Production and Purification of Cellulase from *Aspergillus nidulans* AJSU04 under Solid-state Fermentation using Coir Pith

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The present study deals with the production of cellulase from coir pith accumulated as waste with the aid of *Aspergillus nidulans* AJSU04 and the subsequent conversion of the residual coir pith into suitable biofertilizer for the increased yield of *Solanum lycopersicum*. Alkaline pretreatment using NaOH is used to delignify the feed stock material (coir pith). The experiments were carried out under solid state conditions employing coir pith with 60 % moisture content, pH 5, temperature of 40 °C for 11 days. The extract drawn was purified using ammonium sulphate salt precipitation, dialysis, ion exchange chromatography and gel filtration chromatography. *Aspergillus nidulans* AJSU04 was seen to exhibit endo-β-1,4-glucanase, exo-β-1,4-glucanase and 1,4-β-glucosidase components of cellulase. The residual coir pith was converted into biofertilizer or coir pith waste compost (CWC) using *Azobacter chroococcum, Bacillus megaterium, Bacillus mucilaginosus*

Key words:
Aspergillus nidulans AJSU04, cellulase, solid state fermentation, coir pith, biofertilizer

Introduction

Coir pith is an organic matter which originates from the tropical hemisphere, especially from south-east Asia where coconut oil production is extensive. For professional oil winning companies, the husk of the nut is a waste product. These coconut husks mainly consist of coir pith and coir fibers. Initially, coir pith was considered a waste but now research has established widespread applications for it. Being an agro-waste from the coir industry, coir pith serves as a renewable source.[1] Accumulation of coir pith near coir retting factories causes solid waste pollution problems mainly due to the ligno-cellulosic compounds present in them. The lignin (31 %) and cellulose (27 %) that they contain is responsible for their slow degradation.[2] Over the last few years, environmental concerns have increased attention toward using coir pith as an alternative substrate with orientation towards agricultural needs. Thus, the conversion of such agricultural wastes into useful products may decrease the problems they cause. This study deals with the utilization of coir pith for cellulase production. But the lignin present in the coir pith was observed to interfere with hydrolysis by irreversibly binding the hydrolytic enzymes, thereby blocking access to cellulose. Pretreatment of coir pith increases the crystallinity of cellulose, thus removing lignin and enabling its enzymatic degradation. In addition, pretreatment may increase the surface area of the cellulose thereby enhancing its reactivity with the enzyme and thus its transformation.[3]

Cellulose is commonly degraded by an enzyme called cellulase.[4] Several studies have been carried out to produce cellulolytic enzymes from biowaste using degradation process by using microorganisms including fungi, such as *Trichoderma, Penicillium,* and *Aspergillus* spp.[5] The commercial use of cellulases is dependent on the following: high titer and good enzymatic activity, low production cost and feasible mass production. Cellulases are inducible enzymes and their synthesis is strongly repressed by soluble sugars. Cellulases are imperative enzymes not only for their potent applications in different sectors, like industries of food processing, animal feed production, pulp and paper production, detergent and textile, but also for the significant role in the bioconversion of agricultural wastes into sugar and bioethanol. The cellulase enzyme derived from a thermophilic anaerobe *Clostridium thermocellum*

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has been reported to be resistant towards end product inhibition and repression. Parameters such as control of pH and temperature are critical for the production and release of cellulases.[6] Many species of *Trichoderma* and *Aspergillus* are strongly cellulolytic.[7]

This study deals with the conversion of coir pith into a suitable biofertilizer by a locally isolated culture of *Aspergillus nidulans* AJSU04.[8] Solid-state fermentation (SSF) is an attractive strategy to produce cellulase using a variety of lignocelluloses as substrates.[7] *Aspergillus nidulans* strain appears to be the most promising microorganism; hence it was of interest to examine its role in the breakdown of coir pith for cellulase production.[8] Cellulase obtained from *Aspergillus nidulans* AJSU04 was purified and characterized. The coir pith, after its utilization as substrate for cellulase production, was modified and applied as a biofertilizer for tomato plantations (*Solanum lycopersicum*).

Materials and methods

Microorganism, inoculation and culture conditions

Fifty isolates of fungi were obtained from different soils, decomposing logs and composts collected from Solinganallur-Chennai, Alapuzha-Kerala and Cuddalore district of Tamilnadu. Samples were dispensed into petri plates and brought to the laboratory. The strain *Aspergillus nidulans* was isolated from decayed, outer shell of *Arachis hypogaea* from Cuddalore district of Tamilnadu, India.[8] Solid media containing 2 % malt extract, 2 % agar and 1 % CMC was used to screen the cellulase producing strain, followed by 0.01 % congo-red staining and 0.1% NaCl washing.[9] Isolate having clear orange halo zone on CMC agar plate was preferred in the subsequent studies. Cultures were stored on Malt Extract agar slants at 4 °C and as spore suspension in 10 % glycerol at –20 °C. Spore suspension prepared by washing slant cultures with 5 mL sterilized water was transferred into minimal medium (mg L^{-1}), KH_2PO_4, 0.75; NH_4NO_3, 1.5; Thiamine HCl, 0.01; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 5H_2O$, 0.05 at pH 5. Solid-state fermentation was carried out in a solid-state fermenter containing coir pith as the chief carbon source at 5–25 mm bed height on each tray, autoclaved for 30 minutes at 121 °C, inoculated with three mL of prepared spore suspension in the range of 10^3 to 10^5 spores mL^{-1}. This was then incubated at 50 °C under static conditions for 20 days. The strains *Azobacter chroococcum* AB175653, *Bacillus megaterium* D16273 and *Bacillus mucilaginosus* AF006077 were obtained from American-type culture collection, (ATCC, USA) to act as microbial inoculums for enhancing the residual coir pith properties. *Azobacter chroococcum* AB175653, *Bacillus megaterium* D16273 and *Bacillus mucilaginosus* AF006077 are found to be Nitrogen fixers, Phosphorous solubilizers and Potassium extractors respectively. Na-carboxymethyl cellulose and cellulose were procured from Sigma Chemicals (Mumbai, India). Agar and other medium components were purchased from Hi-media (Mumbai, India). All other analytical grade reagents were supplied by Merck and SD Fine Chemicals (Mumbai, India).

18s rDNA analysis

For the nucleotide sequence analysis, fungal genomic DNA was extracted and purified using the Fungi Genomic DNA Isolation Kit (MTK 08) (Modern Science Co., Nasik, India). Two primers annealing at the 5′ and 3′ end of the 18S rRNA were 5′-GTAACCCGTTGAACCCCATT -3′ and 5′-CCATCCAATCGGTAGTAGCG -3′ respectively. The PCR was run for 35 cycles in a DNA thermal cycler (Thermal Cycler Applied Biosystems 2720, California, USA). Amplified PCR products were then analyzed in a 1 % (w/v) agarose gel and purified. Purified products were cloned into pGEM-T Easy Vector (Promega Co., Madison, USA) and subsequently sequenced using ALF Red automated DNA sequencer (ABI 3130 Genetic Analyzer, California, USA). The 16S rDNA sequence of the isolate was aligned with those in the GenBank database. The sequence data was aligned and analyzed to identify the fungus and its closest neighbours. The ITS rDNA sequence was compared with GenBank and a neighbor joining phylogenetic tree was constructed by the PHYLIP package.[10] The topology of the phylogenetic tree was evaluated by the bootstrap resampling method with 1000 replicates.

Pretreatment and characterization of coir pith

Coir pith obtained from Sakthi coir exports, Pollachi was sun dried for 1 h and hot air oven dried at 80 °C for 2 h, crushed and sieved to obtain particle size of 150 μm. The coir pith was further pretreated using NaOH. The pretreated samples were hot air oven dried at 80 °C and stored for further hydrolysis. The composition of cellulose, hemicellulose and lignin in the NaOH extract free oven dried coir pith was determined using TAPPI standards.[11] The effective humidity of the substrate after sterilization was measured using a capacitive type humidity sensor, Model No. DHI – 400, Nutronics, India.

Solid-state fermentation

Various agricultural substrates or by-products have been used successfully in solid-state fermentation for cellulase production. Solid-state fermentation was performed in a solid-state fermenter employing untreated and pretreated coir pith with 60 % moisture content, pH 5, at temperature 40 °C for 11 days.[12] The solid-state fermenter consists of air spargers arranged one above the other. The substrate was layered on the trays suitable for cultivating microorganisms. These trays prevent the lateral passage of water or air. An orifice and an air outlet are provided on the fermenter lid for the introduction of *A. nidulans* AJSU04 inoculum, and for the passage of air, respectively. The air-inlet is segmented into seven sub-passages. A moisture content of 60 %, pH 5, and temperature of 40 °C was maintained in the fermenter (Fig. 1).

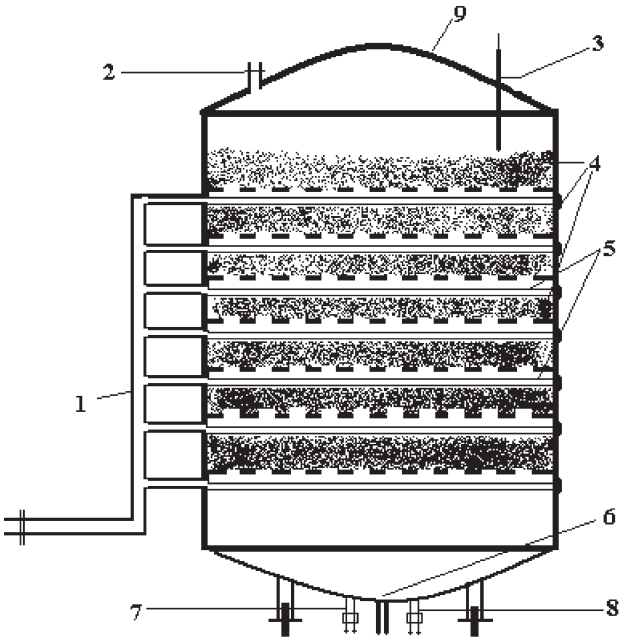

Fig. 1 – *Schematic diagram of experimental setup, solid-state fermenter used for the production of cellulases*

The prepared medium was sterilized and inoculated with 1ml of culture. The culture was assayed for enzyme production after 24 h. Extracts were drawn every 24 h by the addition of 10 mL distilled water to the solid bed, pressing, followed by filtering the extract through nylon cloth. This was followed by assay for cellulases.

Cellulase purification

The supernatant was brought to 80 % saturation with $(NH_4)_2SO_4$ and left overnight at 4 °C, and centrifuged at 10,000 × g for 20 minutes. The precipitate was dissolved in 20 mmol L^{-1} sodium citrate buffer (pH 4.8) and dialyzed against the same buffer for 48 h. Dialysis was performed using cellulose membrane with a molecular weight cut-off of 5 kDa (Sigma). The dialyzed fraction was purified using ion exchange chromatography and gel filtration chromatography using the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia Biotech). For ion exchange chromatography, the crude enzyme preparations were applied to a 5-mL resources Q column (Pharmacia Biotech, USA), which was equilibrated with elution buffer (Sodium citrate buffer, pH 4.8 with 0.5 mol L^{-1} NaCl). Elution was achieved with a linear gradient of 0.1 – 0.5 mol L^{-1} NaCl in equilibration buffer at a flow rate of 0.3 mL min^{-1}. The purified samples were collected using an auto fraction collector. Each 0.5 mL fraction was analyzed for cellulase activity. The active fractions were concentrated using an Amicon ultra-filtration with a molecular weight cut-off at 5 kDa (Millipore). The concentrated enzyme was further processed in a Sephadex G-200 column. The enzyme was eluted with sodium citrate buffer (pH 4.8) at a flow rate of 0.5 mL min^{-1} and the product was collected by an auto fraction collector. Fraction of 0.5 mL was collected, stored at 4 °C, and assayed.

Analytical methods

Assay for endo-β-1,4-glucanase (CMCase) was carried out according to IUPAC recommendation by measuring the release of reducing sugars in the reaction mixture of 1 mL crude enzyme and 1 mL of 0.01 g mL^{-1} CMC solution in 0.05 mol L^{-1} sodium citrate buffer (pH 4.8) incubated at 40 °C for 1 h.[13] One unit of CMC activity is defined as the amount of enzyme needed to liberate one μmol of glucose min^{-1} from 1 mL of culture broth under assay conditions. Filter paper cellulase (Fpase) activity was assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman No. 1 filter paper 50 mg of dimension (1.0 × 6.0 cm) as substrate in 0.05 mol L^{-1} sodium citrate buffer (pH 4.8) at 50 °C for 1 h. One unit of Fpase activity is defined as the amount of enzyme needed to liberate 1 μmol of glucose/min during hydrolysis reaction. β-glucosidase (cellobiase) activity was measured by the release of reducing sugars in a reaction mixture containing 1 mL crude enzyme and 1 mL of 15 mmol L^{-1} cellobiose solution incubated at 50 °C for 1 h. One unit of cellobiase activity is defined as the amount of enzyme needed to liberate 2 μmol of glucose/min during hydrolysis of cellobiose. Glucose in the culture supernatant was analyzed using UV-visible spectrophotometer (Hitachi Model: 100–40) at 540 nm.[14] Protein concentration was determined using the Bio-Rad protein assay kit with Bovine Serum Albumin as the protein standard for

the calibration curve.[15] SDS – PAGE was performed according to the method of Laemmli using 12 % gels.[16] After electrophoresis, the gels were stained by a solution of 0.1 % (w/v) Coomassie Brilliant Blue R-250 in 30 % (v/v) methanol and 10 % (v/v) acetic acid. The molecular mass markers used were those from molecular weight ranges (Bio-rad, USA) including β-galactosidase (120 kDa), Bovine Serum Albumin (91 kDa), Serum Albumin (66 kDa), Glutamic dehydrogenase (56 kDa), Ovalbumin (48 kDa) Carbonic anhydrase (4 kDa), Myoglobin (26 kDa) and Lyzozyme (19 kDa).

Zymogram

Zymogram with CMC was obtained by incorporating 0.1 % (w/v) CMC into the polyacrylamide gel used for electrophoresis. After electrophoresis, SDS was removed by washing the gel at room temperature in a solution of sodium citrate buffer at pH 5, containing 30 % isopropanol for 1 h followed by a solution of sodium citrate buffer at pH 5 for 1 h. Renaturation of the enzyme proteins was carried out by leaving the gel in a solution of sodium citrate buffer at pH 5, containing 5 mmol L^{-1} β-mercaptoethanol and 1 mmol L^{-1} EDTA at 4 °C overnight.[17] The gel was then transferred onto a glass plate, sealed in a film, and incubated at 50 °C for 4 h. The gel was stained in a solution of 0.3 % congo red for 5 minutes and destained in 1 mol L^{-1} NaCl for 1 h. The clear zone in the zymogram clearly indicates the presence of endoglucanase, exoglucanase and glucosidase components of cellulases.

Modification of residual coir pith into coir pith waste compost (CWC)

Residual coir pith obtained after the extraction of cellulase was stored in air tight containers. Amongst biofertilizers *Azotobacter* and *Bacillus* strains play a key role in improving fertility conditions of soil.[18] The residual coir pith was oven dried at 70 °C for 48 h, ground and sieved to pass a 2-mm sieve. It was then autoclaved at 121 °C for 20 minutes. This residual coir pith obtained after the extraction of cellulase was mixed with *Azobacter chroococcum, Bacillus megaterium, Bacillus mucilaginosus* and stored in air-tight containers. This mixture acts as the microbial inoculum.

Preparation of coir pith waste compost (CWC)

The entire mixture comprising of the residual coir pith and the microbial inoculum was composted in trapezoidal windrow piles (0.5 m height × 1 m width × 6 m lenght). Forced aeration was provided during the first 25 days, followed by a maturation period of 110 days. The piles were turned periodically, every 10 days, to maintain adequate O_2 levels by blowing air at the base of each pile during the initial stages of composting. At the end of the composting period, the prepared coir pith waste compost or the biofertilizer was well dried and obtained in the form of powder. The biofertilizer was sealed in sterile plastic bags and stored at 4 °C for field trials.

Field trials using CWC

The study on the applicability of residual coir pith as biofertilizer was conducted under laboratory conditions, by setting up a model experiment with four pots, in which *Solanum lycopersicum* (tomato) plants were chosen to grow.[19,20] Field trials were carried out in four pots, Pot A: Soil + biofertilizer + Seeds (A), Pot B: Soil + Seeds (B), Pot C: Soil + CF + Seeds (C), Pot D: Soil + Autoclaved biofertilizer + Seeds (D). Small pots with perforated bottom were used for growing the seeds.

Results and discussion

Identification of fungal strain

The sequence obtained was compared with the sequence obtained from the nucleotide database of National Center for Biotechnology Information (NCBI). The phylogenetic analysis of the strain AJSU04 using its 18S rDNA nucleotide sequence data showed that this strain had the highest homology (99 %) with *Aspergillus nidulans* strain KCCM60326, *Aspergillus nidulans* strain RGT-S3, *Aspergillus nidulans* strain NRRL 2395, *Aspergillus nidulans* isolate UOA/HCPF 10384 and *Aspergillus nidulans* strain RTMH13.C5. Based on the evolution distance, phylogenetic tree, partial sequencing, ITS sequences and the neighbour-joining method, this strain was identified as *Aspergillus nidulans* (Fig. 2).[21] The strain was submitted to NCBI, National Center for Biotechnology Information with GenBank accession number JN639859.

Chemical composition of PCP

Pretreated coir pith (PCP) was analyzed for chemical composition. 200 g coir pith was treated with 0.8 % NaOH. Pretreatment with 0.8 % NaOH at 8 h was observed to establish optimal conditions. Increasing treatment time beyond 8 h, proved ineffective in modifying the chemical composition of the fiber. At optimum pretreatment conditions (0.8 % NaOH, 8 h) coir pith contained 29.2 % cellulose, 4.1 % hemicelluloses and 4.2 % lignin. There was a 19.2 % decline in the cellulose content, whereas a tremendous turn down of 67.7 % and 91.3 % was observed in the hemicelluloses and lignin content respectively. Amongst cellulose, hemicellulose and

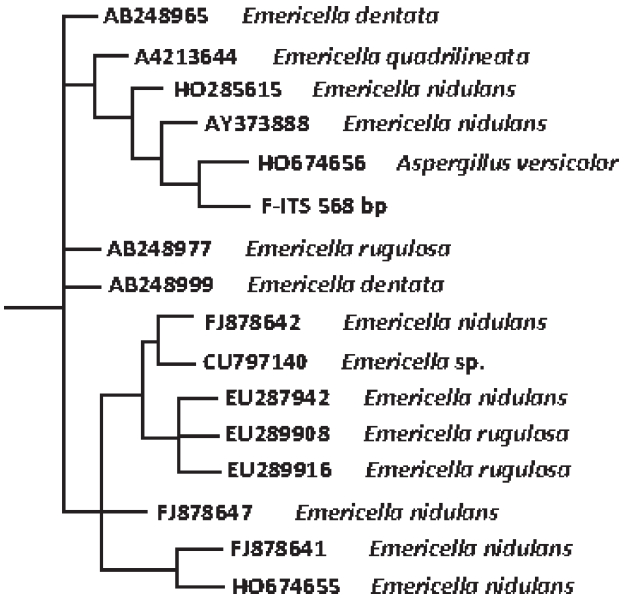

Fig. 2 – *Phylogenetic tree of ITS sequences of the strain Aspergillus nidulans AJSU04 and the fungi most closely related to it*

lignin in the coir pith, cellulose was the least affected by the pretreatment process. A remarkable decrease was observed in the lignin and hemicelluloses content of the coir pith.

Solid-state fermentation

The optimum conditions for solid-state fermentation of PCP were reported earlier.[12] Further, the effective humidity of the substrate after sterilization was found to be 61 %. Solid-state fermentation was performed in a solid-state fermenter employing pretreated and sterilized coir pith with 60 ± 1 % moisture content, pH 5, at temperature 40 °C for 11 days. Under these conditions, *Aspergillus nidulans* AJSU04 produced cellulase with an activity of 60.54 U g^{-1}. The entire solid-state fermentation of PCP by *Aspergillus nidulans* AJSU04 could be scaled up in future studies for the improvement in the yield of endo-β-1–4-glucanase component of cellulase.

Fig. 3 shows the time course of cellulase production by *Aspergillus nidulans,* investigated under optimum conditions. FPase (Exo-β-1–4-glucanase) and CBase (β-glucosidase) activity reached

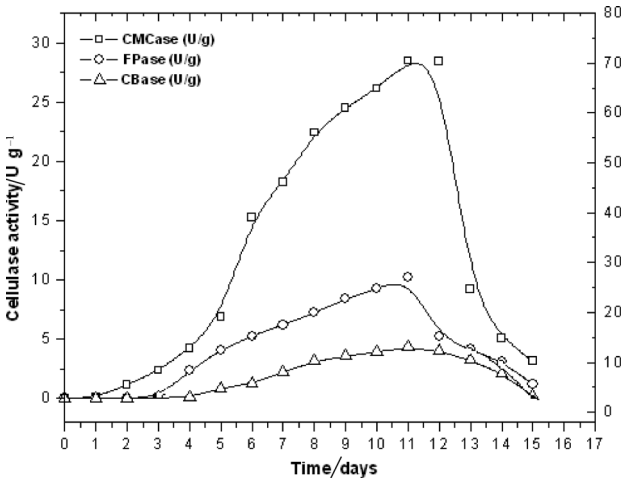

Fig. 3 – *Effect of time on cellulase activity (U g^{-1}) under optimized conditions. [Coir pith 8 g, moisture content 60 %, initial pH 5, temperature 40 °C]*

10.23 U g^{-1} and 4.31 U g^{-1} respectively on the 11th day under optimal conditions. Maximum cellulase activity was revealed on the 11th day after inoculation.[22]

Purification of crude cellulase

The cellulase was purified from the culture broth of *Aspergillus nidulans* AJSU04 following the steps indicated in Table 1. The extract from the culture medium was taken through the two-step purification of the chromatography with an anion exchange HiTrap Q column and a cycle of Sephadex G-200 column.[23] The purification fold of the cellulase was 1.944 times with the recovery yield of 2.44 %. Homogeneous enzyme preparation was obtained as analyzed by SDS-PAGE as shown in Fig. 4. Lane A shows the *Aspergillus nidulans* AJSU04 cellulase in 12 % SDS-PAGE, lane B shows the Zymogram of *Aspergillus nidulans* AJSU04 cellulase and lane M shows the protein standard markers. The molecular mass of the purified exo-β-1,4-cellulase, endo-β-1,4-cellulase and β-glucosidase was estimated to be about 57 kDa, 32 kDa and 23 kDa respectively. The molecular mass of endo-β-1–4-glucanase was closely in association with Cel61A (34 kDa) from a mutant strain of *Trichoderma viride* T 100–14.[24]

Table 1 – *Purification of cellulase from Aspergillus nidulans AJSU04*

Purification steps	Specific activity (U mg^{-1})	Volume of fraction (mL)	Total protein (mg)	Total activity (U)	Recovery %	Purification fold
Crude enzyme	31.17	15	29.13	907.98	100	1
$(NH4)_2SO_4$ precipitation	50.92	10	11.51	586.09	64.55	1.63
Anion exchange HiTrap Q	53.66	1	1.03	55.32	6.09	1.72
Sephadex G-200	60.58	0.5	0.36	21.99	2.42	1.94

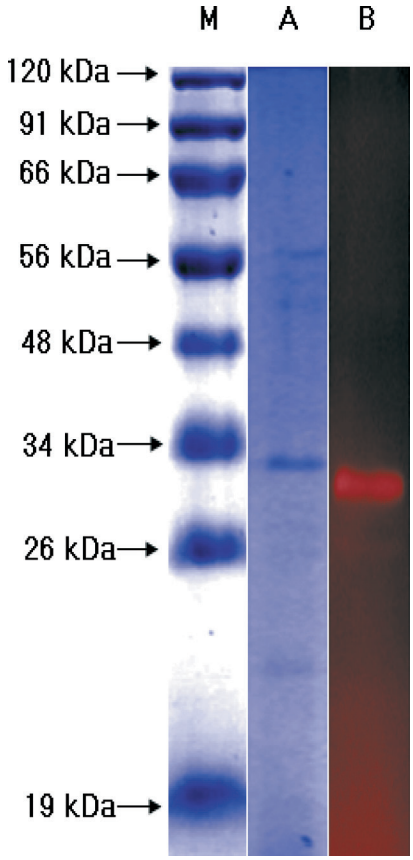

Fig. 4 – *SDS-PAGE and Zymogram of purified cellulase from Aspergillus nidulans AJSU04, Lane A: cellulase in 12 % SDS-PAGE, Lane B: Zymogram of cellulase, Lane M: Protein standard markers.*

Kinetic studies

The Michaelis-Menten kinetic model of single-substrate reaction was investigated for the enzyme produced by *A. nidulans* AJSU04. As enzyme-catalyzed reactions are saturable, their rates of catalysis do not show a linear response to increasing substrate. The Michaelis-Menten equation (1) is given as follows,

$$v = \frac{V_{\max}[S]}{K_m + [S]} \tag{1}$$

If the initial rate of the reaction is measured over a range of substrate concentrations (denoted as [S]), the reaction rate (v) increases as [S] increases, as shown on the right. However, as [S] gets higher, the enzyme becomes saturated with substrate and the rate reaches V_{max}, the enzyme's maximum rate.

The apparent kinetic parameters (V_{max} and K_m) of the cellulase were determined by varying the concentration of CMC from 0.1 to 1.24 mmol L^{-1} in 0.05 mmol L^{-1} sodium citrate buffer (pH 5). The apparent kinetic parameters were determined from the Michaelis-Menten plot (Fig. 5). Carboxy methyl cellulose solutions 0.1 to 1.24 mmo L^{-1} were treated with the

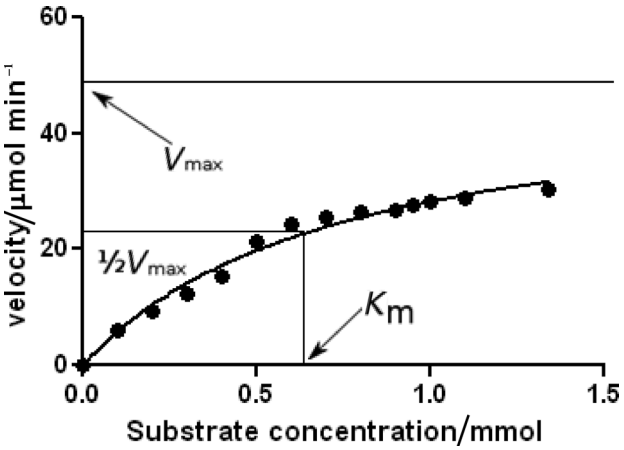

Fig. 5 – *Michaelis-Menten plot*

purified cellulase to investigate the extent of hydrolysis and hence determine the reaction velocities (V). The data was analyzed using Graph Pad Prism software (Version 5.0). Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were found to be 0.62 mmol and 48.9 µmol min^{-1}, respectively.

Field trials

The soil was tilled before transplanting the seedlings outdoors in the pots. The prepared coir pith waste compost was mixed with soil and used for tomato plantations.[25] The plant was generously watered for the first few days and throughout the growing season. It was mulched for five weeks after transplanting them to the pots in order to retain moisture. The fertilizer was applied to the plants for two weeks prior to first picking and this was repeated again after the first picking for two weeks. The raw coir pith and fertilizer prepared using the same is shown in Fig. 6a and Fig. 6b, respectively. The efficiency of the biofertilizer was tested by comparing it with commercially available fertilizer (CF).

The sprouting of *Solanum lycopersicum* seeds supported with soil and biofertilizer, soil alone, soil and commercial fertilizer, and soil and autoclaved biofertilizer are shown in Fig. 7a, Fig. 7b, Fig. 7c and Fig. 7d, respectively. Sprouting of *Solanum lycopersicum* (tomato) seeds occurred in 4 days in the

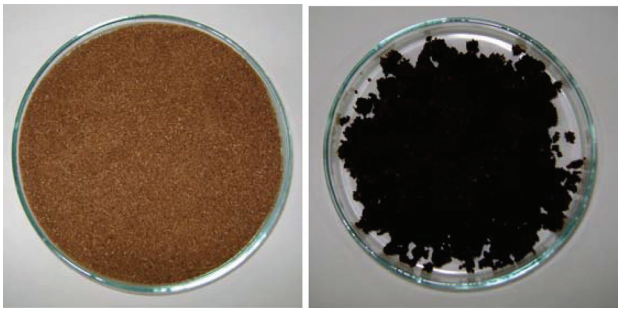

Fig. 6 – (a) *Raw coir pith,* (b) *Coir pith waste compost*

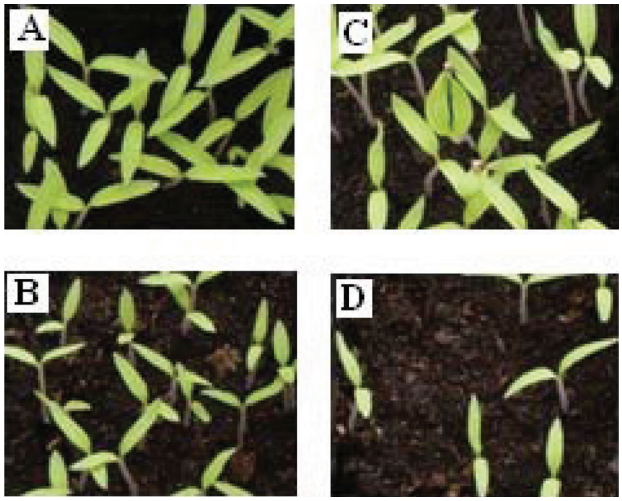

Fig. 7 – *Growth of Solanum lycopersicum seedlings* (a) *Pot A: Soil + biofertilizer + Seeds (after 4 d);* (b) *Pot B: Soil + Seeds (after 10 d);* (c) *Pot C: Soil + CF+ Seeds (after 9 d);* (d) *Pot D: Soil + Autoclaved biofertilizer + Seeds (after 8 d)*

biofertilizer inoculated pot A, while it took 8 to 10 days longer in non-inoculated control Pot B, 7–9 days commercial fertilizer inoculated Pot C and 8 days in the autoclaved sample inoculated Pot D.

A significant enhancement in the growth of the seedling was observed in test sample (Pot A). Out of the 20 seeds inoculated into Pot A, Pot B, Pot C and Pot D, 19, 15, 18 and 6 seeds were observed to sprout after 4, 10, 9 and 8 days, respectively. Further, complete growth was observed for 18, 12, 13 and 3 seedlings of Pot A, pot B, pot C and pot D, respectively. Fig. 8a, Fig. 8b, Fig. 8c and Fig. 8d shows the growth of *Solanum lycopersicum* seedlings in Pot A, Pot B, Pot C and Pot D after 12, 30, 18 and 24 days, respectively. The results of the field study revealed an enhancement in plant height, a significant decrease in sprouting time and a noteworthy fruit yield in the Pot A and Pot C, to which the biofertilizer and CF were applied. The growth of the seedlings was monitored for three months, and the following results were obtained (Table 2). Fruit yield per plant per week in pounds is the weight of the harvested fruit per plant per week. The biofertilizer symbiotically associates with the plant roots. Microorganism functions in long duration, and causes improvement in soil fertility, thereby maintaining the natural habitat of the soil. The biofertilizer was observed to increase crop yield by 17–20 %.

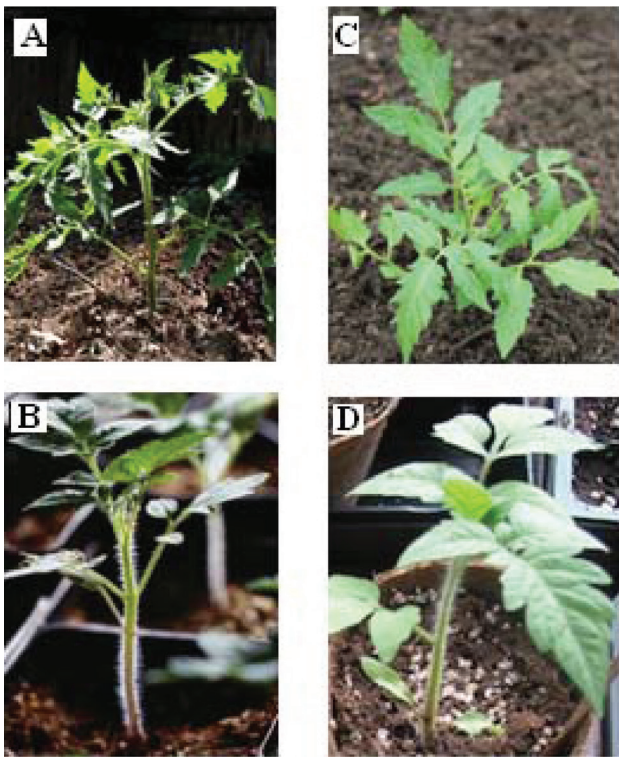

Fig. 8 – *Growth of Solanum lycopersicum seedlings* (a) *Pot A: Soil + biofertilizer + Seeds (after 12 d);* (b) *Pot B: Soil + Seeds (after 30 d);* (c) *Pot C: Soil + CF+ Seeds (after 18 d);* (d) *Pot D: Soil + Autoclaved biofertilizer + Seeds (after 24 d)*

Table 2 – *Effect of biofertilizer on plant height, sprouting time, and fruit yield*

Sl. No.	Field trials	Sprouting time (days)	Plant height after 3 months (m)	Fruit yield per plant per week (lb)
1	Soil + biofertilizer + Seeds (A)	3 ± 1	3.0 ± 0.05	2.5 ± 0.05
2	Soil + Seeds (B)	9 ± 1	2.0 ± 0.03	0.8 ± 0.05
3	Soil + CF + Seeds (C)	8 ± 1	2.5 ± 0.01	2.0 ± 0.05
4	Soil + Autoclaved biofertilizer + Seeds (D)	7 ± 1	1.8 ± 0.02	1.2 ± 0.05

The effect of sprouting time and plant height on yield in Pot A was studied using a statistical tool Design Expert version 8.0 by using central composite design.[26] Table 3 gives the central composite design matrix with experimental and predicted values for yield.

The regression equation shows the yield as an empirical function in terms of coded factors as in equation (1.2).

$$Y_i = 2.50 - 9.5 \cdot 10^{-3} x_1 + 0.021 x_2 + 0.025 x_1 x_2 \quad (2)$$

where Y_i is the predicted yield in lb. Table 4 shows the ANOVA for the model.

The Model F-value of 3.90 implies the model is significant. There is only a 4.89 % chance that a "Model F-Value" this large could occur due to noise.Values of "Prob > F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant. Adequate precision ratio greater than 4 is desirable. The ratio of 6.902 indicates an adequate signal.

Table 3 – *Coded values of variables in central composite design for field trials in Pot A*

Trials	Variables		Yield (lb)	
	X_1 Levels(values)	X_2 Levels(values)	Experimental*	Predicted
1	0(3)	0(3)	2.50	2.51
2	0(3)	0(3)	2.49	2.48
3	1(4)	–1(2.95)	2.48	2.47
4	–1(2)	0(3)	2.50	2.49
5	–1(2)	0(3)	2.51	2.50
6	1(4)	1(3.05)	2.55	2.54
7	1(4)	0(3)	2.46	2.45
8	0(3)	1(3.05)	2.52	2.50
9	–1(2)	–1(2.95)	2.54	2.53
10	0(3)	–1(2.95)	2.43	2.44
11	0(3)	0(3)	2.50	2.49
12	0(3)	0(3)	2.48	2.47
13	0(3)	0(3)	2.49	2.50

X_1: Sprouting time (d); X_2: Plant height (m)
**The observed values of yields were the means of triplicates*

Hence this model can be used to navigate the design space.

Conclusions

Thus, this work showed that the delignification through pretreatment is one of the most important pathways to increase the enzymatic digestibility. Alkali pretreatment using NaOH on coir pith was seen to increase the feasibility of cellulose exposure and hence the production of cellulase with significant activity. This study exploits coir pith as a substrate for cellulase production and the residual coir pith as a carrier material for the preparation of biofertilizer. The adopted approach offers an innovative solution to the need of reusing one of the few dumped wastes 'the coir pith'. The study on coir pith as a biofertilizer on *Solanum lycopersicum* plant and its fruiting characteristics provide a positive indication regarding its application in farmland.

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List of abbreviations and symbols

CBase – Cellobiase or 1,4-β-glucosidase, U g^{-1}
CF – Commercially available fertilizer
CMC – Carboxymethyl cellulose
CMCase – Carboxymethyl cellulase or endo-β-1,4-glucanase, U g^{-1}
CWC – Coir pith waste compost
Fpase – Filter paper cellulase or exo-β-1,4-glucanase, U g^{-1}
PCP – Pretreated coir pith

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Table 4 – *Analysis of variance (ANOVA) and test of significance for yield*

Factor	Sum of squares	Mean squares	df	*F*-value	*P*-value	
Model	6.731E-003	2.244E-003	3	3.90	0.0121	significant
X_1	7.328E-004	7.328E-004	1	1.27	0.0489	
X_2	3.498E-003	3.498E-003	1	6.08	0.0083	
X_1X_2	2.500E-003	2.500E-003	1	4.35	0.0035	
Residual	5.177E-003	5.752E-004	9			
Lack of fit	4.897E-003	9.794E-004	5	0.09	0.2882	Not significant
Pure error	2.800E-004	7.000E-005	4			
Cor total	0.012		12			

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