Synopsis of the Thesis
on

Microbial polysaccharide hydrolases for improvement of lignocellulose hydrolysis process

To be submitted

to

The Maharaja Sayajirao University of Baroda

For the Degree of

Doctor of Philosophy

in

Microbiology

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- **INTRODUCTION:**

➢ **Plant Material for Biofuel:** Recent concerns about future availability of fuels, climate changes and energy independence have invoked interest over unconventional or alternative sources of energy, such as wind, solar, hydropower, geothermal, biomass and nuclear. Plant biomass is supposed to be the largest on the earth when compared with animal biomass. So use of plant biomass for production of biofuel is one of the important aspects of developing new unconventional and renewable energy resources (Zhang et al., 2016). Biofuels includes the two main products, (i) Biodiesel, an alternative to petroleum-derived diesel fuel, and (ii) Ethanol, an additive to petroleum derived gasoline (Hill, *et al.,* 2006). In era of global increase of food and fuel demands, energy conservation and biofuels, which are not food-based, are likely to be of greater importance. The search for additional feedstock was initiated, and potential feedstocks discovered are Jatropha, algae, agricultural plant residues and food waste products (Knothe, 2010; Hossain, *et al.,* 2008; Weng, *et al.,* 2008).

➢ **Ethanol and Plant Biomass:** Plants are rich source of complex lignocellulosic polysaccharides Lignocellulose is a complex matrix of lignin and polysaccharides such as cellulose, hemicellulose and pectin. Plant cell wall harbours majority of these structural complex polysaccharides. These natural, potentially cheap and abundant polymers are found as agricultural waste (wheat straw, corn stalks, soybean residues, sugar cane bagasse, barley straw, sorghum straw and many others), forestry residues, municipal solid waste, wood chips, etc., (McLaughlin, *et al.,* 1996). And hence can be used for production of bioethanol from its cellulose. Ethanol is a fermentation product of simple monosaccharides. Mature technologies for ethanol production are crop based, utilizing substrates such as sugar cane juice, and corn starch. Because silage value and cost of these raw materials are high, now efforts are being focused on utilization of different complex carbohydrates present either as structural or functional polysaccharides, in various plants. The examples of such complex polysaccharide carbohydrates are lignocellulose, cellulose, hemicellulose like xylan, xylo-oligomers, starch, amylopectin and amylose, Pectin, etc. and their derivatives (Ferrer, *et al.,* 2005).

➢ **Structural Complexities of Carbohydrates in plants:** Plant Cell walls are rich source for these structural complex carbohydrates. In monocot plants, the primary cell wall consists of 20-30 % of cellulose, 25 % hemicellulose, 10-30 % pectin and 5-10 % glycoproteins. Pectin forms outer most layer of cell wall (middle lamella) which acts as a cement material and connects two plant cells. It makes wall elastic and together with the glycoproteins and the hemicellulose, forms a matrix in

which the cellulose micro-fibrils are embedded. In dicots when the cell has reached its final size and shape, secondary wall layer is added up, which mainly consists of cellulose microfibrils arranged in layered structures and lignin. Wall matrix polymers (Xyloglucan, pectin and glycoproteins) and lignin covalently linked to one another. The binding of Xyloglucan to cellulose microfibrils results in a non-covalently cross-linked cellulose-hemicellulose network. The cellulose fibrils are embedded in a network of pectin, hemicellulose and lignin.

- **Saccharification of Carbohydrate Polysaccharides, a bottle neck problem:** Enzymatic hydrolysis of such lignocellulosic biomass can increase the availability of simple monosaccharides or oligosaccharides, which can be further used for fermentative production of bioethanol. But cross-linking of this network is believed to result in the elimination of water from the wall and the formation of a hydrophobic composite that limits accessibility of hydrolytic enzymes and is a major contributor to the stiffness of secondary walls (Achyuthan et al., 2010).Hemicellulose and lignin like compounds in biomass exert significant restraints on cellulose hydrolysis, e.g., it binds enzyme components non-productively or it may restrict the access of cellulolytic enzymes by coating cellulose fibers. Sometimes pectin could exert a similar kind of effect. Cellulose crystallinity, porosity, etc. are also important factors affecting enzymatic saccharification. So enzymatic hydrolysis finally depends on structural and spatial confirmation of hemicellulose-cellulose composition, and affinity for lignin. Lignin and hemicellulose removal, reduction of cellulose crystallinity and increase of porosity by pretreatment processes can significantly improve the hydrolytic saccharification (Yoshida et al. 2008).
- **Hydrolases and their role in saccharification:** Enzymatic hydrolysis of lignocellulosic materials needs synergistic action of a group of different functional enzymes. Synergism is defined as a system where the two components when are added together demonstrate the enhanced effectiveness then sum of individual effect. One group 'Core enzymes' such as endo-glucanases, exo-glucanases and cellobiohydrolases acts on cellulosic components of plant cell wall whereas other group 'Accessory enzymes' such as variety of hemicellulases (like xylanases and pectinases), when applied, break the physical interference of hydrophobic composite created by hemicelluloses that covers cellulose, and increase the availability of cellulose to core cellulase for its improvised saccharification (Berlin, *et al.,* 2007; Banerjee et al. 2010). Therefore, instead of individual application, synergistic application of an enzyme cocktail, comprising of "core" and "accessory" enzyme components, is suggested to enhance the saccharification. This synergism can be of two types, (i) additive and (ii) substitutive. In former, second component (accessory enzyme) is added

to first component (core enzyme), while in later, a part of first component (core enzyme) is replaced by certain amount of second component (accessory enzyme). It has been reported that Celluclast, a commercial cellulase from Novozymes, Denmark, was not sufficient to remove all of the xylan, when used on pretreated lignocellulose substrates. Such deficiencies of cellulases can be compensated with supplementation of non-cellulosic accessory hydrolases having appropriate activities with core cellulase mixtures (Hu et al., 2011, Qing and Wyman 2011).

- **Hydrolase Sources:** Glycoside hydrolase is a large family of proteins containing, 130 groups in it. Many of such hydrolases have been found in bacteria and fungi from variety of habitats. Forest and farm soil, where large amount of leaf litter and wood rotting usually occur, can be considered as major sources for obtaining potent carbohydrate hydrolase producing bacterial and fungal isolates. Few invertebrate arthropods like phytophagous beetles and termites possess large number of gut symbionts, which secretes such hydrolases to support the plant digestion. Since plants are food for herbivores majority of ruminant vertebrates, their rumen liquor and fecal material contain microorganisms, which can produce the target enzymes. So microorganisms present in different plant material digesting or decaying niches, do possess a long list of corresponding cellulolytic, hemicellulolytic, amylolytic and lignolytic enzymes (Ferrer, *et al.,* 2005; Sa-Pereira, *et al.,* 2003; Duskova, et al., 2001; Wojciechowic, *et al.,* 1980).
- **Rationale:** Biofuels from plant wastes is a priority area of research in the hunt for renewable energy resources that can replace fossil fuels. The bottleneck to industrial scale production of biofuel from lignocellulosic material lies at the key stone step of hydrolysing this material into soluble fermentable sugars due to its complex arrangement. To improve this saccharification process, it was hypothesised that, addition of so called ‘‘accessory’’ enzymes such as xylanase and pectinase to the “core” cellulase enzymes should stimulate cellulose hydrolysis proficiently by removing non-cellulosic polysaccharide meshwork that coat cellulose fibres. So Isolation of bacteria from variety of niches was aimed for the producers of xylanase and pectinase like “accessory” enzymes. Different agrowastes were studied for higher yield of fermentable mono-disaccharides through enzymatic hydrolysis. Effect of enzyme cocktail of core and accessory enzymes was studied on the substrate in combination of various pre-treatments. Hydrolysis of complex polysaccharide by glycoside hydrolases will lead towards their bioconversion to simpler fermentable mono- or di-saccharides, which will reduce the cost effectiveness and provide the raw material to ease up the fermentation and in turn bioethanol synthesis. The research will solve out

the troubles for industrial fields for optimization and production of bioethanol from agro waste products, and yet contribute to reduce some amount of environmental pollution.

- With the above rationale the objectives of the doctoral research work undertaken are
  - Isolation, screening and identification of bacterial cultures producing complex polysaccharide hydrolases (Xylanase and Pectinase) from different sources.
  - Optimization of production of the polysaccharide hydrolases, their characterization and identification from the selected isolates.
  - Study of enzymatic plant biomass saccharification with enzyme cocktail of accessory enzymes with core commercial enzyme, and effect of various pretreatments on plant biomass saccharification carried out by enzyme cocktail.

- **DETAIL OF WORK DONE:**
  - **Isolation, screening and identification of bacterial cultures producing complex polysaccharide hydrolases (Xylanase and Pectinase) from different sources.**

(A) Isolation and Screening: The work was initiated by collecting the samples from variety of niches like (i) Dung samples of ruminant animals (like Cow, Bull, Buffalo, Camel) who could digest the majority of plant materials taken in their diet, (ii) Rotten wood (Farm yard manure, and wet wood) where plant materials are being decayed and (iii) Winogradsky Column developed for cellulose degradation using various microbial sources. Total of 13 samples were collected and individually enriched for three subsequent subcultures in media with tapered nutrient richness, tapered glucose at each step in presence of polysaccharides as sole carbon source for the production of polysaccharide hydrolase producing bacteria free of catabolite repression. After enrichment total 462 bacterial cultures were isolated and screened individually for Xylanase (X) and Pectinase (P) activities on pure (Beechwood Xylan and Pectin) and crude (Wheat Bran, Citrus Peel) both substrates respectively (Teather and Wood, 1982). Six lab contaminants which grew on these plates were also taken for studies. After selection of 172 isolates positive for one or more than one enzyme(s) from primary qualitative plate screening, 136 isolates were quantified positive for xylanase (DNS Method, Miller, 1959.) and/or pectinase (Nedjma et al., 2001) where 70 of them were above cut-off level for pectinase (11 units) and/or xylanase (3 units). Colony morphology and microscopic analysis narrowed down this count to three groups of (i) 31 isolates with pectinase activity, (ii) 16 isolates for xylanase activity and (iii) 07 isolates showing both xylanase and

pectinase activities. Presence of both xylanase and pectinase activities by seven cultures, M18, M33, M35, R30, R31, J208 and J216, lead towards their selection for further studies.

(B) Identification of isolates: 16S rRNA and gyrB gene sequencing using universal primers and analysis suggested their maximum similarities with *B. safensis*, *B. altitudinis* and *B. pumilus* cultures. 16S rRNA and gyrB gene sequences are submitted to NCBI Genbank with accession numbers KJ373676.1 to KJ373682.1 and KJ809597.1 to KJ809604.1, respectively. ARDRA profile, RFLP analysis, Phenotypic characterization with Biochemical tests, Growth at various temperature-pH conditions and Antibiotic Sensitivity, couldn't discriminate the isolates to the similarities with above stated cultures. For the chosen industrial applications, it is more beneficial to have an enzyme which shows activity under broad range of temperature and pH. So based on the this, all seven cultures were subjected to temperature of 20°C-80°C and pH 4-10 for their activity analysis and three cultures *Bacillus* sp. M35, *Bacillus* sp. R31 and *Bacillus* sp. J208 with highest activity at broad range of pH and temperature were selected for further studies. Species level identification by DNA-DNA hybridization using melt curve method is being carried out.

- **Optimization of production of the polysaccharide hydrolases, their characterization and identification from the selected isolates.**

(A) Optimization of production media using the crude inducer for production of accessory enzymes: Citrus Peel (CP), Cotton Seed Cake (CSC), Molasses (M), Rice Bran (RB) and Wheat Bran (WB) were used in media individually to identify their role as a growth enhancer or an enzyme inducer. When compared for the same on Glucose, WB was found to be the inducer for xylanase and pectinase both while CP was found to induce pectinase. For further studies WB and CP were used as an inducer for enzymes. CP was found to induce pectin lyase (PNL), pectate or polygalacturonate lyase (PL), and polygalacturonate hydrolase (PGL)all three enzyme at a time. Optimization of physical parameters like temperature, pH, aeration rate, chemical parameters like N source, salts etc. is currently under study.

(B) Characterization of Crude Xylanase: Xylanase was produced using WB as an inducer and cell free supernatant was collected as a crude source of enzyme and was characterized further. Optimum condition of incubation was studied using combination of various temperature and pH conditions. Temperature range of 30°C-80°C (with increase of 10 units per set) in combination of pH 4.0-10.0 (with increase of 1 unit per set) was used for incubation condition during assay and enzyme was found to be active at temperature range of 35°C -55°C with pH range of 5.0-9.0. Further pre-

incubation of enzymes individually at various temperature (50°C, 60°C and 70°C up to 3 hours) and at various pH (4.0-10.0) up to 24 hours, with timely estimation of activity revealed that xylanases from all three cultures were able to maintain ~50% activity up to 50°C and at pH 6.0-9.0 with pH. 8.0 was most stabilizing. These results suggest the mesophilic nature of enzymes. Viscometric analysis of xylanase using 3% Xylan was performed to reveal the endo- or exo- action mode of xylanase. The increase in reducing sugar by all the three xylanases from 0.4 to 2.0 µmol of xylose equivalent per ml in initial 10 min time corresponds to an average 25–28% decrease in viscosity. Cleavage of polymer at endo- position only can lead to sudden decrease in viscosity and suggest the endo mode of action of enzyme. When various chemical salt modulators were incorporated in an assay, β-mercaptoethanol and NaCl (1 and 2%) enhanced the activity of all the three enzymes. Other compounds had an inhibitory effect on the activity of enzyme with highest degree of inhibition shown by 150mM of EDTA. Kinetic studies for all three crude xylanases (M35, R31 and J208) were performed and K_m (mg/ml) and V_{max} (µmol/(min*mg)) values were determined as 61.28, 51.04, 55.2 and 49.16, 41.27, 38.29 respectively. Substrate specificity for xylanase was studied using Beechwood and Birchwood Xylan. Xylanase from all three cultures have more activity towards Birchwood Xylan when compared with Beechwood Xylan.

(C) Characterization of Crude Pectinase: Pectinase was produced using CP as an inducer and cell free supernatant was collected as a crude source of enzyme and was characterized further. Optimum condition of incubation was studied using combination of various temperature and pH conditions. Temperature range 30°C-80°C (with increase of 10 units per set) in combination of pH 4.0-11.0 (with increase of 1 unit per set) was used for incubation condition during assay and enzyme was found to be active at temperature range of 40°C-70°C with pH range of 7.0-10.0. Further pre-incubation of enzymes individually at various temperature (40°C, 50°C, 60°C and 70°C up to 5 hours) and at various pH (4.0-10.0) up to 24 hours, with timely estimation of activity revealed that pectinases from all three cultures were able to maintain ~50% activity up to 50°C and ~90% activity at pH 5.0-9.0 with pH. 8.0 was most stabilizing. These results suggest the mesophilic nature of enzymes. Viscometric analysis of pectinase using 3% pectin was performed to reveal the endo- or exo- mode of action of pectinase. Pattern of the α (1→ 4) linkage break down between the D-galacturonic acid residues in chain regulates the drop in viscosity of pectic solution. The internal disruption drops the viscosity down faster with increase in unsaturated oligogalacturonides. Pectinase from all three cultures within initial 10 minutes of incubation revealed 38.29%, 48.93% and 58.29% relative decrease in viscosity for M35, J208 and R31

pectinases respectively, with gradual increase in released endproducts, suggesting the endo acting nature of enzyme. Both, rate of increase in released end products (enzyme activity) and rate of decrease in viscosity, tend to decrease with gradual increase in time because, breakdown of polymer length significantly decreases available endo-action sites for enzymes establishing endo-acting nature of all three pectinases. When various chemical salts modulators were incorporated in assay, most of monovalent and divalent cations showed reduced pectinase Activities. β-mercaptoethanol reduced Pectinase activities while Sodium azide showed increase in pectinase activities. EDTA reduced the activity with increase in its concentration. While detergents did not have any significant effect on activity except C-TAB. Kinetic studies for all three crude pectinases (M35, R31 and J208) were performed and K_m (mg/ml) and V_{max} (µmol/(min*mg)) values were determined as 7.39, 8.15, 7.81 and 94.13, 108.29, 92.59 respectively. Substrate specificity for pectinase was studied using Pectic substrates containing various degree of esterification. The linear backbone of the pectin polymer made by α (1→4) linked D-galacturonic acid residues is called homogalacturonan (HG). The ratio of esterified galacturonic acid groups to total galacturonic acid groups is termed the degree of methyl esterification (DE). HG, with a high DE is referred to as "pectin" whereas HG with a low DE is "pectic acid or polygalacturonic acid". Use of pectins with 0%, 35%, 65% and 65-70% DE suggested that Lyase activity for all three cultures have decreased with increase in the DE.

(D) Identification of Enzyme proteins: Activity staining on native Denaturing SDS PAGE was performed. Based on the position of the band developed in zymogram, and Protein molecular weight marker, loaded and run parallel to protein sample, molecular size of three pectinases were determined ~32kDa for M35 pectinase and ~34kDa for J208, R31 pectinase. Single band in case of both pectin and PGA substrate was observed suggesting a single protein having multiple substrate specificity. Activity staining of xylanase is still under study. MALDI-TOF-MS for protein sequencing will be carried out for the enzyme from the protein band corresponding to the band developed in zymogram.

- **Study of enzymatic plant biomass saccharification with enzyme cocktail of accessory enzymes with core commercial enzyme, and effect of various pretreatments on plant biomass saccharification carried out by enzyme cocktail.**

(A) Plant Biomass Saccharification: Various substrates like Barley Husk (BH), Sugarcane Bagasse (SCB) and Wheat Husk (WH) were used for the enzymatic saccharification. Treatment with

commercial cellulase and individual pectinase and xylanase was carried out. According to synergism definition, treatment with enzyme cocktail in terms of additive synergism was studied for all three substrates. So synergism can be established only when the product released by the enzyme cocktail exceeds the sum of the product released by the individual enzyme when used separately in the same amount as in the cocktail in unit time, moreover the ratio of the two (degree of synergism) should exceed 1.0. The enzyme cocktail studied included Cellulase – Xylanase; Cellulase – Pectinase; Pectinase – Xylanase; and Cellulase - Pectinase – Xylanase was studied. BH, SCB and WH individually were incubated with the above mentioned enzymes and timely the supernatant was estimated for release of reducing sugars. Effectiveness of saccharification was calculated in terms of % saccharification and fold increase in Synergism or degree of synergism.

$$\%\, Saccharification = \frac{reducing\ sugar\ released\ (\frac{mg}{ml})}{substrate\ used\ (\frac{mg}{ml})}$$

$$Degree\ of\ synergism = \frac{\%\ saccharification\ by\ enzyme\ cocktail}{sum\ of\ \%\ saccharification\ by\ individual\ enzyme}$$

Saccharification treatment of these agrowastes individually with Primafast® cellulase (C), xylanases (X) and pectinases (P) revealed synergism. All four cocktail combinations enhanced saccharification, where BH was the highest saccharified substrate showing ~1.8 fold increase in saccharification by CPX. Following this saccharification was observed in WH and SCB. WH and BH being primary animal fodder SCB is the major agrowastes residue available for bioenergy purpose. And hence further studies were carried out using the SCB.

(B) Effect of pre-treatment on plant biomass Saccharification: SCB biomass was subjected to various pretreatment methods, including, 1, 2 and 3% H_2SO_4, 1,2 and 3% NH_4OH, 1, 2, and 3% NaOH, Steam Explosion, and Autoclave. Two different treatments were given for Steam Explosion, (i) 10 PSI (5min*4times) (ii) 15 PSI (5min*2times + 10min*2times). All the remaining treatment were given at 10 PSI (20min*1time). 1.25%, 2.5% and 5% substrate was used for each treatment. Effect of pretreatment on the plant material was determined by (i) estimating release of reducing sugar in pretreated supernatant (ii) release of reducing sugar from plant biomass after applying individual core and accessory enzymes, or applying enzyme cocktail. The minimum release of reducing sugar in pretreated supernatant and maximum fold increase in terms of % saccharification from pretreated biomass were the criteria to determine the most efficient pretreatment and enzymatic

cocktail. Analysis of pretreated supernatant for release of reducing sugar revealed by DNS method indicated that maximum sugar release was observed in case of H_2SO_4 pretreatment. Followed by NaOH, NH_4OH, SE and Autoclave. The simultaneous estimation of decreased dry biomass weight after pretreatment supported the above results, with highest loss in acid treated biomass. The maximum amount will lead us to the most efficient pretreatment. When saccharification was calculated for all above pre-treated substrates, highest % saccharification was observed in case of NaOH pretreated substrate (~40-43%), followed by NH_4OH (~16-18%), SE (8-9%), and Autoclaved (~7-8%). The Degree of synergism or fold increase in saccharification when calculated maximum value observed was 1.3. Further results in details will be discussed in thesis. Determination of pentoses and hexoses with help of HPLC is currently being studied. These results reveals that though native substrate showed the highest degree of synergism, the release of reducing sugar in their case was lower than the pretreated substrates, concluding the application of pretreatment to plant material will enhance its saccharification.

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