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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
BCA	Bicinchoninic Acid
CAZymes	Carbohydrate Active Enzymes
CBH	Cellobiohydrolase
CBM	Carbohydrate Binding Module
DAB	Diaminobenzidine
DP	Degree of Polymerization
DNS	3,5-Dinitrosalicylic acid
EC	Enzyme Commission
GH	Glycoside Hydrolase
IPQC	In Process Quality Control/Check
IPTG	Isopropyl-Beta-D-thiogalactopyranoside
IU	International Unit
KDa	Kilo dalton
LB	Luria Bertani
Ni-NTA	Nickel-Nitrilo TriAcetic acid
OD	Optical Density
PES	Poly Ether Sulfone
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline wth Tween 20
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
VVM	Volume of air (**V**) per unit Volume of medium (**V**) per unit of time (**M**inute)

1. ABSTRACT

Xyloglucan is a hemicellulose polysaccharide mainly found in cell wall of plants. It consists of a backbone similar to cellulose with xylan decoration of β-(1,4)-D-xylanopyranosyl residues. In this study, two previously isolated recombinant strains viz. Xylo and CBM were produced by fermentation wherein Xylo is a recombinant cellulolytic carbohydrate active enzymes (CAZymes) xyloglucan specific endo-1,4-glucanases having molecular mass of **80** kDa which degrades tamarind xyloglucan while CBM is a carbohydrate binding module (CBM46-3) having molecular mass of **25** kDa. Both were purified by Ni-NTA column and their expression, activity and protein content was measured which shows that Xylo is having xyloglucan decomposing activity (to glucose) and can be employed in producing biofuels using lignocellulosic agricultural and industrial waste. Though CBM does not have any xyloglucan degrading activity but have optimal role in increasing local carbohydrate concentrations, binds to insoluble polysaccharides and helps in disruption or modification of carbohydrate structures.

Keywords: Xyloglucan, Xyloglucanase, Carbohydrate binding module (CBM), Biofuels, Lignocellulose, CAZymes.

2. INTRODUCTION

It is well known fact that as the human population is growing, the whole world is facing enormous pressure to meet its need for food, feed, chemicals and energy. The demand and supply needs to be balanced in keeping with environmental concern. The natural gas and oil reserves are depleting very fast for meeting the current demands of global progress. Today, alternate energy sources from environmental wastes and energy crops are the need of hour and becoming high priority research interest worldwide. For the production of bio-commodities from biomass, one of the major hurdles is to perform the efficient and fruitful hydrolysis of lignocelluloses to sugars. Lignocelluloses primarily comprises of cellulose (~30 to 50%), hemicellulose (~20% to 35%), and lignin (~15% to 25%) which may vary differently in different plant species and tissues. The plants alone produces lignocellulosic material of around 10–50 billion tons per annum worldwide which are converted or hydrolyzed to sugars such as pentoses and hexoses which serves as an important requirement for various agricultural and industrial products (Chandrakant and Bisaria *et al.*, 1998; Lynd *et al.*, 2005; Bayer *et al.*, 2007). For such hydrolysis, enzymatic methods are preferred as they produce non-inhibitory and non toxic by-products and effluents. Hydrolytic enzymes like cellulases, hemicellulases and xylanases convert lignocelluloses to sugars which can be fermented by various microorganisms like bacteria and fungi to biofuels and different other value-added products. But, the relatively high cost of these enzymes remains the major barrier to their commercial application in any bio-industry. Although, a lot has been done for significant reduction in the cost of these enzymes but the focus areas of research majorly have been targeting to improve the efficiency of various known enzymes, identify novel enzymes with enhanced activity and to find out optimized enzyme mixes which may be employed for pretreated lignocelluloses along with reduction of the cost associated with the production of enzyme (Merino and Cherry *et al*., 2007). Keeping in view

the same, the present research work entitled **"Production and characterization of recombinant cellulolytic enzymes"** has been undertaken with the aim to produce and characterize two recombinant strains viz. **Xylo and CBM** . The objectives of this research work were:

1. To grow and produce the recombinant bacterial strain viz. Xylo and Carbohydrate binding module (CBM 46-3) at shake flask level and bioreactor level.
2. Purification of fermentation product CBM (carbohydrate binding module) at shake flask level.
3. Purification of Xylo (xyloglucanase enzyme) by affinity chromatography (Ni-NTA) and their characterization based on their xyloglucanase activity.

3. REVIEW OF LITERATURE

The Literature review presents the background and previous research on composition of lignocellulosic material, cellulolytic enzymes and xyloglucanase.

3.1 Biofuels and lignocellulosic material

Fossil-fuel energy resources are depleting at a very rapid pace and their detrimental effects on environment due to their usage have compelled the researchers to shift their focus towards applications of plant biomass and agro-based waste for fuel and chemical production. Though, first generation crops (sugar cane and starch-based plants) have been studied intensively but their usage may lead to steep hike in the price of food and feed of animals. Therefore, it's a demand of time to move towards the development of second generation biofuels which are being produced from non-food biomass. These fuels may prove significant for future bio-refineries (Sims *et al*., 2010). The sources of second generation biofuels are mainly raw materials such as agricultural by-products and waste material generated by plants e.g. wheat straw, corn stover and wood from agricultural and forestry residues which are rich in lignocellulosic material. Following are the components of lignocelluloses viz. cellulose, hemicelluloses and lignin.

3.2 Lignocellulosic Biomass

Lignocellulosic biomass represents a major source of raw materials for bioethanol production having its main characteristics being as renewable. Residues produced by agricultural sector and forest reserves are proved to be a remarkable part for the production of common materials e.g. waste paper, straw *etc*. but taking into consideration of present demand for energy, these residues which are rich in lignocellulosic materials can be a potential resources for production of biofuels. Lignocellulosic biomass is composed of three important fractions viz. cellulose (in range of ~30%–50%), hemicellulose (in a range of ~20%–35%) and lignin in a range of (~15%–25%).

Also, some other polymeric components are also present in low amount e.g. starch, pectin, proteins, minerals, ash, *etc* (Gomez *et al*., 2008; Lynd *et al*., 2008). Celluloses and hemicelluloses connect with lignin to form a three dimensional network which is insoluble.

3.2.1 Cellulose

The most abundant component in the plant cell wall is the cellulose comprising of D-glucose units that are linked together with β-1,4-glycosidic bonds binding together to form its own structure (Fig. 1). Cellulose is having two general forms in biomass viz. crystalline and amorphous. Crystalline cellulose is the main segment of cellulose, while a low amount of cellulose is in the form of an organized amorphous structure. Cellulose is more prone to enzymatic degradation in its amorphous form. Cellobiose is composed of cellulose chains which are stabilized by strong hydrogen bond and van der wall interactions creating microfibrils in plant cell walls consisting of high part of crystalline regions while amorphous regions are present in smaller content. Cellulose chains are held together by the strong hydrogen bonds forming a crystalline structure tightly. Such a structure provides resistance to the degradation of plant (Guo *et al*., 2008).

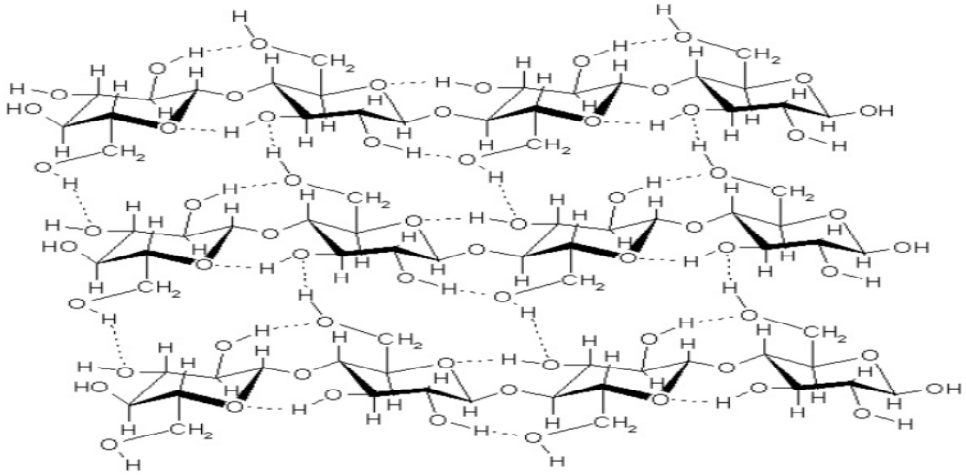


Figure 1 Structure of cellulose [#]

[#] **http://www.doitpoms.ac.uk/tlplib/wood/structure_wood_pt1.php**

There are numerous microorganisms which are capable enough to digest cellulose by consuming cellulose as a source of food e.g. plants, fungi, bacteria, protist *etc*.

3.2.2 Hemicellulose

The second most abundant material in the plant cell wall of monocots and dicots is hemicellulose. The structure of hemicellulose consists of an amorphous chain having many saccharides (Fig. 2). The most common sugars which are present in hemicelluloses are α-D-galactose, β–D-glucose, β–D-mannose, β–D-xylose and α-L-arabinose. Besides, other compounds in smaller amounts are also present in the structure of hemicelluloses like acetic acid and uronic acids. Some other sugars or sachharides are also present in the structure of hemicelluloses like α-L-rhamnose and α-L-fucose. Hemicellulose are mainly classified into four groups: xylans (comprising units of β-1,4-linked D-xylose), mannans (comprising units of β-1,4-linked D-mannose), galactans (comprising units of β-1,3-linked D-galactose) and arabinans (comprising units of α-1,5-linked L-arabinose) (Scheller *et al*., 2010).

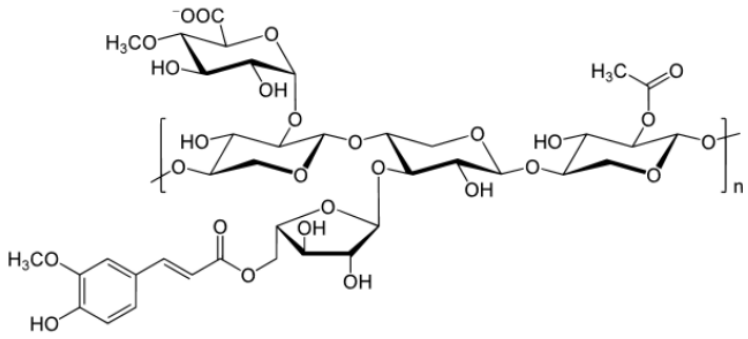


Figure 2. Components of the hemicellulose [#]

[#] **http://upload.wikimedia.org/wikipedia/commons/4/46/Xylan.svg**

The backbone of hemicelluloses is having either of the two structure viz. homopolymer or heteropolymer. Some other short junctions are also present which are linked by β-(1,4)-glycosidic bonds. In some polysaccharides they are linked by β-(1,3)-glycosidic bonds**.** When

celluloses were compared with hemicelluloses it was found that the polymers of hemicellulose are easily digestible into smaller parts as compared to cellulose polymers (Kumar *et al*., 2009).

3.2.2.1 Xylan

The most common polymer from the hemicellulose group range is the xylan. It is a polysaccharide which mainly includes linear chains of β-(1,4)-D-xylanopyranosyl residues. It can be easily substituted by acetyl groups at the position of second or third oxygen atom in the structure. Xylans obtained from higher plants are also easily substituted by 4-*O*-methyl-glucuronosyl residues. There are numerous agricultural plants consisting of xylans as a component like straw, sorghum, sugar cane, corn stalks and husks. Some forest and pulping waste products from hardwoods and softwoods are also rich in xylan (Ebringerova and Hromadkova, 1999). The following xylan groups have been distinguished recently in accordance to their structure. For example: Arabinoxylans: It is a Xylan backbone which is substituted with β-(1,4)-D-xylopyranose at position 2 or 3 with α-(1,3)-L-arabinofuranosyl. They can also be esterified partly with phenolic acids. Arabinoxylans are present in the outer layer of grains and other starchy endosperms. In some parts glucuronoxylans are acetylated and substituted with units of α-(1,2)-4-*O*-methyl-D-glucopyranosyl uronic acids. (Silva *et al*., 2012).

Glucuronoarabinoxylans: These are having substitutions of α-(1,3)-L-arabinofuranosyl next to α-(1,2)-4-*O*-methyl-D-glucopyranosyl uronic acids e.g. softwoods.

Unsubstituted homoxylans: These are the linear polysaccharides which are present in seaweed e.g. **heteroxylans**: They are the one which are substituted with various monosaccharides and/or oligosaccharides. They are mainly found in cereal bran, seed, and gum exudates. Studies and characterization have been extensively carried out on structure of xylans from different plants.

Various xylan extraction methods have been developed and studied thereby providing numerous applications of xylan (Silva *et al*., 2012).

3.2.3 Lignin

Lignin is the hydrophobic component of lignocelluloses which bond covalently to both cellulose and hemicellulose thereby making the structure of lignocellulose more complex. Lignin is regarded as the roughest component of the plant cell wall. It has an important function which adds to the physical characteristics of wood. It also protects lignocellulosic plants against degradation by biological agents (e.g. cellulose degrading microorganisms). Lignin is made up of heterogeneous independent aromatic polymers of hydroxycinnamyl alcohols. Its major components are namely *p*-coumaryl, coniferyl and sinapyl alcohol (Caffall *et al*., 2009). Lignocellulosic biomass contains usually 5-25% of lignin. Lignin increases the rigidness of the cell walls and makes them water impermeable. The quantity and conformation of lignin are diverse in different plant species, cell wall layers and cell types. The concentration of lignin is more considerable in the softwood of coniferous trees (25-33%) than in the hardwood of broad leaved trees (20-25%). In the industrial utilization of lignocelluloses, lignin is affected negatively with the limitation of having access to glycosyl hydrolases (GHs) to the cellulose, hemicellulose and also by irreversible adsorption to GHs enzymes (Sewalt *et al*., 1997; Berlin *et al*., 2006). The hydrogen bonding, electrostatic forces and hydrophobic interactions are mainly responsible for the adsorption of proteins to lignin surfaces. The effect and impact of the inhibition of lignin depends on the composition of lignin as well as on the origin of lignin (Pan *et al*., 2008). Biological degradation of lignin is done by soft white and brown rot fungi. Lignin treatment by the above fungi may be regarded as an efficient method of delignification as it provides an easier access to components of cellulose and hemicellulose (Gupta *et al*., 2011).

3.2.3.1 Inhibition and Hydrolysis of Lignocellulose

The performance of cellulose and hemicellulose degrading enzymes can be reduced during the enzymatic hydrolysis of lignocelluloses. The two possible inhibition processes are described as: 1. steric hindrance of the cellulose exerted by lignin and 2. the reversible or irreversible adsorptions of cellulases on lignin wherein the adsorption between cellulases and lignin happen via ionic, hydrophobic, or hydrogen-bonding interactions (Berlin *et al*., 2006; Pan *et al*., 2008). In order to improve hydrolysis rate of lignocelluloses, the affinity of lignin binding to the enzymes needs to be reduced so as to make the hydrolysis process more economical (Berlin *et al*., 2006). Further other options have been developed to enhance the yield of hydrolysis e.g. application of weak lignin binding enzymes which are mainly produced by fungi. Another way is to intensively increase the temperature that reduces the cellulase-lignin interactions and decreases the cellulases release from the lignin surface (Rahikainen *et al*., 2011). Delignification processes can also improve the digestibility of lignocellulose by removing the lignin (Pan *et al*., 2008).

3.3 Pretreatment of Lignocellulosic Materials

Pretreatment is to digest the plant cell wall and to remove lignin from cellulose and hemicellulose which decrease crystallanity of cellulose and porosity of the lignocellulosic materials (Merino *et al*., 2007). The pretreatment process needs to have some important requirements. Firstly, there is need to improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis. Secondly, it should prevent the degradation or loss of carbohydrate and also prevent the formation of byproducts which are inhibitory to the subsequent hydrolysis and fermentation processes. Lastly, it has to be economical (Galbe *et al*., 2007). Numerous technologies for pretreatment have been studied varying extremely from acidic to quite alkaline in nature (e.g. steam explosion catalysed by acid, ammonia fiber explosion and

solvent extraction), which breaks the composition of the biomass and make it to be in more accessible and digestable form for degrading enzymes. The hydrothermal and steam pretreatment methods are commonly used in pretreatment process. They are supplemented with chemical digestion which improves the accessibility of carbohydrates by the degradation of the tissue structure which modifies the spatial distribution of polymers (Merino *et al*., 2007). Pretreatment extremely improves the enzymatic degradation of plant biomass. However, the pretreated lignocellulosic materials still contains a considerable amount of lignin thereby restricting enzymatic hydrolysis by irreversible conjunction of lignin to glycosyl hydrolases which enhances the cost of the process by increasing the dosage of enzymes and inhibiting the recycling of enzymes (Nakagame *et al*., 2010).

3.4 Degradation of Lignocelluloses by Microorganism

For efficient degradation of lignocellulosic biomass to oligo- and monosaccharides it is required that there should be a cooperation of different microbial enzymes in synergy. Structurally two different enzymes are mainly involved in the degradation of the plant cell wall namely, endo-enzymes and exo-enymes. Endo-enzymes cut the bonds inside the polymer chain. The resultant action of endo-enzymes is the reduction of substrate molecular mass. Exo-enzymes hydrolyse oligomers to monomers and digest the endings of the polymer chain. Numerous protist and polycellular creature are able to produce lignocellulolytic enzymes. Although, the most utilized lignocellulose digesting enzymes are originated from bacteria and fungi (Himmel *et al*., 2010).

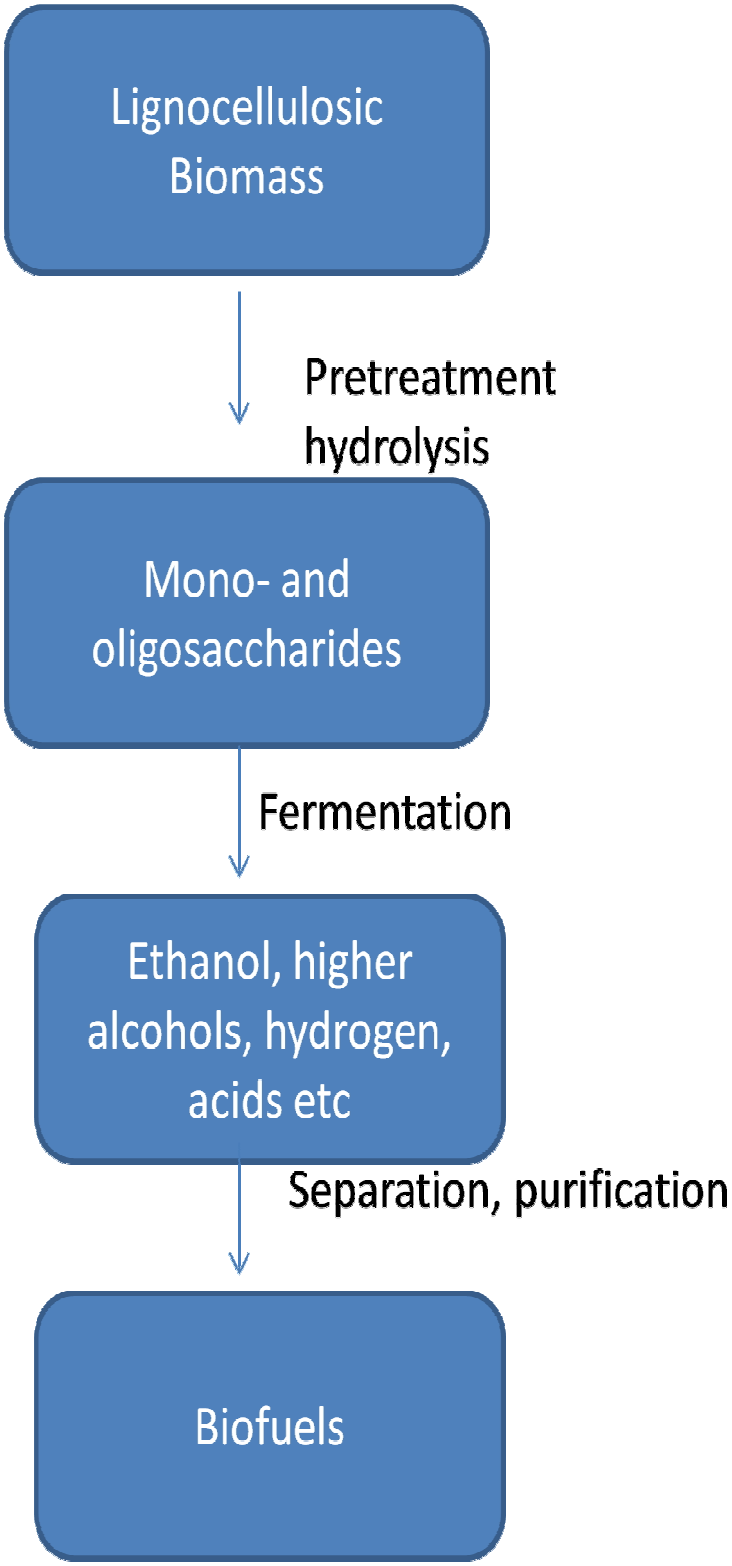


Figure 3. Biofuel production from lignocelluloses (Kumar *et al.*, 2009)

3.4.1 Cellulase

The cellulase enzyme complex consists of three different kinds of enzyme viz., exoglucanases, endoglucanases and β-glucosidases that perform well in degradation of cellulose while working in collaboration (Kubicek *et al*., 2009). They breakdown the cellulose complex and produces glucose which is a fermantable energy source for bioethanol production. Recently used cellulase enzyme preparations are quite efficient in the hydrolysing cellulose**.** But, they are comparatively expensive. Therefore reducing the cost of these enzymes is an important area of study for their commercial usage in biorefineries. Cellulase enzyme complex distinguishes themselves from the hydrolases by the fact that hydrolases break down glycosidic bonds between glucose units (Fig. 4). They have been classified as the number 3.2.1.x by the Enzyme Commission, where x signs the different cellulolytic enzyme type (Lynd *et al*., 2002).

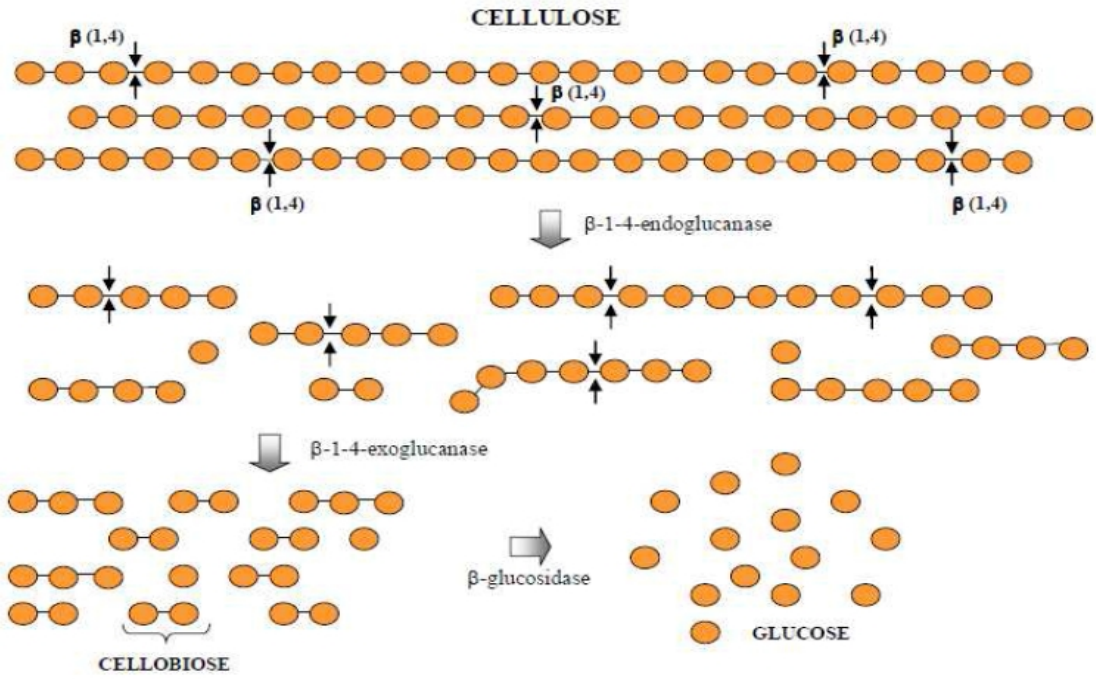


Figure 4. Mode of degradation of cellulose by different enzyme system (Mussatto and Teixeria, 2010)

There are three classes of cellulase enzymes which act in synergy during cellulose hydrolysis:

Endoglucanases: These are represented as the β-1, 4-endoglucanase (E.C. 3.2.1.4). It digests the inner glycoside-linkages of the amorphous cellulose part in a random fashion and liberates polysaccharides having lower polymerization degree (DP). Soluble oligosaccharides have polymerization degree (DP)<7.

Exoglucanases: Cellobiohydrolases (CBH, E.C. 3.2.1.91) and glucanohydrolases (E.C. 3.2.1.74) are the most important enzymes of this group wherein cellobiohydrolase digests the cellobiose from the reducing (CBH I) and non-reducing (CBH II) terminals of a cellulose chain. While, glucanohydrolase liberates glucose units from the ends of the cellulose chain. Both cellobiohydrolases (CBH, E.C. 3.2.1.91) and glucanohydrolases can be inhibited by their hydrolysis products i.e. the glucose (Castro *et al*., 2010; Lynd *et al*., 2002).

β-glucosidases: Another enzymes is β-glucosidases known as β-1,4-glucosidase (E.C.3.2.1.21) which induces the degradation of cellobiose and digests oligosaccharides to glucose. It hydrolyzes the β-1,4 bonds in the cellulose chain at random positions. This enzyme also can be inhibited by glucose (Castro *et al*., 2010; Lynd *et al*., 2002). The most prevalent commercialized products of enzyme for degradation of lignocellulosic biomass are produced by saprophytic mesophilic fungus *Trichoderma reesei* in submerged fermentation**.** *Trichoderma reesei* strain is a very good producer of cellulase enzymes for which several mutants have been studied that increases the productivity of the strain (Mandels *et al*., 1971).

3.4.2 Hemicellulases

The hemicellulose degrading enzymes can be classified in three main groups according to their actions viz. endo-acting enzymes, exo-acting enzymes and other accessory enzymes. The exo-acting hemicellulases are induced by both short and long chain substrates while the endo-acting group has low efficiency only on short oligomers. The accessory enzymes digest the lignin-

linked glycoside bonds thereby liberating different substitutions from the hemicellulose backbone (Himmel *et al*., 2010). Xylan is one of the most commonly known hemicelluloses. The mechanism of degradation of a xylan firstly involves with the release of substituents from the xylan backbone and there after the depolymerization of the xylan chain takes place for the adequate hydrolysis of hemicellulose by a simultaneous action of all these enzymes (Fig. 5). There are various accessory enzymes whose performance and action is required for debranching of the side chains which includes α-L-arabinofuranosidases, α-glucuronidases (E.C. 3.2.1.139), ferulic acid esterase (EC 3.1.1.73), and acetyl xylan esterases (EC 3.1.1.72) (Saha *et al*., 2003). Endo-xylanases (E.C. 3.2.1.8) are required for depolymerisation of backbone that produces unbranched xylooligosaccharides, such as xylotriose and xylobiose, and β-xylosidases (E.C. 3.2.1.37) which split xylobiose and digests the non-reducing terminals of short chain xylooligosaccharides and release xylose (Saha *et al*., 2003).

- ***Endo-xylanases*** hydrolyse the glycosidic bonds of the β-1,4- or β-1,3- linked, unsubstituted xylan backbone thereby producing β-D-xylopyranosyl oligomers.
- ***β-D-xylosidases*** cleave xylooligosaccharides such as xylobiose from the non-reducing terminus and liberates β-D-xylopyranosyl residues.
- ***Acetyl xylan esterase*** liberates the *O*-acetyl groups from β-D-xylopyranosyl of acetylated xylan. It finds an important role in the xylan degradation, because the acetyl groups can inhibit the access of endo-xylanases which digests the backbone.
- ***α-L-arabinofuranosidase*** also named as arabinoxylan hydrolases release α-arabinofuranosyl residues from the β-D-xylopyranose.
- ***α-Glucuronidase*** degrades the α-1,2-bonds between β-D-xylopyranosyl units and the glucuronic acid along with their *O*-methyl ethers.

- ***Ferulic acid esterase*** and *p-coumaric acid esterase* liberates ferulic acid and *p*coumaric acid which is linked to arabinose units. Degradation of xyloglucan and xylomannan requires xyloglucanases (E.C. 3.2.1.151), β-glucosidases, endo-mannases (E.C. 3.2.1.78) and β–mannosidases (E.C. 3.2.1.25) which act as polymerizing enzymes. But α–xylosidases (E.C. 3.2.1.177), α-galactosidases (E.C. 3.2.1.22), α-arabinofuranosidases (E.C. 3.2.1.55) and acetyl esterases (E.C. 3.2.1.6) act as debranching enzymes.

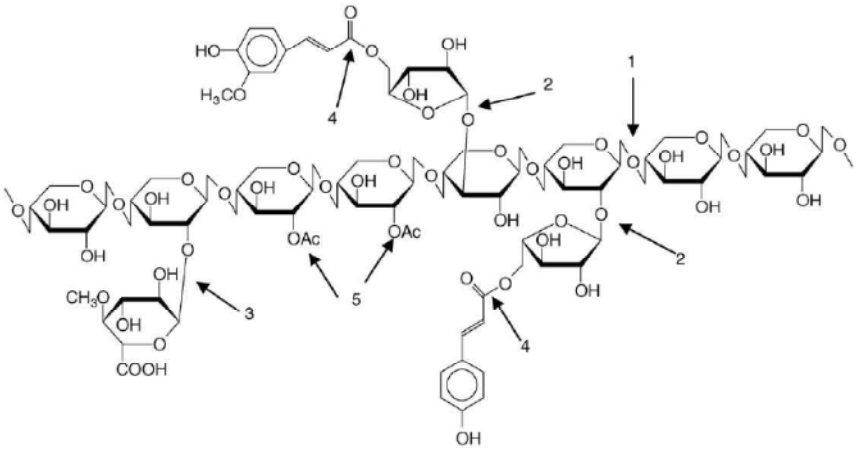


Figure 5. The structure of xylan and performances of the xylan degrading enzymes. 1:endoxylanases; 2: α-L-arabinofuranosidases; 3: α-glucuronidases; 4: feruloyl and coumaroyl esterases; 5: acetyl xylan esterases (Chavez *et al*., 2006)

3.5 Xyloglucanase

Xyloglucanase, also known as xyloglucan-specific endo-beta-1,4-glucanase (E.C No. 3.2.1.151) is an enzyme which glycosidic bond of xyloglucan comprising of β-(1,4)-D-xylanopyranosyl residues. Their catalysis may be represented by following chemical reaction.

Xyloglucan + H_2O $\rightleftharpoons$ Xyloglucan oligosaccharides

The above equation shows that xyloglucan and water are the two substrates of xyloglucanase and its ultimate product is xyloglucan oligosaccharides. It belongs to the family of hydrolases which

specifically linked to the glycosidases that hydrolyse O- and S-glycosyl compounds. The systematic name of xyloglucanase is (1,6)-α-D-xylo-(1-4)-β-D-xyloglucan glucanohydrolase and its other names includes XEG, xyloglucanendohydrolase and xyloglucan endo-β-1,4-glucanase.

3.6 Carbohydrate binding modules (CBM's)

Carbohydrate binding modules (CBMs) are non-catalytic proteins that bind oligosaccharides and polysaccharides. They are associated with carbohydrate-active catalytic domains (CDs) and are classified according to their binding properties viz. type A (surface binding), type B (glycan chain binding), or type C (small sugar binding). CBMs are having few primary roles, firstly they increase local carbohydrate concentrations by the proximity effect and target specific carbohydrates in complex structures such as plant cell walls, or they disrupt or modify carbohydrate structures. CBMs are mainly classified according to the amino acid sequence similarity into families. At present the Carbohydrate-Active enZymes database (CAZymes) lists over 60 CBM families while over 100 unclassified CBM sequences. Of the known families having familiar tertiary structures, the majority are having β-sandwich folds. While, some of the families have β-trefoil, β-barrel, hevein and lectin-like folds. There are many microorganisms which produces hemicellulase enzymes, especially fungi and bacteria. The commonly used xylanolytic fungi are mostly ascomycetes. Filamentous fungi are having a crucial role in the industrial production of xylanase. Commercial xylanase includes formulations mainly from fungi of the genera *Trichoderma, Aspergillus*, and also *Penicillia*. CBM 46 is a module of approximately 100 residues which are found at the C-terminal of several GH5 cellulases. CBM 46-3 is residue of this family which find its application in degradation of carbohydrate structures (Warner *et al*., 2013; www.cazy.org/CBM46.htm).

3.7 Industrial Application of Cellulase and Hemicellulase

A great number of the industrial applications are found for the cellulase and hemicellulase enzyme mixtures. For example, the food and feed industry, pulp and paper industry, textile- and baking industry, laundry industry, waste treatment or alcohol production from biomass. These enzymes function at mild temperature and mild conditions and hence are more economical in comparison to chemical treatment (Castro *et al*., 2010; Saha *et al*., 2003).

Pulp and Paper Industry: Cellulases are applied in biotreatment pulping which helps in considerable energy savings (around 20–40%) during refining and helps in improvement in handsheet strength properties. In addition to it, cellulases are used for dye removal from waste paper (Bhat *et al*., 2000). For example, β-xylanases isolated from filamentous fungi are being used in the biobleaching of cellulose pulp. It has been found that the usage of hemicellulase enzymes can influence or increase the brightness of kraft pulp. This process can replace the use of active chlorine, as it proves to be more eco friendly. Furthermore, these enzymes can be used in the biobleaching of sulfite pulps as well (Bhat *et al*., 2000.).

Textile Industry: Cellulases have also been extensively used for the bio-stoning of jeans and bio-polishing of cotton and other cellulosic textures and fabrics. In the bio-stoning process, cellulases digest the ends of the small fiber from cellulose fabric on the yarn surface thereby making it easy to get small fibres removed by mechanical washing. The benefits of the cellulose are the following: it leads to increase in productivity, is less work-intensive and is environment friendly. The application of xylanases is immense in the textile industry in processing the plant fibres e.g. linen.

Wine and Beer Industry: Glucanases obtained from the microorganisms are successfully and extensively used in fermentation industry producing alcoholic beverages e.g. beers and wines. These enzymes increase both quality and productivity of the different fermented products. Glucanases are extensively used to hydrolyze glucan thereby decreasing the viscosity and filterability (Bamforth *et al*., 2009).

Food Industry: Cellulase enzyme also plays a very important role in the field of food processing industry. For the production of fruit and vegetable juices it helps in treatments for extraction, clarification, stabilization and enhances the yield of juices as well. These digesting enzymes are improving both the yield and process performance without any further substantial investment. In addition to it, usage of xylanases along with amylases, glucose oxidase and proteases is quite prevalent in baking industry. Xylanases degrade hemicellulose in wheat-flour and promote the rearrangement of water and leaves the dough softer and much easier to knead and leads to increase in volume of bread. Also, absorption of water enhances and resistance to fermentation also improves (Camacho *et al*., 2003).

Animal Feed Industry: Cellulose and xylanase enzymes find significant application in the feed industry especially for monogastric animals and broiler chicken. Animal supplements which are treated by xylanases and other enzymes (e.g. glucanases, pectinases, proteases, amylases, phytase, galactosidases and lipases) are more nutritious in feeding monogastric animals (e.g., pigs, broiler chickens). Pretreatment of agricultural silage and grain which are treated by cellulases or hemicellulases increases the quantity of nutritions thereby decreasing the price of the flesh. In addition to it, xylanases degrade arabinoxylans present in the hemicellulose part of the feed and decreases the viscosity of the raw material (Shrivastava *et al*., 2011).

Agricultural Industry: There are a variety of preparations of enzyme (various combinations of cellulases, hemicellulases, and pectinases) which find extensive applications in agriculture. They enhance the yield of crops and decrease plant diseases. Saprophitic fungi cellulases degrade the cell wall of plant pathogens and control the plant diseases. Numerous cellulolytic fungi such as *Trichoderma* species, *Gliocladium* species, *Chaetomium* species, and *Penicillium* species are known for their outstanding performance and contribution in agriculture by promoting seed germination, plant growth and flowering, enhancing the root system and improving crop yields (Bailey *et al*., 1998; Harman *et al*., 1998).

Recycling of Waste: Waste products produced from forest, agricultural land, and agro industries are rich in cellulose sources which contribute to environmental pollution. These unutilized wastes can be used to make valuable raw material for production of products such as enzymes, saccharides, biofuels (e.g. bioethanol), biochemicals, carbon sources for fermentation, enriched animal feeds and nutrients (Kumar *et al*., 2009).

Production of biofuels: The cellulases and hemicellulases are used in the saccharification of lignocelluloses in the process of bioconversion of lignocellulose to biochemicals and biofuels (Saha *et al*., 2003) and play an important role in the hydrolysis of biomass to fermentable mono- and oligosaccharides (Kumar *et al*., 2009).

Kraft Process and Bioleaching: Hemicellulases are used for the removal of residual lignin from Kraft pulp. The most common pulping process is the Kraft process (also known as Sulphate process) which is extensively carried out industrially. Pulp is having a characteristic brown colour due to the presence of residual lignin and other lignin derivatives. The intensity of pulp

colour is a function or directly proportional to the amount and chemical state of the remaining lignin. In order to obtain pulp with high brightness and to remove lignin from the pulp, chemical pulping is more effective than mechanical pulping. However, there is the formation of residual lignin which needs to be removed by the process of bleaching. The residual lignin in pulp is dark in colour because it has been extensively oxidized and being modified in the cooking process. But this residual lignin is difficult to be removed because of its covalent binding to the hemicellulose and to the cellulose fibres. While, bleaching of the pulp is a purification process involving the destruction, alteration or solubilization of the lignin. Conventionally, chlorine is used for bleaching. The effluents that are produced during the bleaching process mainly contribute to water pollution from the pulp paper industry. During the Kraft process, part of the xylan is present on the fibre surfaces in considerable amount after pulping process. Enzymatic hydrolysis of the reprecipitated and relocated xylans on the surface of the fibres apparently renders the structure of the fibre more permeable. One of the leading producers of chemical pulp manufacturers, Lenzing AG, Austria has recovered fibers which contains 3.3% of total xylan. Ligninases and hemicellulases (xylanases) for bioleaching has been tested (Viikari *et al.*, 1994).

4. METHODOLOGY

4.1 Sample

The previously constructed recombinant strains were provided at ICGEB in the form of glycerol stock named as **Xylo and CBM**. LB plates with ampicillin (50µg/ml) were prepared and inoculated with given glycerol stock of Xylo and CBM and kept overnight at 37 ^{0}C (Fig 6 and Fig. 7).

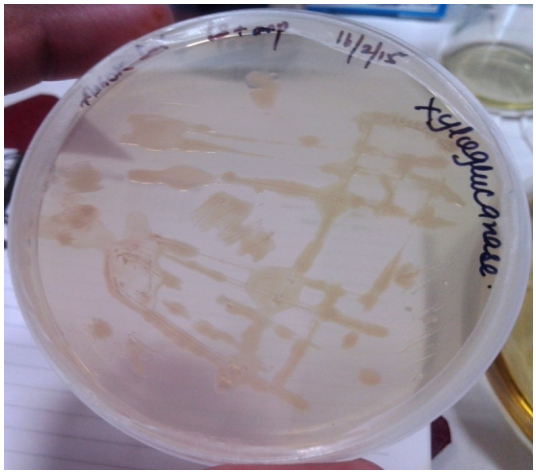


Fig 6. Petri plate containing Xylo recombinant strain

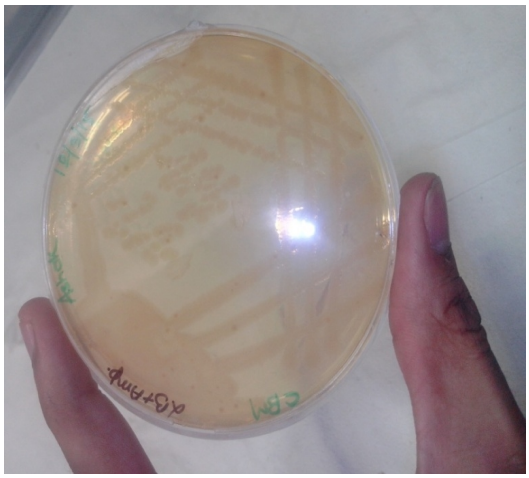


Fig 7. Petri plate containing CBM recombinant strain

4.2 Cultivation of the recombinant strains at shake flask level

To 50ml LB broth with 25 µl of ampicillin (100mg/ml stock) was added and inoculated with a colony of strain from the plate and kept at 37 ^{0}C and 150 rpm for overnight (Seed culture).

4.3 Preparation of mass culture for protein production

To 4.2 Litre LB broth (700ml X 6 flasks), 350 µl ampicillin (100mg/ml stock) was added in each flask. 1% of inoculum i.e. 7ml of culture from seed culture was inoculated in each flask. The flasks were then incubated at 37 ^{0}C, 150 rpm. OD at 600 nm was taken at an interval of 1 hour till it reaches between 0.6-0.8. Then, to the each flask 700 µl of 1M IPTG was added and

incubated for 4-5 hours at 37 ^{0}C, 150 rpm. After completion of the whole process of fermentation, the final optical density was recorded at 600 nm

4.4 Harvesting of recombinant strain

The culture was centrifuged at 4500 rpm for 30 minutes, supernatant was discarded and the pellets were preserved at -80 ^{0}C overnight (Fig. 8). The pellet was then dissolved in lysis buffer pH-7.4 (50 mM sodium phosphate buffer (pH-7.4), 300 mM sodium chloride, 10 mM imidazole) to make suspension of 50 OD cells and kept in ice for sonication.

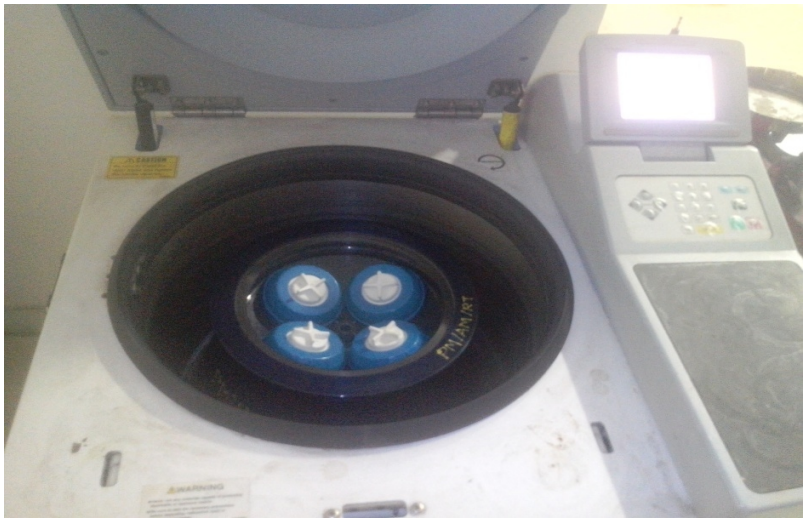


Fig 8. Harvesting of recombinant strain.

4.5 Sonication

The 50 OD cell suspension was lysed by a sonicator (Sonics Vibra Cell) for 45 minutes, 30% amplitude, 4 ^{0}C and 9 sec frequency (beating and resting). After sonication, suspension was again centrifuged at 12000 rpm for 30 minutes (Fig. 9)). Supernatant was taken and pellet was discarded to get cell free enzyme extract.

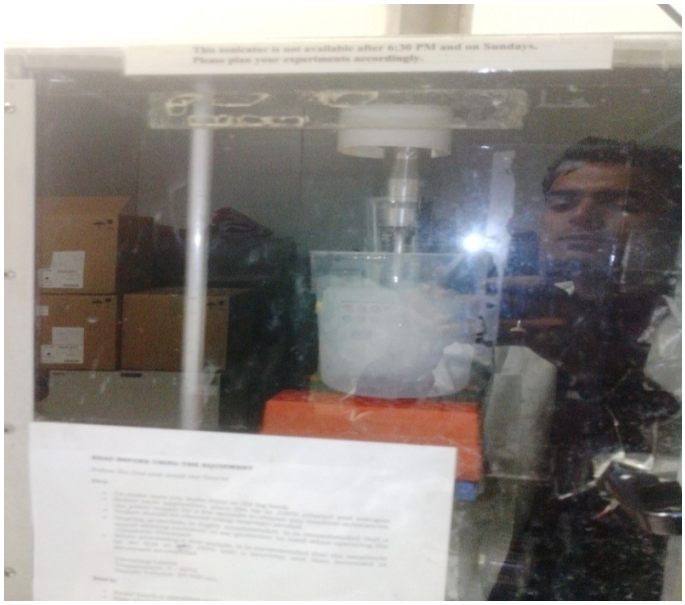


Fig 9. Sonication of sample dissolved in lysis buffer

4.6 Filtration

The supernatant or the cell free extract was then filtered with 0.22 µm poly ether sulfone (PES) filter using the filtration assembly (Fig. 10).

Fig. 10 Filtration of cell free extract with 0.22 µm filter with filtration assembly

4.7 Ni-NTA Column preparation and purification of protein

Column preparation:- Column was washed with 5 ml of distilled water. 1 ml Ni-NTA sepharose was added and allowed to settle down in column and then washed with 5 ml of distilled water. Filtered protein was then added to the column and incubated at room temperature for 1 hour.

Washing and elution of samples:- The sample was then collected from the column, termed as flow through. The column was then washed with 5ml Wash 1 buffer and 5 ml Wash 2 buffer and the wash out samples were collected from the column. The protein was eluted out with elution buffer 1 and elution buffer 2 and fractions of 0.5 ml each were collected. After completion of elution, the column was washed with 5ml distilled water and the column was preserved in 20% ethanol (Fig. 11).

Column regeneration:- Column was washed 3 times with 5ml distilled water followed by washing with 5 ml of regenerating buffer. 2ml of 0.1M $NiSO_4$ was loaded onto the column and then the column was washed thrice by 5ml distilled water. Samples were loaded, incubated for an hour, washed with wash 1 and wash 2 buffers and eluted with elution buffer.

Fig 11. Purification of protein with Ni-NTA Column

4.8 SDS-PAGE and sample preparation of eluted proteins

Sample preparation:- 20 µl of each samples viz. induced sample (with IPTG), preload, flow through, wash 1 elute, wash 2 elute, eluted out fractions were taken in an eppendorf and 5 µl of 2X SDS-loading dye was added and incubated at 100 ^{0}C for 10 minutes.

Uninduced sample preparation:- 200 µl of sample was centrifuged at 13000 rpm for 5 minutes. Pellet was taken and 50 µl of 2X SDS-loading dye was added and vortexed for mixing. Then the sample was incubated at 100 ^{0}C for 10 minutes and then centrifuged at 8000 rpm for 5 minutes. 15 µl of sample was loaded on gel.

SDS-PAGE Gel Preparation:- Plates were placed in gel cast assembly and 4 ml of 12 % resolving gel (as per appendix 1) was added to it. Distilled water was added to the top of gel and allowed to solidify for few minutes. Then removed water using tissue paper and 2 ml of 5% Stacking Gel (as per appendix 1) was added. 1.0 mm comb (10 wells) was placed on the top of gel and allowed to solidify (Fig. 12). 5 µl of 10-180 KDa protein marker and 15 µl other samples were loaded on the gel and gel was run at 110V in 1X TGS buffer (Fig. 13). Then, gel was kept in staining solution for 30 minutes and then in destaining solution for overnight.

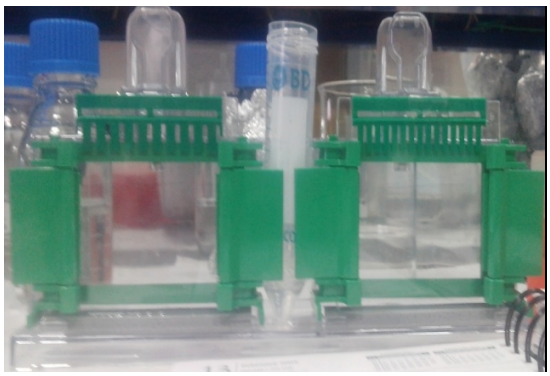


Fig. 12 Gel casting Assembly

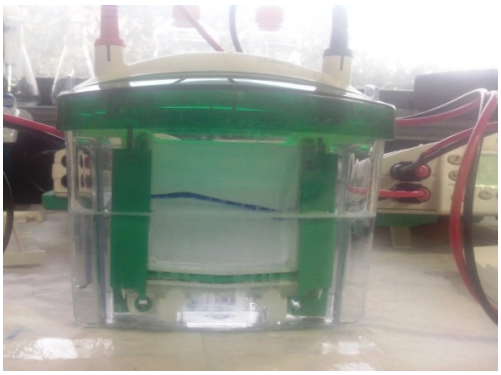


Fig 13. SDS-PAGE Assembly

4.9 Western Blotting

The samples were loaded on SDS gel and run at 110V for 2-3 hours. The proteins were then transferred from the gel to nitrocellulose blotting membrane (0.2 µm Amersham Protran, GE Lifescience). The pads, gel and membrane were dipped in transfer buffer and the nitrocellulose membrane was placed on the pad and SDS gel was placed on to the membrane. Another pad was placed on the gel and the whole system was placed in transfer buffer for 10 minutes. Extra buffer was removed from the system by using a tissue paper gently on the top of the system. System was placed in the western blotting system (Biorad, Trans-blot system) for 15 minutes. After 15 minutes, the nitrocellulose membrane was placed in blocking buffer and stored at 4 ^{0}C for overnight. Next day, the blot was washed with PBST and PBS buffer (10 ml each) twice for 5 minutes on a rocker, one at a time. Then the blot was incubated with primary antibodies for 1 hour. Then the blot was again washed with PBST and PBS buffer twice and then the blot was incubated with secondary antibodies (dissolved in 3% BSA in PBS buffer) for 1 hour. Again, the blot was washed with PBST and PBS buffer twice for 5 minutes each. The blot was then developed with diamino benzidine (DAB) and H_2O_2 solution prepared in PBS buffer (Fig. 14).

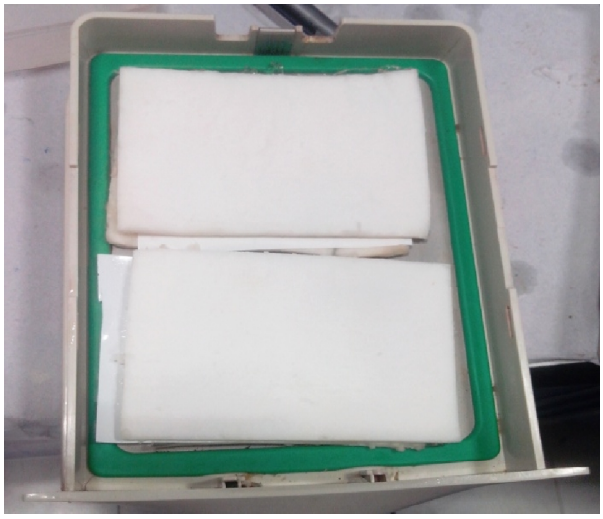


Fig 14. Western blotting with Nitrocellulose Blotting membrane

4.10 Enzyme Assay (DNS Assay)

Preparation of standard curve:- From glucose stock (2mg/ml) different glucose concentration samples between 0-1200 µg/ml were prepared. To each sample 250 µl of DNS solution was added and incubated at 100 ^{0}C for 10 minutes. Absorbance of different concentrations was taken at 540nm.

Substrate Preparation:- 1% Tamarind xyloglucan in 100mM Sodium Phosphate buffer.

Sample preparation:- 125 µl of enzyme (sample) and 125 µl of Substrate (xyloglucan).

Enzyme blank:- 125 µl buffer and 125 µl enzyme.

Substrate blank:- 125 µl buffer and 125 µl substrate.

Buffer Blank:- 250 µl elution buffer .

Method:- All prepared samples and blanks were incubated at 50 ^{0}C for 30 minutes. Samples were then cooled and 250 µl of DNS solution was added and incubated at 100 ^{0}C for 10 minutes. Samples were then cooled and 200 µl of each samples were taken in a microtitre plates (96 wells clear bottom plate) and absorbance was measured using multi reader at 540 nm (Fig. 15). Concentration of glucose is measured by correlating with standard curve.

Fig 15. DNS Assay using microtitre plate

4.11 Protein Estimation using BCA Kit

Preparation of standard curve:- From BSA (Bovine serum Albumin) stock solution of 2mg/ml, different concentrations between 0-2000 µg/ml were prepared. 25 µl of blank, BSA standard concentration solutions were taken on microtitre plate and 200 µl of BCA working solution was added to each well. The plate was then covered with an aluminium foil and incubated at 37 ^{0}C for 300 minutes. The absorbance was measured at 562 nm.

Protein estimation using BCA Method and sample preparation:- All eluted fractions were pooled together in a single falcon tube. Three dilutions viz. 1/5, 1/10 and 1/15 were prepared. 25 µl samples were taken in microtitre plate and 200 µl of working solution was added to each well. Plate was then covered with an aluminium foil and incubated for 30 minutes at 37 ^{0}C (Fig. 16). Absorbance was measured at 562 nm and concentration of sample was calculated by calibrating with standard curve.

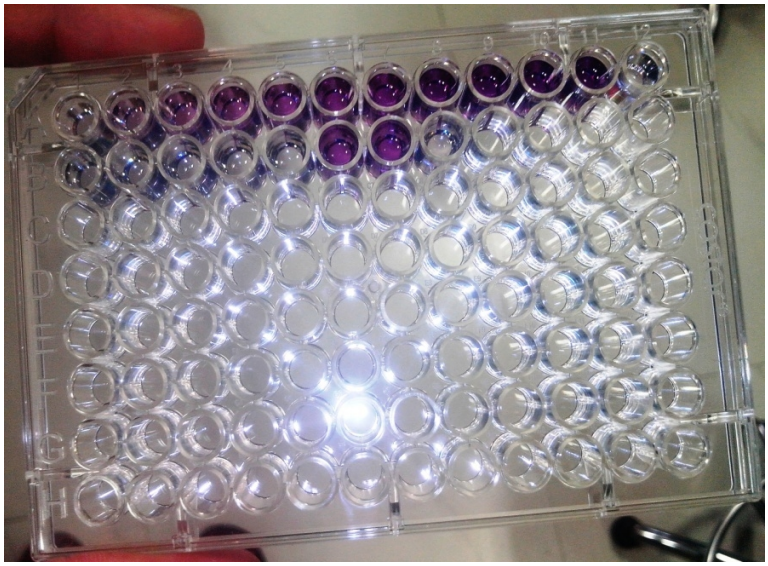


Fig 16. Protein estimation by BCA principle using microtitre plate

Enzyme Activity (IU/ml): One unit of enzyme activity is defined as the amount of enzyme releasing one µmole of reducing sugars (product) per minute.

Specific Activity (U/mg): One unit of specific activity is defined as the amount of enzyme releasing one µmole of reducing sugars (product) per minute per mg of protein.

4.12 Fermentation in a Bioreactor (Xylo)

Inoculum preparation:-

1. Reagents and media were prepared as per appendix- 2.

2. Solution 1 (in 250 ml conical flask) and solution 2 (in 1 liter flask) and rest solutions (in appropriate containers) were autoclaved.

3. To prepare stage-I inoculum, 0.5ml each of solution No. 4,5,6,7,8 & 9 were added to the flask containing solution No.1. Also, 2.5 ml of solution No.10, 100 µl of solution No.13 and 125µl of solution No.11 were added to the culture flask.

4. Transferred 1 ml WCB cryovial (Glycerol Stock of Xylo) into the culture flask and placed in incubator at 37 ^{0}C, 200rpm for 10-12 hours.

5. To prepare stage-II inoculum, 2ml each of solution No.4,5,6,7,8 & 9 were added into the flask containing solution No.2. 10ml of solution No.10, 200 µl of solution No.12 and 0.5 ml of solution No.11 were also added.

6. 10ml of stage-I inoculum from step 4 was transferred into the culture flask (stage-II inoculum) and incubated the flask at 37 ^{0}C, 200rpm for 10-12 hours.

Fermentation:-

7. The fermenter with 3.5 Litre water was sterilized by autoclaving.

8. The pH probe was calibrated using 2-point calibration method with buffers of pH-4.01 and pH-7.

9. Again sterilized the 4.6 litre of solution No.3 in the fermenter vessel. Rest of the solutions No.4, 5, 6, 7, 8, 9 and 13 were pooled into the solution No.10 along with 12.5ml of solution No.11. The content was then transferred to the fermenter in presence of flame.

10. 2N NaOH (autoclaved) (400ml) was attached to the fermenter for automatic delivery.

11. The Dissolved Oxygen probe was calibrated with Nitrogen gas.

12. The fermenter vessel was inoculated with 200ml of grown culture in step 6 (Stage-II inoculum).

13. Started the fermentation with following parameters (Fig. 17(a) and Fig. 17(b)):

a. Initial pH-7

b. Initial stirrer speed-500rpm

c. Initial air flow- 0.6 VVM

d. Initial temperature- 37 degree Celsius

e. Dissolved Oxygen- 40%

14. Collected samples at every 2 hour interval to carry out IPQC analysis for OD 600 nm check.

15. Induced the culture by 1mM IPTG when OD600 nm reaches between 6-8.

16. After 4 hours post induction, the culture was harvested by centrifugation at 4500rpm for 30 minutes at 4 ^{0}C.

17. Supernatant discarded and pellet was kept at -80 ^{0}C for overnight.

18. Pellet was then dissolved in lysis buffer to make 100 OD cells.

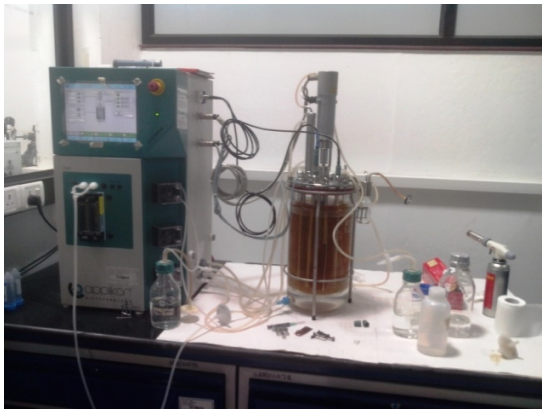


Fig 17. (a) Operational bioreactor

Fig 17. (b) Computerized panel showing various parameters.

Cell lysis by Dyno Mill:-

19. 3 litre 0.5N NaOH was passed in the dyno mill with 150ml/minute as flow rate (Fig. 18).

20. Then, 1.5 litre lysis buffer was passed at the same rate.

21. Harvested cells were passd (3 times) for cell lysis.

22. Again 500ml Lysis buffer was passed to the dyno Mill.

23. Pooled the same lysis buffer with cells.

24. The dyno-Mill was washed with 5 litre water.

25. 3 litre of 0.5N NaOH was then passed.

26. At last 500 ml of 0.1N NaOH was passed.

27. Filteration was done with 0.22 µm filter in the filteration assembly.

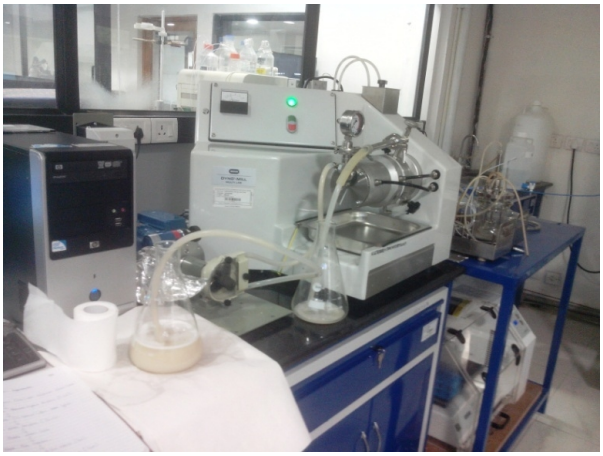


Fig 18. Operational Dyno-Mill

Purification of extracted protein using Ni-NTA column Assembly XK16 with a pump:-

28. 5 ml resin(Ni-NTA sepharose) was loaded on the column (Fig. 19).

29. The column was washed 2 times with 10ml water at the speed of 1-1.5 ml/minute.

30. The column was then equilibrated with 10 ml binding buffer (20mM sodium phosphate buffer, 0.5M NaCl, 10mM imidazole).

31. Sample protein was loaded at the rate of 1-1.5 ml/minute.

32. Washed with 10ml wash buffer1.

33. Washed with 10ml wash buffer2.

34. Eluted 1ml each of 10 vials of protein using elution buffer.

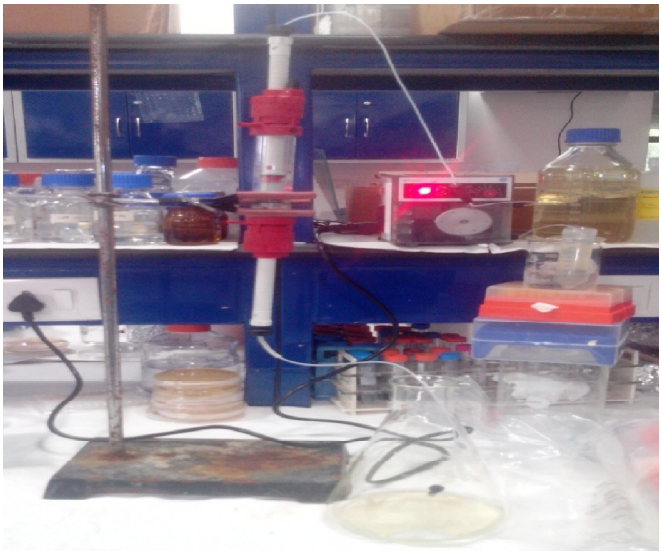
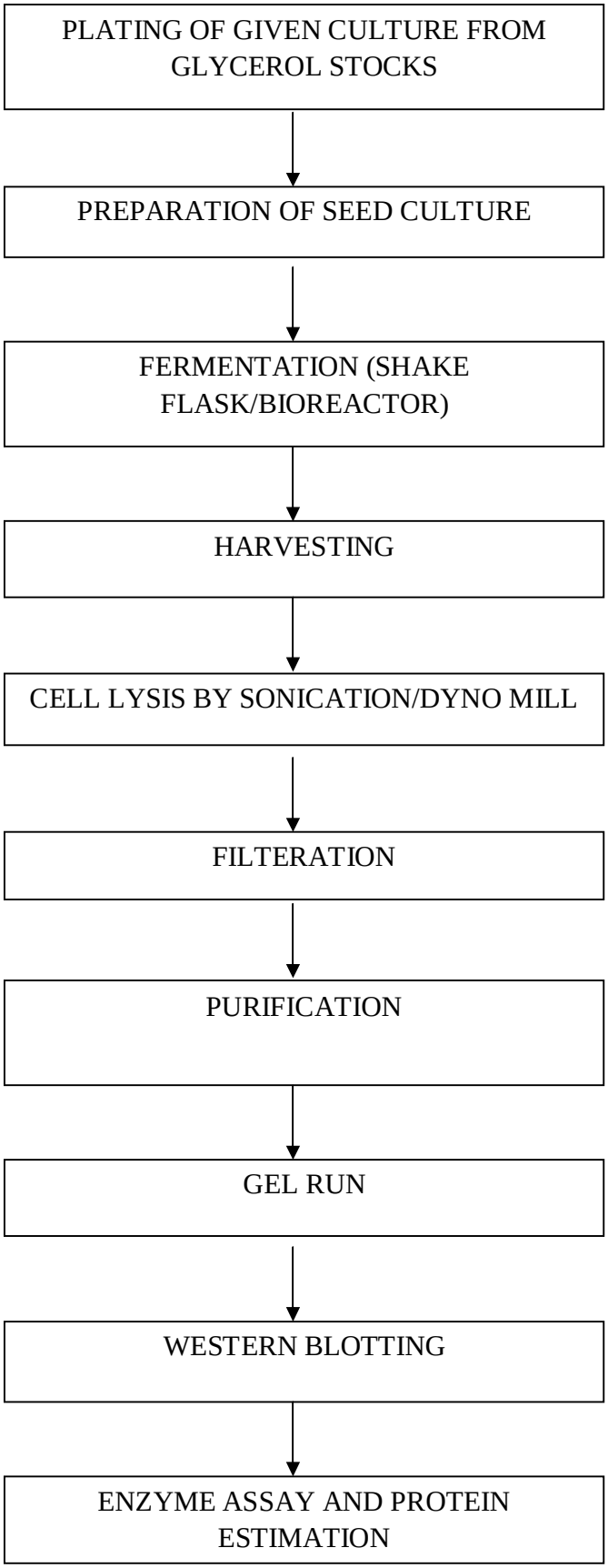


Fig 19. XK16 Column (Ni-NTA) with pump

35. Xyloglucanase activity and protein estimation of the affinity chromatography fractions were done.

4.13 Flowchart of Methodology



5. OBSERVATIONS

5.1 Protein concentration (mg/ml) and activity of Xylo (at shake flask level)

Protein concentration (mg/ml) for the different dilutions of the elutions of the xylo strain fermented at shake flask level viz. 1/5, 1/10 and 1/15 were found to be 1.071, 1.280 and 1.704. The average protein concentration was found to be 1.352 mg/ml. The enzyme activity (IU/ml) of the fractions eluted viz. E1, E2, E3, E4, E5, E6 were found to be 0.164, 0.168, 0.150, 0.060, 0.029 and 0.000 and the specific activity (U/mg) were found to be 0.121, 0.124, 0.111, 0.045, 0.022 and 0.000.

5.2 Protein concentration (mg/ml) of CBM (at shake flask level)

Protein concentration (mg/ml) for the different dilutions of the elution of the CBM strain fermented at shake flask level viz. 1/5, 1/10 and 1/15 were found to be 1.166, 1.266 and 1.471. The average protein concentration was found to be 1.301 mg/ml.

5.3 Protein concentration (mg/ml) and activity of Xylo (at shake flask level)

Protein concentration (mg/ml) for the fractions eluted out viz. E1, E2, E3, E4, E5, E6, E7, E8, E9 and E10 for xylo strain fermented at bioreactor level were found to be 0.838, 0.713, 0.536, 0.406, 0.324, 0.299, 0.266, 0.297, 0.215 and 0.188 . The enzyme activity (IU/ml) of the fractions eluted viz. E1, E2, E3, E4, E5, E6, E7, E8, E9 and E10 were found to be 0.177, 0.176, 0.161, 0.150, 0.118, 0.097, 0.092, 0.052, 0.032 and 0.023 and the specific activity (U/mg) of the fractions were found to be 0.211, 0.246, 0.300, 0.369, 0.364, 0.325, 0.345, 0.176, 0.151 and 0.120.

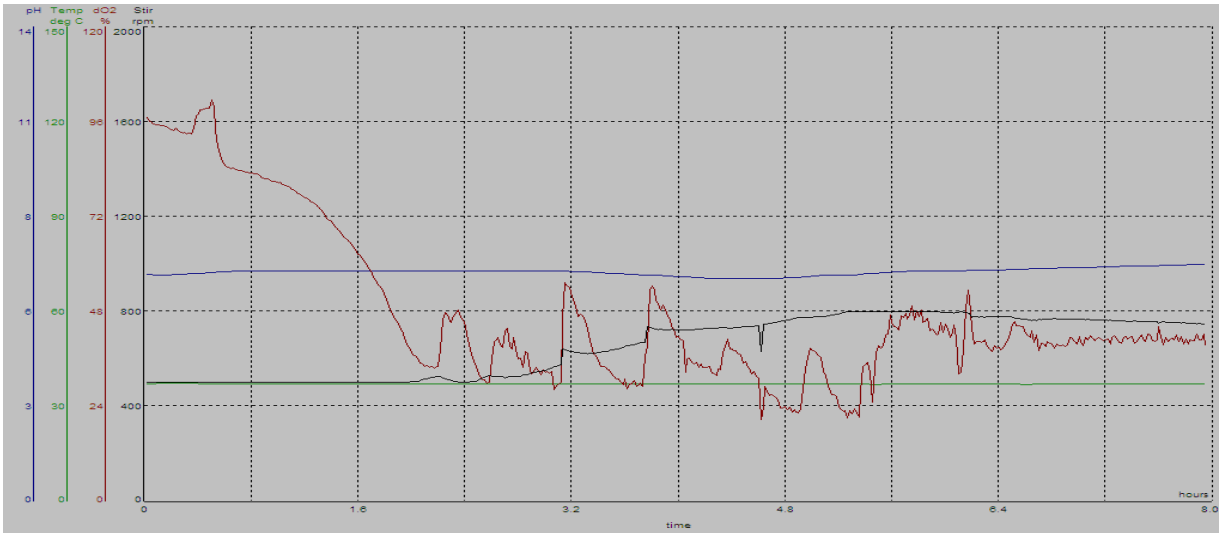


Fig 20. Chart showing various parameters of bioreactor during whole operation.

6. RESULTS AND DISCUSSION

6.1 Xylo (Shake flask level)

Xylo was produced at 4.2 Litre shake flask level and the final OD600 nm at the time of harvesting was 1.562. The harvested cells were dissolved in 130 ml of lysis buffer to prepare 50 OD cells. The cells were lysed by sonication and purified through Ni-NTA chromatography. All the samples were separated on SDS-PAGE Gel (Fig.21). The fractions which showed band at ≈ 95 KDa were corresponding to Xylo. Also it was found that protein of interest Xylo eluted out along with residual proteins which shows that only partial purification of proteins was achieved through Ni-NTA column.

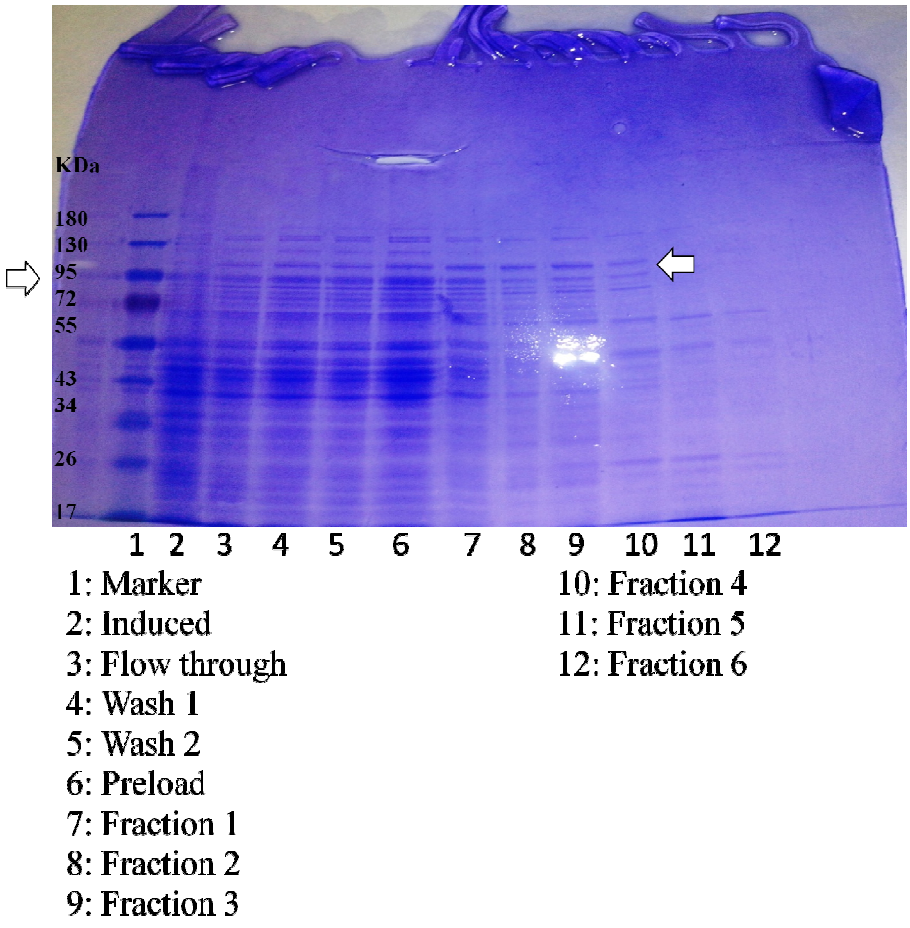
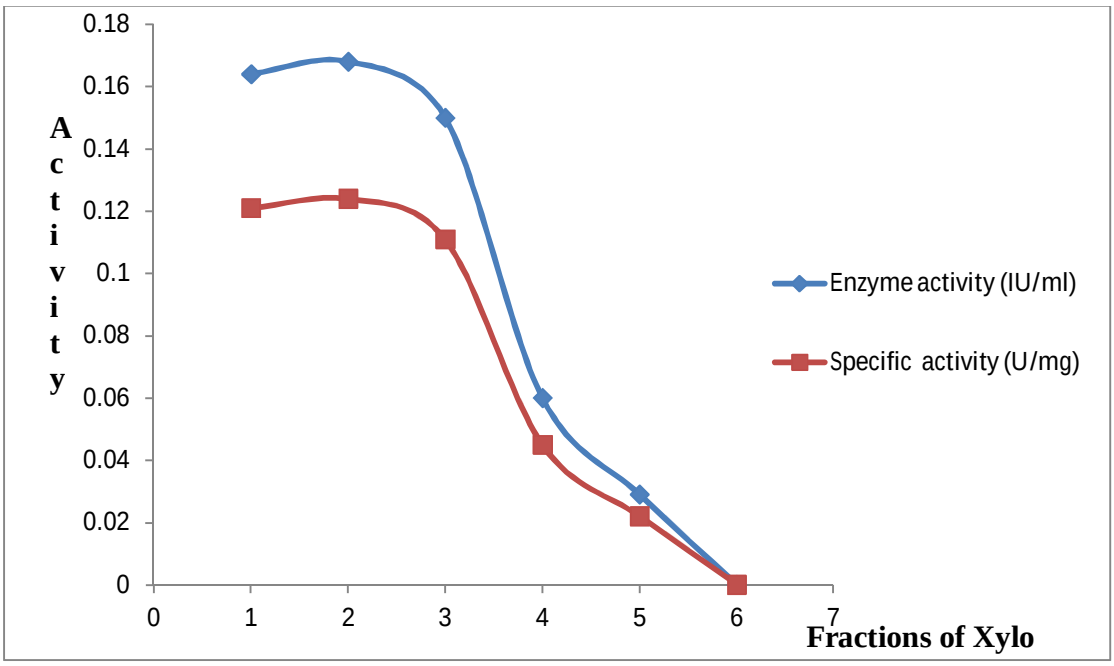


Fig 21. SDS-PAGE of Ni-NTA chromatography fractions exhibiting xylo activity at shake flask level

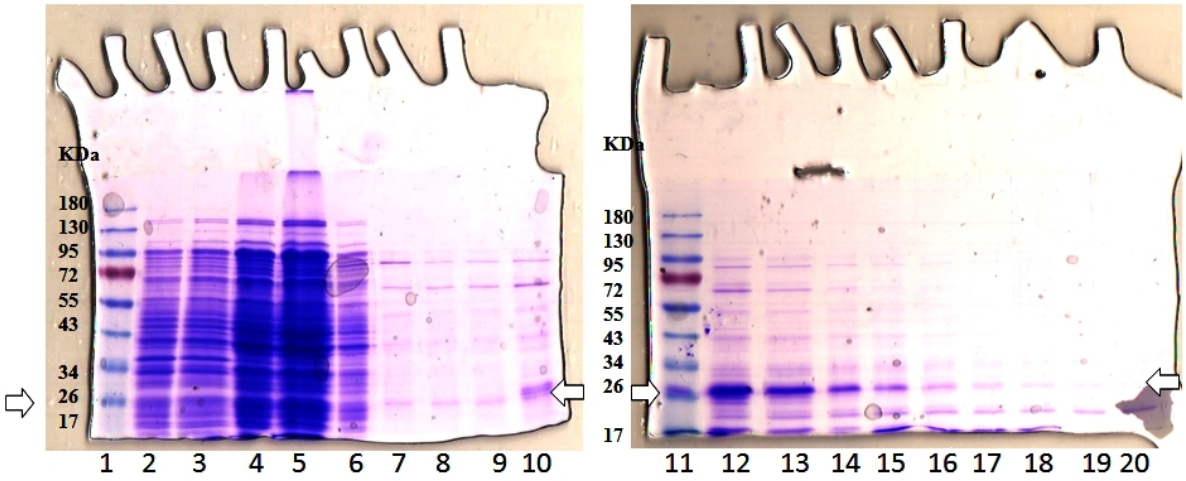
Fig 22. Plot showing enzyme activity (IU/ml) and specific activity (U/mg) of different elutions of Xylo (Shake Flask level)



The activity (IU/ml) of all the fractions viz. E1, E2, E3, E4, E5 and E6 were- 0.164, 0.168, 0.150, 0.060, 0.029, 0.000 while specific activity (U/mg) were 0.121, 0.124, 0.111, 0.045, 0.022, 0.000 (Fig. 22) which shows that they were having low amount of xyloglucanase activity as compared to the one reported by Yaoi and Mitsuishi *et al.* (2004) from *Geotrichum sp.* (0.8 U/mg). So, again the fermentation of the Xylo strain was performed in bioreactor with more optimized and controlled conditions along with enriched media.

6.2 CBM (Shake flask level)

CBM (46-3) was also produced at 4.2 Litre shake flask level and the final OD600 nm at the time of harvesting was 1.423. The 50 OD cells were prepared by dissolving the pellet in 120 ml of lysis buffer obtained after harvesting 4.2L culture. The cells were lysed by sonication and purified through Ni-NTA chromatography. All the fractions exhibiting activity were separated on SDS-PAGE gel (Fig.23). The fractions which showed band at ≈ 26 KDa were corresponding to CBM (46-3). Fraction 4-6 exhibits high CBM concentration after chromatographic separation with Ni-NTA column.



1: Marker
2: Uninduced
3: Induced
4: Preload
5: Flowthrough
6: Wash 1
7: Wash 2
8: Fraction 1
9: Fraction 2
10: Fraction 3

11: Marker
12: Fraction 4
13: Fraction 5
14 : Fraction 6
15: Fraction 7
16: Fraction 8
17: Fraction 9
18: Fraction 10
19: Fraction 11
20.: Fraction 12

Fig 23. SDS-PAGE analysis of CBM eluted through Ni-NTA column

Western Blot of CBM

Western blot of the eluted samples of CBM (46-3) showed that CBM was expressed in fair manner (Fig.24). Though, CBM does not have any xyloglucanase activity but it helps in the degradation of carbohydrates. They increase local carbohydrate concentrations by the proximity effect, target specific carbohydrates in different complex structures such as plant cell walls and disrupt carbohydrate structures (Warner *et al*., 2013).

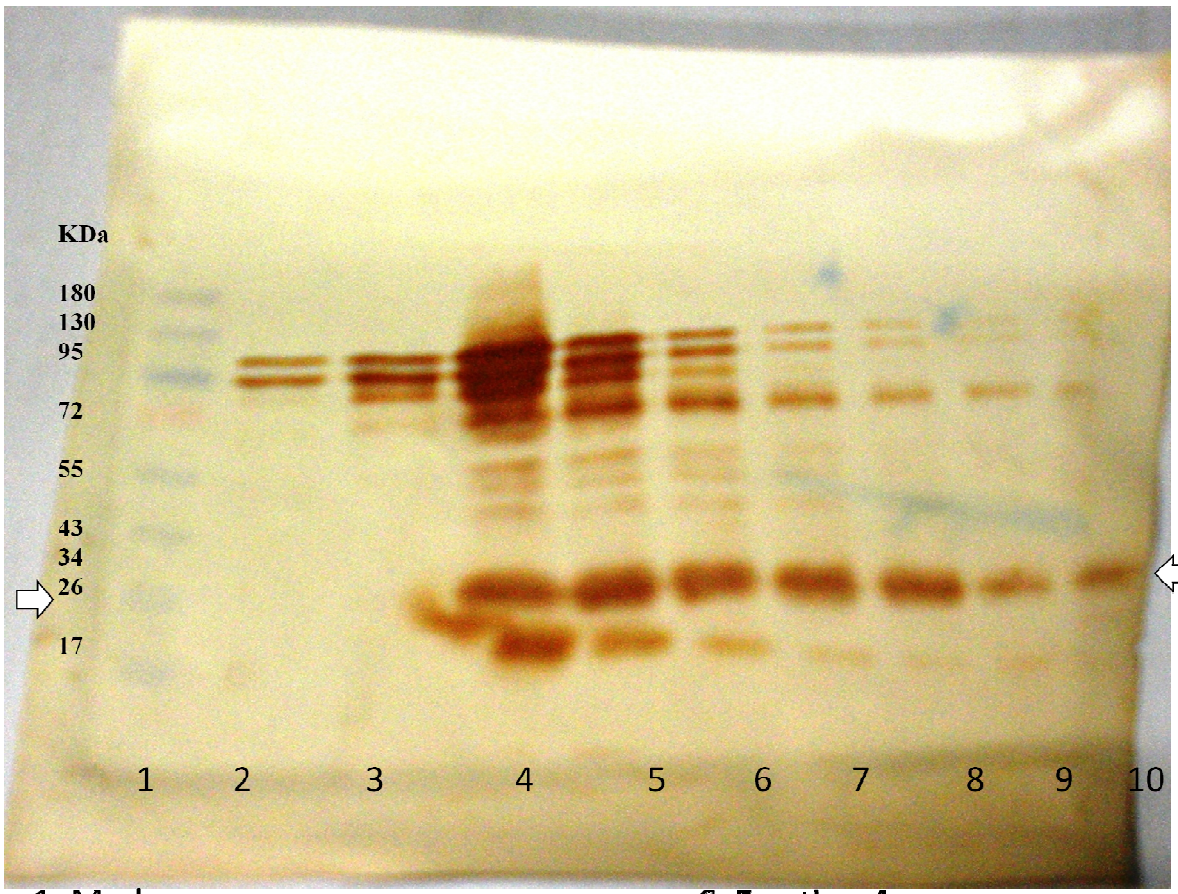


Fig 24. Western blot of CBM

6.3 Xylo (Bioreactor)

Xylo was grown at Bioreactor level (5 litre) under controlled conditions and the final OD600 nm at the time of harvesting was 44.250.The 100 OD cells were prepared by dissolving the pellet obtained after harvesting 5 Litre culture in approximately 2.210 litre of lysis buffer. The cells were lysed by sonication and purified through Ni-NTA chromatography taking advantage of histidine tag present in the protein. All the samples were separated on SDS-PAGE gel (Fig.25). The fractions showed band at ≈ 95 KDa were corresponding to Xylo. Here also it was found that protein of interest Xylo eluted out along with the residual proteins which was also previously seen at the shake flask level. Fraction No. 2-6 exhibit higher concentration of Xylo as compared to other samples.

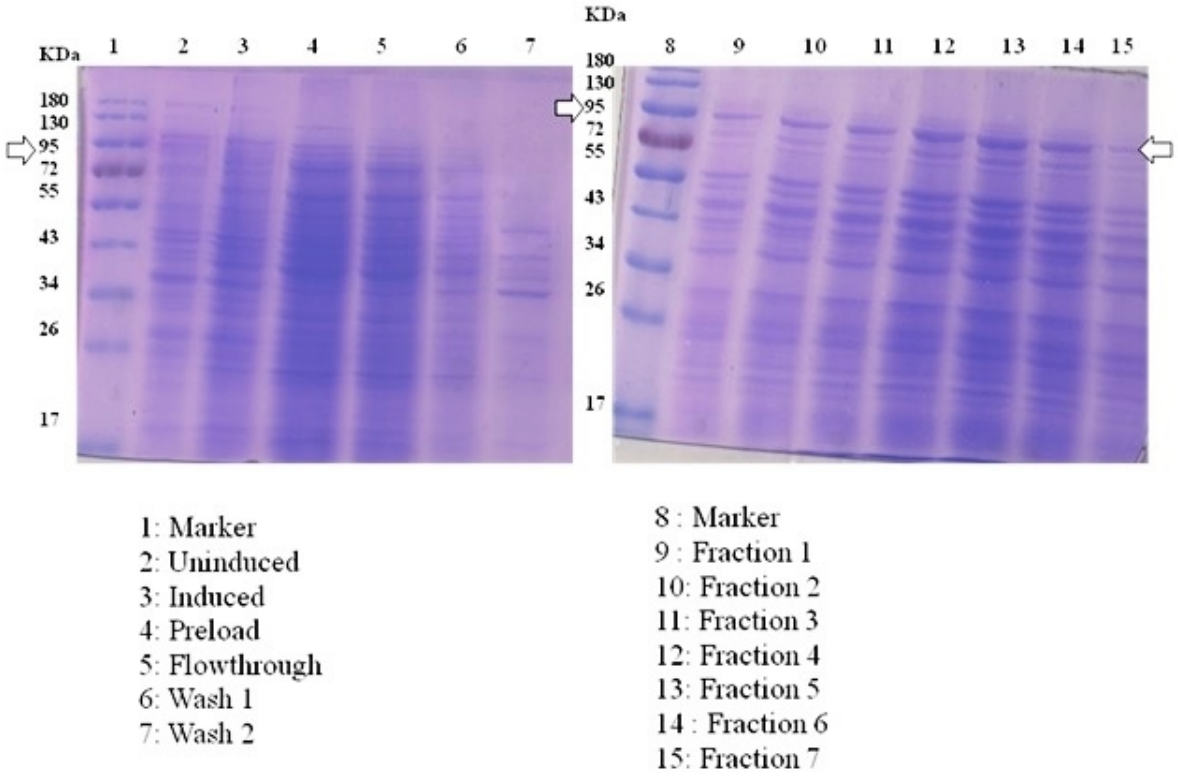
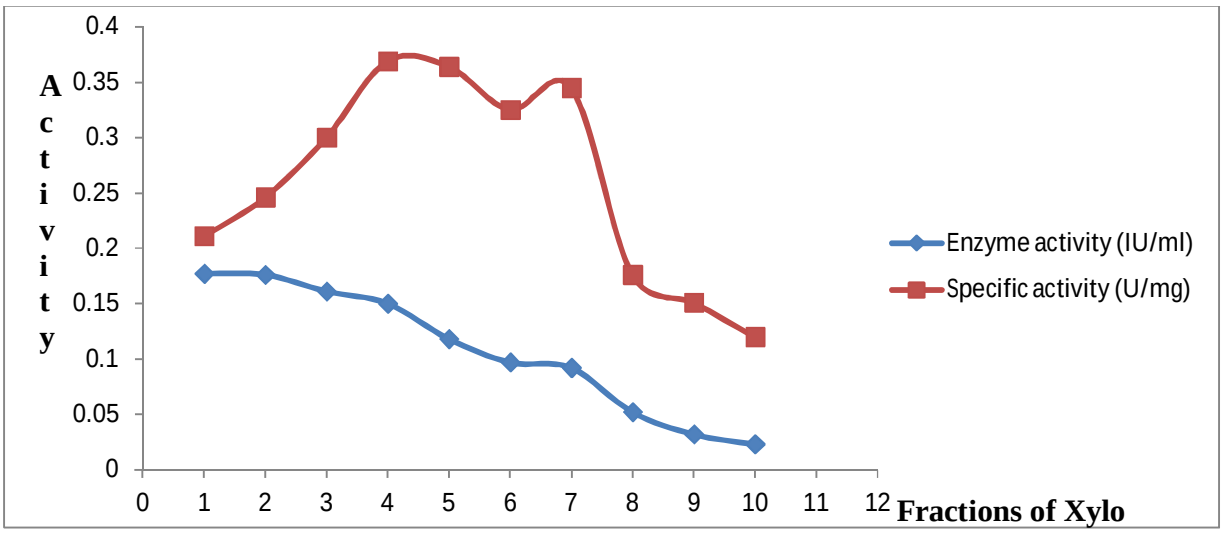


Fig 25. SDS-PAGE analysis of samples (xylo) through Ni-NTA chromatography at bioreactor level

Fig 26. Plot showing enzyme activity (IU/ml) and specific activity (U/mg) of different fractions of Xylo (at Bioreactor level)



The activity (IU/ml) of all the fractions viz. E1, E2, E3, E4, E5, E6, E7, E8, E9 and E10 were found to be- 0.177, 0.176, 0.161, 0.150, 0.118, 0.097, 0.092, 0.052, 0.032, 0.023 and the specific activity (U/mg) were found to be- 0.211, 0.246, 0.300, 0.369, 0.364, 0.325, 0.345, 0.176, 0.151, 0.120 (Fig. 26) which was found to be better than the one done at shake flask level. Rashmi *et al*., (2012) have reported xyloglucanase activity of 0.82 U/mg from *Aspergillus terreus* and Yaoi and Mitsuishi *et al*., (2004) exhibited activity of 0.8 U/mg from *Geotrichum sp*. Hence it was concluded that the expression and purity level of xyloglucanase produced was still low because of the presence of other residual proteins. Hence, further purification is required for getting purified protein or studies on expression in some other host can be undertaken to improve the protein expression of xyloglucanase.

6.4 COMPARISION BETWEEN FERMENTION OF XYLO AT SHAKE FLASK LEVEL AND BIOREACTOR LEVEL

Fig 27. Comparison of enzyme activity (IU/ml) of Xylo fermentation at shake flask and bioreactor level.

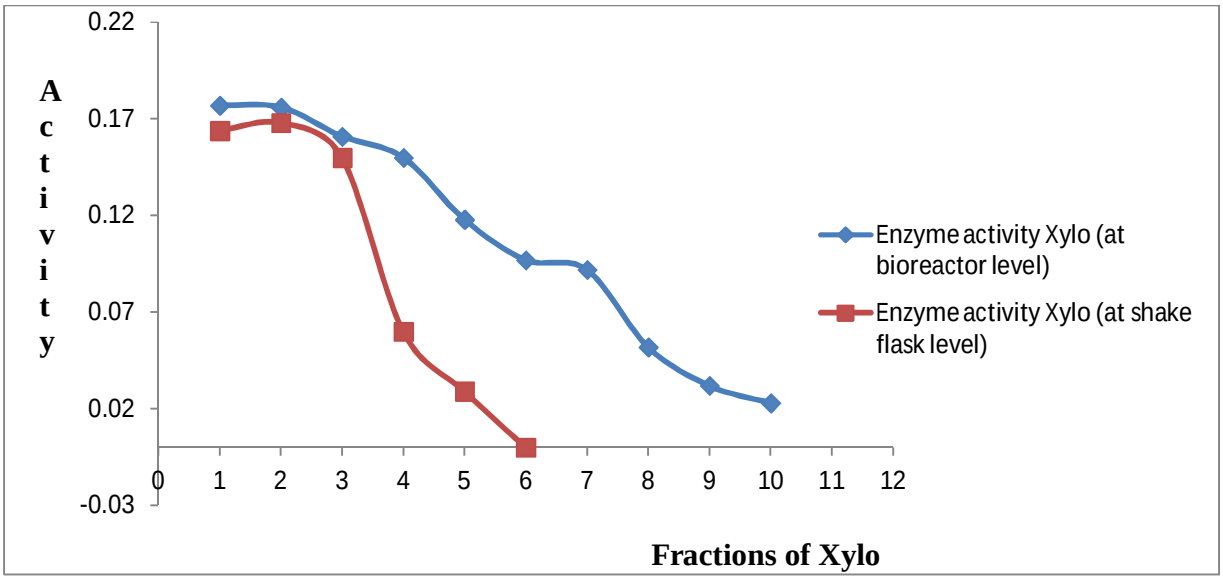
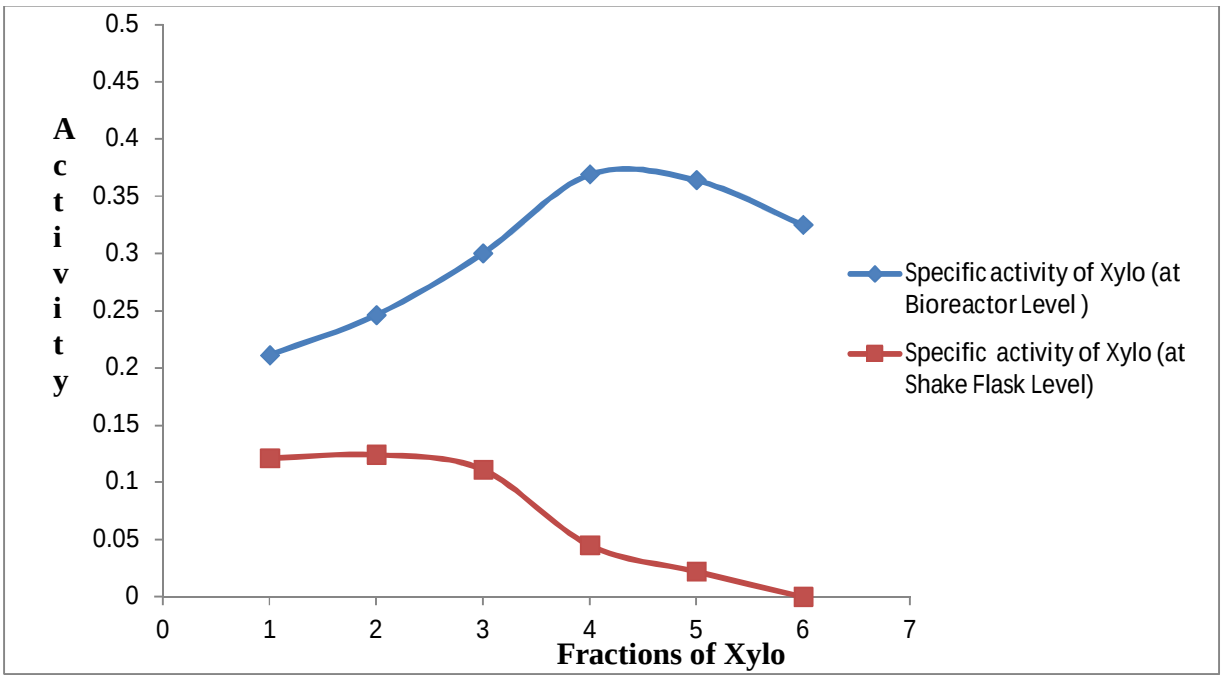


Fig 28. Comparison of specific activity (U/mg) of Xylo fermentation at shake flask and bioreactor level.



The comparison of enzyme activity (Fig 27) and specific activity (Fig 28) of Xylo between shake flask and bioreactor level shows that enzyme activity (IU/ml) and specific activity (U/mg) of enzyme increased at bioreactor level as compared to the shake flask level. In bioreactor more enriched media was used and controlled and optimum conditions were provided as compared to the fermentation at shake flask level. It might have led to the increased biomass production and better enzyme activity.

7. CONCLUSION AND FUTURE PERSPECTIVES

Biomass production was performed for both Xylo and CBM strains and the activity analysis was performed for Xylo at shake flask level, though only Xylo was further studied at bioreactor level under controlled conditions. Proteins were purified through affinity chromatography (Ni-NTA) taking advantage of the histidine tag present in these proteins. Xyloglucanase activity was assayed for Xylo suggesting that this protein has been folded accurately in the heterologous system. The recombinant strain Xylo exhibited fair amount of activity at both shake flask and bioreactor level and hence can be used further to assess the hydrolysis of polysaccharides from the lignocellulosic biomass. Enzyme activity (IU/ml) and specific activity (U/mg) of the eluted out fractions of Xylo at bioreactor level were better as compared to the elutions (fractions) of shake flask level due to the presence of more enriched media and optimized conditions for fermentation. But the specific activity of the Xylo strain both at shake flask level and bioreactor level were low when compared to the previously reported research by other researchers due to lower expression and low purity of the proteins which needs further purification and optimization of the process. Further characterization of the given recombinant strain Xylo can be done by studying and analysing its enzyme kinetics, temperature optima, pH optima and substrate inhibition characteristics. Also, studies on its expression in other host can be undertaken.

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9. APPENDIX

9.1 APENDIX-1

LB Broth

LB Media	25gms
Distilled Water	1000 ml

LB Agar

LB Agar	40gms
Distilled Water	1000 ml

DNS Reagent

Distilled Water	283.2 ml
3,5-Dinitrosalicylic Acid	2.12 g
NaOH	3.96 g
Sodium Potassium Tartarate	61.28 g
Phenol	1.52 ml
Sodium Metabisulphite	1.66 g
Total Volume	300 ml

1 M Sodium Phosphate Buffer

1 M Sodium Phosphate (Monobasic)	138 g/Litre
1 M Sodium Phosphate (Dibasic)	142 g/Litre

Taken 77.4 ml of 1 M Sodium Phosphate (dibasic) and 22.6 ml of 1 M Sodium Phosphate (Monobasic) and make up volume to 1 litre using Milli Q water.

Lysis Buffer (Ph-7.4)

1M Sodium Phosphate Buffer (pH-7.4) (50mM Working)	5 ml
5 M Nacl (300mM Working)	6 ml
1 M Imidazole (10mM Working)	1 ml
Milli Q Water	88 ml
Total Volume	100 ml

Wash Buffer 1 (pH-7.4)

1 M Sodium Phosphate Buffer (pH-7.4) (50mM Working)	5 ml
5 M Nacl (300mM Working)	6 ml
1 M Imidazole (20mM Working)	2 ml
Milli Q Water	87 ml
Total Volume	100 ml

Wash Buffer 2 (pH-7.4)

1 M Sodium Phosphate Buffer (pH-7.4) (50mM Working)	5 ml
5 M Nacl (300mM Working)	6 ml
1 M Imidazole (40mM Working)	4 ml
Milli Q Water	85 ml
Total Volume	100 ml

Elution Buffer 1 (pH-7.4)

1M Sodium Phosphate Buffer (pH-7.4) (50 mM Working)	5 ml
5 M Nacl (300mM Working)	6 ml
1 M Imidazole (250mM Working)	25 ml
Milli Q Water	64 ml
Total Volume	100 ml

Elution Buffer 2 (pH-7.4)

1M Sodium Phosphate Buffer (pH-7.4) (50 mM Working)	5 ml
5 M Nacl (300mM Working)	6 ml
1 M Imidazole (500mM Working)	50 ml
Milli Q Water	39 ml
Total Volume	100 ml

12% Resolving Gel

Distilled water	3.3 ml
30% Acrylamide	4 ml
1.5 M Tris Hcl pH-8.8	2.5 ml
10% SDS	100 µl
10% APS	100 µl
TEMED	10 µl

5% Stacking Gel

Distilled water	4.0 ml
30% Acrylamide	1 ml
1.5 M Tris HCl pH-6.8	0.75 ml
10% SDS	60 µl
10% APS	60 µl
TEMED	6 µl

Commassie Staining Solution

Distilled water	450 ml
Methanol	450 ml
Commassie Brilliant Blue	2.5 g
Glacial Acetic Acid	100 ml
Total Volume	1000 ml

Destaining Solution

Distilled water	500 ml
Methanol	400 ml
Glacial Acetic Acid	100 ml
Total Volume	1000 ml

Ni-NTA Column Regenerating Buffer

1 M Sodium Phosphate Buffer (pH-7.4) (20mM Working)	2 ml
5 M Nacl (0.5M Working)	10 ml
0.5 M EDTA (pH-8) (0.05M Working)	10 ml
Milli Q Water	78 ml
Total Volume	100 ml

Composition of Loading Dye 4X

SDS (10% Working)	2 g
1 M Betamercaptoethanol (10 mM Working)	200 µl
Glycerol (10% Working)	4 ml
1 M Tris HCl (0.2M Working)	4 ml
Bromophenol Blue(0.05% Working)	0.01 g
Total volume	20 ml

1X PBS Buffer – 10ml of 10X PBS in 100ml.

1X PBST – 0.05% Tween-20 in 100ml of 1X PBS.

10X Transfer Buffer – 30.2 g Tris Base, 188 glycine in 1000 ml distilled water.

1X Transfer buffer – 10 ml of 10X transfer buffer, 20ml Methanol and 70 ml Distilled Water.

Developing Solution – 10mg of diaminobenzidine (DAB) and 10 µl H_2O_2 in 10 ml of 1X PBS

BCA Working Solution – Combined 0.1 ml Copper Solution with 4.9 ml BCA Solution to make 5 ml working solution.

0.5N NaOH – Dissolved 20g of NaOH pellet in 1 Litre distilled water.

0.1N NaOH – Dissolved 4g of NaOH pellet in 1 Litre distilled water.

9.2 APPENDIX-2

Reagents and media for Fermentaion in a bioreactor (5Litre)

SNo.	Solution Name	Concentration	Required
1.	Yeast Extract for inoculum I	10g/L	0.5g/50ml
2.	Yeast Extract for inoculum II	10g/L	2X(2g/200ml)
3.	Yeast Extract for Fermenter	10g/L	50g/4.6L
4.	KH2PO4	100X	20g/50ml
5.	K2HPO4	100X	20g/50ml
6.	Na2HPO4.7H2O	100X	26g/50ml
7.	(NH4)2SO4	100X	6g/50ml
8.	NH4Cl	100X	1g/50ml
9.	MgSO4.7H2O	100X	19g/50ml
10.	Batch Glucose	0.4g/ml	100g/250ml
11.	Trace Element solution: a. $COCl_2.6H_2O$ - 30mg b. $MnCl_2$ - 114mg c. $CuCl_2.2H_2O$ - 18mg d. H_3BO_3 - 36mg e. $Na_2MoO_4.2H_2O$ - 30mg f. $Zn(CH_3COO)_2.2H_2O$ - 156mg g. Fe(III) Citrate - 1.2g	400X	30ml
12.	Ampicillin for inoculums I & II	1000X	100mg/ml
13.	Ampicillin for Batch	1000X	500mg/5ml

9.3 APPENDIX-3

Table.1 :- Glucose standard curve

Glucose stock solution – 2mg/ml

SNo.	Glucose from stock solution (µl)	Water (µl)	Concentration (mg/ml)	OD1 540nm	OD2 540 nm	OD Mean
1.	0	100	0	0.108	0.114	0.111
2.	100	900	0.2	0.612	0.608	0.610
3.	200	800	0.4	1.468	1.488	1.478
4.	300	700	0.6	2.127	2.138	2.133
5.	400	600	0.8	2.726	2.722	2.724
6.	500	500	1.0	3.229	3.225	3.227
7.	600	400	1.2	3.522	3.506	3.514

Table.2 : Protein Standard curve

Protein stock solution(BSA)- 2mg/ml

SNo.	Protein (BSA) from stock solution (µl)	Water (µl)	Concentration (mg/ml)	OD1 562nm	OD2 562 nm	Mean OD
1.	0	100	0	0.160	0.164	0.162
2.	100	900	0.2	0.285	0.273	0.279
3.	200	800	0.4	0.407	0.401	0.404
4.	300	700	0.6	0.530	0.532	0.531
5.	400	600	0.8	0.664	0.674	0.669
6.	500	500	1.0	0.871	0.873	0.874
7.	600	400	1.2	1.025	1.049	1.037
8.	700	300	1.4	1.256	1.252	1.254
9.	800	200	1.6	1.300	1.308	1.304
10.	900	100	1.8	1.377	1.376	1.376
11.	1000	0	2.0	1.488	1.498	1.493

Table 3: Protein estimation of sample of different dilutions (OD 562nm) Xylo (at Shake flask level)

SNo.	Dilutions	Sample OD1	Sample OD2	Mean OD	Protein Conc. (mg/ml)
1.	1/5	0.182	0.179	0.1805	1.071
2.	1/10	0.104	0.106	0.1050	1.280
3.	1/15	0.092	0.088	0.090	1.704

Average Protein Concentration Xylo: 1.352 mg/ml

Table 4: Glucose estimation for estimating xyloglucanase activity Xylo (at shake flask level).

SNo.	Sample	OD1 540nm	OD2 540nm	Mean OD	Corrected OD	Glucose Conc. (mg/ml)	Enzyme Activity (U/ml)	Specific Activity (U/mg)
1.	E1	3.132	3.138	3.135	2.812	0.883	0.164	0.121
2.	E2	3.256	3.220	3.238	2.886	0.907	0.168	0.124
3.	E3	2.926	2.922	2.924	2.594	0.809	0.150	0.111
4.	E4	1.470	1.484	1.477	1.15	0.325	0.060	0.045
5.	E5	0.914	0.915	0.914	0.658	0.159	0.029	0.022
6.	E6	0.266	0.270	0.268	0.006	0.000	0.000	0.000

OD 540nm for : Buffer Blank=0.111, Substrate Blank=0.113, E1 Blank=0.099, E2 Blank= 0.128, E3 Blank=0.106, E4 Blank= 0.103, E5 Blank= 0.058, E6 Blank= 0.038

Table 5: Protein estimation of sample of different dilutions (OD 562nm) (at Shake flask level) CBM

SNo.	Dilutions	Sample OD1	Sample OD2	Mean OD	Protein Conc. (mg/ml)
1.	1/5	0.196	0.192	0.194	1.166
2.	1/10	0.107	0.101	0.104	1.266
3.	1/15	0.083	0.075	0.079	1.471

Average Protein concentration CBM: 1.301 mg/ml

Table 6: Protein estimation of sample of different dilutions (OD 562nm) Xylo (at Bioreactor level)

SNo.	Sample	Sample OD	Protein Conc. (mg/ml)
1.	E1	0.738	0.838
2.	E2	0.649	0.713
3.	E3	0.524	0.536
4.	E4	0.432	0.406
5.	E5	0.374	0.324
6.	E6	0.357	0.299
7.	E7	0.333	0.266
8.	E8	0.355	0.297
9.	E9	0.297	0.215
10.	E10	0.278	0.188

Table 7: Glucose estimation for estimating xyloglucanase activity Xylo (at bioreactor level).

SNo.	Sample	OD1 540nm	OD2 540nm	Mean OD	Corrected OD	Glucose Conc. (mg/ml)	Enzyme Activity (U/ml)	Specific Activity (U/mg)
1.	E1	3.311	3.308	3.309	3.025	0.954	0.177	0.211
2.	E2	3.323	3.315	3.319	3.012	0.949	0.176	0.246
3.	E3	3.025	3.091	3.058	2.767	0.867	0.161	0.300
4.	E4	2.921	2.822	2.871	2.590	0.808	0.150	0.369
5.	E5	2.351	2.363	2.357	2.078	0.636	0.118	0.364
6.	E6	2.018	2.024	2.021	1.745	0.524	0.097	0.325
7.	E7	1.897	1.929	1.913	1.661	0.496	0.092	0.345
8.	E8	1.272	1.268	1.270	1.027	0.283	0.052	0.176
9.	E9	0.940	0.936	0.938	0.703	0.175	0.032	0.151
10.	E10	0.801	0.787	0.794	0.547	0.122	0.023	0.120

OD 540nm for : Buffer Blank=0.111, Substrate Blank=0.113, E1 Blank=0.060, E2 Blank= 0.083, E3 Blank=0.067, E4 Blank= 0.057, E5 Blank= 0.055, E6 Blank= 0.052, E7 Blank= 0.028, E8 Blank=0.019, E9 Blank=0.011, E10 Blank=0.023

Table 8: Data showing parameters of bioreactor during whole operation

Time (hr:min)	Ph	Temp Degree Celsius	dO2 (%)	Stir (rpm)
0:00				
1:00	6.8	37.1	80.4	500
2:00	6.8	37	37.6	503
3:00	6.79	37	32.5	551
4:00	6.63	37.1	41.6	722
5:00	6.64	37	38.1	775
6:00	6.8	37	44.8	795
7:00	6.89	37	41.6	767

APPENDIX-4

Fig 29. Standard curve for glucose estimation DNSA between Glucose concentration (mg/ml) and OD 540nm

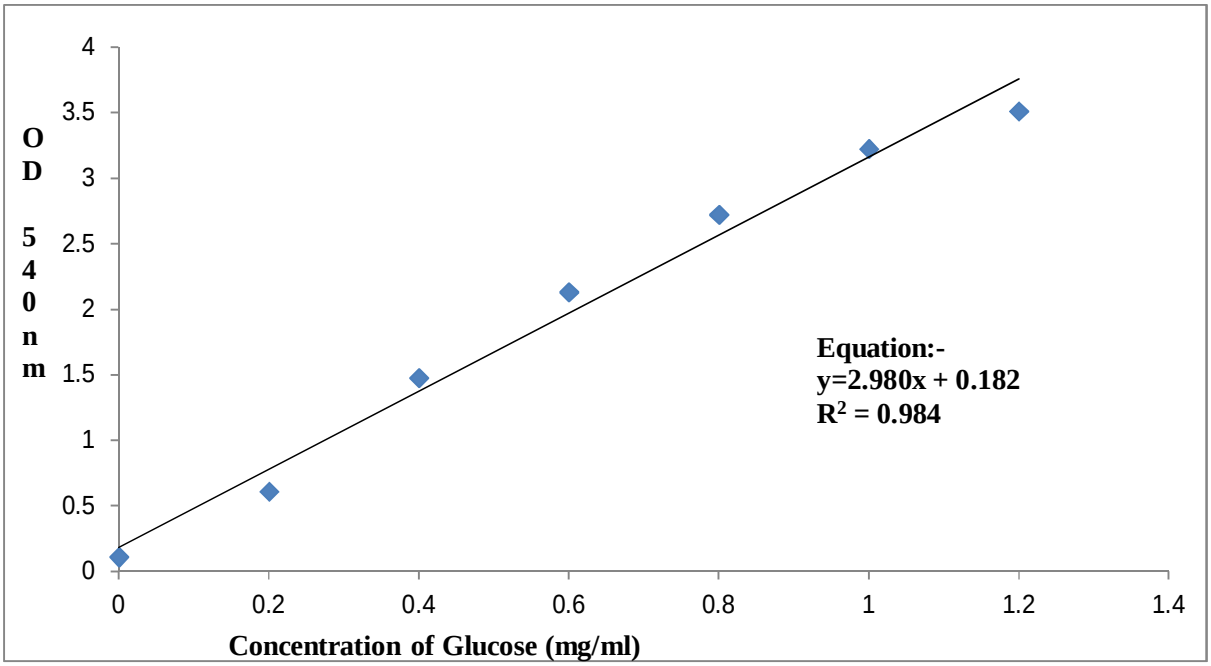

Fig 30. Standard curve for protein estimation between conc. of BSA protein (mg/ml) and OD 562nm