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BIOPROSPECTING XYLANASE ENZYMES FROM DIVERSE ECOLOGICAL HABITATS

Payal Das[1], Prateek Kumar[1], Munendra Kumar[1], Renu Solanki[2] and Monisha Khanna Kapur[1]

[1] Acharya Narendra Dev College, University of Delhi, Govindpuri, Kalkaji, New Delhi110 019
[2]Deen Dayal Upadhyaya College, University of Delhi, Sector 3, Dwarka, Opp NSIT, New Delhi 110078

ABSTRACT

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 yield. Microbial xylanases are important group of industrial enzymes that are used for various commercial purposes. Wide variety of bacteria in the environment permits screening for efficient xylanase producing strains to help overcome the current challenges. In an attempt to discover new xylanase producing strains, more than 600 actinomycete isolates representing varied ecological habitats were screened. By using birchwood xylan as substrate, diameter of zones of hydrolysis was measured and it ranged from 17-40 mm. Among the isolates tested, colonies 169, 126, and 202 along with the positive controls NRRL B-24314 (Streptomyces thermocoprophilus) and NRRL B-24916 (Streptomyces mexicanus) showing appreciable zones of clearance were selected for quantitative screening under the submerged state fermentation. Enzyme from respective colonies was partially purified by ammonium sulfate precipitation and dialysis. Enzyme activity ranged from 6.72- 15.0 IU/ml (in crude) and 11.15-25 IU/ml (in partially purified). The highest enzyme producer, colony 169 was further purified to homogeneity by ion exchange chromatography. Activity estimated in purified fraction was 32.12 IU/ml. Colony 169 showed maximum xylanase activity

16.12IU/ml at pH range of 7.0-7.5, 16.22IU/ml activity at temperature range of 35^{0}C-40^{0}C and 16.44IU/ml substrate concentration of 1-1.5%. The fraction showed presence of two bands of approximately 50-55kDa and 60-65kDa as determined by SDS gel electrophoresis. Xylanase enzyme from colony 169 was analyzed with the help of proteomics MS/MS database. It was found that the enzyme type was endo-1, 4-beta-xylanase. Molecular analysis showed that the gene encodes a protein of 493 amino acid residues. Comparison of deduced amino acid sequence to other xylanases in the database indicated that the enzyme showed 63% similarity with Streptomyces lividans endo-1, 4-beta-xylanase and belongs to Glycoside hydrolase family 10. For construction of protein structure, homology modeling was done followed by structure verification. The efficiency of colony 169 in biodegradation of wastes was also studied.

Keywords - Actinomycetes, Biodegradation, Enzymatic Purification and Activity, Homology Modeling, MS/MS Analyses, Zone of Hydrolysis.

1. Introduction

Enzymes are proteins that act as catalysts in living organisms, regulating the rate at which chemical reactions proceed, without themselves being altered in the process. Extracellular enzymes are economically important as they have widespread applications in industries (Viikari et al., 1994; Sharma et al., 2014).

Bacteria are well known for production of valuable secondary metabolites. Actinomycetes are free living, saprophytic bacteria, ubiquitously present in nature (Mohan and Charya, 2012; Khanna and Solanki, 2012). They act as major recyclers of organic matter and are invaluable for production of extracellular enzymes, antibiotics, antitumor agents, enzyme inhibitors, etc (Mohan and Charya, 2012; Sharma et al., 2014; Khanna et al., 2011; Selvam et al., 2013). Among diverse actinomycete genera, *Streptomyces*, *Cellulomonas* and *Thermomonospora* are widely tapped groups for production of xylanase (Ninawe et al., 2006). Exploitation of unexplored ecosystems is highly necessary for the discovery of new strains. Advancement in the field of modern biotechnology has opened up unexpected new horizons for discovering novel actinomycete strains from various habitats as producers of xylanase enzyme (Balagurunathan et al., 2010; Deepthi et al., 2012).

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 by fine tuning fermentation parameters. This has led to efficient degradation of substrates and selection of

advanced and more active enzymes for enhanced enzyme yield (Manivasagana et al., 2014; Akoh et al., 2004). At the molecular level, protein engineering has served as one of the principal means for designing new enzymes or proteins with novel or desirable functions (Antikainen et al., 2005; Enkhbaatar et al., 2016). 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 amino acids for desired enzymatic properties (Das et al., 2014; Cheng et al., 2015). Furthermore, recombinant DNA technology has become an important tool for enhancing enzyme production.

With the advancement in industrialization and urbanization, dumping of enormous amount of materials as wastes has become a nuisance. The conventional techniques for biodegradable waste management are becoming increasingly expensive and energy inefficient. Also, the chemical treatment methods are hazardous to both environment and humans. As a result, search for more sustainable approaches becomes important for conversion of wastes into by-products that can be directly used for commercial purposes. One such approach is bioremediation, which makes use of the enzymatic potential of micro-organisms present in the environment for effectual degradation of biodegradable wastes. Waste contains substances like cellulose, starch and lignin, which are susceptible to microbial degradation. It is an eco-friendly process which decomposes the wastes into useful raw materials (Karigar and Rao, 2011; Joutey et al., 2013).

2. Materials and Methods

2.1 Collection of soil samples and isolation of Actinomycetes

Soil samples were collected from various ecological habitats and actinomycetes colonies were isolated by plate dilution method. Single actinomycete colonies were purified by restreaking on Yeast extract-Malt extract agar plates and stored as 20% glycerol stocks at -20 ^{o}C/-80^{0}C (Khanna et al., 2011; Bredholt et al., 2008; Kumar et al., 2016).

2.2 Primary screening or qualitative analyses of isolates for production of xylanase enzyme

Screening of isolates for production of xylanase enzyme was done by spot inoculating the cultures on mineral salt agar medium (Ramakrishnan and Narayanan, 2013) supplemented with 1% birchwood xylan (Sigma), (pH 7.2). The clear zone diameter was measured by subtracting the inoculum size from the total zone diameter in order to select the xylanase

producing isolates (Das et al., 2014; Ramakrishnan and Narayanan, 2013).

2.3 Secondary screening or quantitative analyses of enzyme activity

Strains showing maximum zones of clearance during primary screening were selected for subsequent secondary screening.

2.3.1 Standard inoculum preparation under submerged fermentation process

The isolates showing maximum zone of clearance were inoculated in 25ml of 148G medium (Sriyapai et al., 2013; Schupp and Divers, 1986). CFU's/ml was calculated (18, 51-52). The inoculum having an average viable count of 10^5 to 10^7 CFU's/ml was transferred in production medium (Das et al., 2014; El-Sersy et al., 2010; Shaikh et al., 2013).

2.3.2 Estimation of xylanase activity in crude culture broth by DNS method

Enzyme activity was measured in crude cell free supernatant using Dinitrosalicyclic acid method (Sriyapai et al., 2013; Akoh et al., 2004; Ramakrishnan and Narayanan, 2013; Wood and Bhat, 1998). Enzyme activity was calculated by using the formula:

$$\textbf{Enzyme activity (IU/ml)} = \frac{\text{Concentration of glucose} \times \text{dilution factor}}{\text{Time of incubation (min)} \times \text{volume of enzyme}}$$

Where,

$$\textbf{Glucose concentration} = \frac{\text{Actual absorbance (OD)}}{\text{Slope from graph}}$$

And Actual OD= Test OD- (Enzyme blank OD + Substrate blank OD)

2.3.3 Estimation of protein content in cellulase and xylanase crude culture broth

Protein concentration in crude enzyme was determined by Lowry's method (Sriyapai et al., 2013; Lowry et al., 1951) with BSA (Bovine serum albumin) as a standard.

2.3.4 Optimization of fermentation parameters (pH, temperature and substrate concentration) for enzyme production in crude extract

Different cultural conditions like medium pH (6 to 8.5), incubation temperature (20°C to 50°C) and substrate concentration (0. 25% to 2.5%; w/v) were optimized for enhanced production of enzyme in submerged fermentation process (Kuddus and Ahmad, 2013; Rajnisz et al., 2016).

2.3.5 Statistical analyses of enzymatic activity using SPSS software

The data obtained after optimization of fermentation conditions (pH, temperature and substrate concentration) was statistically analysed using one way ANOVA and Multiple Comparison test (Post-Hoc test) at significance level of $p < 0.05$. Both the tests were performed by using IBM SPSS Statistics 19 software (Fatokun et al., 2016; Yassien et al., 2014).

2.4 Purification of enzymes and enzyme assay of purified products

2.4.1 Purification by ion exchange chromatography

The highest enzyme producers were subjected to partial purification. 250 ml of crude enzyme solution of the cultures was saturated by sequential ammonium sulphate followed by dialysis and concentration (Shanmugapriya et al., 2012 ;). Enzyme activity and protein content was estimated in partially purified samples of colonies. The concentrated sample of colony 169 was purified further by ion exchange chromatography using DEAE Bio-Gel A column. Both unbound and bound fractions were determined for xylanase activity. Active fraction was used as purified enzyme solutions. The fractions were loaded on SDS-PAGE gel for verification of presence of purified enzyme and enzyme activity as well as protein content was estimated in purified fractions.

2.4.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For observing the protein profile of enzyme samples, denaturing SDS- PAGE was used (Murugan et al., 2007; El-Sersy et al., 2010).

2.4.4 Analysis of kinetic parameters

Kinetic factors of colony 169 was studied at a substrate concentration of 0.5-10mg/ml. A Lineweaver-Burk plot was obtained by plotting 1/v against 1/s. Kinetic parameters (K_m and Vmax) was estimated by linear regression from Lineweaver-Burk plot.

2.5 Characterization of enzyme by molecular approach

2.5.1 Protein identification by mass spectrometry analyses

Slices of interest from the semi-denaturing PAGE were cut and in gel digestion was performed. The final samples were submitted for MALDI-MS and LC-ESI-MS/MS analyses respectively. Raw data from both the analyses were transformed in mz.data

format and used to query non-redundant protein databases with a licensed version of MASCOT 2.1 (Matrix Science, Boston, USA).

2.5.2 Analysis of structure and catalytic sites

For studying the structure and catalytic site analysis of xylanase, the peptide sequence for colony 169 obtained by MS/MS analysis was matched against the NCBI database and the FASTA protein sequence database (Mascot search). Then the Blastp was performed to study the homology among the various xylanases. As a result a list of the sequence coverage of the peptide for *Streptomyces* strains was obtained which was used for further studies.

For constructing the structure for the enzymes, N-terminal sequences of colony 169 was submitted in SWISS-MODEL. The software search the database for similarity with the query structure. The best suited structure was chosen by the SWISS-MODEL and based on the sequence of that model a tentative three dimensional structure was made

Homology modeling approach was adopted for structural and functional study of catalytic site of colony 169. The pBLAST program conducted on obtained amino acid sequences revealed about the three dimensional structure of Endoxylanase (Altschul et al., 1990). The most suitable high-resolution protein structure of xylanase was selected as the template protein. The multiple sequence alignment of target and template protein sequence was performed by ClustalW (multiple sequence alignment program for DNA and proteins). Modeling was performed with the help of MODELLER version 9.11 (Schwede et al., 2003; Arnold et al., 2006). After aligning, the target and template sequences were used as input in MODELLER, it derives the restraints automatically from related known structure provided as template (Kim et al., 2010; Shi et al., 2014).

The final 3D model of xylanase were verified by the Structural Analysis and Verification Server (SAVES) which used PROCHECK software. Ramachandran Plot was constructed and analysed to check the percentage of rresidues present in most favored, allowed, generously allowed and disallowed regions respectively (http://nihserver.mbi.ucla.edu/SAVES). The compatibility of the 3D model with its own amino acid sequence (1D) was done by VERIFY 3D program (Bowie et al., 1991; Eisenberg et al., 1997). Identification of active sites that are responsible for substrate binding was done by using Catalytics Site Atlas (CSA) database of European Bioinformatics Institute (http:// www.ebi.ac.uk/thorntonsrv/ databases/CSA/) (Das et al.,

2014; Hoell et al., 2006; Cheng et al. 2015; Shi et al., 2014).

2.5.3 Circular Dichroism for protein secondary structure analysis

Circular dichroism measurements was performed on a Chirascan spectropolarimeter (Applied Photophysics). The CD spectra were recorded from 190nm to 260nm. The results were analyzed by Graphpad Prism processing software (Wang et al., 2010; Sreerama and Woody, 2004; Greenfield, 2006).

2.6 Role of extracellular enzymes in bioremediation of wastes

2.6.1 Collection of waste samples from various sites

Biodegradable waste samples such as agricultural wastes (rice straw and wheat bran) was collected from various sites.

2.6.2 Pre-Treatment of waste samples for obtaining powdered substrates

Various pre-treatment methods has been followed to covert the raw wastes into powdered substrates (Brodeur et al., 2012; Saritha et al., 2012; Karimi and Taherzadeh, 2016). They can be divided into different categories: physical (e.g. milling, grinding and irradiation), chemical (e.g. alkali, dilute acid, oxidizing agents and organic solvents), physicochemical (e.g. steam pre-treatment/autohydrolysis, hydro-thermolysis and wet oxidation) and biological (combined use of lignin degrading enzymes like peroxidases and laccases)

2.6.3 Compositional analysis of wastes

Estimation of the composition (cellulose, hemicellulose, lignin, ash and moisture content) in raw and pretreated wastes (rice straw and wheat bran) was done for getting an insight for the efficient degradation of these wastes using the TAPPI protocol.

2.6.4 Primary and secondary screening of isolates for degradation of wastes

For qualitative screening of selected strains for their ability to degrade wastes, colony 169 was spot inoculated on mineral salt agar medium supplemented with either 0.4% wheat bran or rice straw was prepared. The clear zone diameter was measured by subtracting the inoculum size from the total zone diameter in order to observe the potential of isolates in degradation. For quantitative screening of enzyme activity in crude extracts under solid state fermentation, inoculum having an average viable count of 10^4 to 10^5 CFU's/ml was transferred in mineral salt broth supplemented with specific substrates. Enzyme activity was

estimated in crude cell free extract by DNS method (as mentioned earlier).

3. Results and Discussion

3.1 Collection of soil samples and isolation of Actinomycetes

In the course of our study, a total of 646 actinomycete bacterial colonies were isolated as mentioned in the Table 1.

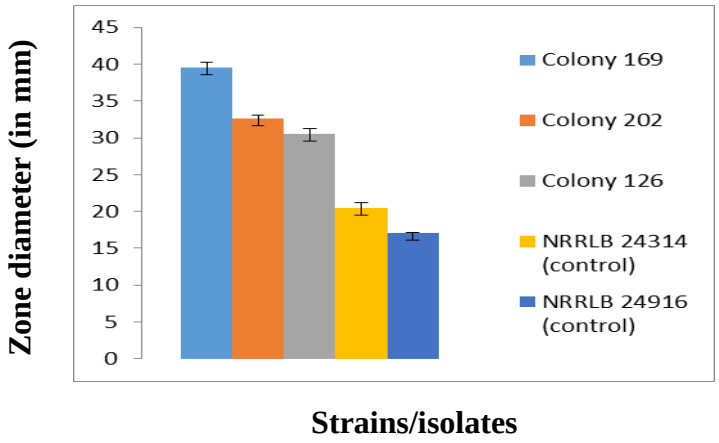
Table 1: Number of isolates from different ecological habitats

S.No.	Habitat	Total no. of colonies
1.	AGRICULTURAL SOILS	
	Agricultural soil, Dhanaura, U.P	86
	Agricultural soil, Yamuna Bank, Delhi	37
	Agricultural soil, Kashipur, Uttarakhand	17
2.	INDUSTRIAL SOILS	
	Sugar Plant, Dhanaura, U.P	7
	Chemical Plant, Faridabad	10
3.	LANDFILL SOILS	28
	Dumping site, Sarai Kale Khan, Delhi	
4.	RIVER/LAKE SOILS	
	Yamuna Bank, Delhi	6
	Lake soil, Purana Quila, Delhi	14
5.	DIVERSITY PARK SOILS	
	Diversity Park, Sarai Kale Khan, Delhi	8
	Great Himalayan National Park, Teerthan Valley, H.P Great Himalayan National Park near a narrow spring, Teerthan Valley, H.P	42 38
6.	SEA/BEACH SOILS	
	Catamaran Beach Hotel, Colombo, Sri Lanka	20
	Havelock Islands and carbon Islands, Andaman & Nicobar Islands	76 and 98
7.	FOREST SOILS	
	Killingpong 4000ft, Kolkata, West Bengal	44
	Pine Forest, Teerthan Valley, H.P	102

Isolates from different ecological habitats were subjected to primary screening to select actinomycetes having potential for production of xylanase enzyme which are commercially important. Isolation of actinomycetes from various ecological environments has been reported by researchers for identifying the extracellular enzyme producers (Mohanta, 2014; Manivasagana et al., 2014; Das et al., 2015; Sriyapai et al., 2013; Ramakrishnan and Narayanan, 2013).

4.2 Primary screening or qualitative analyses of isolates for production of enzyme

27 strains (including isolates and controls) were tested for preliminary xylanase enzyme production. Between 70-80% cultures showed xylanase activity. Based on the results of primary screening, colony no 169, 126, 202 and *Streptomyces mexicanus* (NRRLB 24916) *and Streptomyces thermocoprophilus* (NRRLB 24314) which showed maximum xylanase activity and representing different ecological habitats were selected for further analyses (Table 2, Figure 1). Comparison of xylanase activity of different isolates is shown in histogram 1.



Histogram 1: Comparison of activity of different xylanase producing isolates

* Error bars presented mean values of ± standard deviation of triplicates of three independent experiments, SD determined was in the range of 0.07-0.7.

Table 2: Clear zone produced by isolates due to production of xylanase

S.NO.	Strains	Clear zone diameter (mm)
1.	Colony 169 (Sugar plant, Dhanaura, U.P.)	40
2.	Colony 126 Lake soil, Purana Quila, Delhi	33
3.	Colony 202 (Chemical Plant, Faridabad)	30
4.	NRRLB 24314 *Streptomyces thermocoprophilus (xylanase control)*	21
5.	NRRLB 24916 *Streptomyces mexicanus (*xylanase control)	17

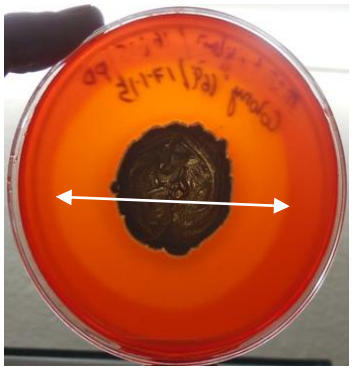


Figure 1.1: colony 169 culture showing zone of clearance on mineral salt agar medium with xylan (congo red staining)

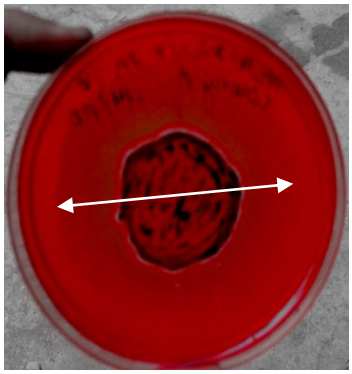


Figure 1.2: Colony 126 culture showing zone of clearance on mineral salt agar medium with xylan (congo red staining)

Figure 1.3: Colony 202 culture showing zone of clearance on mineral salt agar medium with xylan

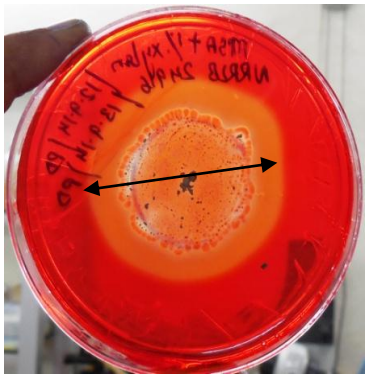


Figure 1.4: NRRLB 24916 (Positive Control) culture showing zone of clearance on mineral salt agar medium with xylan (congo red staining)

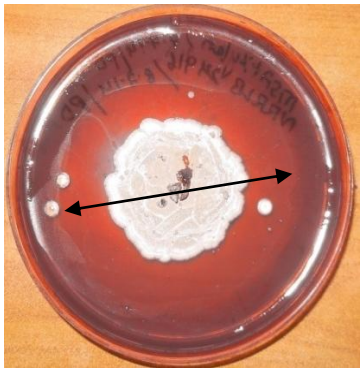


Figure 1.5: NRRLB 24314 (Positive Control) culture showing zone of clearance on mineral salt agar medium with xylan

(**Arrows showing zone of clearance)

Figure 1: Plates showing zone of clearance of isolates due to production of xylanase enzyme

The results obtained during primary screening were comparable with data reported in literature. Boroujeni *et al*., 2012 randomly selected 18 actinomycete isolates form a total of 30 isolated strains and subjected them to primary screening to analyze xylanase, pectinase and cellulase production. Among these, 13 isolates were found to produce extracellular enzymes. Production of xylanase was found to be highest in isolate no. 7 with zone size of 15mm. Porsuk *et al*., 2013 subjected 65 actinomycete isolates for xylanase activity screening by plate assay. It was found that 33 isolates were able to produce xylanase enzyme. Based on the zone diameter, strains were classified as weak/medium/strong xylanase producers. Out of the 33 strains, 4 strains were found to be strong xylanase producers.

3.3 Secondary screening or quantitative analyses of enzyme activity

It is evident from the results of primary screening that maximum xylanase activity was observed in isolate nos. 169, 126, 202, *S. mexicanus* (NRRLB 24916) and *S. thermoprophilus* (NRRLB 24314). Therefore, these cultures along with the controls were selected for subsequent secondary screening. Submerged fermentation process was used for production of enzymes in culture broth. During secondary screening, protein content in culture extract was calculated by extrapolating from BSA standard curve. For enzyme activity, protein content in all samples was equalized. The concentration of glucose released in culture extracts was calculated by extrapolating from glucose standard curve. The results are shown in Table 3.

Table 3: Xylanase enzyme activity and protein content in crude culture extract

S.NO.	Cultures	Absorbance at 575 nm	Concentration of glucose (mg)	Protein content (mg/ml)	Protein content after equalization (mg/ml)	Enzyme activity (IU/ml)
1.	Colony 169	1.101	1.348	0.88	0.49	14.12
2.	Colony 126	0.987	1.130	0.81	0.48	12.72
3.	Colony 202	0.861	0.996	0.76	0.48	11.1
4.	NRRLB 24314 (control)	0.672	0.858	0.59	0.47	8.42
5.	NRRLB 24916 (control)	0.521	0.629	0.45	0.44	6.72

The results obtained during secondary screening were compared with literature. Porsuk *et al*., 2013 evaluated the xylanase producing actinomycete strains using oat spelt xylan as substrate and by DNS method. The liquid medium was inoculated with culture and incubated at 30 °C for 120 h and then centrifuged to obtain crude cell free extract. Out of the 19 isolates tested, only 5 isolates showed activity of approximately 50U/ml. Muthusamy *et al*., 2016 estimated xylanase activity in crude supernatant of four positive xylanolytic actinomycete strains using DNS method using birchwood xylan as substrate. The production broth in each case was inoculated with 5ml of respective culture and incubated at 30^0C for 3-5 days. Total protein content and specific activity measured in highest enzyme producing strain SSA3 was 145.29mg/ml and 35.85IU/mg respectively. Bhosale *et al*., 2011 and El-Gendy and Ahmed, 2014 have done similar work using oat spelt xylan and birchwood xylan as substrates.

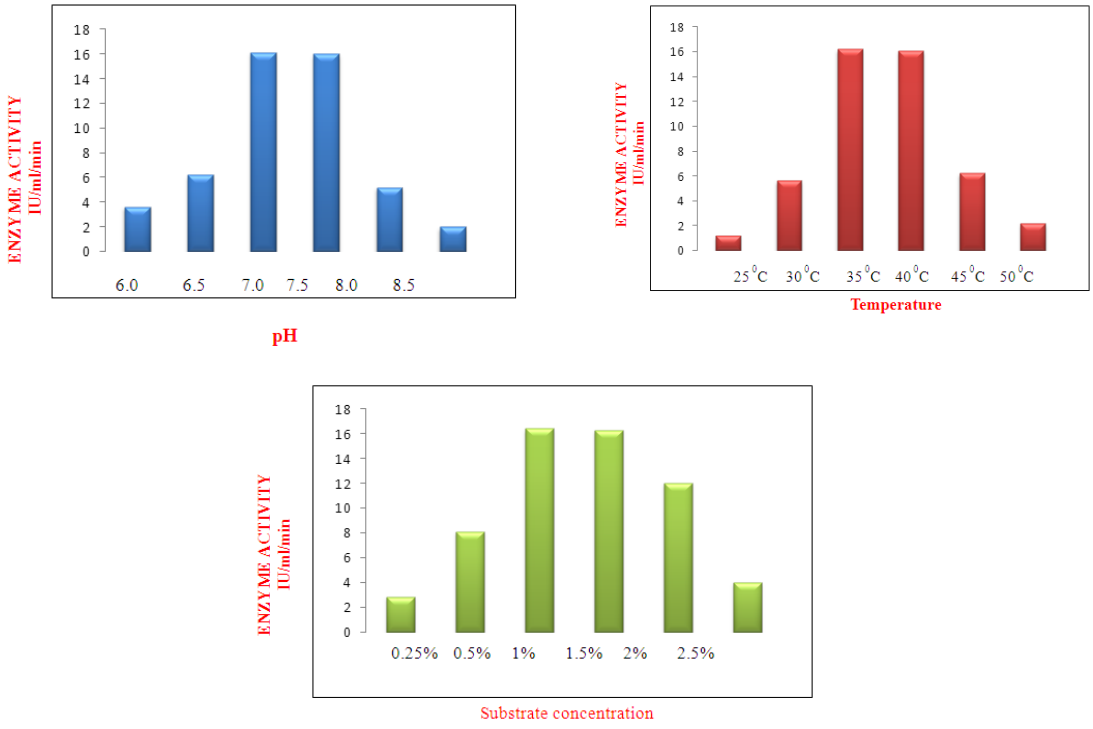
3.3.1 Optimization of fermentation parameters (pH, temperature and substrate

concentration) for enzyme production in crude extract

Based on the results of primary screening, colony 169 was selected for secondary or quantitative analyses by submerged fermentation process at a range of temperature, pH and substrate concentration conditions to determine maximum enzyme activity. Colony 169 showed maximum xylanase activity, 16.12IU/ml at pH range of 7.0-7.5, 16.22IU/ml activity at temperature range of 35^0C-40^0C and 16.44IU/ml substrate concentration of 1-1.5% (Table 4, Graph 1).

Table 4: Optimization of fermentation conditions (pH, temperature) for highest xylanase producer (in crude), colony 169 utilizing birchwood xylan as a substrate

Cultures	pH	Enzyme activity (*IU/ml)	Temp.	Enzyme activity (*IU/ml)	Substrate concentration (%)	Enzyme activity (*IU/ml)
Isolate 169 (Sugar Plant, Dhanaura) Uttar Pradesh	6.0	3.61	25^0C	1.19	0.25	2.85
	6.5	6.21	30^0C	5.62	**0.5**	**8.12**
	7.0	**16.12**	**35^0C**	**16.22**	**1**	**16.44**
	7.5	**16.00**	**40^0C**	**16.08**	1.5	16.32
	8.0	5.21	45^0C	6.22	2	12.02
	8.5	2.0	50^0C	2.16	2.5	4.01



Graph 1: Comparison of fermentation conditions for highest xylanase producer (in crude at different pH (A), temperature (B) and substrate concentration (C)

3.3.2 Statistical analyses of enzymatic activity using SPSS software

Statistical analyses of fermentation conditions (pH, temperature and substrate concentration) using one way ANOVA showed that there is a significant effect of pH, temperature, substrate concentration on the enzyme activity shown by colony 169. The values obtained was $F(5,12) = 66581.475$, $p = .000$ for pH, $F(5,12) = 137621.778$, $p = .000$ for temperature and $F(5,12) = 111638.172$, $p = .000$ for substrate concentration. This was also proved by Post Hoc test (Turkey HSD) analyses which demonstrated that there lies a statistically significant difference in the activity observed at different pH and temperature. This means with the increasing pH and temperature, activity initially increases, attains a maximum level then gradually decreases thus produces a bell shaped curve.

3.4 Purification of enzyme and enzyme assay in purified products

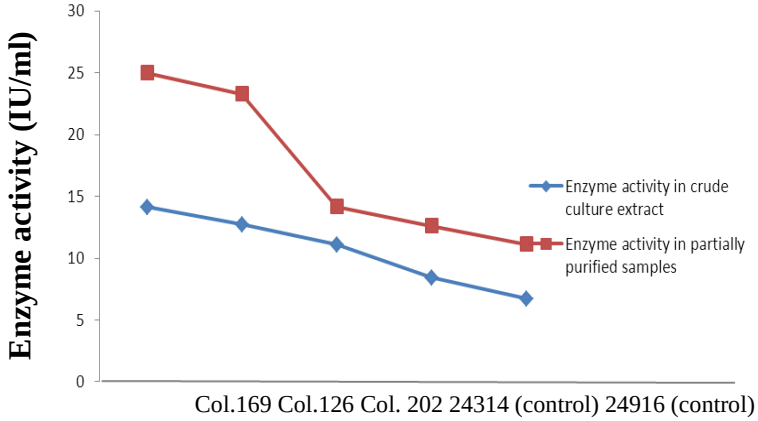
3.4.1 Purification by ion exchange chromatography

Xylanase enzyme activity and protein content were determined in the partially purified protein samples using birchwood xylan and BSA standard curves, respectively as already mentioned in the analyses of crude extracts. Xylanase enzyme activity was maximum in

colony 169 followed by colony 126, colony 202, NRRLB 24314 and NRRLB 24916 (Table 5). Same results of activities were also observed in crude culture extract but there was a proportionate enhancement of xylanase activity in the partially purified samples as shown in Table 5 and Graph 2. The highest xylanase producing colony 169 was selected for further purification by ion exchange column chromatography. Enzyme activity in purified fraction was found to be 32.12 IU/ml.

Table 5: Protein content and xylanase enzyme activity in partially purified protein samples

S.No	Cultures	Protein content (mg/ml)	Protein content after equalization (mg/ml)	Enzyme activity (IU/ml) in partially purified	Enzyme activity (IU/ml) as observed in crude cell free extracts
1.	Colony 169	1.101	0.49	25.0	14.12
2.	Colony 126	0.987	0.48	23.31	12.72
3.	Colony 202	0.861	0.48	14.17	11.1
4.	NRRLB 24314 (control)	0.672	0.47	12.60	8.42
5.	NRRLB 24916 (control)	0.521	0.44	11.15	6.72



Graph 2: Comparison of xylanase enzyme activity of crude culture extracts and partially purified samples

The results obtained after purification were compared with data reported in literature. Deesukon *et al.*, 2011 performed purification of XynSW2A and XynSW2B from *Streptomyces sp.* SWU10 by ultrafiltration, dialysis and then by using different sets of columns like DEAE-Toyopearl, Resource PHE, Superdex 75 HR 10/30 and Mono QR-H 5/5. The result showed that in case of XynSW2A, total activity (U), total protein content (mg) and specific activity (U/mg) recorded was 1300U, 3200mg, 0.41U/mg in ultrafiltration sample, 180U, 73mg, 2.5U/mg in DEAE-Toyopearl purified sample, 56U, 1.7mg, 33U/mg in Resource PHE purified sample and 7.4U, 0.2mg, 37U/mg inSuperdex75 purified sample. Similarly in case of XynSW2B, total activity (U), total protein content (mg) and specific activity (U/mg) recorded was 410U, 110mg, 3.7U/mg in DEAE-Toyopearl purified sample, 160U, 28mg, 5.7U/mg in Mono-Q purified sample and 24U, 0.4mg, 60U/mg in Resource PHE purified sample. El-Gendy and Ahmed, 2014 purified xylanase from *Streptomyces* sp. ESRAA-301097 by 70% ammonium sulphate saturation followed by dialysis. The resultant dialyzed sample was then purified by DEAE-cellulose column, Sephadex G-200 and G-100 columns respectively. Total activity, protein content and specific activity observed during ammonium suplhate precipitation was 17395.42U, 67.00mg, and 25.93U/mg respectively, and in column chromatography (DEAE-cellulose and Sephadex G-200, G100) were 11736.86U, 151.11mg and 77.67U/mg. 9.88.65U, 23.62mg and 384.79U/mg and 7145U, 14.18mg and 493.48U/mg respectively.

3.4.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The partially purified samples for each enzyme were run on denaturing SDS–PAGE for observing the protein profile. The number of bands observed were more than 20 bands of different sizes (Figure 4.4.2.1). The purified fractions was run to estimate the band size and observe the extent to which the samples were purified (Figure 2(a)). The fraction showed presence of two bands of approximately 50-55kDa and 60-65kDa corresponding to purified xylanase enzyme (Figure 2(b)). The purified band from the gel was eluted and processed for further studies.

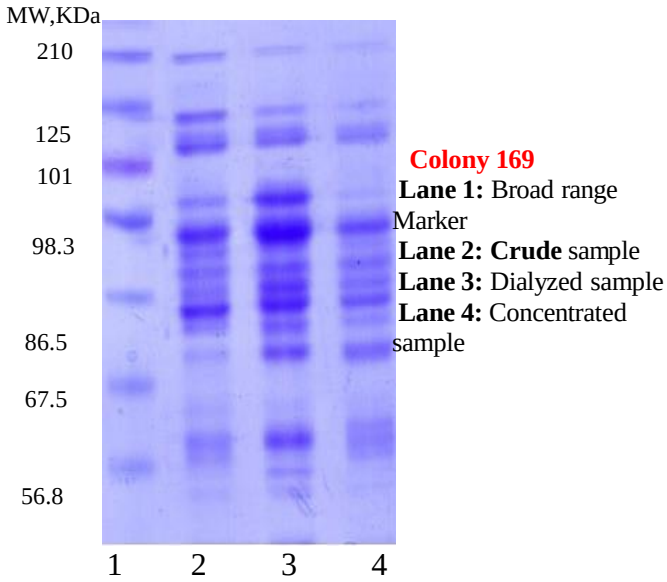


Figure 2(A): SDS-PAGE profile of Colonies 194, 169, 130 and 165

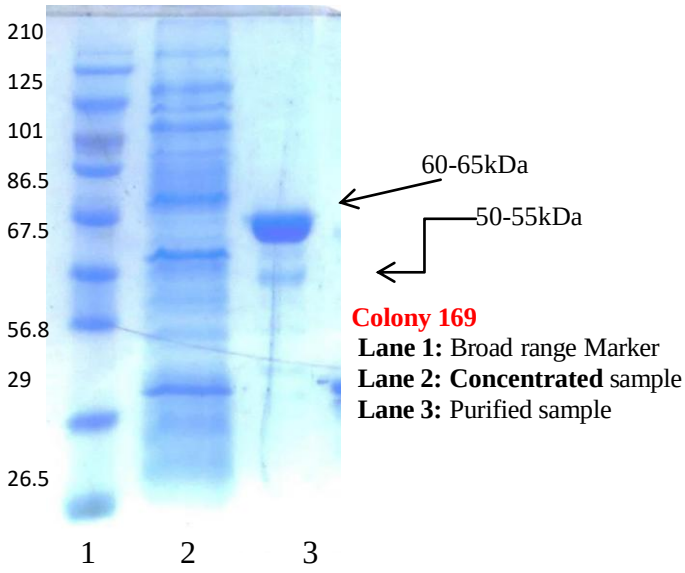


Figure 2(B): SDS-PAGE profile of Colony 169 purified fractions

The molecular weight of xylanase from *Streptomyces lividans*, *S. thermoviolaceus* OPC-520, *Streptomyces* sp. QG-11-3, *Streptomyces* sp B-12-2, *Streptomyces chartreusis* and *S. viridisporus* T7A reported in literature were (Wang et al., 2010; Akoh et al., 2004; Lowry et al., 1951) 20.5, 23.8-40.5, (Akoh et al., 2004; Lowry et al., 1951; Altschul et al., 1990) 31.6 and 15-36kDa (El-Gendy et al., 2014; Roberge et al., 1999; Mansour et al., 2003).

3.4.5 Analysis of kinetic parameters

Kinetic factors of xylanase and chitinase producing colony 169 was studied at a substrate concentration of 0.5-10mg/ml. A Lineweaver-Burk plot was obtained by plotting 1/v against

1/s by linear regression. The K_m and V_{max} values of the purified fraction in case of xylanase (colony 169) were 56.03mg/ml and 2.04µg/ml/min (Figure 3).

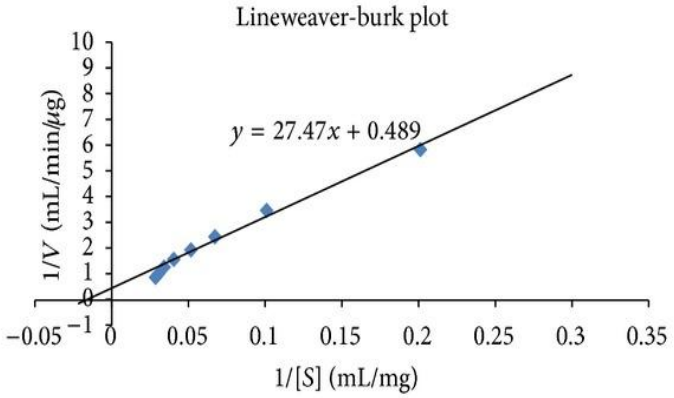


Figure 3: Lineweaer-Burk Plot for Colony 169

3.5 Characterization of enzymes by molecular approach

3.5.1 Protein identification by mass spectrometry

The sequences obtained after MS/MS both for xylanase was assembled using MASCOT. ESI-MS spectra of colony 169 is shown in Figure 4. The total assembled amino acid sequence obtained for colony 169 was 495aa (Figure 4). The assembled sequences were then used for further analyses.

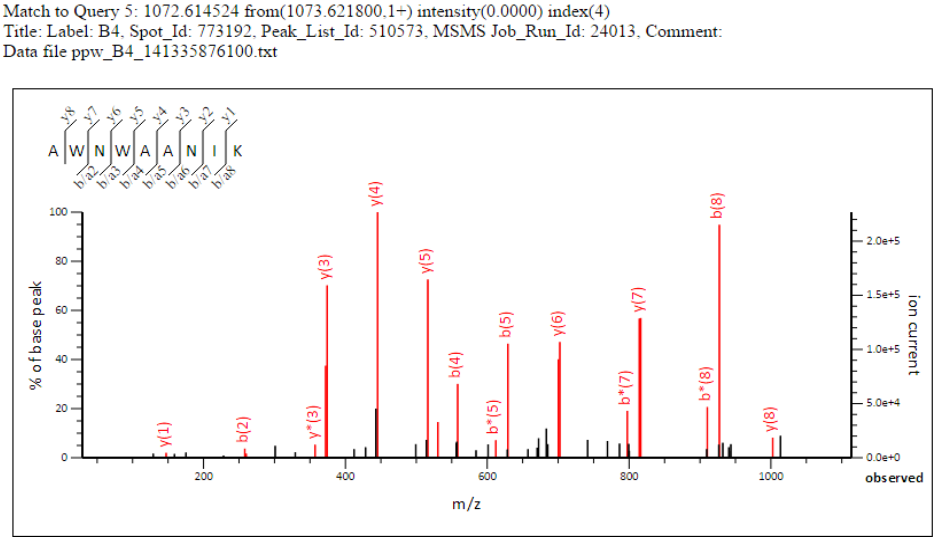


Figure 4: ESI-MS Spectra for Colony 169

3.5.2 Analysis of structure and catalytic sites

For identifying the type of protein in colony 169, the assembled amino acid query sequence was searched using pBLAST against Protein data base (PDB) database. The target sequence of colony 169 showed high identity (65%) with chain A of endoxylanase family 10 from *Streptomyces lividans* (PDB id: 1v0k). Multiple sequence alignment of N-terminal sequence of colony 169 was done with N-terminal sequences of known endoxylanase producing *Streptomyces sp*. using CLUSTALW software. (Figure 5)

Figure 5: Multiple sequence alignment of target colony 169 and template *Streptomyces lividans* (PDB ID: 1vv0k). The important residues from active site point of view are highlighted in red and hydrophobic residues in blue.

For constructing the structure for the enzymes, N-terminal sequences of colony 169 was submitted in SWISS-MODEL. The resultant structure showed that the 495 amino acid residues of colony 169 was folded into a domain (β-jelly roll) structure comprising two parallel β-sheets and a single α- helix (Figure 6), like other enzymes belonging to the glycoside hydrolase family 10. Due to the availability of the 3D protein structure of 1v0k from *Streptomyces lividans* it was selected as a template for homology modeling using MODELLER 9.11 for endoxylanase from colony 169. The superimposed modelled structure has been shown in Figure 7. After the homology modeling, Ramachandran plot drawn through PROCHECK program validated the model with 72.2% of total residues confined in the core region (allowed region) (Figure 8). This confirms that the protein back bone dihedral angles phi (φ) and psi (ψ) occupied reasonably accurate

positions in the 3-D model. The Verify 3D comparison results for colony 169 showed that in a 3D/1D profile, 79.67% of the residues had an average 3D-1D score >=0.2. The obtained data was also compared against protein database (PDB) using DaliLiteV3.1 server. RMSD and Z score values for the top 5 matches were in the range of 1.0-1.2 and 22.9-25.6. This data further confirmed and validated the modelled structures obtained for both the colonies.

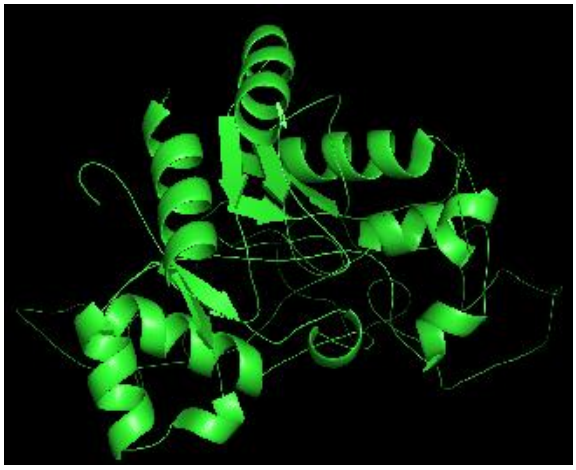


Figure 6: Modelled structure of Colony 169 Using SWISS-MODEL

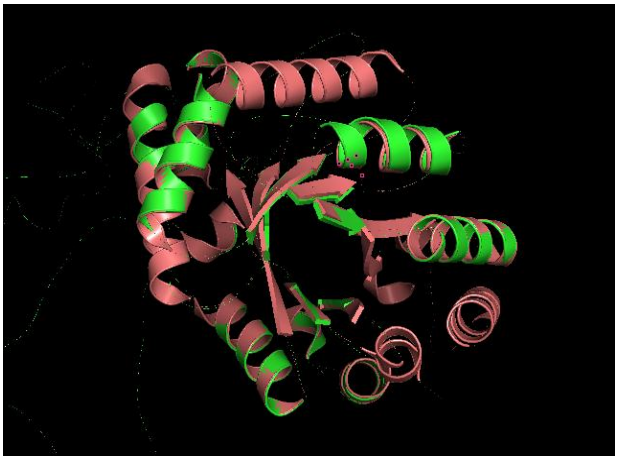
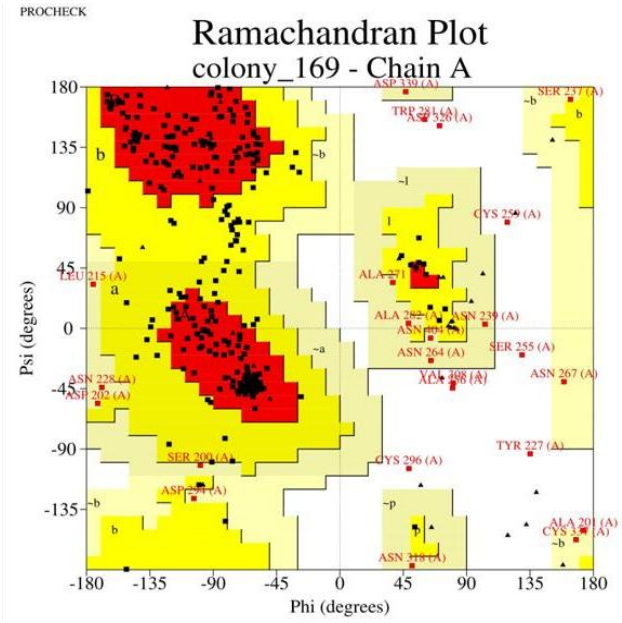


Figure 7: Superimposed modelled structure of Colony 169 By MODELLER (Green: 1v0k; Salmon: Colony 169)



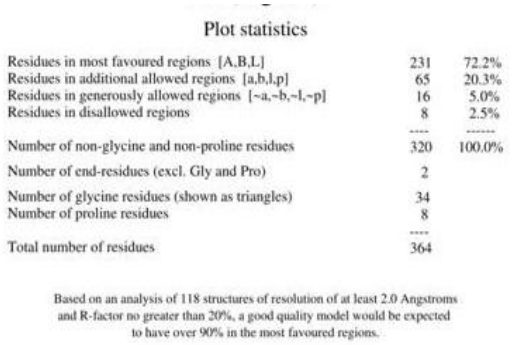


Figure 8: Ramachandran plot of modelled Colony 169 obtained by PROCHECK validation package

The information about the active site was obtained through superimposing 3-D model structure of the target enzyme with that of template protein from *Streptomyces lividans* which provided accuracy of homology between the structures, and also helped in positioning the conserved active site residues. Overlapping of colony 169 xylanase with the template of 1v0k stipulates the amino acid residues (Tryptophan (W) at position 185, Tyrosine (Y) at position 172, Tryptophan (W) at position 266 and Tryptophan (W) at position 274 constitute active site of the enzyme and out of these, (Tryptophan (W) at position 185, Tyrosine (Y) at position 172 were shared by both colony 169 and *S. lividans* (Figure 9).

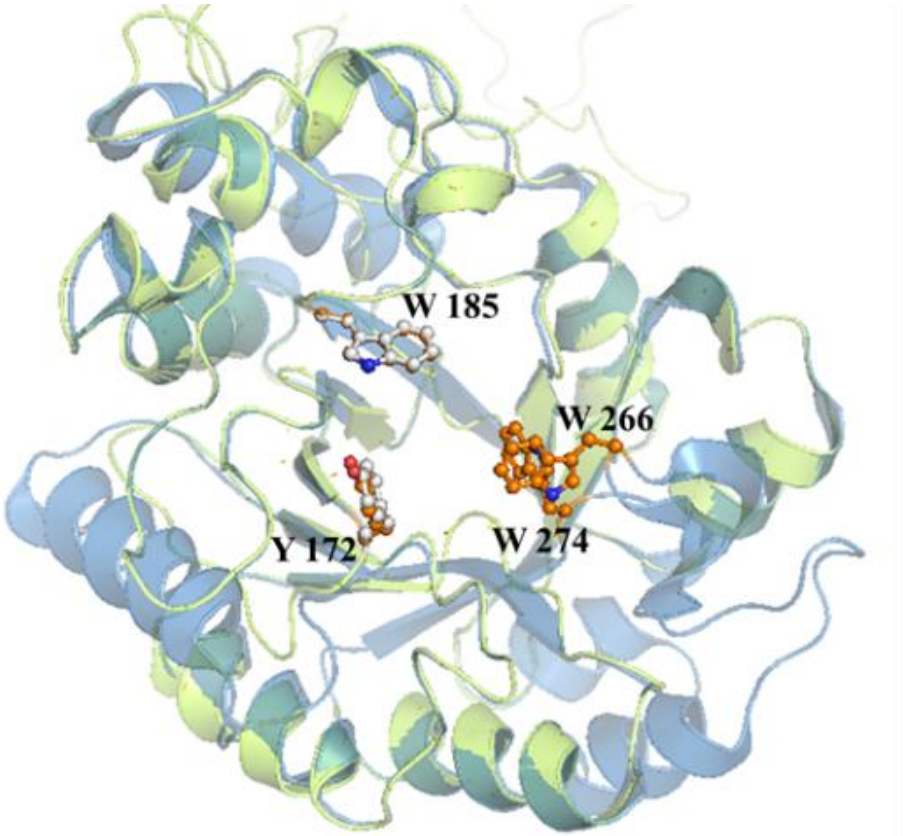


Figure 9: Superimposition of active site residues of modelled xylanase and template 1v0k. (Blue and green color ribbons modelled and template proteins, respectively).

3.5.3 Circular Dichroism for protein secondary structure analysis

The far-UV CD spectrum of colony 169 exhibited a pronounced maximum and minimum at 195 nm and 222 nm which are characteristics of β-sheet and α-helix structures in aqueous solution, respectively (Figure 10). Thus, colony 169 endoxylanase are an autonomous

structural protein that contains both α-helix and β-sheet secondary structures as predicted from homology modelling.

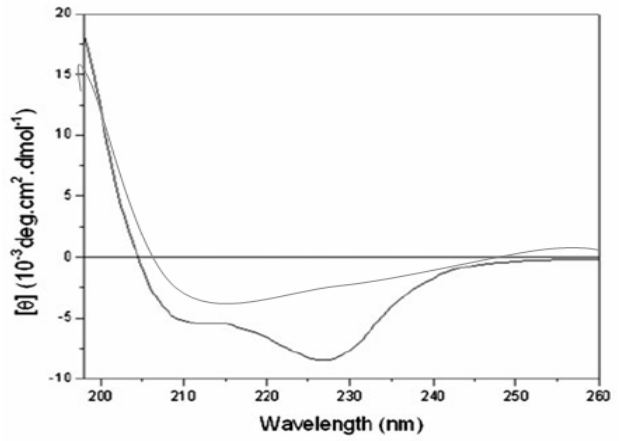


Figure 10 Far-UV CD spectra of Colony 169

The results obtained during secondary screening were compared with literature. Cheng *et al*., 2009 identified xylanase of *Streptomyces thermonitrificans* NTU-88 by mass spectrometry. The spot identified by 2D electrophoresis was excised and subjected to in-gel digestion using trypsin. The samples were submitted for mass spectrometry analysis. The results obtained were compared with NCBI and SwissProt databases using the Mascot search algorithm. Significant protein scores ($p<0.05$) were obtained from Mascot search. Three trypsin-digested peptides showed similarity with amino acid sequence of xylanase A of *S. coelicolor* A3 and belonging to family GH10 (accession number: NP_733679). The alignment of the sequence obtained for xylanase of *S. thermonitrificans* NTU-88 with *S. coelicolor* A3 xylanaseA showed one consensus pattern of GH10 family. Hence, it was concluded that the type of xylanase present in *S. thermonitrificans* NTU-88 was an endoxylanase belonging to GH10. Enkhbaatar *et al*., 2016 determined the molecular masses of β-1, 4-endoxylanase from *Streptomyces coelicolor* A3(2) using micrOTOF-Q II and mass spectra obtained were in the range of in a 120–3000-m/z value. Molecular mass obtained from MS showed m/z values of 305.24 and 701.59 corresponding to xylobiose and xylopentose which indicated that a specific endoxylanase from *Streptomyces coelicolor* A3(2) cleaves the β-1,4 linkage of xylan to yield xylobiose

and xylopentose respectively. Similar results were reported by Grabski *et al*., 1993 for β-(1-4) endoxylanases from *Streptomyces roseiscleroticus* and Deesukon *et al.,* 2011 for XynSW2A and XynSW2B from *Streptomyces sp.* SWU10 using LC/IT/TOF MS. Kim *et al*., 2010 worked on the 3D structure of purified XylG from *Streptomyces thermocarboxydus* using the Modeler and Discovery Studio 2.0. Homology modeling was used to generate the tentative structure of the query protein by alignment with template sequences. A BLAST search of the PDB database using the XylG sequence exhibited the highest homologies with two endo-1, 4-xylanases (1E0V and 1V0K) from *Streptomyces lividans*. The homology model of XylG indicated that the enzyme may form a $(\beta/\alpha)_8$-barrel with active site loops arranged to form a deep cleft, which is similar to other GH10 endo-β-1,4-xylanases. The residues of Glu95, Trp136, Ser138, Gln139, Asn180, Glu181, His260, Glu289, Asp291, Arg293, Trp333, Trp341, and Val342 in the active site of XylG were predicted to be within the cleft.

Wang et al., 2015 performed multiple-sequence alignment of *Streptomyces sp.* xylanase XynAS9 and six GH10 xylanases using ClustalW to identify the key residues responsible for substrate binding and thermophilic properties. The 3D of XynAS9 and its five mutants were modeled using Discovery studio 2.5.5 software. Circular dichroism (CD) measurements were also done to determine the secondary structure of the protein. Based on the MSA results, a regular pattern was identified from consensus sequences. The template strains had proline at sites 81 and 185 and glutamic acid at sites 82 and 186 whereas in case of XynAS9 valine and aspartic acid and glycine and serine were present.

3.6. Role of extracellular enzymes in bioremediation of wastes

3.6.1 Collection of wastes samples from various sites

An important application of the work done during the investigation was to study the role of xylanase enzyme in biodegradation of waste samples. For this, agricultural wastes such as rice straw and wheat bran were collected from sites- Matloda, Hisar, Haryana and Local atta chakki, Govindpuri.

3.6.2 Pre-Treatment of waste samples for obtaining powdered substrates

Raw waste samples were converted into powdered substrates using combination of pre-treatment methods.

3.6.3 Compositional analysis of wastes

Estimation of the composition (cellulose, hemicellulose, holocellulose, ash and moisture content) in rice straw and wheat bran wastes was done for getting an insight for the efficient degradation of these wastes using the Technical Association of the Pulp and Paper Industry (TAPPI) protocol (Table 6).

Table 6: Composition of wastes determined by TAPPI protocol

Waste material used as substrate	Composition expected	Composition observed in raw wastes	Composition observed in pretreated powdered substrates
Wheat Bran	38.5% Cellulose, 34.4% Hemicellulose, 21.0% Lignin 6.1% Ash Total=100%	38.3% Cellulose, 34.1% Hemicellulose, 21.0% Lignin, 6.0% Ashes Total=99.4%	48.3% Cellulose, 46.3.1% Hemicellulose 1.25% Lignin, 0.2% Ashes Total= 96.05%
Rice straw	38.7% Cellulose, 36.5% Hemicellulose, 19.9% Lignin 4.9% Ash Total=100%	38.6% Cellulose, 36.3% Hemicellulose 19.6% Lignin 4.5% Ash Total=99%	42.8% Cellulose, 52.3% Hemicellulose 0.8% Lignin 0.5% Ash Total=96.4%

3.6.4 Primary screening of isolates for degradation of wastes

Screening of isolates for production of extracellular enzymes and degradation of wastes was done by spot inoculating colony 169 on media supplemented with 0.4% of powdered wastes. Results showed that colony 169 efficiently degraded wheat bran (6mm) and rice straw (10mm) (Figure 11). Comparison of primary screening results obtained for during degradation of commercial substrates and biodegradable wastes is shown in histogram 2.

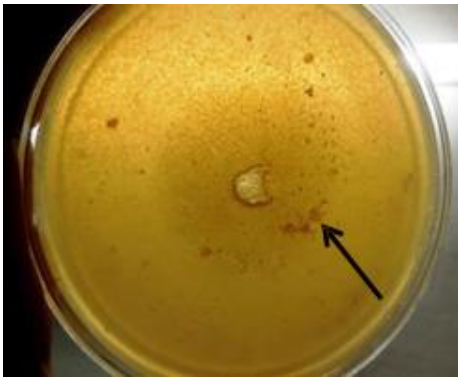
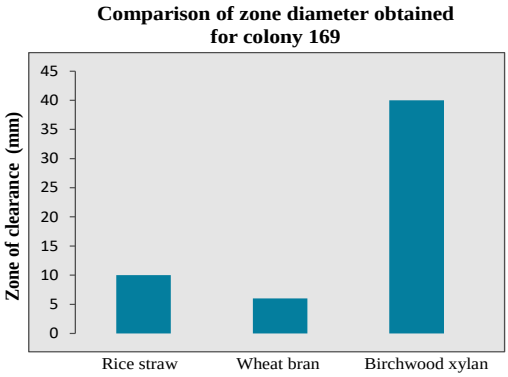


Figure 11: Primary screening results for Colony 169

Colony 169 (control) on MSA + wheat bran plate



Histogram 2: Comparison of primary screening results obtained for during degradation of commercial substrates and biodegradable wastes

3.6.5 Secondary screening or quantitative analyses

For quantitative analyses of enzymatic activity, the production supplemented with 0.4% wastes were inoculated with culture. Enzyme activity was measured under the solid state fermentation by using various methods such as (DNS) method Activity observed was 7.12IU/ml (with wheat bran) 10.11IU/ml (with rice straw).

These bacterial cultures can be converted into powdered form by lyophilization and can be packed in containers along with nutrient supplements. The sample can be dissolved in water to prepare a formulation and sprayed on to the waste materials for degradation. However, optimization of environmental parameters (pH, temperature and nutrients) is required to allow microbial growth and speed up the process of metabolism. Hence initially the ex-situ degradation of the waste samples can be done under controlled conditions.

As per literature, Nadia *et al*., 2010 tested xylanase activity of *Streptomyces lividans* by using corn cobs and wheat straw along with the commercial substrates. The waste samples were collected from Giza farm, Egypt, air dried at 70^0C and grinded to obtain fine powder. The production media containing wastes were inoculated with culture spores and incubated at 34^0C. Total enzyme activity and total protein content was measured by DNS method and Lowry's method respectively in crude cell free supernatant under two conditions: static and shaking. Activity and protein content recorded were 0.980mg/l protein and 4.65U/ml activity under static conditions and 1.040 mg/ml protein and 3.55U/ml activity under shaking conditions with corn cobs. Similarly with rice straw as substrate, 0.920mg/l protein and 7.85U/ml activity under static conditions and 0.980 mg/ml protein and 6.62U/ml activity under shaking conditions was observed. El-Gendy and Ahmed, 2014 collected waste samples like bagasse, rice straw, corncob, banana stalk, wheat straw, sorghum stalk, barely bran and maize stalk from local markets in Dokki, Giza, Egypt. The samples were cut, dried, grinded, sieved and evaluated as substrate support. Production media containing 1% of the individual wastes were inoculated with culture (10^6spores/ml) and incubated at 28^0Cfor 7 days. After fermentation, the culture broth was centrifuged to obtain the crude cell free supernatant. Xylanase activity was measured by DNS method on each day of fermentation period. Activity was found maximum on 5^{th} and 6^{th} day with range of 8.50U/ml to 47.30U/ml respectively.

4. Conclusions

In an attempt to discover new extracellular enzyme producers, more than 600 actinomycete isolates were isolated from varied ecological habitats. 105 random isolates were subjected to qualitative screening to select colonies having potential for production of cellulase, xylanase, chitinase and phosphatase enzymes which can be commercially important. During primary screening, 80% showed xylanase. Based on the results of primary screening, colonies which showed substantial xylanase activity and representing diverse ecological habitats were selected for quantitative screening under the submerged state fermentation. Protein content in all the crude samples was first measured and equalized followed by measurement of enzyme activity in each case. Initially all the enzyme producers were subjected to partial purification and enzyme activity in each was estimated. However, colony 169 was selected for further purification by ion exchange chromatography. Enzyme activity was found to increase from crude extract to partially purified and purified samples. SDS-PAGE profile showed 2 bands of sizes approximately 50-55kDa and 60-65kDa. The effect of fermentation conditions (pH,

temperature and substrate concentrations) on enzymatic activity of xylanase under submerged fermentation process in crude was determined and the data obtained were verified by ANOVA and Multiple Comparison test (Post-Hoc test). The Km and Vmax values of the purified fraction of colony 169 were 56.03mg/ml and 2.04µg/ml respectively.

Analyses of bands run on SDS-PAGE gel, which were responsible for enzymatic activity was done by molecular approach. The N-terminal amino acid sequence obtained after ESI-MS was 495aa. For studying the structure and catalytic site analysis for both the enzymes, the N-terminal peptide sequence for colony 169 by ESI-MS was matched against the NCBI database. Results showed that the target sequence of colony 169 showed 65% identity with chain A (PDB id: 1v0k) of Streptomyces lividans β 1-4 endoxylanase belonging to family 10. For homology modelling, the MODELLER software used the target sequences of colony 169 and superimposed them on the template structure of *Streptomyces lividans* (PDB Id1v0k) and *Streptomyces plicatus* (PDB id-1hp4) respectively. The obtained data was also compared against protein database (PDB) using DaliLiteV3.1 to obtain RMSD and Z scores. The final 3D model of

Xylanase and chitinase was verified using PROCHECK (Ramachandran plot) and Verify 3D. The 495 amino acid residues of colony 169 were folded into a domain (β-jellyroll) structure. The active site analysis showed that colony 169 and *S. lividans* shared Tryptophan (W) at position 185, Tyrosine (Y) at position 172 responsible for substrate binding. CD analysis was conducted to check the secondary-structure present in colony 169. The far-UV CD spectrum of both the colonies exhibited a pronounced maximum and minimum at 195nm and 222 nm, respectively, which are characteristics of β-sheet and α-helix structures in aqueous solution, respectively.

An important application of the work done during the investigation was to study the role of extracellular enzymes in biodegradation of waste samples such as agricultural wastes (wheat bran and rice straw). Biodegradable waste samples were collected from various sites. As the raw wastes cannot be used directly for biodegradation, the wastes were first pretreated using a multitude of different pretreatment methods. Estimation of the components such as cellulose, hemicellulose, holocellulose, ash and moisture in different wastes was then done for getting an insight for the efficient degradation of these wastes. The primary screening of isolates for production of extracellular enzymes and degradation of wastes was done by spot inoculating colony 169 on media supplemented with 0.4% of powdered wastes. Results showed that colony 169 efficiently degraded wheat bran (6mm) and rice straw (10mm).

During the secondary analyses, enzymatic activity observed in case of colony 169 was 7.12IU/ml (with wheat bran) and 10.11IU/ml (with rice straw). These bacterial cultures can be converted into powdered form by lyophilization and can be packed in containers along with nutrient supplements. The sample can be dissolved in water to prepare a formulation and sprayed on to the waste materials for degradation. However, optimization of environmental parameters (pH, temperature and nutrients) is required to allow microbial growth and speed up the process of metabolism. Hence initially the ex-situ degradation of the waste samples can be done under controlled conditions.

5. Acknowledgements

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