated proteins produced from *S. cerevisiae* are primarily responsible for the hyper-antigenic nature of these proteins making them particularly unsuitable for terapeutic use. The flexibility of the *Pichia* expression system makes it an ideal tool for laboratory research as well as for industrial application. Previous papers have been reports that xylose reductase gene from pentose-fermenting yeasts were cloned and expressed in *P. pastoris* (Dahn *et al.*, 1996; Handumrongkul *et al.*, 1998).

Table 5 Enzymes used in DNA cloning

Enzymes	Use
Alkaline phosphatase	Remove phosphate form 5'–ends of double-or Singal-stranded DNA or RNA
DNA ligase	Joins sugar - phosphate backbones of dsDNA with a 5'-phosphate and a 3'-OH in an ATP-dependent rection. Requires that the ends of the DNA be compatible, i.e. blunt with blunt, or complementary cohesive ends.
DNA polymerase I	Synthesizes DNA complementary to a DNA template in a 5' to 3' direction beinging with a primer with a free 3'-OH. The Klenow fragment is a truncated version of DNA polymerase I which lacks the 5' to 3' exonuclease activity.
Polynucleotide kinase	Adds phosphate to 5-OH end of double or single stranded DNA or RNA in an ATP- dependent reaction. If [γ-^{32}P]ATP is used, then the DNA will become radioactively labled.
Restriction enzymes	Cut both strands of dsDNA wthin a (normally symmetrical) recognition sequence. Hydrolyze sugar-phosphate backbone to give a 5'-phosphate on one side and a 3'-OH on the other. Yield blunt or 'sticky'ends (5'-or3'-overhang).
Rnase A	Nuclease which digests RNA, but not DNA.
Rnase H	Nuclease which digests the RNA strand of an RNA-DNA heteroduplex.
Taq DNA polymerase	DNA polymerase derived from a thermostable bacterium (*Thermus aquaticus*). Operates at 72°C and is reasonably stable above 90°C. Used in PCR.
Terminal transferase	Adds a number of nucleotides to the 3'-end of linear- or double stranded DNA or RNA. If only GTP is used, for example, then only Gs will be added.

Taken from: Turner *et al.*, 2001

2.9 Xylose reductase

Xylose reductase (XR) is commonly found in yeasts and filamentous fungi, often with several isozymes in one species (Neuhauser *et al.*, 1997). Xylose reductase catalyses the first step of D-xylose metabolism, reducing xylose to xylitol with concomitant NAD(P)H oxidation. Based on sequence and structure similarities, xylose reductase belongs to the aldose reductase family (Woodyer *et al.*, 2005). The majority of its family members are monomeric, but some have quaternary structural organisations, including xylose reductase, which has a unique dimeric interface.

Xylose reductase has gained interest because of their importance both in the fermentation of plant biomass to ethanol and in the production of xylitol, a low-calorie anticariogenic natural sweetener. The genetic sequences of many xylose reductase has been determined and several have been cloned and expressed in a variety of hosts (Schneider, *et al.*, 1989; Ho *et al.*, 1990; Amore *et al.*, 1991; Takuma *et al.*, 1991; Billard *et al.*, 1995; Kuhn *et al.*, 1995; Yokoyama *et al.*, 1995a&b; Bolen *et al.*, 1996; Neuhauser *et al.*, 1997; Walfridsson *et al.*, 1997; Hacker *et al.*, 1999; Mayr *et al.*, 2000; Anderlund *et al.*, 2001; Bernd *et al.*, 2001; Kang *et al.*, 2003; Lee *et al.*, 2003; Nidetzky *et al.*, 2003; Woodyer *et al.*, 2005).