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3.1 Materials and equipments used

Maps/plot plan; safety first aids (band aid, disposable gloves, etc); survey equipment or global positioning system (GPS, Garmin, USA) to locate sampling points; tape measure/scale; survey stakes or flags; digital camera (NIKON D5300, Japan); Stainless steel, plastic, or other appropriate homogenization bucket; bowl or pan; 50 ml capacity sample containers (TARSONS, Kolkata, India); ziplock plastic bags; logbook; field data sheets and sample labels; coolers; Ice; canvas or plastic sheet; spade or shovel; spatula; scoop; plastic or stainless steel spoons; trowels; soil sampler outfit; pH testing strips (MO BIO Laboratories, USA); sifter (different pore sizes); well-maintained dark room (4°C); 90 mm size petridish (TARSONS, Kolkata, India); inoculation loop (1µl to 3µl); spirit lamp; disposable L-shaped spreader; spreading stands; Laminar airflow (Kenz Flo); colony counter; Stereo-zoom microscope; Bunson burner; gas cylinder; refrigerator; conical flask of different capacity (Borosil, Kolkata, India); measuring cylinder; weighing balance; oven with the capacity to maintain 25 to 50°C temperature; aluminium foils; weighing paper; pipettes and its tips of different capacity; polytene bags; conical flask; test tubes; test-tube stands; mixer grinder (USHA, Gurgaon, India); Spectrophotometer (Thermoscientific, Waltham, MA, USA); stationery and shaker incubators of different temperature limits like adjustable incubators from Sanyo Osaka, Japan; temperature regulated shaker incubators (Pelican, Chennai, India); water bath of medium range temperature (ThermoScientific, Waltham, USA); High
temperature water-bath (ThermoScientific, Waltham, USA); Centrifuge (Eppendorf
Centrifuge R2050, Hamburg, Germany); PCR (Touch 1000, Bio-rad®, Hercules,
CA, USA); Mortar and Pestle; Isopropanol, Cool box.; ChemiDoc™ MP Imaging
System (Bio-rad®, Hercules, CA, USA); Horizontal Electrophoresis system;
PROTEAN i12 IEF Cell system (Bio-rad®, Hercules, CA, USA); PROTEAN II xi
cell system (Bio-rad®, Hercules, CA, USA); Trans-Blot® Turbo (Bio-rad®,
Hercules, CA, USA); Gel Rocker; UltrafleXtreme MALDI-TOF/TOF analyser
(Bruker Daltonics, Billerica, MA); LTQ Orbitrap Discovery-MS (Thermoscientific,
Waltham, MA, USA); AKTA prime plus protein purification system (GE
Healthcare, Buckinghamshire, UK); BioLogic DuoFlo (Bio-rad®, Hercules, CA,
USA); Fraction collector, Econo-glass column (Bio-rad®, Hercules, CA, USA);
Savant ModulyoD lyophilizer/freeze dryer (ThermoScientific, Waltham, USA);
beakers; 2 ml capacity syringe; dialysis system; concentrator (Merck®, Bangalore,
India), C 10/10 column (GE Healthcare, Buckinghamshire, UK); HiPrep DEAE
FF prepacked column (GE Healthcare, Buckinghamshire, UK); rice straw;
sugarcane bagasse; sawdust etc.

3.2 Chemicals and reagents

All the chemicals used were of high quality and purchased from reputed brands.
Sodium Nitrate (Merck®, Bangalore, India); Di-potassium hydrogen phosphate
(Merck®, Bangalore, India); Magnesium Sulphate (HiMedia®, Mumbai, India);
Potassium Chloride (Merck®, Bangalore, India); Cellulose powder (Sigma
Aldrich®, Saint Louis, MO, USA); Ferrous Sulphate (HiMedia®, Mumbai, India);
Agar (Merck®, Bangalore, India); Ammonium Sulphate (Merck®, Bangalore, India); Calcium carbonate (HiMedia®, Mumbai, India); distilled water; Phosphate Buffer Saline; Cibacron Blue 3GA (Sigma Aldrich®, Saint Louis, MO, USA); Reactive Orange 12 (Sigma Aldrich®, Saint Louis, MO, USA); Hydroxyethylcellulose (Sigma Aldrich®, Saint Louis, MO, USA); 1,4-butanediol diglycidyl ether (Sigma Aldrich®, Saint Louis, MO, USA); Whatman Filter Paper (type 1 & 42); carboxymethylcellulose (HiMedia®, Mumbai, India); boric acid grade Peptone (HiMedia®, Mumbai, India); Dinitrosalicylic acid (DNS); Potassium Sodium Tartrate (HiMedia®, Mumbai, India); Potato-Dextrose Broth (HiMedia®, Mumbai, India); Molecular grade ethyl alcohol; Silica gel mesh 200 (Merck®, Bangalore, India); Bradford reagent (Bio-rad®, Hercules, CA, USA); Tris-HCL; Sodium Chloride (Merck®, Bangalore, India); Ethylenediaminetetraacetic acid (Merck®, Bangalore, India); Sodium Dodecyl Sulphate (Merck®, Bangalore, India); Agrose; Ethidium Bromide (Sigma Aldrich®, Saint Louis, MO, USA); Tracking dyes (Sigma Aldrich®, Saint Louis, MO, USA); (NH₄)₂SO₄; Coomassie blue R-250 (SRL, Mumbai, India); Triton X–100 (Sigma Aldrich®, Saint Louis, MO, USA); β–Mercaptoethanol; BCIP/NBT (5–Bromo, 4–Chloro, 3–Indoly1 phosphate/Nitrobluetetrazolium) (Sigma Aldrich®, Saint Louis, MO, USA); Tween-20 (Sigma Aldrich®, Saint Louis, MO, USA); Bovine Serum Albumin (BSA) (Bio-rad®, Hercules, CA, USA); ReadyPrep 2D Cleanup Kit (Bio-rad®, Hercules, CA, USA); Immobilized pH gradient (IPG) strips (11 cm, pH 3 to 6; Bio-rad®, Hercules, CA, USA); Sephacryl S-200 HR; Tris Buffer; AffiGel 15 (Bio-rad®, Hercules, CA, USA); dNTPs (Sigma Aldrich®, Saint
Louis, MO, USA); Taq polymerases (Sigma Aldrich®, Saint Louis, MO, USA); ITS primers (Sigma Aldrich®, Saint Louis, MO, USA); DNA standard ladders (Sigma Aldrich®, Saint Louis, MO, USA); Protein molecular weight standards (Bio-rad®, Hercules, CA, USA); Secondary antibody (Goat anti-rabbit IgG coupled to alkaline phosphatase from Bio-rad®); anti-β-glucosidase and anti-cellulases primary antibodies (Acris Antibodies GmbH, Germany) etc.

3.3 Soil sampling

The extent of the sampling effort, methods to be employed, and the types and amounts of equipment and supplies required were planned and determined well ahead of carrying the actual works. The materials and equipments to be used were sterilized and checked to ensure they were in working order. Proper schedules were made for collection of sample and was well coordinated with staff and regulatory agencies. A general site survey was made prior to entry into the site of sample collection in accordance with the site specific health and safety plan. Flagging of the spots was done to identify and mark all sampling locations. Collection of samples from near-surface soil were accomplished with tools such as spades, shovels, trowels, and scoops. Accurate, representative samples were collected with this procedure depending on the care and precision required to be followed. A flat, pointed mason trowel was used to cut a block of the desired soil maintaining its undisturbed profiles. The top layer of soil or debris to the desired sample depth with a pre-cleaned spade was carefully removed. Using a pre-cleaned, stainless steel scoop, plastic spoon, or trowel, remove and discard a thin layer of soil from
the area which came in contact with the spade. From the cleared soil surface, soil sample to a depth of 15 cm was carefully cut out using flat mason trowel. Three replica soil samples were collected from each site and the spots were 3 feet apart and were assumed to represent the collection spot. Similarly, three spots were chosen, each about 100 feet apart to represent the collection sites in three replicates. The samples were put into appropriate homogenization container, and mixed thoroughly to obtain a homogenous sample representative of the entire sampling interval. Then, the samples were placed into appropriate, labelled containers and the caps were secured tightly. Geographical location of the sample collection site was noted using GPS equipment. The latitude, longitude and altitude of each sample collection spot were recorded in logbook or on field data sheets. The time of sample collection, season, month of the year and also the weather condition of the site on the day of collection was noted. The physical condition of the sample was briefly described and the collection site (Figure 3.2.1) was also briefly described in the logbook.
3.4 Processing of soil samples

The soil samples were carried to the laboratory from field in cooler boxes and stored at 4°C until further analysis. Prior to isolation of the microorganisms from these samples, the soil samples were sieved through 2 mm size sifter to remove the coarse materials. The replicates of each spot were mixed together to bring about the homogeneity of the collection spot.

3.5 Preparation of soil sample for isolation

Five gram of soil sample, previously sieved through 2 mm pore size sifter was mixed with 45 ml of phosphate buffered saline (PBS). The soil water suspension was agitated in a shaker incubator for 1 h to release the microorganisms from the soil sample. Serial dilution was made in the same buffer.
3.6 **Isolation on three selective media**

Three selective media viz. Omeliansky’s agar (OA) \[ g/l: (NH_4)_2SO_4 1.0; K_2HPO_4 1.0; MgSO_4 0.5; CaCO_3 2.0; NaCl 0.1; Cellulose powder 3.0; Agar 17.0 \], Cezpak Dox agar (CDA) \[ g/l: NaNO_3 2.0; K_2HPO_4 1.0; MgSO_4 0.5; KCl 0.5; Fe_2SO_4 0.01; Cellulose powder 3.0; Agar 17.0 \] with pH adjusted to 5.3 using filter sterilized tartaric acid and Kenknight and Munier’s agar (K&M) \[ g/l: NaNO_3 0.1; KH_2PO_4 1.0; MgSO_4 0.1; KCl 0.1; Cellulose powder 3.0; Agar 17.0 \] were prepared for isolation of bacteria, fungi and actinobacteria, respectively. The composition of these selective media were prepared in 500 ml capacity conical flask each containing 200 ml of the media and autoclaved at 121°C, 15 psi for 15 min. The autoclaved media were cooled to luke warm and poured onto sterile 90 mm petridishes inside a laminar airflow. The media were than allowed to solidify and then packaged into sterile polytene bags and stored in cool room until future use. 100 μl of each serially diluted sample was pour plated on the modified selective media and spread evenly over the plate with L-spreader. The plates were incubated at 20°C and 30°C for 3–7 days.

3.7 **Microbial colony count and grouping of colonies**

The incubated culture plates were observed for microbial growth every 12 h. With the observance of homogenous distinct colonies, they were counted under colony counter. If the density of the colonies were more, the equivalent half count of the plate was made and later summed up to give the full plate count. The dilution plate which had the average count of 300 colonies were taken as the count of population.
of the sample. For, OA plates, only the bacterial colony counts were taken, fungus growing in this plates were omitted. However, measures were taken to prevent growth of fungal colonies in OA plates. For example, anti-fungal antibiotics were added and pH of the media was maintained above 6. Only fungal colony counts were taken for CDA media plates and only actinomycetes for K&M agar plates. Actinomycetes colonies grew profusely in OA medium and hence the count was undertaken. The time, day and year of count was also noted on the data sheet. In each plate, size, shape and other morphological features of colonies were observed and grouped based on similarities. Each group was considered to be one type. Thus, number of group types were taken as a measure of diversity of cellulolytic microorganisms from different site samples. One representative colony from among similar group was sub-cultured, purified and then stored in appropriate condition.

The population of the sample was derived based on the following formula.

\[
\text{Population of microorganisms} = \frac{\text{Plate count} \times \text{dilution factor of sample}}{0.1 \text{ ml} \times \text{sample dry weight}}
\]

3.8 Sub-culturing of microorganisms

Distinct colonies on the plates were marked on the culture plates. The marked bacterial colonies were scraped using 1µl capacity loops and streaked on to fresh
selective media plates. The sub-culturing of the distinct colonies was carried until a pure distinct colony was obtained. For fungal and actinomycetes colonies, a small lump of agar along with edge of a distinct colony were scooped out with loops and placed on to fresh plates. These were then streaked only for the first time. In the subsequent sub-culturing, no streaking was made for fungal colonies, instead a small lump of colony was placed at the center of a fresh CDA plate. While for actinomycetes sub-culturing with streaking was carried out until pure distinct colonies were obtained. The colony morphology of pure isolates were photographed using stereo zoom microscope and digital camera and described in details in the data sheets. The pure distinct isolates were coded as to keep the identity of the isolate. Majority of the pure isolates were deposited in the Microbial Repository Centre (MRC), IBSD, Imphal and accession numbers obtained.

3.9 Screening for cellulolytic property of the isolates

Though the isolates were obtained from soil samples using selective media which had cellulose as the sole carbon source, further screening was carried out using qualitative and quantitative assays to discard the non-cellulolytic microorganisms.

3.9.1 Insoluble chromogenic substrate medium

Insoluble blue HE-cellulose (HE-cellulose-blue) and orange HE-cellulose (HE-cellulose-orange) were prepared from HE-cellulose using the dyes: Cibacron Blue 3GA and Reactive Orange 12, respectively. To a HE-cellulose (2 g in 30 ml distilled water), 10 ml of 2 M NaOH, 1.9 g of Cibacron Blue 3GA and 1.2 ml of 1,4-butanediol diglycidyl ether were added, stirred for 5 min and left standing at
room temperature. Similarly, HE-cellulose- Reactive Orange 12 was followed in the same procedure. After 48 h, the mixture solidified into a gel. The gel was mixed with 100 ml of distilled water and was ground by a blender for 15 s. To remove the unbound dye, the ground particles were washed with boiling water and filtered (Whatman, type 1) repeatedly until the filtrate was colourless. The wet (highly hydrated) product was used as a supplement to agar medium. The content of the chromogenic substrate in the product was 0.89% (Ten et al., 2004b).

3.9.2 Soluble substrate and color developed using reagents

Qualitative plate assay was carried out using carboxymethylcellulose agar ([NaNO₃ 2.0; K₂HPO₄ 1.0; KCl 0.5; MgSO₄ 0.5; carboxymethylcellulose (CMC) 2.0; Peptone 0.2; Agar 17.0]) as a growth media for the isolated microorganisms. After some days of incubation, the culture plates were flooded with Gram’s iodine for 3 to 5 min. Positive activity was determined based on the relative cellulolytic activity index (RCAI) which compares the diameter of the clearing zone with the diameter of microbial colony (Kasana et al., 2008b).

3.9.3 Estimation of total cellulase activity

Filter paper assay (FPase) was carried out to determine cellulase activity. The amount of reducing sugar released during the assay was finally quantified by Dinitrosalicylic Acid (DNS) method (Miller, 1959b). Filter paper assay was determined by incubating 32 mg punched pieces of filter paper in 1 ml of 0.05 M Sodium citrate buffer (pH 4.8) with 1 ml of culture supernatant in 50 ml capacity centrifuge tube at 50˚C. After 1 h, 3 ml of Dinitrosalicylic acid (DNS) reagent was
added and kept in boiling water bath for 5 min to stop the reaction and then 1 ml of 40% Rochelles salts solution was added. Blanks, without filter paper and with filter paper (without culture supernatant) was used as negative control (Eveleigh et al., 2009b).

3.9.4 Estimation of individual cellulase activities

Endo-β-1, 4-glucanase activity assay, and β-glucosidase activity assay were carried out following the protocol of Thygesen et al. (2003). Exoglucanase (EC3.2.1.91) activity was measured using 1% avicel (Sigma Chemical, St. Louis, USA) in 100 mM sodium acetate buffer (pH 4.8), as described by (Wood and Bhat, 1988d). One unit (IU) of filter paper, endoglucanase and exoglucanase activity was defined as the amount of the enzyme that released 1 µmole of glucose per minute from the original substrate, at the experimental conditions described above. For β-glucosidase activity, one unit (IU) is defined as the amount of the enzyme that releases 1 µmole of p-Nitrophenol per minute from the p-Nitrophenyl β-D-glucopyranoside.

3.10 Identification of potential isolates and their optimal cellulolytic parameters

Comparing the qualitative and quantitative cellulase assays, only three fungal isolates which showed high correlation between qualitative and quantitative cellulase activities and potential to degrade cellulose were selected identification and further study.
3.10.1 *Genomic DNA isolation and rDNA amplification*

The MNPf3, BMf4 and SPf7 isolates were grown in 30 ml potato-dextrose broth medium for 72 h at 30°C. The mycelial mat was pelleted by centrifugation (Eppendorf Biofuge R2050, Hamburg, Germany) at 3,000 rpm for 20 min. Genomic DNA was isolated following the rapid extraction method for PCR amplification of Cenis (1995). For sequence analysis of the ITS1-5.8S-ITS2 rDNA region, PCR was performed in a C1000™ Touch Thermal Cycler (BIORAD, USA) using the primer set: 5’-CTTGGTCATTTAGAGGAAGTAA-3’ and 5’-TCCTCCGCTTATTGATATGC-3’ according to standard protocol (White *et al.* 1990). The run was programmed with an initial denaturation at 95°C for 5 min, followed by amplification for 34 cycles at the following conditions: 1 min at 95°C, 1 min at 50°C and 2 min at 72°C. A final 5 min extension at 72°C and infinite hold time at 12°C completed the program run. The amplified products were profiled in 1.5% agarose gel and visualized with ethidium bromide in ChemiDoc™ MP Imaging System (Bio-rad®). Amplicons of about 450-580 bp were purified and sequenced in ABI370X1 Cycler Sequencer (ABI, USA). The sequences were automatically trimmed and assembled in DNAbaser 3.5.3 software. Following annotation, sequences were assigned to species based on 98-100% sequences similarity threshold in the GenBank. The rDNA sequences of MNPf3, BMf4 and SPf7 were submitted to GenBank® with Accession Numbers KC937053, KC937054 and KC937055, respectively.
3.10.2 Phylogenetic dendrogram construction

Sequence alignment was performed using Clustalo program (Sievers et al., 2011). Program Bioedit was used to assess the entropy within the alignment and validate the alignment (Hall, 1999). Phylogenetic analysis with maximum likelihood method was conducted in MEGA5 software (Tamura et al., 2011). All positions containing gaps and missing data were eliminated. Bootstrap resampling analysis for 1000 replicates was performed to estimate the condense of tree topologies.

3.10.3 Optimum pH and temperature on growth

Modified CDA plate medium of pH gradient: 3.3, 4.3, 5.3, 6.3, 7.3 and 8.3 adjusted with filtered sterilized 10% tartaric acid and 1 M NaOH were prepared. Each set of pH graded media plates were inoculated with one mycelia disc (each 0.3 cm dia.) of MNPf3, BMf4 and SPf7 isolates and incubated at 30˚C in stationary incubator for 60 h. The growth in diameter was measured every 12 h interval. Media plates of pH 5.3 containing the same composition were inoculated with a mycelia disc (each 0.3 cm dia) of the isolates and triplicate sets of the inoculated media plates were incubated at different temperature ranges: 15˚C, 25˚C, 30˚C and 37˚C.

3.10.4 Optimum pH and temperature on enzyme production

The effect of pH on enzyme production was analysed by varying the pH (3.3 to 8.3) of modified Czapek Dox Broth (CDB) medium supplemented with cellulose powder (3 g/l). Triplicate sets of Erlenmeyer flask containing 30 ml of CDB were inoculated with six mycelial discs (each 3 mm dia.) of MNPf3, BMf4 and SPf7 isolates and incubated in an orbital shaker (120 rpm) at 30˚C for 8 days. Similarly,
the effect of temperature was studied at 15°C, 25°C, 30°C, 35°C, 40°C and 45°C, keeping the pH of the culture medium at pH 5.3.

3.11 In-depth study on the cellulolytic characteristics of T. verruculosus SGMNPf3

Amongst the three identified fungal isolates, T. verruculosus SGMNPf3 was chosen for analysis of its secretory enzymes based on the availability of limited scientific reports on its cellulolytic potential.

3.11.1 Time course of cellulase production

The cellulase production pattern against incubation period was analysed by incubating cultures for a period of 18 days. Culture flasks (100 ml capacity) each containing 30 ml modified CDB medium were inoculated with three mycelial discs (each 0.3 cm dia.) and incubated at 30°C in shaker incubator (Sanyo, Osaka, Japan). Cellulose powder (Sigma Aldrich®, Saint Louis, MO, USA) was the sole carbon source in the medium. Triplicate sets were analysed for the production of enzyme every two days interval by FPase activity assay.

3.11.2 Enzyme production on different substrates

Cellulase enzymes production was determined using commercial and natural cellulose substrates. Commercial cellulose substrates were avicel (Fulka), cellulose powder (Sigma Aldrich), carboxymethylcellulose (CMC) and filter paper (Whatman–I). The natural cellulose substrates used were rice straw, sugarcane bagasse and saw dust. The natural substrates, including filter paper were powdered
using a grinder (USHA, India). Modified CDB media was supplemented with 5 g l$^{-1}$ of commercial substrates and with 1 g l$^{-1}$ of filter paper and natural substrates. The enzyme production was determined by estimating the FPase activity of the culture supernatant after 5 days of incubation. The extracellular proteins from the cultures were precipitated and run on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

3.11.3 Precipitation of secretory proteins of *T. verruculosus* SGMNPf3

The culture supernatant was obtained by centrifugation of culture broth at 1500 g. (NH$_4$)$_2$SO$_4$ was added to the clear culture supernatant to give 85% saturation at 4°C. After standing overnight the precipitate formed was collected by centrifugation (Thermo Scientific Biofuge Primo R) at 10,000 g for 30 min, and dissolved in 50 mM sodium acetate buffer, pH 5.3. The dissolved sample was dialyzed against the same buffer and concentrated by lyophilisation (Thermo Scientific). The protein obtained was quantified using Bradford method using Bradford reagent (Bio-rad$^\text{®}$).

3.11.4 Polyacrylamide gel electrophoresis and protein profile

The gel was prepared with 0.1% SDS in 12.5% separating gels and 5.0% stacking gels. Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli (1970a). Samples to be analysed were treated with sample buffer and boiled for 3 min prior to application to the gel. Electrophoresis was run at a constant 80 V at room temperature in a Mini-Gel Electrophoresis unit.
Molecular weight standards were used to determine the subunit molecular weight of the enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie blue R-250 in Chemi Doc system.

3.11.5 Extracellular and intracellular protein profile

The extracellular proteins from the culture supernatant were precipitated by (NH₄)₂SO₄ at 85% saturation. After standing overnight, the precipitate formed was collected by centrifugation at 10,000 g for 30 min (Thermo Scientific Biofuge Primo R) and dissolved in 50 mM sodium acetate buffer (pH 5.3). The dissolved sample was dialyzed against the same buffer and concentrated by lyophilisation (Thermo Scientific). Quantification of crude protein precipitate was carried out by Bradford method. SDS–PAGE was performed according to the method of Laemmli (1970b) using 12.5% gels. After electrophoresis, the gels were stained by a solution of 0.1% (w/v) Commassie blue, 40% (v/v) methanol and 10% (v/v) acetic acid. The gels were destained by a solution of 40% (v/v) methanol and 10% (v/v) acetic acid and then gels were kept in 7% (v/v) acetic acid. Molecular weight standards were used. To determine the intracellular nature of the cellulases produced by the fungus, the hyphae from CDB grown culture was collected in 50 ml centrifuge tube. The collected residues were cleaned thrice with 50 mM citrate buffer pH 5.3. One set of the culture broth residues were sieved through 60 µm pore sized sifter to free it from cellulose powder remnant. 1 g each of the set were crushed using motor and pestle with a pinch of 200 mesh silica gel (Merck) and 500 µl 50 mM citrate buffer pH 5.3. Then, 5 µl of the clear supernatant was spotted on CDA medium plates and incubated at 50°C for 1 h. The plates were flooded with Gram’s Iodine.
for 2–3 min and then observed for clearing zone as described in screening method for cellulase activity (Kasana et al., 2008c).

3.11.6 Zymogram for cellulase enzyme determination

To determine the protein bands with cellulytic activity, regular SDS–PAGE was performed with slight modifications: 12% acrylamide gel was supplemented with 0.3% CMC, the sample was not boiled and β–Mercaptoethanol was omitted from the sample buffer. After the run, SDS was removed by incubating the gel in 1% TritonX–100 for 30 min (repeated twice). The gel was washed extensively in distilled water to remove the excess TritonX–100 and then again incubated for 1 h at 50°C in 50 mM Sodium Citrate buffer at the optimum pH of the enzyme (Karnchanatat et al., 2008). The gel was then stained with Gram’s Iodine.

3.11.7 Dot Blot analysis for cellulase and β-glucosidase

The analysis was carried out by dot blotting 2 µl of protein (3µg) each of the samples {viz. cellulase and β-glucosidase (Sigma Aldrich, USA), primary antibodies, BSA and secretory protein of T. verruculosus SGMNPf3 (test sample)} per dot on a PVDF membrane. Three strips of the membrane were made. On the first strip, test sample, commercial cellulase and BSA were dotted. On the second strip, test sample, β-glucosidase and BSA were dotted. On the third strip, test sample, secondary antibodies and BSA were dotted. The preparation was allowed to dry for 1 h and then blocked with 3 % BSA in TBS-T solution for 3 h. Overnight incubation was carried out with primary antibodies (first strip with anti-cellulase, second strip with anti-β-glucosidase and third strip with secondary antibodies) in
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TBS-T solution containing 0.1 % BSA. Then washed thrice with TBS-T solution in 50 ml centrifuge tube in a shaker. Preparation was then incubated with Alkaline Phosphatase conjugated secondary antibody in TBS-T/BSA (0.05%) solution for 30 min at RT and then washed thrice. The development was carried out in BCIP-NBT solution.

3.11.8 Western Blot analysis for β-glucosidase

To detect the protein band having β-glucosidase activity, 3 μg of crude protein samples were separated on a 12% gel under native conditions. The proteins were transferred onto a 0.2 μm polyvinylidene fluoride (PVDF) membrane (Bio-rad®). The blot was performed in Trans-Blot® Turbo (Bio-rad®) at 1.3 A, 25 V, for 28 min. Thermo Scientific PageRuler Plus Pre-stained Protein Ladder was used for calibrating the immunoblotting assays. The membrane was blocked with 3% BSA in 1X Tris buffered saline (8 g NaCl, 0.2 g of KCl, 3 g of Tris base, pH adjusted to 7.5 with 1N HCl) containing 0.05% v/v Tween-20 (TBS–T) for 1 h to reduce non-specific binding. The membrane was then incubated with anti-β glucosidase primary antibody (Acris Antibodies GmbH, Germany) overnight at 4°C in TBS–T buffer containing 0.1% BSA, followed by incubation with secondary antibody (1:15,000 dilutions in TBS–T) of Goat anti-rabbit IgG coupled to alkaline phosphatase for 30 minutes. Each step after primary antibody coupling was followed by thorough washing of membrane with TBS buffer (100 mM tris HCl pH 9.5, 100 mM NaCl and 5 mM MgCl). Finally, the membrane was incubated in alkaline phosphate buffer and developed with BCIP/NBT (5–Bromo, 4–Chloro, 3–
Indolyl phosphate/Nitrobluetetrazolium). The process was performed on a rocking shaker.

3.11.9 *Two-dimensional electrophoresis of secretory proteins*

Extracellular protein harvested from culture broth supplemented with rice straw as the sole carbon source was processed for 2D electrophoresis (2DE) using the ReadyPrep 2DE Cleanup Kit (BIO-RAD, USA) following the company’s instructions. The cleaned protein was dissolved in ReadyPrep Rehydration buffer (Bio-rad®). Immobilized pH gradient (IPG) strips (11 cm, pH 3 to 6; Bio-rad®) were rehydrated overnight with approximately 50 µg (200 µl) protein and then subjected to isoelectric focusing (IEF). IEF was performed with a PROTEAN i12 IEF Cell system (BIO-RAD, USA) at 20°C with a 3-phase gradient program: 500 V for 0.25 kVh, 4000 V for 5.25 kV h, and 4000 V for 28 kVh. Following IEF, each strip was equilibrated for 12 min in 2 ml of equilibration buffer-I [50 mM Tris-HCl, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue] containing 1% (w/v) dithiothreitol. A second equilibration step with equilibration buffer-II containing 2.5% (w/v) Iodoacetamide in the SDS equilibration buffer-I was then followed. The strips were then loaded onto 12.5% homogeneous polyacrylamide gel. The second dimension of the separation was performed using the PROTEAN II xi cell system (Bio-rad®) operating at 15°C and with the following 2-phase gradient program: 90 V for 15 min and 200 V for 4 h. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) R250
(for spot-picking experiments). The 2DE gels were documented in the ChemiDoc (Bio-rad®) and the protein spots were analysed using PD Quest software.

3.11.10 MALDI-TOF and LC-MS/MS protein identification

A total of 17 protein spots which were dominant on the 2DE gel were excised with a clean scalpel and cut into small cubes (1 mm). The in-gel digestion for peptide preparation was carried out according to the protocol of Shevchenko et al. 2006. The samples were desalted and concentrated using C18 zip-tips (Eppendorf, Hamburg, Germany) and eluted onto an AnchorChip sample plate (Bruker Daltonics, Billerica, MA) with 1 µl of matrix (1 mg ml⁻¹ α-cyano-4-hydroxy cinnamic acid in 90% [vol/vol] acetonitrile, 0.1% [vol/vol] trifluoroacetic acid). Matrix-assisted laser desorption ionization (MALDI)–mass spectrometry (MS) was performed using an UltrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Billerica, MA) analyzer. The spectra were acquired in reflector mode in the mass range of 700 to 3,500 Da and Peak lists were generated by Bruker Daltonics flexAnalysis (Bruker Daltonics, Billerica, MA) and were searched against fungal proteins in the NCBI nonredundant database (NCBI 20140323 ) and NCBI (Fungi) using the Mascot search engine (Matrix Sciences, London, United Kingdom). Protein identifications were assigned where the Mowse scores were significant ($P < 0.05$). All proteins in NCBI (Fungi) and peptides matching to known contaminants (e.g. trypsin, keratin and collagen) were eliminated from the analysis. Similarly for LCMS analysis, the digested peptides were reconstituted in 15 µl of 2% ACN with 0.1% formic acid and 1 µl of the same was injected on to
the column. Digested peptides were subjected to 70 min RPLC gradient, followed by acquisition of the data on LTQ Orbitrap Discovery-MS (Thermo scientific). Generated data was searched for the identity using MASCOT (http://www.matrixscience.com) as search engine against the *Talaromyces verruculosus* database of Swiss prot and TrEMBL. For the LC-MS/MS data, a mass error of 0.8 Da was allowed for both the MS and MS/MS mode and variable modifications were set as for the database searches with the MALDI/MS data. To validate the results, false-discovery rate (FDR) was calculated using a decoy database (http://www.matrixscience.com/help/decoy_help) at FDR cu-off of 1%.

### 3.12 Preliminary attempt to scale up enzyme production

Three different approaches were made to determine the best process to increase the production of cellulolytic enzymes from *T. verruculosus* SGMNPf3.

#### 3.12.1 Submerged Fermentation (SF)

Submerged Fermentation (SF) for production was carried out using the CzD broth medium (g/L: [NaNO₃ 2.0; K₂HPO₄ 1.0; MgSO₄ 0.5; KCl 0.5; Fe₂SO₄ 0.01; filter paper 3.0]). The substrate used as sole carbon source was filter paper and cellulose powder. Six mycelial discs (each 3 mm dia) of the fungal colony was inoculated uniformly on the solid substrates. The preparation was incubated in a shaker incubator at 30°C.
3.12.2 Solid State Fermentation (SSF)

8.2 Solid State Fermentation (SSF) was carried out on two solid cellulose substrates namely rice straw and filter paper. The rice straw and filter paper were first cut into small pieces and then grinded using a Mixer Grinder (USHA, Gurgaon, India). The grounded rice straw was then autoclaved with water. Then sieved through a sifter and later dried. This final product was used as a substrate. 1 g of processed rice straw or grinded filter paper was autoclaved in 100 ml capacity conical flask. These were then moistened with CDB minimal broth media under the sterile platform of laminar air flow. Six mycelial discs (each 3 mm dia) of the fungal colony was inoculated uniformly on the solid substrates. The cotton plugs of the preparation was wrapped with aluminium foil. The preparation was incubated in a stationary incubator at 30°C.

3.12.3 Bioreactor /Fermentor

Fermentor (New Brunswick BioFlo) was used to produce cellulolytic enzymes from \textit{T. verruculosus SGMNPf3}. 6 l of CDB medium was prepared in which grinder filter paper (5g/l) was the sole carbon source. The whole tank was autoclaved with the medium inside it and with all the outlet of the system wrapped with aluminium foil at 120°C, 15 psi for 20 min. To obtain the pre-inoculum, six mycelial discs (each 0.3 cm dia) were inoculated in 250 ml conical flasks containing 100 ml of culture medium and were incubated at 30°C, 120 rpm for 72 h. Cellulase production was performed batch-wise in an instrumented bioreactor (New Brunswick BioLogic Flo), which was inoculated with 5 and 10% v/v of pre-
inoculum. Grinded Filter paper (30 g/l) was used as a carbon source for cellulase production. The nominal and working volumes were 5 and 7 l, respectively. The temperature, agitation and pH were maintained at 30°C, 200rpm and 5.3, respectively. Air was sparged at 0.5–1 vvm to provide dissolved oxygen concentrations within 20–40% of the saturated level. Samples were withdrawn every 12 h for determination of of FPase activity. Total protein content was measured using the Bio-Rad protein assay (Bio-rad®), which is based on the Bradford method. BSA was used as standard.

3.13 Preliminary attempt for purification of its individual cellulase enzymes

Purification of extracellular individual cellulolytic enzymes was attempted using size exclusion, ion exchange and affinity based chromatographic techniques on i) AKTA prime plus protein purification system (GE Healthcare, UK) and BioLogic DuoFlo (Bio-rad®) and normal gravity flow chromatography.

3.13.1 Size exclusion chromatography

The enzyme solution injected through injection valve of the system into gel filtration matrix containing Sephacryl S-200 HR, packed into a C 10/10 column. The column was pre-equilibrated with the 20 mM Tris Buffer containing 20 mM NaCl. The column was eluted with the same buffer at a flow rate of 3 ml/min. Each fraction of 6 ml was collected, and the active fractions were pooled, dialyzed, and concentrated by lyophilization and stored at -20°C.
3.13.2 Ion exchange chromatography

The ion exchange chromatography was carried out based on the information of the sample having pI below 6. HiPrep DEAE FF (GE Healthcare, UK) pre-packed columns were used to purify the individual cellulase enzymes on AKTA prime plus (GE Healthcare, UK) protein purification system. The enzyme solution was injected through the injection valve to the column previously equilibrated with 20 mM Tris buffer and a flow rate adjusted to 3 ml/ min. After the column was washed thoroughly with the buffer, the elution was carried out with the buffer containing NaCl in a linear concentration gradient from 0.0 to 2.0 M. Fractions of 8 ml each were collected and those fraction that fell within the same peak were pooled, concentrated by dialysis cum concentrator (Merck®, Bangalore, India) and then dissolved with Sodium citrate buffer pH 5.3 buffer.

3.13.3 Antibody based affinity chromatography

Normal gravity flow based chromatography using AffiGel 15 (Bio-Rad) matrix was performed. The Wizard SV Minicolumn (Merck®, Bangalore, India) was used to wash off the storage acetone reagent completely with de-ionised water from the AffiGel 15 matrix. The wet cake was then transferred to a fresh 5 ml eppendorf tube where the reagent containing the antibodies and the samples were added. The preparation was incubated overnight and then loaded on to Econo-glass column (Bio-rad®) position along with a fraction collector. The matrix was washed several times till no residue was observed at A280 nm. The target protein was eluted with MOPS of different pH gradient.
3.14 Statistical analysis

The values are presented as mean ± SD (standard deviation). Comparison between two groups was done by t-test and multiple comparisons between more than two groups were performed by one-way ANOVA supplemented with Tukey’s HSD test using StatPlus software (AnalystSoft Inc., Alexandria, VA, USA). Values at $P < 0.05$ were considered to indicate statistical significance.