Chapter 2

Isolation, screening and identification of bacterial isolates for production of plant polysaccharide hydrolyzing enzymes

2.1. Introduction:

On the long path of evolution, most of non-photosynthetic organisms have developed the ability of utilizing sugars by their oxidation through the central energy-yielding pathway. Therefore, a range of simple to complex carbohydrate polysaccharide compounds can serve as a source of food and energy (Nelson et al., 2008). In nature, the easiest consumable carbohydrates are simple monosaccharides, which in turn get complex when polymerized to form di-, oligo- and poly-saccharides. The complexity in structural composition of homopolysaccharides (e.g., amylose, glycogen, cellulose, chitin, etc.,) or heteropolysaccharides (e.g., xylomannan, arabinoxylan, xyloglucan, glucomannan, etc.,) arises due to involvement of various pentose and hexose monomers of aldo-sugars and keto-sugars having diverse substitutions in their structures, (Glasser et al., 2000; Nelson et al., 2008).

As mentioned in Chapter 1, Section 1.3, plant cell wall is one examples of the complex structures majorly comprising polysaccharide macromolecules. The two adjacent plant cells are cemented together through the middle lamella, made up of calcium pectate, a pectic (or rhamnogalacturonic) polysaccharide. Inner to this middle lamella there is a deposition of structural homo and heteropolysaccharide materials such as lignocellulose and hemicellulose to form the primary and secondary plant cell wall. Animals which feeds on these complex carbohydrate polymers as a source of their food and energy, are dependent on the symbiotic microorganisms inhabiting in their gut for the digestion process (Dehority, 2002; Brune, 2014; Brune and Dietrich, 2015). These microorganisms play an important task to break these polymers down to their oligo or monosaccharides and bring them back to the nutrition and elemental cycles.

In last few decades functional metagenomic studies from diverse metagenome samples have revealed a presence of numerous enzymes from various Glycosyl Hydrolase (GHs) families and are secreted by diverse microorganism. Such examples include various rumen liquors and dung or faecal samples of ruminant herbivore animals like cow, goat, buffalo, etc., and other herbivores i.e., panda, monkeys etc.; gut liquors of certain wood feeding insects, such as termite, beetle, cockroach, etc.; water samples viz., hot water spring, drainage samples, fisheries and paper industries effluent channels; various soils such as arctic, mangrove, compost, etc., These enzymes from these samples includes endoglucanases, cellobiohydrolases, cellodextrinases, β-glucosidases, amylases, acetyl xylan esterases, phenolic acid esterases, endoxylanases,

xylogalcturonases, polygalacturonases, pectin lyases, polygalacturonate lyases, arabinofuranosidases, and several others (Cruden and Markovetz, 1979; Duskova and Marounek, 2001; Ferrer et al., 2005; Zhu et al., 2011; Scully et al., 2013; Sathya and Khan, 2014). Breakdown of diverse complex polysaccharides to simpler end-products are completed with the help these enzymes. Involvement of more than one types of these biocatalysts promotes and eases the process.

Plant cell wall degrading enzymes, majorly hydrolases and lyases, produced by microorganism like bacteria and fungi, can be used for hydrolysis of lignocellulose, which gives fermentable reducing sugars, a fundamental raw material for biofuel (bio-alcohols) industry. Out of these, the cellulase is a major enzyme family that yields glucose as a product which is widely used as fermentable sugar. The core group of cellulase comprises endo-glucanase, cellobiohydrolases and β-glucosidease for complete breakdown of cellulose to glucose. But the action of cellulase is hindered by the presence of hemicellulose which coats the cellulose fibres. Different core cellulase groups e.g. Celluclast, Novozyme and Accellerase has been immensely studied and characterized for their commercialization with this aspect. Their supplementation with other commercial Multifect xylanases and Multifect pectinases in enzyme cocktails to remove the covering of hemicellulose and improve performance of cellulase has been studied and yet newer combinations of cocktails are required (Berlin et al., 2007; Delabona et al., 2013; Li et al., 2014b; Reyes-Sosa et al., 2017). So, there is a room for enhancement of saccharification by core cellulase group of enzymes by supplementing it with diverse accessory enzymes. This necessitates the study of the role of diverse xylanase and pectinase enzymes which can further improve the yield of biomass saccharification.

Microorganisms from diverse polysaccharide degrading niches have been isolated, identified and such enzymes from these organisms have been characterized for their activities. *Bacillus mojavensis* AG137, isolated from cotton farm soil (Kashan-Iran) has been characterized for production of alkaline xylanase (Sepahy et al., 2011). *Bacillus altitudinis* DHN8, isolated from soil sample was characterized for its thermo-alkali-solvent stable xylanase (Adhyaru et al., 2014). *Brevibacillus borstelensis* P35 isolated from hot water spring sample has been characterized for alkaline pectin lyase (Demir et al., 2014). *Pectobaterium carotovorum* subsp. *carotovorum* has been isolated from diseased brinjal and characterized for three types of pectinases i.e., pectin lyase, pectate lyase and polygalacturonase (Maisuria et al., 2010; Maisuria and Nerurkar,

2012). Table 2.1 enlists the details about few of the isolated microorganisms and extracted metagenomes, their source samples and polysaccharide degrading enzyme(s) obtained from them. Based on these and several other reports it was decided to focus on isolation of xylanase and pectinase producing bacteria which further can be characterized for these enzymes for their accessory role to commercialize in plant biomass saccharification.

Table 2.1. List of some polysaccharide degrading enzymes reported from diverse sources:

Source	Enzyme (Source microorganism)	Reference
Soil	Alkaline pectinase (*Bacillus subtilis* ZGL14)	Yu et al., (2017)
Soil of food processing unit	Pectin lyase (*Bacillus* sp. DT7)	Kashyap et al., (2000)
Brazilian Atlantic forest soil	Pectin lyase (*Aspergillus giganteus*)	Pedrolli and Carmona, (2014)
Lab isolate	Pectinolytic complex (*Aspergillus niger* MIUG 16)	Dinu et al., (2007)
The cut aged flue-cured tobacco (FCT) leave	Pectate lyase (*Bacillus subtilis* PB1)	Zhou et al., (2017)
NM[#]	Xylanase (*Bacillus stearothermophilus*)	Khasin et al., (1993)
NM[#]	Xylanase (*Caldicellulosiruptor bescii*)	An et al., (2015)
Soil from active compost pit	Xylanase (*Bacillus altitudinis DHN8*)	Adhyaru et al., (2014)
Soil samples under decaying tree fibers	Xylanase (*Paecilomyces themophila*)	Li et al., (2006)
Pulp from paper mill	Xylanase (*Geobacillus thermoleovorans*)	Sathya and Khan, (2014)
Cow dung	CMCase or endoglucanase (*Bacillus* sp. C1)	Sadhu et al., (2013)
Metagenome sample	Enzyme(s)	Reference
Metagenome from rumen content of a dairy cow	β-1,4-xylanase; putative xylanase; endo-1,4-β-glucanase; acetylxylan esterase; mannanase; cellulase; α-amylase; pectin acetylesterasse; etc.,	Ferrer et al., (2005)
Metagenome of wood boring beetle gut	β-glucosidase; β-galactosidase; β-mannosidase; β-glucuronidase; exo-β-1,4-glucanase; xylan 1,4-β -xylosidase; β -N-acetylhexosaminidase; glucan 1,3-β -glucosidase; Endo-β -1,4-glucanase; exo-1,3-1,4-glucanase; α-L-arabinofuranosidase; etc.,	Scully et al., (2013)
Giant panda gut metagenome	Cellulase, β-glucosidase; xylan 1,4-β-xylosidase; endo-1,4-β-xylanase; β-1,4-endomannanase; β-1,3-glucosidase; polygalacturonase; rhamnogalacturonase; etc.,	Zhu et al., (2011b)

NM[#]: Not Mentioned.

In this chapter, bacteria producing responsible enzymes for breakdown of one or more complex plant cell wall structural polysaccharides were isolated from the dung

samples of various herbivore ruminants, decaying wood and manure samples. The isolates were screened qualitatively and quantitatively for production of one or more enzymes such as cellulase, pectinase and xylanase. Further cellulase free xylanase-pectinase (accessory) enzyme producers were selected for their identification and application studies. The emphasis of the studies that followed in the subsequent chapters was focused on the xylanase and pectinase enzymes from the selected strains, to confirm their ability as accessory enzymes for plant biomass saccharification in a commercial cellulase mediated enzyme cocktail and their characterization.

2.2. Materials and Methods:

2.2.1. Chemicals, crude polysaccharide sources and bacterial type strains:

All required chemicals, purchased from HiMedia (Mumbai, India) or Sigma-Aldrich (Missouri, USA) or SRL Pvt. Ltd. (Mumbai, India), were of assay grade. Orange fruits (*Citrus reticulata*, common Indian cultivar of orange) were purchased from local market, and their peels were collected. The peels were freeze dried to avoid structural damage as well as fungal contamination and finely ground in a grinder. Wheat Bran, the outermost fibrous layer of wheat seed (*Triticum aestivum*) was procured from the local industrial market Manjusar GIDC, Vadodara. Both Citrus Peel (CP) powder and Wheat Bran (WB) were stored separately at room temperature in airtight containers and henceforth they were referred as CP and WB respectively throughout this chapter. The type strains of three *Bacillus* spp, i.e., *B. safensis* FO-36b^T, *B. pumilus* ATCC 7061^T and *B. altitudinis* 41KF2b^T were procured and used as reference strains for further identification studies.

2.2.2. Sampling for isolation of polysaccharide hydrolase producer bacteria:

Different niches, where lignocellulosic materials were being digested or decayed by microorganisms, were prioritised for sampling sites. Accordingly, selected samples included fresh dung deposits of the herbivore ruminants, partially decayed wood sample, farm yard manures and cellulose as well as vegetable waste containing Winogradsky columns seeded with pond water and sewage sludge. All the samples were collected in sterile sample tubes or containers, kept at 4 ºC till their processing by the following approaches.

2.2.3. Enrichment and isolation from collected samples:

Enrichment culture technique was performed prior to isolation for the fresh dung deposits, partially decayed wood and manure samples. Whereas, samples

collected from Winogradsky columns were directly subjected to dilution and isolation.

2.2.3.1. Enrichment of polysaccharide hydrolase producing bacteria:

0.5 gm of each of dung, manure and decaying wood samples individually was subjected to enrichment in a series of Enrichment Media (EM, composition mentioned as below). Samples were inoculated in 50 ml volume of EM1 broth in 250 ml Erlenmeyer flask and incubated for 24 h at 37 °C on New Brunswick Scientific (NBS) shaker at 180 rpm. Two serial transfers of 1% v/v were subsequently made from EM1 to EM2 to EM3 in sequence where each transfer was followed by the same incubation conditions as were used for EM1.

Enrichment Media (EM) Components: Three different media were comprised of following components mentioned as % w/v unless specified.

- **EM 1**: Nutrient rich medium: Containing Nutrient broth (NB) (0.65%) with beechwood xylan, pectin, cellulose and glucose (0.5% each).
- **EM 2**: Minimal Salt Medium: Bushnell Haas Medium (BHM) (0.327%) with beechwood xylan, pectin, cellulose and glucose (0.5% each).
- **EM 3**: Minimal Salt Medium: Bushnell Haas Medium (BHM) (0.327%) with beechwood xylan, pectin and cellulose (0.5% each).

2.2.3.2. Preparation of Winogradsky column:

Winogradsky columns were prepared in a traditional way using mud supplemented and mixed with shredded cellulose papers and vegetable waste materials and layered with water samples from pond water as well as domestic sewage or waste water treatment plant containing diverse microflora. Carbonate and phosphate salts were added to the column as buffers. The columns were enriched for several days and on seventh day samples from both columns were collected and directly subjected for serial dilutions in 0.85% w/v saline and spreaded as mentioned ahead in Section 2.2.3.3.

2.2.3.3. Isolation of polysaccharide hydrolase producing bacteria:

Enriched samples were subjected to dilutions using 0.85% w/v saline. 100 µl of each dilution was spread on plates containing Nutrient broth (NB, 1.35% w/v) with Agar (2% w/v) and incubated for 24 h at 30 °C. Obtained colonies with different morphologies were further sub-cultured on fresh NB agar plates in multiple sets. The obtained isolates were preserved at 6-8 °C on the NB agar plate after 24 h incubation at 37 °C and maintained through subculture after every 15 days on the same media.

2.2.4. Primary screening of isolates for polysaccharide hydrolase production:

Primary screening involved qualitative assays for observation of zone of hydrolysis or clearance on different substrate containing solid media. Following the enrichment strategy, isolated cultures were further screened for qualitative production of xylanase, pectinase and cellulase enzymes on both pure and crude substrates as mentioned below. Isolates producing one or more enzymes were selected.

2.2.4.1. Screening of xylanase producers:

Screening media for xylanase production contained following components (% w/v): 0.2% Congo red (CR) was mixed with either 0.5% of pure substrate beechwood xylan or 0.5% crude substrate WB separately along with 0.65% NB and 2% agar. Media were autoclaved at 10 lbs for 20 min. To develop clear zones, media were washed with 5.0% NaCl; further flooded with 1N HCl for 5-10 min; and incubated at 4 °C for 12-16 h (adapted and modified, Teather and Wood, 1982).

2.2.4.2. Screening of pectinase producers:

Screening media for pectinase production contained following components (% w/v): 0.5% of pectin as pure substrate and 0.5% CP as crude substrate separately each along with 0.65% NB and 2% agar. 2x NB-agar and 2x pectic substrate were autoclaved at 10 lbs for 20 min separately and then mixed aseptically before pouring in Petri dishes. For observation of the zone of clearance 1% Cetyl Pyridinium Chloride (CPC) or 1% Cetyl Trimethyl Ammonium Bromide (CTAB) was flooded on the plates and kept for 45-60 min (Hadj-Taieb et al., 2011).

2.2.4.3. Screening of cellulase producers:

Screening media for cellulase production contained following components (% w/v): 0.5% of Carboxy Methyl Cellulose (CMC) as pure substrate and 0.5% Whatman filter paper (WFP) #1 as crude substrate separately each along with 0.65% NB and 2% agar. WFP was shredded in small pieces and swirled in water on 160 rpm up to 1 h to loosening the fibres and then used for media preparation. Media was autoclaved at 10 lbs for 20 min. To develop the zone of clearance Lugol's iodine was flooded on the plates and kept till the contrast appeared (adapted and modified, Kasana et al., 2008).

2.2.5. Secondary screening of the isolates for xylanase-pectinase production:

Secondary screening was done by quantification of xylanase and pectinase activities. Positive cultures selected from primary qualitative tests were subjected for

quantitative analysis. Both xylanase and pectinase were produced separately by using pure and crude sources of xylan and pectin added to BHM-YEP media and activities were quantified using the assay systems as mentioned ahead.

2.2.5.1. Media for qualitative screening of xylanase and pectinase:

Overnight grown culture at 37 °C, 160 rpm in 5.0 ml NB medium was used as inoculum. Optical Density (OD_{600}) was set to 0.4 using sterile NB as blank and diluent, 25 µl of this culture was inoculated in a test tube having 5 ml of BHM-YEP medium containing (g/L) Bushnell Haas Medium (BHM) 3.27, Yeast extract (YE) 0.25 and Peptone (P) 0.75. To this 0.5% of (a) beechwood xylan as pure or WB as crude xylan source, and (b) pectin as pure or CP as crude pectin source were amended individually. The media were incubated at 37 °C for 48 h in shaking condition at 160 rpm. Grown cultures were then centrifuged at 10,000 rpm for 10 min, and cell free supernatants (CFS) were analysed for xylanase and pectinase activities.

2.2.5.2. Xylanase Activity:

50 µl of individual CFS was added to 250 µl of 50 mM Tris-Cl pH 7.0 buffered beechwood xylan (0.5% w/v) and incubated in water-bath at 40 °C for 10 min. Reaction was stopped by addition of 300 µl Di-Nitro Salicylic acid (DNS) reagent, and incubated in boiling water-bath for 10 min. Once the system was cooled down to room temperature, volume was made up to 1.5 ml by adding distilled water (DW) to each reaction system and absorbance was measured at 540 nm by using spectrophotometer (adapted and modified, Miller, 1959; Ghose and Bisaria, 1987). The amount of enzyme required to release an end product equivalent to 1 µmol of xylose in reaction mixture per unit time in optimum incubation conditions was considered as 1 unit of xylanase activity.

2.2.5.3. Pectinase Activity:

50 µl of individual CFS was added to 250 µl of 50 mM Tris-Cl pH 7.0 buffered pectin (0.5% w/v) and incubated in water-bath at 40 °C for 30 min. Enzyme action was terminated by addition of 500 µl 1 N NaOH solution followed by incubation at 76 °C for 10 min. To this 600 µl of 1 N HCl followed by 500 µl of 0.04 M 2-Thiobarbituric acid were added and incubated at 76 °C for 10 min. Developed pink color was estimated at 550 nm by using spectrophotometer (adapted and modified, Nedjma et al., 2001). The amount of enzyme required to increase the A_{550nm} of the reaction mixture by 0.01 value per unit time in optimum incubation condition was measured as 1 pectinase unit.

2.2.6. Screening based on colony and cell morphology:

24 h old pure bacterial cultures grown on NB agar plates at 37 °C were used to study the colony morphology and characteristics. Appearance on solid media, shape, margin, elevation, surface texture, consistency, opacity and pigmentation were the characteristics for the colony to be observed (Harley and Prescott, 2002; Cappuccino and Sherman, 2005). Most commonly studied morphological characters like staining nature (Gram stain), size, shape, arrangements, sides, endospore and their arrangements etc were observed for isolates. Cultures were grown in 1.3% NB and Gram staining and endospore staining were performed with mthem (Harley and Prescott, 2002; Cappuccino and Sherman, 2005).

2.2.7. Molecular characterization:

Further screening and identification of the isolates was carried out by molecular characterisation using 16S rRNA gene sequencing (along with ARDRA) and *gyrB* gene sequencing (along with RFLP).

2.2.7.1. Genomic DNA extraction:

Based on the criteria mentioned in Sections 2.2.4, 2.2.5 and 2.2.6, 31 pectinase producers, 16 xylanase producers, 7 cellulase free xylanase-pectinase producers as well as three type strains *Bacillus safensis* FO-36b^T, *Bacillus pumilus* ATCC 7061^{T}and *Bacillus altitudinis* 41KF2b^T were selected for further screening. Genomic DNA was extracted from 16-18 h old culture (grown in NB) by using modified CTAB method (Sambrook et al., 2001; Wilson, 2001). 2 ml culture was pelleted down by centrifugation at 10,000 rpm for 5 min and resuspended in 330 µl of $T_{25}E_{25}S_{300}$ (25 mM Tris Cl pH 8.0, 25 mM EDTA pH 8.0, and 300 mM Sucrose). After adding 6.6 µl of 1% lysozyme (1M in Tris-cl pH 8.0) mixture was incubated at 37 °C in water bath for 1 h. 6 µl of 2% Proteinase K (in DW) and 20 µl of 10% SDS (in DW) were added to mixture and mixed by several inverts. Incubation was done at 55 °C in water bath for 1 h. This mixture was cooled down to room temperature and 70 µl of 5 M NaCl (in DW) was added and mixed by several inversions. To this 50 µl of 10% CTAB (in 0.7 M NaCl) was added and incubated at 65 °C for 10 min. Mixture was cooled down to room temperature. 400 µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to above mixture, after several inversions, organic and aqueous phase were separated by centrifugation at 10,000 rpm for 20 min. Upper aqueous phase was extracted and 300 µl of Chloroform: Isoamyl alcohol (48:2) was added. After several inversions and

centrifugation at 10,000 rpm for 20 min upper aqueous phase was extracted. To the extracted aqueous phase 1/10th volume of chilled 3 M Sodium acetate (in DW) and double the volume of absolute ethanol were added and incubated at chilled temperature, for 1 h. Centrifugation for 10,000 rpm, for 10 min was done. Supernatant was discarded. Pellet was resuspended in 300 µl of 70% ethanol and centrifuged at 10,000 rpm for 10 min. Supernatant was decanted, and pellet was air dried and resuspended in 25 µl of deionized nuclease free water. 1 µl of 0.01% RNase (in DW) was added to it and incubated at 65 °C for 10 min and preserved in cold condition. Presence of genomic DNA was detected via Agarose Gel Electrophoresis by running the 2 µl sample on a 0.8% agarose gel containing 0.5 µg/ml of Ethidium Bromide (EtBr) in 1x TBE buffer, visualized and photographed at 295 nm. DNA samples were properly diluted and stored at 4 °C for further use.

2.2.7.2. 16S rRNA gene amplification and analysis:

16S rRNA gene sequences from 54 isolates and three type strains were amplified using the Polymerase Chain Reaction (PCR) technique in Eppendorf Thermocycler. Forward (27F) and reverse (1541R) universal eubacterial 16S rDNA primers were used to amplify the 16S r DNA region from the chromosome (Zhou et al., 1995; Löffler et al., 2000; Shivaji et al., 2000). Each reaction system (30µl) contained 3µl of 10x reaction buffer A, 1.5 µl of each primer (0.5 µM), 10 µl of dNTPs (2.5 µM each), 0.5µl of Taq DNA polymerase (1.3 units) and 1.5 µl of properly diluted (30 ng/µl) template DNA. The amplification was performed as follows: initial denaturation for 5 min at 94 °C, denaturation for 30 sec at 94 °C, annealing for 45 sec at 58 °C and primer extension for 90 sec at 72 °C, and final extension for 10 min at 72 °C. The presence of 1.5 kbp amplicons was confirmed by electrophoresis of the PCR product with 500 bp StepUp DNA ladder marker on 0.8% agarose gel containing 0.5 µg/ml of EtBr in 1x TBE buffer, visualized and photographed at 295 nm.

27F: 5' GAG AGT TTG ATC CTG GCT CAG 3'

1541R:5’AAG GAG GTG ATC CAG CCG CA 3’

2.2.7.2a. Amplified ribosomal DNA Restriction Analysis (ARDRA):

Confirmed 16S rDNA amplicons from the selected 54 isolates and three type strains *B. safensis* FO-36b^{T}, *B. pumilus* ATCC 7061^{T}and *B. altitudinis* 41KF2b^{T} were digested by restriction enzymes namely *Alu*I, *Msp*I and *Hha*I. 7.0 µl of each PCR product was digested for 12 h at 37 °C with 0.3 µl of each restriction endonuclease

individually. Restriction fragments, separated on a 2% agarose gel containing 0.5 µg/ml of EtBr in 1x TBE buffer, were visualized and photographed at 295 nm. The restriction profiles were analysed and fragment sizes were estimated using a low range, 100 bp StepUp DNA ladder as marker. The band patterns were observed and their nucleotide base pair size determination was done using Alpha Ease FC software. Grouping of isolates was performed by a visual comparison of the restriction digestion gel patterns. With the help of MS Office 2010 and NTSYSpc 2.0 software phylogenetic tree was constructed.

2.2.7.2b. 16S rRNA gene sequence analysis:

Seven xylano-pectinolytic enzymes producers, M18, M33, and M35 from camel dung, R30 and R31 from cow dung and J208 and J216 from buffalo dung were selected for further genotypic analysis. 16S rRNA gene sequence was amplified using the PCR technique and confirmed as mentioned in Section 2.2.7.2. The PCR amplicons were sequenced using the universal eubacterial primers, 27F, 1107R and 1541R from Xcelris Lab (Ahmedabad, Gujarat, India). Obtained raw sequence data was further analysed.

27F: 5’ GAG AGT TTG ATC CTG GCT CAG 3'

1107R:5’ GCT CGT TGC GGG ACT TAA CC 3’

1541R:5’AAG GAG GTG ATC CAG CCG C A 3’

2.2.7.2c. 16S rRNA gene analysis for type strains:

Type strains *Bacillus safensis* FO-36b^T and *Bacillus pumilus* ATCC 7061^T were provided by Dr. Kasthuri Venkateswaran from Jet Propulsion Laboratory (JPL), NASA, USA) for the identification and analysis of our isolates and *Bacillus altitudinis* 41KF2b^T was purchased from MTCC India. For analysis of 16S rRNA gene sequence, FASTA files were procured from EZ-Taxon, Bio-cloud database or NCBI database, while genomic DNA was extracted from these type strains and ARDRA was performed as mentioned in Section 2.2.7.1 and 2.2.7.2a.

2.2.7.3. *gyrB* gene amplification and analysis:

gyrB gene sequence was amplified from the selected seven isolates and three type strains *B. safensis* FO-36b^T, *B. pumilus* ATCC 7061^{T}and *B. altitudinis* 41KF2b^T using the PCR technique in Eppendorf Thermocycler. Forward (UP-1) and reverse (UP-2r) degenerate universal primers were used to amplify the *gyrB gene* from the chromosome (Yamamoto and Harayama, 1995). Each reaction system (30 µl) contained 3 µl of 10x Reaction buffer, 1.5 µl of each primer (0.5 µM), 10 µl of dNTPs (2.5 µM

each), 0.5 µl of Taq DNA polymerase (1.3 units) and 1.5 µl of suitably diluted (30 ng/ µl) template DNA. The amplification was performed as follows: (i) initial denaturation for 5 min at 94 °C, (ii) 30 cycles of denaturation for 1.0 min at 94 °C, annealing for 1.0 min at 54.5 °C and extension for 2.0 min at 72 °C, and (iii) final extension for 15 min at 72 °C followed by hold at 4 °C. The presence of 1.2 kbp amplicons was confirmed by electrophoresis of the PCR product with 500 bp StepUp DNA ladder marker on a 0.8% agarose gel containing 0.5 µg/ml of EtBr in 1x TBE buffer which were visualized and photographed at 295 nm.

UP-1: 5' GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AAR TTY GA-3'

UP-2r:5'AGC AGG GTA CGG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT-3'

where degenerate codes were Y=C+T, N=A+T+G+C, R=A+G.

2.2.7.3a. Restriction Fragment Length Polymorphism (RFLP) analysis:

The *gyrB* gene amplicons from seven selected isolates were digested by restriction endonuclease enzymes (REs) namely *Alu*I, *Msp*I, *Hha*I and *Rsa*I. Single digest from (*Alu*I), double digest, (*Msp*I, *Rsa*I) and triple digest (*Hha*I, *Msp*I and *Rsa*I) were performed. 7.0 µl of each PCR product was digested for 12 h at 37 °C with 0.3 µl of each restriction endonuclease and restriction fragments were separated on a 2% agarose gel containing 0.5 µg/ml of EtBr in 1x TBE buffer, visualized and photographed at 295 nm. The restriction profiles were analyzed and fragment sizes were estimated using a low range, 100 bp StepUp DNA ladder marker. The band patterns were observed and their nucleotide base pair size determination was done using Alpha Ease FC software. Grouping of isolates was performed by a visual comparison of the restriction digestion patterns. Phylogenetic tree was constructed with the help of Microsoft Office 2010 and NTSYSpc software version (2.0).

2.2.7.3b. *gyrB* gene sequence analysis:

The *gyrB* gene amplicons of seven xylano-pectinolytic enzymes producers and the type strain *B. altitudinis* 41KF2b^T were sequenced using forward (UP-1S) and reverse (UP-2Sr) universal sequencing primers for *gyrB* gene (Yamamoto and Harayama, 1995) from Xcelris Lab (Ahmedabad, Gujarat, India). The raw sequence data obtained was further analyzed. The *gyrB* sequences for *B. safensis* FO-36b^T and *B. pumilus* ATCC 7061^T were available and procured in FASTA format from NCBI

database for sequence analysis, while for *B. altitudinis* 41KF2b^T, *gyrB* sequence was not available hence it was sequenced.

UP-1S: 5'- GAA GTC ATC ATG ACC GTT CTG CA-3'

UP-2Sr: 5'- AGC AGG GTA CGG ATG TGC GAG CC-3'

2.2.7.4. Sequence submission:

Offline data submission tool Sequin was used to generate the ".seq" format for submission of the nucleotide sequences for 16S rRNA and *gyrB* genes. Sequences have been submitted to NCBI database.

2.2.8. Phenotypic characterization:

Growth phenotypes, utilization of certain substrates for growth (Bochner, 2009) and antibiotic sensitivity tests (Corona and Martinez, 2013) were performed for phenotypic characterisation.

2.2.8.1. Growth phenotypes at various physicochemical parameters:

Growth phenotype at various physical conditions such as anaerobic and aerobic conditions, growth in presence of various salt concentrations (2,5,7 and 10% NaCl), growth at various pH (4.0 to 10.0 with increase of one unit), and growth at various temperatures (8, 15, 28, 37 and 45 °C) at static and shaking conditions were observed for selected seven isolates.

2.2.8.2. Biochemical characterization of selected isolates:

Several conventional biochemical phenotypic tests were performed for further characterization of the selected *Bacillus* spp. Media was prepared for individual culture as mentioned by MacFaddin (1976) and Zimbro et al., (2009) and results were analysed using Bergey's manual of Systemic Bacteriology volume three The Firmicutes, (De Vos et al., 2009) and the reported results for the type strains (Satomi et al., 2006; Shivaji et al., 2006), Indole production, urea utilization, Voges Proskauer, citrate utilization, lysine decarboxylase, nitrate reduction, gas from nitrate, phenylalanine deamination, catalase, malonate, ONPG (β-galacrosidase), β-glucosidase or esculin hydrolysis, arginine dihydrolase, starch hydrolysis, gelatin hydrolysis or liquefaction, casein hydrolysis, utilization of D-glucose, D-xylose, D-arabinose, sucrose, mannitol, trehalose etc and and acid formation form the same sugars were observed. Cellulose, pectin and xylan hydrolysis were also considered as a biochemical phenotype for isolates.

2.2.8.3. Antibiotic sensitivity test:

Antibiotic sensitivity tests were done using HiMedia octadisc. Antibiotics tested included Bacitracin (B, 10U), Chloramphenicol (C, 30mcg), Co-trimoxazole (CoT, 25mcg), Penicillin G (P, 10 U), Polymyxin B (PB, 300U), Gentamicin (Gen, 10mcg), Neomycin (N, 30mcg), Tetracycline (Tc, 30mcg) on octadisc GVII Plus. The disc was kept on a Mueller Hinton agar medium, with the overnight grown individual culture spread over the agar surface. Incubation was done at 37 °C for 24 h and result for zone of growth inhibition was observed, diameter was measured and the sensitivity and graded as Sensitive (S), Intermediate (I) and Resistant (R) based on the analysis reference table given by HiMedia.

2.2.9. Screening based on xylanase and pectinase activities in different pH and temperature conditions:

Overnight grown individual cultures were grown on NB and inoculated in media containing WB or CP as mentioned in Section 2.2.5.1. CFS was used as crude source of enzyme. Assay for xylanase and pectinase was performed as mentioned in Sections 2.2.5.2 and 2.2.5.3 with the following modifications.

2.2.9.1. Xylanase and pectinase activities at different pH:

Assay was performed by incubating crude enzymes at 40 °C with the 0.5% substrates prepared in 50 mM buffers with different pH conditions. Buffer solution(s) used for different pH were pH 4.0, pH 5.0, pH 6.0, Citrate-Phosphate buffer; pH 7.0, pH 8.0, pH 9.0, Tris–Cl buffer and pH 10.0 Carbonate-Bicarbonate buffer.

2.2.9.2. Xylanase and pectinase activities at different temperatures:

Assay was performed by incubating crude enzymes with the 0.5 % substrates prepared in 50mM Tris-Cl buffer pH 7.0 at 20, 30, 40, 50, 60, 70 and 80 °C.

2.2.10. Characterization of selected isolates:

The selected three *Bacillus* isolates M35, R31 and J208 were further characterized on the basis of xylanase and pectinase production on WB and CP respectively and growth patterns. SEM was performed to study cell morphology.

2.2.10.1. Scanning electron microscopy (SEM):

Three of the selected *Bacillus* isolates M35, R31 and J208 were grown on NB medium for 16 h. Cells were pelleted at 2,000 rpm and washed with Phosphate buffered saline (PBS) pH 7.2 ± 0.2. The culture was fixed in 2.5% v/v glutaraldehyde, again

washed with PBS and dehydrated in a series of increasing acetone concentrations i.e., 10, 25, 50, 75 and 100% for 10 min each (Rheims et al., 1999). 5 µl of the cell suspension in absolute acetone was spotted on the copper foil fixed on metal stub and sputter coated with Platinum (Pt) in Auto Fine Coater (JEOL-JFC-1600) and cells were observed with FEG-SEM (JEOL, JSM-7600F) at SAIF, IIT-Powai, Mumbai, India.

2.2.10.2. Time course production of xylanase-pectinase enzymes:

The three isolates were inoculated in 10 ml NB and incubated till OD_{600} reached 0.2 unit and 0.5% inoculum was transferred to BHM-YEP media containing 0.5% WB or CP and incubated as mentioned in section 2.2.5.1. At every 12 h 0.5 ml samples were withdrawn, centrifuged at 10,000 rpm and WB and CP CFS were assayed for xylanase and pectinase assays respectively as mentioned in Section 2.2.5.2 and 2.2.5.3.

2.2.10.3. Growth curve:

The three isolates were inoculated in 10 ml NB and incubated till OD_{600} reached 0.2 unit. Then 0.5% inoculum was transferred to 100 ml NB. 1.1 ml aliquots were withdrawn at each 1 h, 0.1 ml from that was used to prepare dilutions in 0.85% saline and plated to estimate Colony Forming Unit (cfu) /ml and remaining sample was used to estimate the OD_{600}.

2.2.11. Strain deposition:

Isolates, *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were submitted to National Centre for Microbial Repository (NCMR, formerly known as Microbial Culture Collection - MCC, Pune, India) in the form of streaked pure cultures on NA plates.

2.2.12. Data analysis:

- All the Quantitative estimation experiments were performed in triplicates and data and error values are represented as Mean ± Standard Error of Mean (SEM) either in GraphPad Prism 6.0 or Origin 2017 software.
- ARDRA and RFLP band patterns were observed and their nucleotide base pair size determination was done using Alpha Ease FC software. Presence or absence of a particular sized band after analysing various digestion pattern was considered as positive (1) or negative (0) values as binary traits respectively. Distance matrix was generated using Simqual method and cluster analysis was done based on this matrix using simple matching according to the unweighted pair group method with arithmetic

averages (UPGMA) in the NTSYSpc software (version 2.0; Exeter Software, USA).

- For phylogenetic analysis of 16S rRNA and *gyrB* gene, raw data sequences obtained from Xcelris were checked for quality of the base call, and reverse sequences were reverse complemented and aligned with forward sequences and contig was generated using Chromas Pro software 2.0. The generated contigs of 16S rDNA sequences were analysed using EZ-Taxon bio-cloud database of 16S rRNA gene sequences, while contigs of *gyrB* gene sequences were analysed using BLASTn tool at NCBI database to determine the maximum similarity for identification. A distance matrix was generated using the maximum composite likelihood model and the evolutionary distances are in the units of the number of base substitutions per site. The evolutionary history was inferred and tree was constructed using the Neighbour-Joining method for bootstrap value of 1000. The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

2.3. Results and Discussion:

Three *Bacillus* cultures, able to produce high cellulase free xylanase and pectinase enzymes, were selected from 468 bacterial isolates during these extensive polyphasic studies. The isolation, screening, identification and characterisation with respect to the application of the selected isolates is presented in this chapter.

2.3.1. Sample collection, enrichment and isolation:

Enrichment culture technique, where environmental conditions can be controlled and imposed on microorganisms to create a selective environment (Stanier et al., 1993), and Winogradsky column were setup to enrich plant polysaccharide degrading bacteria from different sources. Based on reasons cited in introduction Section 2.1 the samples were collected from the habitats where plant cell wall degrading enzyme producers were expected to inhabit abundantly. Therefore, ruminant dung, decaying wood, farm yard manure and Winogradsky column were used for isolation.

- **Enrichment culture technique:**

Enrichment culture technique was designed in such a way that in the final step bacteria able to produce complex plant polysaccharide degrading enzymes such as xylanase, pectinase and cellulase could be selected. A three-step enrichment procedure was followed in three different media as listed in Section 2.2.3.1. In first step, EM1 medium was supplemented with nutrient broth along with crude plant substrates to

allow the growth of all possible microbes. To create a selective pressure for carbon source, during the second step, in EM2 minimal salt medium, low concentration of glucose was supplemented along with polysaccharides. Those which can utilize glucose would shift on polysaccharides once glucose was depleted. In the third step, EM3 minimal salt medium was not supplemented with glucose to further create pressure for production of enzymes for utilization of one or many of the polysaccharide substrates as carbon source. After enrichment, the samples were diluted and subjected to isolation.

- **Winogradsky column:**

Since the salts and carbon substrates (if any) used in the column were selected with such a criteria that they favour the enrichment of certain selective traits and once the crude enrichment system was established, pure cultures can be pursued from them (Madigan et al., 2011; Willey et al., 2013). Hence, the cellulose papers and vegetable wastes were used as major substrates in the column for enrichment of polysaccharide hydrolase producing organism. The Winogradsky column itself was an enrichment system and hence after incubation of seven days the samples were collected from the column and subjected to dilution and isolation.

Ruminants are mammals which acquires their nutrition from plant-based food by fermenting it in a specialized four lobbed stomach. During this process of rumination, they regurgitate and chew the ingested food again and again, to break it down in smaller size as this process increases the surface/volume ratio and enhances the microbial fermentation of the food. Since there are reports of isolation of microorganisms with various cellulase, xylanase and other polysaccharide hydrolase activities from the ruminant dung samples (Girija et al., 2013; Sun et al., 2015), it was decided to collect samples of different ruminant dungs. Herbivore ruminants like cow, bull, buffalo, camel etc., feeding mostly upon grass and plant materials available from surroundings were selected for sampling and fresh dung samples of these animals were collected. Farm yard manure was considered as a sample because it is a mixture of faecal matter of ruminants and dried plant parts like grasses, leaves etc., hence it develops a suitable environment for polysaccharide hydrolase producing bacterial and fungal microorganisms to flourish. Similarly, decaying wood has a likelihood of plant cell wall degraders and was selected for isolation. Winogradsky column spiked separately with vegetable wastes and waste paper were set up to enrich microorganisms that produce cell wall degrading enzymes. The details of sample types and specificities

for their collections is mentioned as below in Table 2.2.

Table 2.2. Sampling details- source of samples and specifications for sample collection:

Sample	Sample source and description
(a) Dung samples of herbivores fed on locally harvested dried crops and grass	
01 RN	Bull dung-BLD*
02 RN	Cow dung-CWD*
03 LMP	Cow dung-CWD*
04 MND	Camel dung-CMD[#]
05 JNG-1	Buffalo dung-BFD*
06 JNG-2	Buffalo dung-BFD*
(b) Partially decayed wood samples	
07 BKH-3	Termite mound near decayed wood-TMW
08 BKH-4	Moistened and partially decayed woody mass -PDW
(c) Farm yard manure containing grass, plant parts and animal dung	
09 JNG-5	Farm yard manure dry-FYMD
10 JNG-6	Farm yard manure wet with water-FYMW-1
11 JNG-7	Farm yard manure wet with water-FYMW-2
(d) Winogradsky Column	
12 WCP	Pond water from garden, mud and raw vegetable waste
13 WCS	Sewage from domestic treatment plant, mud and waste paper

*Grass and plant waste material from harvested crops used as fodder, [#]Acacia and other plant material available nearby used as fodder

Table 2.3. Distribution of plant polysaccharide degrading isolates obtained from individual samples:

Sample Name	Code given according to sample type	Isolates count from individual sample	Total	
RN	BLD	R01-R35 (35)	113	468
	CWD	R36-R113 (78)		
LMP	CWD	L01-L136 (136)	136	
MND	CMD	M01-M36 (36)	36	
JNG	BFD	J101-J117 (17)	100	
	BFD	J201-J217 (17)		
	FYMD	J501-J517 (17)		
	FYMW	J601-J615 (15)		
	FYMW	J701-J734 (34)		
BKH	TMW	B301-B323 (23)	47	
	PDW	B401-B424 (24)		
WC	WCP	WS01-WS15 (15)	30	
	WCS	WC01-WC15 (15)		
LC	LC	LC01-LC06 (06)	6	

The core enzyme group comprising various cellulases and the accessory enzyme group comprising diverse xylanases and pectinases are the major ones which can perform plant biomass saccharification and were of major interests. Therefore, the collected samples were further subjected to enrichment of organisms which can breakdown complex plant polysaccharides enzymatically and use the products for their growth. Hence, the enriched samples were expected to harbour a population of microorganisms producing one or more enzymes to breakdown the polysaccharides substances provided in media. After enrichment and isolation process, 462 isolates from 13 different samples were obtained. Six other laboratory contaminants were also taken in consideration since they grew on the complex polysaccharide containing agar plates. So as depicted in Table 2.3, the total of 468 isolates were obtained and were further subjected to primary quantitative screening studies.

2.3.2. Primary screening of isolates producing polysaccharide hydrolase:

The 468 isolates were replica gridded and screened on the plates containing either pure or crude polysaccharide substrate sources for production of extracellular xylanase, pectinase and cellulase activities. Beechwood xylan, pectin and Carboxy Methyl Cellulose (CMC) are the pure polysaccharides and commercially available. WB and CP have been reported as a good source of xylan and pectin respectively (Immerzeel et al., 2014; Kanmani, 2014) while WFP is a known cellulose source and hence these were used as crude polysaccharides in media for growth and enzyme production. The screening method for all three enzymes was based on agar diffusion method, where the secreted and diffused extracellular enzyme gave an activity zone.

2.3.2.1. Screening of xylanase producing bacteria:

Qualitative screening for xylanase activities of the bacterial isolates individually on pure (beechwood xylan) and crude (WB) substrate is represented in Figure 2.1(A) and 2.1(B) respectively. CR binds strongly to β-1,4-xylosidic linkages present in xylan, which are also active sites for most of xylanases. Enzyme produced by the isolates, diffused around the colonies, hydrolysed the substrates producing a zone of clearance. Excess CR was washed off from the plate with NaCl and pale or light orange halo in dark red background was observed. Since in acidic conditions (at pH < 3.5) CR changes its colour from red to blue, the plate was further flooded with 1 N HCl and blue halo in purple background was observed for xylanase activity. A similar method was used by Teather and Wood (1982) and also by Ruijssenaars and Hartmans (2001).

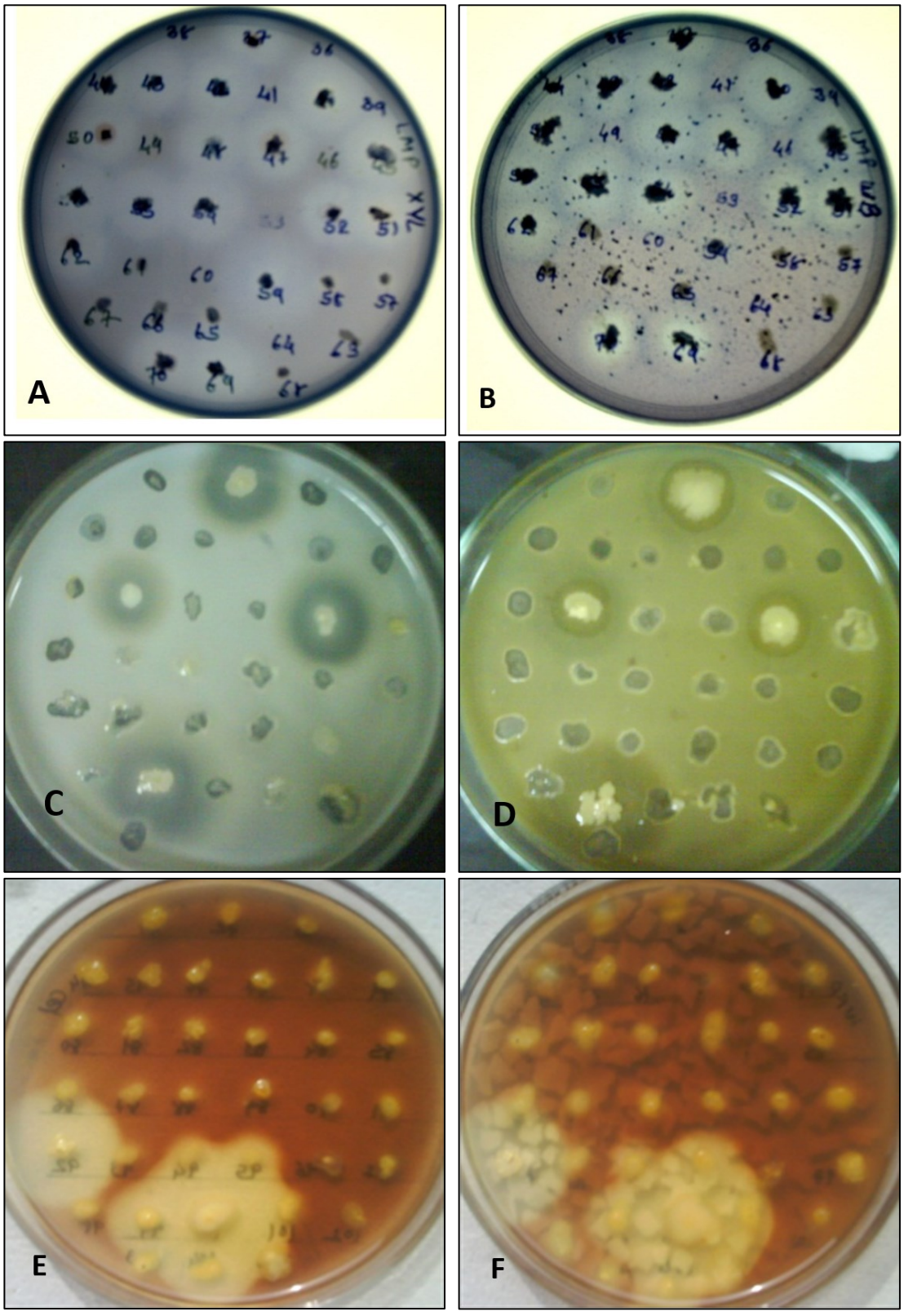


Figure 2.1. Representative images of primary (qualitative) screening for xylanase, pectinase and cellulase enzymes:
(A) Xylan-CR, **(B)** WB-CR, plates flooded with HCl to show xylan hydrolysis by xylanase; **(C)** Pectin, **(D)** CP, plates flooded with CPC to show pectin hydrolysis by pectinase; **(E)** CMC, **(F)** WFP#1, plates flooded with Lugol's Iodine to show cellulose hydrolysis by cellulase.

2.3.2.2. Screening of pectinase producing bacteria:

Qualitative screening for pectinase activities of the bacterial isolates individually on pure (pectin) and crude (CP) substrate has been represented in Figure 2.1(C) and 2.1(D) respectively. Pectin or pectic acid is the acidic polymer having anionic charges in dissolved condition. When cationic detergent CPC was added to pectic acid, they interacted with anionic charged pectic polysaccharide substrates and precipitated them creating an opacity on the plate where enzymatic digestion of the pectic substrate was observed as clear halo surrounding the colonies due to pectinase activity. Hadj-Taieb et al., (2011) had reported a similar method for development of pectinase zymogram.

2.3.2.3. Screening of cellulase producing bacteria:

Qualitative screening for cellulase activities of the bacterial isolates individually on pure (CMC) and crude (WFP#1) substrate has been represented in Figure 2.1(E) and 2.1(F) respectively. Lugol's iodine binds strongly to β-1,4-glycoosidic linkages present in cellulose, which are also active sites for most of the cellulase enzymes. Enzyme produced by the isolated colonies, diffused around them and hydrolysed the substrates. After flooding the plate with Lugol's Iodine, the clear zone of hydrolysis was observed in brown background for cellulase production. Kasana et al., (2008) has proved this method to be more efficient than the CR staining method for cellulase.

A comparative analysis for presence of zones on all substrates unveiled diverse ability of isolates for production of one or more kind of enzymes. Although majority of non-producers, it was observed total of 174/468 isolates were able to produce either one or two or all three enzymes. Sample wise details of enzyme producers and non-producers has been depicted in Figure 2.2(A) while a cumulative overview of total producers from all samples for their number count and percentage based on diverse enzyme production has been depicted in Figure 2.2(B).

Out of 174, 20 isolates produced only single kind of enzyme which included 9, 10 and 1 isolates producing cellulase, xylanase and pectinase respectively. 116 isolates were producers of all the three enzymes. While only one isolate could produce both cellulase and pectinase. Second predominant group was cellulase and xylanase producers which was also in accordance with the reports suggesting that many fungal and bacterial isolates can concomitantly produce cellulase and xylanase enzymes (Sizova et al., 2011; Chutani and Sharma, 2016) or multifunctional enzymes having

xylanase activity along with any of β-glucosidase, cellobiohydrolases, endoglucanase, arabinofuranosidases feruloyl esterase activities (Van Dyk and Pletschke, 2012). Eight isolates produced both xylanase and pectinase but not cellulase which is the group of interest for present studies. *B. pumilus* AJK has been reported for production of cellulase free xylano-pectinolytic enzymes (Kaur et al., 2010). Thus, count of 468 was narrowed down to 174 isolates positive for one or more types of polysaccharide hydrolase production and they were subjected to further studies.

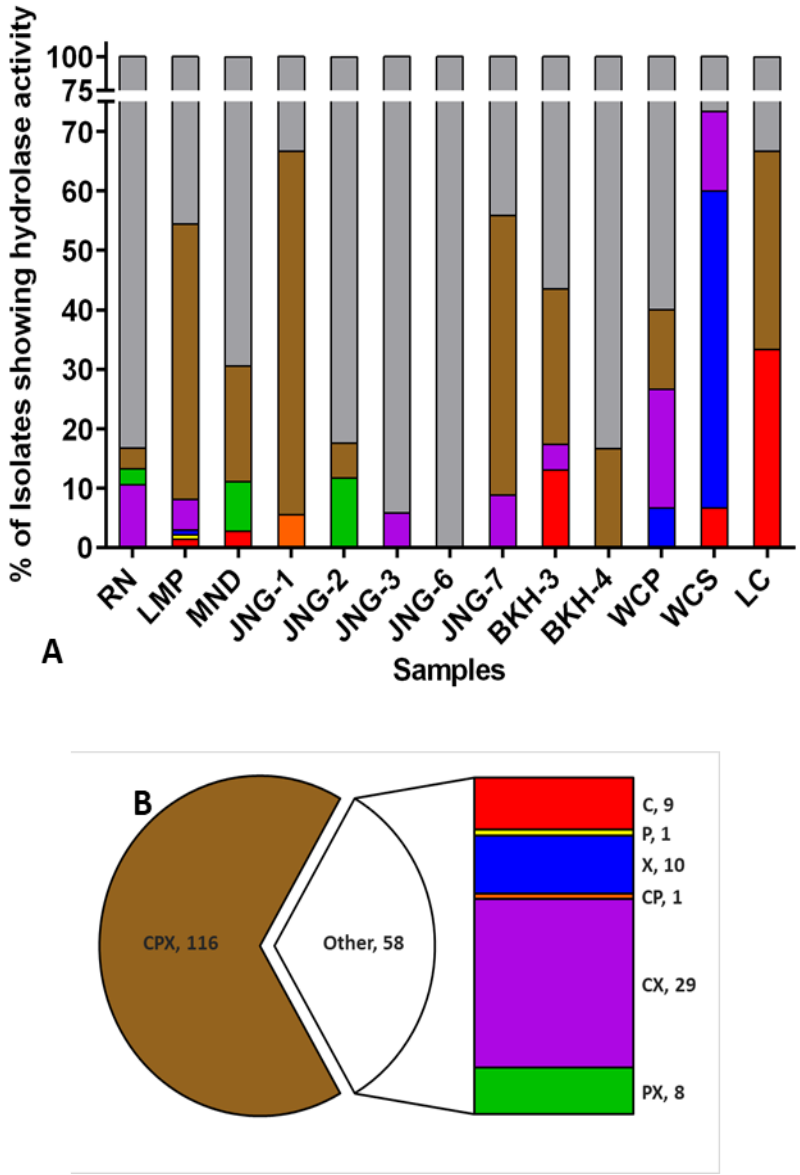
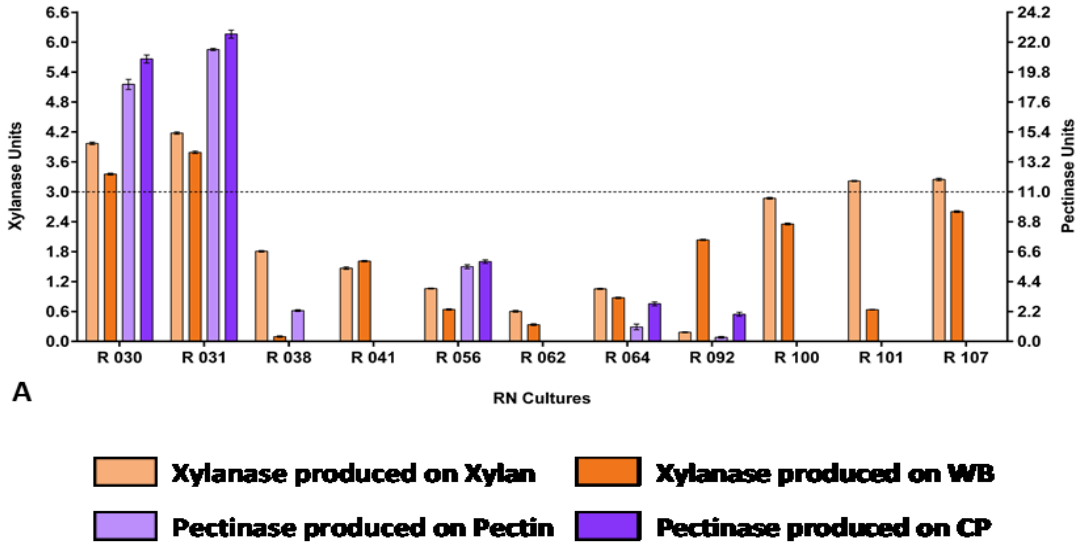


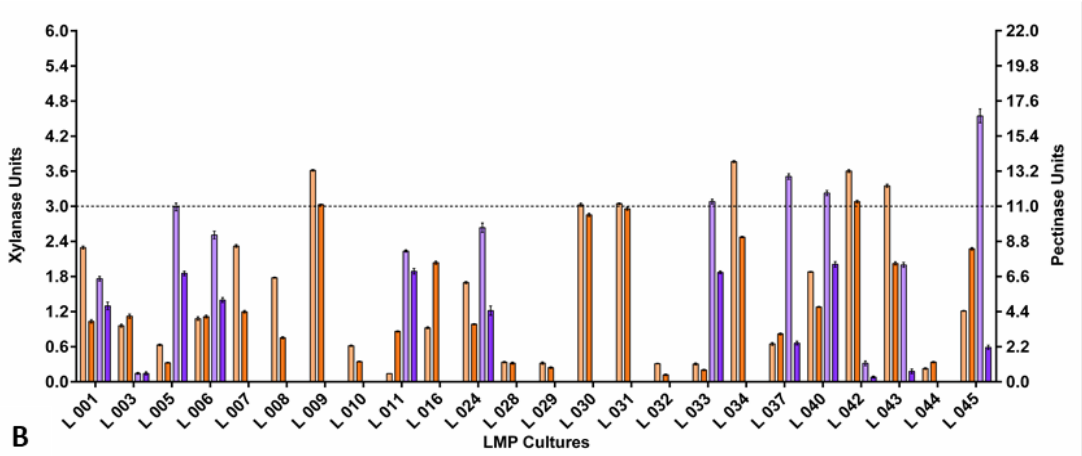
Figure 2.2. Summary of qualitative screening for polysaccharide hydrolase production by the bacterial isolates:
(A) Sample wise representation of polysaccharide hydrolase enzyme producers and nonproducers; **(B)** Total number of enzyme producers, combined from all the samples based on production of one enzyme: cellulase (C), pectinase (P), xylanase (X); two enzymes: cellulase and pectinase (CP); cellulase and xylanase (CX); pectinase and xylanase (PX); and three enzymes: cellulase, pectinase and xylanase (CPX); enzyme non-producers.

2.3.3. Quantitative screening of xylanse-pectinase producing bacteria:

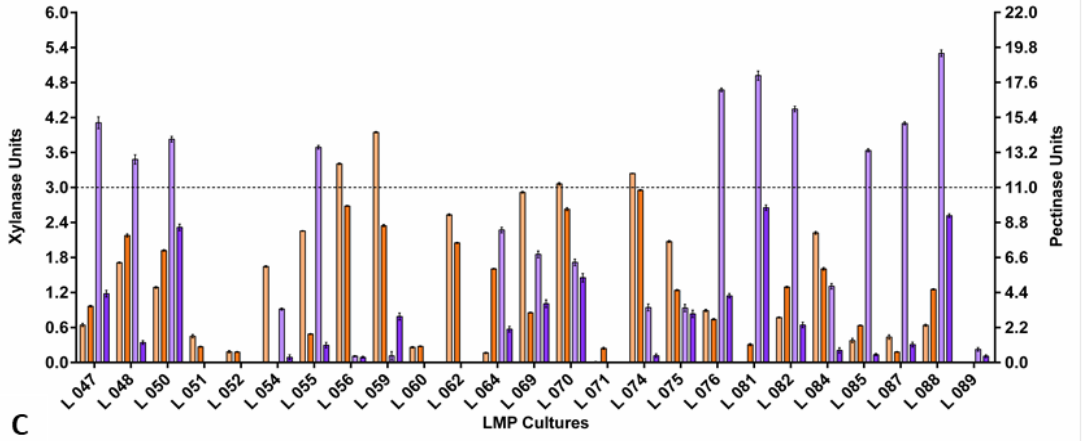
Xylanase and pectinase activities were quantified from 174 isolates. The isolates were grown on both pure and crude polysaccharides individually. The xylano-pectinolytic activities obtained after growth on pure and crude polysaccharide containing media by individual cultures were calculated according to the unit definitions provided in Section 2.2.5. Though the standard unit definitions were followed for the enzyme activity calculations, when it comes to a comparison of enzyme unit calculations with reported units from other laboratories, use of different types of substrates obtained from different sources and degrees of substitution in substrates, different estimation methods, types of enzymes and their mode of actions etc., are the parameters which offer poor comparability of reported xylanase activities (Bailey et al., 1992). Same problem was observed in case of pectinase estimations and hence realistic comparison between the methods in practice was extremely difficult. Therefore, the cut-off values of 3 and 11 units respectively for xylanase and pectinase were decided based on the observed activities.

Following is the Figure 2.3 (A-H) which represents comparison of xylanase and pectinase activities of all the isolates in pure and crude polysaccharide containing media. As observed from Figure 2.3 R101, R107, L009, L030, L070, L127, L134, J115 etc., are few of the 19 isolates with xylanase activities above the 3 units whereas L005, L045, L096, L108, L136, M001, M008, M031, J102, J108, J504, W008, W010 etc., are few of the 44 isolates with pectinase activity above 11 units. Out of these 63 isolates, most of the isolates exhibited xylanase or pectinase activities above cut-off levels in the CFS obtained from the production media containing pure polysaccharides.

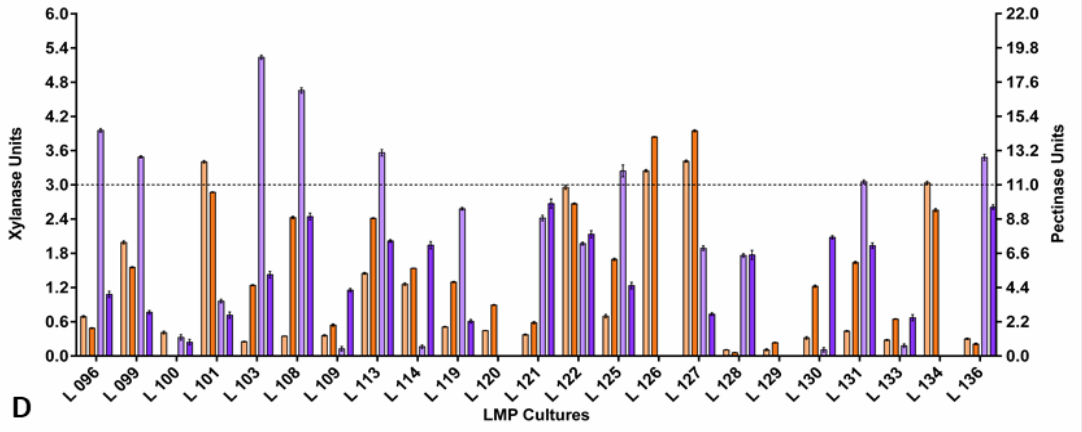



Xylanase Units
Pectinase Units
L 001 L 003 L 005 L 006 L 007 L 008 L 009 L 010 L 011 L 016 L 024 L 028 L 029 L 030 L 031 L 032 L 033 L 034 L 037 L 040 L 042 L 043 L 044 L 045
LMP Cultures

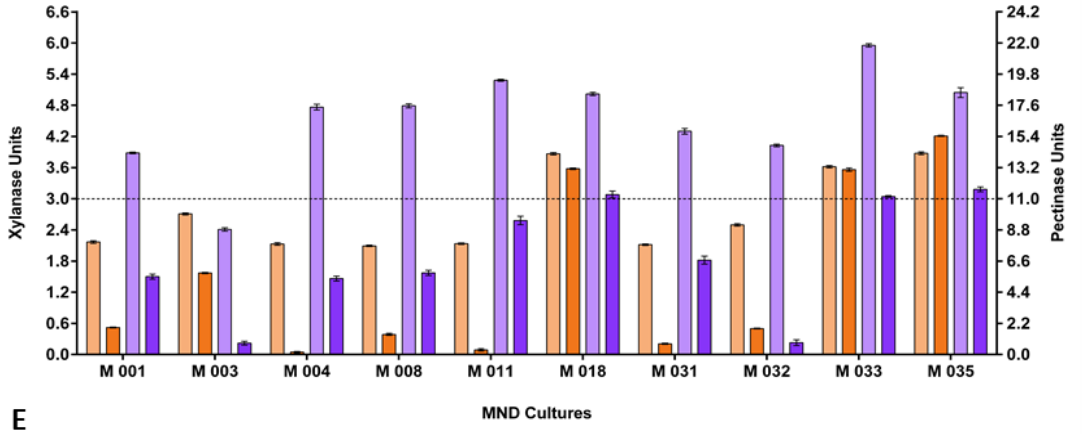
B


Xylanase Units
Pectinase Units
L 047 L 048 L 050 L 051 L 052 L 054 L 055 L 056 L 059 L 060 L 062 L 064 L 069 L 070 L 071 L 074 L 075 L 076 L 081 L 082 L 084 L 085 L 087 L 088 L 089
LMP Cultures

C


Xylanase Units
Pectinase Units
L 096 L 099 L 100 L 101 L 103 L 108 L 109 L 113 L 114 L 119 L 120 L 121 L 122 L 125 L 126 L 127 L 128 L 129 L 130 L 131 L 133 L 134 L 136
LMP Cultures

D


Xylanase Units
Pectinase Units
M 001 M 003 M 004 M 008 M 011 M 018 M 031 M 032 M 033 M 035
MND Cultures

E

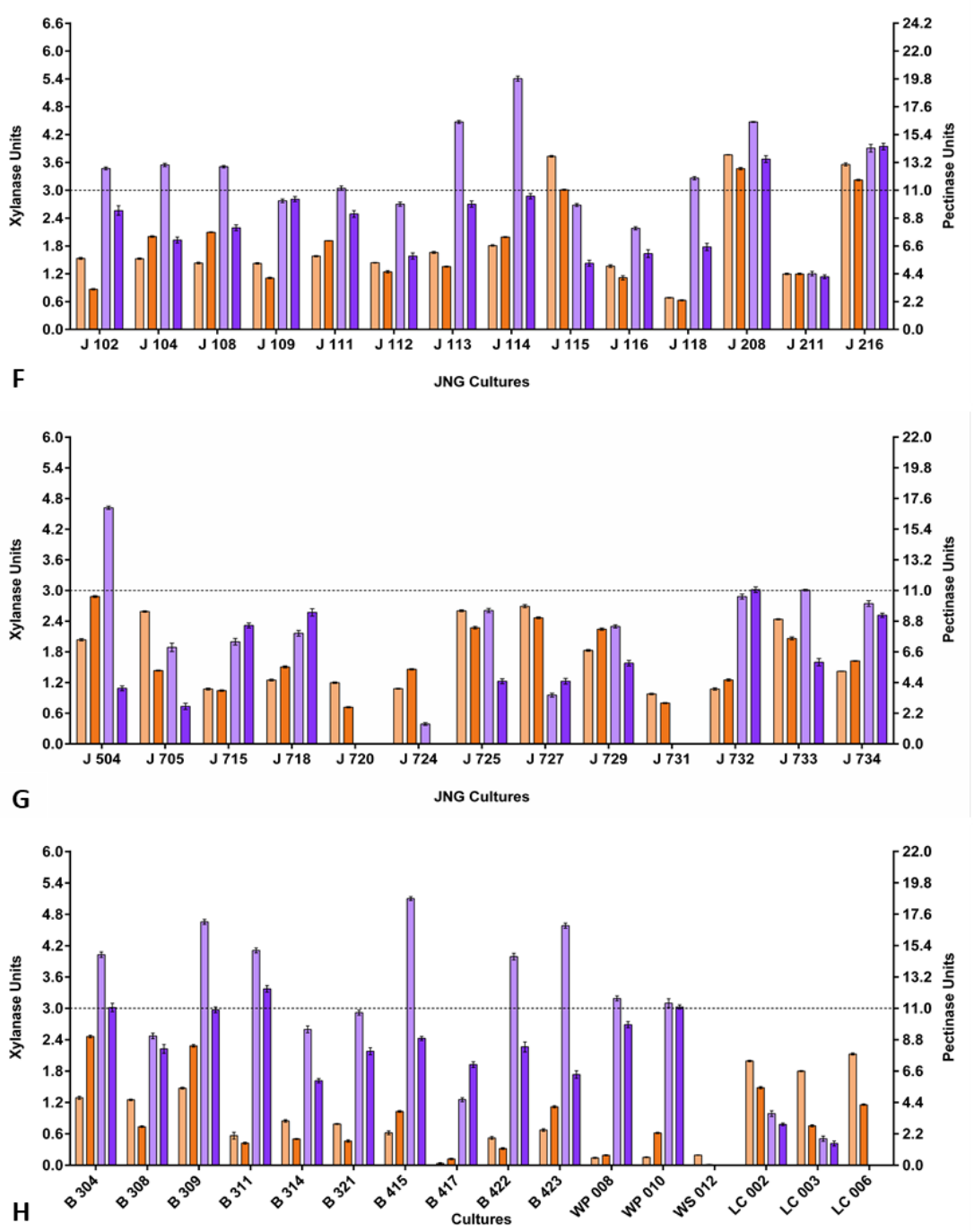


Figure 2.3. Secondary (quantitative) screening of xylanase and pectinase activities:
Image represents the comparison of xylanase (on left Y-axis) and pectinase (on right Y-axis) activities of individual isolates obtained from various samples as **(A)** RN: cow and bull dung sample, **(B, C and D)** LMP: cow dung sample, **(E)** MND: camel dung sample, **(F)** JNG: buffalo dung sample, **(G)** JNG: farm yard manure, **(H)** BKH, Winogradsky column sample and Lab contaminants; Column and error bars represents Mean ± Standard Error of Means for n=3. (Refer Table 2.2 and 2.3 for sampling details).

Only seven cultures M18, M33, M35, R30, R31 J208 and J218 exhibited both xylanase and pectinase activities above the cut-off values of 3 and 11 units respectively from the CFS obtained from the production media containing both pure and crude polysaccharides. Based on the observed activities and cut-off levels, the isolates again

can be sorted as represented in Table 2.4. This screening narrowed down the isolates count further to 70 from 174. Out of which seven isolates were cellulase free xylanase and pectinase producers. While 44 were pectinase producers with either xylanase and/or cellulase producing ability, 19 were xylanase producers with either pectinase and/or cellulase producing ability.

Table 2.4. Bacterial isolates showing xylanase and pectinase activities above cut-off level from different samples:

Categories based on cut-off values for xylanase (3 units) and pectinase (11 units)	RN	LMP	MND	JNG	BKH	WC	LC	Total
Xylanase above 3 units and pectinase above 11 units	02	--	03	02	--	--	--	**07**
Xylanase above 3 units and irrespective of pectinase	02	14	--	03	--	--	--	**19**
Pectinase above 11 units and irrespective of xylanase	--	22	06	08	06	02	--	**44**
Xylanase below 3 units and pectinase below 11 units	07	36	01	14	04	01	03	**66**
Total	11	72	10	27	10	03	03	**136**

A comprehensive analysis of qualitative and quantitative screening led to the conclusion that, as the samples were collected from different niches where plant materials are being digested or decayed, bacterial isolates from these sample had the ability to produce one or more than one type of complex polysaccharide hydrolase enzymes. The selected 70 isolates were further studied for their morphological features.

2.3.4. Morphological and microscopic characterization of isolates:

The results of microscopic analysis of each of 70 isolates for Gram nature, cell size and shape, sporulation, shape and location of spore etc., and along with their enzyme activity these isolates were screened further. Figure 2.4 represents the microscopic images of Gram staining and endospore staining.

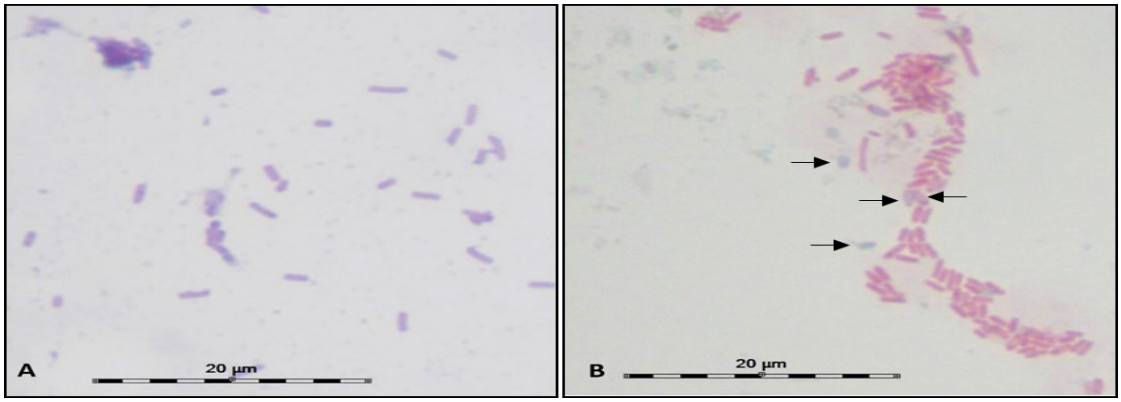


Figure 2.4. Representative brightfield micrographs of isolate J208:
(A) Gram staining and **(B)** Endospore staining (Endospores indicated with black arrows).

And the observations regarding colony morphology of isolates and microscopy are reported in the Table 2.5. Thus, after screening at qualitative, quantitative, and morphological level, the selected 70 isolates were further reduced to 54 isolates which were distributed in three categories based on xylanase and pectinase production. The isolates were distributed as 31 pectinase producers, 16 xylanase producers and seven cellulase free xylanase and pectinase producers. The selected aerobic, Gram positive, spore forming rods were classified into order *Bacillales*. Some of the literature reports citing cellulase, xylanase and pectinase production by different *Bacillus* spp. are shown in Table 2.6.

Table 2.5. Morphological and microscopic characteristics observed for isolates:

Feature of colony morphology	Common observations
Shape	Circular, irregular circular
Margin	Entire, lobate, serrated, undulate
Elevation	Raised, convex, flat, capitate
Surface texture	Smooth, alveolate, corrugated, punctate, rough,
Opacity and pigmentation	Opaque, translucent, transparent, chalky white, pale yellow, etc.
Gram nature	Gram positive
Cell shape and size	Rods with1.5 $\pm$ 0.2µm length and 0.6 $\pm$ 0.1µM width.
Endospores	Cylindrical Endospores located centrally of sub-terminally with swollen sporangia in most cases

Table 2.6. *Bacillus* spp. reported for production of xylanase, pectinase and cellulase enzyme(s):

Microorganism	Enzyme activities*			References
	Xylanase	Pectinase	Cellulase	
Bacillus subtilis AJK	✓	✓	-	Kaur et al., (2011)
Paenibacillus xylanolyticus	✓	✓	✓	Giacobbe et al., (2014)
Bacillus altitudinis DNH8	65	ND	ND	Adhyaru et al., (2014)
Bacillus circulans	400	ND	0.05	Subramaniyan and Prema, (2000)
Bacillus stearothermoohilus T-6	2.3	ND	--	
Bacillus sp.	120	ND	--	
Braviabaciilus borstelensis	ND	5.2	--	Demir et al., (2014)
Bacillus sp. DT7	ND	15.2	ND	Kashyap et al., (2000)
Bacillus sp. TS44	ND	✓	ND	Jayani et al., (2005)
Bacillus marcerans	ND	✓	ND	
Bacillus sp.	ND	✓	ND	
Bacillus sp. KSM-P410	ND	✓	ND	

*Mention of enzyme: either presence (✓) or absence (--) of enzyme in references; Values represent enzyme units reported; ND: Not Determined

2.3.5. Genotypic characterization of selected isolates:

These selected 54 isolates were carried further for 16S rRNA gene and *gyrB* gene analysis. The method reported by Wilson (2001) for the gDNA extraction was modified to include a step of lysozyme treatment since the isolates were Gram positive. The results obtained from type strains *B. safensis* FO-36b^T, *B. pumilus* ATCC 7061^{T}and *B. altitudinis* 41KF2b^T were included in the studies from here onwards although these specific species were chosen only after the confirmed identification of the finally selected isolates.

2.3.5.1. Characterization by 16S rDNA analysis:

Amplification of 16S ribosomal gene fragment (16S rDNA) from the gDNA of the isolates as well as three *Bacillus* type strains was carried out as mentioned in Section 2.2.7.2. Figure 2.5(A) represents the 1.5 kb sized amplicon of the 16S rRNA gene. The amplicons were further analysed by ARDRA and sequenced.

2.3.5.1a. ARDRA analysis:

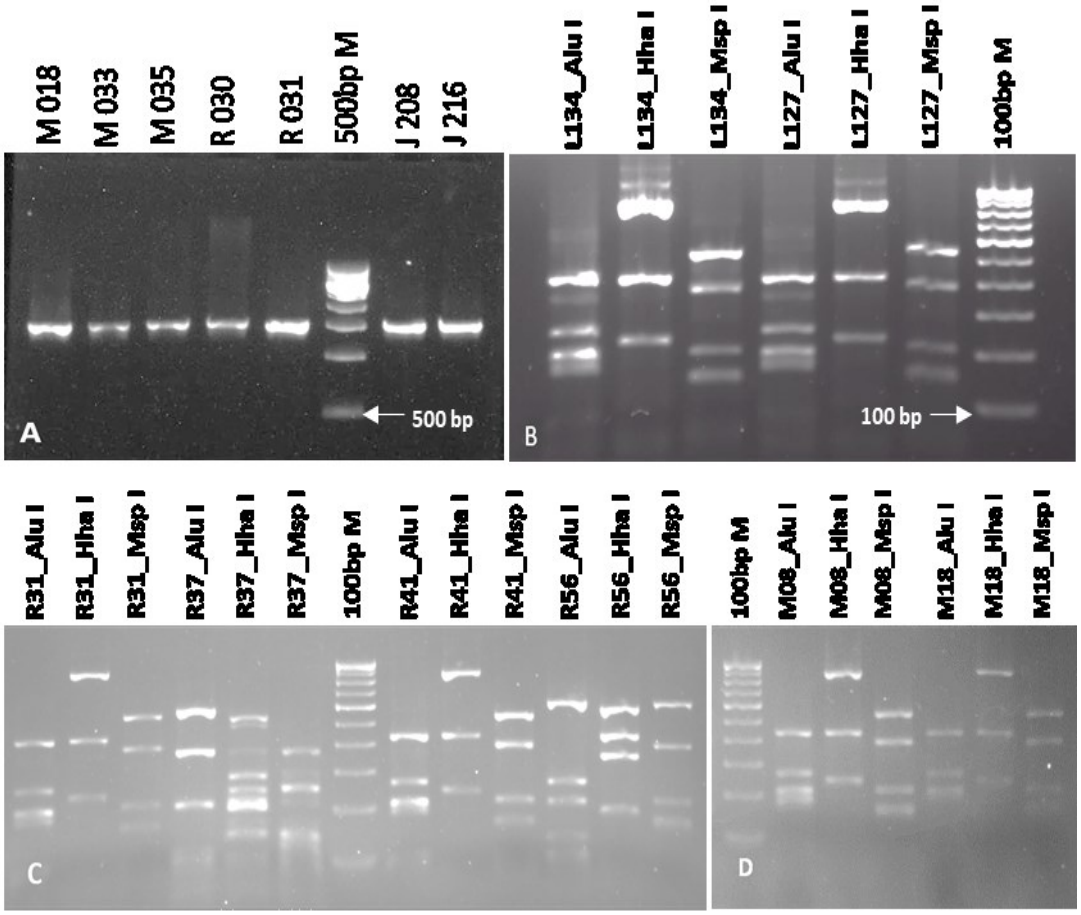


Figure 2.5. Representative image of 16S rRNA gene amplification and ARDRA pattern: **(A)** ~1.5 kb amplification product for 16S rRNA gene; **(B, C** and **D)** Band patterns of RE digested 16S rRNA gene; Labels indicates name of culture and RE used for digestion; M:500bp DNA StepUp ladder marker for **(A)**; M: 100bp DNA StepUp ladder marker for **(B, C** and **D)**.

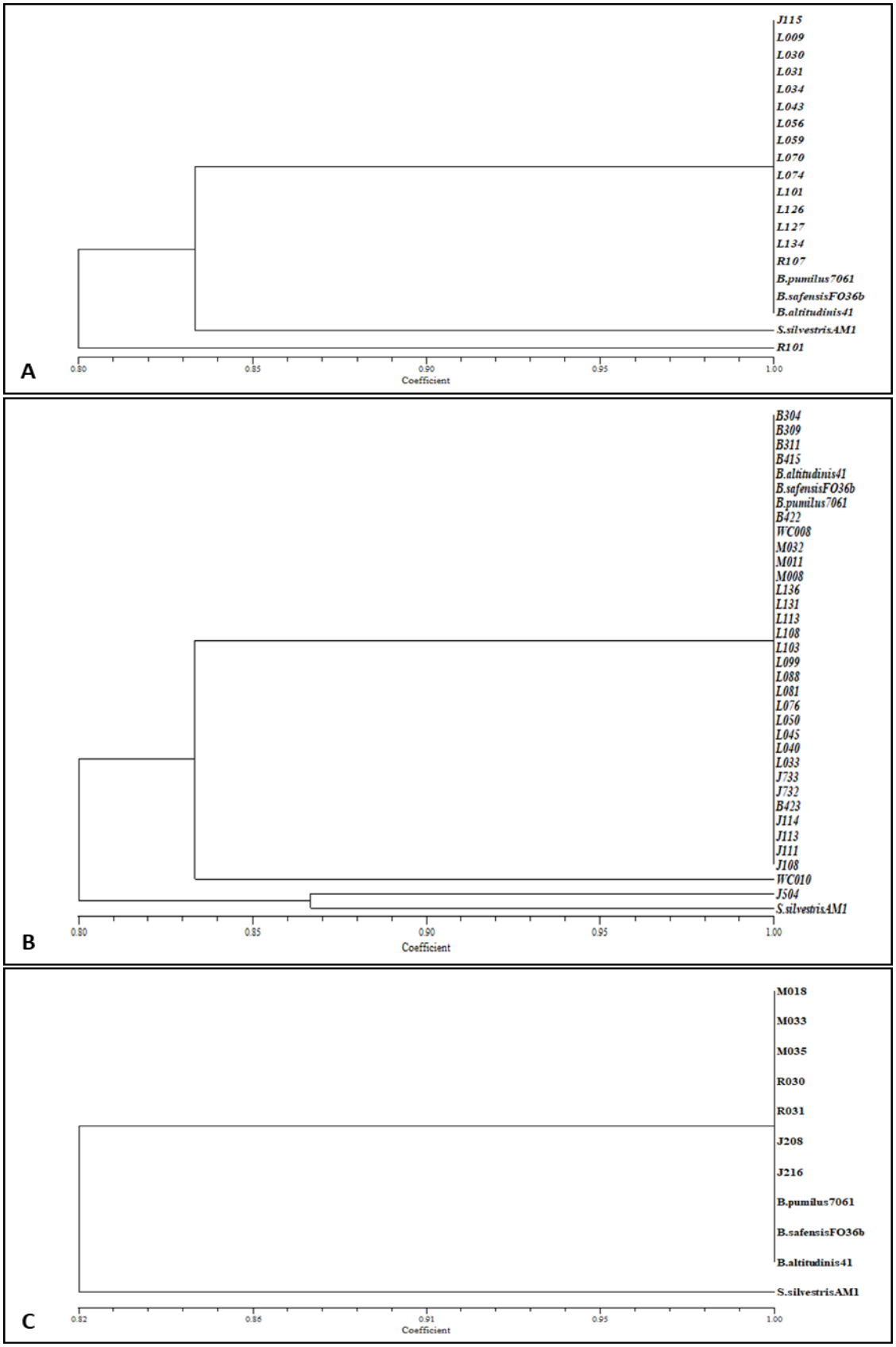


Figure 2.6. Dendrograms of 54 selected isolates with three type strains *B. safensis* FO-36b^T, *B. altitudinis* 41KF2b^T and *B. pumilus* ATCC 7061^T:
(A) 16 Xylanase producing isolates; **(B)** 31 Pectinase producing isolates and **(C)** 7 Cellulase free xylanase-pectinase producing isolates.

Figure 2.5 (B, C and D) represents ARDRA analysis of representative isolates by three different enzymes viz. *Alu*I, *Msp*I and *Hha*I. The ARDRA band pattern depicted as a dendrograms in Figure 2.6 upon analysis with NTSYSpc software V2.0 helped to group the isolates in one single cluster, which was not useful in further differentiation.

The seven cellulase free xylano-pectinolytic enzyme producers were therefore picked for further studies. They were interesting because as per our knowledge only one bacterial isolate *Bacillus pumilus* AJK MTCC 10414 has been reported for production of cellulase free xylanase-pectinase enzymes with respect to their industrial applications (Singh et al., 2015; A. Kaur et al., 2016). Though *Paenibacillus xylanolyticus* has been reported for production of xylanase (Park, 2013) and pectinase (Giacobbe et al., 2014), production of endoglucanase (Park et al., 2012) and β-glucosidase (D.J. Park et al., 2013) from the same has also been reported. Therefore, this category of isolates was considered important for their attribute of producing cellulase free accessory enzymes with diverse application and were taken up for further identification studies.

Subsequently, based on their genotypic 16S rRNA gene identification discussed in the next section, three type strains *B. pumilus* ATCC 7061^T, *B. altitudinis* 41KF2b^T and *B. safensis* FO-36b^T having close identity with the cellulase free xylanase-pectinase enzyme producing isolates were procured and their ARDRA analysis was performed and included in the dendrograms in Figure 2.6 (A, B and C).

2.3.5.1b. 16S rRNA gene sequence analysis:

The ~1.5 kb size sequences amplified from the isolates were further analysed for the quality of nucleotide base reads, and a single contig of approximate 1450-1500 nucleotides was constructed from forward and reverse sequences using Chromas Pro software for individual isolates. The contig sequences were uploaded on Ez-Taxon database available on www.ezbiocloud.net (Yoon et al., 2017). It is a web-based open access database tool for the identification of prokaryotes based on 16S rRNA gene sequences of type strains. Maximum sequence similarities with the type strain cultures were identified for all seven isolates. The isolates M18, M33 and M35 exhibited maximum similarity values of 99.7%, 100% and 100% with *B. safensis* FO-36b^T, while *B. pumilus* 7061^T and *B. altitudinis* 41KF2b^{T}were next in list with more than 99% similarities in order. The isolates R30, R31, J208 and J216 exhibited 99.9, 100, 99.4

and 100% similarities with *B. altitudinis* 41KF2b^T, while *B. safensis* FO-36b^T and *B. pumilus* 7061^T were next in list with more than 99% similarities.

The 16S rRNA gene reference sequences from identical or closely matching type strains were downloaded from NCBI database and their phylogenetic analysis along with that of the isolates was performed using MEGA 6.0 software. Figure 2.7(A) represents the phylogenetic tree of seven isolates with closely identical type strains (Thite and Nerurkar, 2015).

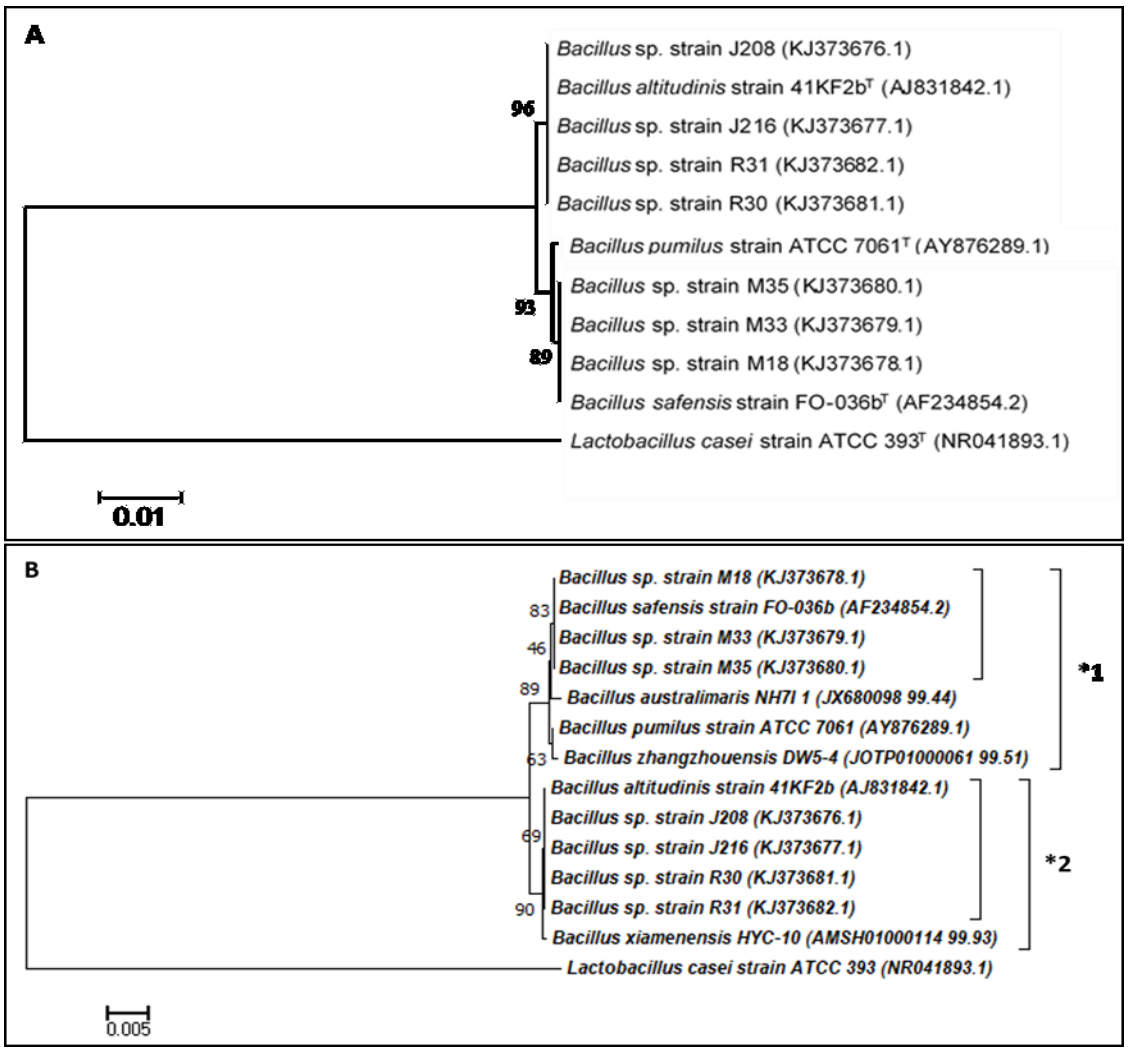


Figure 2.7. Phylogenetic tree based on 16S rRNA gene sequences of the seven isolates and closely related type strains:
(A) Phylogenetic tree of seven isolates and three closely related *Bacillus* spp. type strains. *B. pumilus* ATCC 7061^T, *B. altitudinis* 41KF2b^T and *B. safensis* FO-36b^T; **(B)** Revised phylogenetic tree of seven isolates with six closely related type strains of *Bacillus* spp. *B. zhangzhouensis* DW5-4^T, *B. australimaris* NH71-1T, *B. xiamenensis* HYC-10^T, *B. pumilus* ATCC 7061^T, *B. altitudinis* 41KF2b^T and *B. safensis* FO-36b^T; The percentage of replicate trees in which the associated taxa clustered together in the boot strap analysis (1000 replicates) are shown next to the branches; Their evolutionary distances were computed using Maximum Composite Likelihood method and are in units of the numbers of base substitutions per site; *1 and *2 on the right side indicates the *B. safensis* and *B. altitudinis* clusters further expanded in APPENDIX I.

In recent years the other three *Bacillus* spp. *B. zhangzhouensis* DW5-4^T, *B. australimaris* NH71-1^T (Liu et al., 2016) and *B. xiamenensis* HYC-10^T (Lai et al., 2014) were added to *Bacillus pumilus* group. And the group now covers 6 taxa in total. The revised phylogenetic tree based on 16S rRNA gene sequences to include these new species is represented in Fig 2.7(B) where the identity with the *B. safensis* and *B. altitudinis* type strains remains persistent and the newly added strains were arranged in individual clades. On the basis of the phylogenetic tree showing the maximum similarity between the selected seven cellulase free xylanase-pectinase producing bacteria and three type strains *B. pumilus* ATCC 7061^T, *B. altitudinis* 41KF2b^T and *B. safensis* FO-36b^T it is observed that the seven isolates are grouped as two clusters, where one included isolates R30, R31, J208, J216 showing identity with *B. altitudinis* and another comprised isolates M18, M33, M35 showing identity with *B. safensis*. *B. pumilus* occupied intermediate position between the two clusters.

Literature cited suggested that although the strains possess >99% 16S rRNA gene sequence similarity, they may not belong to the same species (La Duc et al., 2004; Satomi et al., 2006). Many *Bacillus* spp. possessed similar clustering pattern and still were classified as new species under *B. pumilus* group as *B. safensis* FO-36^T (Satomi et al., 2006), *B. altitudinis* 41KF2b^T (Shivaji et al., 2006), *B. zhangzhouensis* DW5-4^T, *B. australimaris* NH71-1^T (Liu et al., 2016) and *B. xiamenensis* HYC-10^T (Lai et al., 2014) based on *gyrB* gene analysis, DNA-DNA Hybridization (DDH) studies and other phenotypic analysis. These reports support the observation by La Duc et al., (2004) stating that the *gyrB* gene sequence based phylogenetic topology could be highly discriminative as compared to 16S rRNA gene sequence.

DNA gyrase or type II DNA topoisomerase is a universally distributed important enzyme protein for alteration in DNA topology. Presence of highly conserved motifs in *gyrA* and *gyrB* gene sequences, which encodes DNA gyrase protein, also provide a useful tool for bacterial identification and diversity (Das et al., 2014). The greatest advantage of using gyrB sequences in identification practices is that, the average base substitution rate of 16S rRNA gene is 1% per 50 million years, whereas, the rate is estimated to be 0.7–0.8% per one million years in the case of gyrB (Chun and Bae, 2000). Hence, those species having completely identical 16S rDNA sequences can be differentiated using *gyrB* gene sequences in addition to the 16S rDNA sequence data (Das et al., 2014). As mentioned above variation of *gyrA* gene sequences has been found to discriminate among *Bacillus subtilis* group whereas *gyrB* is useful to

discriminate among members of *Bacillus cereus* and *Bacillus pumilus* group and therefore selected seven isolates were further analyzed for *gyrB* gene.

2.3.5.2. *gyrB* gene analysis:

Amplification of *gyrB* gene sequence was carried out for all seven isolates and three type strains from their genomic DNA as mentioned in Section 2.2.7.3 and Figure 2.8(A) represents the ~1.2 kb sized amplicon of the *gyrB* gene sequence. The amplicons were further analysed as below.

2.3.5.2a. RFLP analysis of *gyrB* gene:

The amplified products (Figure 2.8 A) were digested with single, double and triple restriction enzymes and the digestion band pattern for double digest by *Msp*I and *Rsa*I is represented in Figure 2.8(B) and the triple digestion pattern by *Msp*I, *Hha*I and *Rsa*I is given in Figure 2.8(C).

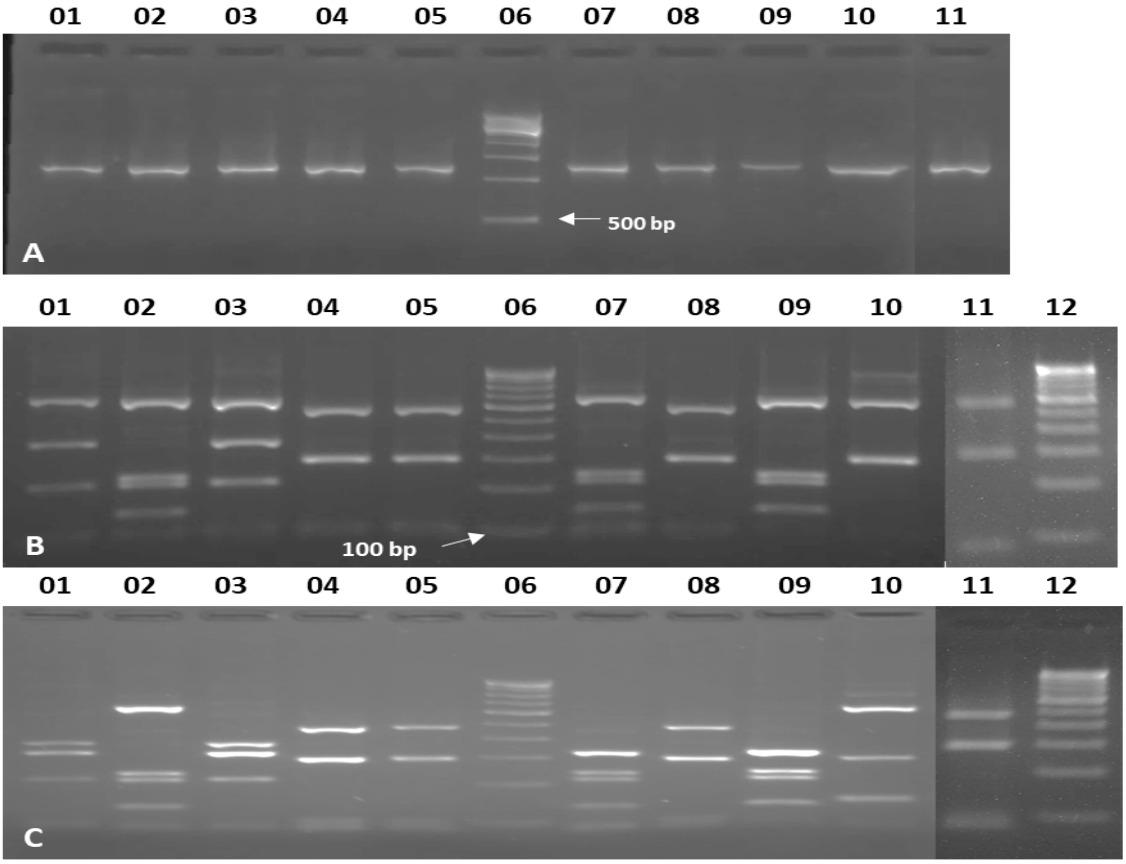


Figure 2.8. *gyrB* gene amplification and restriction digestion pattern analysis of the seven closely related type strains:
(A) ~1.2kb amplification product for *gyrB* gene; **(B** and **C)** Images of band patterns of DNA fragments after digestion with two (*Msp*I and *Rsa*I) and three (*Msp*I, *Hha*I and *Rsa*I) restriction enzymes respectively; Number above each lane indicates- 1: M18, 2: M33, 3: M35, 4: R30, 5: R31, 7: J208, 8: J216, 9: *B. safensis* FO-36b^T, 10: *B. altitudinis* 41KF2b^T, 11: *B. pumilus* 7061^T; **(A)** 500 bp DNA StepUp ladder marker in 6 and in **(B** and **C)** 100 bp DNA StepUp ladder marker in 6 and 12.

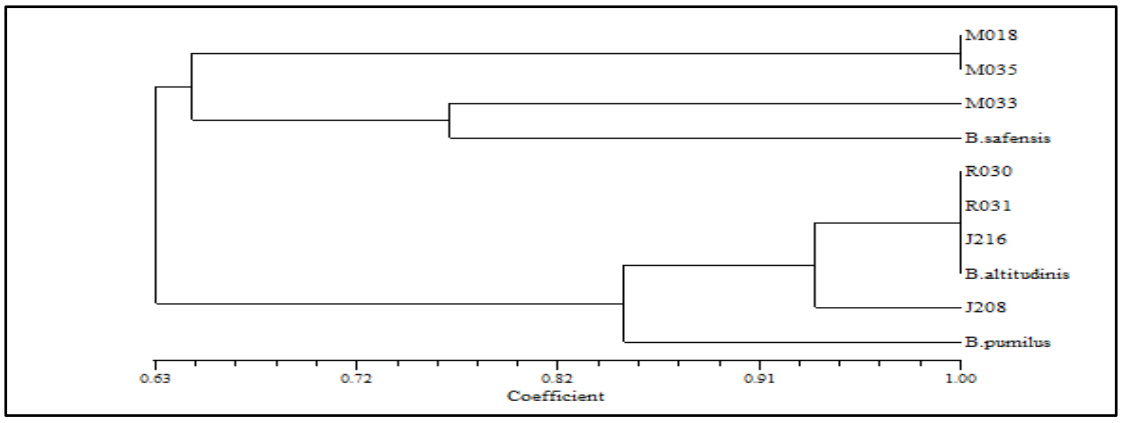


Figure 2.9. Dendrogram of seven isolates and three type strains *B. safensis* FO-36b^T, *B. altitudinis* 41KF2b^T and *B. pumilus* ATCC 7061^T based on the RFLP analysis of *gyrB* gene:

The restriction enzyme digested fragments length (RFLP) pattern was analyzed for similarity and dendrograms were constructed with NTSYSpc software V2.0. The dendrogram depicting clustering patterns for seven cultures with three type strains is given in Figure 2.9. The results here also support the maximum identity observed in 2.3.5.1, where isolates M18, M33 and M35 clustered together with *B. safensis* FO-36b^T, while the other four isolates R30, R31, J208 and J216 clustered with *B. altitudinis* 41KF2b^T, keeping *B. pumilus* 7061^T on a separate clade.

2.3.5.2b. *gyrB* gene sequence analysis:

Amplified *gyrB* gene fragments from these seven isolates and *B. altitudinis* 41KF2b^T were also sequenced and sequence data was analyzed using NCBI BLASTn tool. *gyrB* gene sequences of *B. safensis* FO-36b^T and *B. pumilus* 7061^T was obtained from database and phylogenetic analysis with seven isolates and *B. altitudinis* 41KF2b^T *gyrB* sequences was performed using Mega 6.0 software. Figure 2.10 represents the phylogenetic analysis for isolates based on *gyrB* gene sequence.

Results of *gyrB* gene sequence analysis indicate again the similar type of clustering, where isolates M018, M033, M035 showed identity with *B. safensis* FO-36b^T, and rest four isolates R030, R031, J208, and J216 showed identity with *B altitudinis* 41KF2b^T, whereas *B. pumilus* 7061^T was positioned on the single clade separate from both the clusters. The only difference observed in 16S and *gyrB* sequence phylogeny was that in 16S gene sequnece analysis *B. pumilus* 7061^T was closer to *B. safensis* cluster while in *gyrB* gene sequence analysis it shifted towards *B. altitudinis* cluster as reported by (Liu et al., 2013), although on a separate clade sugesting the isolates M18, M33 and M35 were *B. safensis* and R030, R031, J208, and J216 were *B. altitudinis*.

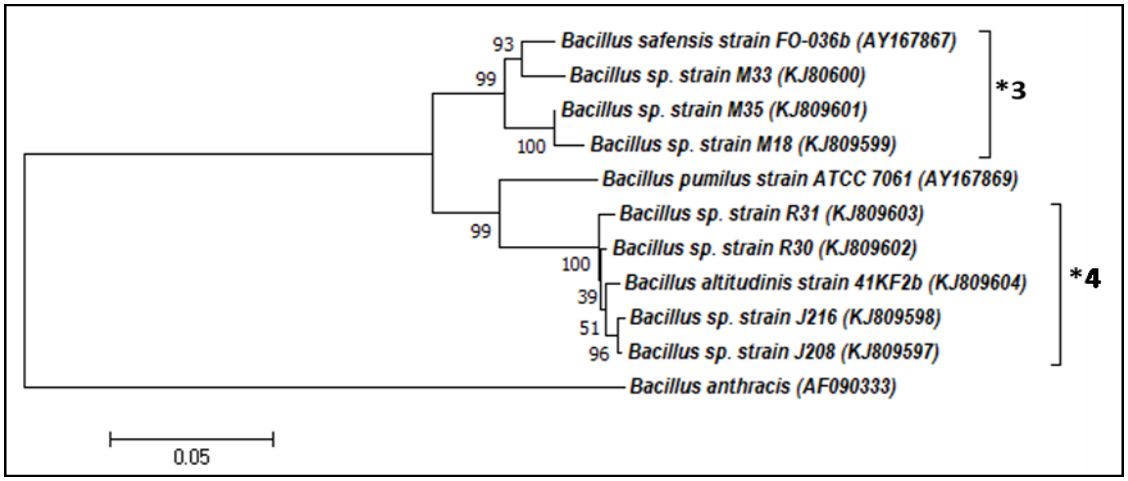


Figure 2.10. Phylogenetic tree of seven selected *Bacillus* spp. with their closely matched type strains:
The percentage of replicate trees in which the associated taxa clustered together in the boot strap analysis (1000 replicates) are shown next to the branches. Their evolutionary distances were computed using Maximum Composite Likelihood method and are in units of the numbers of base substitutions per site. Numbers *3 and *4 on the right side indicates the *B. safensis* and *B. altitudinis* clusters further expanded in APPENDIX I.

2.3.5.3. Sequence submission:

.seq format for seven 16S rRNA and eight *gyrB* gene nucleotide sequences were submitted to NCBI database and after analysis the sequences were made available on database with accession numbers indicated as below in Table 2.7.

Table 2.7. Accession numbers assigned by NCBI to submitted gene sequences:

Organism	Accession number at NCBI database	
	16S rRNA gene sequences	***gyrB* gene sequences**
B. safensis M18	KJ373678.1	KJ809599.1
B. safensis M33	KJ373679.1	KJ809600.1
B. safensis M35	KJ373680.1	KJ809601.1
B. altitudinis R30	KJ373681.1	KJ809602.1
B. altitudinis R31	KJ373682.1	KJ809603.1
B. altitudinis J208	KJ373676.1	KJ809597.1
B. altitudinis J216	KJ373677.1	KJ809598.1
B. altitudinis 41KF2b^T	--	KJ809604.1

2.3.6. Phenotypic characterization:

Phenotypes like growth at various incubation conditions, ability to utilize certain compounds and sensitivity against antibiotic etc., were observed as below.

2.3.6.1. Growth phenotypes:

Growth phenotypes of selected seven isolates with three type strains in NB were reported in Table 2.8. When grown under anaerobic and aerobic condition, sparse growth was observed under anaerobic condition suggesting that the isolates were aerobic, as observed for the type strains. All the isolates were able to grow up to 7% NaCl concentrations although decreased growth was observed with increased saline concentrations. The isolates were able to grow from mild acidic to moderate alkaline conditions, but with decreased growth at pH 5.0 and 10.0 and no growth at pH 4.0. The temperature optima were about 28-37 °C in static and shaking conditions for all isolates.

Table 2.8. Growth phenotypes of selected seven isolates and three type strains:

Cultures (Isolate / Type strains)		*B. safensis*				*B. altitudinis*					*B. pumilus*
		M18	**M33**	**M35**	**Fo-36b^T**	**R30**	**R31**	**J208**	**J216**	**41KF2b^T**	**ATCC 7061^T**
In static NB	Aerobic	+	+	+	+	+	+	+	+	+	+
	Anaerobic*	-	-	-	-	-	-	-	-	-	-
In shaking incubation with NaCl	2%	+	+	+	+	+	+	+	+	+	+
	5%	+	+	+	+	+	+	+	+	+	+
	7%	+	+	+	+	+	+	+	+	+	+
	10%	-	-	-	-	-	-	-	-	-	-
In shaking incubation at pH	4.0	-	-	-	-	-	-	-	-	-	-
	5.0	+	+	+	+	+	+	+	+	+	+
	6.0	+	+	+	+	+	+	+	+	+	+
	7.0	+	+	+	+	+	+	+	+	+	+
	8.0	+	+	+	+	+	+	+	+	+	+
	9.0	+	+	+	+	+	+	+	+	+	+
	10.0	+	+	+	+	+	+	+	+	+	+
In static/shaking incubation at	8 °C	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	15 °C	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
	28 °C	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
	37 °C	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
	45 °C	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

Signs: "+" Positive (Growth present); "-" Negative (Growth absent)

2.3.6.2. Biochemical phenotypes:

Results for various biochemical phenotype characterization were presented in Table 2.9. The phenylalanine deaminase and lysine decarboxylase tests are differentiating between *B. altitudinis* which gives positive results and *B. safensis* which gives negative results.

Table 2.9. Biochemical phenotypes of selected seven isolates and three type strains:

Cultures (Isolate/Type strains)		*B. safensis*				*B. altitudinis*					*B. pumilus*
		M18	M33	M35	Fo-36b^T	R30	R31	J208	J216	41KF2b^T	ATCC 7061^T
Indole		-	-	-	-	-	-	-	-	-	-
Urea		+	+	+	-	+	+	+	+	-	+
Lysine decarboxylase		-	-	-	-	+	+	+	+	+	-
Gas from nitrate		-	-	-	-	-	-	-	-	-	-
Phenyl alanine deamination		-	-	-	-	+	+	+	+	+	-
Voges-Proskauer		+	+	+	+	+	+	+	+	-	+
Catalase		+	+	+	+	+	+	+	+	+	+
Malonate		-	-	-	-	-	-	-	-	-	-
Citrate utilization		+	+	+	-	+	+	+	+	-	+
(β-Galactosidase)		+	+	+	+	+	+	+	+	+	+
Nitrate reduction		-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase		-	-	-	-	-	-	-	-	-	-
B-Glucosidase		+	+	+	+	+	+	+	+	+	+
Hydrolysis	Starch	-	-	-	-	-	-	-	-	-	-
	Gelatin	-	-	-	-	-	-	-	-	-	+
	Casein	+	+	+	+	+	+	+	+	-	+
	Xylan	+	+	+	+	+	+	+	+	+	+
	Pectin	+	+	+	+	+	+	+	+	+	+
	Cellulose	-	-	-	-	-	-	-	-	-	-
Sugar utilization	Sucrose	+	+	+	+	+	+	+	+	+	+
	Glucose	+	+	+	+	+	+	+	+	+	+
	Arabinose	+	+	+	+	+	+	+	+	+	+
	Mannitol	+	+	+	+	+	+	+	+	+	+
	Trehalose	+	+	+	+	+	+	+	+	+	+
	Xylose	+	+	+	+	+	+	+	+	+	+
Acid production	Sucrose	+	+	+	+	+	+	+	+	+	-
	Glucose	+	+	+	+	+	+	+	+	+	+
	Arabinose	-	-	-	-	-	-	-	-	-	-
	Mannitol	+	+	+	+	+	+	+	+	+	+
	Trehalose	+	+	+	+	+	+	+	+	+	+
	Xylose	+	+	+	+	+	+	+	+	+	+

Signs: "+" Positive (Growth present); "-" Negative (Growth absent);

Out of the seven isolates the three *B. safensis* and four *B. altitudinis* strains gave identical results to type strains. All the isolates were positive for VP, catalase, β-galactosidase and β-glucosidase, and hydrolysis of casein, xylan & pectin, while negative for indole test, nitrate reduction, gas production from nitrate and arginine dihydrolase and hydrolysis of starch, cellulose & gelatin. All the isolates were able to grow up on sucrose, glucose, xylose, arabinose, mannitol, trehalose and produced acid in all cases except arabinose. Unlike the type strain of *B. safensis* and *B. altitudinis*, the seven isolates were positive for urea hydrolysis and citrate utilization like *B. pumilus*. The results supported their identification as *B. safensis* or *B. altitudinis*.

2.3.6.3. Antibiotic sensitivity test:

Antibiotic susceptibility test revealed that all the seven isolates, can be distinguished from *B. pumilus* as it was sensitive for all the used concentrations of antibiotics while *B. safensis* and *B. altitudinis* displayed either intermediate or resistant phenotype for Bacitracin, Penicillin G and Neomycin. All the isolates were sensitive to the antibiotics used except, Penicillin G and Neomycin. All isolates were either intermediate or resistant to Neomycin, while four isolates showing identity with *B. altitudinis* were resistant to Penicillin G; whereas those identified as *B. safensis* were sensitive to Penicillin G. Table 2.10 represents the sensitivity of isolates and type strains towards the antibiotics in terms of resistant (R), intermediate (I) and sensitive (S). Figure 2.11 shows representative antibiotic susceptibility tests results.

Table 2.10. Antibiotic sensitivity test for seven isolates and three type strains:

Cultures (Isolate / Type strains)	***B. safensis***				***B. altitudinis***					***B. pumilus***
Antibiotics *	**M18**	**M33**	**M35**	**Fo-36b^T**	**R30**	**R31**	**J208**	**J216**	**41KF2b^T**	**ATCC 7061^T**
B	S	S	S	I	S	S	S	S	I	S
C	S	S	S	S	S	S	S	S	S	S
CoT	S	S	S	S	S	S	S	S	S	S
P	S	S	S	R	R	R	R	R	R	S
PB	S	S	S	S	S	S	S	S	S	S
Gen	S	S	S	S	S	S	S	S	S	S
N	I	I	I	I	I	R	R	I	R	S
T	S	S	S	S	S	S	S	S	S	S

***Antibiotics (concentration):** **B**: Bacitracin (10 units); **C**: Chloramphenicol (30 mcg); **CoT**: Co-Trimoxazole (25 mcg); **P**: Penicillin G (100 units); **PB**: Polymyxin B (300 units); Gen: Gentamycin (10 mcg); **N**: Neomycin (30 mcg); **T**: Tetracycline (30 mcg)
***Response:** "**S**" - Sensitive, "**I**" - Intermediate and "**R**" – Resistant

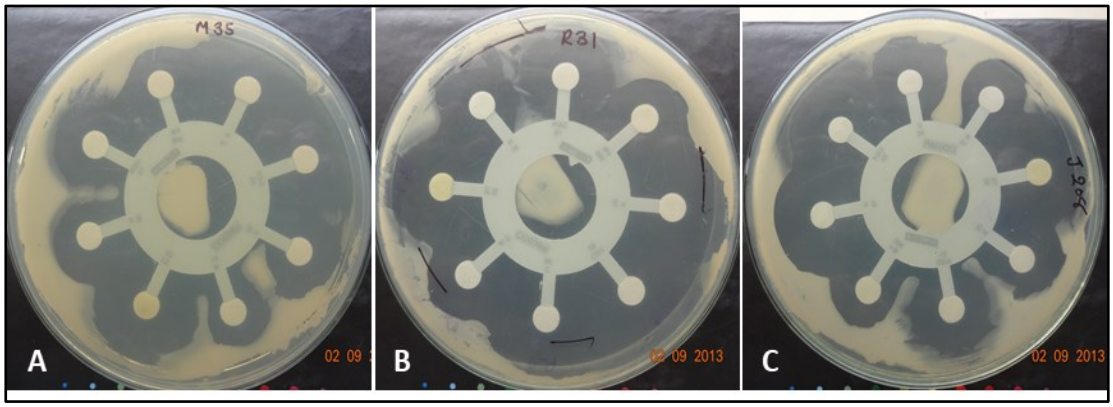


Figure 2.11. Representative images of isolates showing antibiotic sensitivity tests: (A) *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208F.

Phenotypic characterization based on growth phenotypes, biochemical phenotypes and antibiotic sensitivity as depicted in Section 2.3.6 broadly supports the results of identification by 16S rRNA and *gyrB* gene analysis. This confirms the isolates to be the *B. safensis* and *B. altitudinis*, and hence the species names were allotted to them as: *B. safensis* M18, *B. safensis* M33, *B. safensis* M35, *B. altitudinis* R30, *B. altitudinis* R31, *B. altitudinis* J208, *B. altitudinis* J216.

2.3.7. Screening of the isolates based on xylanase and pectinase activity at various incubation conditions:

Since the further application of these selected isolates was for plant biomass saccharification, next screening step was based on the ability of the isolates to produce xylanase and pectinase enzymes that are active at broad range of pH and temperature.

2.3.7.1. Crude xylanase and pectinase activities of selected seven *Bacillus* isoaltes at various pH conditions:

The efficiency of the crude enzyme to work at different pH was analysed as mentioned in 2.2.9.1. Figure 2.12(A) and 2.13(B) represents the crude xylanase and pectinase activities respectively at pH range of 4.0 to 10.0 when incubated at 40°C. Since in unbuffered condition xylan has pH in between 5.0-6.0 and pectin has pH 4.0 or less, unbuffered substrates were also included in the study. These results suggested that among the seven isolates screened, three isolates *B. safensis* M35, *B. altitudinis* R031 and *B. altitudinis* J208 retained more than 50% of relative activity between pH range 4 to 10 for xylanase while between pH 6-10 for pectinase. The broad range of pH for the crude xylanases and pectinases is an important attribute for their application in plant waste saccharification.

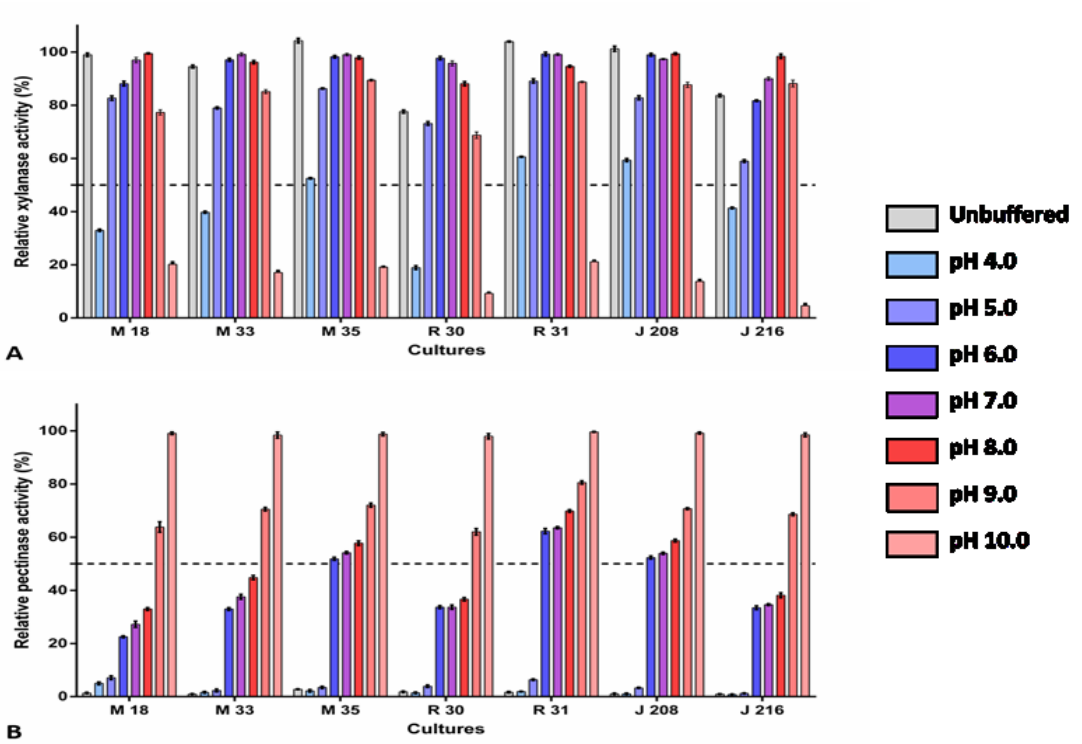


Figure 2.12. Crude xylanase and pectinase activities of *B. safensis* M18, M33 & M35 and *B. altitudinis* R30, R31, J208 & J216 at different pH:
(A) Crude xylanase activities from seven isolates, **(B)** Crude pectinase activities from seven isolates; Column and error bars represents Mean ± Standard Error of Means for n=3.

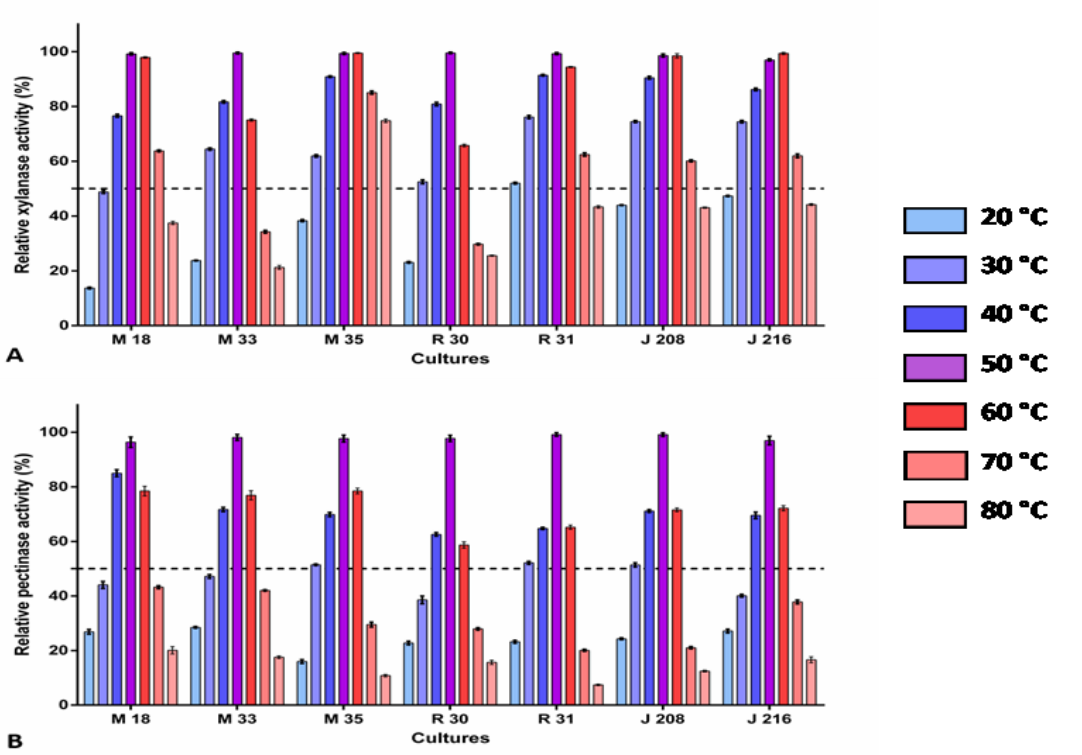


Figure 2.13. Crude xylanase and pectinase activities of *B. safensis* M18, M33 & M35 and *B. altitudinis* R30, R31, J208 & J216 at different temperatures:
(A) Crude xylanase activities from seven isolates, **(B)** Crude pectinase activities from seven isolates; Column and error bars represents Mean ± Standard Error of Means for n=3.

2.3.7.2. Crude xylanase and pectinase activities of selected seven *Bacillus* isolates at various temperatures:

The activity of the crude enzyme from the seven isolates at different temperatures was analyzed as described in 2.2.9.2. Figure 2.13(A) and 2.13(B) represents the crude xylanase and pectinase activities respectively in the range of 20 to 80°C when incubated at pH 7.0. These results suggested that among seven isolates screened, three isolates *B. safensis* M35, *B. altitudinis* R031 and *B. altitudinis* J208 retained more than 50% of relative activity at temperature range of 30-60°C for xylanase as well as pectinase. It was also seen that 40-50°C was the optimum temperature for both the enzymes.

So, based on these activities at broad range of pH and temperature the three isolates, *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were selected for further characterization studies and their scanning electron micrographs are shown in Figure 2.14. The approximate cell size was calculated to be 1.6 ± 0.2 µm in length and 0.6 ± 0.1 µm in width.

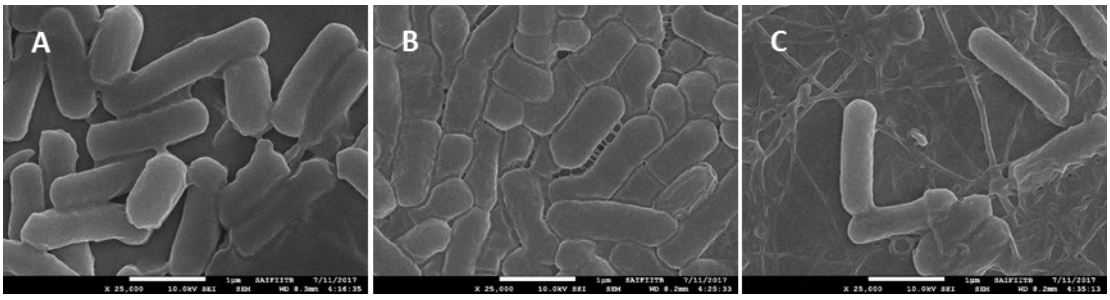


Figure 2.14. Scanning electron micrograph of selected three isolates:
(A) *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208; Images were captured at 25,000X magnification with electron beam of 10.0 kv voltage at distance of 8.2-8.3mm; white bar at base of each images measures for 1µm distance.

2.3.8. Characterization of selected three isolates *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208:

Three selected isolates, *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were further characterized for their growth and time course enzyme production.

2.3.8.1. Time course studies for production of xylanase and pectinase:

The isolates *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were subjected to analyze the production of xylanase and pectinase as depicted in Figure 2.15(A) and 2.15(B) respectively. Observations after every 12 h indicate that, the maximum production of xylanase was achieved by 24 h in case of all three enzymes while it took 36-48 h for pectinase to achieve maximum production level. From figure 2.15(A) it seems that xylanase production is maximum by R31. But as the time span

increases the amount of activity shown by secreted enzymes decreases for all three, but this decrease is comparatively lesser for M35. Whereas in case of pectinase, as shown in Figure 2.15(B) maximum production is shown by R31 within 24 h, followed by J208, and M35 which persisted throughout incubation.

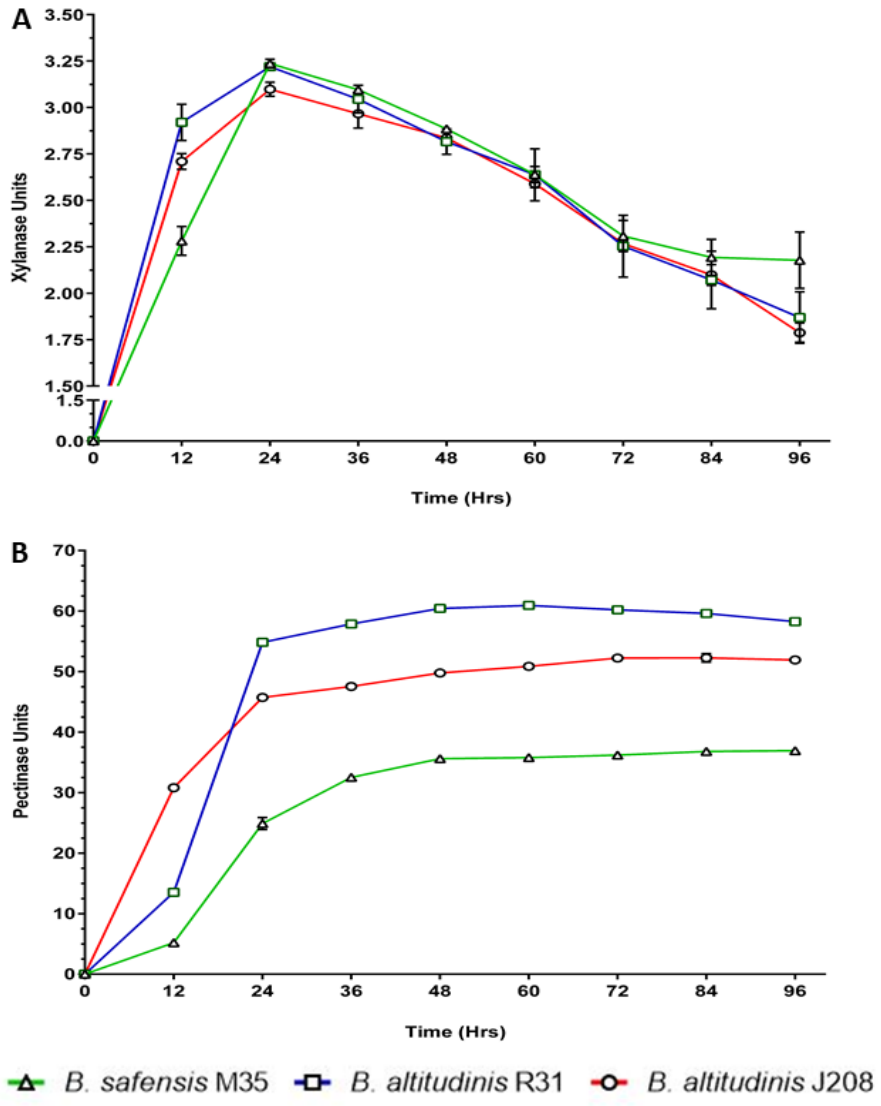


Figure 2.15. Time course production of xylanase and pectinase enzymes:
(A) Xylanase production and **(B)** pectinase production; Line and error bars represents Mean ± Standard Error of Means for n=3.

2.3.8.2. Growth pattern:

Three selected isolates were analyzed for their growth patterns and their growth curves in terms of CFU/ml and OD_{600nm} were plotted as shown in Figure 2.16. It was observed that for all three cultures the growth pattern was sigmoid curve and the cultures entered in log phase after a short lag phase of 1 h as depicted by cfu/ml count and achieved stationary phase by approximately 7 -8 h. 0.2 units of OD_{600} corresponds to approximate value of $1x10^6$ cfu/ml.

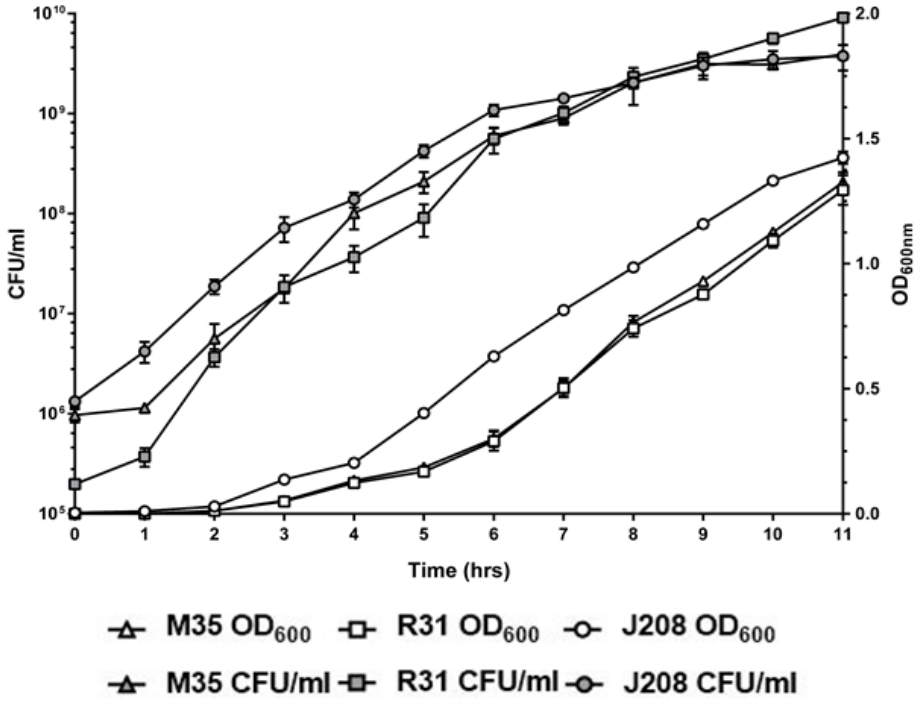
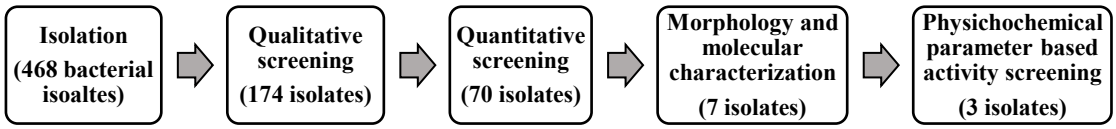


Figure 2.16. Growth curve of selected three isolates:
Left Y axis represents values of CFU/ml whereas right Y axis represents values of OD_{600nm}; Line and error bars represents Mean ± Standard Error of Means for n=3.

Thus, to summarize the work from this chapter,



These three isolates *B. safensis* M35, *B altitudinis* R31 and *B. altitudinis* J208 were isolated from three different ruminant dung samples viz., camel, bull and buffalo. Interestingly, the isolates were able to produce cellulase free xylanase and pectinase enzymes. This attribute is not commonly found in the enzyme producers and imparts distinct advantage from the viewpoint of industrial scale production. Additionally, the enzymes were active over broad pH and temperature range and were produced within 36-48 h of incubation. The xylanases from three isolates were active in pH range of 6.0 to 9.0 and temperature range of 20-60 °C. While pectinase enzymes from three isolates were active in pH range of 6.0 to 10.0, and temperature range of 30-70°C. Thus, same culture producing both the enzymes with broad active range of temperature and pH conditions and their cellulase free nature are attributes which give them a huge

potential for biotechnological applications, in present studies agrowaste saccharification in bioethanol production and in paper-pulp, textile and food industries too. *B. safensis* M35, *B altitudinis* R31 and *B. altitudinis* J208 were used in the studies discussed in subsequent chapters.

2.3.9. Strain submission:

B. safensis M35, *B. altitudinis* R31 and *B. altitudinis* J208 were verified by National Centre for Microbial Repository (NCMR, Pune, India), accepted for deposition and allotted strain accession numbers are *B. safensis* M35 (MCC 3305), *B. altitudinis* R31(MCC 3308) and *B. altitudinis* J208 (MCC 3307).