REVIEW OF LITERATURE

Alarming concern about the environment sustainability and security has drifted efforts towards substituting the chemical oriented practices with the environment friendly practices. In all the areas of development, there has to be replacement of fossil energy with the green energy. However, an appreciable initiative in this direction started years back when application of enzymes in various industrial processes was introduced. With time, use of enzymes as tremendous biocatalyst has gained momentum and currently they are efficiently driving diverse industrial processes. With an objective of leading to a "green future", researchers have shown keen interest in lignocellulases (cellulases, hemicellulases and lignases) in the past few decades. This class of enzyme plays vital role in valorization of the lignocellulosic residues which are an abundant and renewable source of bio-energy. About 500 Million tons of agricultural waste is generated in India, per year [18]. However, the recalcitrant composition of lignocelluloisc residues is a major hindrance and thus requires potential lignocellulases, for being converted into value-added products. Vast exploitation of lignocellulases has been done so far and is still under progress to overcome the existing research gaps. In this context, the following chapter is focused on xylanases, the type of hemicellulase which breaks down the most common form of hemicellulose i.e. xylan. Currently, thermostable xylanases have specifically grabbed much attention due to its compatability with the industrial conditions. Thus, the chapter includes discussion about the diverse sources of xylanase, its production processes, factors affecting the production of xylanase, immobilization of xylanase and its application in various industries.

2.1 Xylanase definition

Xylanase breaks down the complex backbone of polysaccharide "xylan" to release simple sugars in the form of xylose and xylooligsaccharides. Officially, xylanase is called as endo-1,4-β-xylanase. The nomenclature clearly indicates that xylanase specifically breaks 1,4-β-D- xylosidic bonds present between the xylose monomers of the linear chain of xylan (Fig. 2.1). Originally, xylanases were named as pentosanases when they were reported for the first time in 1955 [19]. Whereas, the enzyme commission number of xylanase (EC number) i.e. EC 3.2.1.8 was assigned in 1961 by the International Union of Biochemistry and Molecular Biology (IUBMB) [1].

2.2 Xylan backbone

Xylan being the major constituent of hemicellulose accounts for about one-third of all renewable organic carbon on earth [20].The backbone of xylan comprises of a linear stretch of xylose units joined together by 1,4-β-xylosidic linkage [Fig. 2.1]. Often, it is accompanied by branching at random distances due to the presence of side chain residues; intervening the continuous xylose stretch. The side chain residues are composed of arabinose, glucuronic acid (GlcA), acetyl and 4-O-methylglucoronic acid [21]. However, there are literature reports that evident plant species having xylan with no side chain residues [22, 23, 24].

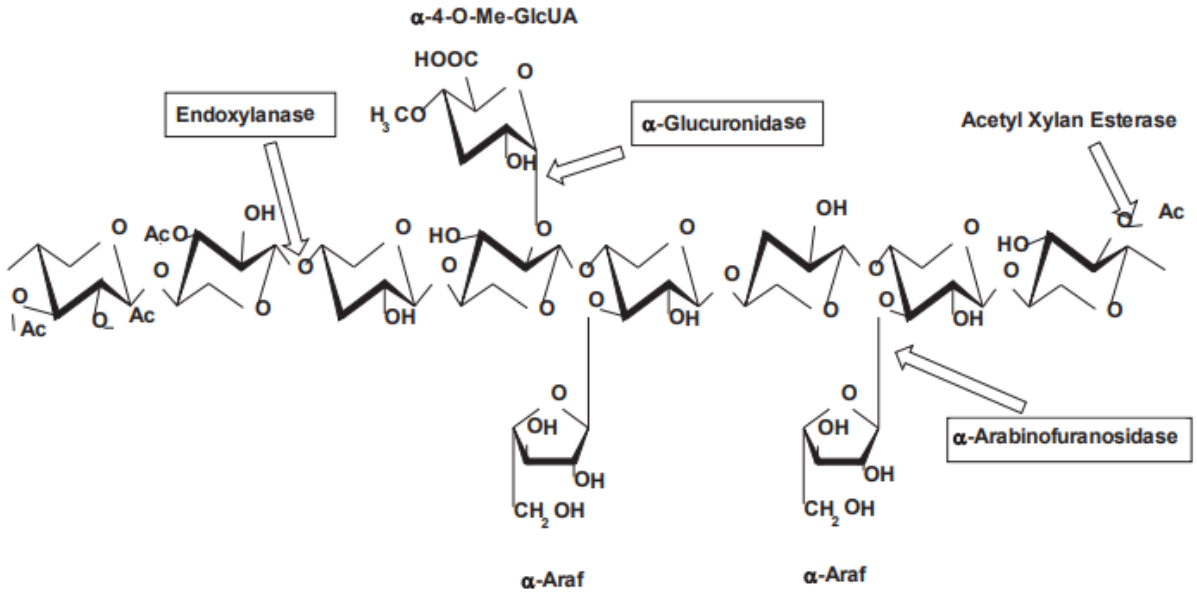
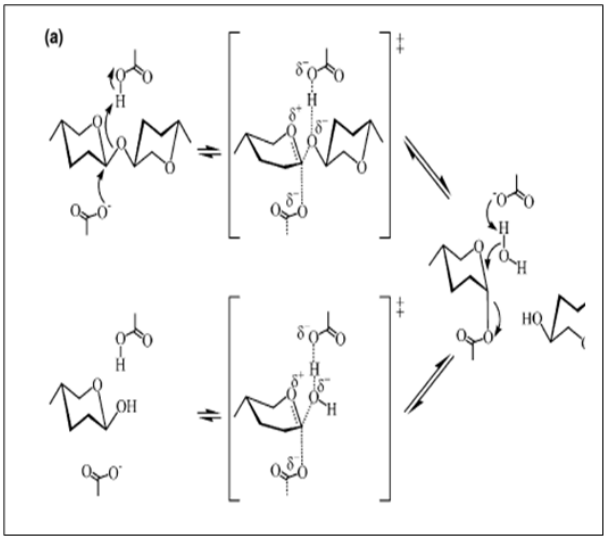


Figure 2.1: Structure of xylan and various xylanolytic enzymes involved in its degradation. Ac: Acetyl group; α-Araf: α-arabinofuranose; α-4-O-Me-GlcA: α-4-O-methylglucuronic acid [25].

2.3 Localization and mode of action of xylanase

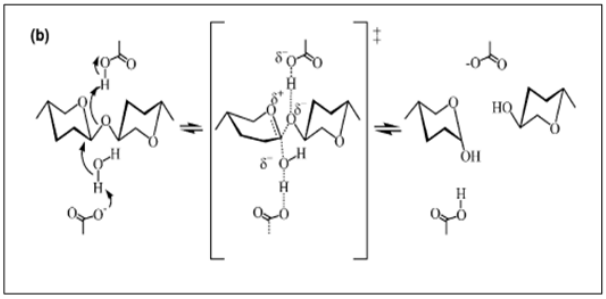
Majority of xylanases are extracellular in nature and are excreted into the environment outside the cell because the large sized substrate cannot penetrate into the cell. However, currently the production of xylanase is believed to be induced by their own products from hydrolysis [7, 25, 26]. Constitutively expressed xylanases generate xylo-oligomers which may be transported into the cell. Inside the cell intracellular xylanases and β-xylosidases conduct further degradation of xylo-oligomers [27, 28], and induction of xylanase synthesis. Mechanism of hydrolytic action of xylanases differs among xylanases from six different glycosyl hydrolase families, namely GH5, GH7, GH8, GH10, GH11 and GH43. Xylanases from families 5, 7, 10 and 11 carry out

hydrolysis with retention of configuration at the anomeric center and the two glutamate molecules are the catalytic residues (Fig. 2.2 and Table 2.1). The other mechanism involves inversion of anomeric center, glutamate and aspartate in catalytic mechanism (Fig. 2.2). This mechanism is followed by xylanases from families 8 and 43 (Table 2.1).



Retention mechanism:

- Double-displacement mechanism which involves formation of a covalent glycosyl-enzyme intermediate and subsequent hydrolysis via oxocarbenium-ion-like transition states.
- Two carboxylic acid residues of the active site are involved in the formation of the intermediate; one acts as a general acid catalyst by protonating the substrate, while the second performs a nucleophilic attack which results in the departure of the leaving group and the formation of the α-glycosyl enzyme intermediate (inversion β to α).
- In second step, the first carboxylate group now functions as a general base, abstracting a proton from a nucleophilic water molecule which attacks the anomeric carbon. This leads to a second substitution in which the anomeric carbon again passes via an oxocarbenium-ion-like transition state to give to a product with the β configuration (inversion α to β).



Inversion mechanism:

- Single displacement reaction in which one carboxylate provides for a general acid-catalyzed leaving group departure and the second functions as a general base, activating a nucleophilic water molecule to attack the anomeric carbon, thereby cleaving the glycosidic bond and leading to an inversion of the configuration at the anomeric carbon.

Figure 2.2: Mechanism of hydrolysis by xylanases (a) retention mechanism (b) inversion mechanism.

2.4 Classification of xylanases

Xylanases have been categorized on the basis of: (a) molecular weight (MW) and isoelectric point (pI) (b) crystal structure and kinetic properties and (c) substrate specificity and product profile [29]. However, complete classification of all the xylanases could not comply with the criteria based on MW and pI. So, later on classification related to structural based and comparative analysis among various catalytic domains was introduced. All the updated information regarding xylanases classification and characteristics is found in the Carbohydrate-Active Enzyme (CAzy) database. This database classifies xylanases into various glycoside hydrolase families namely 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62. Out of these, only families 5, 7, 8, 10, 11 and 43 have fairly distinct catalytic domain with endoxylanase activity

[1]. Rest of the families including 16, 51 and 62 have bifunctional nature with two catalytic domains whereas families 9, 12, 26, 30 and 44 may have residual or secondary xylanase activity. Overall differentiating features of the families 5, 7, 8, 10, 11 and 43 have been discussed in the Table 2.1.

Table 2.1: Characteristics and mechanism of hydrolysis of xylanases classified under different families.

Glycoside Hydrolase (GH) Family	Classifying Characteristics	Hydrolysis mechanism	References
GH 5	Largest glycoside hydrolase family, only seven amino acids including nucleophile and general acid/base residue, are strictly conserved among all members, activity affected by the presence of substituent along the main chain, produces products shorter than those produced by family GH 7.	Retaining	[1]
GH 7	High molecular weight, low pI, small substrate binding sites contains approximately four subsites with catalytic site in the middle.	Retaining	[1]
GH 8	Composed of cellulases, chitosanases, lichenases and endo-1,4-β-xylanases, cold adapted xylanases, hydrolyze xylan to xylobiose and xylotetarose, have large substrate binding clefts, six xylose binding residues with catalytic site in the middle.	Inverting	[1, 30, 31]

GH 10	High molecular mass, low pI, display an (α/β)-8 barrel fold, four to five small substrate binding sites, composed of endo-1,4-β-xylanases and endo-1,3-β-xylanases, also capable of hydrolyzing the aryl β-glycosides of xylobiose and xylotriose at the aglyconic end. Exhibit greater catalytic versitality and lower substrate specificity in comparison to GH 11, activity less hampered by presence of side chains.	Retaining	[1, 26, 31]
GH 11	Low molecular weight and basic pI, small size, high substrate specificity, high catalytic efficiency, wide range of optimum values for pH and temperature, considered as true xylanases as they exclusively act on D-xylose containing substrates, can hydrolyze aryl β-glycosides of xylobiose and xylotriose at the aglyconic end but are inactive on aryl cellobiosides, have larger substrate binding clefts (minimum seven subsites), prefer cleavage of unsubstituted regions, forms consists of two large β-pleated sheets and a single α-helix that forms a structure similar to a partially closed right hand.	Retaining	[32-34]
GH 43	Display five blade β-propeller fold, have glutamate and aspartate as catalytic residues, grouped with family 62 in clan GH-F, also demonstrated in family 8 enzymes.	Inverting	[1, 31]

2.5 Sources of xylanase

Bacteria, actinomycetes, fungi, anthropods, algae, gastropods and protozoa, gastropods and anthropods are the reported xylanase producers [1]. Since, microorganisms have extremely high rates of multiplication and can be easily cultivated under controlled conditions; they are the most preferred source of enzyme producers [28]. Among the diverse microflora, bacteria and fungi are in the limelight [35], however, currently bacteria are the most desired producers of enzyme in various industrial applications. Bacterial sources put forth various advantages over fungal sources such as rapid growth rate and mass production, less space requirement, easy maintenance, and higher accessibility for genetic modifications [36]. Xylanases from bacteria have tremendous potential for industrial application as they render high thermostability and alkaline stability. Bacteria form the genus *Bacillus* and *Geobacillus* have grabbed utmost attention for industrial exploitation as they are potent producers of diverse extracellular enzymes, endowed with thermophilic activities [2, 37, 38].

2.6 Xylanase from extremophiles

Xylanases from mesophilic bacteria and fungi cannot withstand the extreme environments such as high acidity (acidophiles) or alkalinity (alkalophiles), high temperature (thermophiles) or extreme low temperature (psychrophiles) and high salinity (halophiles). Thus, extremophiles are looked upon as a reliable source of xylanases that can combat such extremities [1]. Some of the themostable xylanases produced by thermophilic bacterial and fungal sources have been listed in the Table 2.2.

Table 2.2: Thermostable xylanases from thermophilic bacterial and fungal sources.

Source	Name of microorganism	References
Bacterial	*Geobacillus* sp. WSUCEF1	[39]
	Herbivorax saccinola	[40]
	Herbinix hemicellulosilytica	[41]
	Bacillus oceanisediminis	[42]
	G. stearothermophilus KIBGE-IB29	[4]
	Rhodothermus marinus	[43]
	Geobacillus sp. DUSELR 13	[44]
Fungal	*Clostridium thermocellum*	[45]
	Melanocarpus albomyces	[46]
	T. lanuginosus DSM 10635	[47]
	Humicola insolens	[48]
	Remersonia thermophila	[49]
	Thermoascus aurantiacus	[50]
	Thermomyces lanuginosus	[7]

Industries majorly crave for thermostable xylanases, as the industrial processes are carried out under robust conditions usually not bearable by thermolabile xylanases.

2.7 Isolation of xylanase producing microbial strains

For isolation of potent microbial strains, sample collection is an indispensable crucial step. The relevant site, correct way of sample collection followed by storage (sterile conditions) and safe transportation are vital for efficient sampling. The geographical area being selected for sample

collection has relevance to the category of microorganism required. If thermophilic microbial producer is the priority, high temperature areas such as hot springs, mud pots and fumaroles will be best suited for sample collection. Similarly for mesophilic microorganisms, area with an ambient temperature would be preferred. Literature reports evident collection of diversity of sample for isolation of xylanolytic microorganisms which includes soil, water, lignocellulosic residues, cow feed etc. [4, 35, 51]. For selective isolation, enrichment of sample is done to increase the fraction of desired microbial population. For isolation of xylanolytc bacteria, enrichment of the sample is done in a medium which is incorporated with xylan. Sample from the enriched medium is plated on xylan nutrient agar (NA) plates under specific conditions. Further, the isolates are screened for xylanase activity through Congo red assay and Dnitrosalicylic acid method [52].

2.8 Xylanase production

Xylanase production is carried out by different fermentation methods which can be categorized as SSF and SmF. Both practices have been widely exploited by the researchers for xylanase production. However, 90% of the total xylanase production is performed through SmF because there is homogenized distribution of microbial biomass, nutrients and oxygen in the liquid medium [5, 6]. SmF is advantageous in terms of easy scale up and maintenance of uniform conditions throughout the liquid production medium [53]. On the other hand, SSF has a limitation that it is more appropriate only for production using fungal source as it supports better mycelia growth on the solid substrate. Thus, majorly till date, SmF is preferred over SSF for xylanase production [54, 55]. As per literature reports, several *Bacillus* and *Geobacillus* sp. render xylanase production through SmF [4, 35, 38, 56, 57]. Accordingly in the current research work, xylanase was produced through SmF using *Geobacillus thermodenitrificans* X1 (GTX1).

2.9 Parameters influencing the xylanase production

Besides the method of fermentation, there are several physiological and nutritional parameters that hold great significance in the enzyme production. Factors such as inoculume size, incubation time, temperature, pH, substrate concentration, nitrogen sources/concentration, agitation speed rate and carbon sources were taken under consideration to study their impact on xylanase production. The mentioned factors have also been explored in earlier reports [58, 59]. For

efficient and maximum yield of xylanase, these influencing parameters need to be optimized with respect to the production medium.

2.9.1 Inoculum size

The inoculum size defines the initial microbial population used for initiating the xylanase production. It plays a critical role as the population has to be in synergy with available environmental conditions to avoid the depriving of nutrients and oxygen in the production medium. Any deteriorating impact on the microbial population, eventually affects the production of enzyme. Thus using an optimum inoculum size is important for attaining maximum xylanase production. Usually, inoculum size in the range of 1- 2% (v/v) has been reported to be optimum for xylanase production in the literature reports [60, 61]. Higher inoculum is not preferred because it leads to depletion of nutrients at faster rate and thus minimizes the xylanase yield [62].

2.9.2 Incubation time

The optimum time period required for maximum xylanase production varies among different sources and strains of the microorganisms used for the production. Usually, fungal producers require longer incubation time for xylanase production in comparison to bacterial producers. In recent studies, maximum production of xylanase from *Aspergillus foetidus* through SmF was attained after incubation time of 168 h [58]. However, variations have also been observed among various bacterial producers. *Anoxybacillus kamchatkensis* NASTPD13 has been reported to produce maximum xylanase within an incubation period of 24 h [56] whereas studies by Muhammad Irfan and his coworkers, reported optimum incubation time of 48 h for production of xylanase from *Bacillus subtilis* [63]. Other factors such as feedback inhibition by accumulation of the end product, nutrient depletion and variations in the pH of the production medium have correlation with incubation time [64].

2.9.3 Temperature

Different temperature optima are exhibited by microbial producers for their growth and production of enzymes [4]. This parameter has an important influence on xylanase production and need to be optimized according to the microbial producer used in the production medium. Variations in the temperature affect the microbial protein structure and properties and eventually

enzyme production [65]. Temperature below and higher to the optimum, results in decreased xylanase production as the significant metabolic pathways involved in the secretion of xylanase are negatively affected [66]. Earlier literature reports also show variations in the temperature optima for xylanase production among different microorganisms. Optimum temperature for maximal xylanase production from *Geobacillus* sp. Strain DUSELR13 was 60°C. It's been reported that temperature higher to optima is more deteriorating than the lower temperature. That is why, at 70°C, decrease in xylanase production was observed for *Geobacillus* sp. DUSELR13. Temperature beyond the optima could affect the growth of microbial culture by denaturing the essential metabolic enzymes required by cell. Eventually, due to reduced microbial cell growth, enzyme production also gets reduced [44]. Also, at temperature higher than the optimum, membrane proteins associated with the secretion of extracellular protein are negatively affected. Thus, the production of extracellular enzyme is decreased [67]. Other thermophilc bacteria such as *Anoxybacillus kamchatkensis* NASTPD13 [56] and *Bacillus amyloliquefaciens* [68] have also shown maximum xylanase production at high temperatures of 65°C and 50°C, respectively. On the contrary, being mesophilic bacteria *Bacillus cereus* SAMRC-UFH1 and *Bacillus pumilus* VLK-1, both rendered maximum production of xylanase at 30°C [62, 69].

2.9.4 pH

pH of the production medium vastly affects the enzyme yield because of its impact on the transportation of nutrients and enzymes across the microbial cell membrane, substrate accessibility and synthesis and activity of microbial proteins [70, 71]. The internal environment of the microbial system is influenced by the pH of the external environment comprising of the production medium [4]. Thus, pH is an influential parameter to be taken under consideration while optimizing xylanase production. Literature reports have shown variations in the pH optima for different microbial producers of xylanase. This is because; every microorganism has its specific pH optima for growth, which consequently affects the enzyme production by it. In 2016, Irfan et al. observed impact of initial pH on xylanase production from *Bacillus* sp. (*subtilis* and *megaterium*) , within a range of pH 4-10. For both the *Bacillus* sp. production medium with initial pH 8 rendered maximum production of xylanase [63]. Neutral pH (7) was favored by *Bacillus vallismortis* RSPP-15 for maximum xylanase production [35]. Whereas, acidic pH was

found optimum for xylanase production from *Geobacillus* sp. DUSEL13 [44], *Geobacillus stearothermophillus* KIGBE-IB29 [4] and *Bacillus altitudinis* DHN8 [72].

2.9.5 Type of Substrate and its concentration

The lignocellulosic composition of the substrate has a key role to play. Since, xylanase production is induced in the presence of respective substrate (xylan), so the hemicellulosic content of the substrate used should be high enough to induce xylanase production. Diverse carbon sources such as glucose, birchwood xylan, xylose, wheat bran, rice straw, orange peel, wheat straw, sugarcane bagasse etc. have been exploited for xylanase production. However, for cost-effective production of enzyme, birchwood xylan is usually replaced with the cheaper lignocellulosic substrates. There is variation in the hemicellulosic content of various lignocellulosic residues; 26-32% in wheat straw, 23-28% in rice straw, 19-24% in bagasse etc. Like other parameters, choice of substrates varies among different microbial producers. Birchwood xylan resulted in maximum xylanase production from *Bacillus halodurans* MTCC 9512 [73]. No xylanase production was observed from *Geobacillus stearothermophilus* KIBGE-IB29 when maltose and xylose were fed into the production medium as sole carbon source. This result was attributed to the inducible nature of xylanase [4]. In context of combating the cost constraints of xylanase production, many cost effective lignocellulosic substrates (wheat straw, corn cob, wheat bran etc.) have been used in recent studies [74, 75]. Some pretreatment studies have also been reported to open up the recalcitrant structure of lignocellulosic substrates for increasing the accessibility to microbial culture and thus enhance the xylanase production. In a study by Bandikari and coworkers in 2014, enhanced xylanase production (2,869.8 ± 0.4 IU/g) was achieved from *Trichoderma koeningi* on using pretreated (alkaline pretreatment) corn cob [76]. Whereas, the untreated corn cob showed lower xylanase production with xylanase activity of 1,347.2 ± 0.7 (IU/g). Similarly in another study, sugarcane bagasse was pretreated and used as substrate for xylanase production from *Penicillium echinulatum* 9A02S1. Pretreated bagasse resulted in higher production in comparison to untreated one [77]. However, to avoid usage of chemicals and keep the production cost to minimum, usually mechanically treated biomass is used. Besides the choice of substrate, its concentration is a deciding factor for the amount of carbon and energy supply to the production medium for the synthesis of xylanase. At low substrate concentrations, low xylanase yield is attained due to lack of adequate energy supply for

the microbial producers [78]. Whereas, high substrate concentrations decrease the xylanase yield due to substrate inhibition and non-uniform circulation of oxygen and nutrients because of the high viscosity of the medium [64, 79].

2.9.6 Nitrogen sources/concentration

The influence of nitrogen sources and their respective concentrations have also been found to vary among different microorganisms. Nitrogen sources have significant impact as they affect the availability of precursors for the synthesis of proteins required for microbial growth and enzyme production [80]. Nitrogen sources mainly used in the basal medium for xylanase production constitute peptone, beef extract, yeast extract, malt extract, gelatin, casein, urea, sodium and potassium nitrate and ammonium salts [35]. Peptone and yeast extract have growth factors and traces of nitrogen compounds that stimulate production of xylanase and microbial growth [81]. However, variations in the choice of best nitrogen source have been reported in the literature. Xylanase production from *Bacillus subtilis* sp. BS04 [63] and *Geobacillus thermolevarans* [82] was maximum in presence of tryptone as an organic source, whereas use of tryptone (0.2%) decreased xylanase production from *Geobacillus stearothermophilus* KIBGE-IB29 [4]. Similarly, urea has been observed to show inhibitory action on xylanase production from *Bacillus vallismortis* RSPP-15 [35] and *Bacillus aerophilus* KGJ2 [71] whereas it enhanced the production in case of *Trichoderma harzianum* 1073 D3 [83] and *Aspergillus terreus* [84].

2.9.7 Agitation speed rate

Enzyme production through submerged microbial fermentation is greatly affected by the agitation speed rate. It affects the oxygen mass transfer and uniform mixing of nutrients; such that it is adequately available for microbial growth [72, 85]. An optimal rate of agitation speed is significantly required for maximal xylanase production. At lower agitation, there is improper mixing of components of the production medium and poor oxygen transfer [69] whereas high agitation has been deleterious for xylanase production because of the shearing stress subjected to the microbial cells [86, 87].

2.10 Optimization studies for xylanase production

Optimization studies of various influencing factors is indispensable for attaining maximum xylanase yield from the fermentation process. OFAT is a conventional strategy opted for

optimizing the parameters one by one. The method is however, time consuming and tedious, but literature reports that it is reliable in terms of increasing the xylanase yield. Xylanase yield from *Geobacillus* sp. strain DUSELR 13 was increased from 6 to 19.8 U/mL after optimization by OFAT [44]. Similarly, optimization through OFAT resulted in enhanced xylanase yield from 402 to 4986 U/mL during production from *Bacillus pumilus* VLK-1 [62]. Other than OFAT, statistical optimization through RSM is practiced for enhancing the enzyme yield and analyze interactions between two factors. Usually OFAT is initially performed to screen the significant parameters and then followed by statistical analysis of those selected parameters. By performing RSM, one can study large number of parameters with minimum number of experimental trials and also saves time and resources. In comparison to OFAT, RSM resulted in 3.7 fold increases in the xylanase production from *Bacillus tequilensis* strain ARMATI [88]. Similarly, the xylanase yield increased from 19 to 31 U/mL after RSM optimization in case of *Geobacillus* sp. strain DUSELR 13 [44]. The two designs namely Central Composite Design (CCD) and Box-Bhenken Design (BBD) are practiced under the category of RSM [59, 89].

2.11 Purification and gene expression of xylanase

Purification is an important aspect to be considered for commercialization of an enzyme. However, it is required as per its eventual application on large scale. Food industries and pharma industries seek higly purified xylanases while industrial processes of paper pulp bleaching and biomass conversion prefer the crude form of xylanase. Various techniques such as ammonium sulphate precipitation, ion exchange chromatography, gel permeation chromatography and utrafilteration have been employed for purification of xylanases [90] [53].

Xylanase gene expression through recombinant DNA technology is significant in enhancing the xylanase production and its hydrolytic activity. The technology leads to strain improvement such that the recombinant strain has better functional properties in comparison to the native one [8]. Through these molecular techniques, xylanases with the desired industrial traits could be produced. Xylanase genes from different microorganisms vary in product length. XlnA gene from *Bacillus circulans* constitutes 185 amino acid (a.a) length whereas that from *Pseudoalteromonas haloplanktis* is of 405 a.a [91]. Many research reports have presented the expression of xylanase gene; both homologous and heterologous gene expression. *E. coli*, Yeast, *Lactobacillus* sp. and *Bacillus* sp. have been reported as efficient heterologous hosts for the

expression of xylanase gene. These hosts are generally regarded as safe (GRAS); which makes them a reliable option [65]. *E.coli* has been used as an expression host for many bacterial and fungal xylanases from *Bacillus brevis* [92], *Bacillus pumilus* [93], *Aspergillus niger* [94], *Neocallimastix sp.* GMLF7 [95] etc. However, xylanases expressed in *E. coli* tend to have lower specific activity and stability [96]. In this context, *Bacillus* sp and *Lactobacillus* sp. are reliable alternatives for higher xylanase production [97]. Moreover, yeasts have ability of growing in high cell densities and secreting the protein extracellularly in fermentation medium; which makes them an attractive host too. *Pichia pastoris* is known as an efficient host for xylanase production on commercial scale because it exhibits very high expression of enzyme under its own specific promoter [98]. High xylanase activity of 1760 U/mL was attained on extracellular expression of xylanase II gene from *Aspergillus usamii* in *Pichia pastoris* [99]. Similarly, xylanase gene PoXyn2 from *Penicillium occitanis* Pol6 was expressed in *P. pastoris* [100].

2.12 Characterization of xylanase

The characteristic features of an enzyme justify its potential for industrial exploitation. For characterization, various parameters such as optimum pH and temperature, thermostability, pH stability, metal ion effect, isoelectric point, molecular weight, Km/Vmax and solvent tolerance are determined. Xylanases from various sources and their respective characteristics have been summarized in the Table 2.3.

Table 2.3: Biochemical characterization of xylanases from different sources.

Organism	Xylanase activity (U/mL)	Optimum pH/ temperature	Km/Vmax (mg/mL)/ (µM/min/ mg)	Molecular weight (kDa)	References
Bacillus amyloliquefaciens	42.5	9/50°C	5.6/433	50	[68]
Geobacillus thermodenitrificans C5	994.5	6/60°C	NA	~44	[102]
Geobacillus thermodenitrificans TSSA1	2.8	7.5/70°C	2.85/45.45	43	[57]
Melanocarpus albomyces	415	6.6/65°C	0.30/311	38	[46]
Malbranchea flava MTCC 4889	164	9.0/70°C	1.25/1666	25.2	[103]
Aspergillus fumigates R1	208	5/50°C	11.66/87.6	24.5	[104]
Bacterium-OKH	28.14	9/37°C	4.2/0.31	55	[105]
Penicillium chrysogenum	4.56	6.5/45°C	2.3/731.8	22.1	[106]

For estimating molecular weight of xylanases; techniques such as SDS-PAGE and PAGE-Zymography are being used [44, 63]. Metal ions also influence the activity of xylanase; either inhibitory or stimulating (co-factors). In 2018, Bibra et al reported increased xylanase activity under the influence of metal ions Cu^{+2}, Zn^{+2}, K^{+}, Fe^{+2}, Ca^{+2}, Mg^{+2}, and Na^{+} [44]. Similarly, activity of xylanase from *Bacillus amyloliquefaciens* also increased in presence of Ca^{+2}, Mn^{+2} and Fe^{+2} [68]. Whereas some studies reported strong inhibition of xylanase activity by metal ions such as Mn^{+2}, Hg^{+2}, Fe^{+2} and Pb^{+2} [101]. Hydrolysis profile of xylanases has also been studied by many researchers to estimate the biomass conversion efficiency of xylanase. For this, end products (xylose and xylooligosaccharides) from substrates hydrolyzed by xylanase have been analyzed [39, 57].

2.13 Immobilization of xylanase

The application of enzymes at industrial scale is restricted by factors such as lack of operational and functional stability, tedious recovery and poor reusability [107]. Immobilization of enzyme has appeared as a reliable method to overcome the limitations confronted for industrial application of enzymes. On the same line, for increasing the commercial and industrial viability of xylanases, various kinds of immobilization methods (carrier bound or carrier-free) have been followed in the recent years for the immobilizing xylanases. In 2006, Pal and coworkers immobilized xylanase from *Aspergillus terreus* using barium alginate entrapment method [108]. Similarly, Chang and co-workers reported entrapment of xylanase as an efficient immobilization method to enhance its efficiency for industrial applications [109]. Also, there are literature studies reporting immobilization of xylanase on supports such as Eudragit TM L-100, Eudragit L-100, Eudragit S-100, polysulfone membranes, amine active poly (ethylene glycol) monoacrylate (PEGMA) and many other matrices [110-113]. Kapoor and coworkers carried out comparative studies among different methods for immobilization of xylanase. They practiced physical adsorption on chitin, entrapment using gelatin, covalent binding by HP-20 beads and ionic binding with Q-sepharose [114]. However, certain disadvantages are also associated with the immobilization methods such as leakage from the carrier material used for adsorption, poor interactions between the reactive groups of carrier support and enzymes, high expenses of modifications of the carrier support etc. [11, 115]. Many of these limitations have been overcome by preparation of xylanase cross-linked enzyme aggregates (CLEAs).

2.14 Xylanase CLEAs (Xy-CLEAs)

Application of CLEAs for industrial applications is a cost-effective approach as the process of their preparation is very simple, could be prepared by directly using the crude form of enzyme and do not occupy much space in the reactors, which is otherwise being occupied by the immobilization support [116]. Similarly, for enhancing the efficiency of xylanases in industrial processes like biomasss conversion, xylo-oligosaccharide production and paper pulp bleaching, preparation of Xy-CLEAs have been practiced by many researchers. In recent study, J.S. Hero and coworkers, prepared cross-linked aggregates of xylanase and exploited it for the bioconversion of lignocellulosic biomass [14]. For the similar application, another group reported preparation of Magnetic-CLEAs (M-CLEAs) and Calcium-magnetic CLEAs of

xylanase [117]. In 2015, Bhattacharya and Pletschke, synthesized combi-CLEAs of xylanase-mannase for its application in carrying out hydrolysis of lignocellulosic biomass [118]. The concept of modifications in CLEAs such as M-CLEAs and porous CLEAs (p-CLEAs) is attributed to provide ease of separation of CLEAs and overcome mass-transfer limitations, respectively. Through magnetic decantation, there is easy separation of M-CLEAs and also it prevents squeezing of CLEAs which promotes better mass transfer [12, 119]. Despite various advantages offered by CLEAs certain challenges are faced while using them. The size of the aggregates formed cannot be controlled, desired stability and flexibility cannot be achieved. Majorly, it is the loss in activity that has been observed after cross-linking [120]. Structural alterations after cross-linking have been considered as the reason for affecting the activity of the enzyme. These alterations are not under control; rather use of some additives as Bovine Serum albumin, cations (sodium and calcium ions), surfactants and ionic polymers has been reported as an alternative to overcome the activity loss [121-124].

2.15 Preparation of Xylanase CLEAs

Two main steps are involved in the preparation of CLEAs as shown in Fig. 2.3, (a) Formation of enzyme aggregates through precipitation ;using precipitating agents such as salts, organic solvents etc. (b) Formation of CLEAs with the aid of cross-linker (bifunctional: glutaraldehyde or multifunctional: dextran polyaldehyde) [125]. The cross linkers react with amino groups on the enzyme surface. Various factors play significant role in the preparation of CLEAs. That is why; optimization of such factors is a prerequisite necessity to synthesize CLEAs with the desired efficiency.

2.16 Factors affecting CLEA preparation

Various factors affecting the preparation of CLEAs include cross-linker, precipitating agent, cross-linking time, concentration of precipitating agent, protein concentration etc. In order to have maximum CLEA recovery for the respective enzyme, optimization of the above mentioned factors is an indispensable step in the process of CLEA preparation. These factors eventually influence the characteristics and functional properties of CLEAs formed. These significant factors and their impact on CLEA preparation has been discussed as follows:

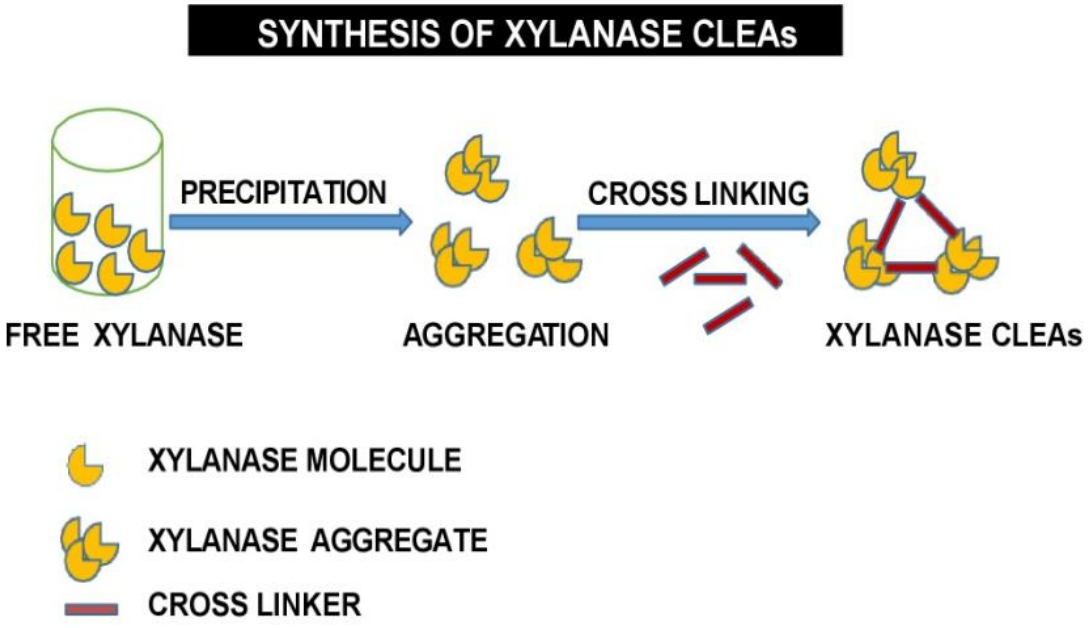


Figure 2.3: Synthesis of xylanase Cross-linked Enzyme Aggregates (CLEAs) by carrier free immobilization using cross-linker.

2.16.1 Type of precipitating agent and its concentration

The type of precipitant being used for precipitation of enzyme, affects the catalytic activity of CLEAs. Different precipitants induce the conformational alterations in the protein structure of the enzyme aggregates in a different manner [126]. That is why, for every enzyme, different precipitants need to be evaluated for their consequences on the catalytic activity. For evaluating the type of precipitant, the aggregates formed by it are assayed for the catalytic activity after being re-dissolved in the buffer [127]. Various precipitating agents used are organic solvents (acetone, isopropanol, ethanol, acetonitrile etc.), non-ionic polymers (poly-ethylene glycol) and neutral salts (ammonium sulphate) [125, 128]. For the synthesis of Xylanase CLEAs, precipitation by ammonium sulphate resulted in better activity recovery in comparison to isopropanol [14]. In another study, highest activity was retained with acetone after precipitation but after cross-linking, eventually the CLEAs formed by xylanase aggregates precipitated by ammonium sulphate showed better results [118]. The addition of precipitants does not harm the tertiary structure of protein and form aggregated supramolecular structures through non covalent bonding. The aggregation and precipitation by the precipitant is induced by changing the hydration state of the enzyme molecule or creating alterations in the dielectric constant of the

solution [129]. From commercial point of view, precipitant used should be inexpensive and should not react with the enzyme or buffer used for synthesis of CLEAs. Furthermore, the concentration of precipitant is also impactful for maximum CLEA recovery. Literature reports that a high concentration of precipitant promotes maximum retention of CLEA activity. This is because at high concentrations, precipitation rate is so fast that that chances of denaturation of enzyme gets fairly reduced due to the competition between denaturation and aggregation of enzyme molecules [130]. Increase in the retention of CLEA activity with increasing concentration of ammonium sulphate has been observed during immobilization of lipase [131], invertase [132] and acetyl xylan esterase [133]. Similar effect was observed on increasing concentration of acetone and PEG600 for lipase [134] and lipozyme [135], respectively.

2.16.2 Type of cross-linker and its concentration

The cross-linker creates a network between the enzyme aggregates by covalently binding to the lysine residues present on the enzyme surface. The bifunctional cross-linker glutaraldehyde is most commonly used for cross-linking due to its eaem of availability on large scale and low price. Cross-linking using glutaraldehyde as cross-linker occurs via schiff's base recation [125, 136]. However, complete loss of catalytic activity after cross-linking with glutaraldehyde has been observed for certain immobilized enzymes. The small size of glutaraldehyde renders its easy penetration into the internal structure of enzyme molecules, where it may react with the catalytically essential amino residues. Binding of the cross-linker to such amino residues, eventually has negative impact on the catalytic activity of CLEAs. Using large size cross-linker instead, such as dextran-polyaldehyde has been reported as an alternative. For cross-linking of nitrilases aggregates, Mateo et al reported 10-90 times higher activity with polyaldehyde as cross-linker in comparison to glutaraldehyde [137]. Similarly, β-mannanase CLEAs formed using dextran-polyaldehyde showed 16 times higher activity than those prepared using glutaraldehyde as cross-linker [138]. Besides this, other cross-linkers such as *p*-benzoquinone, polyethylenimines and L-lysine have also been explored in some studies for the preparation CLEAs [139-141]. However, for attaining maximum activity in CLEAs, optimization of the cross-linker concentration is equally important as the choice of cross-linker. Very low concentration may not be sufficient to form CLEAs while at very high concentration of cross-linker there is loss of flexibility of enzyme structure which minimizes the catalytic activity of

CLEAs. Due to the rigid protein structure, the substrate molecules could not enter through the CLEAs and react with the catalytically active amino residues on the enzyme surface [142-144]. The required amount of particular cross-linker varies according to the available amino residues for cross-linking on the enzyme surface. Study by Bhattacharya and coworkers found optimum concentration of glutaraldehyde to be 150 mM for preparation of X6-CLEAs and 100mM for X7-CLEAs [118].

2.16.3 Cross-linking pH, time and temperature

Maximum cross-linking is achieved at an optimum pH, as it depends on the acid-dissociation constant of the lysine residues present on the enzyme surface and also on the reactivity of glutaraldehyde at a particular pH. Generally glutaraldehyde has been found to show highest reactivity and thus maximum CLEA recovery at around neutral or slightly alkaline pH [145]. Also, glutarladehyde exists in either monomeric or polymeric form in accordance with the pH and also results in different end product, respectively [136]. The duration of cross-linking is also significant for the efficient recovery and activity of CLEAs. Cross-linking reaction conducted for very short period leads to inadequate cross-linking which causes operational and functional loss of CLEAs recovered. Whereas, prolonged cross-linking is also detrimental as it causes rigidity in the structure which results in reduced or complete loss of activity [107]. Some studies report that on increasing cross-linking time, an increased thermostability and reusability of CLEAs was observed but at the cost of great activity loss (50-100%) [146]. Another significant parameter that influences the recovery of CLEAs is temperature during cross-linking. Xylanase-mannanase combi-CLEAs were prepared at 37°C [118] whereas, Combi-CLEAs of glucose oxidase-versatile peroxidase [147] and lipase-protease [148] were prepared at 30°C and 4°C, respectively. Usually low temperature (4°C) is preferably the choice during cross-linking to prevent the denaturation of enzyme due to high temperature [149, 150]. However, at very low temperature it may require long cross-linking time.

2.16.4 Protein concentration and additives

Protein concentration directly affects the amount of lysine residues available for efficient cross-linking. If the concentration is too low, the cross-linked network would not be formed by the cross-linker due to lack of sufficient binding to the amino residues. Whereas very high

concentration results in activity loss of enzyme aggregates due to lack of structural mobility, caused by overcrowding of the enzyme molecules [151]. When there is scarcity of sufficient lysine residues for cross-linking, certain additives such as BSA, surfactants and ionic polymers are used to cause co-aggregation and increase the number of available lysine residues. Also these additives protect the enzyme active sites from the deteriorating effects of cross-linkers. Lipase CLEAs with BSA as an additive retained 100% activity in comparison to that without BSA, which retained only 0.4% activity [121]. Similar, results were found for preparation of laccase and amino-acylase in presence of the additive [152, 153]. However, the catalytic activity of CLEAs is not significantly improved after using additives, as observed in case of amylase CLEAs prepared alongwith BSA [154].

2.17 Morphology and characteristics of Xy-CLEAs

Morphology and size of CLEAs has significant impact on the mass transfer and tolerance towards shear stress [155, 156]. Xy-CLEAs reported in literature are known to exhibit both type I (~ 1µm diameter) and type II (< 0.1µm diameter) morphology [14, 117]. Xy-CLEAs from *Conhella* sp. AR92 rendered two sizes of CLEAs; clustered aggregates of CLEAs with 300-400 nm diameter as well as detached spherical structures with 100-200 nm diameter. Also some exposed cavities were observed in the close packed structure of Xy-CLEAs after SEM analysis [14]. Such cavities have been reported to be beneficial in increasing surface area and facilitating better accessibility to substrate molecules [157]. So far the literature survey depicts that the immobilization of xylanase in the form of CLEAs have characteristic improvement in terms of thermostability, reusability and storage stability. CLEAs of xylanase from *Conhella* sp. AR92 showed 25% increase in relative activity at 50°C in comparison to free xylanase [14]. Similarly, half life of xylanase activity in a commercial preparation (Pectinex™ Ultra SP-L) was increased to 82 min from 22 min at 60°C after CLEAs formation [158]. Mag-CLEAs and calcium-mag-CLEAs from *Bacillus geletanii* ABBP-1 rendered 1.35 fold increased xylanase activity as compared to free xylanase. Also the half lives were increased from 52 h (free xylanase) to 136 h, 214 h and 310 h for CLEAs, mag-CLEAs and calcium-mag-CLEAs, respectively [117]. Reusability is another characteristic which undergoes tremendous improvement after CLEA formation. Xylanase CLEAs from *Conhella* sp. AR92 exhibited 50 % relative activity after five

consecutive cycles. Whereas, 100% xylanase activity was observed for CLEAs from Pectinex™ Ultra SP-L after three consecutive cycles [14, 158].

2.18 Industrial applications of xylanases

Xylanases serve diverse industries including the sectors related to paper, food, feed, biofuel and pharmaceutical (Fig. 2.4).

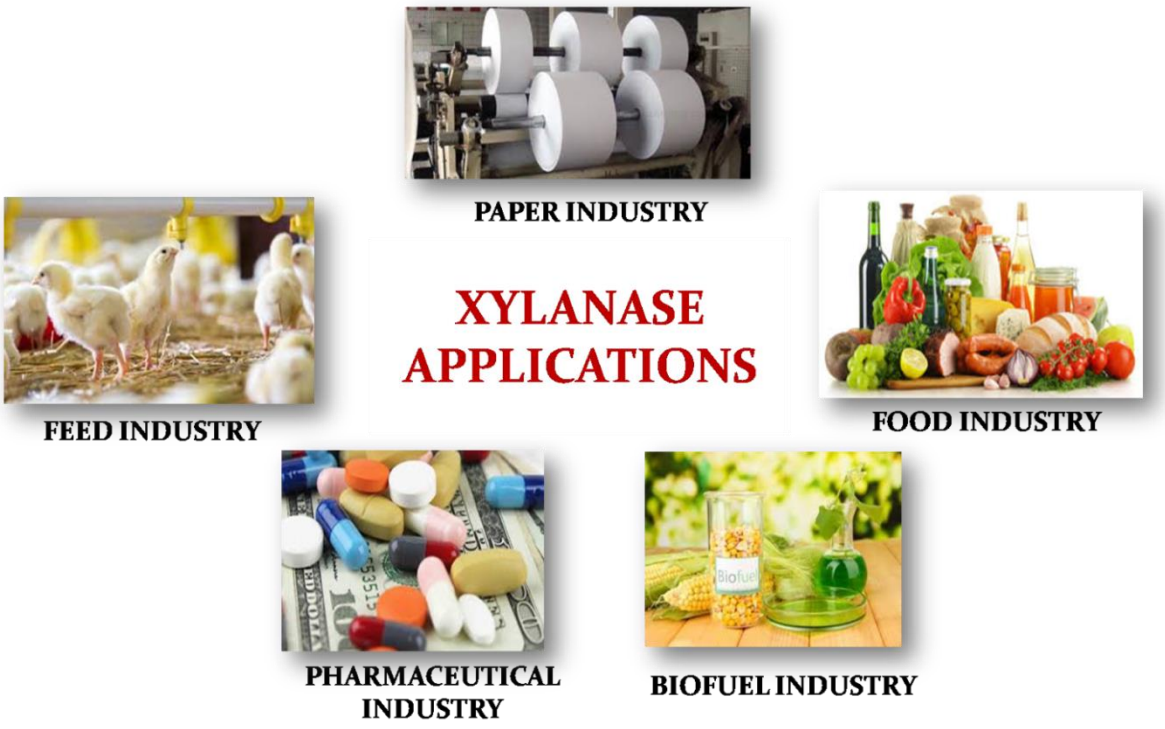


Figure 2.4: Application of xylanases in different industries.

Several xylanases have been commercialized and are applied in various industrial processes. Some of the commercial xylanases and their respective producers have been summarized in Table 2.4.

Figure 2.4: Application of xylanases in different industries.

Commercial Xylanase	Producer	Application
Bleachzyme F	Bicon, India	Paper industry
Pulpzyme	Novozymes, Denmark	Paper industry
Ecopulp	Rohn Enzyme 0Y, Primlaco, Finland	Paper industry
Ecozyme	Thomas Swan, UK	Paper industry
Rholase 7118	Rohm, Germany	Food industry
Sanzyme X	Sankyo, Japan	Food industry
Ceremix	Novo Nordisk, Denmark	Food industry
Multifect XL	Genencor, Netherlands	Food industry
Ecosane	Biotech, Thailand	Feed industry
Biofeed	Novo Nordisk, Denmark	Feed industry
Allzym PT	Alltech, America	Feed industry
Amano 90	Amano, Japan	Pharmaceutical industry

However, researchers are still looking for xylanases with better functionality and stability, with respect to the robust industrial processes. Moreover, efforts are being made to lower down the production cost of enzyme to make their applications on large scale; an economical one.

2.18.1 Food industry

Xylanases are used for clarification of juice in combination with amylases, pectinases and cellulases. They hydrolyze the turbidity causing substances and reduce the viscosity for clearing the juices [6]. In bakery, they are used for increasing the bread volume and softness of the dough by breaking the hemicellulose content of wheat flour [159]. They also improve the nutritional properties of food and prevent starch retrogradation [160]. Cereals containing arabinoxylans are given xylanase pretreatment to reduce the viscosity of their water extract. This avoids problems

of slow filtration rate and haze formation during brewing [161]. Xylanses are also applied for separating gluten from wheat starch [162].

2.18.2 Feed industry

Xylanases are used in feed industry to increase the nutritional value of feed. Addition of xylanases in poultry diet is helpful in increasing the feed conversion efficiency and weight gain of the chicks [15]. Xylooligosaccharides (XOs) produced from hydrolyzed xylan are used in many feed products [163]. Improvement in absorption of nitrogen and fiber and food transmit time has been reported by Babalola and coworkers [164]. Usually, xylanase is used in combination with phytase and β-glucanase as poultry diet supplement.

2.18.3 Pharmaceutical industry

Xylanases are helpful in improving health due to their prebiotic effects. They stimulate the growth and activity of beneficial microflora inhabiting the colon. XOs produced by xylanases have numerous pharmaceutical applications. Micro or nanoparticles for drug delivery are prepared from XOs [165]. Likewise XOs have many other properties which constitutes of anti-cancerous and anti-microbial effect, growth regulation, immunomodulation, antioxidant, ant-allergic, anti- inflammatory and anti-hyperlipidaemic activity etc [166].

2.18.4 Biofuel industry

For generation of second generation biofuels, efficient recovery of fermentabe sugars from the lignocellulosic biomass is big challenge. Lignocellulases play significant role in bioconversion of the lignocellulosic biomass for biofuel production. Xylanases in combination with other hydrolytic enzymes (cellulases and laccases) are used in the biomass conversion process [29, 167]. After delignification, xylanases hydrolyze the hemicelluloses content and increase the porosity and swelling of the lignocellulosic biomass. This makes the cellulose content easily accessible to cellulases for conversion into sugars and further fermentation into ethanol (biofuel) [16].

2.18.5 Paper industry

Application of xylanases for bio-bleaching of pulp has significnatly minimized the usage of chemical bleaching agents in the the paper industry. Consequently, the chemical load (hazardous chlorine derivatives) on the effluents being released into the environment has also reduced [17]. Xylanases are mainly used for their pre bleaching effect, which means that they are not directly involved in the removal of lignin form the pulp. Schematic presentation of xylanase application as bio-bleaching agent has been shown in Fig. 2.5. The residual lignin is responsible for imparting the brown color to the pulp. Thus its maximum removal is necessary for achieving a quality paper with satisfactory brightness and whiteness [168]. Xylanases work by hydrolyzing the xylan and thus breaking its linkage with the lignin. The free lignin is thus easily accessible for the other bleaching agents to act upon and remove it. However, xylanases to be used in pulp bleaching processs must comply with certain charactersistics such as thermotability, alkaline stabilty and cellulase free. Redution in chlorine dosage after biobleaching of pulp using xylanase has been reported in the litertaure. Pretreatment of wheat straw pulp by xylanase from *C. cellulans* CKMX1 resulted in 12.5% chlorine reduction [8].

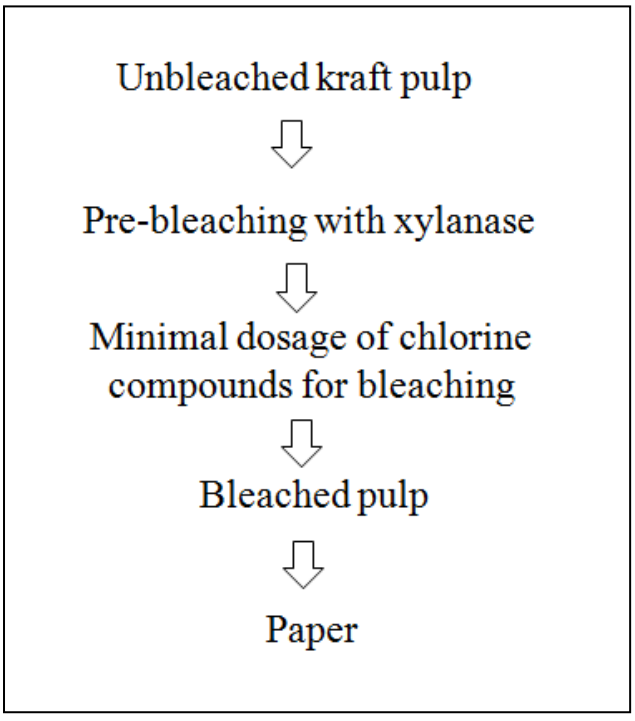


Figure 2.5: Schematic presentation of xylanase application in bio-bleaching of pulp during paper manufacturing.

Similalry, 16% chlorine reduction was observed after bio-bleaching of pulp using xylanase from *Thielaviopsis basicola* [169]. Besides chlorine reduction bio-bleaching of paper pulp with xylanase also has impact on the strength properties of the paper generated. Properties such as tear factor, burst index, breking length, freeness etc. decide paper quality. The impact of xylanase pretreatment on paper varies; depending on the dosage and quality of pulp used for paper making. Usually, pretreatment with xylanase either enhances or has neutral effect on the paper properties. Decreae in the strength properties of paper after xylanase pretreatment is only in the case where xylanase has associated cellulase activity. This implies that the xylanase to be used for bio-bleaching necessarily has to be cellulase free, to prevent deterioration of the cellulosic structural integrity of paper. Thus, cellulase free thermo-alkali-stable xylaanases are in great demand by the paper industry, as it is an eco-friendly approach and has promising potential to completely substitute the chemical bleaching agents. In context of such industrial applications of xylanase, efforst are being made to screen efficient sources of xylanases for maximum xylanase production in cost-effectiev manner. This will elevate the viability of xylanases to be used on large scale.