Chapter 1

General introduction and Review of Literature

1.1 Introduction

A wide range of biotechnologically important products used in the food, chemicals, pharmaceutical and allied industries are produced by enzymatic biocatalysis of several organic compounds (Uday *et al.* 2016, Bhunia *et al.* 2013, Ratledge and Kristiansen 2008). According to a report, the approximate world market of enzymes was US $30–60 billion, in the year 2010 (Savitha *et al.* 2007). A/S (Denmark), Danisco/Genencor (Denmark and USA), BASF (Germany) and DSM (Netherlands) are the major industries that control about 73% of enzyme market in the world (Uday *et al.* 2016, Jaouadi *et al.* 2008, Ratledge *et al.* 2008). The largest group of industrially important enzymes (75%) belong to the class hydrolases and among these glycoside hydrolase class of enzymes have been marked as the second largest group of industrially important enzymes produced by wide range of bacteria, fungi, animal and plant cells (Bhat 2000). Cellulases and hemicellulases are the two important enzymes of glycoside hydrolase group that can depolymerize cellulose and hemicellulose, respectively. The above two polymers are generally found in lignocellulosic components of plant cells (Bhat 2000). In nature, hemicellulose is the most abundant polysaccharide after cellulose, that mostly contains xylan made up of β-D-xylopyranosyl residues linked via β-1, 4-glycosidic bonds (Saha 2003, Beg *et al.* 2001). The exact amount of xylan present in plant cell wall differs from plant to plant; however, it comprises approximately 30-35% of the total dry weight of plant (Beg *et al.* 2001). In India, an agriculture based country, various agro residues and byproducts such as wheat bran, sugar cane bagasse, corn cobs, rice bran, water hyacinth, corn fiber etc., are generated throughout the year (Cano *et al.* 2007). The improper management of these agro residues creates environmental pollution (Uday *et al.* 2016). Therefore, these inexpensive and easily processed agro residues can be used for production of value added glycoside hydrolase group of enzymes. Due to its immense industrial applications such as in animal feeds, release pentose sugars, bio bleaching of wood pulps, food additives in baking industry, production of xylitol, ethanol, ingredients in laundry detergents or fabric care compositions etc., xylanase production from agro residues has been reported in various work (Khanahmadi *et al.* 2018, Adhyaru *et al.* 2014). Endo-xylanases (EC 3.2.1.8) are important group of industrially applicable enzymes that act in association with β-xylosidase, α-L-arabinofuranosidase and α-glucuronidase for complete breakdown of plant cell

wall polysaccharide to xylo-oligosaccharide and xylose (Collins *et al.* 2005, Chavez *et al.* 2004). Bacteria, fungi, actinomycetes and yeast are reported to produce different family of xylanases (Adhyaru *et al*. 2016, Irfan *et al.* 2016, Thomas *et al.* 2014). Bacteria, due to their faster growth rate and easy to cultivate, are widely used as large scale producers of extracellular xylanases (Aarti *et al.* 2015, Nagar *et al.* 2013). Among the xylanase producing bacteria, *Bacillus* sp has been studied extensively for industrially important xylanase production (Khusro *et al.* 2016, Irfan *et al.* 2016, Thomas *et al.* 2014, Adhyaru *et al.* 2014). On the other hand, filamentous fungi due to their higher rate of production over yeast and bacteria are used in various work as source of xylanase (Ribeiro *et al.* 2014, Sakthiselvan *et al.* 2014, Knob *et al.* 2013, Polizeli 2005).

Xylitol is a naturally occurring sugar alcohol, with sweetness equivalent to sucrose but less calorie content (Edelstein *et al.* 2008, Maguire and Rugg-Gun, 2003). Xylitol has some exclusive health promoting effects such as it can prevent tooth decay and ear infection in infants, used as a substitute of sucrose for diabetic patients due to its non involvement with insulin metabolic pathway, has a low glycemic index, its non fermentable nature retard the formation of bacterial dental plaque in oral cavity and anti cariogenic property and lack of anti nutritional effect on food (Ritter *et al.* 2013, Uittamo *et al.* 2011, Zacharis *et al.* 2012, Chen *et al.* 2010, Khalid *et al.* 2012, Islam 2011). In the recent past, demand for xylitol has steadily increased by 6% per annum owing to its multiple benefits, primarily in the food markets. Two major factors have contributed to the escalating demand; firstly, health concerns (driving the growth of alternative sweetener market) and secondly, increasing awareness of naturally derived low calorie sugar free chemicals. Globally, xylitol has vast market comprising an annual demand of 161.5 million metric tons (MMT) equivalent to a value of US$670 million (in 2013), and forecasted to reach 250 MMT by 2020, nearly 1.5 times its current requirement with an overall market value of US $1 billion (http://www.prnewswire.com). 70% of the global xylitol consumption accounts for manufacturing chewing gums and confectionery products (http://www.marketresearch.com/product/42sample-8164119.pdf). M/s DuPont Danisco, is the leading xylitol manufacturer globally, with plants located in Finland, USA and China (http://www.dupont.com), and followed by M/s Xylitol Canada Inc. in terms of production capacity (Dasgupta *et al.* 2017). Industrially xylitol is produced by catalytic hydrogenation of pure D-xylose solution under high temperature and pressure. With requirement of high purity feed and severe reaction conditions, the process is very expensive and energy intensive. As a counter measure, alternative cost effective production strategies through biotechnology route have been undertaken by the scientific fraternities with attention towards cheap source of xylose and its bio catalytic conversion into xylitol under ambient conditions. Lignocellulosic biomass

from agro-industrial waste was considered to be a potential raw material in this regard (Cheng *et al.* 2014, Franceschin *et al.* 2011).

In the present study, microorganisms isolated from environmental samples were screened for xylanase production followed by characterization of the best producers. Xylanase production by the selected microorganisms were optimized with respect to growth conditions and media composition, using agro residues as sole carbon source under submerged fermentation (SmF). Furthermore, the xylanases producer *B. subtilis* xym4 and *P. citrinum* xym2 were employed in for optimization of sacchrification of agro residues to fermentable sugars. Finally, the reducing sugars obtained after saccharification were used for xylitol production using *E. coli* xyl6. Moreover, wheat bran was subjected to simultaneous saccharification and fermentation (SSF) by *Bacillus* and *E. coli* for xylitol recovery.

1.2 Objectives

1. To isolate the xylanase producing microorganisms from garden soil or other environmental samples.
2. To determine the identity of the isolates with biochemical and molecular techniques.
3. To determine the effect of cheap agro residues on xylanase production under submerged fermentation (SmF) and comparison with production in birchwood xylan, by the isolates.
4. To optimize the culture conditions for enhanced xylanase production with step wise modification of culture media under SmF of agro-residues.
5. To optimize the culture conditions for enhanced xylanase production using statistical model.
6. To purify xylanase from selected isolate by using methods based on solubility and chromatographic techniques and characterization of the enzyme.
7. To optimize the parameters for saccharification of agro residues by xylanase.
8. Isolation and identification of xylitol producing microorganism using biochemical and molecular techniques.
9. Application of xylitol producing microorganism for xylitol production from saccharified broth of agro residues under submerged fermentation.
10. Simultaneous saccharification and fermentation (SSF) of agro residue with xylanase and xylitol producing microorganisms for xylitol production.

1.3 Review of Literature

1.3.1. Xylan structure

Lignocelluloses are mainly composed of cellulose (45-55 %), hemicelluloses (25-35 %) and lignin (20-30%). All these components are major polymers of plant cell wall. Being the second most abundant plant polymer after cellulose, hemicelluloses comprise 25-35 % of weight of terrestrial plants and agro-residues (Mosier *et al.* 2005, Saha 2003, Sun and Cheng 2002). Structurally hemicellulose is a heteropolysaccharide with β-1,4 linked D-xylopyronoside residues as back bone called xylan. In soft wood, xylans make 5-15 % of the total plant dry weight. Depending on the plant varieties and plant tissues the xylose unit of the β-1,4 linked back bone chain often substituted with O-acetyl groups at O-2 and/or O-3, branched (1-3 units/branch) with L-arabinofuranoside (arabinoxylan), or D-glucuronic acid (glucuronoxylan) residues, which can be further linked with O-acetylated or O-methylated at C-4. D-galactose and ferulic acid moieties are also found linked with L-arabinose residues of xylan (Ebringerova 2006, Mosier *et al.* 2005, Saha 2003, Scheller and Ulvskov 2010). Representative chemical structure of xylan is illustrated in the figure 1.1.

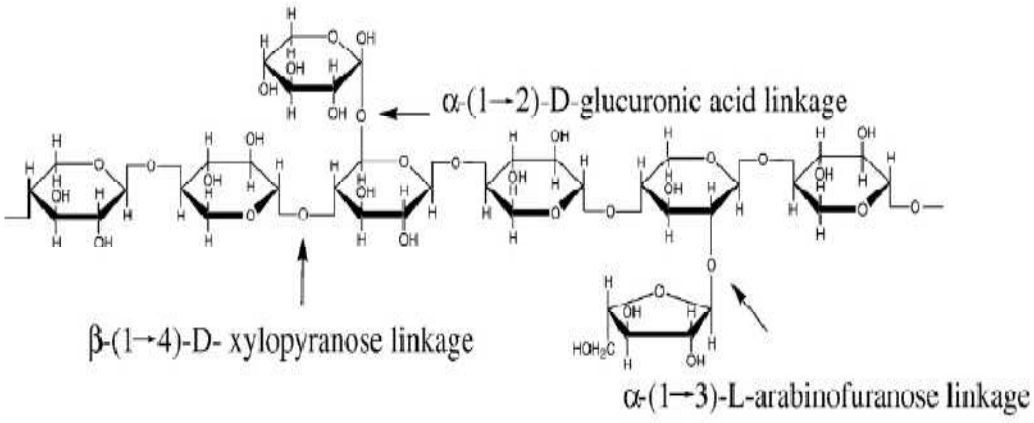

Fig.1.1. Chemical structure of xylan with different side chains attached (Bastawde 1992).

1.3.2. Xylanase and their mode of action

An array of enzymes with vast specificities and mode of actions are required for complete and efficient hydrolysis of xylan heteropolymer. Endo-1,4-β-D-xylanase (E.C. 3.2.1.8) cleaves the xylan back bone randomly and liberates xylooligosaccharides, xylan-1,4-β-xylosidase (EC

3.2.1.37) cleaves xylooligosaccharides from the non reducing end and release xylose monomers while the phenolic and acetyl side groups are removed by the catalytic action of α-L-arabinofuranosidases (EC 3.2.1.55), α–D-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.6) and ferulic acid esterases (EC 3.1.1.73). All these enzymes function synergistically on heteroxylan for its efficient depolymerization (CoIlins *et al.* 2005, Beg *et al.* 2001, de Vries *et al.* 2000, Uffen 1997).

1.3.2.1. Endo-1,4-β D-xylanase (E.C. 3.2.1.8)

The most important xylan degrading enzyme is endo-1,4-β-D-xylanase and it catalyzes the hydrolysis of internal glycosidic bonds in a highly specific manner. The catalytic activity of the enzyme depends on the length and the degree of branching or the presence of specific substituents in the xylan polymer (Coughlan *et al.* 1992).

1.3.2.2. Xylan 1,4-β-xylosidase (E.C. 3.2.1.37)

This is an exo acting enzyme that mainly hydrolyses xylobiose or xylooligosaccharides, and its specific affinity with disaccharides is higher than that of oligosaccharides (Panbangred *et al.* 1984). Hydrolysis of 1,4-β-D-xylooligosaccharides from the non reducing ends by the enzyme liberates xylose monomers. This enzyme has been reported to be present in the microorganisms as monomeric, dimeric or tetrameric forms with molecular weight between 26-360 kDa (lUBMB, 1982).

1.3.2.3. α-L-arabinofuranosidases (E.C. 3.2.1.55)

The terminal, non reducing α-L-arabinofuranosyl groups of arabinans, arabinoxylans, and arabinogalactans are hydrolysed by α-L-arabinofuranosidases (EC3.2.1.55) and liberate arabinose (Gomes *et al.* 2000). Wide range of microorganisms including fungi, actinomycetes and other bacteria has been reported to produce α-arabinosidases (Saha 2000).

1.3.2.4. α–D-glucuronidases (E.C. 3.2.1.139)

The enzyme catalyzes the hydrolysis of α-1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan. Due to the stability of α-1,2 linkages over β-1,4 linkages the enzymatic reaction has been recognized as most crucial for xylan degradation. A number of microorganisms are reported to produce α–D-glucuronidases (Puis 1997).

1.3.2.5. Acetyl xylan esterases (E.C. 3.1.1.6)

Baiely *et al.* (1986) first reported the production of acetyl xylan esterase by cellulolytic and hemicellulolytic fungi, *Trichoderma reesei, Aspergillus niger, Schizophyllum commune* and *Aureobasidum pullulan*. It acts on both xylan and xylooligomers and removes the O-acetyl group from the C-2 and C-3 position of xylose residues (Degrassi *et al.* 2000, Joselau *et al.* 1992).

1.3.2.6. Ferulic acid esterases (E.C. 3.1.1.73)

Removal of ferulic acid moiety from arabinoxylans is catalysed by ferulic acid esterases. Among the fungal species *A. oryaze* was found to be an efficient producer of ferulic acid esterases (Tenkanen *et al.* 1995). Being an acidic monomeric protein with pI 3.6 and mol. wt. 30 kDa, it also liberated p-coumaric and acetic acids from heat treated wheat straw arabinoxylan. The enzyme also hydrolyse of ester bond between hydroxycinnamic acids and sugars in the plant cell walls (Crepin 2004). Figure 1.2 depicts the mode of action of different kinds of xylanases on xylan polymer.

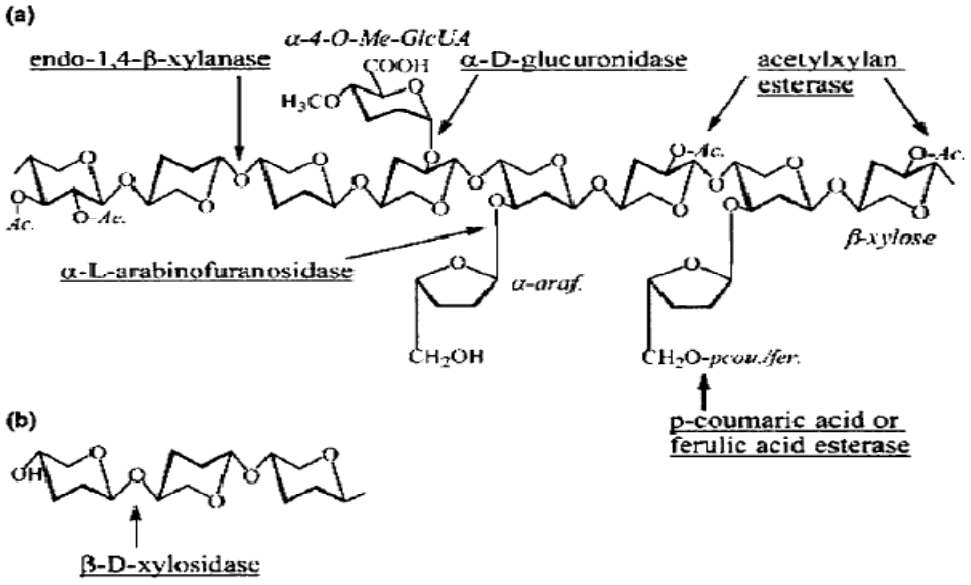

Fig 1.2. (a) Xylan structure of cereals, and mode of action by different xylanolytic enzymes (b) Mode of action of β-xylosidase (Collins *et al.* 2005).

1.3.3. Classification of xylanase

Carbohydrate active enzyme (CAZy) database (http://www.cazy.org) categorize xylanases (EC 3.2.1.8) under glycoside hydrolase (GH) families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62 and 98. Families 16, 51 and 62 are composed with bi-functional xylanase enzymes with two catalytic domains, whereas families 5, 7, 8, 10, 11 and 43 are composed of enzymes having a true single catalytic domain with endo-1,4-β-xylanase activity (Collins *et al.* 2005). The residual or secondary xylanase activity was recorded for the enzymes belonging to GH families 9, 12, 26, 30 and 44. However, based on the hydrophobic cluster analysis of the catalytic domains and amino acid sequences similarities, xylanases have been primarily grouped into GH families 10 and 11 (Verma *et al.* 2012). Although the members of these two families have been studied extensively, the information regarding the catalytic activity and chemical properties of the members of other GH families (5, 7, 8 and 43) are very recent and limited (Taibi *et al.* 2012). Collins *et al.* (2005) observed that the members of families 5, 7, 8, 10, 11 and 43 are widely different with respect to their structure, mode of action, substrate requirement and physicochemical properties. The GH 10 family contains the enzymes having high molecular mass, cellulose binding domain (CEBD) and a catalytic domain (CD). The CEBD and CD are connected by a linker peptide which has the isoelectric point (pI) between 8 and 9.5. This family usually has a $(\beta/\alpha)_8$ fold TIM barrel. On the other hand, GH 11 family enzymes have low molecular weight and low pI values. GH family 11 is further subdivided into alkaline and acidic enzyme groups, based on their isoelectric points (Buchert *et al.* 1995, Ahmed *et al.* 2009, Juturu *et al.* 2012).

1.3.3.1. Family 5 glycoside hydrolase

Despite having very limited information the characteristic endo mode of action GH family 5 has been extensively investigated. α-1,2-linked glucuronate moiety directed site specific catalytic hydrolysis of β-1,4 xylan chain is a distinctive ability of this family. The cloning of GH 5 xylanase C (XynC, 90.86 kDa) from *Bacillus subtilis* 168 and its overexpression and crystallization have been done. The crystallographic study revealed its exceptional specificity and catalytic depolyerisation of glucuronoxylan (John *et al.* 2009). Figure 1.3 depicts the biological assembly image of XynC.

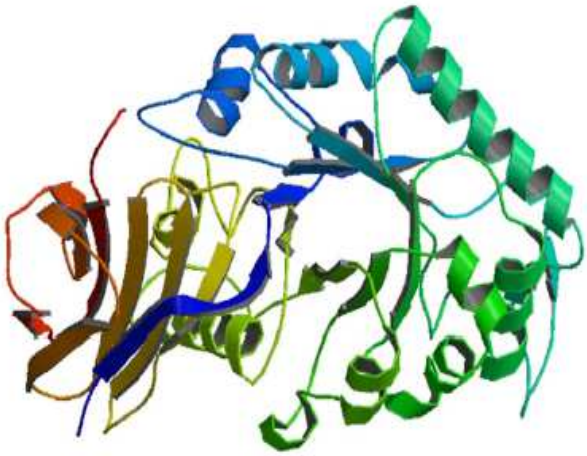

Fig. 1.3 Model assembly image of crystal structure of XynC isolated from *Bacillus subtilis* 168. Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient (http://www.rcsb.org/pdb/home/home.do).

1.3.3.2. Family 8 glycoside hydrolase

The capability to hydrolyse the β-1,4 xylopyranoside chain of xylan is also a characteristic mode of action of this family. The substrate binding to the enzyme active site is highly specific and follows classical induced fit mechanism. The catalytic events are followed by conformational changes of enzyme in number of times due to the proper shiftment of proton donar into a more catalytically competent position. The bacterium *Pseudoalteromonas haloplanktis* TAH3a, isolated from the Antarctic, has been thoroughly studied for its pXyl, the GH family 8 xylanase (Fig. 1.4.) The X-ray chrytallographic study of the enzyme pXyl (kDa 47.34) complex with its substrate xylopentaose and product xylotriose, reveled that subsites from -3 to +3 are mainly responsible for the structure function relationship. Moreover it has been described in the report that the xylan hydrolysis by GH 8 family is preceded by conformational changes with out the ground state conformation of substrate (Vos *et al*. 2006).

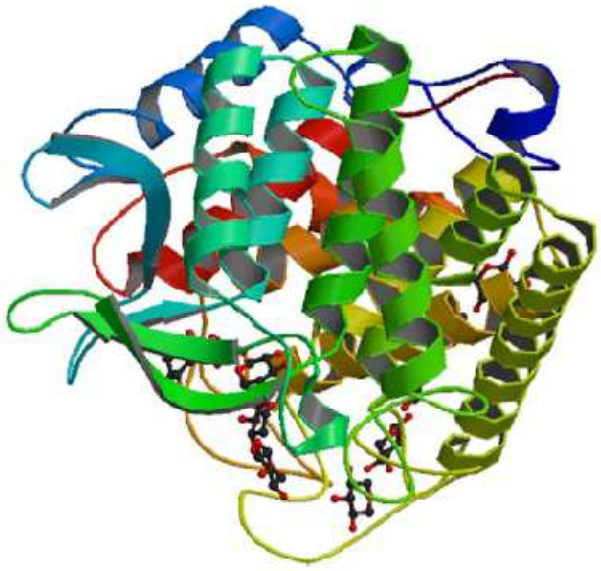

Fig. 1.4. Model assembly image for a cold adapted GH 8 family xylanase in complex with substrate. Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient) (http://www.rcsb.org/pdb/home/home.do).

1.3.3.3. Family 10 glycoside hydrolase

Santos *et al.* (2010) isolated the TpXyl10B (81.62 kDa), a GH 10 family xylanase, from the hyperthermophilic bacterium *Thermotoga petrophila* RKU-1 (Fig. 1.5). Biochemical characterization and crystallographic studies of the enzyme complex with xylobiose in native state at high temperature revealed that there are two catalytic subunits available for bonding with the substrate. Due to the coupling effect of temperature induced structural change the enzyme showed a temperature dependent mode of action. Molecular dynamics simulations method further confirmed the temperature dependent mode of action and also suggested that the release of xylobiose at high temperature is tightly regulated by the significant modification of the catalytic loop Trp297-Lys326 (Santos *et al.* 2010).

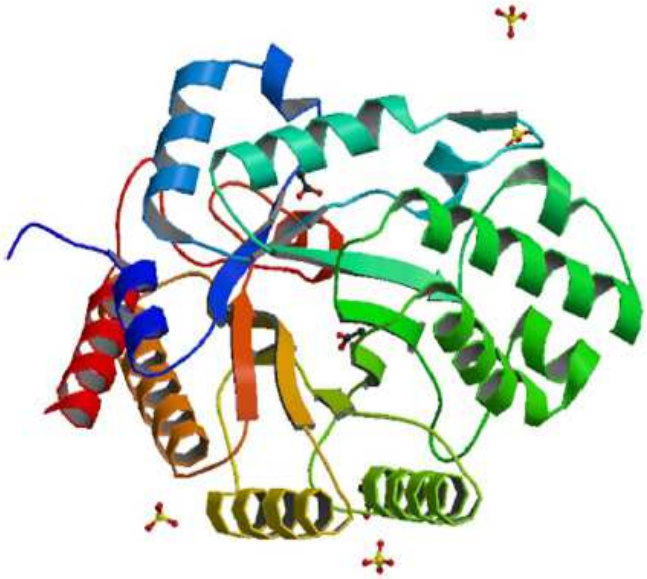

Fig. 1.5. Model assembly image for crystal structure of native xylanase 10B from *Thermotoga petrophila* RKU-1. Protein chains are coloured from the N-terminalto the C-terminal using a rainbow (spectral) colour gradient) (http://www.rcsb.org/pdb/home/home.do).

1.3.3.4. Family 11 glycoside hydrolase

Xylanase enzyme BCX (20.7 kDa) isolated from *Bacillus circulans* by Joshi et al. (2000) was found to be a member of GH family 11 (Fig. 1.6). The enzyme bears an asparagines residue at position 35 and found to influence the pH dependent activity change. This is due to the ionization of the glutamate acid residue at position 78 and 172 with respective pKa values of 4.6 and 6.7. When the asparagines residues at position 35 was substituted with aspartic acid residues, the resulting enzyme N35D BCX showed 20 % increment in its activity as compared to BCX and it was also observed that the optimum pH was shifted from 5.7 to 4.6. The pKa valuesof the enzyme N35D BCX were recorded in the range of 3.5-5.8 and found to follow the double displacement mode of action like that of the native BCX. In this mechanism glutamate at position 78(Glu 78) act as a nucleophile whereas aspartic acid residue at position 35 (Asp 35) and glutamate at position 172 (Glu 172) function together as the general acid/base catalyst. The higher catalytic activity of the mutant N35D BCX was due to the fact that it could catalyzes the reaction through reverse protonation where protonated Asp35 and deprotonated Glu78, stimulate the activity (Joshi *et al.* 2000).

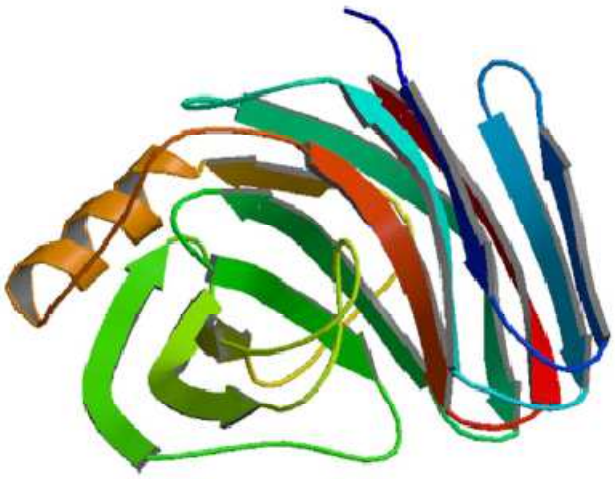

Fig. 1.6. Model assembly image for a GH 11 family xylanase. Protein chains are coloured from the N-terminal to theC-terminal using a rainbow (spectral) colour gradient) (http://www.rcsb.org/pdb/home/home.do).

1.3.3.5. Family 19 glycoside hydrolase

Biochemical analysis and crystal structure study of Xyl-ORF19 (kDa 41.18), an enzyme of GH family 19, was isolated by Han *et al.* (2013) from bacteria isolated from the termite (*Globitermes brachycerastes*) gut. Two domines were reported for the Xyl-ORF19, the catalytic daomin, made up with a C-terminal GH 10 and a non catalytic bacterial Ig-like (Big 2) domain (Fig 1.7). The catalytic domain has a $(\beta/\alpha)_8$ barrel which is similar to the barrel found in the enzymes of GH10 fmily.In addition to that two extra β-strands are also present in that domain. The non catalytic domain is closely similar to that of the immune glubuline like domain of intimins. If the non catalytic domain of the enzymes was removed the catalytic efficiency of the enzymes in terms of kinetic parameters was greatly reduced and alteration its biochemical parameters interms of pH and temperature profiles was altered (Han *et al.* 2013).

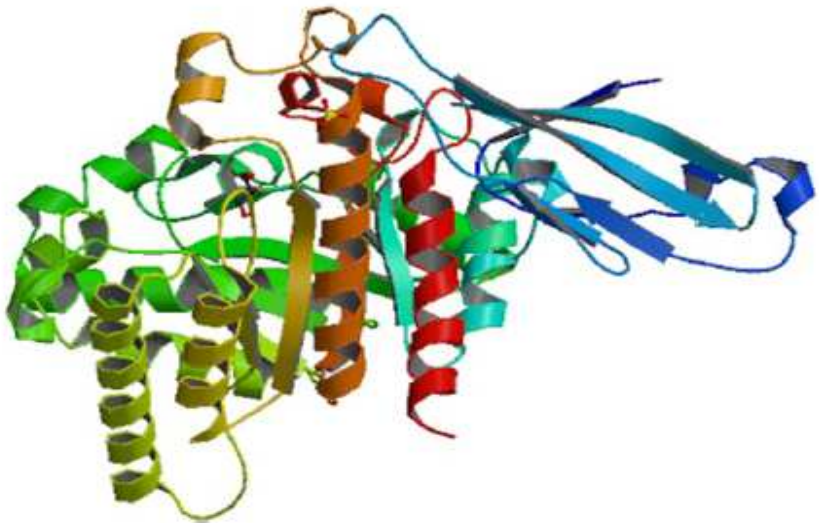

Fig. 1.7. Model assembly image for GH 19 xylanase from bacteria of termite gut. Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient) (http://www.rcsb.org/pdb/home/home.do).

1.3.3.6. Family 30 glycoside hydrolase

The enzyme glucurono xylanase, Xyn30D (kDa 44) belongs to the glycoside hydrolase family 30. It contains a catalytic domain having a carbohydrate binding module (CBM) closely related with CBM35 family (Fig. 1.8). The enzymes contains an $(\beta/\alpha)_8$ barrel as a catalytic domain similar to that of GH family 10 xylanases with an additional β-peptide structure. Two calcium ions are present between the β-sandwich of CBM35 domain. These two domain fold in an independent manner and they are much flexible. The flexibility is due to the presence of linker polypeptide region between them which makes a polar interaction with the catalytic domain. The binding affinity of the enzyme (Xyn30D-CBM35) is highly specific for the substrate with conjugated glucuronic acid moiety. This is occurring due to presence of two successive aromatic amino acid residues in the catalytic pocket. In this pocket a non conserved glutamate residues at position 129 (Glu 129) is also present which binds to the calcium ions (Sainz-Polo *et al.* 2014).

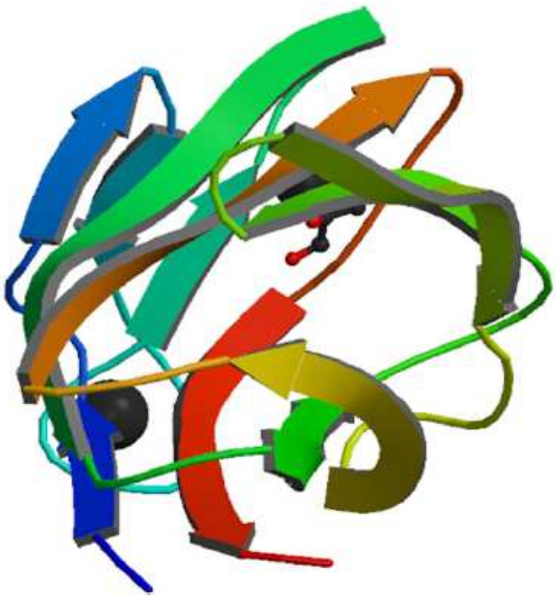

Fig.1.8 Model assembly image for GH family 30 xylanases of CBM35 from *Paenibacillus barcinonensis* (protein chains are coloured from the N-terminal to the C-terminalusing a rainbow (spectral) colour gradient). (http://www.rcsb.org/pdb/home/home.do).

1.3.4. Sources of xylanase

Xylanase, an important group of carbohydrate active enzyme are produced by both prokaryotic and eukaryiotic organisms. It has been reported from marine and terrestrial bacteria, fungi, algae, protozoa, snails, insects and germinating seeds of terrestrial plants (Walia *et al.* 2013a). Apart from soil bacteria, several marine bacteria and cyanobacteria are also reported as xylanase producers (Annamalai *et al.* 2009). Information regarding the presence of xylanase in Japanese pear fruits and in higher animal such as mollusks has also been cited in literature (Yamaura *et al.* 1997). Certain uncommon sources such as immature cucumber seeds and germinating barley were reported as a source of xylanase enzyme (Sizova *et al.* 2011).

1.3.4.1. Bacterial xylanase

A numbers of *Bacillus* species are potential producer of xylanase. *B. circulans, B. stearothermophilus, B. amyloliquefaciens, B. subtilis, B. pumilus, Geobacillus thermoleovorans* and *B. halodurans* were cited in various reports as a significant and considerable producer strain (Subramanian and Prema 2002, Verma and Satyanarayana 2012, Banka *et al.* 2014, Thomas *et al.* 2014, Gupta *et al.* 2015). The bacterial strains *Bacillus* sp, *Stenotrophomonas maltophila, Rhodothermus marinus, Thermotoga* sp, *Clostridium thermocellum* and *Streptomyces* sp, were

found to produce thermostable xylanases that were active in the temperature range 60 - 70 ^{o}C (Kumar and Satyanarayana 2013, Thomas *et al.* 2014). Psychrotrophic or cold adapted xylanase was found to be produced by the bacterial strains such as *Flavobacterium frigidarium* and *Clostridium* sp. PXLY1 (Akila and Chandra 2003, Humphry *et al.* 2001). In terms of industrial production of xylanase, bacterial sources have certain advantages over that of fungi, like optimum pH of the bacterial xylanases is in neutral or alkaline range, whereas for fungal xylanase optimum pH lies in the acidic range thus requiring additional steps in subsequent stages of industrially important process. Moreover, higher growth rate of bacteria increases the volumetric productivity of enzyme in short time (Verma and Satyanarayana 2012). Table 1.1 represents the list of some potential xylanase producing bacteria along with the biochemical properties of the enzyme produced by them.

1.3.4.2. Fungal xylanase

Due to the extracellular secretion of xylanase in the fermentation media in higher titer in comparison to that of bacteria, fungi had been extensively used as a potent source of xylanase enzyme (Polizeli *et al.* 2005). Table 1.2 represents fungi with the ability to produce xylanase and the biochemical characteristics of the enzyme produced by them. Several mesophilic as well as thermophilic fungi are reported to produce xylanase. Among the mesophilic fungi, genera *Aspergillus* and *Trichoderma* and among the thermophilic fungi, genera *Thermomyces* are being the dominant xylanase producers (Kumar *et al.* 2017). Xylanases with high temperature optima were recorded for several fungal strains such as *Aspergillus terreus*, *Penicillium* sp, *Trichoderma* sp, *Thermomyces lanuginosus*, *A.nidulens* etc (Ryan *et al.* 2003, Ghanen *et al.* 2000, Taneja *et al.* 2002). The optimum pH range varied between 4 -7 for xylanase produced by *Gloeophyllum trabeum*, *Humicola insolens* etc. (Kim *et al.* 2014, Du *et al.* 2013) whereas the enzyme from *Malbranchea flava*, *Thermoascus aurantiacus* var.*levisporus* KKU-PN-I2-1 had alkaline pH optima (Ryan *et al.* 2003, Ghanen *et al.* 2000).

Table 1.1
Biochemical characteristics of xylanase produced by bacteria

Bacteria	Substrate	pH	Temp (°C)	Km	Amount	References
Sorangium cellulosum S09733-1	OSX	7	30	38.13	4.11 U/mg	Wang (2015)
Bacillus sp. SN5	Bex	7	40	0.6	104.7 U/mg	Bai *et al.* 2012
Paenibacillus xylanilyticus KJ03	Bix	7.4	40		33U/mg	Park *et al.* 2013
Bacillus sp SV-345	Bix	6.5	50	3.7	2803.1 U/mg	Mittal *et al.* 2012.
Streptomyces sp 76	WB	6	50		79.43U/mg	Bajaj and Singh 2010.
Bacilus sp GRE7	OSX	7	70	2.23	191.1U/mg	Kiddinamoosthy 2008.
Arthrobacter Sp	WB	9	100	0.9	162U/mg	Khandeparkar and Bhosle 2006.
Geobacillus thermoleovorans	Bix	8.5	80	2.6	10.2 U/mg	Verma and Satyanarayan 2012.
Stenrophomonas maltophilia	WB	9	80		82.40U/mg	Raj *et al.* 2013.
Bacillus sp.	OSX	9	55		31.27 U/mg	Annamalai *et al.* 2009.
Actinomadura sp. Strain CPT20	OSX, Bex	10	80		51.06	Taibi *et al.* 2012.
Bacillus brevis	WS	7	55		4380	Goswami *et al.* 2014.
B. pumilus SV-205	WB	10	60		7382.7 IU/ml	Nagar *et al.* 2012.
B. haloduran	CM		80		69 U/ml	Kumar and Satyanarayan 2013.
B. subtilis BS05	SB	5	50		439 IU	Irfan *et al.* 2012.
Paenibacillus macerans		4.5	60		4170 U/mg	Dheeran *et al.* 2012.
Paenibacillus sp.		9	50		24.60 IU/ml	Pathania *et al.* 2012.
Provideacia sp.		9	60		36.3IU/ml	Raj *et al.* 2013.

BiX (birchwood xylan), BeX (Beechwood xylan), OSX (Oat spelt xylan), WB (Whear bran), WS (Wheat straw), CM (Cane molasses), SB (Sugarcane baggase).

Table 1.2
List of fungi producing xylanase with their biochemical characteristics

Fungi	Mw (kDa)	pH	Temp (^{o}C)	Family	References
Aspergillus nidulans KK-99		8	55		Taneja *et al*. 2002
Aspergillus terreus		7	50		Ghanen *et al.* 2000
Acrophialophora nainiana	22	7	55		Salles *et al*. 2000
Myceliophthora sp. IMI 387099	53	6	75		Chadha *et al.* 2004
Penicillium brasilianum	31				Jorgensen *et al.* 2003
Penicillium capsularium	22	3.8	48		Ryan *et al.* 2003
Penicillium sp. 40	25	2	50		Kimura *et al.* 2000
Thermomyces lanuginosus	24.7	6-6.5	70		Singh *et al.* 2000
Gloeophyllum trabeum	50	4-7	70	GH10	Kim *et al.* 2014
Humicola insolens	23	7	60	GH11	Yang *et al.* 2014
Humicola insolens Y1	39-44	6-7	70-80	GH10	Du *et al.* 2013
Malbranchea flava MTCC(MFXI)	25	9	60	GH11	Sharma *et al.* 2010
Malbranchea flava MTCC(MFXII)	30	9	60	GH10	Sharma *et al.* 2010
Thermoascus aurantiacus var. *levisporus* KKU-PN-I2-1	27	9	60		Chanwicha *et al.* 2015
Aspergillus tubingensis FDHN1					Adhyaru *et al.* 2016
Aspergillus tamarii Kita		5.5-6	60		Heinen *et al*. 2017

1.3.4.3. Xylanases from metagenomics libraries

The isolation of novel xylanase enzyme by conventional technique based on the selection of xylanolytic microorganism from environmental samples. But according to the research conducted by Amann *et al.* (1995) it had been obtained that only a small fraction (0.1-1%) of microorganisms were culturable using standard microbiological methods. Whereas with the help of metagenomic approaches a huge genetic diversity was extracted from the environmental samples in short time without laborious microbial culture techniques. Recently, the genes

encoding thermostable xylanases had been extracted from metagenomic library of various echological niches (Table 1.3).

Table 1.3
List of xylanase obtained from metagenomic libraries

Source	Expression host	GH family	Temperature (^{o}C)	pH	References
Volcano crater	*E. coli*	GH10	95	6.8	Mientus *et al.* 2015
Cowdung	*B. megaterium*	GH10	75	7.0	Sun *et al.* 2015
Sugarcane bagasse	*E. coli*	GH11	80	6.7	Kanokratana *et al.* 2015
Hot spring	*E. coli*	GH10	100	5.5-7.0	Sunna and Brqquist 2003.

1.3.5. Xylanase production strategies

Wide range of organisms such as bacteria, fungi, plant and animal cells produce xylanases that are either extracellular or intracellular in nature (Collins *et al.* 2005, Ahmed *et al.* 2009, Knob *et al.* 2010). Due to the several inabilities of plant and animal xylanases, the interest on microbial xylanases has increased to meet the current crisis of energy demand in the world. The improvement of xylanase enzyme production by microorganisms has been based on two major strategies, either through optimization of process parameters for enzyme production by natural wild organisms or by generating recombinant strains.

1.3.5.1. Production using wild strains

Due to the potential industrial applications of xylanase obtained from fungal and bacterial sources, extensive research are being carried out by the scientific communities to improve the xylanase production using wild producers. The process involves preliminary selection of desired microorganism(s) having the potential for novel xylanase production. Also, the enzyme should be free from toxin and undesired products that are produced along with the enzyme. The availability of potent xylanase producer and its applicability as a robust industrially important strain are the main challenges with the wild producer. Although xylanase production by wild producers shows lesser batch to batch variation in xylanase yield, the lower yield using wild producer is a major concern. Moreover, the isolation, screening and production of xylanase with the wild producers through classical microbiological approach is a tedious process. Therefore, the microbial diversity in several ecological niches of hemicellulolytic degradation demands the

proper evaluation for isolation, and screening of new microbial strains with improved industry compatible xylanase production. Several progressive research had been carried out in past for production of xylanase using wild fungal or bacterial cells such as *Burkholderia* sp. (Mohana *et al.* 2008), *Bacillus pumilus* (Battan *et al.* 2008, Poorna *et al.* 2007), *S. actuosus* (Wang *et al.* 2003), *S. cyaneus* (Ninawe *et al.* 2008), *S. matensis* (Yan *et al.* 2009), *Aspergillus niger* (Dobreva *et al.* 2007), *P. thermophila* (Yan *et al.* 2008), *T. longibrachiatum* (Azin et al., 2007), *T. languginosus* (Li *et al.* 2005). Among the microbial strains, filamentous fungal species are reported as potent xylanase producers for commercial applications as they secrete very high amount of xylanase in the fermentation medium. Mesophilic and thermophilic microorganisms were found to secrete significantly high quantity of extracellular xylanase in the medium (Polizeli *et al.* 2005). *Aspergillus*, *Trichoderma* and *Penicillium* species are mesophilic xylanase producers (Taneja *et al.* 2002, Jorgensen *et al.* 2003, Adhyaru *et al.* 2016). Very recently studies have been conducted on extraction of xylanase from thermophilic fungi and as the growth temperature of such fungi is higher, it can be inferred that the enzyme obtained from them also have the high thermostability (Adhyaru *et al.* 2016). Bacterial and fungal xylanases are mainly classified into the glycoside hydrolase family 10 and 11, respectively (Liu *et al.* 2011). Bacterial or fungal endo xylanases are generally produced as monomeric protein with molecular weight ranging between 8.5 to 85 kDa. The enzymes are glycosylated with isoelectric point (pI) ranging between 4.0 and 10.3 (Polizeli *et al.* 2005). Structural comparison between bacterial and fungal xylanases through *in silico* modeling reveals the differences in the secondary structure especially in the loop areas. Xylanase produced by *B. circulans* and *B. subtilis* contained β-pleated sheets in the loop region, whereas the enzyme produced by *Aspergillus niger* mainly contained α-helix in the loop regions. Inspite of having differences in secondary structures both these xylanases were found to contain the glutamic acid residue as nucleophile and proton donor (though the position may change) and thus belong to GH family 11 (Mathur *et al.* 2015). Downstream processing steps are often elabaorative for fungal xylanases as they are produced along with cellulases; on the other hand bacterial xylanases are produced mostly alone and thus shortening the course of downstream processing (Subramanian and Prema 2002).

1.3.5.2. Production using recombinant producer

Recombinant DNA technology (RDT) has been used for modifying the xylanase genes followed by their expression in suitable host. In this strategy the xylanase enzymes with suitable characteristics like theromostability, acid/alkai stability etc. were selected for industrial applications (Li *et al.* 2000). Microorganisms also have the capability to induce xylanase production through synthesis of various kinds of inducers. Therefore, engineering of microbial regulatory pathway for xylanase production happens to be one of the recent and advanced

approach that can improve the xylanase productivity and specificity. The gene encoding xylanase enzyme PoXyn2 (GH 11, 320 aa) was obtained from the cDNA library of *Penicillium occitanis* Pol6, was sub-cloned into the pGAPZαA vector to construct recombinant xylanase (Driss *et al.* 2012). The recombinant PoXyn2 cDNA was ligated to His-tag at the N-terminal end. The resulting construct was further integrated into the genome of *Pichia pastoris* X-33 under the constitutive promoter of glyceraldehyde 3-phosphate dehydrogenase (GAP). The expression of the recombinant xylanase enzyme was confirmed by the activity assay followed by SDS-PAGE analysis. The recombinant enzyme was purified from the cell free supernatant by affinity chromatography on Ni-NTA resin. The engineered *P. pastoris* X-33 was cultivated in the medium containing oat spelt xylan and found to overexpress the PoXyn2 xylanase with specific activity of 8549.85 U/mg (Driss *et al.* 2012).

Overexpression of a xylanase (Xyn 186) from *Alternaria* sp. HB186 in *P. pastoris* GS115 was conducted by Mao *et al.* (2012). Cloning and sequence analysis of the Xyn 186 revealed the presence of an intron of 52 bp within 748 bp nucleotide sequence. The non coding sequence was removed by the restriction digestion using Dpn I and protein encoding cDNA fragment was cloned into the vector pHBM905A. The recombinant vector was inserted into the *P. pastoris* GS115 and the gene copy number was evaluated using real time PCR technique (Mao *et al.* 2012).

1.3.6. Genetic regulation of xylanase

Effect of several carbohydrates along with xylan on genetic regulation and expression of xylanase enzyme has been reported in various scientific reports. It was observed that the microbial strains accomplished xylanase production when they were cultivated in presence of xylan, explaining the induction effect of xylan on xylanase (Biely 1986). An interesting finding was made that xylan having large bulky structure could not directly enter inside the cell to induce the xylanase gene expression. However, some sort of physical contact between the inducer and regulatory apparatus of the cell is required to accomplish xylanase expression and secretion into the fermentation medium. Next, the extracellular enzyme hydrolyses the complex polymers of xylan and liberates the short chain less complex xylooligosaccharides. These products were then transported inside the cell through β-xyloside permeases. The small oligosaccharides now induce the xylanase enzyme expression in a concentration dependent manner (Fig 1.9). In support of the above hypothesis Polizeli *et al.* (2005) observed that not only the xylanase expression but also the permease activity of the cell was increased in presence of xylanolytic inducer. Regulation mechanism for xylanase production has been extensively studied with *Aspergillus* spp. The respective xylanase and β-xylosidase genes xlnA, xlnB, xlnC and xlnD were expressed by the

Aspergillus spp. in presence of xylan and xylose (Colabardini *et al.* 2012). Stricker *et al.* (2008) reported that expression of these genes are under tight transcriptional regulation by transcription factor XlnR and also subjected to catabolite repression mediated by CreA transcription repressor. The repressor protein CreA encodes a 415 amino acid long polypeptide chain having the characteristic features of DNA binding proteins. The presence of zinc fingures motif, alanine rich domain and the occurrence of SPXX and TPXX motifs explaining the strong affinity of this protein to the specific cis acting sites of DNA (Ruijter and Visser 1997). The CreA repressor binds to the CreA binding sites present in the genome of *Aspergillus* spp. (Degraaff *et al.* 1994). The regulation of xylanolytic enzymes by XlnR and CreA ensures a double check mechanism for enzyme expression.

Bacillus subtilis str168 has been studied extensively to understand the regulatory mechanism of xylanase expression. Whole genome sequence analysis of the strain was able to locate the genes for xylanolytic enzyme as well as the transporter protein for xylose uptake. The gene xynA (1241 bp), xynB (1601 bp), xynC (641 bp), and xynD (1241 bp) have been reported as the principal xylanase encoding gene of *B. subtilis* str168 (Fig.1.10a). Endo-1,4-β-xylanase that depolymerises heteroxylan to glucurono xylotetraose and xylooligosaccharides is encoded by xynA gene and is a member of GH 11 family. β-xylosidase is encoded by xynB whereas xynC encodes GH30 family endoxylanase. The product of xynD was found to be active on arabinoxylan. Constitutive expression of xynA has been recorded in contrast to the other extracellular enzymes especially during the logarithmic phase of the bacteria. Expression of β- xylosidase is tightly regulated by the inducer xylose and xylan. The role of xylose as inducer has been previously explained in the study conducted by Gartner et al. (1988) and Hastrup (1988). In a study it was reported that xylA (1337 bp) encodes xylose isomerase while xylulose kinase are encoded by xylB gene. A glycoside pentoside hexuronide transporter protein encoded by xynP (1392 bp) has been found to be present upstream to the xynB gene. The utilization of xylose by xynCB and xylAB has been found to be strongly controlled by transcriptional regulatory protein XylR coded by xylR (1053 bp). The higher amount of XylR negatively regulates the expression of xylAB (Gartner *et al.* 1988, Hastrup *et al.* 1988). Presence of xylanolytic genes both in chromosome and plasmid are reported for *Clostridium acetobutylicum* ATCC 824. Figure 1.10b represents the organization of xylanase encoding genes in the plasmid of *Clostridia*. In addition to the principal xylanolytic genes xynB (957 bp) and xynD (1376 bp), the bacteria contains two homologus copies of xynC (CAP_0118, 1766 bp and CAP_0119, 1758 bp). Involvement of xylulokinase (XylB, CAC2612), a xylose proton symporter (XylT, CAC1345), and a transcriptional regulator (XylR, CAC3673) for xylose utilization by *Clostridium acetobutylicum* has also been noticed (Chakdar *et al.* 2016).

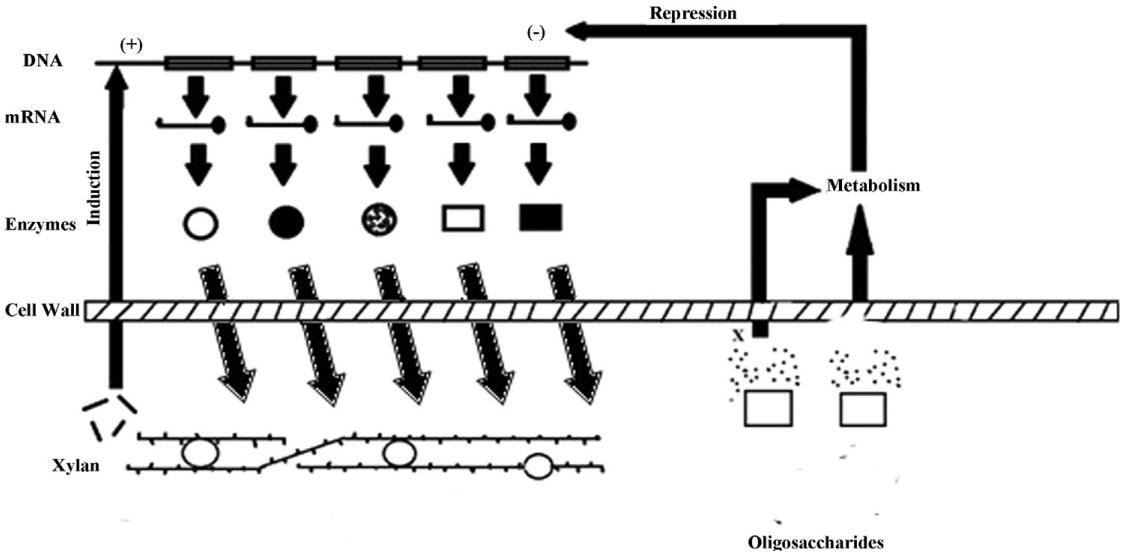

Fig. 1.9. Cellular recognition, genetic regulation and expression of xylanase (Uday *et al.* 2016)

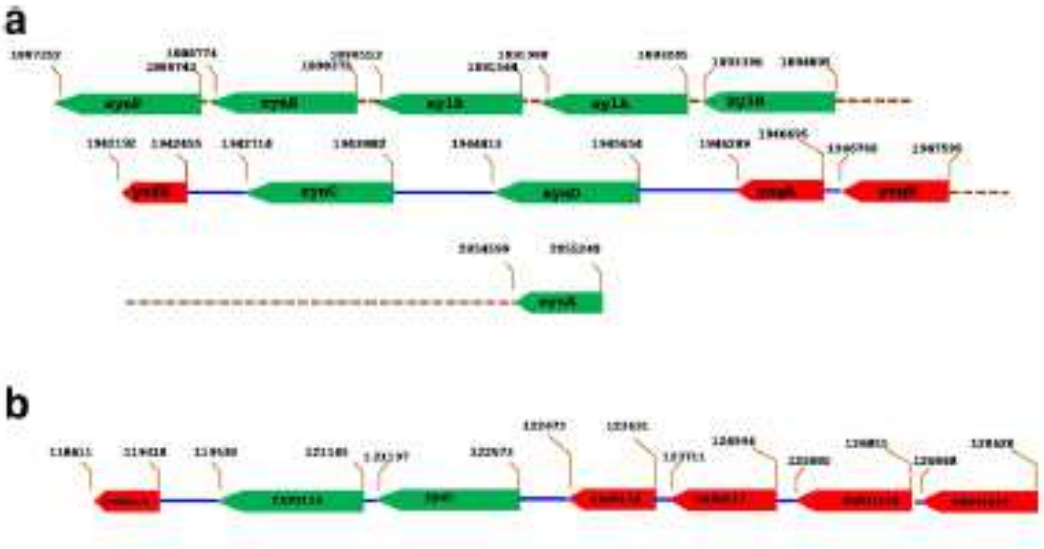

Fig.1.10. (a) Xylanase gene arrangement on chromosome of *B.subtillis* str168 (b) Arrangement of plasmid coded xylanase genes of *C. acetobutylicum* ATCC824 (Chakdhar *et al.* 2016)

1.3.7. Fermentative production of xylanase

Raw materials, cell biomass, media parameters and conditions are crucial factors for efficient fermentation by microorganisms. The sterilized medium inoculated with the starter organism placed/fed into the bioreactor or fermentor, well equipped with agitators, sparger, temperature, pH, and dissolved oxygen sensors, for fermentation. Industrially it has been found that the cost effective production of the final value added product can be achieved by using cheap raw materials (Cheng *et al.* 2014, Franceschin *et al.* 2011).

1.3.7.1. Optimization of media parameters

For maintaining a balance between the medium components and final product yield, medium composition and cultural conditions are required to be optimized. It helps to maximize the effect of each medium component and condition for higher product recovery. Several research have been carried out to evaluate the effect of cost effective agro residues, additional carbon sources, N_2 sources, metal ions, medium pH, incubation temperature, incubation time, agitation speed and inoculum concentration on the yield of xylanase enzyme. Since the nutritional requirements and growth conditions greatly vary between various microorganisms, reports on defined medium and growth conditions for highest xylanase yield are lacking (Bhunia *et al.* 2013).

Using different range of metabolites and transporter proteins microorganisms are capable of using wide variety of carbon and N_2 sources. Two kind of metabolism are generally recorded for microorganism, the primary and secondary metabolism. Primary metabolism helps the organism to go through the exponential phase of growth and the primary metabolites serve as a splitting points of biosynthetic reactions and lead to the production of secondary metabolites in stationary phase. Most of the extracellular enzyme production is reported to be enhanced during the stationary phase and subjected to regulation by various medium components as well as by catabolite repression and feedback inhibition mechanisms (Uday *et al.* 2016).

Although the xylanases can be produced by submerged fermentation (SmF) and solid state fermentation (SsF) processes, environmental parameters during aerobic stage are controlled better in SmF than SsF. Hence, past research works on xylanases production mainly used SmF. Submerged fermentation also produced higher amount of purified enzymes.

A variety of cheap agro residues, such as wheat bran, rice bran, soy meal, sugarcane bagasse, etc., had been found to be effective substrate for xylanase production (Kumar *et al.* 2009, Ras *et al.* 2013). In a study conducted by Sepahy *et al.* (2011) using oat bran as sole carbon source *Bacillus mojavensis* AG137 produced 249.308 IU/ml xylanase under SmF. Saw

dust on fermentation with *Arthrobacter* sp. MTCC6915 under SsF produced 117 U/ml of xylanase (Murugan et al. 2011). Adhyaru *et al.* (2016) reported the xylanase production using sorghum straw by *Aspergillus tubingensis* FDHN1 while Heinen *et al.* (2017) showed the xylanase production by *Aspergillus tamarii* Kita using barley bagasse. Xylanase production from wheat bran, wheat straw, rice husk, saw dust, gram bran, groundnut, and maize bran under SsF by *Bacillus subtilis* ASH has also been observed (Sanghi *et al.* 2008). Production of xylanase using wheat bran as potential substrate has been reported in various research (Kumar *et al.* 2013, Nagar *et al.* 2010, Kapoor *et al.* 2008, Sanghi *et al.* 2008). The higher xylanase productivity from wheat bran could be due to its enriched nutrient content comprising 54% carbohydrates (pentoses and hexoses) and 14% protein, minerals, amino acids, and vitamins (El-Sharnouby *et al.* 2012).

1.3.7.2. Media design by statistical approaches

Fermentation media designing is an important process for the development of microbiotechnological industrial process. The volumetric productivity and the product concentration were greatly affected by fermentation medium. To minimize the overall cost of the process, the medium components must have low cost. Optimization of media components through classical microbiological approach happens to be time consuming, as each of the possible combination of media variables have to be tested in step wise manner. On the other hand statistical approach could identify the significant media variables and study their interaction effect in very short period of time to maximize the yield of response variables. Khusro *et al.* (2016) studied the interaction effect between birchwood xylan concentrations, yeast extract concentration, incubation temperature and incubation time with the help of central composite design (CCD) based response surface methodology (RSM) for optimizing the production of xylanase by *Bacillus tequilensis* ARMATI. The result showed that highest production of xylanase was obtained with the fermentation conditions of birchwood xylan (1.5% w/v), yeast extract (1% w/v), incubation temperature (40°C) and incubation period (24 h). It was also noticed that optimization of medium by RSM caused approximately 3.7 fold enhancement in xylanase activity as compared to that optimized through one factor at a time approach (OFAT). Rosmine *et al.* (2017) conducted a study on xylanase production optimization through Plackett-Burman (PB) design and RSM techniques using *Streptomyces* sp. The significant media variables screened out with the help of PB design were further employed in RSM to maximize the xylanase production. The model predicted the xylanase activity of 10292 U/ml with the optimum concentrations of olive oil, xylan, agitation speed and inoculum age at 33.10 mg/l, 0.37%, 42.87 RPM and 21.05 h, respectively. The observed activity of 10292 U/ml was recorded which was close to the model predicted response (Rosmine *et al.* 2017). Xylanse production by the *A. niger* LPB 326 through SsF using sugarcane bagasse and soybean meal as cheap carbon

and nitrogen source, respectively, was optimized by factorial design. The media containing 6.5 g of sugarcane bagasse, 3.5 g of soybean meal and 85% initial water content showed the maximum xylanase (3099 IU/g of dry matter) production (Maciel *et al.* 2008). Adhyaru *et al.* (2017), using sorghum straw as a principal carbon source in the medium overproduce *Bacillus altitudinis* DHN8 xylanase of 492 IU/ml through RSM technique which was comparable to the xylanase (245 IU/ml) produced in OFAT method as reported in the authors previous study (Adhyaru et al., 2014).

1.3.8. Applications of xylanase

The bacterial xylanases are generally alkali stable and thermostable; moreover, they are devoid of cellulase activity. Due to the above features the bacterial xylanases have played a wide range of role and are industrially important. Along with the bacterial xylanases their production processes were also patented for several industrial applications and have been marketed globally by various commercial companies. Commercial fungal xylanases were also produced in large scale using mainly *Aspergillus niger*, *Trichoderma* sp. and *Humicola insolens* in Japan, Finland, Germany, Republic of Ireland, Denmark, Canada and the USA. Some of the major xylanase producing companies and their purposes are presented in the table 1.4.

1.3.8.1 Paper and pulp industry

The xylanase enzymes active at high temperature and alkaline pH are used for biobleaching of paper pulps without depolymerizing the cellulose polymers (Polizeli *et al.* 2005). Xylanase from various microbial sources has also been employed for bleaching of wood pulp. Xylanse from several bacteria such as *Streptomyces* sp. (Beg et al. 2000; Georis et al. 2000); *Streptomyces galbus* (Kansoh and Nagieb 2004), *Bacillus pumilus* (Bim and Franco 2000; Duarte et al. 2003); *Bacillus circulans* (Dhillon et al. 2000) and from fungus such as *Aspergillus niger* (Zhao et al. 2002); *Aspergillus nidulans* (Taneja et al. 2002); *Aspergillus fumigatus* (Lenartovicz et al. 2002); *Thermomyces lanuginosus* (Haarhoff et al. 1999); *Trichoderma reesei* (Oksanen et al. 2000); *Acrophilophora nainiana*, *Humicola grisea* (Salles et al. 2004) etc. being widely used for biobleaching of paper pulp. There were two proposed mechanisms for biobleaching of paper pulp using xylanase enzyme. According to one mechanism, the industrial scale cooking of primary wood pulp lowers the pH that precipitate plant cell wall xylan over lignin polymers and the depolymerisation of xylan using xylanase expose the lignin and make it more sensitive for successive stages of pulp processing (Viikari *et al.* 1994). On the other hand, second mechanism proposed by Buchert *et al.* (1992) and Paice *et al.* (1992) state that lignin usually form complex with the hemicellulosic material specially with xylan and some of the bonds are alkali resistant

and thus could not been hydrolysed during kraft process. Cleavage of these bonds are catalyzed by microbial xylanases and opening the structure of cellulose fibrils of the paper pulp.

Table 1.4
Commercial products of microbial xylanases

Sl No.	Product	Company	Source	Application
1.	Nutri Xylanase Enzyme	Ultra Biologics Inc., USA	*Bacillus subtilis*	Feed additive
2.	Bleachzyme F	Biocon India, Bangalore	-	Bleaching of pulp
3.	Bacterial Xylanase XBK BX9	Leveking, China	Bacteria	Bakery
4.	Panzea	Novozyme, Denmark	*B. licheniformis*	Bakery
5.	Belfeed B1100	Agrimex, Belgium	Bacteria	Feed additive
6.	Allzym PT	Alltech	*Aspergillus niger*	Animal feed improvement
7.	Bio-Feed Plus	Novo Nordisk	*Humicola insolens*	Animal feed
8.	Solvay pentonase	Solvay Enzymes	*T. reesei*	Starch and bread-making industries
9.	Xylanase GS35	Iogen	T. reesei	Cellulose pulp bleaching, animal feed
10.	Cartazyme	Clariant, UK	*Termomonospora fusca*	-

1.3.8.2. Deinking of waste paper

Deinking is considered as one of the important process for recycling the waste papers. Chemical treatments with chlorine, chlorine based derivatives, sodium hydroxide, sodium carbonate, sodium silicate, hydrogen peroxide, hypochlorites, and chelating agents are being used for deinking purpose which generate large amount of toxic effluents and creating environmental pollution (Maity *et al.* 2012). Removal of ink from the effulent generated from paper and pulp industries had been carried out using xylanase in combination with laccase (Dhiman *et al.* 2014, Chandra and Singh 2012). Synergistic activity of xylanase and laccase had been successfully

employed for recycling the news paper and observed to increase the brightness (11.8 %), whiteness (39 %) and physical properties like breaking length (34.8 %), burst factor (2.77 %), and tear factor (2.4 %) of the recycled paper (Gupta *et al.* 2012).

1.3.8.3. Improvement of feed quality used for livestocks

Digestibility of the feeds for live stock can be improved by xylanase. Certain cereals, such as maize and sorghum have shown very low viscosity due to the presence of arabinixylans which remain undigested in the animal stomach and act as antinutrients. Xylanase treated cereals were digested properly in the upper digestive tract of animal and helped to extract more energy from the feed (Harris and Ramalingam 2010). Diet rich in xylanase treated cereals was found to reduce the level of unwanted residues such as phosphorus, nitrogen, copper and zinc in the excreta and thus playing an important role to minimize the environmental pollution (Polizeli *et al.* 2005).

1.3.8.4. Bakery industry

Wheat, the key ingredient of baking industry contain substantial amount of arabinoxylans. The treatment of wheat flour with xylanase helps to solubilize the water unextractable arabino xylan (WU-AX) and thus improved the do ugh quality (Courtin and Delcour 2002). Butt *et al.* (2008) observed that the viscosity of dough was reduced with treatment of xylanase from *B. subtilis*. Moreover in the same study the negative effect of xylanase on gluten aglomeration was also reported.

1.3.9. Conversion of hemicellulose to fermentable sugar

Heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids together comprises the hemicelluloses. Xylans are mostly present as hardwood hemicelluloses where as glucomannas are the predominant molecule of soft wood hemicelluloses . The hetero polysachharide xylan chains are mostly made up with β-1,4- linked D-xylopyronoside residues along with arabinose, glucuronic acid or its 4-O-methyl ether, and acetic, ferulic, and p-coumaric acids. Different plants varieties such as grasses, cereals, softwood, and hardwood, showed great variation on the content and type of xylan present in the cell wall. Birch wood xylan was found to compose with 89% xylose, 1% arabinose, 1.4% glucose and 8.3% anhydrouronic acid (Kormelink *et al.* 1993), whereas 65.8% xylose, 33.5% arabinose, 0.1% mannose, 0.1% galactose, and 0.3% glucose were found to be the monomeric sugar of wheat xylan (Gruppen *et al.* 1992).Hence, these complex hemicelluloses were treated with xylanolytic enzymes for recovery of several monomeric fermentable sugar.

1.3.9.1. Pretreatment of hemicellulose

Agro residues such as the straws, hulls, stems, stalks etc. of different plant species are the considerable lignocellulosic biomass. Although the composition varied from plant to plant and tissue to tissue, roughly cellulose (35-50%) is the main component which is followed by hemicellulose (20–35%) and lignin (10–25%) (Saha 2003). Disruption of polymeric matrix to monomers is the key chalange for the utilization of lignocellulosic biomass. Thus various research works had been designed aiming to develop an efficient pretreatment method that would assit the enzymatic catalysis of agro residues with higher digestibility (Jorgensen *et al.* 2007). Several numbers of pretreatment methods had been developed such as treatment with dilute acid or alkali, steam explosion, lime treatment, microwave irradiation, wet oxidation, H_2O_2 oxidation, ammonia mediated freeze explosion etc. to make the lignocellulosic residues more sensitive to enzymatic catalysis (Wyman 1994). Removal of hemicelluloses or lignin, reduction of the crystallinity of cellulose and increase the surface area are the main purpose of pretreatment method (Singh *et al.* 2010). Pretreatment with microwave radition found had a positive role on digestion of ligonecllulosic biomass (Maa *et al.* 2009). However, the drawback of the pretreatment procedures is the generation of some microbial growth inhibitory compounds. Degradation of lignin yields phenolic compounds, sugar degradation generates furfural and 5-hydroxymethylfurfural (HMF), and certain aliphatic acids such as acetic acid, formic acid and levulinic acid were found to have microbial growth inhibitory capabilities (Jonsson *et al.* 2016, Ko *et al.* 2015). Microbial growth inhibitory capabilities of furfural and HMF were well documented in the research conducted by Zheng *et al.* (2014). The authors also noticed that these compounds had a negative effect on the production of bacterial nano cellulose. Moreover, the bioconversion of bacterial nono cellulose to oxidized or reduced products was also inhibited by HMF (Zheng *et al.* 2014). Various method of detoxification had been developed, such as filtration of the pretreated broth through ion exchanger resins, charcoal, treatment with laccase, fermentation by filamentous fungus, extraction with organicsolvents etc (Saha 2003, Palmqvist *et al.* 2000). However, another alternative approach was based on the isolation of some microorganism which was resistant to these compounds and could efficiently ferment the released sugar and produced value added products (Jonsson *et al.* 2016). New resistant strains of *K. xylinus* ATCC 23770 had been isolated which showed significant growth on furan aldehydes and phenol and also produced 14.78 g/l bacterial nano cellulose. The conversion of these toxic compounds to less toxic products either by oxidation or reduction was also reported for the *K. xylinus* ATCC 23770.

1.3.9.2 Enzymatic saccharification of hemicellulose

Conversion of polymeric carbohydrates like celluloses and hemicelluloses into monomeric sugars was crried out by the enzymes sucha as cellulase, xylanase, xylosidase etc. obtained from various microbial sources. Among the various ligonocelluosic biomas depolymerizing enzyme producer fungi play crucial role.In addition to the production of cellulolytic and xylanolytic enzymes filamentous fungi also produced hydroxyl radical which oxidized the plant cell wall (Yarbrough *et al.* 2017). Many microorganisms were also reported for production of complete xylanolytic enzyme system such as *Penicillium capsulatum, Talaromyces emersonii* etc. (Filho *et al.* 1991). The thermophilic actinomycete *Thermomonospora fusca* produced an array of endo-xylanase, β-xylosidase, α- arabinofuranosidase and acetyl xylan esterase. These enzyme cocktail was found to act on lignocellulosic biomass synergistically and liberated the fermentable monomeric sugar (Saha 2003).

Bhalla *et al.* (2015) observed that 68.9% birchwood xylan was converted to monomeric sugar when it was acted upon by xylanase from *Geobacillus* sp. WSCUF1. Whereas conversion rate was much lower using commercial enzymes such as Celic HTec2 (49.4 %) and Accelerase XY (28.92 %), at 70 ^{o}C. The enzymatic saccharification of agro residues supplemented with xylanase increase the production of reducing sugar which further fermented for biethanol production. Genetically modified microorganisms were employed for conversion of pentose sugar to ethanol inaddition to the fermentation of hexose sugar generated from lignocellulosicresidues. But as the pentose sugar can simply be converted by microorganism to xylitol, this approach was accssed by many researchers as a promising approach for utlilization of agro residues (Cheng *et al.* 2014, Franceschin *et al*. 2011).

1.3.10. Xylitol, the sweetener

Xyliol ($C_5H_{12}O_5$) is a five carbon sugar alcohol and generally used as sweetener. It was first chemically synthesized by Emil Fisher and Stahel in 1891 (Fischer and Stahel 1891). Its structural formula is shown in Fig. 1.11. Sweetening power of xylitol (sugar alcohol of xylose) is similar to that of sucrose, nearly three times of mannitol (sugar alcohol of mannose) and twice that of sorbitol (sugar alcohol of glucose). As the calorie content of xylitol is less than sucrose, it has the potential to replace sucrose to make low calorie products. The physical and chemical properties of xylitol are listed in Table 1.5. Due to some exclusive properties of xylitol it has been widely used in pharmaceuticals, nutraceuticals, food and beverage industries (Maguire and Rugg-Gun 2003).

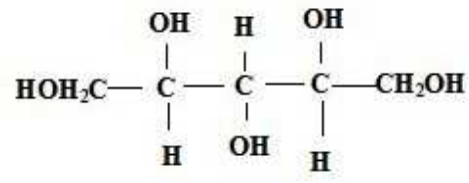

Fig.1.11. Chemical structure of xylitol.

Table 1.5 **Physico-chemical properties of xylitol (Rehman *et al.* 2013)**	
Formula	$C_2H_{12}O$
Molar mass	152.15 g/mol
Appearance	White, crystalline powder
Odour	None
Solubility in water (20^{o}C)	100 g/l
pH in water (1g/10ml)	5-7
Melting point	92 to 96^{o}C
Boiling point	345.39^{o}C
Density	1.52 g/l
Caloric value 4.06 cal/g	16.88 J/g
Density (specific gravity) of aqueous solution (20 °C) 10% 60%	 1.03 1.23
Relative sweetness	Equal to sucrose; greater than sorbitol and mannitol

1.3.10.1. The applications of D-xylitol

D-xylitol is used in chewing gums, mouth rinser and in tooth pestes as an effective safe tooth decaying preventive agent (Ritter *et al.* 2013, Uittamo *et al.* 2011). Most of the human dental decay was found to be associated with *Streptococcus mutans*. This bacterium attached to the enamel pellicle, forms bioflim and interacts directly with salivary components. The intake of xylitol containing products by people suffering from dental plaques caused by *S. mutants*, resulted in decrease of the lactic acid production by the strain. Moreover, xylitol is more alkaline than other sugar thus increasing the salivary pH. High pH facilitates the deposition of calcium and phosphate salts into the exposed enamel parts where it is lacking.

Additionally, the potentiality of saliva in preventing plaque is enhanced due to the acid buffering capacity of xylitol (Chen *et al.* 2010). Xylitol also reduces the bioflim forming ability of *S. mutans* in the enamel (Ritter *et al.* 2013). Uittamo et al. (2011) reported that xylitol at a concentration of 5% to either epithelial cells or pneumococci or both, reduced the adherence of *Pneumococci*. Growth inhibition of *Pneumococci* was found to be xylitol mediated and occurred via fructose phosphotransferase system. In the liver and red blood cell of healthy human beings xylitol is converted to glucose-6-phosphate and this pathway is not regulated by insulin. Glucose-6-phosphate later converted to glucose in a slow catalytic reaction thus raising the insulin level very slowly in the blood. Thus the patients suffering from insulin deficiency are being treated with xylitol. Moreover, due its low calorie content and little thermogenic effect, xylitol appears to be attractive compounds for insulin independent diabetic patient (Chen *et al.* 2010).

1.3.10.2. Chemical method of xylitol production

After extraction and chromatographic separation of D-xylose from woody plant, chemical conversion of xylose to xylitol was first initiated in Finland, during the period of 1970s. Chemically D-xylose was catalyzed in presence of hydrogen with high temperature and pressure for production of D-xylitol. Due to the requirement of pure D-xylose, this process required several purification steps. The process of xylitol production was categorized into four different steps. Firstly, the different biomasses were chemically depolymerised into a mixture of different sugars, mainly rich in pentoses (Kumar *et al.* 2009). Second steps involve the purification of xylose from the chemical hydrolysates with the help of ion exchange resins (Zamani 2015). Activated charcoals were also employed in this step for the removal of salts, metal ions, unpleasant odour and colour (Wei *et al.* 2010). Third, the purified xylose was reacted with hydrogen in presence of Ni as catalyst, under high temperature (140–200 ^{o}C) and high pressure (50–60 bar) until the xylitol production take place (Su *et al.* 2013). In the final step of the process, the xylitol was purified and crystallized (Martinez *et al.* 2015, Sampaioa *et al.* 2006). Although the chemical process of xylitol production showed high yield and high conversion efficiency, but it has some major drawbacks, such as it requires expensive equipments, purification steps, recovery of product etc. which make the overall process energy demanding and expensive.

1.3.10.3. Biotechnological production of xylitol

Due to the major drawbacks and expensive product cost obtained from chemical process creates an urge for intervention of biotechnological process for low cost production of xylitol. As the biotechnological processes operate at relatively mild condition and can be

employed on relatively crude mixture of lignocellulosic hydrolysates, overall cost is greatly reduced. Biotechnological production mainly depends on the live cell of wild microorganisms such as bacteria, fungi or their recombinant strains. Although immobilized cell free catalysts were also employed for bio-conversion of xylose to xylitol, but this process was industrially not economical (Park *et al.* 2005).

1.3.10.4. Microorganisms & metabolic pathway

Microorganisms capable of fermenting xylose to xylitol are cited in various scientific reports and most of them belong to yeast whereas few bacteria are also reported (Rafiqul and Sakinah 2013). Low yielding filamentous fungi were also reported. Table 1.6 represents the list of microorganisms capable of xylitol production. D-xylose is reduced by an intracellular enzyme xylose reductase (XR, EC 1.1.1.21) to xylitol in a single step. The xylitol may either secrete in the fermentation broth or may act upon by xylitol dehydrogenase (XDH, EC 1.1.1.9) and oxidized to xylulose. The cofactor requirement of both the enzyme is just opposite, NADH/NADPH act as a cofactor for XR while XDH requires NAD^+/$NADP^+$ (Granstrom *et al.* 2007). Pentose phosphate pathway recycles the D-xylulose for the production of several biosynthetic precursors and the generation of reducing equivalents. Xylose utilization in bacteria which were devoid of XR and XDH occur through the activity of xylose isomerase enzyme (XI; EC 5.3.1.5) which convert xylose to xylulose and xylulose later enter into the pentose phosphate pathway to support the growth (Dasgupta *et al.* 2017). The pathway for xylose utilization and its conversion to xylitol by microorganisms is presented in figure 1.12. Only few bacteria were reported for xylitol production, such as *Enterobacter liquefaciens* (Ghindae *et al.* 2010, Yoshitake *et al.* 1973), *Corynebacterium* sp. (Rangaswamy and Agblevor 2002). These bacterial strains showed very low productivity and required high incubation period. Due to these features, these strains were not applied for xylitol production in industrial scale (Dasgupta *et al.* 2017). On the other hand due to the stable expression of XR and XDH, and higher rate of pentose conversion to xylitol, yeasts were preferred for xylitol fermentation (Rafiqul and Sakinah 2013). The role of *Candida* sp. as whole cell biocatalysts has been reported by various researchers (Ping *et al.* 2003, Silva *et al.* 2006). Tamburini *et al.* (2015) reported a *Candida* sp. which had approximately 86% conversion efficiency of xylose to xylitol, and showed a considerable amount of volumetric productivity (0.63 g/l/h). Although well known for ethanol production, *Pichia spitis* was also reported as a potential xylitol producer (Rodrigues *et al.* 2008). The diary yeast *Kluyveromyces marxianus* was found ferment pentose sugars under high temperature and reported to produce 0.6 g/g xylitol (Mueller *et al.* 2011). Inspite of its inability to assimilate pentose sugar, *Saccharomyces cerevisae* was

genetically modified for fermentation of xylose to xylitol due to the available vast knowledge about its genomic constitution and scientific data (Kim *et al.* 2002).

1.3.10.5. Genetic modifications of microorganism for high xylitol yield

Major problems with the existing xylitol fermenting yeast are associated with their low xylitol recovery. Genetic stability of microorganisms, their growth patterns, level of expression of proteins, product yield and finally the product recovery are the important factors regarding the production of certain chemical industrially (Ghindae *et al.* 2010). After gradual enrichment of genetic information and knowledge of metabolic pathways in many non *Saccharomyces* yeasts, the product volume and yield of xylitol has increased through specific gene manipulations. Factors influencing the several gene manipulations are briefly discussed in the following sections. Gene manipulations such as gene level optimization of xylose transporter increased the xylose uptake inside the cell, over expression of XR leads to high rate conversion of xylose to xylitol, whereas overexpression of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6-PGD) increase the level of NADPH for xylose reduction by XR and deletion of XDH coding gene stoped the further conversion of xylitol to xylulose (Fig 1.13).

Table 1.6
List of xylitol producing microorganisms.

Microorganism	Temperature (^oC)	pH	Substarte	Xylitol (g/l)	Yield (g/g)	Volumetric productivity (g/l/h)	References
Bacteria *Enterobacter liquefaciens 553*	30	7	Xylose	33.3	0.33	0.35	Yoshitake *et al.* 1973
Corynebacterium sp. B-4247	30	7.2	Xylose	40	0.4	0.8	Rangaswamy and Agblevor 2002
C. guilliermondii FTI 20037	-	-	Rice straw	37.6	0.62	-	Roberto *et al.* 1996
Candida parapsilosis	-	-	Corn cob	36	0.72	-	Kim *et al.*1999
Candida tropicalis KCTC 7221	30	6.5	-	110	0.81	5.4	Kim *et al.* 2004
Pichia sp.	28	-	-	25	0.58	0.5	Rao *et al.* 2006
Kluyveromyces marxianus IMB2	45	4.5	-	7.78	0.42	0.24	Mueller *et al.* 2011
Kluyveromyces marxianus CCA510	30	6	-	12.27	0.50	0.17	De Albuquerque *et al.* 2015
Fungus *Penicillium crustosom CCT 4034*	30	6	-	0.52	-	0.005	Sampaio *et al.* 2003

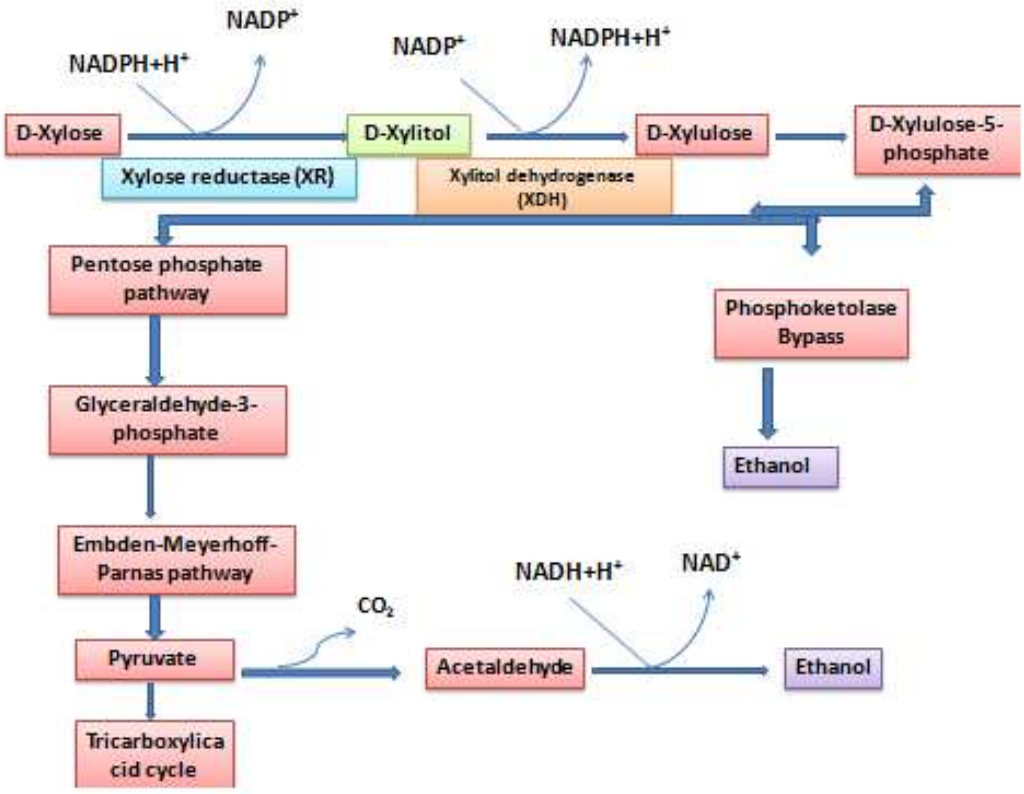

Fig. 1.12. Metabolic pathway of xylose utilization in microorganism (Granstrom *et al.* 2007).

1.3.10.5.1. Transport optimization

Pentose sugars are mainly transported into the cell through the hexose transporter proteins such as Hxt4, Hxt5, Hxt7 and Gal2 in yeast (Jeffries *et al.* 2007). These transporter proteins are neither selective for xylose nor for other sugars (Farwick *et al.* 2014). In hemi ascomycetes yeasts transporters Hxt 7 and Hxt5 are mainly responsible for xylose uptake when it is present as sole carbon source in the media. Fernandes and Murray (2010) successfully cloned and expressed the native transporter proteins of yeast involving in xylose uptake. *Candida* sp xylose symporter GFX1 and GXS1 and xylose symporter SUT from *Pichia* sp has been expressed in *Saccharomyces* and *Kluyveromyces* and found to be functional (Zhang *et al.* 2015). However, the modified transporters suffer from low xylose uptake hence low xylitol yield (Apel *et al.* 2016). In order to regulate the xylose uptake and flux control, a group of transporter proteins which act cooperatively might be essential

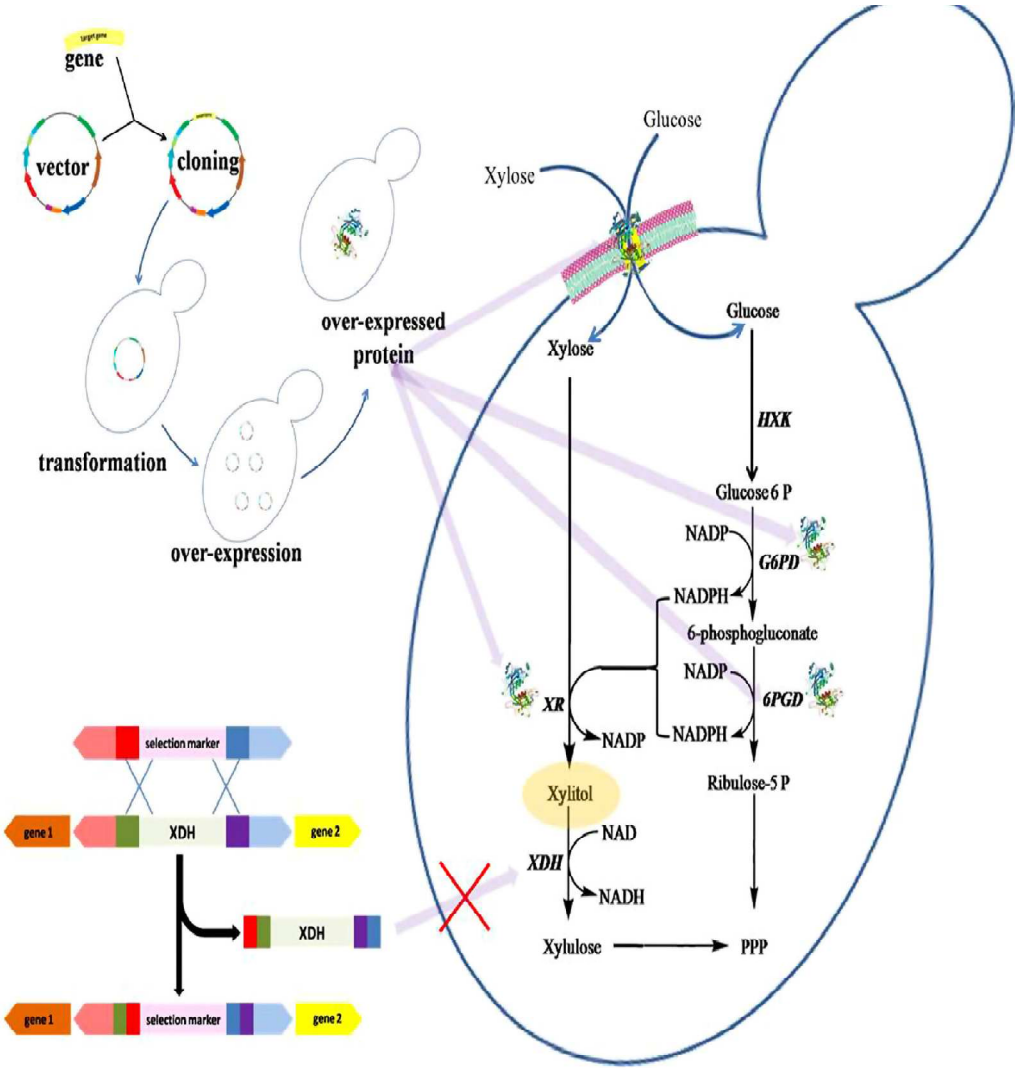

Fig 1.13. Several types of gene manipulation conducted in yeast for high xylitol yield (Dasgupta *et al.* 2017).

1.3.10.5.2. XR overexpression

The principal enzyme for xylitol production pathway is xylose reductase. Most of the xylitol producing strains often suffer from low expression level of XR and hence low xylitol yield. Native XR enzyme coding genes were modified through recombinant DNA technology for their stable expression or exogenous XR genes might be overexpressed in the endogenous

host under a strong constitutive promoter. In *S. cerevisae* XR genes from different sources were expressed after modification of the codon usage according to the *S. cerevisae*, and had shown to improve the xylose reduction rate to xylitol (Kogje and Ghoshalkar 2016). Baker's yeasts harbouring the exogenous XR genes of *Candida* and *Pichia*, under constitutive promoter, showed the production of 0.86 g/g and 0.9-1.0 g/g of xylitol, respectively (Bae *et al.* 2004, Kim *et al.* 2002).

1.3.10.5.3. Cofactor supply and engineering

Xylose to xylitol conversion by XR enzyme required reduced form of NAD in phosphorylated or unphophorylated state. Product yield become greatly reduced due to the unavailability of reducing equivalent. To enhance the product yield, reduced cofactors might be externally added to the media. But due to the high cost of the reductant overall cost of the process becomes expensive. Xylitol production pathway in *C. utilis* CBS-621 has been studied thoroughly and found that accumulation of NADPH/NADH principally depends on hexoses monophosphate pathway (HMP) and the redox imbalance between XR and XDH (Bruinenber *et al.* 1983). Berg et al. (2002) observed that regeneration of NADPH occurred in oxidative pentose phosphate pathway, during the generation of cell biomass. Two key enzymes were involved in this mechanism G6PD (glucose-6-phosphate dehydrogenase) and 6-PGD (6-phospho-deleta gluconolactonase). Over expression of these proteins in the presence of fermentable sugars helped to regenerate the cofactors (Uppada *et al.* 2014). Recombinant *Saccharomyces* BJ3505, harbouring XR gene from *P. stipitis,* was modified by genetical tools to overexpress the endogenous G6PD gene that found to improve the NADPH accumulation. Moreover, the strains showed 6 fold increase in XR activity and xylitol volumetric productivity was increased from 1.6 to 2 g/l/h (Kwon *et al.* 2006).

1.3.10.5.4. Gene deletion

Downstream to the xylose metabolism pathway, XDH is a key enzyme. In absence of the XDH enzyme their might be accumulation of xylitol provided cofactors were regenerated through some other route. Knocking out the XDH gene pairs, using the disruption cassettes (URA3 and His3) resulted the formation of mutant version of *C. tropicalis* ATCC 20913 which showed approximately 98% xylose conversion efficiency to xylitol (Ko *et al.* 2006). In a separate experiment *Candida* sp. was subjected to UV mutagenesis and a mutant strain was found to have point mutation in the XDH gene. The mutant accumulated 1-2 fold more xylitol as compared to wild type strain (Kumar *et al.* 2010, Rao *et al.* 2006).

1.3.10.6. Bioprocess

Xylitol production through biological process depends on several factors such as, low cost raw materials or carbon source, nitrogen source, incubation temperature, media pH, aeration, reaction mode, inoculum stage and volume and finally the product recovery with downstream processing. Lignocellulosic agro residues were widely used as low cost raw materials for xylitol production. These complex polymers were initially depolymerised with physical, chemical and enzymatic treatment for liberation of monomeric sugars (Binder and Raines 2010). Xylose from lignocellulosic residues was converted to xylitol, thus the process of xylose production from lignocellulosic wastes through hydrolysis using xylanolytic enzyme system proved to be a promising approach. In addition to being a law cost feed stock, lignocellulosic agro residues require mild pretreatment and moderate detoxification was required for removal of toxic compounds from saccharified hydrolysates. Microbial xylitol fermentation route via xylose reduction also has the process advantage. There is no requirement of extra pure D-xylose, as required in chemical process. Thus mild detoxification with over liming or by activated charcoal removed the toxic compound from sachharified hydrolysates and makes the broth suitable for microbial fermentation (Tanifuji *et al.* 2013). Figure 1.14 represents the overall scheme of xylitol production from agro residues.

1.3.11. Future prospects and conclusions

Production of xylitol from lignocellulosic materials involved three principal steps, which are pretreatment, saccharifion and fermentation. The main focus of the process happens to be the degradation of complex carbohydrate polymers into fermentable sugars. Among the several methods of pretreatment, dilute acid or alkali pretreatments were used as method of choice. The pretreated agro residues were subjected to enzymatic hydrolysis using xylanolytic enzyme system. In addition to fermentable sugar, pretreatments led to the generation of undesirable toxic compounds in the hydrolystaes, such as phenolics, furfuryl, HMF etc. The toxic compounds can be removed by over liming or activated charcoal treatment. Moreover, some microorganisms are also reported to ferment the pentoses in presence of these toxic compounds. Industrially xylanases are mainly produced by some filamentous fungi and bacteria. Large scale industry compatible xylanase enzyme can be produced by the genetic modification and site specific mutagenesis of the endogenous xylanase coding gene as well as by optimization of media components and process parameters through OFAT and statistical approaches. Xylanases produced in these processes had wide range of applications; especially in remarkable sachharification of agro residues.

Although the xylanase mediated fermentable sugars were employed for xylitol production, the global need of xylitol has been met mostly through chemical hydrogenation process. However, in terms of energy expense and environmental compatibility the biological process of xylitol production is very much warranted. This biological process not only helps to produce value added products but also recycle the renewable agro wastes. Although majority of works in this field was carried out in laboratory scale, to meet up the global need the process should be conducted in industrial scale through the intervention of chemistry, chemical engineering and biotechnological aspects. Hence validation of bioprocess has been required for the development of a economic industrial process. Lignocellulosic agro residues will be proved as economic feedstock for generation of xylitol in cost effective manner and to meet the world need of xylitol demand. Although many fungus are reported that can be used in bioconversion of xylose to xylitol, the number of reported bacterial isolates are very few. Due to their rapid growth rate, bacteria can be used as effective producer with high volumetric productivity of xylitol in shorter period of time and hence, lowering down the production cost. Moreover, bacteria resistant to the phenolic inhibitory compound can also be used without much genetic alternation for xylitol production.

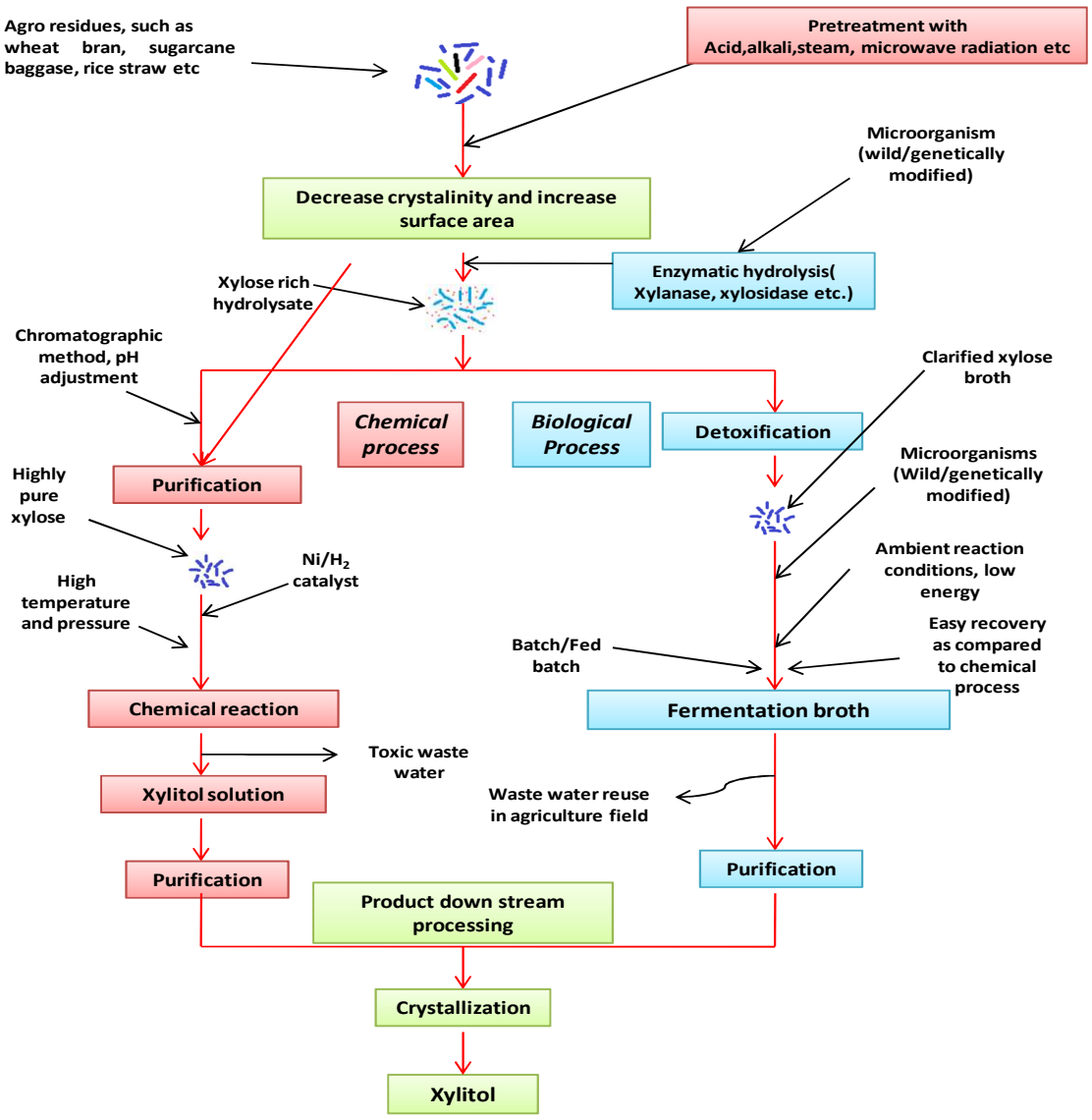

Fig.1.14. Overall scheme of xylitol production from agro residues by chemical and biological process