Characterization of Extra Cellular Enzymes from Soil Actinomycetes: A Molecular Approach

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Abstract— *Extracellular enzymes from microbes are important biocatalysts with their widespread applications in several industries such as textile, biorefineries, food, pulp and paper, agriculture, detergent and pharmaceuticals. Actinomycetes are group of gram positive soil microbes, well known as producers of extracellular enzymes. Among the various genera of actinomycetes, Streptomyces has been a widely exploited group for production of these enzymes. Primary and secondary screening of isolates for identifying the highest enzyme producers followed by estimation of enzyme activity is the primary step. Immense work has been done for identification of genes, understanding the role of structural domains responsible for enzyme activity by X-ray crystallography, mass spectroscopy and NMR studies. This is followed by analyses of protein sequences, phylogenetic tree construction based on amino acid differences among species and recombinant studies for identification of protein families for hyper-producing the enzyme yield.*

Keywords— Extracellular enzymes from actinomycetes, Primary and Secondary screening, Structural analysis by X-ray crystallography and Mass spectroscopy, Gene and protein phylogeny, Recombinant studies

I. INTRODUCTION

Enzymes that alter the rate of chemical reaction reactions [1] can broadly be divided into intracellular enzymes which are retained within the cells that produce them and extracellular enzymes that are produced within the cells but are secreted outside the membrane [2], [3]. Soil is a rich source of complex organic matter. Those micro-organisms which cannot transport complex molecules inside their cytoplasm depend on the action of extracellular enzymes for breakdown of these molecules into useful nutrients [2]-[4].

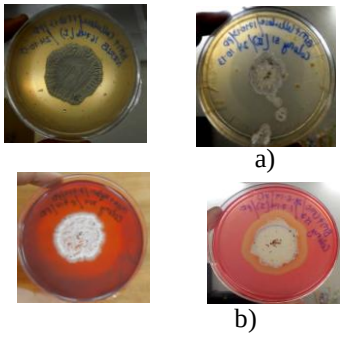
Extracellular enzymes have gained increased attention due to their large scale applications in various industries such as paper, textile, detergent, cosmetics, biosensors, pharmaceuticals, agricultural, bioremediation and biorefineries [5]-[11]. Microbial enzymes are widely used in industrial processes due to their low cost, extensive productivity, environmental protection, stability and vast availability. In order to exploit and improve the metabolic versatility of micro-organisms, the classical area of research has been to optimize the production of extra cellular enzymes for efficient degradation of substrates and selection of advanced and more active enzymes for enhanced enzyme stability and yield.

Wide variety of bacteria in the environment permits screening for efficient extracellular enzyme producing strains to help overcome the current challenges. Actinomycetes are an important group of gram positive, filamentous soil bacteria, extensively distributed in aquatic and terrestrial habitats [5], [12]. These are known degraders of organic materials and producers of different antibiotics [13]-[15] and extracellular enzymes [6]-[11], [16]-[22]. Among diverse actinomycete genera, *Streptomyces, Cellulomonas and Thermomonospora* are widely exploited groups for production of extracellular enzymes [6]-[9], [16], [21]-[26] (Table 1).

II. RESEARCH STATUS OF ENZYME TECHNOLOGY

A. Isolation and screening of isolates: Classical approaches to enzyme characterization

Actinomycetes have been isolated by researchers from diverse ecological habitats for identifying strains having potential for production of extracellular enzymes [16], [44]-[50]. The strains are subjected to plate assay by spot inoculating them on different media supplemented with specific substrates. The zone of clearance produced due to hydrolysis of substrate by the enzyme is measured (Fig.1) [16], [46], [48], [50], [51].



a)

b)

Fig. 1: Screening of isolates for production of extracellular enzymes. Images showing zone of clearance a) without staining and b) with congo red staining

Based on the results of primary screening, strains showing substantial zones of clearance are subjected to secondary screening by submerged fermentation process [16], [45], [47], [49], [52], [53]. Effect of carbon sources, temperature, pH, nitrogen sources and incubation time is tested in crude and purified samples for determining the optimum conditions for maximum enzyme stability and activity [21], [51]. The crude supernatant is then purified by ammonium sulphate precipitation followed by dialysis [16], [27], [45], [46], [52], [54] and through specific column chromatography methods or HPLC/FPLC purification techniques [27], [45], [46], [52]-[57]. Cellulase and xylanase activity (IU/ml/min) in crude and purified extracts is determined by dinitrosalicyclic acid (DNS), chitinase activity (IU/ml) by p-dimethylaminobenzoic acid (pDMAB) method and phosphatase activity (measuring inorganic phosphate) by molybdate blue method [16], [45], [47], [49], [52], [53]. Protein content is quantified by Lowry's method [39], [52]. SDS-PAGE profiling is done to determine the protein profile of the samples and molecular sizes of the visible bands on the gel (Fig. 2) [18], [28], [58], [59].

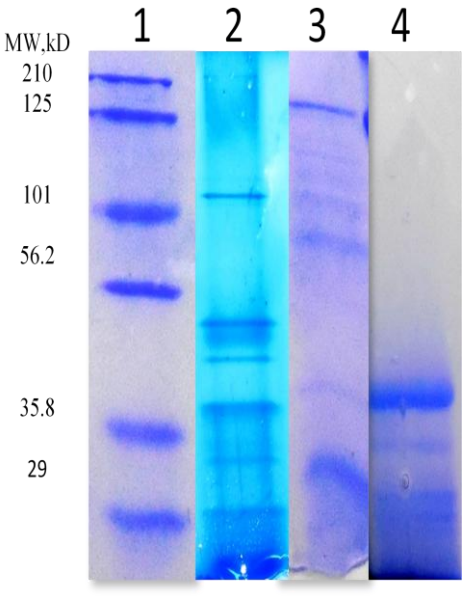


Figure 2: SDS-PAGE protein profile of partially purified samples of cellulase and chitinase enzymes.

Table 1: Extracellular enzymes from actinomycetes

MAJOR GROUP	MICRO ORGANISM	TYPE OF ENZYME PRODUCED	INDUSTRIAL APPLICATIONS	REFERENCES
ACTINOMYCETES	*Cellulomonas fimi, Cellulomonas bioazotea, Cellulomonas uda, Cellulomonas flavigena*	Cellulase	Pulp and paper, Textiles, Biorefineries, Animal feedstocks, Wine and brewing, Baking	[6], [7], [23], [24]
	1) *Streptomyces drozdowiczii; S.lividans; Streptomyces longispororuber, Streptomyces rutgersensis, Streptomyces sp.* B-PNG23			[6], [7], [27], [28], [29], [30], [31], [32], [33]
	Thermonospora fusca; T. cuvata			[34]
	Actinoplanes missouriensis	Sugar isomerases	Textiles, Animal feedstocks, Wine and brewing, Baking	[20]
	Streptomyces hygroscopicus; Streptomyces collinus subsp. *Albescens*	Chitinase	Agricultural, Pharmaceuticals, Food industry	[21], [22], [35], [36]
	Streptomyces avermitilis Streptomyces sp. SLBA-08; Streptomyces strain A3; Streptomyces rochei BTSS 1001	Amylase	Starch, Detergent, Food, Textile, Medicine	[8], [37], [38]
	Streptomyces roseiscleroticus; Streptomyces cuspidosporus; Streptomyces sp. QG-11-3, *Streptomyces sp.* CS624	Xylanase	Pulp and paper, Textiles, Biorefineries, Animal feedstocks, Wine and brewing, Baking industry	[7], [30], [39], [40], [41]
	Saccharomonospora virdis			[42]
	Streptomyces albidoflavus; Streptomyces sp. MAB18	Protease	Leather industry, Food industry, Detergent industry	[9], [43]
	Streptomyces galbus; Streptomyces platensis	Phosphatase	Agricultural	[10], [11]

The protein sample can also be subjected to 2D gel electrophoresis, in order to identify the protein of interest based on the pI and molecular size of the protein. SDS-PAGE/ 2D protein profiles can be further subjected to MS/ LC-MS analysis [54], [60], [61]. Alternatively, enzyme activity confirmation in purified fraction can be done by zymogram analysis for pinpointing the bands responsible for activity (Fig. 3). Zymogram analysis can be done in gel or on plate, both supplemented with the substrate of the respective enzyme being analysed [31], [52], [62].

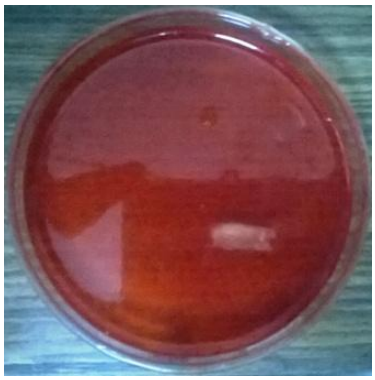


Figure 3: Zymogram analysis, on plate (stained with congo red) showing zones of clearance around bands corresponding to partially purified chitinase enzyme

III. Molecular and Biotechnology Approaches

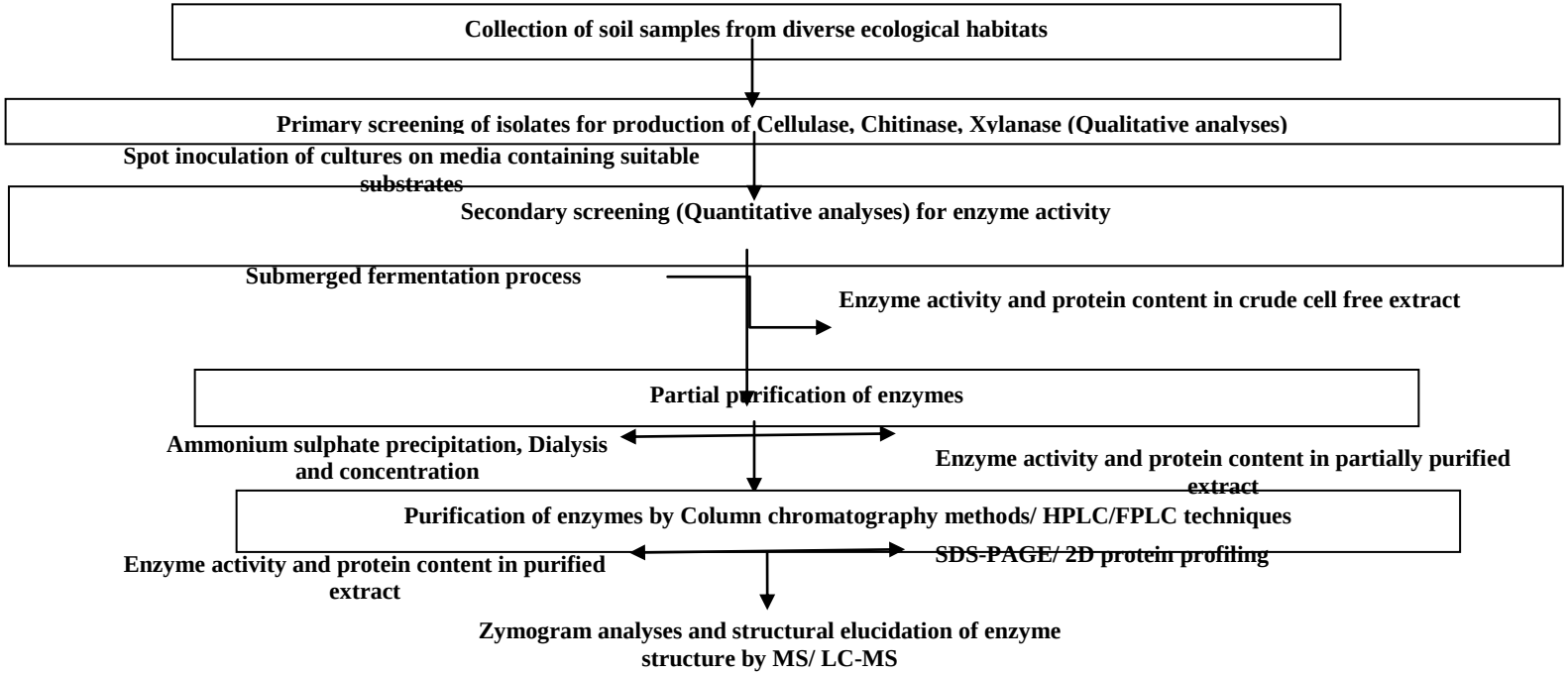
Once the protein bands have been identified by MS/ LC-MS and activity has been confirmed through zymogram analyses, they are initially subjected to protein engineering by X-ray crystallography, fluorescence studies and NMR studies for understanding the modes of catalysis and the role of various structural domains [34], [62], [63], [64] and subsequently used for analyses of protein sequences, phylogenetic tree construction based on amino acid differences among species and recombinant studies for identification of protein families [36], [52], [65].

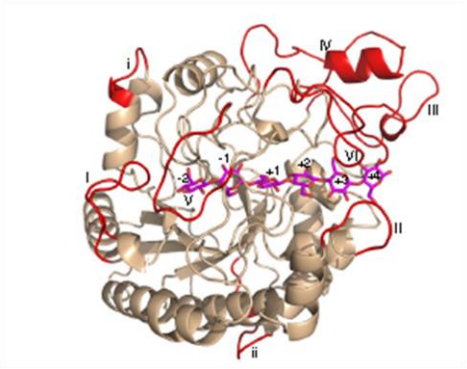
A. Protein engineering by X-ray crystallography and mass spectroscopy studies

Protein engineering has served as one of the principal means of examining the active site of an enzyme and to identify the roles of specific residues in catalysis. The identification of active site residues by chemical modifications or mutations followed by X-ray crystallography data provides basic information regarding the structure-function correlation of the enzymes. These studies form the basis for protein engineering of enzymes and allow specific manipulation of the associated gene for desired enzymatic properties.

The X-ray crystal structure of the catalytic domain of the CBH Cel6B has been reported by Sandgren et al., 2013 [34] from the soil bacterium *Thermobifida fusca*. When the enzyme structure of cellobiose from Cel6B was compared with its fungal counterparts, they observed that the substrate-binding site of Cel6B enzyme is much more extended and its tunnel exit is completely closed by a 13-residue loop, which is not present in fungal GH6 enzyme. However, the tunnel length of Cel6B was comparable to that of fungus *Hypocrea jecorina* belonging to GH7 CBHs family (Fig. 5).

Figure 4: Classical approaches for characterization of extracellular enzymes





Gene coding for *T. fusca* Cel6B (TfuCel6B) was cloned and protein was expressed in *Streptomyces lividans*

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The N-terminus of the expressed enzyme was sequenced

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Crystallization of TfuCel6B catalytic domain was carried out

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TfuCel6B structure was determined with molecular replacement softwares and a search model based on a combination of five different GH6 structure models

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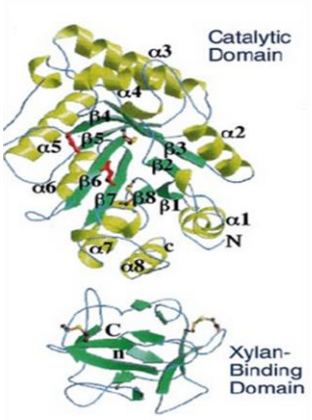
Structure models were compared and structurally aligned based on Cα atoms using the available softwares

Figure 5: a) Backbone of ligand-bound *T. fusca* Cel6B protein molecule A with cellohexaose. Sections I–VI, i and ii are indicated and the residues corresponding to the sections are colored red (TfuCel6B) and blue (HjeCel6A) [34]; b) Flow chart showing the steps followed for obtaining the structure.

Crystal structure of glycoside hydrolase 10 (GH10) xylanase from *Streptomyces* sp. 9 (XynAS9) has been demonstrated by Chen et al., 2014 [63]. It has already been reported that the GH10 xylanase from *Streptomyces sp*. 9 (XynAS9) is active at various pH and temperature range. In order to enhance the enzyme thermotolerance and thermostability, they prepared XynAS9 mutants by altering many residues (such as E82, E62), in comparison with the known GH10 conserved sequences of thermophilic xylanases. The crystal structures of mutants and the wild type were compared, to study the mechanism responsible for improved temperature stability. In mutant XynAS9, it was observed that mutation did not changed the overall protein folding, the structures still resembled the classical GH10 $(\beta/\alpha)_8$ TIM-barrel fold (Fig. 6). However, the mutated E82 residue of the mutant showed many interactions with the side chains of the proteins and neighbouring residues which was absent in the wild type, resulting in protein flexibility and increase in thermal stability

Hoell et al., 2006 [64] reported the crystal structure and enzymatic properties of a family 19 chitinase, Chi G from *Streptomyces coelicolor* A3 (2). It was revealed that the structure and enzymatic activity of family 19 chitinases differs from that of plant chitinases. Comparison of the crystal structures of chitinase G and of the related ChiC from *S. griseus* HUT6037 with chitinases from plants showed that the difference lies in the deletion of 13-residue loop between two catalytic glutamate residues, resulting in decreased size of substrate binding domain (Fig. 7). Apart from structural correlation, analysis of chito-oligosaccharides, amorphous and crystalline chitin degradation by chitinase G was also observed.



Cloning of gene coding for xylanase (XynAS9) in *Streptomyces sp.* 9 and preparation of XynAS9 mutants

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Production and purification of mutants

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The N-terminus of the expressed enzyme was sequenced

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Crystallization of gene for identifying the catalytic and xylan binding domain was carried out

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The structure was determined with molecular replacement softwares and a search model based on a combination of eight different GH6 structure models

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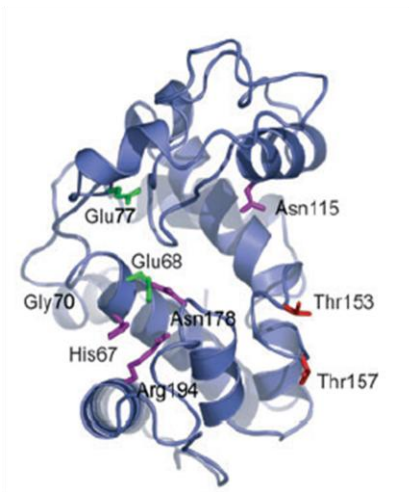
Structure models were compared and structurally aligned based on Cα atoms using the available softwares

Figure 6: a) Ribbon model of XynAS9 from *Streptomyces* sp. 9; showing catalytic domain and xylan binding domain. β-strands Cβ1- Cβ8 and α-helices Cα1- Cα8 build up the (β/α) barrel of the catalytic domain and the catalytic residues are shown in red. [63]; b) Flow chart showing the steps followed for obtaining the structure.



The chiG gene was amplified from genomic DNA of *S. coelicolor* with: primer Chi-Gul_S.coeli-F and primer ChiG_S.coeli-R

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Cloning of chiG gene and preaparation of chiG mutants

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Production and purification of recombinants

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High-quality diffracting crystals of ChiG were obtained by the vapor diffusion method

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Complete X-ray data were collected at beamline ID14-EH3 at the ESRF in Grenoble, equipped with an ADSC Q4R detector. Diffraction images were processed

Figure 7: a) Structure of chitinase G (ChiG) of *Streptomyces coelicolor*. The side chains of the catalytic residues are shown in green. The side chains of residues that are deleted (Trp72, Trp82), mutated (Thr69/Gly70) or mutated and relocated (Lys165/Thr157 and Gln162/Thr153) in ChiG are shown in red. The side chains of four fully conserved residues in subsites) 2 (right side) to +2 (left side) are shown in purple [64]; b) Flow chart showing the steps followed for obtaining the structure.

B. Strategies used for enhancing enzyme production

Amplification, recombinant DNA technology and gene studies for commercial realization and economic viability of enzyme production, there is a necessity to implement techniques which can hyper-produce these enzymes to meet the current demands in various industries. Recombinant techniques are important tools of enhancing enzyme production. Identification of genes involved in the production of particular enzyme is the first step in this procedure followed by cloning and analyses of recombinant gene [36], [52], [65]. The two methods that have been followed till date are (i) rational design, which requires detailed information about an enzyme, such as its 3-D structure, information about its protein domains and mode of enzymatic action. This information helps in site directed mutagenesis of amino acid sequence in the recombinant variants. Comparison of the new enzyme obtained during the screening procedure, to that of the large number of enzymes available in the databases, helps in designing methods for improving the properties of enzymes by modifying the active site structures and construction of new catalytic residues [34], [63], [64], (ii) combinatorial methods, in which recombinants are made through random mutagenesis and screened for their improved activities without having prior knowledge of the enzyme structure and function [36], [52], [65].

Various cellulase, xylanase and chitinase genes have been reported from *Streptomyce*. These include casA gene from *Streptomyces sp.* (strain KSM-9) (359 aa), cel1 from *S. reticuli* (746 aa), celA1 from *S. halstedii* (321 aa) encoding cellulases, xln A, xln C from *Streptomyces lividans* encoding xylanases and chtA from *Streptomyces plicatus*, *stx*I from *Streptomyces thermonitrificans* NTU-88 encoding chitinases respectively.

1. Cellulases: Nacke et al., 2012 [65] reported novel cellulolytic genes from soil metagenomes. Genes encoding cellulolytic enzymes were recovered from soil samples. Three large DNA insert libraries were prepared and the recombinant clones were sequenced. Domain analyses, ORF and coding sequences were examined. Function driven screening led to identification of a novel gene, cel01 (for cellulase) and xyn01 and xyn02 genes (for xylanase). The cel01 gene was amplified from pLC01 plasmid by the primer set containing an ATG start codon- 5'-GCGTTCGTTGAAACGC-3' and 5'-CACCATGCAGGAAATGCTCGCGCCC-3'.
Sequence and protein domain analyses showed that Cel01 (831 aa) belongs to the GH9 family and contains a family 9 carbohydrate binding domain and Xyn 01 (170 aa) and Xyn02 (255 aa) are GH family 11 members. Further activity based screening of cellulase genes was done. The recombinant clones was

found to show high activity as compared to the crude strain.

Apart from primer based domain analyses, genes have also been reported to be used as polymorphic markers as demonstrated by Munar et al., 2014 [66] who showed that partial coding genes of *celB, celS2* and *celS*, can be used as potential polymorphic markers to discriminate *S. coelicolor* M145 strain from other cellulase-producing *Streptomyces.*

2. Xylanases: An extracellular thermophilic xylanase-producing *Streptomyces* sp. strain THW31 isolated from rubbish compost sites in Thailand was identified by Sriyapai et al., 2013 [52]. Chromosomal DNA was extracted, purified and was partially digested with *Sau*3AI. The pZErO-2 vector digested with *Bam*HI was then ligated to the Sau3AI digested chromosomal DNA. The ligated product was transformed into *E. coli* TOP10 competent cells. Plasmids which were found to contain the xylanase gene (pZErO-X3) were selected. The nucleotide sequences of the inserted DNA, ORF, homology search, prediction of signal peptide and generation of three dimensional models of xylanase gene was done by using different softwares respectively. The complete ORF of xylanase gene was found to be 999bp, containing a single peptide of 123 bp and coding nucleotide sequences of 876 bp. The predicted xylanase gene was amplified using primer set (F1, 5'-CGGGATCCGGCACGGTCGTCACGACCAAC-3' and R1, 5'-CCAAGCTTTCAGCCCGCGCTGCAGGAGACC-3'; which contains the *Bam*HI and *Hind*III restriction sites. The primer sets were designed based on the ORF sequence of xylanase gene. The amplified gene was then expressed in *E. coli*. The final result showed identification of a 999 bp ORF termed as *XlnW31*. The XlnW31 protein was found to contain 41 aa signal peptide, and 292 aa of mature xylanase. XlnW31 was classified into family 11, based on the predicted nucleotide and amino acid sequences containing a Gly-rich linker and a type 2 CBD.

Ribosome engineering was used to introduce mutation in gene rpsL (encoding ribosomal protein S12) in marine *Streptomyces viridochromogenes* which produces thermostable xylanase. Ribosomal protein S12 plays an essential role in the ribosome decoding function, in recognizing the codon– anticodon pairings in ribosomal sites and showing resistance against streptomycin [67]. Mutated 30S ribosomal subunit was created by altering the codon-anticodon pairings, resulted in the mutant M11-1(10) of *S. viridochromogenes* strain M11. The mutant showed 14% higher xylanase activities than that of the wild-type strain.

Diaz et al., 2014 [68] reported how a single mutation of residues affects the activity of xylanase Xys1Δ from *Streptomyces halstedii* JM8. The *xysA* gene region from *S. halstedii* JM8, corresponding to the entire Xys1 catalytic domain was amplified by using each of the primers MRGII (5′-GATATACATCATATGGCTCAGAATCCCCCGGTC-3′, with a *NdeI* site) and MRGI (5′-GTGATGCAGCTCGAGCGCGGCGAGCACCGC-3′, with a *Xho*I site). The amino acid was mutated and is then cloned and a library was constructed. Activity analyses of both wild type and mutant showed an increased enzymatic activity ranging from 8-126U in mutants.

3. Chitinases*: Streptomycete* chitinases form two clusters. Family 18 group A bacterial chitinases encompass *chi63* of *Streptomyces plicatus*, *chiC* of *Streptomyces lividans*, *chi40* of *Streptomyces thermoviolaceus* and *chiC, chiD* and *chiE* from *Streptomyces coelicolor*. Similarly group B bacterial chitinases include *chiA* and *chiB* of *S. lividans, chi01* of *Streptomyces olivaceoviridis* and *chiA* and *chiB* from *S. coelicolor* .

Brzezinska et al., 2013 [36] isolated **c**hitinolytic strain *Streptomyces albidoflavus f*rom agricultural soil of Lwowska 1, Torun, Poland. The purified protein was identified by mass spectrometry. Fragments of the amino acid sequences identified by MS were found to be similar to the sequence of chitinase from *Streptomyces albus* J1074. A part of the chitinase gene from *S. albidoflavus* was amplified using the primers: F: 5'-CGGTGAAGGTCACCACCACCGG-3' and R: 5'- TCAAGATGGGCTACTTCACCAACT-3'. The resulting fragment was cloned and sequenced. The obtained nucleotide sequence (750 bp) after conversion into the amino acid sequence was also very similar to the sequence of *S. albus* J1074. The deduced amino acid sequence with DXDXE motif (399-403 out of 628 aa) of the chitinase gene of *S. albidoflavus* indicates that it belongs to GH 18 family.

In order to progress in developing better enzyme expression systems and to solve the difficulties with the expression of heterologous recombinant proteins, two different approaches have been used by researchers. The first method includes the use of systems biology approaches, in which one can improve the yields of active proteins by using bacterial variants or by selecting different promoters or sequence optimization. A second approach includes shuffling from multi-domain cellulase expression to single domain proteins expression, which solves the problem of low yields due to folding or processing errors. More recently, Datta et.al, 2013 [69], reported the immobilization of enzymes on nano/ micro-sized particles, as an alternative technique for increasing enzyme stability and activity.

IV. FUTURISTIC CONSIDERATIONS

In order to deal with future challenges, there is a need to enhance enzymatic hydrolysis of different cellulosic materials available in environment by cellulase enzyme system. Conventional cellulase is active up to a temperature range of around 50^0C, therefore innovative bioprocesses for identification of the thermophilic/extermophilic strains from various habitats for the production of new generation of enzymes are needed. Creation of novel and effective cellulase system by genetic engineering can further improve enzyme production and activity. Secondly, use of low- cost lignocellulosic/ genetically engineered biomass can further reduce the production cost of enzymatic hydrolysis. Efforts are needed to make use of combinatorial methods for producing variants with enhanced enzyme activity.

For cost-efficient bulk production of xylanase enzyme for fulfilling the rising demands of various industries, there is a need to emphasise on economical utilization of abundantly available xylan in the ecosystem. Low cost lignocellulosic wastes can be used as ideal substrates for these enzymes. Till now, birchwood xylan, oat spelt xylan, wheat bran, sugarcane bagasse have been used as a substrate. Apart from use of molecular techniques for enhancing enzyme production, there is a requirement of strain improvement as well as fine tuning of industrial processes including enzyme recovery and downstream processing. Genes coding for thermostable and alkaline form of xylanases can be cloned for industrial purposes. Metagenomic approaches can be used for recovering novel xylanases from complex soil samples.

In the near future, there is a possibility of generating novel chitinases. Further identification of active enzyme sites and the functions associated with them can be targets for protein engineering techniques for increased production of chitinases with exclusive functions. By understanding the biological roles of different chitinases, new therapeutic approaches can be developed for treatment of several diseases. The use of chitinases for the biocontrol of plant pathogens, and for developing transgenic plants is their other major applications. The potential of chitinases can be enhanced by combinatorial strategies, such as by combining them with other bioactive peptides and lytic enzymes, such as glucanases. Thus, special emphasis should be made of the use of combinatorial techniques. The enormous potential of genetic engineering will allow us to combine the natural responses of plants with transgenes of microbial and insect chitinases, other bioactive peptides and improved microbial bio-control agents. CHIs are inducible (adaptive) enzymes, i.e., they are expressed only under certain conditions induced by a certain factor(s) and are regulated by a repressor/inducer system such as glucose. One can find out the different repressor/inducer systems for increasing the yield of chitinases.

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