Evaluation of gut bacterial population from *Capra aegagrus hircus* and *Sus domesticus* for their production of holocellulose degrading enzymes and their applications

Dissertation submitted in fulfillment of the requirements for the degree of Master of Philosophy in Biotechnology

By

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CERTIFICATE

This is to certify that the dissertation entitled **"Evaluation of gut bacterial population from *Capra aegagrus hircus* and *Sus domesticus* for their production of holocellulose degrading enzymes and their applications"** submitted to the Mizoram University for the award of Master of Philosophy in Biotechnology by **Dhaneshwaree Asem** *Registration no. MZU/M.Phil./ 356 of 26.05.2017,* Research scholar in the Department of Biotechnology, is a record of research work carried out by her during the period from 2016 to 2017 of study, under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma or other similar titles in this University or any other University or institution of higher learning.

It is further certified that the scholar fulfilled all the requirements as laid down by the University for the purpose of submission of M.Phil. dissertation.

Date: **Dr. Bhim Pratap Singh**

Place: **(Signature of the supervisor)**

DECLARATION

I, **Dhaneshwaree Asem**, hereby declare that the dissertation entitled **"Evaluation of gut bacterial population from *Capra aegagrus hircus* and *Sus domesticus* for their production of holocellulose degrading enzymes and their applications"** submitted to Mizoram University for the award of degree of Master of Philosophy (M.Phil.) in Biotechnology is a bonafide record of work carried out by me during the period from 2016 to 2017 under the guidance of **Dr. Bhim Pratap Singh** (Supervisor), Department of Biotechnology and has not formed the basis for the award of any other degree, diploma or other similar titles in this University or other University or institution of higher learning.

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Dedicated to

my parents and my family

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Date: **Dhaneshwaree Asem**

COURSE WORK CERTIFICATE

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Master of Philosophy First Semester Award-Sheet

The following is the assessment record of ***Dhaneshwaree Asem,*** *Roll No.* ***BT/M. Phil/16/07,*** *Registration No.* ***7034 of 2014*** *in the M. Phil. First Semester Examination held in December,* ***2016***

SEMESTER EXAMINATION						Total Grade Point	Grade Point Average	Grade
LS-601 Research Methodology		LS-602 Instrumentation: Tools & Techniques		LS-603(D) Microbial Diversity and Systematics				
Grade Point	Grade	Grade Point	Grade	Grade Point	Grade			
7.2	'O'	6.9	'A'	8.5	'O'	22.6	7.53	'O'

First Tabulator | Second Tabulator | Controller of Examinations

Date: 27 MAR 2017

EVALUATION : *The following indicates the corresponding grades of grade points :*

'O'	'A'	'B'	'C'
7.00-10.00	6.00-6.99	5.00-5.99	Below 5.00

The performance of the scholar shall be evaluated in the following grades : ***'O'*** *– Outstanding,* ***'A'*** *and* ***'B'*** *with grade point valuation in the 10 point scale, i.e., 7.00-10.00 corresponding to* ***'O'*** *grade, 6.00-6.99 corresponding to* ***'A'*** *grade, 5.00-5.99 corresponding to* ***'B'*** *grade. Those securing less than 5.00 points shall be graded as* ***'C'****. A scholar will be eligible for the award of M. Phil Degree if he/she secures at least a 'B' grade in each course.*

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Chapter I

Introduction

Introduction

Owing to the concerns of depletion of non-renewable fossil fuels, universal call for cleaner environments and global warming, bio-energy has gained immense interest (Sun and Scharf, 2010; Lynd et al., 1991; Lynd et al., 2008). Plant biomass, which is mostly abundant in lignocellulosic content, does represent the most copious organic matter in nature, made of interlinked matrix of cellulose and hemicellulose along with glycosylated proteins and lignin polymer moieties (Wang et al., 2011). Lignocellulose biomasses, like agricultural and forest residues are considered as a potential feedstock for the generation of renewable bioenergy (Leo et al., 2016; Lopez-Linares et al., 2015). Utilizing lignocellulosic biomass can serve as an economical source of fermentable sugar for the production of second generation bioethanol production (Stephanopoulos, 2007; Warnecke et al., 2007). Significant challenges still exist for reducing the enzyme cost, optimizing biorefinary processes and developing simultaneous saccharification, although various strategies have been developed to improve the biomass conversion efficiency. Studies on natural biocatalyst systems will help us to resolve these challenges since many natural biocatalyst systems have evolved to convert lignocellulosic biomass with high efficiency (Mohanram et al., 2013; Sweeney and Xu 2012; Jonsson and Martin 2016). Larger animals generally lack the enzymes capable of degrading lignocellulosic biomasses, and hence rely on their gut microbial communities to provide such hydrolytic enzymes to digest the complex polymers (Dar et al., 2015). Gut residing microbes from the herbivorous animals will be of substantial usage, if their harvested, characterized and applied for their lignocelluloytic activities.

Chapter I: Introduction

Cellulose is the most common, abundant and renewable biopolymer on earth and considered as one of the most important sources of carbon on this planet but its domestic waste materials from agriculture representing about 1.5 x 10^{12} tons of the total annual biomass production (Klemm et al., 2002; Bhat, 2000; Nowak et al., 2005). Cellulose hydrolysis emerge as the subject of intense research and industrial interest as the value of cellulose as a renewable source of energy rises (Bhat et al., 2000). Cellulases produced by microorganisms have been classified into three major classes: cellobiohydrolase (EC 3.2.1.91); endo-glucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) (Hong et al., 2007). Cellulases possessed several biotechnological applications such as bioconversion of agricultural wastes, in textile industry, in paper industry and as an additives in laundry detergents (Howard et al., 2003). Furthermore, in animal feeds for improving the nutritional quality and digestibility, fruit juices processing, baking etc. (Tolan and Foody, 1999). Bacterial cellulases has several advantages over fungal originated ones regardless of being used commercially for the production of cellulase because bacteria have faster growth rate results in ease growing in high cell densities. Enzyme expression system in bacteria is more convenient and they can withstand conditions like extreme temperature and pH as well (Maki et al., 2009).

Xylan, a major component of hemicellulose found in plant cell walls and second most abundant renewable polysaccharide in nature after cellulose which accounts for approximately one third of the total plant biomass (Beg et al., 2001). The backbone of xylan composed of xylopyranosyl residues, linked through β-1,4-$_D$-glycosidic bonds, that can be substituted with acetyl groups or glucuronopyranosyl, 4-O-methyl-$_D$-glucuronopyranosyl, α-$_L$-arabinofuranosyl (Collins et al., 2005). Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) that has the

ability to hydrolyze the β-1,4-$_D$-glycosidic linkages of the xylan backbone (Biely, 1985). Complete hydrolysis of xylan incoporate an enzyme system consisting of endo-1,4-β-xylanases (EC 3.2.1.8), β-$_D$-xylosidases (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), and ferulic/coumaric acid esterases (EC 3.1.1.73) (Adav et al., 2010; del-Pulgar and Saadeddin, 2014). Biodegradation of xylan using xylanases (endo-1,4-β-$_D$-xylanohydrolase) [EC 3.2.1.8] produces xylooligosaccharides (Bakri et al., 2013) which can be used in cosmetics, biofuel, pharmaceuticals, as ingredients of functional food, etc. (Hauli et al., 2013; Sunna et al., 2000).

Xylanase have widespread applications in different industrial areas such as pulp bleaching, baking and brewing, animal feeding, waste-treating and bio-energy conversion (Li et al., 2009). Desirable property of xylanase as catalysts in pulp and paper industries is the tolerance to alkaline pH and high temperature (Techapun et al., 2003) but most of the xylanase known to date are optimally active at temperatures below 50 ºC and are mostly active in acidic or neutral pH (Ryan et al., 2003; Wang et al., 2003). Conversely, only a few xylanase are reported to be active and stable at alkaline pH and high temperature (Dhillon et al., 2000). Bacterial, fungal and actinobacterial xylanase have fascinated the research interest due to their potential applications in recovery of fermentable sugars from hemicellulose, bio-bleaching of pulp and paper industry and more (Wong et al., 1988; Kang et al., 1996). Several *Streptomyces* species were found to be proficient in degrading lignocellulosic biomass and were reported to produce xylanase (Jiang et al., 2011).

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The perennial grasses are considered as a rich source of lignocellulosic biomass, making it a second generation alternative energy source and can be diminish the use of fossil fuels. Traditional forage grasses, e.g., *Saccharum arundinaceum*, *Panicum antidotale*, *Thysanolaena latifolia*, and *Neyraudia reynaudiana*, are among the candidates having the potential to produce energy. Moreover, all of them were abundantly available and can be harvested minimum three times in a year, which makes them a suitable plant for the production of energy (Bor, 1960; Nair and Sekharan, 2009). The cellulases and xylanases are mainly used in textile industry, in pulp and paper, baking along with animal feed industries respectively. Eventually, microorganisms that produce hydrolytic enzymes such as cellulases and xylanases are probable candidates for making second-generation bio-fuel thereby using low cost abundantly available substrates, such as perennial grasses (Leo et al., 2016).

Intestines, or more commonly known as gut, have been a major reservoir for diverse mutualistic microorganisms associated with animals (Savage, 1977). Gut microorganisms benefit the host as the energy accumulates from the fermentation of undigested carbohydrates of plant origins (Cumming and MacFarlane, 1997; Wynne et al., 2004). Recent researches have showed that the gut microbiomes composed of four dominant phyla: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Khanna and Tosh 2014). Among these, most prominent genera include *Bacteroides, Clostridium, Facecailibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus* and *Bifidobacterium*, and to lesser extent *Escherichia* and *Lactobacilllus* (Vendatam and Hecht, 2003). Superior biological efficiency has been asserted for the goat (Fitzhugh. 1981). Lately, isolation and characterization of many rumen microbes has been done by sequence analysis of 16S rRNA (Faridha et al., 2013). The enzymes obtained from gut residing

bacteria are considered as potential candidate for the breakdown of cellulose (Oyeleke and Okusanmi, 2008). Numerous studies reported the occurrence of several cellulolytic and xylanolytic bacterial genera from compost (Archana and Satyanarayana, 1997), swine waste (Liang et al., 2009) and hot springs (Mawadza et al., 2000).

Gut bacteria has been cited to show antagonistic activity against pathogenic bacteria and fungi (Chellaram et al., 2012). The antimicrobial activity of the cow's intestinal Lactic acid bacteria (LAB) was determined against *Escherichia coli* and *Klebsiella* spp. and were able to inhibit the growth of the test organisms with the largest zone of inhibition (Adeniyi et al., 2015).

In Mizoram, majorly swine's were fed with a special cellulose rich diet (chayote, *Sechium edule*), and effect of feeding chayote (*Sechium edule*) meal on growth and nutrient utilization in indigenous pig (Zovawk) was reported (Lalthansanga and Samanta, 2015). However, isolation of bacterial population having potential of holocellulolytic from gut of indigenous goat and swine has not yet been reported. Thus, the present study aimed to isolate the gut residing bacterial strains from the indigenous goat and swine having cellulolytic and hemicellulolytic potential. The potential bacterial isolates were targeted to identify the ability to degrade the biomass by the production of hydrolytic enzymes for the generation of second-generation biofuels. Meanwhile it is intended to examine the antimicrobial biosynthetic potential against various Gram positive and Gram negative bacterial pathogens and to detect the genes (Halo, CYP, NRPS and *phz*E) responsible for their secondary metabolites production.

To understand the diversity of holocellulolytic gut bacteria and search for noble biomass degradation agents for sustainable energy, following objectives were kept for this study.

- Isolation and identification of holocellulose degrading bacteria from gut extract of Black Bengal (*Capra aegagrus hircus*; domesticated goat) and Zovawk (*Sus domesticus*), an indigenous pig of Mizoram.
- Optimization and quantification of cellulase and xylanase production from selected isolates of holocellulose degrading bacteria.
- Bioconversion potential of locally available biomass by selected isolates.
- Determination of antimicrobial activity and detection of biosynthetic genes in selected strains.

Chapter II

Review of Literature

2.1. Lignocellulose: Structure and composition

Lignocellulose is the major component of biomass, representing the most abundant renewable organic resource in soil comprising around half of the plant matter produced by photosynthesis (Isikgor and Becer, 2015). It is composed of three types of polymers: cellulose, hemicellulose and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages (Fig. 1) (Pérez et al., 2002).

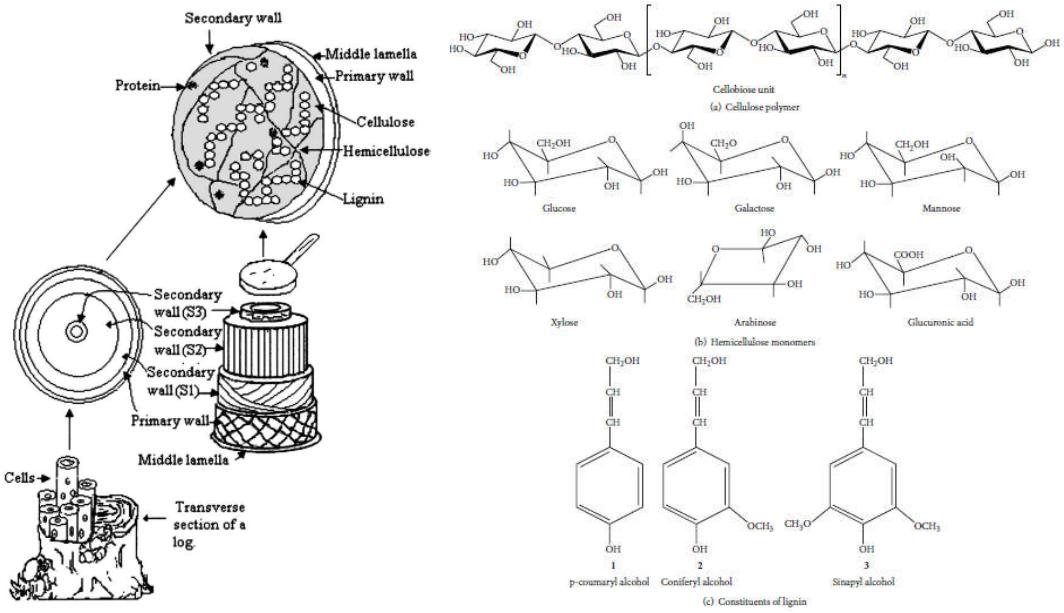

Fig. 1 Composition and chemical structure of lignocellulosic residues (Adapted from Saini et al., 2015; Sánchez, 2009)

Cellulose, a linear polymer that composed of $_{D}$-glucose subunits linked by β-1,4-glycosidic bonds formed the dimer cellobiose thereafter, these form long chains linked together by hydrogen bonds and van der Waals forces. Cellulose is usually present as a crystalline form and amorphous cellulose and later is more susceptible to enzymatic degradation (Pérez et al., 2002).

Biodegradation of lignocellulosic residues

Only a scarce amount of cellulose, hemicellulose and lignin produces as by-products in agriculture or forestry is been used, rest being considered as waste. Many microorganisms have vast potential of degrading and utilizing cellulose and hemicellulose as carbon and energy sources. Moreover, lignin, the most recalcitrant component of plant cell walls were degraded to CO_2 by a much smaller group of filamentous fungi particularly white-rot fungi, has been reported. Lignocellulosic residues are predominantly degraded by actinobacteria among bacteria (Saini et al., 2015). The natural process of degradation by the help of microorganisms requires the production of different enzyme systems.

Cellulases

Cellulolytic enzymes are a group of glycosyl hydrolases classified into different families based on their sequence homologies. They are generally divided into exoglucanases (EC 3.2.1.74), endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (del-Pulgar and Saadeddin, 2014; Sadhu and Maiti, 2013). The mechanisms of action and substrate specificities varies but exoglucanases act on reducing or non-reducing ends of cellulose chains releasing glucose units, whereas endoglucanases hydrolyse β-1,4-glycosidic bonds randomly inside the cellulose chains releasing dextrans of variable lengths (Kuhad et al., 2011). At non-reducing ends, cellobiohydrolases cleave glycosidic bonds and release cellobiose units. Complete hydrolysis of cellulose involves synergistic effect of all the enzymes (Lynd et al., 2002). Cellulosomes are characteristics of anaerobic bacteria, consisting of multi enzyme complex protuberances from cell surface stabilized by dockerin and adhesion proteins. In aerobic bacteria, cellulases are non-complexed or free and are secreted extracellularly using specific

secretion pathways including most of the actinomycetes. Among Cellulase producing actinobacteria, *Cellulomonas fimi*, *Microbispora bispora*, and *Thermobifida fusca* have been deliberated extensively (Lynd et al., 2002; Wilson, 1992). *Cellulomonas fimi* is a facμltative anaerobe; it produces free cellulases (Christopherson et al., 2013). Similarly, facultatively anaerobic *Cellulomonas flavigena* also secrets free cellulases (Christopherson et al., 2013). Both carry out efficient hydrolysis of celluloses and hemicelluloses. The cellulase enzyme systems in *Cellulomonas fimi* also consist of six cellulases: three endocellulases, two exo-cellulases and a processive endo-celullases (Christopherson et al., 2013; Lynd et al., 2002). These cellulases are primarily secreted by sec dependent pathway and, therefore, do not require intracellular folding or cofactors for their activity.

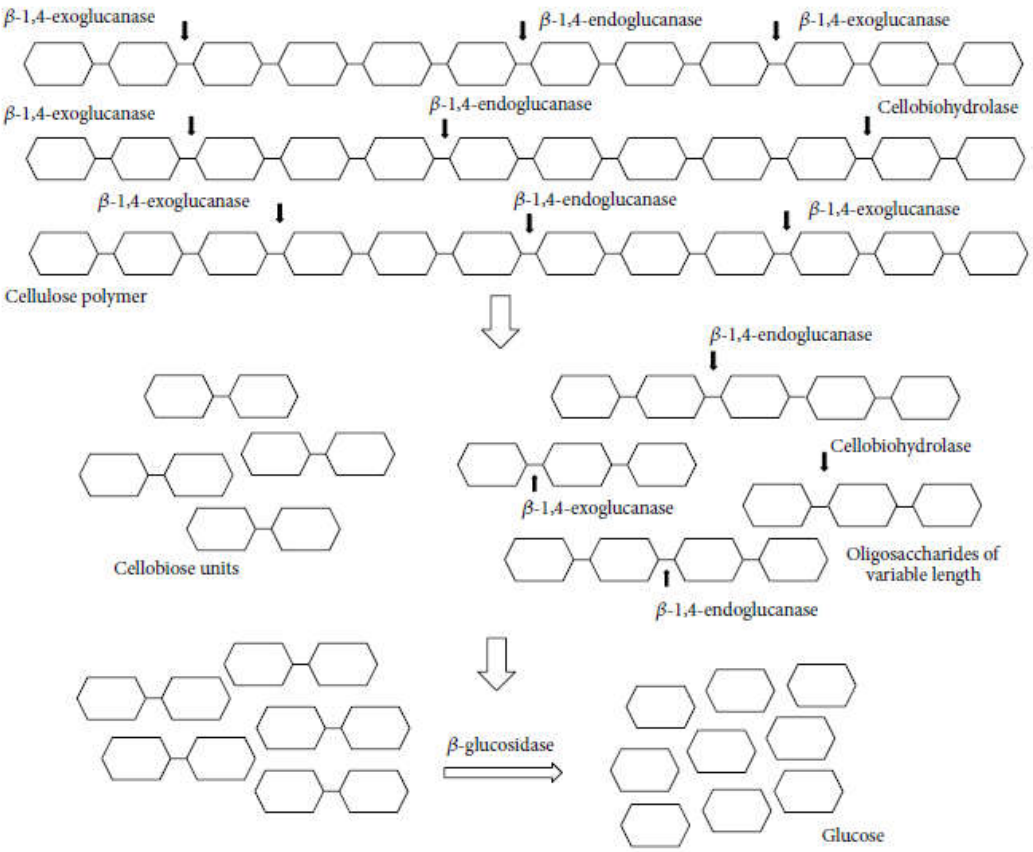

Fig. 2 Scheme of cellulose hydrolysis (Adapted from Saini et al., 2015).

In *C. fimi* ATCC 484 and *C. flavigena* ATCC 482, another enzyme cellobiose phosphorylase has also been discovered (Christopherson et al., 2013). *Microbispora bispora* also shows synthesis of six different cellulases, showing exo-exo and endo-exo synergism (Wilson, 1992). Genomic studies of *Streptomyces* sp. SirexAA-E, isolated from pineboring woodwasp *Sirex noctilio*, have also shown genes for endocellulase (Takasuka et al., 2013). The expression of cellulolytic genes in *T. bifida* is induced by cellobiose, whereas easily utilizable sugar glucose shows catabolite repression as in most of the other cellulolytic microbes (Lin and Wilson, 1987).

The extracellular cellulases are secreted by actinomycetes using either one or both of the common bacterial systems for secretion of extracellµlar proteins, that is, *sec* general secretion system and *sec* independent twin-arginine translocation (TAT) systems. The general *Sec*retion route catalyses transmembrane translocation of proteins in their unfolded conformation, whereas TAT system translocates secretory proteins in their native folded state. In *T. bifida* both of these systems were discovered, whereas *S. coelicolor* mainly utilizes TAT systems for protein export (Lykidis et al., 2007).

Hemicellulases

Hemicellulases are generally synthesized along with cellulases (del-Pulgar and Saadeddin, 2014; Lynd et al., 2002). Xylan and mannan are most abundant components of hemicellulose. Complete hydrolysis of xylan involves an enzyme system consisting of endo-1,4-β-xylanases (EC 3.2.1.8), β-$_D$-xylosidases (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), and ferulic/coumaric acid esterases (EC 3.1.1.73). Mannan is hydrolysed primarily by synergistic action of mannanases (EC 3.2.1.78), β-mannosidases (EC 3.2.1.25), and α-galactosidases (EC 3.2.1.22) (Adav et al., 2010; del-Pulgar and Saadeddin, 2014). Mannanases hydrolyze β-1,4-glycosidic bonds internally, β-mannosidase cleave β-1,4 linked mannose from nonreducing ends, and α-galactosidase removes terminal $_D$-galactosyl residues linked by α-1,6 linkages (Gilbert, 2010). Degradation of mannan and xylan also enhances cellulose hydrolysis as they are known to inhibit cellulase activities (del-Pulgar and Saadeddin, 2014). Most of the hemicellulases belong to glycosyl hydrolases families; however, some enzymes involved in hemicellulose hydrolysis belong to glycosyl transferases (EC 2.4.1.x) (Lynd et al., 2002). Studies

have indicated production of several xylanases by *T. bifida* and other actinobacteria. *T. bifida* has been found to have potential to produce β-1,4-endoxylanases, xylosidases, α-L-arabinofuranosidases, xyloglucanases, β-1,3-glucanases and α-N-arabinofuranosidases (del-Pulgar and Saadeddin, 2014; Lykidis et al., 2007). *Cellulomonas fimi* synthesises extracellµlar endo- as well as exo-Xylanases: xylan binding domain, β-mannanase, mannosidase, and xyloglucan specific β-1,4-glucanase (Christopherson et al., 2013; del-Pulgar and Saadeddin, 2014). *Cellulomonas flavigena* ATCC 482 are known to synthesize an unusual mixture of 19 endoxylanases, along with β-xylosidase, α-arabinofuranosidase, α-glucuronidase and β-glucanase (Christopherson et al., 2013). *Streptomyces flavogriseus* has shown production of β-1,4-glucan glucanohydrolase (Ishaque and Kluepfel, 1980). Xylanase genes have also been found in *Streptomyces* sp. SirexAA-E (Takasuka et al., 2013).

Lignolytic Enzymes

Lignin degradation is mediated by a complex of enzymes containing three principal enzymes laccases (EC 1.10.3.2), manganese peroxidases (MnP, EC 1.11.1.13), and lignin peroxidases (LiP, EC 1.11.1.14) (Mason et al., 2001; Plácido and Capareda, 2015). Laccases are the oxidoreductases which degrade polyphenol, the principal recalcitrant component in the lignocellulose (Abdel-Hamid et al., 2013; Madhavi and Lele, 2009). They are extracellular inducible enzymes which employ simple oxygen as an oxidizing agent as well as cofactor. Low substrate specificity of laccases enables them to degrade wide variety of compounds. Manganese and lignin peroxidases are together known as heme peroxidases containing protoporphyrin IX as a prosthetic group. Lignin peroxidases can specifically degrade high redox potential compounds and are known to oxidize phenolic as well as nonphenolic aromatic rings, which make up around

90% of the lignin polymer. They require H_2O_2 for their activity. Veratryl alcohol is an attractive substrate for LiP, which oxidises other substrates by acting as the redox mediator for indirect oxidation. Manganese peroxidases are low redox potential heme peroxidases requiring H_2O_2 for their activity. They can be manganese dependent or versatile peroxidases (Fisher and Fong, 2014; Plácido and Capareda, 2015).

Xylanases

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyze the endohydrolysis of 1,4-β-$_D$-xylosidic linkages in xylan (Collins et al., 2005), leading to the formation of a sugar hemiacetal and the corresponding free a glycone (nonsugar compound remaining after replacement of the glycoside by a hydrogen atom)(Esteban et al., 1982). Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes endo-1,4-β-$_D$-xylanases (EC 3.2.1.8) randomly cleave the xylan backbone, β-$_D$-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose while removal of the side groups is catalysed by α-L-arabinofuranosidases (EC 3.2.1.55), α-$_D$-glucuronidases (EC3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC3.1.1.73) and p-coumaric acid esterases (EC 3.1.1). Indeed, complete xylanolytic enzyme systems, including all of these activities, have been found to be quite widespread among fungi (Benacic et al., 1995; Sunna et al., 1997), actinomycetes (Elegir et al., 1994) and bacteria (Sunna et al., 1997) and some of the most important xylanolytic enzyme producers include Aspergilli, Trichodermi, Streptomycetes, Phanerochaetes, Chytridiomycetes, Ruminococci, Fibrobacteres, Clostridia and Bacilli. The ecological niches of these microorganisms are diverse and widespread and typically include environments where

plant material accumulate and deteriorate, as well as in the rumen of ruminants. Xylanases have been classified in at least three ways: based on the molecular weight and isoelectric point (pI), the crystal structure and kinetic properties or the substrate specificity and product profile.

Xylanases have been reported from *Bacillus*, *Streptomyces* and other bacterial genera that do not have any role related to plant pathogenicity (Esteban et al., 1982). The optimum pH of bacterial xylanases are, in general, slightly higher than the optimal pH of fungal xylanases (Katapodis et al., 2003), which is a suitable characteristic in most industrial applications, especially the paper and pulp industries. Noteworthy producers of high levels of xylanase activity at an alkaline pH and high temperature are *Bacillus* spp. (Grepinet et al., 1988). When considering only temperature, a handful of xylanases that show optimum activity at higher temperatures have been reported from various microorganisms. These include *Geobacillus thermoleovorans*, *Streptomyces sp.* S27, *Bacillus firmus*, *Actinomadura* sp. strain Cpt20 and *Saccharopolyspora pathunthaniensis* S582, all of which produce xylanases that show activity between 65 and 90 °C (Li et al., 2000). One xylanase, reported from *Thermotoga sp.* (Bray et al., 1992) has been shown to exhibit a temperature optima between 100 and 105 °C.

In addition to human gut isolates, NRPS family were found in isolates from the chicken gut (*Bacteroides barnesiae* DSM 18169) and bovine rumen (*Methanobrevibacter ruminantium* M1) (Donia et al., 2015). The CYP gene, which encodes cytochrome P450 hydroxylase, in polyene antibiotics biosynthesis could be detected in several strains of Janibacter, Salinibacterium and Arthrobacter genera without antimicrobial activity (Lee et al., 2006). Increased expression of cytochrome P450 genes show its role in the detoxification of monoterpenes released by this insect's host trees (Cano-Ramírez et al., 2013). Halogenases (Halo) have plays a significant role in biosynthesis and introducing the bioactivity of many

halogenated secondary metabolites (Gao and Huang, 2009). Phenazines were found to produced by diverse group of bacteria including actinobacteria which represent a large group of nitrogen containing heterocyclic compounds. Streptomyces were found to be potential for phenazine production (Karuppiah et al., 2015).

Chapter III

Materials and methods

3.1. Sample collection

Gastro-intestinal (GI) fluid was collected from Zovawk (*Sus domesticus*), an indigenous pig of Mizoram and Black Bengal (*Capra aegagrus hircus*; domesticated goat) from slaughterhouse in Vaivakawn, Mizoram, India (23° 43' 27" N and 92° 43' 2" E). As described and recommended, both the organisms were fed with chayote (*Sechium edule*) meal of G4 treatments for a month (Lalthansanga and Samanta, 2015). Total GI contents (500 ml) of the representative samples were collected immediately after slaughter. The collected GI was rinsed thoroughly by subsequent washing with sterile water and dissected using sterile surgical scissor and the content was transferred to a sterilized vessel. Using two layers of muslin cloth, the GI fluids and GI solids were separated (Dhakal et al., 2014). In a sterile vessel, the total fluids were placed and brought in an icebox to the laboratory and processed immediately for isolation for bacteria.

3.2. Enumeration of total culturable bacterial population

Isolation of bacterial culture was done by serial dilution method and spread plate techniques by using Tryptone Soy Agar (TSA); Actinomycete Isolation Agar (AIA); Starch Casein Agar (SCA) and modified BPS-CX media (additional 1% Lignin) agar. Briefly, 1 ml of GI fluid was diluted with 90 ml double distilled water and mixed in an oscillation water bath at room temperature, for 30 min followed by gradient dilution (10^{-2} to 10^{-6}). The culture media was supplemented with 80 µg/ml of filter sterilized actidione (cycloheximide) to inhibit the growth of fungi. Plates were incubated at 37ºC for 24h for eubacteria growth and 10 to 15 days for actinobacteria and the appearance of colonies was observed periodically. The pure cultures were obtained after successive sub-culturing on respective media by streaking and were preserved on

slants at 4ºC. For long term storage the slants were stored in 20% glycerol at -80^{0}C for subsequent studies.

3.3. Identification and phylogenetic affiliation

Genomic DNA was extracted using Pure-link Bacterial DNA Purification Kit (Invitrogen, USA) according to the manufacturer's protocol. Amplification of 16S rRNA gene was carried out using bacteria-specific universal primers [PA (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer PH (5'-AAG GAG GTG ATC CAG CCG CA-3')] (Qin et al., 2009). The PCR reaction was carried out using Veriti thermal cycler (Applied Biosystems, Singapore) in a total volume of 25 µl consisting of 1.0 µl genomic DNA (50 ng), 0.5 µl of each primer (10 pmol), 2.0 µl of deoxynucleotide triphosphates (2.5 mM each), 2.5 µl of 1X PCR buffer, 1.0 µl of Taq DNA polymerase (1 U/µl) and 17 µl MilliQ grade water. The thermocycling steps used were as follows: initial denaturation at 94 ºC for 5 min, followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 57 ºC for 1 min and extension at 72 ºC for 1.2 min with a final extension at 72 ºC for 10 min. The amplified PCR products were checked by electrophoresis through 1.2% agarose gels made in TAE buffer. The PCR bands were visualized under gel documentation system (Bio-rad Gel DocXR+, USA) and were purified using Purlink PCR Purification Kit (Invitrogen, USA), and sequencing was done commercially at Sci-Genom Pvt. Ltd., Cochin, India.

DNA Sequence data were compared with those in the GenBank and EMBL databases using BlastN and BlastX search programs, and the sequences were aligned using EMBL ProtPro (Thompson et al., 1997). Based on lowest BIC ("Bayesian Information Criterion") and highest AIC ("Akaike Information Criterion") values, evolutionary models were selected using MEGA

6.0 version. Analysis of the 16S rRNA gene sequences used the model K2+I (R = 1.24) for construction of neighbor-joining tree. Phylogenetic trees were constructed based on the neighbor-joining method using the Kimura 2 parameter models with MEGA 5.05 (Tamura et al., 2011; Saitou and Nei 1987). The robustness of the phylogenetic trees was evaluated by bootstrap analysis with 1000 resamplings using p-distance model (Felsenstein, 1985). Trees were viewed and edited by using program FigTree 1.3.1 (2012).

3.4. Screening for cellulolytic and xylanolytic activity of bacteria isolates

Pure cultures of bacterial colonies were re-streaked individually on CMC induction agar plates (K_2HPO_4–0.5%, NaCl-0.5%, Yeast extract- 0.25%, Peptone-0.5%, Agar Agar -1.5% and CMC-1%) and xylan induction agar plates (K_2HPO_4, 0.5%; NaCl, 0.5%; yeast extract, 0.25%; peptone, 0.5%; agar, 1.5%; MilliQ H_2O, 75%; WSOSX-1 (Water Soluble Xylan from Oats for Screening Xylanase), 25%) and incubated at 30 °C for 3-5 days. On attaining sufficient growth, the cellulase and xylanase-induced bacterial isolates were streaked in screening agar plates supplemented with 0.5%(w/v) CMC and 25% (v/v) Oat Spelt Xylan for cellulase and xylanase screening respectively at pH 7 for a period of 3 to 5 days. Using Congo red assay, all the bacterial isolates were screened for their cellulase and xylanases production (Teather and Wood, 1982). The plates were incubated at 30 °C for 3-5 days (Leo et al., 2016) and were flooded with Congo red (0.5%) for 5 min followed by destaining (1M NaCl) for 15 min. Zone of clearance and the diameter of clear zone (Halos) formed around the isolates was observed and is indicative of the magnitude of the cellulase and xylanase production (Teather and Wood, 1982). Hydrolysis capacity was calculated as the ratio of the diameter of zone of clearance divided by the diameter of the colony based on the efficiency of the halo zone (Hendricks et al., 1995). For further

quantitative estimation of xylanase and cellulase production, all cultures showing hydrolysis capacity ≤ 1cm was selected.

3.5. Quantification and optimization of enzyme production conditions

The potential isolates showing hydrolytic zones in screening plate assays were subjected to SmF in BPS-CX induction broth at 150 rpm (Leo et al., 2016). The effect of incubation time on cellulase and xylanase production was determined by growing the selected bacterial isolates for 6 days (0, 12, 24, 48, 72, 96, 120 and 144 h) at 30˚C and pH 7. Based on the obtained optimal incubation range , the effect of pH (5, 5.5, 6, 7, 8 and 9) and temperature (25ºC, 30ºC, 37ºC , 45˚C, 50ºC, 55ºC and 60ºC) were optimized for the maximum xylanase and cellulase production.

3.6. Cellulase and xylanase enzyme assays

Based on enzyme optimization and quantification, FPase, CMCase and xylanase assays were undertaken for those isolates that showed maximum cellulase and xylanase production respectively. Cellulase quantification assays: the filter paper activity (FPA) and CMCase assay were performed according to (Ghose, 1987) with certain amendments from (Camassola and Dillon, 2012). The xylanase assay was performed according to (Bailey et al., 1992) with 1% xylan (w/v) supplemented as substrate. All the enzymes activities were represented in U/ml and One unit (1U) of enzyme activity was defined as the amount of enzyme that released 1µmol of reducing sugar in a minute reaction. All samples were analyzed in triplicate and mean values were taken.

3.7. Bioconversion

Based on cellulase and xylanase enzyme assay, the best two strains were selected and grown in 100 ml of BPS-CX broth at 30^0C, 140 rpm for 3-4days. For bioconversion studies, *Thysanolaena latifolia* (BPS-G104), an abundantly available common perennial grass, was selected and processed (Leo et al., 2016). The raw biomass (BPS-G104) was grinded to a particle size of 0.5 to 5.00mm and made into slurry with 20:1 ratio (biomass: sterile water) and further treated with 1% NaOH for 12h at 55˚C to obtain a pulpy crude biomass (BPS-G104a) (Leo et al., 2016; Xu et al., 2010). Later using Muslin Cloth, the remaining supernatant that might possess hydrolysis inhibitor elements was also removed and homogenized in BPS-YM media (1% peptone and 0.5% yeast extract in phosphate buffer pH 7). Determination of the lignocellulosic components for the raw and the treated samples was done according to the methodology of the National Renewable Energy Laboratory (NREL–TP–510–42618) to verify the effectiveness of alkaline treatment. Using *T. latifolia* pulp (BPS-G104a) as the major carbon substrate alone for strain DBT87 (BPS-M2), Submerged Fermentation (SmF) was carried out and similar treatments were done for strain DBT4 (BPS-D2). The SmF media contained 500ml of BPS-YM media along with 20% biomass for BPS-M2 and BPS-D2. At optimized temperature (55˚C) and pH (pH 8) with continuous agitation of 150 rpm, SmF was carried out for a period of 6 days with aliquots sampled on alternate days for enzyme assays. Sterile nylon cloth was used inorder to filtered out the supernatant from the biomass pulp which was later dried at 55˚C for 48 h. Later, the supernatant BPS-E4, BPS-E87 was used as crude enzyme solution, obtained from BPS-D2 and BPS-M2 treatments respectively. The Relative Dry Weight (RDW) was calculated with the alkali pre-treated (BPS-G104a) serving as the control (Leo et al., 2016; Haruta et al., 2002).

3.8. Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR spectra of pretreated and treated biomass (BPS-M2 and BPS-D2) were obtained at room temperature, using a JASCOFT/IR-6800 Spectrometer (Jasco, Japan) equipped with an Attenuated Total Reflectance unit. Spectral data between 650 and 4000 cm^{-1} were collected averaging 64 scans at a resolution of $4cm^{-1}$. Know It All ID Expert (Biorad,USA) was was used for Principal components analysis (PCA).

3.9. Morphology of Biomass after Decomposition Using Scanning Electron Microscopy (SEM)

Using scanning electron microscopy (SEM), cell surface morphology of the pretreated and treated biomass (BPS-M2 and BPS-D2) was observed as done earlier (Leo et al., 2016).

3.10. Kinetics study

The most effective crude extract from biomass utilization study (DBT4) was evaluated against varying substrate concentration of filter paper (0.1% - 3%) and Birchwood xylan (10mg-100mg) for FPase and Xylanase assays for its cellulase and xylanase activity. The K_m and V_{max} was calculated from the Michaelis-menten plot using Graph Pad Prism 5 software.

3.11. Screening for Antimicrobial Activity

Antimicrobial screening was carried out against *Staphylococcus aureus* (MTCC-96), *Bacillus subtilis* (MTCC-2097), *Micrococcus luteus* (MTCC-5262), *Pseudomona aeruginosa* (MTCC-2453), *Escherichia coli* (MTCC-739) and *Candida albicans* (MTCC-3017), all obtained

from Microbial Type Culture Collection (MTCC), Chandigarh, India. All the isolates were inoculated in nutrient broth and incubated at 28 ^{0}C, 150 rpm for 7-10 days. The grown cultures were allowed for cells harvesting by centrifugation at 8000 rpm for 3 min and the supernatant was collected into a fresh tube and used for antimicrobial activity by the agar well diffusion method (Saadoun and Muhana, 2008). The test pathogenic microbes were spread on nutrient agar plate and using sterile cork borer, wells of 6 mm diameter were prepared. In each of the plates, 50 µl of clear supernatant of bacterial cultures were dispensed into each wells and the plates were incubated at 28 ± 2◦C for 24 h. All experiments were analyzed in triplicate. A clear zone of inhibition around each well indicates the antimicrobial activities of the isolates.

3.12. Amplification of Biosynthetic Gene Fragments (NRPS, *phz*E , CYP and Halo)

The potential antimicrobial isolates were used for the amplification of biosynthetic genes for non-ribosomal peptide synthetase (NRPS), aminodeoxyisochorismate synthase (*phz*E), CYP (cytochrome hydroxylase) and Halo (halogenase). Non-ribosomal peptide synthetase (NRPS) gene fragments were amplified using degenerate primers: A3F 5′-GCSTACSYSATSTACACSTCSGG-3′ and A7R 5′-SASGTCVCCSGTSGCGTAS-3′ (Ayuso-Sacido and Genilloud, 2005): *phz*E (phzEf, phzEr) 5’-GAAGGCGCCAACTTCGTYATCAA-3’ 5’-GCCYTCGATGAAGTACTCGGTGTG-3’ (Schneemann et al., 2011): CYP (PEH-1, PEH-2) 5′-TGGATCGGCGACGACCGSVYCGT-3′ 5′-CCGWASAGSAYSCCGTCGTACTT-3′ (Lee et al, 2006) Halo (FW, RV) 5′-TTCCCSCGSTACCASATCGGSGAG-3′ 5′-GSGGGATSWMCCAGWACCASCC-3′ (Hornung et al., 2007). The action was carried out in the Veriti thermal cycler (Applied Biosystems, Singapore) with some modifications from previously described. The components and reaction conditions of the PCR mixture are as

follows:

NRPS (50 µL): 3 µL template, 5 µL 10× buffer, 1 µL $MgCl_2$ (25 mM), 1 µL DMSO (10%), 5 µL dNTP (2.5 mM), 2 µL each primer (10 mM) and 5 U *Taq* DNA polymerase; 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 2 min at 59°C and 4 min. at 72°C, followed by 10 min extension at 72°C.

***phz*E** (25 µL): 2 µL template, 2.5 µL 10× buffer, 1.5 µL $MgCl_2$ (25 mM), 2 µL BSA (1 mg/mL), 2 µL dNTP (2.5 mM), 0.8 µL each primer (10 mM) and 2 U Taq DNA polymerase; 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, followed by a 8 min extension at 72°C.

CYP (25 µL): 2 µL template, 2.5 µL 10× buffer, 1.5 µL $MgCl_2$ (25 mM), 2 µL BSA (1 mg/mL), 2 µL dNTP (2.5 mM), 0.8 µL each primer (10 mM) and 2 U Taq DNA polymerase; 5 min at 95 °C, followed by 40 cycles of 1 min at 95 °C, 40 s at 58 °C and 1min at 70 °C, followed by a 5min extension at 70°C.

Halo (50 µL): template 2 µL, 5 mL 10× buffer, 1 µL $MgCl_2$ (25 mM), BSA (1 mg/mL) 2.5 µL, dNTP (2.5 mM) , 2 µL each primer (25 µM) and 2 U Taq DNA polymerase; 5 min at 94 °C, followed by 35 cycles of 1 min at 94°C, 1.5 min at 55 °C and 1 min at 72 °C, followed by a 8 min extension at 72 °C.

A negative control reaction mixture without DNA template of gut bateria was also included with each set of PCR reactions. The PCR products were visualized under gel documentation system.

Chapter IV

Results

4.1. Isolation and identification of bacterial population

From the gastro-intestine (GI) content of ruminant Zovawk (*Sus domesticus*) and Black Bengal (*Capra aegagrus hircus*), eighty one bacterial morphotypes were isolated and were molecularly identified based on 16S rRNA gene amplification. All the sequences were deposited in NCBI Genbank. Out of 81 isolates, 29 (35.80%) were isolated from pig GI extract and 52 isolates (64.19%) were recovered from goat GI content. Actinomycetes isolation agar (AIA) was the most suitable media among the nutritional media used, followed by starch casein agar (SCA), Luria Bertani (LB) media and tryptone soya agar (TSA) which results in the recovery of 29, 25, 19 and 8 isolates respectively (Table 1).

Table 1 Frequency of the bacterial isolates obtained from the gastro-intestinal extract of the tested animals using various growth media

Organisms	**Media**			
	AIA	SCA	LB	TSA
Black Bengal (*Capra aegagrus hircus*),	19	16	13	4
Zovawk (*Sus domesticus*)	10	9	6	4

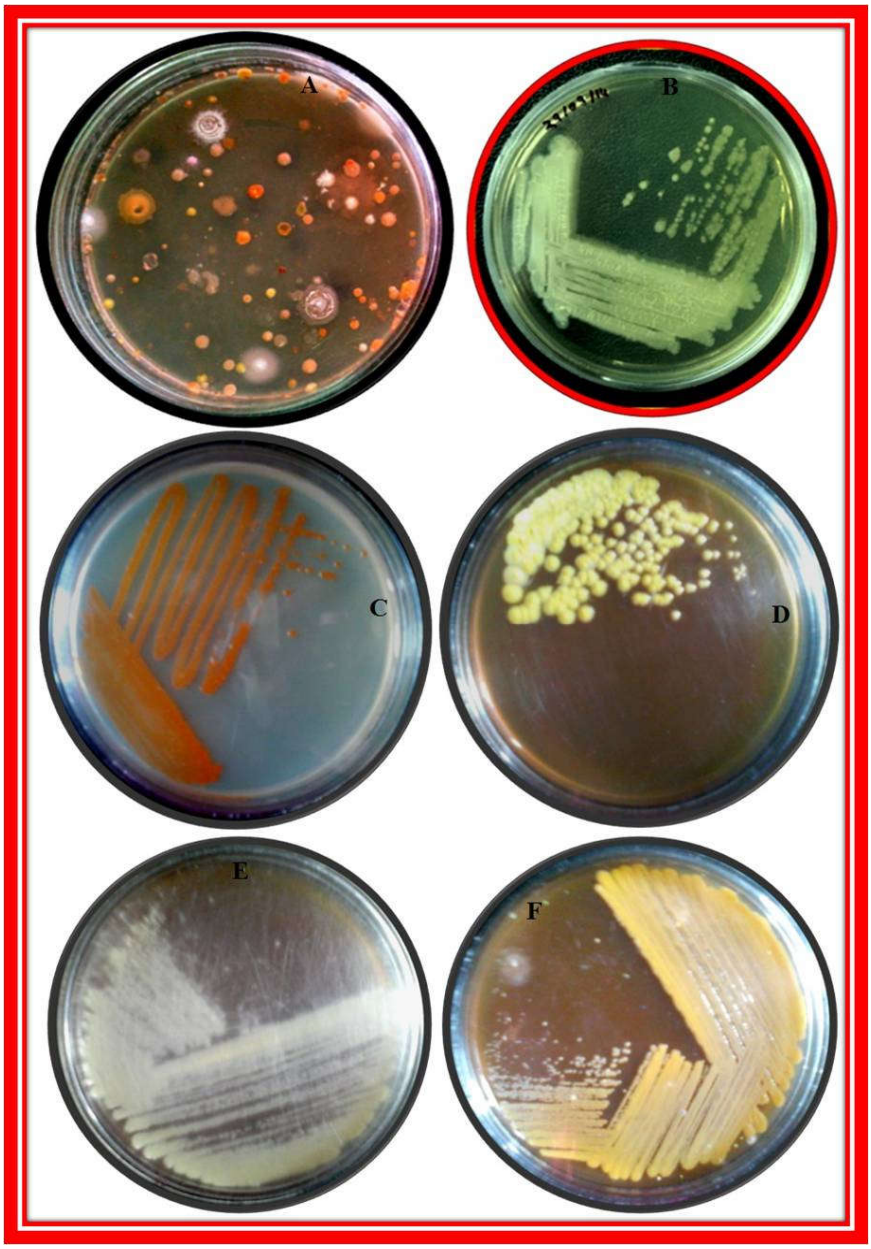

Fig 3 Morphological characteristics of bacterial isolates isolated from goat and swine (A: mixed culture and B, C, D, E and F: Pure bacterial strain)

4.2. Phylogenetic affiliation of the obtained isolates

The 16S rRNA sequence of all the isolates were identified and was sequenced commercially at Sci-genome labs Pvt. limited, Cochin, India. The desired amplicon size of about 1500 bp was obtained and the sequences were aligned by BLAST analysis along with the type strains downloaded from EzTaxon-database. The results revealed that all the isolates were grouped into 12 families belongs to *Bacillaceae* (n=21; 25.9%) followed by *Paenibacillaceae* (n=20; 24.6%), *Alcaligenaceae* (n=14; 17.2%), *Enterobacteriaceae* (n=6; 7.4%), *Streptomycetaceae* (n=5; 6.1%), *Micrococcaceae* (n=4; 4.9%), *Burkholderiaceae* (n=3; 3.7%), *Pseudomonadaceae* (n=3; 3.7%), *Micromonosporaceae* (n=2; 2.4%), *Sphingomonadaceae* (n=1; 1.2%), *Mycobacteriaceae* (n=1; 1.2%) *and Nocardiopsaceae* (n=1; 1.2%). The 16SrRNA gene sequence analysis by BlastN with 97–100% similarity results in 25 isolates grouped into gram-negative and 56 isolates into gram positive bacteria. The phylogenetic tree was constructed based on Neighbor-joining method with Kimura 2-parameter model (R=1.24) according to lowest BIC (5976.12) and highest AIC (4435.59) values using Mega 6.06 **(**Fig 1). Gaps were treated by pair wise deletion and the estimated Transition/Transversion bias (R) is 1.30. All the gut intestine bacterial strains were clustered into three clades (Clade I, Clade II and Clade III). In clade I, all the gram negative bacterial genus *Alcaligenes, Burkholderia, Pseudomonas, Achromobacter, Enterobacter*, *Serratia* and *Sphingomona* are closely clustered with their type strains under high bootstrap value of 77%. Similarly, in clade II, all the actinobacterial genus *Streptomyces, Microbacterium, Micromonospora, Micrococcus, Nocardiopsis, Arthrobacter* and *Pseudoarthrobacter* are closely

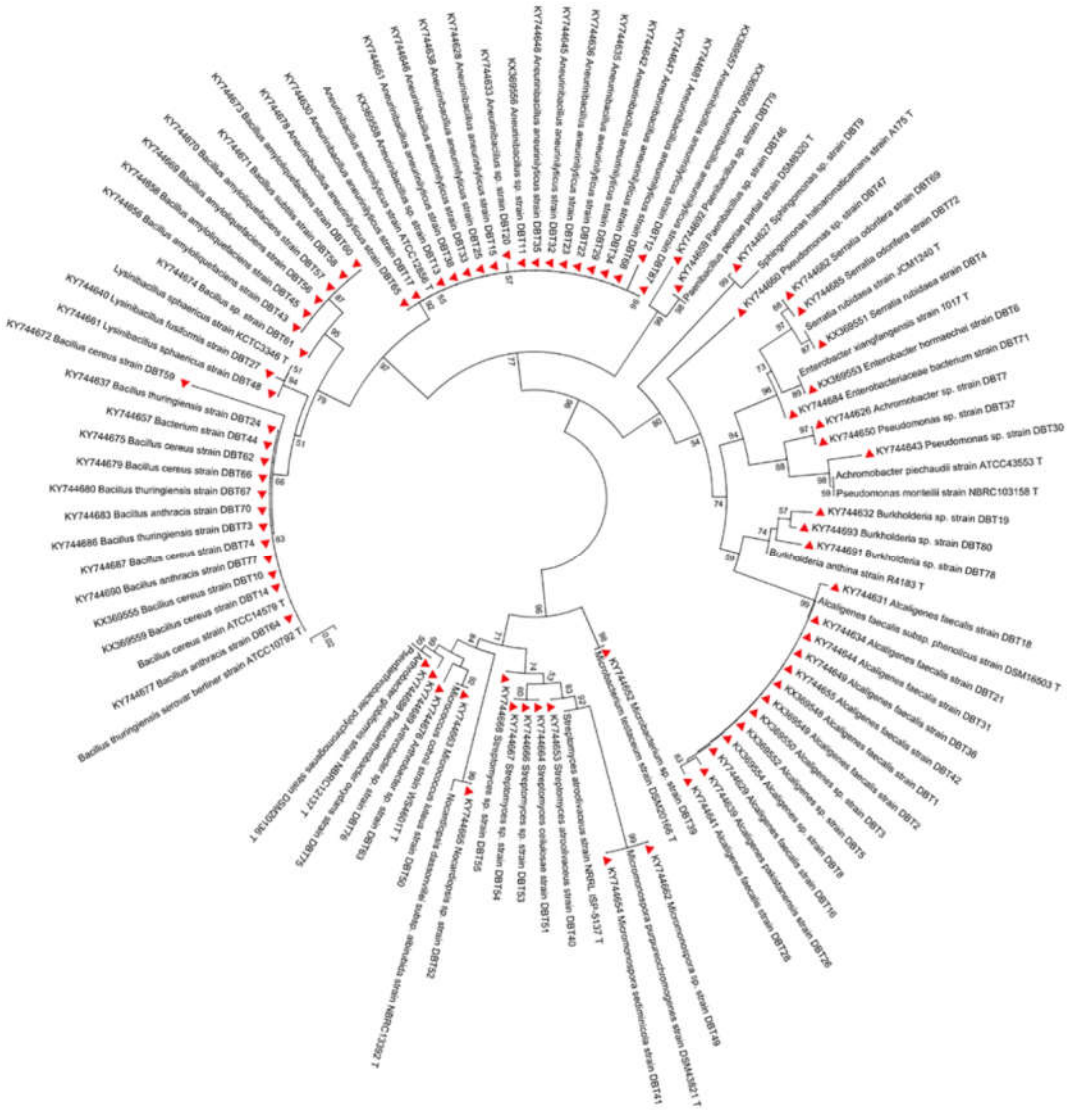

Fig 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene of the bacterial population associated with gastro intestine extract of swine and goat. Numbers at branches indicate bootstrap values of neighbour-joining analysis (>50%) from 1,000 replicates.

clustered with their type strains retrieved from EzTaxon database under high bootstrap value of 97%. In clade III contains 39 isolates which belongs to *Paenibacillus sp., Aneurinibacillus aneurinilyticus, Aneurinibacillus sp., Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus sp., Lysinibacillus fusiformis, Lysinibacillus sphaericus, Bacillus cereus, Bacillus thuringiensis, Bacillus anthracis* and *Bacterium* under bootstrap supported value 100%. Isolates *Aneurinibacillus aneurinilyticus* strain DBT87 and *Bacillus cereus* strain DBT10 was closely clustered with their type strain *Aneurinibacillus aneurinilyticus* ATCC12856^T and *Bacillus cereus* ATCC14579^T under bootstrap supported value of 99% and 78% respectively. In this present study, *Proteobacteria* was found dominant class (n=69; 85.18%) followed by *Actinobacteria* (n=12; 14.8%).

4.3. Screening for Cellulase and Xylanase production

Out of 81 isolates, 5 isolates were positive for cellulase production while 13 isolates showed promising hydrolysis zone for xylanase by using Congo red assay (Table 2).

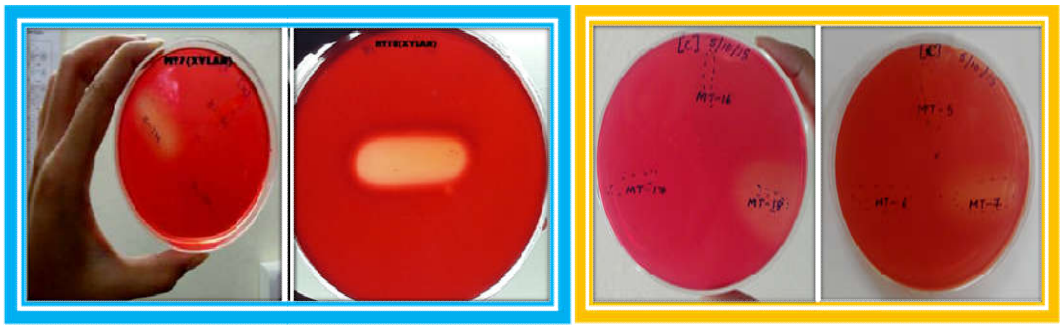

Fig 5. Screening of xylanase and cellulase for bacterial isolates from gastro intestine extract of goat and swine.

Table 2. Production of cellulase and xylanase using Congo red assay by selected bacterial isolates

Isolate	[A] Size of the colony (cm)	[B] Destained Zone (cm)	[B/A] Hydrolysis capacity
	Cellulase		
DBT4 (*S. rubidaea*)	1.5	1.2	0.8
DBT87 (*A. aneurinilyticus*)	2.0	0.2	0.1
DBT9 (*Sphingomonas* sp.)	1.7	0.7	0.4
DBT15 (*A. aneurinilyticus*)	1.9	0.2	0.1
DBT14 (*B. cereus*)	1.6	0.4	0.8
	Xylanase		
DBT1 (*A. faecalis*)	2.5	1.0	0.4
DBT2 (*A. faecalis*)	2.0	1.0	0.5
DBT3 (*Alcaligenes* sp.)	2.0	0.5	0.2
DBT10 (*B. cereus*)	1.5	1.2	0.8
DBT5 (*Alcaligenes* sp.)	2.0	1.0	0.5
DBT6 (*E. hormaechei*)	1.6	0.4	0.2
DBT11 (*Aneurinibacillus* sp.)	1.5	0.3	0.2

DBT8 (*Alcaligenes* sp.)	2.0	1.0	0.5
DBT12 (*A. aneurinilyticus*)	2.4	1.2	0.5
DBT13 (*Aneurinibacillus* sp.)	2.9	0.6	0.2
DBT7 (*Achromobacter* sp.)	2.4	1.2	0.5
DBT4 (*S. rubidaea*)	1.0	2.0	2.0
DBT87 (*A. aneurinilyticus*)	1.0	1.0	1.0

Among 13 xylanase positive bacteria, 9 and 4 isolates were isolated from goat and swine GI tract extracts respectively. Similarly, among the cellulase positive isolates, 3 and 2 isolates were from swine and goat GI extract respectively. Amongst all positive strains for cellulase and xylanase production, four strains (DBT4, DBT87, DBT14 and DBT10) were selected for the consequent studies based on their positive activities of cellulase and xylanase. Among them, only 2 isolates *Serratia rubidaea* strain DBT4 and *Aneurinibacillus aneurinilyticus* strain DBT87 showed both cellulase and xylanase activity. The selection of these strains was also based on highest enzyme production in submerged state fermentation, which is been discussed in later section.

4.4. Phylogenetic affiliation of hollocellulolytic bacterial strains

The 16S rRNA gene sequence analysis showed that the strains DBT4, DBT87 DBT14 and DBT10 were related phylogenetically to the genus *Serratia, Aneurinibacillus* and *Bacillus* respectively. The isolates were designated as *Serratia rubidaea* DBT4, *Aneurinibacillus*

aneurinilyticus DBT87, *Bacillus cereus* DBT14 and *Bacillus cereus* DBT10 based on their sequence similarity with the type strains in NCBI gene bank. To the finest of our information and literature search, this is the first report of two genera *Micrococcus luteus* strain DBT50 and *Pseudoarthrobacter oxydans* strain DBT75 isolated from gut intestine of Goat.

4.5. Quantification and optimization of enzyme production conditions

The four strains (*S. rubidaea* DBT4, *A. aneurinilyticus* DBT87, *Aneurinibacillus* sp. DBT14 and *Bacillus cereus* DBT10) based on their *in vitro* enzymatic assays were selected for further characterization of their enzyme production capabilities using submerged fermentation (SmF) with different substrates.

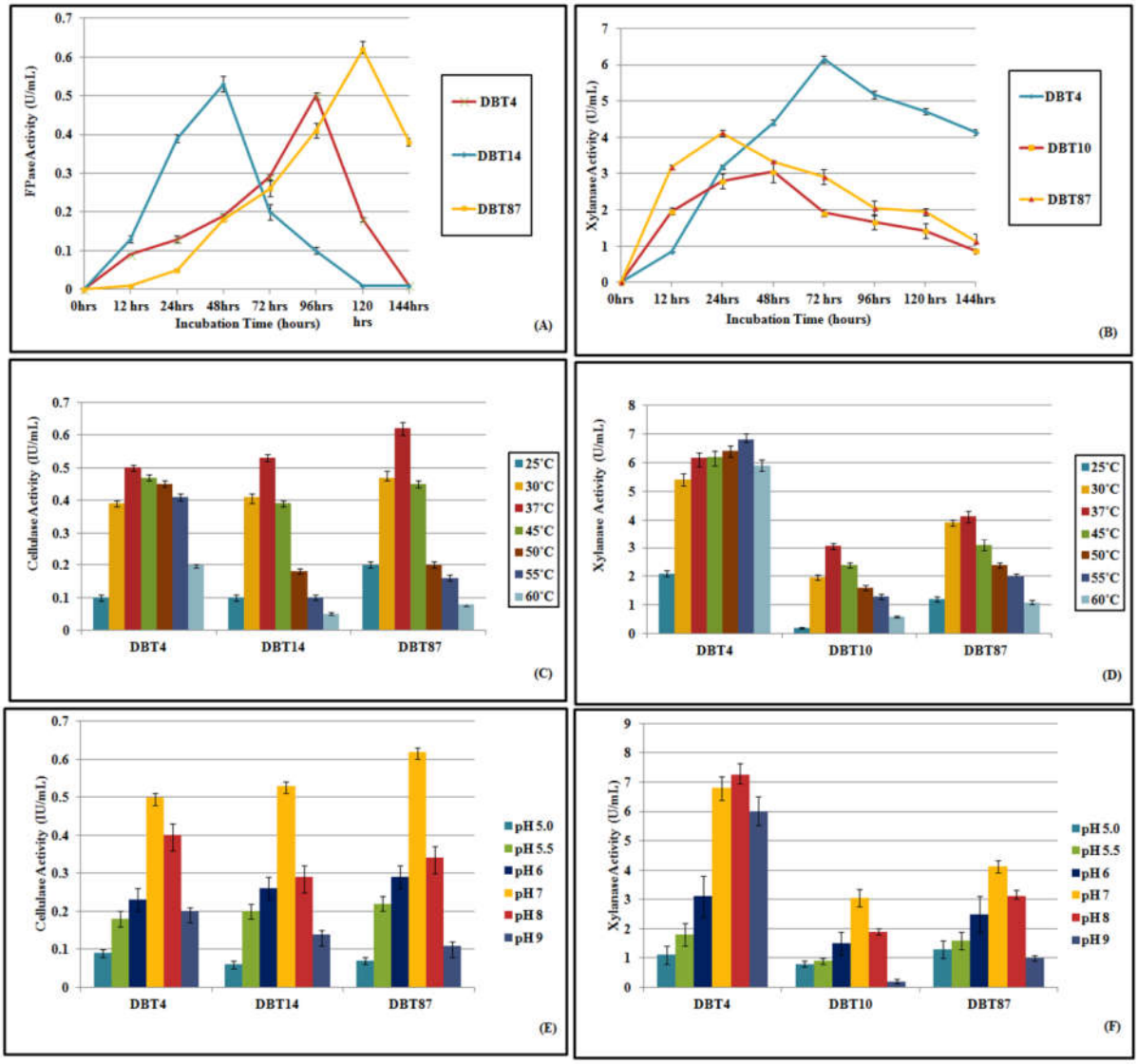

Fig 6. Effect of incubation time, temperature and pH on FPase activity (A, C, E) and on xylanase activity (B, D, F) activity by using three selected bacterial isolates.

4.6. Effect of incubation time on cellulase and xylanase production

The SmF study at varying incubation time was carried out at ambient temperature and neutral pH to evaluate the growth parameters of the selected bacterial strains and concurrently their optimal day of enzyme productions was investigated. Among the cellulase producers, *A. aneurinilyticus* DBT87 exhibited maximum FPase activity of 0.62 U/ml at 120 h, followed by *Aneurinibacillus* sp. DBT14 and *S. rubidaea* DBT4 having optimal FPase activity of 0.53 and 0.5 U/ml at 48 and 96 h respectively (Fig 6A). The xylanase assay showed that *S. rubidaea* DBT4 exhibit maximum xylanase activity of 6.15 U/ml at 72 h, followed by *A. aneurinilyticus* DBT87 (4.12 U/ml at 96 h) and *Bacillus cereus* DBT10 (3.05 U/ml at 48 h) (Fig 6B). Meanwhile, the two bacterial strains (*S. rubidaea* DBT4 and *A. aneurinilyticus* DBT87) showed both, cellulase and xylanase activities where the xylanase was produced earlier (72 and 48 h) followed by cellulase (120 and 96 h) implying that they might be an potential microbes for biomass utilization as the xylanase expression initially will let on the hydrolyzing of hemicelluloses linkages which in turn help in freeing up the cellulosic entities, which later could be acted upon by the cellulases.

4.7. Effect of temperature on cellulase and xylanase production

The temperature profile was established at their optimal incubation time and at neutral pH. The results revealed that for the three cellulase producing strains, their optimal temperature for enzyme production was at ambient 37˚C itself. Interesting, *S. rubidaea* DBT4 had FPase activity of 0.41 U/ml observed at 55˚C (Fig 6C). Similar results were observed in two xylanase producing

strains (*A. aneurinilyticus* DBT87 and *Bacillus cereus* DBT10); while *S. rubidaea* DBT4 showed an enhanced xylanase activity of 6.8 U/ml at 55˚C compared to 37˚C, which further lowered down at 60˚C (Fig 6D). This results suggested that *S. rubidaea* DBT4 might be a thermo-tolerant bacterial strain, with both xylanase and cellulase producing capabilities. There are very few report found in Uniport database for xylanase like β-xylosidase (http://www.uniprot.org/uniprot/A0A126VGJ6), Endo-1, 4-beta-xylanaseZ (http://www.uniprot.org/uniprot/A0A126VIN5) as well as cellulase gene 6-phospho-beta-glucosidase (http://www.uniprot.org/uniprot/A0A126VIF7) in strain S. *rubidaea*.

4.8. Effect of pH on cellulase and xylanase production

Finally, the initial pH was varied under optimized incubation time of growth and temperature for SmF conditions. The best pH for growth of two cellulase and two xylanase producers remained still at pH 7. While, *S. rubidaea* DBT4, the xylanase production was further optimized at pH 8 (7.25 U/ml) and FPase activity of 0.4 U/ml (Fig 6E and Fig 6F) was obtained at this alkaline condition. This result is the indicative that the strain *S. rubidaea* DBT4 showed the capability of producing enzymes with substantial activity at an alkaline pH 8.

4.9. Biomass compositional analysis.

Given the pulpy nature of this grass variety, utilizing of this grass as a biomass was designed in such a way that the pulpy nature could be retained. In-order to generate a homogenized pulpy biomass the alkali treatment was done and the effect was confirmed using compositional analysis. The results showed an increased in the cellulose content, while the hemicelluloses and lignin contents decreased in the alkali pretreated biomass as compared to raw biomass. Such increase in cellulosic content on alkali treatment (from 48.74% to 55.85%) might be due to the decrease in the hemicellulosic entities (17.74% to 12.22%) on treatment (Table 3).

Table 3. Compositional analysis of BPS-G104 (raw *T. latifolia*) and BPS-G104a (alkaline pretreated pulpy *T. latifolia*)

Sample name	Cellulose (%)*	Hemicellulose (%)*	Lignin (%)*	Moisture content (%)*	Extractives (%)*	Ash (%)*	Reference
BPS-G-104	48.74 ± 0.65	17.74 ± 0.71	8.7 ± 0.62	8.12 ± 0.12	16.26 ± 0.34	0.44 ± 0.01	Leo et al., 2016
BPS-G-104a	55.85 ± 0.13	13.22 ± 0.32	6.92 ± 0.81	5.47 ± 0.23	12.3 ± 0.34	0.25 ± 0.53	This study

***Each value represents three replicates, Mean (±) SD**

With such decreased lignin content and majorly cellulosic contents, this biomass in its pulpy form will be an ideal non edible biomass feedstock.

4.10. Biomass utilization potential

Both the potent strains, *A. aneurinilyticus* DBT87 and *S. rubidaea* DBT4, having both cellulase and xylanase activity were selected to evaluate their biomass utilization potential using a perennial grass, *T. latifolia* (BPS-G104a). Treatment BPS-D2 was the most efficient with maximum xylanase (11.98U/ml) and cellulase (0.5U/ml) production on 3rd and 4th day respectively. It showed consistent xylanase production at 55ºC and pH 8 which ranges from 8.13 to 11.98 U/ml from 2nd - 5th day of incubation. This infers to the fact that *S. rubidaea* DBT4 is capable of utilizing the hemicelluloses. In all the treatments, a 40 to 60% loss in dry weight was observed, with a maximum of 60% using BPS-D2 followed by BPS-M2 compared to the alkali pretreated biomass that served as the control Meanwhile, a slight increase in FPase (0.4 to 0.5U/ml) activity was also observed in BPS-D2 which further indicates the bioconversion potential of the strain In treatment M2, cellulase production was more prominent with an optimal FPase production of 0.70U/ml on 5th day, even though the xylanase production was not prominent as BPS-D2 (Fig 7A). With the steady capability of xylanase and cellulase production at thermophilic and alkaline conditions, *S. rubidaea* DBT4 will be an exploitable source for the bioconversion of pulpy biomasses like BPS-G104 with less lignin content. Loss of 40 to 60% in dry weight was observed in all the treatments, with the maximum of 60% using BPS-D2 followed by BPS-M2 in comparison to the control which is alkali pretreated biomass (Fig 7B).

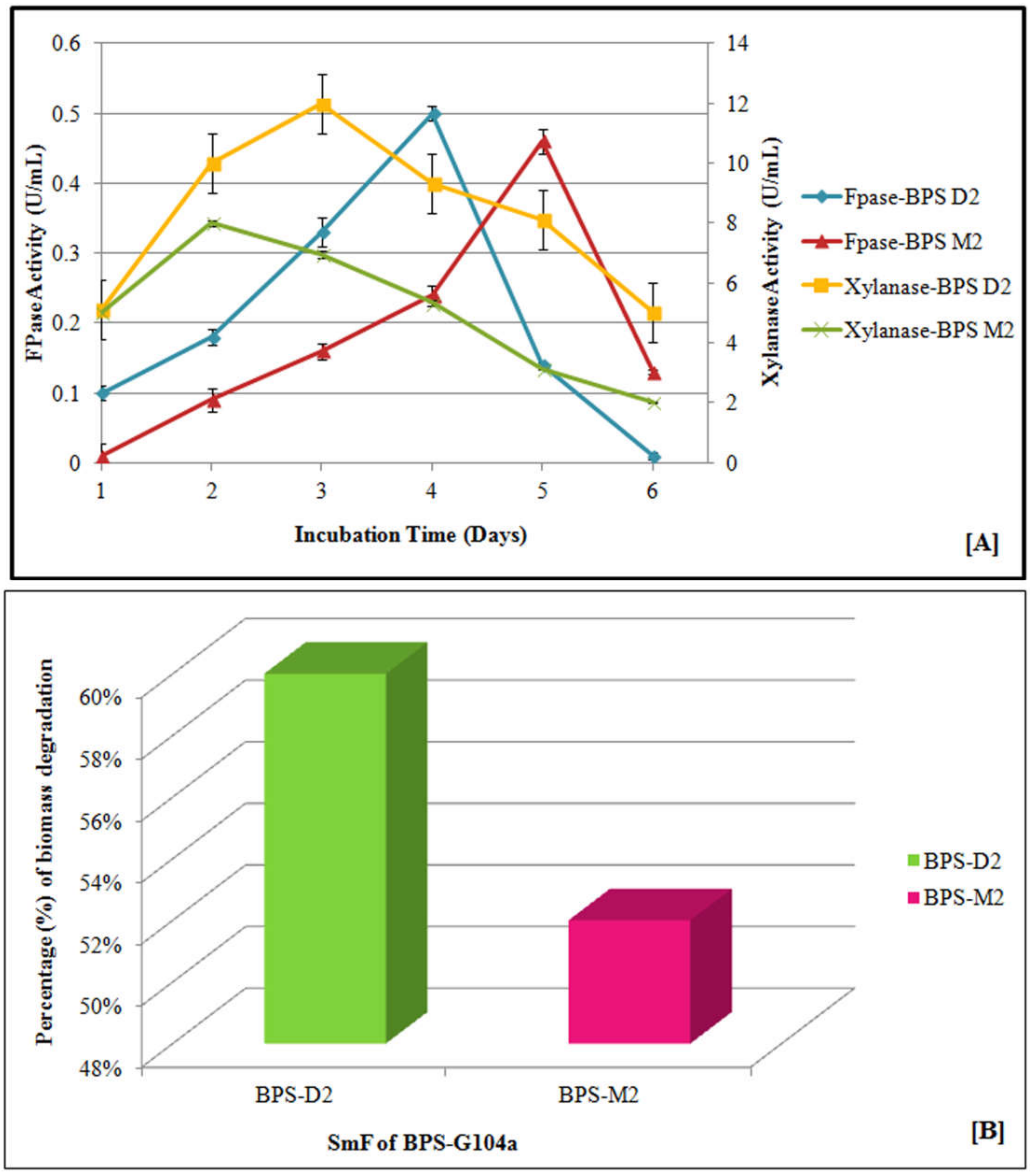

Fig 7. (A) Effect of incubation time on FPase and xylanase activity of treatment BPS-D2 and BPS-M2 under submerged fermentation (SmF) at optimized temperature and pH. (B) Percentage of biomass degradation by using different treatments.

4.11. FTIR analysis

In order to decipher the structural alterations that might have occurred during the two most efficient treatments (BPS-D2 and BPS-M2) with respect to the alkali pretreated biomass, they were analyzed using FTIR spectral readings ranging from 700 to 3000 cm^{-1}. Relatively significant variation in the peak location corresponding to 1053 cm^{-1} indicating C-O stretching for crystalline cellulose or hemicelluloses was observed both BPS-D2 and BPS-M2 treatments. At 1370 cm^{-1} which is the characteristic of crystalline cellulose symmetric C-H deformation, BPS-D2 treatment showed more prominent reduction in peak in comparison with BPS-M2 treatment. Similarly it was observed at 1240 cm^{-1} that indicates the C-O stretching for xylan. Characteristic assignment of hemicelluloses (xylan) denoted at 1735/1730 cm^{-1} (unconjugated C=O stretching) with considerable variation in peak reduction in BPS-D2 treatment (63%) as compared to BPS-M2 (76%) (Fig 8). This showed that the xylan has been utilized efficiently in BPS-D2 treatment which is line with our biomass utilization assay results. The C-H stretching of lignocelluloses components detected at 2890/2923 cm^{-1} also varied between BPS-D2 and BPS-M2 treatments where BPS-D2 being more effective. Thus the FTIR analysis aimed at finding the impact of BPS-D2 and BPS-M2 treatments on the chemical structure build up of holocellulosic components revealed that the BPS-D2 treatment was more successful in reducing the biomass macromolecules. In the current study, FTIR readings were found slight variation at 1053, 1240, 1735/1730 cm^{-1}. The chemical components that increase the biomass are tremendously important in determining their viability as a potent substrate for biofuel production.

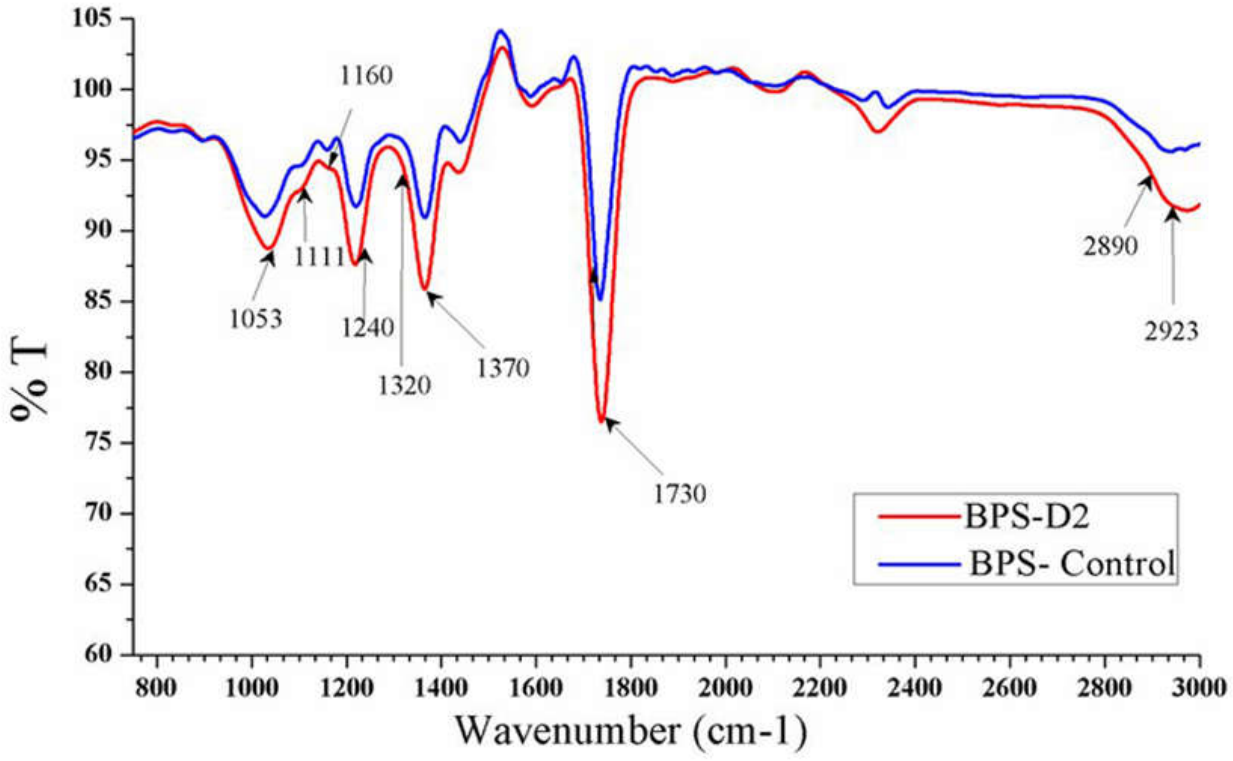

Fig 8. FTIR analysis of the most potential treatment (BPS-D2) as compare to the pre-treated control(BPS-Control).

4.12. Scanning Electron Microscopy (SEM) Analysis

For topology analysis of the two best treatments, SEM analysis were chosen using alkali pre-treated biomass as control after 6 days of SmF. Both treatments showed disintegration and breakdown of the fibrous hollo-cellulosic components and also gaps and sutures on the biomass layer (Fig 9). The SEM analysis confirmed its effect on biomass morphology and the extent of enzyme action on the biomass.

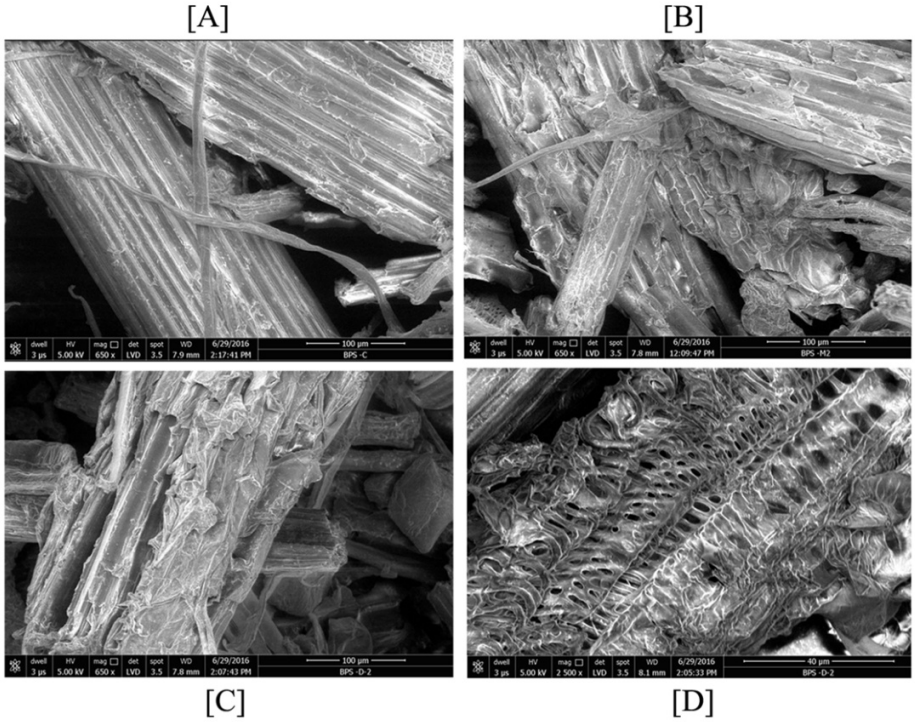

Fig 9. Surface destruction images of T. latifolia (BPS-G104) using SEM. (A) pre-treated biomass as control (BPS-C); (B) biomass treated with *Aneurinibacillus aneurinilyticus* strain DBT87 (BPS-M2); (C) and (D) biomass treated with *Serratia rubidaea* strain DBT4 (BPS-D2).

4.13. Kinetic study on the BPS-DBT4 xylanase enzyme

The kinetics capablity of the optimised crude xylanase obtained from the SmF treatment on BPS-G104 by *S. rubidaea* DBT4 was verified under varying substrate (Beechwood Xylan) concentration (0.1, 0.2, 0.5, 1, 2 and 3%) in xylanase DNS assay. The kinetic study on *S. rubidaea* DBT4 xylanase revealed that the V_{max} as high as 12.56 U/ml and a stable K_m for the enzyme at 0.58 (Fig 10).

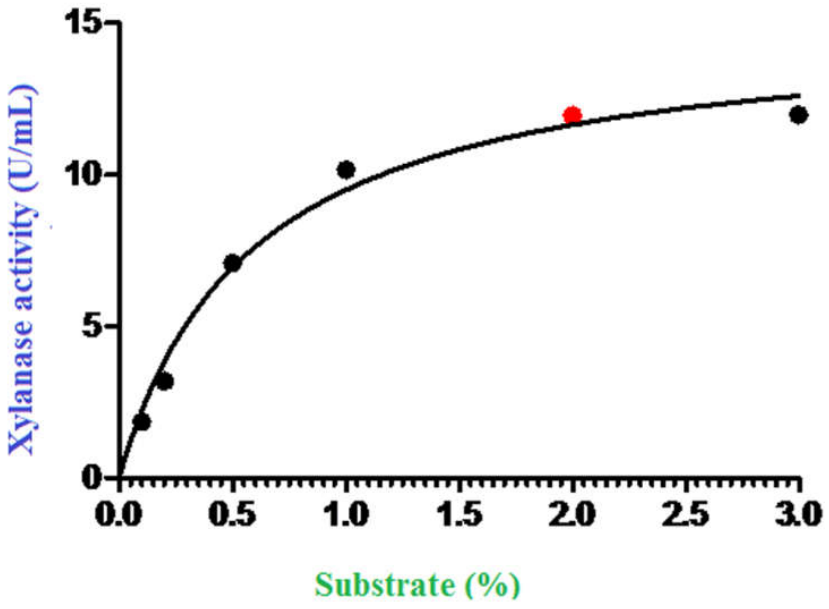

Fig 10. Kinetic analysis of xylanase obtained from submerged fermentation of treatment BPS-D2 and its hydrolyzing effect on varying concentrations of beechwood xylan .

4.14. Determination of Antimicrobial Activity

All the 81 isolates were tested for their antimicrobial activity against five bacterial pathogens *P. aeruginosa, S. aureus, E. coli, M. luteus, B. subtilis* and one yeast *C. albicans*. Out of 81 isolates, 23 (28.3%) exhibited antagonistic activity against at least five of the six tested pathogens and all of them were found positive against *S. aureus, M. luteus* and *E. coli* (Table 4). Isolates DBT32 (*Aneurinibacillus aneurinilyticus*), DBT33 (*Aneurinibacillus aneurinilyticus*) and DBT34 (*Aneurinibacillus aneurinilyticus*) exhibited broad spectrum antimicrobial activities and are considered the most promising isolates for further studies. DBT32 showed activity against *E. coli* (14.8 mm) and *M. luteus* (12.6 mm), antimicrobial activity of DBT33 was also found to be high against *E. coli* (14.9 mm) and *M. luteus* (14.5 mm), DBT34 showed the highest antimicrobial activity against *E. coli* (15 mm) and *M. luteus* (12.7 mm). Interestingly, DBT18 (Alcaligenes faecalis) showed the highest activity against yeast pathogens, *C. albicans* (12.6 mm).

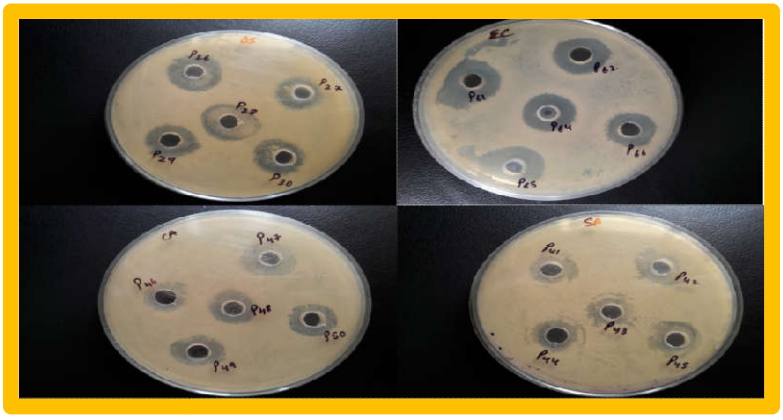

Fig 11. Formation of inhibition zone by strains against pathogens.

Table 4. Antimicrobial activity shown by bacterial isolates obtained from the gastro-intestinal extract of the tested animals

Isolates	Antimicrobial activity (zone of inhibition in mm) Each value represents the value of three replicates, Mean (± SD)					
	B. subtilis	***E. coli***	***C. albicans***	***P. aeruginosa***	***S. aureus***	***M. luteus***
DBT16	12±0.015	10±0.2	10±0.17	6±0.2	11.5±O.057	10±0.17
DBT17	0.0	11±0.015	10±0.2	7±0.17	13±0.2	10±0.015
DBT18	12.5±O.057	10.5±0.17	12.6±0.17	10±0.2	10±0.17	10.5±0.2
DBT19	11±0.2	11±0.015	11±0.015	6±0.015	6±0.015	10±0.015
DBT20	11±0.17	10±0.17	5±0.2	5±0.17	9±0.2	10±O.057
DBT21	10±0.015	14.5±0.015	12±0.015	0.0	12±0.015	12.5±0.015
DBT22	12±0.17	14.5±O.057	12±0.17	0.0	10±0.17	10±0.2
DBT23	10±0.015	14.5±0.17	12.5±0.2	0.0	12±0.2	6±0.17
DBT24	10±0.17	14.6±0.015	11±0.015	0.0	10±0.015	10±0.2
DBT25	15±0.2	14.3±0.2	12±0.17	0.0	12±O.057	20±0.015
DBT27	8.5±0.015	5±0.17	12.5±0.2	10±0.2	10±0.015	10.5±0.17
DBT29	9±0.17	12±0.015	12.5±0.015	12±0.17	10±0.17	10±0.015
DBT31	9.5±O.057	14.6±0.17	11±0.2	12±0.015	10±0.015	12±0.17
DBT32	**10±0.015**	14.8±O.057	11.5±0.17	12.5±0.17	10±0.2	12.6±0.2
DBT33	**9±0.17**	14.9±0.015	12.5±0.015	10±0.015	10±0.17	4.5±O.057

DBT34	**12±0.015**	15±0.17	12±0.17	10±0.17	10.2±0.2	12.7±0.17
DBT35	10±0.17	15±0.2	11.5±0.2	11±0.2	7±0.17	11±0.17
DBT40	0.0	10±0.2	12±0.015	2±0.2	10±0.17	9±0.2
DBT58	4±0.015	14.8±O.057	0.0	12.6±0.17	10±0.015	7±0.015
DBT62	5±0.2	9±0.015	12.5±0.015	8±0.015	6±0.2	9±0.17
DBT64	6±0.015	9±0.17	9±0.2	8.5±0.17	7±0.17	11±0.2
DBT66	5±0.17	9±0.2	8±0.2	5.5±0.2	7±0.17	6±0.17
DBT74	6±0.015	6±0.2	0.0	5±0.015	2±0.2	2±0.2

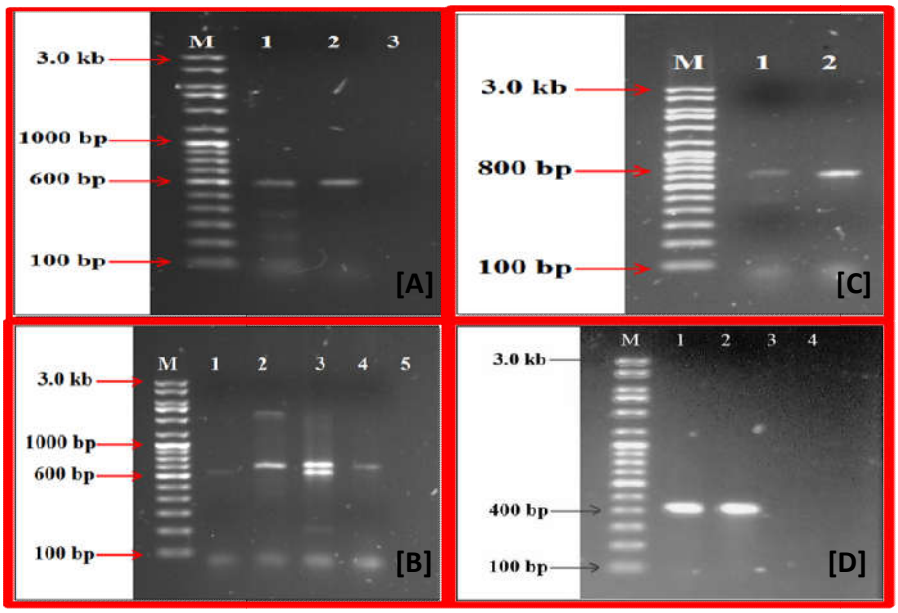

Fig 12. Detection of [A] *phz*E, [B] Halo, [C] NRPS and [D] CYP gene among selected antagonistic isolates.

4.15. Amplification of genes encoding NRPS, CYP, Halo and *phz*E

The presence of genes encoding NRPS, CYP, Halo and *phz*E were detected in 8 isolates (Fig 12). Strain DBT23 showed positive amplification products with both the CYP and NRPS primers whereas DBT25 showed in both Halo and *phz*E primers. Two strains DBT23 and DBT25 showed the presence of PCR products although they have limited antagonistic activity (Fig 12). Biosynthetic gene NRPS were also detected in DBT33, which exhibited significant antimicrobial activities against all tested pathogens indicates the existence of biosynthetic gene clusters which accounts for antimicrobial secondary metabolite productions. These results intrigued that some of the factors such as climatic or environmental conditions may have a role in some of the pathways encoding antimicrobial genes which lead to non-functional.

Chapter V

Discussion

Chapter V: Discussion

In tropical countries, the ruminants are fed on lignocellulosic agricultural by products. Some researchers suggested that ruminants can digest the plant materials in the presence of microbes which are found in the rumen and present inside the gut as host to supply nutrients, partially in the form of volatile fatty acids and microbial protein (Miron et al., 2001; Pope et al., 2012; Jami and Mizrahi, 2012). The surroundings of ruminants are associated with microbes that possess the complex lignocellulosic degradation system for the microbial attachment and digestion of plant biomass. Though, a single species have very difficult to break complex plant cell wall. Hence, most of the important microorganisms and gene occupied the efficient hydrolysis of plant cell wall which is associated with the fiber portion of the rumen digest (Forsberg et al., 1997). Plant cell wall has a complex structure which surrounded area covered by cellulose, hemicelluloses, pectin and protein, cell types, and stages of maturity (Cosgrove, 2005). Presence of gut microbes inside of herbivorous animals having capacity to degrade enzymes. These bio-catalytic enzymes help hydrolyze the materials thereby helping the host garner energy from the digested plant biomass (Cumming and MacFarlane, 1997; Wynne et al., 2004). Even though, these microbes having high efficiency, specificity to digest plant materials as well as having ability to survive at certain conditions like at lower pH and higher temperature inside the gut system. Thus, it is prime importance to study on lignocelluloses degrading enzyme production.

The actinomycetes isolation agar (AIA) media was the best nutritional media (n=29; 35.8%) for the isolation of bacteria from the gastro-intestine (GI) of ruminant's goat and non-ruminant's Zovawk pig. This finding was similarly reported by Patil et al., (2010) who stated that 13 bacteria recovered from honeybees gut. Among them, eight bacteria belong to *Streptomyces* sp. and five belongs to *Bacillus* sp. They are identified by 16S rRNA gene with 97% similarity.

Moreover, Yang et al., (2014) reported that Strain *Bacillus subtilis* BY-2 was isolated from the intestine of Tibetan pig using Luria Bertani (LB) media which showed that strain BY-2 was rapidly grown in respective media and produced high amount of cellulase. Many previous researchers suggested that in the gastro-intestines of the warm blooded animals, *Bacteroidetes* and *Firmicutes* are the predominant phylum (Kim and Issacson, 2015, Jiao et al., 2015). In our study, *Proteobacteria* was found dominant class (n=69; 85.18%) followed by *Actinobacteria* (n=12; 14.8%). From the gut of swine and goat varieties, *Proteobacteria* was also reported as dominant phylum followed by *Actinobacteria*, *Spirochaetes* and *Synergistetes* (E.G. et al., 2004; Stojanovic et al., 2007; Han et al., 2015; Min et al., 2014).

All isolates were identified by PCR amplification of the 16S rRNA gene upto subspecies level and phylogenetic tree was constructed using neighbor joining method. 16S rRNA gene sequences of all gut intestine bacterial isolates showed 97-100% identity with a reference sequences in NCBI GenBank. *Bacillaceae* was the dominant family followed by *Paenibacillaceae, Alcaligenaceae, Enterobacteriaceae, Streptomycetaceae, Micrococcaceae, Burkholderiaceae, Pseudomonadaceae, Micromonosporaceae, Sphingomonadaceae, Mycobacteriaceae and Nocardiopsaceae*. All the gram positive and gram negative bacteria was clustered separately in different clade which is similarly reported by Vivero et al., (2016) who stated that all the different bacterial genera like *Enterobacter, Pseudomonas and Acinetobacter, Ochrobactrum, Lysobacter, Microbacterium, Bacillus, Streptomyces and Rummeliibacillus* isolated from the digestive tracts of adult *Lutzomyia evansi* are clustered separately in Neighbour joining phylogenetic tree. Further, some genera like *Aneurinibacillus, Serratia* and *Bacillus cereus* formed a separate cluster with their type strains. This findings with similarly reported by previous

researchers like Yang et al., (2014); Vivero et al., (2016). To the best of our information and literature search, this is the first time reported two genera *Pseudoarthrobacter oxydans* strain DBT75 and *Micrococcus luteus* strain DBT50 isolated from gut intestine of Goat.

Gut residing microbes may plays a major role in supporting the host. Now days, many researchers has been focused to study on Cellulose and Xylanase degrading bacteria (Tomsic et al., 2007; Reyes et al., 2016). In our study, out of 81 isolates, 13 isolates was found to be positive in xylanase whereas, five isolates showed cellulase production by forming clear halo zone on cellulase congo red agar media. Among them, only two isolates *Serratia rubidaea* strain DBT4 and *Aneurinibacillus aneurinilyticus* strain DBT87 showed both cellulase and xylanse activity. This finding was in consistent with the finding of Anand and Sripathi, (2004) who stated that 14 bacterial isolates was obtained from gut intestine of the Indian flying fox (*Pteropus giganteus*). Among them, eight isolates showed both cellulase and xylanase activity and also reported that strain *Serratia liquefaciens* have produced both cellulase and xylanase enzymes. Moreover, Gupta et al., (2011) reported that eight isolates was found to be positive in cellulase degrading bacteria which have obtained from four different invertebrates (termite, snail, caterpillar, and bookworm). In our study, the maximum hydrolytic capacity of cellulase zone was seen in *Aneurinibacillus* sp. strain DBT14 (0.87 cm) followed by *S. rubidaea* strain DBT4 (0.8 cm) and *A. aneurinilyticus* strain DBT87 (0.1 cm). Similarly, Gupta et al., (2011) reported that the highest clear zone around the colony with diameter of 4.5 cm and 5.0 cm were showed by strain CDB 8 and CDB 10 with the hydrolytic value of 9 and 9.8, respectively.

In our study, four isolates (*S. rubidaea* strain DBT4, *A. aneurinilyticus* strain DBT87, *Aneurinibacillus* sp. strain DBT14 and *Bacillus cereus* strain DBT10) were selected based on their

zone of inhibition in cellulase and xylanase production. Enzyme assay for cellulase production on filter paper (FPase) activity was found highest in isolates *A. aneurinilyticus* strain DBT87 (0.62 U/mL) at 120 h followed by *Aneurinibacillus* sp. strain DBT14 and *S. rubidaea* strain DBT4 with 0.53 U/mL and 0.5 U/mL at 48 h and 96 h respectively. This finding was similarly reported by Yang et al., (2014) who stated that strain *Bacillus subtilis* BY-2 showed maximum cellulase production (CMCase) of 3.56 U/ml within 48 h which has obtained from Tibetan Pig's intestine. On the other hand, Gupta et al., (2011) reported that eight bacterial isolates (CDB1, 2, 3, 6, 7, 8, 9 and 10) showed cellulase production with ranges from 0.012 to 0.196 IU/mL for FPC (filter paper cellulase) activity which is isolated from four different invertebrates (termite, snail, caterpillar, and Bookworm gut**).** Rastogi et al., (2009) reported that *Bacillus* sp. strain DUSELR13 exhibited highest CMCase activity (0.12 U/ml) on days 9, when the culture had reached the dying phase. During stationary phase, cellulolytic enzyme production was decreased due to metabolite repression by molecules released after the hydrolysis such as glucose or cellobiose (Saratale and Oh, 2011). Further, *Brevibacillus* sp. strain DUSELG12 and *Geobacillus* sp. strain DUSELR7 showed maximum FPase activity of 0.027 and 0.043 U/mL on days 7 and 8, respectively as per Rastogi et al., (2010). To best of our knowledge, this is the first time reported strain DBT4 showing highest FPase activity as compare to previous studies. The Xylanase assay revealed that *S. rubidaea* strain DBT4 was the best enzyme producers with maximum xylanase activity of 6.15 U/mL at 72 hrs, followed by *A. aneurinilyticus* strain DBT87 and *Bacillus cereus* strain DBT10 having optimal xylanase activity of 4.12 U/mL and 3.05 U/mL on 96 h and 48 h respectively. Garg et al., (2009) reported that alkalo-thermophilic bacteria, *Bacillus halodurans* strain MTCC 9512 was isolated from dung which indicated highest xylanase production at 42 h.

Chapter V: Discussion

In our study, the effect of temperature on FPase activity of the crude enzyme was determined over a temperatures range of 37 ^{0}C to 60 ^{0}C at pH 7.0. Isolate *S. rubidaea* strain DBT4 exhibited FPase activity of 0.41 U/mL at 55˚C. Similar results were observed in isolates *A. aneurinilyticus* strain DBT87 and *Bacillus cereus* strain DBT10 which showed xylanase activity of 6.8 U/mL at 55˚C and slowly decreased at 60 ˚C. This finding was similar with the finding of Seo et al., (2013) who stated that *Bacillus licheniformis* strain JK7 showed maximum xylanase activity (0.44 U/ml) at 55˚C. The optimum temperature for xylanases have also been reported from various *Bacillus* sp. in previous literature (Archana and Satyanarayana, 1997; Ko et al., 2010; Ko et al., 2011, Yang et al., 1995; Yin et al., 2010). Moreover, Choi et al., (2009) reported that *Bacillus licheniformis* strain KCTC1918 showed xylanase activity at 47 ^{0}C. As per Rastogi et al., (2009) *Bacillus* strain DUSELR13 also demonstrated maximum endoglucanase activity at 75 ^{0}C. In our presence study, we have displayed that isolate *S. rubidaea* strain DBT4 exhibited both xylanase and cellulase activity at 55˚C and also might be a potential thermo-tolerant bacterial strain. Many researchers reported that some different bacterial species like *Geobacillus thermoleovorans*, *Streptomyces* sp. strain S27, *Bacillus firmus*, *Actinomadura* sp. strain Cpt20, *Saccharo polyspora pathunthaniensis* S582 and *Thermotoga* sp. having xylanase producing capabilities at different temperature ranges from 65 ^{0}C to 105 ^{0}C (Li et al., 2000; Bray and Clarke, 1992). Previous researchers described that strain S. *rubidaea* is used as phytopathogen as well as an invasive pathogen in certain animals (Escobar et al., 2001; Ursua et al., 1996). There are limited reports found in Uniport database of xylanases like β-xylosidase (http://www.uniprot.org/uniprot/A0A126VGJ6), Endo-1, 4-beta-xylanaseZ (http://www.uniprot.org/uniprot/A0A126VIN5) as well as cellulase gene 6-phospho-beta-glucosidase (http://www.uniprot.org/uniprot/A0A126VIF7) in strain S. *rubidaea*. Thermophilic

cellulose degrading enzymes have great potential for the textile, leather, biofuel, food and agriculture industry given that high temperatures are often required in these processes (Rastogi et al., 2009; Trivedi et al., 2011).

The effect of pH on enzyme activity was examined at various pH levels ranging from a pH of 5.0 to 9.0 as shown in our study under Smf conditions. The optimal pH for cellulase enzymes was 7.0 and their activities in isolates BPS-D7, BPSM9 and BPSM18 at that pH were 0.62, 0.52 and 0.5 U/ml respectively. Immanuel et al., (2006) reported that the cellulolytic enzyme, endoglucanase from *Cellulomonas*, *Bacillus*, and *Micrococcus* sp., isolated from the estuarine coir netting effluents hydrolyzes substrate in the pH range of 4.0 to 9.0, with maximum activity at pH 7.0. Isolate *Bacillus* sp. MVS3 showed maximum FPase production at pH 7.0 (Acharya and Chaudhary, 2012). Isolate *S. rubidaea* strain BPS-M18 showed highest FPase and xylanase activity at optimal pH 8.0 & 9.0. There are very scanty reports available for cellulase and xylanase enzyme production in *Serratia rubidaea* (Abd-Alla et al., 2011; Doddapaneni et al., 2007). This findings was similary reported by Anand and Sripathi (2004) who stated that *Serratia liquefaciens* from the intestine of Indian flying fox (*Pteropus giganteus*) having cellulase and xylanase activity at pH 8.0. Further, Garg et al., (2009) reported that isolate *Bacillus halodurans* obtained from dung showed maximum xylanase production in temperature 55°C at pH 9.5. Moreover, Anand et al., (2010) mentioned that *S. liquefaciens* isolated from digestive tract of *Bombyx mori* was able to utilize three polysaccharides including CMcellulose, xylan and pectin, with FPase activity of 0.4 U/ml (400 mU/ml), CMCase of 0.45 U/ml (450 mU/ml) and xylanase of 0.1 U/ml (100 mU/ml) at pH 8. Isolate *Bacillus cereus* strain BPS-D7 showed highest xylanase activity (1.2 U/ml) at pH 5.0. Similarly, Seo et al., (2013) stated that *Bacillus licheniformis* isolated from the rumen Korean goat

exhibited maximum xylanase activity of 1.08 U/mL at pH 5.0. To best of our knowledge, this is first report that isolates *Bacillus cereus* strain BPS-D7 obtained from gut intestine of goat having highest xylanase activity at low pH 5.0.

FTIR spectral data is generally useful for chemical analysis of pulp and wood products (Ferraz et al., 2000; Chen et al., 2010). The use of microorganisms that produce hydrolytic enzymes such as cellulases and xylanases and low cost abundantly available substrates, such as perennial grasses are possible candidates for making second-generation biofuel (Leo et al., 2016). Biomass conversion and utilization studies, especially pulp and wood are mainly depends on FTIR analysis to verify the variations in compositions incurred after treatments (Ferraz et al., 2000; Chen et al., 2010). In our study, the FTIR analysis of the best two treatments (D2 and M2) was compared with untreated alkali pretreated BPS-G104a biomass which indicated significant variations in their FTIR data peaks, especially for the intensities corresponding to holocellulose components. This finding was similarly reported by Lima et al., (2013) who stated that the effect of enzymatic hydrolysis on eucalyptus barks by FTIR analysis. In the current study, FTIR readings was found slightly variation at 1053, 1240, 1735/1730 cm^{-1} which have closely similar with other studies (Leo et al., 2016); Komolwanich et al., 2016). The chemical components that increase the biomass are tremendously important in determining their viability as a potent substrate for biofuel production. This study envisages an insight into the potential of gut or ruminal bacteria for holocellulose degrading enzymes. These enzymes thus produced could also have the additional benefit of surviving in harsher conditions (alakaline, thermophillic) and being substrate specific (holocellulosic biomass).

Chapter V: Discussion

In this study, isolates DBT32 (*Aneurinibacillus aneurinilyticus*), DBT33 (*Aneurinibacillus aneurinilyticus*) and DBT34 (*Aneurinibacillus aneurinilyticus*) exhibited broad spectrum antimicrobial activities and are considered the most promising isolates for further studies with similar findings with Zothanpuia et al., 2016. Two strains DBT23 (*Aneurinibacillus aneurinilyticus*) and DBT25 (*Aneurinibacillus aneurinilyticus*) showed the presence of PCR products although they have limited antagonistic activity. Biosynthetic gene NRPS were also detected in DBT33, which exhibited significant antimicrobial activities against all tested pathogens indicates the existence of biosynthetic gene clusters which accounts for antimicrobial secondary metabolite productions. These results intrigued that some of the factors such as climatic or environmental conditions may have a role in some of the pathways encoding antimicrobial genes which lead to non-functional.

Chapter VI

Summary and Conclusion

The microbial population in gastro-intestinal (GI) of ruminant and non-ruminant animals that are capable of utilizing lignocellulosic plant biomass was explored. In this study, an indigenous swine, Zovawk (*Sus domesticus*) and a domesticated goat, Black Bengal (*Capra aegagrus hircus*), were investigated to isolate the bacteria having plant biomass degrading enzymes. Total of eighty one bacterial isolates were obtained and enzyme screening assay revealed 13 xylanase and 5 cellulase positive cultures. Quantification and optimization of growth parameters revealed *Serratia rubidaea* DBT4 and *Aneurinibacillus aneurinilyticus* DBT87 as the most potent strains showing both cellulase and xylanase production. Biomass utilization study showed that D2 treatment (alkaline pre-treated pulpy biomass, *Thysanolaena latifolia*) under Submerged Fermentation (SmF) using strain DBT4 was the most efficient with the maximum xylanase (11.98U/ml) and FPase (0.5U/ml) activities (55˚C, pH 8). This was substantiated by the 60% loss in dry weight after D2 treatments and by Scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR) which showed variations in structural and chemical constituents of the biomass treated. Strains DBT32 (*Aneurinibacillus aneurinilyticus*), DBT33(*Aneurinibacillus aneurinilyticus*) and DBT34 (*Aneurinibacillus aneurinilyticus*) exhibited broad spectrum antimicrobial activities and are considered the most promising isolates for further studies. Biosynthetic gene NRPS were also detected in DBT33 (*Aneurinibacillus aneurinilyticus*), which exhibited significant antimicrobial activities against all tested pathogens.

The present study demonstrated the ability of bacterial strains residing in the gastro-intestinal region of ruminant and non ruminant swine as a promising source for lignocellulose degrading microorganisms that could be used for biomass conversion and typifies potential targets for novel enzymes with industrial applications. Further, the present study revealed the existence of biosynthetic gene clusters which accounts for antimicrobial secondary metabolite productions and could be a promising source for the discovery of novel bioactive metabolites.

Conference/Seminars/Workshops Attended

- **Dhaneshwaree Asem,** Vincent Vineeth Leo, Mary Vanlalhruaii Tonsing, Surendrakumar Thingnam, Ajit Kumar Passari and Bhim Pratap Singh*. Presented a poster entitled "Screening and Identification of Xylanolytic Bacteria from the Rumen of a domesticated common Goat *Capra aegagrus hircus* and Investigation for Xylanase Production*"*. In the 57th Annual Conference of AMI and International Symposium on "Microbes and Biosphere: What's New What's Next"-from 24th Nov to 27th Nov, 2016 organized by Department of Botany, Gauhati University and IASST, Guwahati, Assam, India.

- Workshop on "**National Level Workshop on Biostatistics and Bioinformatics**" (1st -7th September, 2016) organized by Department of biotechnology, Mizoram University.

- Attended Science Communication Workshop (SciComm 101) held on 6th June, 2017 at Mizoram University, Aizawl.

ABBREVIATIONS

U, ml	Unit, milliliter
µl	Micro Liter
ng	Nanogram
mM	Micromolar
pmol	Pico-Mol
pH	Potential of Hydrogen
h	Hours
min	Minutes
AIA	Actinomycetes Isolation Agar
SCA	Starch Casein Agar
TSA	Tryptone Soy Agar
LB	Luria-Bertani
LAB	Lactic acid bacteria
GI	Gastro-intestinal
TAE	Tris Base, Acetic Acid and EDTA
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
16S rRNA	16S-Ribosomal Ribonucleic Acid
CMC	Carboxymethyl cellulose
WSOSX-1	Water Soluble Xylan from Oats for Screening Xylanase
FPA	Filter Paper Activity
K_2HPO_4	Potassium Phosphate Monobasic
NaCl	Sodium Chloride
H_2O	Water
SmF	Submerged Fermentation

DNS	Dinitro Salicyclic Acid
RDW	Relative Dry Weight
w/v	Weight by Volume
v/v	Volume by Volume
FTIR	Fourier Transformed Infrared Spectroscopy
SEM	Scanning Electron Microscopy
ISP1	Tryptone Yeast Extract Broth medium 1
NRPS	Non-Ribosomal Peptide Synthetase
***phz*E**	aminodeoxyisochorismate synthase
CYP	Cytochrome P450 hydroxylase
Halo	Halogenase
MTCC	Microbial Type Culture Collection
mm	Millimeter
cm	Centimeter
Blast	Basic Local Alignment Search Tool
EMBL	European Molecular Biology Laboratory
NCBI	National Center of Biotechnology Information
BIC	Bayesian Information Criterion
AIC	Akaike Information Criterion