



Production and Partial Purification of Cellulase from a New Isolate, *Penicillium verruculosum* BS3

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Authors' contributions

This work was carried out in collaboration between all authors. Author SB designed the study, wrote the protocol and edited the first draft of the manuscript. Author S. Sajith managed the analysis of the study, wrote the first draft of the manuscript and literature searches. Authors S. Sreedevi, PP and KNU helped in analysis and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study describes the production of cellulases by a newly isolated fungus, *Penicillium verruculosum* BS3 on lignocellulosic biomass with special emphasis on endoglucanase.

Study Design: Isolation, screening and characterization of cellulolytic fungus. Production of cellulase by submerged and solid state fermentation. Partial purification of cellulase by ammonium sulphate precipitation, dialysis and spin column. Characterization by Native PAGE and SDS-PAGE.

Place and Duration of Study: Enzyme Technology Laboratory, School of Biological Sciences, University of Calicut, Kerala - 673635, India, between October 2013 and November 2014.

Methodology: The lignocellulolytic fungus was characterized and identified by lacto-phenol cotton blue staining and 28S rRNA gene sequencing. Preliminary confirmation of cellulase production was done by iodine plate assay method. Production of cellulase was carried out on synthetic and natural raw substrates employing submerged and solid state fermentation strategies. The produced cellulase was purified by ammonium sulphate precipitation, dialysis and spin column followed by

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characterization using native-PAGE and SDS-PAGE.

Results: Primary screening on carboxymethyl cellulose (CMC) agar plates showed cellulase production potential of *P. verruculosum* BS3. Production of cellulases was quantitatively estimated on basal salt medium (BSM) supplemented with 1% CMC by submerged fermentation (SmF). Among cellulases, endoglucanase showed the highest yield (930.9 U/ml), which was focused on the subsequent studies. The CMC in BSM was replaced by the natural flour (1%) of banana, tapioca, potato or banana peel, of which tapioca flour supported the maximum production of endoglucanase (598.3 U/ml). In solid state fermentation (SSF), *P. verruculosum* BS3 was grown on sawdust supplemented with 10% banana flour as a solid support, moistened with BSM and the maximum production of endoglucanase (3214.1 U/gds) was observed on day 9 of incubation at 28°C. The partially purified (9.1 folds purification with 2.1% yield) cellulase was then characterized by native PAGE and SDS-PAGE. Upon staining with Congo-red (1%), on the CMC (0.25%) impregnated native gel, the zymogram showed a major cellulase fraction as yellow opaque zone. From the SDS-PAGE profile, the approximate MW of cellulase was estimated as 17 kDa.

Conclusion: This study signifies the industrial importance of the cellulase produced by *P. verruculosum* BS3 on cheap agro-products, and that the low MW cellulase has added importance as it offers potentials for protein engineering.

Keywords: *Penicillium verruculosum* BS3; endoglucanase; lignocelluloses; solid-state fermentation; purification.

1. INTRODUCTION

In the recent years, lignocellulosic substrates have gained more attraction for the manufacture of value-added products through bioprocess technology. Lignocelluloses are mainly composed of lignin, cellulose and hemicelluloses, among which cellulose contributes the major part of its bulkiness. Cellulose, the major structural polysaccharide in plants, is the most abundant organic compound in nature, and its terrestrial production crosses over 30 billion tons per year. Cellulose is an un-branched glucose polymer composed of glucose units linked by β -1,4-D glycosidic bond [1,2]. Cellulose has huge potential as sustainable and renewable source of energy, as it offers reduced exploitation of fossil fuels and environmental pollution [3]. For the past few years, there has been significant advances in identifying new cellulase resources that can efficiently and economically convert cellulose into fermentable sugars. Cellulase is a complex enzyme consists of endo- β -glucanase (EC 3.2.1.4), exo- β -glucanase (EC 3.2.1.91) and β -glucosidase (EC 3.2.1.21), and the synergistic action of all these three enzyme components is required for the hydrolysis of cellulose [4,5].

Among the groups of cellulolytic microorganisms, fungi are the most studied member with respect to degradation of cellulose and production of cellulases [6]. Fungal cellulases have been used for several years in food processing, feed preparation, waste water treatment, detergent

formulation, textile production, and a host of other areas. Members of the ascomycota, basidiomycota, and deuteromycota, coupled with some chytrids inhabiting the rumen are well known producers of cellulases [7]. Even though there are many reports on cellulolytic fungi producing cellulases such as *Aspergillus*, *Trichoderma*, *Penicillium*, and *Phanerochaete chrysosporium* [8], only a few have proved higher activities for commercial use.

Cellulolytic fungi can efficiently transform the cellulosic biomass into a wide range of platforms for the production of free sugars, fuels, and value added products by applying suitable strategies of submerged fermentation (SmF) or solid state fermentation (SSF) [9]. Many studies were reported on the production of cellulase by SmF, but the high cost of downstream processing hinders its economic applications in industries [10]. Now-a-days, the SSF strategy is gaining more interest for the enzymatic hydrolysis of biomass, because of its cost effectiveness and easier processing [11]. Moreover, it offers effective methods for the decomposition of cellulosic wastes. Upon this background, in the present study, we focused on isolation and characterization of novel mycelial fungi from the centuries-old wood-yards on the Kallai river belt; screen them for the cellulolytic activities; explore the potentials for the production of cellulase by SmF and SSF; and to purify and characterize the cellulase produced.

2. MATERIALS AND METHODS

2.1 Chemicals and Media

Analytical grade chemicals and reagents were used in this study. Potato-dextrose agar (PDA) and carboxymethyl-cellulose (CMC) were procured from Hi-Media Laboratories Pvt. Ltd. India. All other chemicals and reagents were procured from Sigma (USA), Hi-Media (India) or Merck India. Basal mineral salt medium (BSM) with composition (g/l): 2 NaNO₃; 1 K₂HPO₄; 0.5 MgSO₄.7H₂O; 0.5 KCl and 2 protease peptone (pH 5) was the basic medium (synthetic) used throughout the study. Raw carbon sources flour of banana, tapioca, potato or banana peel were also used as supplement to the BSM, as described in the respective sections. Before inoculation, all the media were autoclaved at 15lbs, 121°C for 20 min.

2.2 Isolation of Fungi

Samples were collected from different localities from the wood-yards on Kallai river belts as we described which was suspended in sterile distilled water and serially diluted by conventional dilution methods [12,13]. Suspensions after serial dilution were spread on PDA medium containing (g/l): 200 potato infusions; 20 dextrose and 15 agar. Repeated sub culturing was accomplished for confirming the purity of the isolated fungal cultures, and preserved subsequently on PDA slants.

2.3 Morphological Characterization

The macro morphological characterization of the isolate was done by observing colony characteristics such as colour, texture and spore structures, according to the hand book for the identification of fungi [14]; and micro morphologically by employing conventional lactophenol cotton blue method.

2.4 Molecular Characterization

The molecular level characterization of the isolate was confirmed by PCR-amplification using forward and reverse rDNA sequencing primers, *i.e.*, with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The D1/D2 region of LSU (Large subunit of 28S rDNA) gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score, first ten sequences were selected and the

phylogenetic tree was constructed using MEGA 4 (Xcelris Labs, Ahmedabad, India).

2.5 Screening for Cellulase Activity

The iodine plate assay method was used for the qualitative determination of cellulolytic activity of the culture on CMC-agar medium [15]. Five day old culture from PDA slant was cultured on BSM-CMC-agar plates. After 4 days of incubation, the plate was flooded with iodine solution (1% iodine crystals and 2% potassium iodide) and then incubated at 30°C for 15 min, and excess stain was drained off for visual observation of clear zone around the mycelial growth.

2.6 Inoculum Preparation

Fungal spores were harvested aseptically from 5 day old PDA slants. Spores were mixed in sterile ddH₂O and shaken vigorously by vortex mixture for preparing a uniform suspension to be used as inoculum. Haemocytometer was used for calculating the number of spores ($\sim 5.9 \times 10^7$ spores/mL) in 1 mL of suspension.

2.7 Production Conditions of Submerged Fermentation (SmF)

2.7.1 Production of cellulase by CMC as carbon source

To examine cellulase activity in liquid medium, with "fungal isolate was cultured in BSM with 1% CMC as carbon source in 100 mL Erlenmeyer flasks. After sterilization, the medium was inoculated with 100 μ L spore suspension ($\sim 5.9 \times 10^7$ / mL) under aseptic conditions. All flasks were incubated at 28°C in an environmental shaker for 8 days, and samples were withdrawn (whole flask) for various assays at 24h interval. After centrifugation (9000 \times g), the supernatant was used as crude enzyme extract for endoglucanase, exoglucanase, and β -glucosidase assays.

2.7.2 Production of cellulase on natural flours

BSM was supplemented with 1% banana peel flour (BPF), tapioca flour (TF), banana flour (BF) or potato flour (PF) instead of CMC as carbon source in 100 mL Erlenmeyer flasks. After sterilization, the medium in the flasks were inoculated with 100 μ L spore suspension ($\sim 5.9 \times 10^7$ / mL) under aseptic condition. All flasks were incubated at 28°C in an environmental shaker for

8 days, and samples were withdrawn (whole flask) at 24 h interval for various assays. The supernatant was used as crude enzyme extract for assays.

2.8 Production Conditions of Solid State Fermentation (SSF)

To analyse the production on solid support, sawdust or banana peel flour independently or sawdust (from the heart wood of teak) supplemented with 10% (w/w) flours of tapioca, banana or potato was used. SSF was carried out in 100 mL Erlenmeyer flasks. One gram of substrate was taken in individual flask and 10 mL BSM was added (pH 5). The flasks were sterilized by autoclaving at 15lbs, 121°C for 20 min. Then the flasks were inoculated with 100 μ L spore suspension ($\sim 5.9 \times 10^7$ / mL) in sterile condition. The contents in the flasks were mixed thoroughly to ensure uniform distribution of the inoculum. All flasks were incubated at 28°C for 15 days under static condition. The samples (whole flask) were withdrawn at 2 day interval, and the enzyme was extracted with 10 mL (0.1M) citrate buffer (pH-4.8) by stirring for 10 min under chilled condition. The supernatant (9000 \times g, 10 min, 4°C) was used as crude enzyme extract.

2.9 Quantitative Estimation of Cellulase Production

Quantification of cellulolytic enzymes, *i.e.*, endoglucanase, exoglucanase and β -glucosidase was accomplished by dinitrosalicylic acid (DNS) method [16] using CMC (Himedia, India), filter paper (Whatman, GE Healthcare U.K., Ltd.) and salicin (Himedia, India), respectively as substrates. For the estimations of endoglucanase and β -glucosidase, the reaction mixture used contained 0.5 mL of 1% (w/v) substrate in 0.1 M citrate buffer (pH 4.8) and 0.5 mL of culture supernatant. The mixture was incubated at 50°C for 30 min, which was stopped by adding 3 mL DNS. For the exoglucanase activity assay, rolled filter paper strip (1 \times 6 cm, 50 mg) was saturated with 0.5mL of 0.1 M citrate buffer (pH 4.8), and equilibrated for 10 min at 50°C, followed by the addition of 0.5 mL culture supernatant. Subsequently, the reaction mixture was incubated at 50°C for 60 min and the reaction was stopped by adding 3 mL DNS. Then, the mixture was incubated for 5 min in boiling water bath, and cooled rapidly. The absorbance was measured against the reagent blank at 540 nm using UV-vis spectrophotometer

(Shimadzu UV-1601, Japan). The quantity of reducing sugar liberated was quantified using glucose as standard. One unit of cellulase is defined as the quantity of enzyme required for liberating 1 μ mol of glucose equivalents per min under the assay conditions.

Cellulase activity for SmF and SSF was calculated using the formula, $\frac{\Delta E \times V_f}{\Delta t \times \sum \times V_s \times d}$ and $\frac{\Delta E \times V_f \times V_s}{\Delta t \times \sum \times gds \times d}$, respectively. Where, ΔE = absorbance at 540 nm, V_f = final volume of reaction mixture including DNS, V_s = crude supernatant (mL) containing cellulase used, Δt = incubation time for of hydrolysis, \sum = extinction coefficient of glucose (0.0026), gds = grams dry substrate, d = diameter of cuvette.

2.10 Partial Purification of Cellulase

Crude enzyme extract (after SSF with sawdust-banana flour-BSM) was precipitated with ammonium sulfate (0-20%, 20-40%, 40-60% and 60-80%) up to 80% saturation. The precipitate obtained after each saturation point was dialyzed in a cellulose membrane bag against 20 mM sodium citrate buffer (pH 4.8) for 24 h at 4°C. After dialysis, the precipitate was collected by centrifugation (9000 \times g for 10 min at 4°C), and the 40-60% fraction, which showed maximum cellulase activity was subjected to spin column purification (Vivaspin 6, Sweden) with a MW cut-off of 45 kDa. The fraction below 45 kDa obtained from spin column was used for further characterization studies. The estimation of protein content was done by the method of Lowry et al. [17].

2.11 Characterization of Cellulase

Native-PAGE and SDS-PAGE were employed for the determination of approximate molecular weight of the cellulase. For the native PAGE, the separating gel (10%) was impregnated with CMC (0.25%), and stacking gel was 5%. After electrophoresis, the gel was immersed in 100 mM sodium citrate buffer (4.8) for 30 min at 28°C. Congo-red (0.1%) was used for staining the gel and 1M NaCl was used for de-staining, which helped for visualizing the yellow active zone on the gel [18]. For SDS-PAGE, 12% separating and 5% stacking gels were used [19]. Reference marker proteins (Bioscience, Merck, India) were used. After electrophoresis, the gel was stained by coomassie-brilliant blue R 250 (0.1%).

2.12 Statistics

All experiments were conducted in triplicate and the values were given as mean±SE. Graphs were draw by Microsoft Excel. Adobe Photoshop CS5 was used for setting the images.

3. RESULTS

3.1 Identification of Culture

The fungus used in this study was identified as *Penicillium verruculosum*, for which BS3 was assigned as strain name (GenBank accession number: HQ876770) by morphological and molecular characterizations. The fungus grew as grayish green in 2 to 3 days of incubation on PDA agar, and soon spread all over the surface of the plate as green mycelial mat. Surface of the colony was smooth and velvet in nature (Fig. 1A). The growth of fungus was very fast and micro-morphological observation by lacto-phenol cotton blue stain showed brush like

conidiophores sprout on the mycelia, bearing individually constricted conidiospores (Fig. 1B). After initial identification, the species level confirmation was done by molecular characterization using PCR-amplification of D1/D2 region of LSU (large subunit) of the 28S rRNA gene from the isolated genomic DNA, based on nucleotide homology and phylogenetic analysis. Its similarity to other related taxa was also analyzed, thus the name was confirmed (Fig. 2).

3.2 Cellulolytic Activity

The fungus *P. verruculosum* BS3 showed cellulolytic activity on BSM-CMC agar medium. After incubation, the fungus on the medium (in the plates) was flooded with Gram's iodine and kept for 5 min for the development of clear zone; the fungus showed clear and distinct zone around the mycelial colonies against the bluish black coloration with unhydrolyzed area of cellulose, indicating cellulase production (Fig. 3).

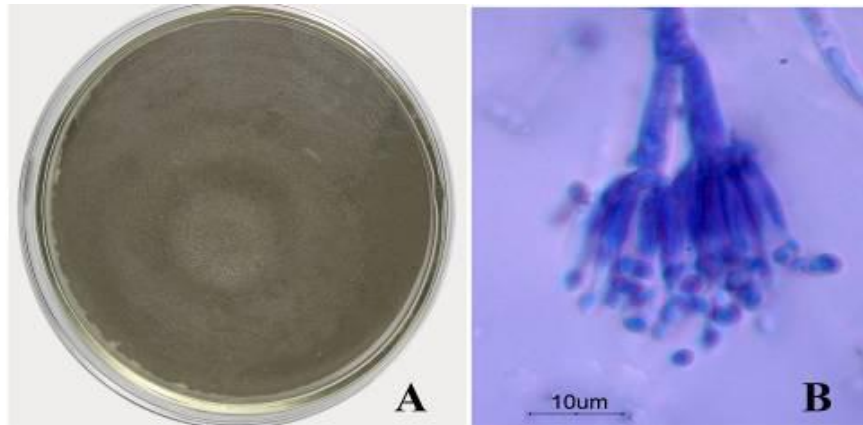


Fig. 1. Morphology of *Penicillium verruculosum* BS3: A. *Penicillium verruculosum* BS3 growing on PDA medium (Digital image, DSLR Canon 450 D, Japan); B. Fruiting body (Phase Contrast Microscope, Scale bar = 10 µM, Leica M80, Germany)

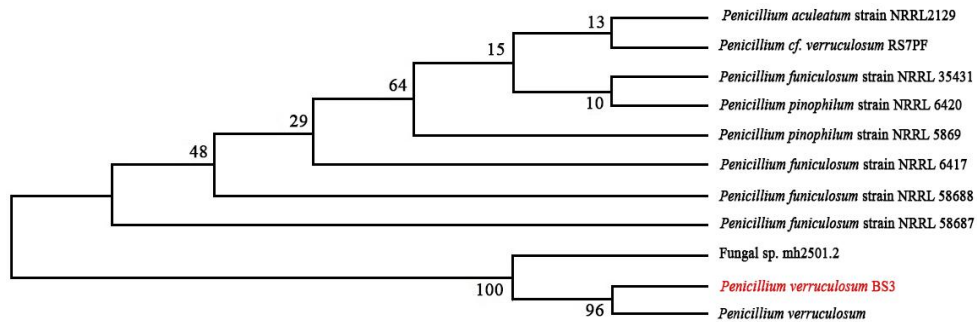


Fig. 2. Evolutionary relationship among different species of fungi

3.3 Production of Cellulase by SmF

Production of cellulases (endoglucanase, exoglucanase and β -glucosidase) by *P. verruculosum* BS3 was conducted in BSM supplemented with different percentages of CMC (0.5%, 0.75%, 1%, 1.25, 1.5%). Among this, 1% CMC showed the maximum production of endoglucanase (Fig. 4A). Hence, the ensuing studies were mainly focused on the production of endoglucanase. The production of endoglucanase was also quantified by supplementing natural flours (banana, banana peel, tapioca or potato) as carbon source, instead of CMC (Fig. 4B). This study showed that CMC as the selective carbon source for the production of cellulase.

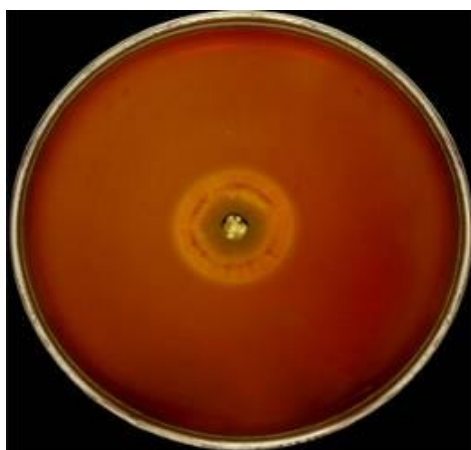


Fig. 3. Cellulolytic activities of *Penicillium verruculosum* BS3: A. iodine plate assay showing clear zone around the colony indicating cellulase activity on 4th day of incubation at 28°C

P. verruculosum BS3 showed the production of endoglucanase, exoglucanase and β -glucosidase in BSM supplemented with 1% CMC (w/v) at 28°C, pH-5 and 150 rpm. At this condition, *P. verruculosum* BS3 showed the maximum production of endoglucanase and β -glucosidase on day 6, whereas the maximum production of exoglucanase was on day 5 of incubation, which was about 930.9 U/mL, 380.8 U/mL, and 485.3 U/mL, respectively. By replacing CMC with 1% (w/v) natural flours, comparable production of endoglucanase was observed (Table 1). Among the natural flours, tapioca flour supported the maximum production of endoglucanase on day 6 of incubation whereas flours of banana, banana peel or potato supported the maximum production on day 5 at

28°C. The potato flour supported the least production of endoglucanase, whereas flours of banana and banana peel supported moderate production. These results indicate the efficacy of cheap agro-products as carbon source for the production of cellulase by *P. verruculosum* BS3.

3.4 Production of Cellulase by SSF

The production of endoglucanase by SSF was carried out according to the method of Sajith et al. [7]. For this, *P. verruculosum* BS3 was grown in raw sawdust or banana peel flour alone moisturized with BSM (w/v) or sawdust-flour mixture combination (90% sawdust + 10% flour of banana, tapioca or potato; w/w) moisturized with BSM. To check the optimum cellulase production, ratio (w/v) between the solid content to the moisture (BSM) (1:1, 1:2 or 1:3 ratios) was also tested. Among this, the maximum cellulase production was observed at 1:2 combination i.e., one part solid substrate (i.e., saw dust + flour) and 2 parts BSM (Fig. 5).

From the second day of incubation, *P. verruculosum* BS3 started growing on substrate which showed production of cellulase on sawdust; from the day 3 onwards, production of cellulase was increased at regular interval and reached the maximum on day 12 of incubation, whereas banana peel flour supported the maximum production of endoglucanase on day 3 of incubation. The mixture of substrates like 10% flours of banana, tapioca or potato in sawdust (10:90; w/w) was used for enhancing the production of cellulase. *P. verruculosum* BS3 started growing from the day 2 onwards, and the maximum production of cellulase was supported by sawdust supplemented with banana flour, which was on day 12 of incubation; whereas tapioca flour and potato flour supported the maximum production of endoglucanase on day 3 of incubation (Table 1). These results depict that the natural flours can act as inducer for the enhanced production of cellulase.

3.5 Partial Purification and Characterization of Endoglucanase

The partial purification of endoglucanase was done from the culture filtrate obtained by SSF (sawdust-BF-BSM) of *P. verruculosum* BS3. Ammonium sulphate precipitation, dialysis and spin column separation were carried out for the purification of endoglucanase. After ammonium sulfate precipitation and dialysis, 40-60% saturation showed the maximum cellulase

activity (3.77 folds purified with 15.2% yield), which was subjected to spin column purification with molecular weight cut off of 45kDa that resulted in 9.1 folds purification with 2.1% yield (Table 2). Native PAGE and SDS-PAGE analyses were used for the qualitative characterization of the cellulase. By native-

PAGE, the zymogram (0.25% CMC impregnated in gel) showed yellow opaque zone, which indicated the cellulolytic activity (Fig. 6). By SDS-PAGE analysis, the approximate molecular weight of cellulase was estimated as 17kDa (Fig. 6), which was observed as yellow opaque zone in the native gel (Fig. 6).

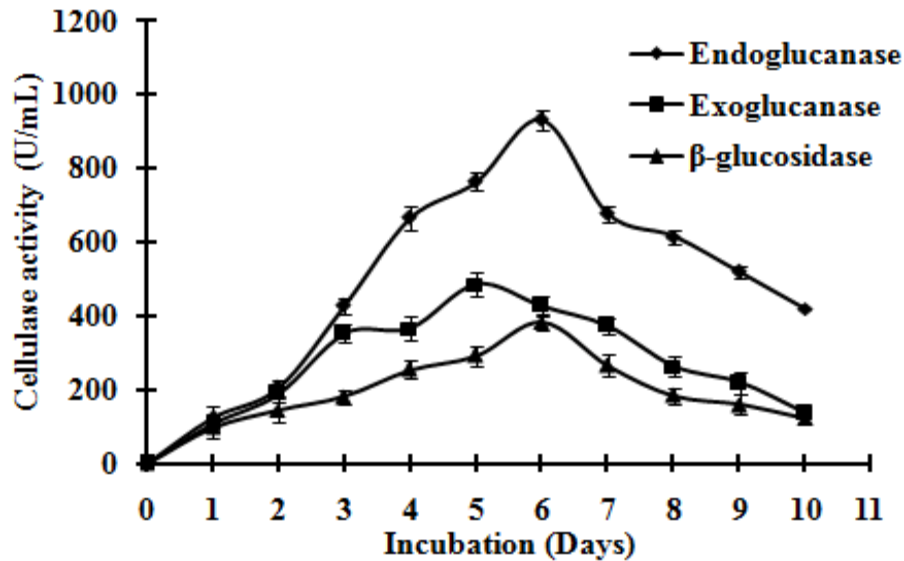


Fig. 4A. Cellulase production by *P. verruculosum* BS3 in SmF: endoglucanase, exoglucanase and β -glucosidase production in BSM supplemented with CMC at 28°C. Assay was done by DNS method spectrophotometrically at 540 nm (Shimadzu-UV1601, Japan)

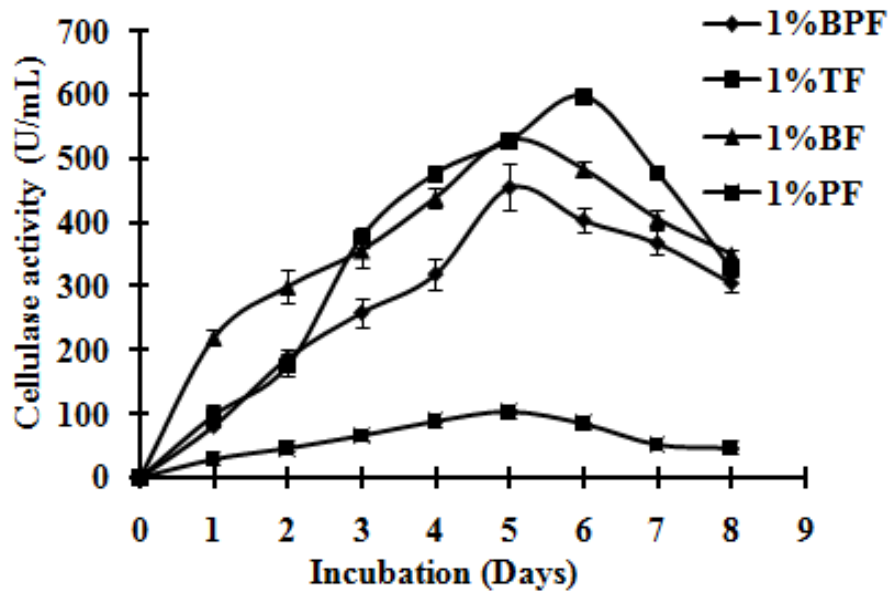


Fig. 4B. Endoglucanase production by *P. verruculosum* in SmF: production of cellulase in BSM supplemented with 1% of different substrates [banana flour (BF), tapioca flour (TF), potato flour (PF), banana peel flour (BPF)] at 28°C

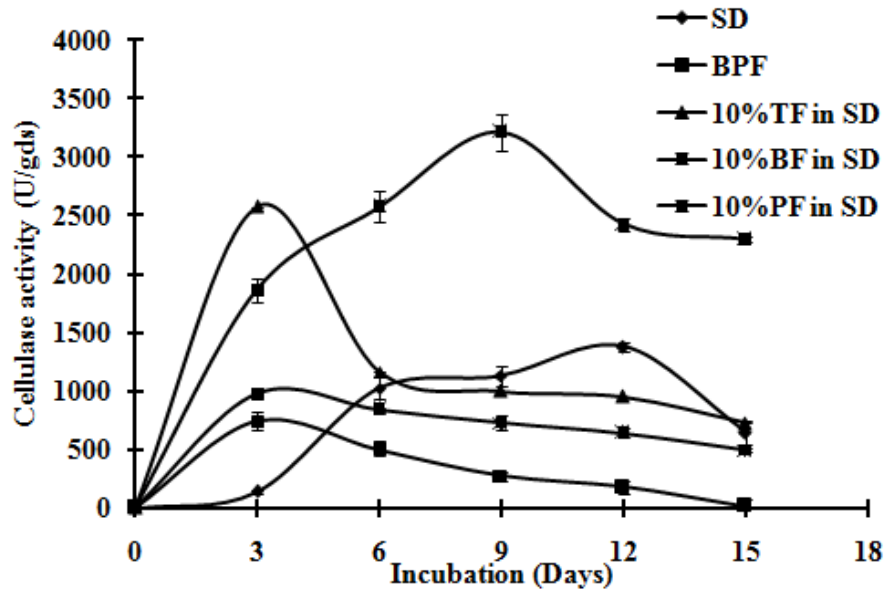


Fig. 5. Endoglucanase production by *P. verruculosum* BS3 in SSF: production of cellulase in sawdust (SD), banana peel flour (BPF) and SD supplemented with 10% TF, 10% BF and 10% PF respectively and moistened with BSM in the ratio of 1:2 at 28°C

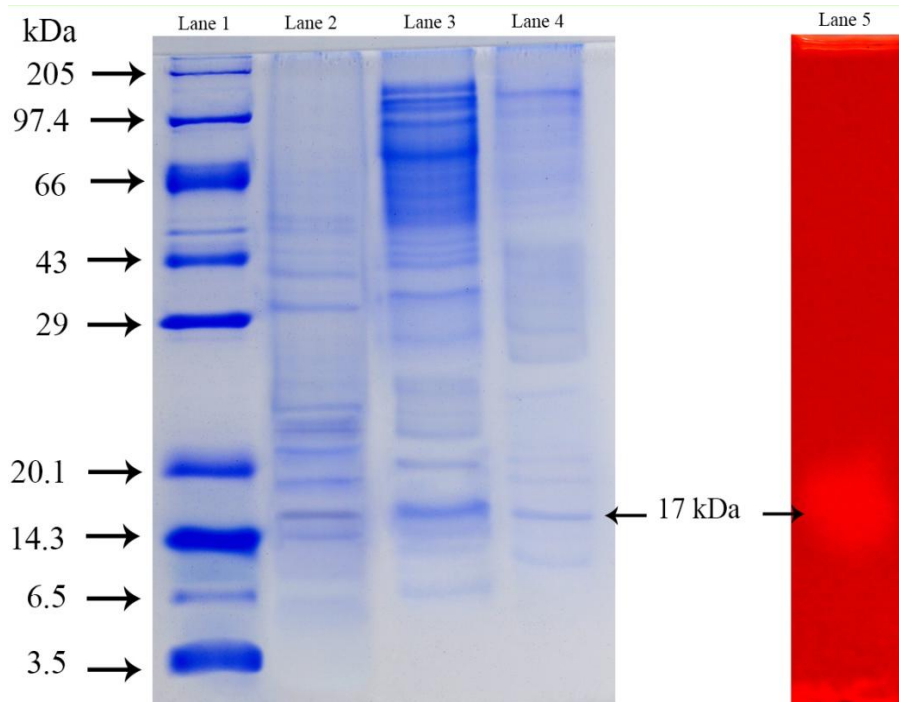


Fig. 6. Profile of partially purified endoglucanase by *P. verruculosum* BS3 in SDS and native PAGE: Lane 1: Reference molecular weight marker, Lane 2: Spin column fraction of partially purified endoglucanase, Lane 3: $(\text{NH}_4)_2\text{SO}_4$ (40-60%) fraction, Lane 4: crude sample, Lane 5: partially purified endoglucanase showing yellow opaque zone in native polyacrylamide gel

Table 1. Production of endoglucanase in SmF and SSF

Fermentation strategy	Substrate	Endoglucanase activity	Incubation (Days)
Submerged fermentation (U/mL)	Bananapeel flour (BPF)	455.2	5
	Tapioca flour (TF)	598.3	6
	Banana flour (BF)	529.0	5
	Potato flour (PF)	104.0	5
Solid state fermentation (U/gds)	Sawdust (SD)	1378.5	12
	BPF	744.4	3
	10% TF in SD	2570.9	3
	10% BF in SD	3214.1	9
	10% PF in SD	980.6	3

Table 2. Summary of purification fold of endoglucanase produced by *P. verruculosum* BS3

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Crude	243822.7	95.69	2548.1	100	1
(NH ₄) ₂ SO ₄ (40-60%)	37078.89	3.85	9622.47	15.2	3.77
Spin column	5155.95	0.223	23098.95	2.1	9.1

4. DISCUSSION

This work is a part of our ongoing programme for characterizing bacteria and fungi with high lignocellulolytic activities inhabiting the wood-yards on Kallai river banks in Kozhikode city, Kerala, India where the wood processing is being practiced for centuries. Several potent fungi [7] and bacteria [20] were already characterized from this region. Thus, this is a new report in the series.

The biodegradation of lignocellulosic biomass in the ecosystem is primarily carried out by microorganisms. Among these microorganisms, cellulolytic activity of fungi enabled them to play remarkable role in the biodegradation of cellulose, the major component of lignocellulose. In this study, we introduced a highly active cellulase producing fungus - *P. verruculosum* BS3 - utilizing cheap lignocellulosic biomass as the carbon source. During plate assay, the Gram's iodine spread over the plate and formed bluish-black complex with the native cellulose against the hydrolyzed cellulose, which indicated clear and distinct zone around the mycelial colony within a short period of time [15]. The plate assay method is one of the rapid techniques used for the primary screening of enzymes [20].

SmF and SSF were mainly employed in this study for the production of cellulase. Since cellulases are more or less inducible enzymes, the yield highly depends on the nature and

conditions of substrates used during fermentation [21]. Of the supplements (flours), some of them acted as suitable inducer for maximizing the production of cellulase. When CMC was used as the substrate in SmF, the yield of endoglucanase (930.9 U/mL) was higher than other 2 cellulases: exoglucanase (380.8 U/mL) and β -glucosidase (485.3 U/mL). Therefore, in subsequent studies only the production of endoglucanase was focused. Natural substrates supplemented in BSM as carbon sources also supported reasonably higher production of cellulase. Till date, several studies were reported on the production of cellulases using natural carbon sources. For instance, Picart et al. [22] reported 7.1 U/mL and 3.8 U/mL of endoglucanase on rice straw by *Penicillium* CR-316 and *Penicillium* CR-313, respectively. It was reported that *P. citrinum* (MTCC 6489) an extremophilic fungus effectively utilized rice bran and only 2 U/mL cellulase was produced [23]. *Trichoderma viride* showed the maximum production of cellulase (173 U/mL) in BSM supplemented with 1.25% of CMC under optimized conditions at 28°C and pH 4 [24]. In another study, *T. harzianum* produced 146 U/mL cellulase in the presence of 0.5% CMC in BSM [25]. Jadhav et al. [26] studied the production of cellulase in lignocellulosic biowastes like rice husk, millet husks, banana peels, wheat bran, coir waste and saw dust by different fungal strains such as *Aspergillus niger*, *A. oryzae*, *A. flavus*, *P. chrysogenum* and *Fusarium moneliforme*. Of them, *A. niger* showed the maximum cellulase production (12.4 U/mL) on banana peel after 4 days of incubation. In

comparison to all the above reports, it is clear that the cellulolytic activity of *P. verruculosum* BS3 is much higher in CMC (931 U/mL) as well as natural flour supplemented media.

In SSF, fermentation was carried out on solid support under limited moisture condition. SSF claims many advantageous over SmF because of its easier processing, low production cost and easily availability of substrate. Large volume of productivity by SSF with less wastage and water requirement makes the method more suitable for the production of industrial enzymes. In this study, low cost substrate, i.e., sawdust was used as the base solid substrate, and natural carbon source (flour) was supplemented as additional nutrient and inducer for the production of cellulase under SSF by *P. verruculosum* BS3. The maximum production of cellulase (3214 U/gds) was on sawdust-banana flour-BSM medium. Many fungi were showed as efficient producer of cellulase using lignocellulosic biomass. In SSF, moisture is a key factor, and the optimum moisture content may augment the production of cellulase, and that higher moisture content may limit the growth of fungi [27]. Agricultural biomasses such as wheat straw, wheat bran, corn stover, bulrush straw rapeseed straw and sugar cane bagasse were commonly used for the production of cellulase by fungi. Kim et al. [28] studied the production of cellulase (606 IU/g) using oil palm empty fruit bunch by *Penicillium* sp. GDX01; and *A. terreus* M11 efficiently produced cellulase (581 U/g) utilizing corn stover as substrates at 80% moisture [29]. *P. roqueforti* showed (53 U/gds and 49 U/gds endoglucanase production on wheat bran alone and wheat bran-pumpkin oil cake mixture, respectively [30]. Singhania et al. [8] studied the production of cellulase on both raw and pretreated agro-residues such as cassava bagasse, sugarcane bagasse, wheat bran and rice straw by *T. reesei* NRRL11460, which resulted in the maximum production of 158U/gds with pre-treated sugarcane bagasse at 72h of incubation, 66% initial moisture, pH-7 and 28°C. We already reported that the sawdust supplemented with inducers can effectively enhance the production of endoglucanase. For instance, *A. flavus* BS1 produced 5408 U/gds endoglucanase on sawdust medium supplemented with tapioca flour [7]. Similarly, in the present study, it is evident that the sawdust supplemented with banana flour supported the maximum production of endoglucanase by *P. verruculosum* BS3. From the above reports it is

clear that the *P. verruculosum* BS3 can efficiently produce endoglucanase on a low cost substrate by SSF, which is of industrial significance.

In the present study, the native gel (0.25% CMC impregnated gel) was used for the qualitative detection of endoglucanase. The presence of yellow opaque zone in the gel confirmed the activity of endoglucanase. Similar results were obtained in zymogram with the endoglucanase produced by *Fusarium oxysporum* [31]. Three proteins produced by *T. viride* T 100-14 showing endoglucanases activity were detected in 0.2% CMC impregnated gel [32]. Similarly, cellulase produced by *Penicillium* CR-313 and *Penicillium* CR-316 showed several activity band in the zymogram which exhibits the endoglucanase activity [22]. SDS PAGE was used for the detection of apparent molecular weight of endoglucanase produced by *P. verruculosum* BS3 which was found to be 17KDa. Different sp. of *Penicillium* showed broad range of isoenzymes in SDS PAGE. It was reported that the endoglucanase produced by *Penicillium citrinum* (MTCC 6489) exposed two bands with different molecular weight which was found to be 90kDa and 38 kDa respectively in the zymogram analysis [23]. Similarly, the molecular weights of two endoglucanases produced by *Penicillium occitanis* Mutant Pol 6 were about 31 kDa and 28 kDa respectively [33].

5. CONCLUSION

In recent years, bioconversion of lignocellulosic materials by enzymatic hydrolysis have gained much attention because of its practical application in various agro industrial processes like bio fuel production, delignification of paper pulp, detergent industry etc. The newly isolated strain *Penicillium verruculosum* BS3, is an industrial useful fungus which can effectively utilizes cheap and freely available carbon sources for the large scale production of cellulase. The endoglucanase (3214.1 U/gds) produced by the *P. verruculosum* BS3 was the highest ever reported by the *Penicillium* sp. under SSF. Apart from this, it helps in effective bioremediation of lignocellulosic wastes there by providing environmental and strategic benefits to the society. Hence, this study suggests an appropriate method for the bioconversion of lignocellulosic biomass and the strain can be used for industrially exploration for the production of value added products.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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