

**Isolation of efficient cellulase producing *Aspergillus unguis* UCSC324 and
determination of the kinetic properties of its crude cellulase
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Abstract

Bioengineering of cellulolytic enzymes with enhanced catalytic efficiency and thermostability is important in the commercialization processes. This study describes the isolation of efficient cellulase producing fungi and determination of the kinetic properties of the crude cellulase. Among the fungal strains isolated from cow dung, hot rice water, water used in autoclave and decaying coconut wood, the strains growing on decaying coconut wood was selected for this study because of the higher amount of cellulase production measured by the rate of zone of clearance on the Carboxymethylcellulose (CMC)sodium salt agar plates by Congo red test. The three isolated fungal strains isolated from coconut wood were identified and confirmed as *Aspergillus niger* FL17, *Aspergillus oryzae* CBS108.24 and *Aspergillus unguis* UCSC324 based on the morphological studies and molecular analysis done by amplifying the ITS5.8SrDNA sequence, PCR amplification and multiple sequence alignment. Since there had been no reports recorded about the production of cellulase from *Aspergillus unguis* UCSC324, kinetic properties of the cellulase from this fungal strain were studied. Fermentation medium contained (g L^{-1}) 2.0g cellulose; 3.0g carboxymethyl cellulose; 0.3g ammonium sulphate and 100mL of distilled water was used at an optimal conditions of temperature $20 \pm 1^\circ\text{C}$, pH7.0 for 5 days at 100rpm. Crude cellulase showed zero order kinetics for 5 minutes. When the activity of cellulase was measured at different temperatures ranging from 20°C to 75°C at pH 7.0, the optimum temperature for the enzyme activity was 50°C . When the pH of the media was changed from 2.0 to 8.0, while temperature was kept at 50°C with 1g/100mL cellulose substrate, highest cellulase activity was observed at pH 5.0. Michaelis constant and the V_{max} of the cellulase enzyme to soluble cellulose by Lineweaver-Burk Plot were $4.545 \times 10^{-2} \text{ moldm}^{-3}$ and $26.66 \text{ mgml}^{-2}\text{mins}^{-1}$ respectively at pH 5.0 and 50°C . The crude enzyme was stable for at least 90 minutes at pH 5.0 and at 50°C . Since the cellulase enzyme from *Aspergillus unguis* was active in moderately acidic pH and showed better stability at 50°C , it could be a good candidate for the cellulase dependent industrial applications.

Keywords: *Aspergillus unguis*, Cellulase, Decaying coconut wood, Kinetic properties.

Introduction

Cellulases are inducible enzymes that are synthesized by microorganisms during their growth on substances containing cellulose. Cellulase is a synergetic enzyme which is accustomed to split cellulose into glucose and/or different oligosaccharide compounds (Gao et al., 2008). Oligosaccharide compounds are subdivided into endoglucanase, cellobiohydrolase or exoglucanase and β -glucosidase (Kim et al., 2008). Commercial cellulases are of two types: crude and pure enzymes. The crude one constitutes a

complete enzyme system and contains three enzymes usually responsible for cellulose hydrolysis. Most of the cellulolytic microorganisms produce several cellulases that show huge differences in their physiochemical characteristics.

Cellulose molecule is composed of lot of unbranched long chains with repeating β -1,4-linkages of D-glucose sub units. Large number of parallel glucan chains form compact microfibrils held together tightly

by the hydrogen bonds, providing the external surface of cellulose hydrophobic and recalcitrant to different enzymatic reactions (Habibi et al., 2010). The cellulase production has been possible from a wide range of bacterial genera (Immanuel et al., 2006) and fungi (Anita et al., 2009). Filamentous fungi have been extensively used for the production of diverse extracellular enzymes including amylases, proteases, xylanases and cellulases (Bakri et al., 2003). Fungi are mainly used for the lignocellulolytic enzymes on large scale industrial application under solid-state fermentation system, since they grow on diverse solid substrates with limited water availability (Sette et al., 2008). Fungal elongated hyphae would create mechanical pressure on the cellulose structure and stimulate them to supply massive amounts of cellulolytic enzymes. Furthermore, fungal strains are capable of producing higher quantities of cellulases than the other microorganisms (Kaushal et al., 2012). Since most of the fungi of genus *Aspergillus* synthesize cellulases, genus *Aspergillus* has been most commonly employed for the production of some of these enzymes.

Cellulases are the key and primary enzymes that react on the lignocellulosic substrates for the bioconversion of lignocellulosic biomass in order to produce efficient biofuels (Philippidis, 1994). Cellulases have a wide range of potential applications in the food processing, animal feed production, cloths, chemical, waste-water management, detergent preparation and pharmaceutical industries (Anita et al., 2009). Applications of cellulases further include the wine, beer and fruit juice production. Due to increasing worries due to the greenhouse effect, depleting oil reserves and raising oil prices, as well as interest in renewable fuels, such as bioethanol, cellulose enzymes have become very important to keep the green environment active. Therefore, several steps have been taken to transform diverse cellulosic biomasses into fermentable

sugars using cellulase enzymes and for further conversion to ethanol, which is one of the efficient renewable fuel. Due to the presence of higher amount of recalcitrance of cellulose, bioconversion needs high quantity of enzyme, aiming to increase yield and reduce the cost of production. Considering the importance and application of the cellulases, this study was aimed to isolate efficient cellulase producing fungi and to determine the kinetic properties of the crude cellulases.

Materials and Methods

Microorganism

Fungal strains isolated from cow dung, hot rice water, water waste after autoclaving and decaying coconut wood picked from different areas of Sri Lanka were chosen. Among them three fungal strains isolated from decaying coconut wood were selected for this study based on the higher amount of cellulase production.

Isolation of Cellulase producing fungi

Inocula were plated on Potato Dextrose Agar medium (PDA) plates supplemented with 2% Carboxymethyl cellulose (CMC) sodium salt as a carbon source and Tetracycline (20 µg/mL) to inhibit the bacterial contamination and incubation was done at 28 ± 1°C for 5-7 days. After the incubation process, the fungal species were purified and sub-cultured on CMC agar and Czapek agar plates and used. Secondary screening was done to select the best extracellular cellulase producer by culturing on CMC agar. Actively growing mycelia (3-4 days old) were removed from the growing edge of the fungal isolates by using sterile cork borer of 6 mm diameter, the discs were inoculated to the pre-welled CMC agar (pH 7.0) plates and incubated at 28°C for 6-7 days. After incubation, the plates were flooded with Congo red solution (1mg/mL sterile distilled water) for 20 min, decant the dye and flooded with 5 M NaCl for 30 minutes and

decanted it. Carboxymethyl cellulase (CMCase) producing fungal strains would develop a pale orange to clear colour (Teather and Wood, 1982).

Morphological characterization of fungal genera

The isolates were grown on PDA and transferred several times on the same medium and further incubated at 30°C for 7 days to obtain pure cultures. The isolated fungal colonies were sub-cultured and maintained on PDA slants and stored at 4°C in the refrigerator. All the three fungal isolates that produced higher cellulase enzyme, were maintained on PDA slants at 4°C and re-cultured on proper intervals and screened by the modified method explained by Wood and Bhat (Wood and Bhat, 1988). For morphological identification, the isolated fungi were grown aseptically on the PDA drop located on the surface of sterile glass microscope slides placed in sterile petridishes. Fungal isolates were then identified conventionally according to their morphological features to determine their genera (Devenathan et al., 2007).

Molecular characterization

All the three selected fungal genera were subjected to molecular identification by amplifying the ITS5.8SrDNA sequence, PCR amplification and multiple sequence alignment followed by phylogenetic analysis (MBSU, University of Alberta, Canada). Modified method used by Balajee and co-workers (Balajee et al., 2009) was used. All the fungal isolates were thawed, sub-cultured on either PDA or CMC agar and confirmed their purity by careful visual observation before the commencement of molecular characterization. Genomic DNA was extracted from 48 h grown culture plates by using a DNeasy kit (Qiagen, CA). Universal fungal primers directed to the ITS1-5.8S-ITS2 and the β -tubulin regions were employed to amplify DNA from

all the isolates. The resultant PCR amplicons were purified from the excised gel pieces using QIAquick gel extraction kit (QIAGEN Inc. Mississauga, ON, Canada) and quantified using manufacturer's protocols. Both the strands were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the same forward and reverse primers. All the sequencing reactions were done on a thermocycler (GeneAmp PCR system 9700) and the PCR conditions were 1 cycle at 94°C for 5 min, 40 cycles at 94°C for 40 s, 50°C for 40 s, and 72°C for 2 min, followed by 1 cycle at 72°C for 5 minutes. The products were purified, dried, resuspended in 0.1 mM EDTA, and run on a DNA analyzer (3730 Applied Biosystems) using manufacturer's protocols. The resultant nucleotide sequences were edited by using the Sequencer program (Gene Codes Corporation, Ann Arbor, MI) and aligned by using the program CLUSTAL W. Sequences derived from the ITS1-5.8S-ITS2 and the β -tubulin regions of all the fungal isolates were compared with the sequences of the GenBank database in order to identify the fungal isolates.

Chemicals and media

All the chemicals used were from standard sources. The activation medium contained glucose 0.4g and nutrient broth 0.8g. Fermentation medium contained (gL⁻¹) cellulose 2.0g; 3.0g carboxymethyl cellulose; 0.3g Ammonium sulphate and 100mL of distilled water and incubated at 121°C 15lbs for 20 minutes and 200rpm based on the experiment.

Preparation of inoculum

The inocula were prepared by growing the organism in 250 mL Erlenmeyer flask with 100 mL of PDA broth containing g/L of sucrose, 30; sodium nitrate, 3; K₂HPO₄, 1; MgSO₄, 0.5; KCl, 0.5; FeSO₄, trace; agar, 15. This medium was used for the fermentation process.

Production of cellulase

To the fermentation medium, the fungal inocula (size 6×10^6 conidia) were added and incubated at 35°C in a rotatory shaker (100rpm). Samples were taken after 6 days at the end of the fermentation and centrifuged at 6000 rpm for 15 min. The Supernatant was treated as cellulose source and used for the kinetic studies.

Kinetic studies

Activity of cellulase with time

Soluble cellulose 10 mL (1g/100mL, pH 5.0, 0.25mL) was mixed with cellulase (0.25mL) from *Aspergillus unguis* at 50°C and the amount of glucose produced was monitored.

Effect of temperature

The effect of temperature on the cellulase enzyme activity of *Aspergillus unguis* was determined by incubating the appropriately diluted enzyme (pH 7.0) for optimized amount of time with 0.5mL of soluble cellulose at pH 7.0 (1g/100mL) in triplicates and at different temperatures 20°C , 35°C , 40°C , 45°C , 50°C , 55°C , 60°C , 65°C , 70°C and 75°C .

Effect of pH

The effect of pH on cellulase activity was measured by preparing 1g/100mL soluble cellulose in phosphate buffers at different pH values (pH 2.0 to 8.0) in triplicates and incubated at optimized conditions.

Effect of substrate concentration

Different concentrations of cellulose solutions were prepared (0.20 to 0.5 mL) with citrate -phosphate buffer in triplicates at optimum pH and they were allowed to react with the diluted crude enzyme from *Aspergillus unguis* at optimum temperature

for optimized time. The Michaelis constant and V_{max} for the cellulase to cellulose were calculated by using Lineweaver - Burk plot.

Effect of stability of cellulase

Effect of the stability of the enzyme at 50°C and pH 5.0 was also studied by incubating the enzyme in triplicates at pH 5.0 and at 50°C and the residual activity was determined using 10 ml (1g/100mL) cellulose, under optimized conditions.

Analytical methods

Cellulase ((filter paperase (FPase) and carboxymethylcellulase (CMCase)) activities were assayed according to the modified method described by Ghose (1987). The glucose produced was measured by Dinitrosalicylic acid (DNSA) method. One unit of cellulase activity is defined as the amount of enzyme ((filter paperase (FPase) and carboxymethylcellulase (CMCase)) that produces one μmol of reducing sugar in one minute at pH 5.0 and 50°C with 10 mL (1g/100mL) cellulose. The values of enzymatic activity were expressed as U/mL.

Statistical analysis

All the experiments were made in triplicates and the average values were used to plot the graphical representation. Means and standard error of the mean were calculated for each parameter measured for the three biological replicates of experiments in each case. Statistical analyses were performed using R2.15.3 (R Development Core Team, 2010). The data were analyzed using ANOVA. Tukey's multiple comparison test was used to determine significant differences at $p \leq 0.05$.

Results and Discussion

The indigenous microflora from decaying tree wood could be rich source of cellulases thus lead to the production of industrially important cellulolytic enzymes. Cellulose is one of the most abundant and renewable energy source of earth (Guedon et al., 2002). Since it is a rich assemblage of cellulolytic microorganisms owing to the nutrient and cellulosic constituents, the coconut wood was chosen as a source for cultivating cellulose degrading fungi. Conversion of cellulose to soluble sugars is an enzymatic hydrolysis facilitated by cellulases that gives opportunity for achieving large amount of benefits of biomass utilization (Himmel et al., 1999). Cellulase assays helped to enumerate and isolate prominent extracellular cellulase producing fungal strains from diverse natural environments (Banakar and Thippeswamy, 2011).

Isolation and screening of cellulolytic organism

The decaying coconut wood collected from coir retting zone area was serially diluted in saline and was spread on to the PDA agar plates. The plates were incubated at 30°C for 3-5 days. Potential cellulase producing fungal colonies were selected after flooding the plates with congo red (0.1% w/v), followed by destaining with 0.1 M NaCl for the present study. Among the 21 different strains isolated, only nine were potential cellulase producers based on the flooding plate method of screening. Among the nine potential cellulase producing strains, three fungal colonies that showed largest halo forming zones were selected.

Identification of potential fungal species

Colony morphology: Three selected strains were subculture on PDA medium. Initially all the colonies were white and changed to dark, as culture matured. When immature, the colonies were covered with white

colour aerial mycelia, while mature colonies showed the formation of black circular spores.

Microscopic and molecular biological studies

One week old matured colonies were subjected to lacto phenol cotton blue staining test and microscopic examination was also carried out. Microscopic observation confirmed that all three colonies had the hyaline septate hyphae (Figure 1). Fungal colonies are generally fast growing, white, yellowish brown, brownish black or shades of olive green, consisting of a dense erect conidiophores. The conidial head was large and appeared black in colour to brownish black. The conidiophores were hyaline or brownish black near the vesicle. Each vesicle was globose in shape and cover with dark sterigmata on the entire surface in two series. Conidiophores terminate in a vesicle attached with either a single palisade-like layer of phialides (uniseriate) or a layer of subtending cells (metulae) that bear small whorls of phialides (biseriate). The vesicle, phialides and conidia all these form the conidial head. Conidia are one celled, rough walled, pigmented, are capable of producing long chains that may be radiate or clustered in compact columns (columnar).

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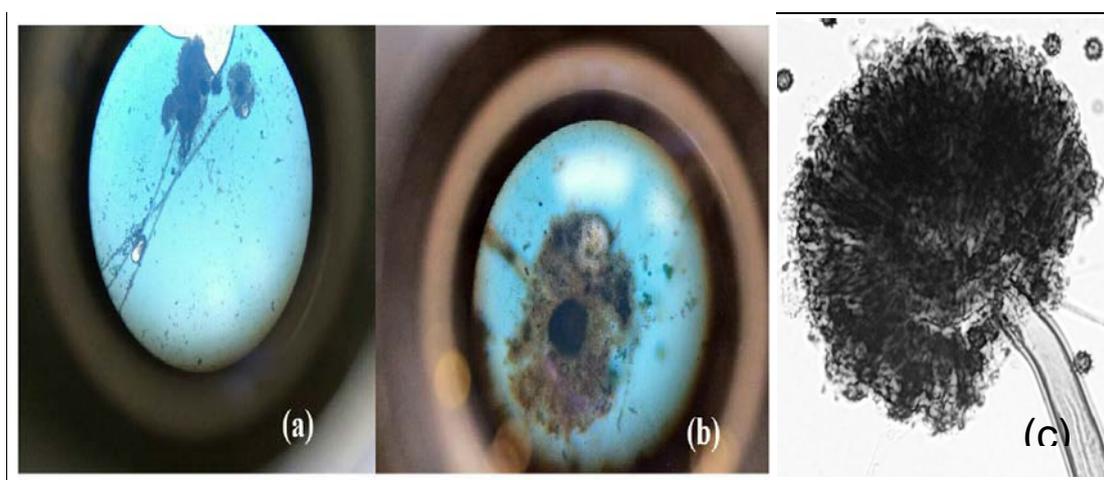


Fig. 1: Structure of *Aspergillus unguis* a) Conidiophore with conidia b) Conidia under high power of the Light Microscope (c) Conidial heads are medium sized in length columnar and uniseriate

Based on the colony morphology studies, the genus of the three strains were identified as *Aspergillus*. Then a series of molecular analysis such as amplifying the ITS5.8SrDNA sequence, PCR amplification, multiple sequence alignment and phylogenetic analysis were done to all these three strains and were identified and confirmed as *Aspergillus niger*FL17, *Aspergillus oryzae* CBS108.24 and *Aspergillus unguis* UCSC324 respectively (Percentage of similarity of the sequences found with the GenBank sequences were 94%, 96% and 94%). Since there was a good correlation between morphology and comparative sequencing result, it was decided to identify the fungal species by employing the ITS regions. Since there had been no reports recorded about the

production of cellulase from *Aspergillus unguis*UCSC324, it was decided to study the kinetic properties of the cellulase from this fungal strain.

Activity of cellulase with time

Cellulase from *Aspergillus unguis* strain showed zero order kinetics for 5 minutes. Therefore the incubation time for the kinetic studies was fixed as 5 minutes for the rest of the experiments.

Effect of temperature

The cellulolytic activity of the cellulase from *Aspergillus unguis* was assayed at different temperatures ranging from 20–75°C at neutral pH (Fig. 2). The activity of

cellulase increased up to 50°C and further increase in temperature decreased the enzyme activity. Cellulase exhibited a temperature profile with a significantly higher activity at 50°C (43.58 mgml⁻²mins⁻¹) and showed activity between 20 – 75°C. Optimum temperature for the cellulase from *Aspergillus unguis* was fixed as 50°C for further studies due to the significantly higher cellulase activity obtained at this temperature.

The activity of the crude cellulase enzyme obtained from *Aspergillus unguis* growing on decaying coconut wood increased up to 50°C and further increase in the temperature decreased the enzyme activity. Cellulase exhibited a temperature profile with the sharp peak of maximal activity at 50°C. Therefore, this moderately higher temperature could be considered for the usage in commercial applications. However, after a certain value, all of these activities occur at overly high rates, and enzymes start to denature (Abdul et al., 2014; Kapilan and Arasaratnam, 2014; Kapilan, 2015). An extracellular carboxymethyl cellulase enzyme produced from a wild type strain of *Aspergillus niger* showed optimal activity at 40°C (Coral et al., 2002). An active strain *Anoxybacillus* sp. 527 that can grow on crystalline cellulose showed optimal activity at 70°C (Liang et al., 2010). Research on the crude cellulase from local *Trichoderma viride* showed optimal cellulase activity at 50°C (Abdul et al., 2014). Temperature affects the microbial metabolism, regulates growth, spore formation, germination and product formation. At lower temperatures, molecules move slower, enzymes cannot mediate chemical reactions. As the temperature increases, molecules move faster, enzymes speed up metabolism and cells rapidly increase in size (Okoye et al.,

2013). However, after a certain value, all of these activities occur at overly high rates, and enzymes start to denature. Cellulase activity of the *Aspergillus unguis* obtained from decaying coconut wood increased up to pH 5.0 and further increase decreased the enzyme activity. Even though this enzyme is moderately acidic, it was active in wide pH ranges. The kinetic characteristics of crude β -d-glucosidase from *Aspergillus wentii* showed maximum activity over a pH range of 4.5–5.5 (Srivastava et al., 2015).

Effect of pH

Though the cellulase activity was observed for the crude cellulase between the pH ranges 2.0 and 8.0, significantly higher and lower cellulase activities were measured at pH values 5.0 and 2.0 respectively (Fig. 3). Optimum pH for the cellulase from *Aspergillus unguis* was fixed as 5.0 for further studies due to the significantly higher cellulase activity obtained at this pH value. Most of the cellulases from fungi are acidophils and active at a temperature range from 40 to 70°C in nature. The optimum temperature for the cellulase enzyme produced by *Aspergillus niger* IFO31125 was 70°C at pH 6.0 and the enzyme was stable for 2 hours (Akiba et al., 1995). The optimum pH and the temperature for the extracellular cellulase produced by *Aspergillus terreus* M11 2.0 and 60°C, respectively and 60% of the enzyme activity retained for more than 60 minutes at 70°C (Gao et al., 2008). *Aspergillus oryzae* secretes a highly glucose tolerant cellulase with the optimum activity at 50°C and pH 5 (Riou et al., 1998). Cellulase activity of the fungal strain *Aspergillus oryzae* S/92Gbr was optimum at pH 5.0 and 45°C (Begum and Alimon, 2011).

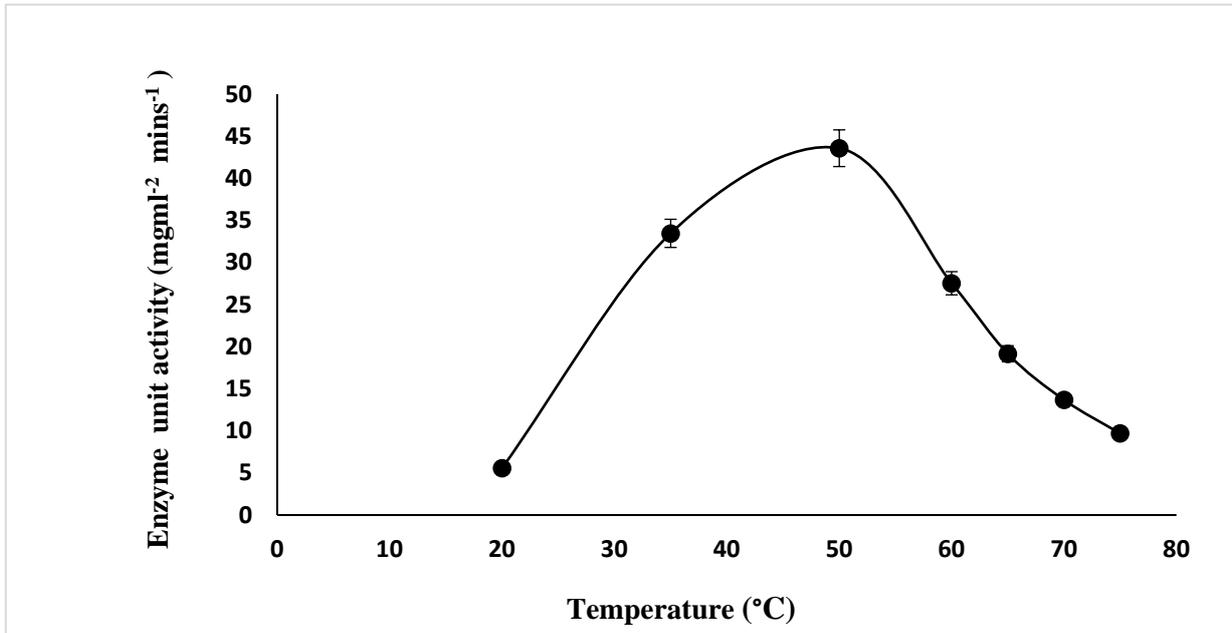


Fig. 2: Effect of external temperature on the crude cellulase enzyme activity from the fungal strain *Aspergillus unguis* UCSC324 isolated from decaying coconut wood

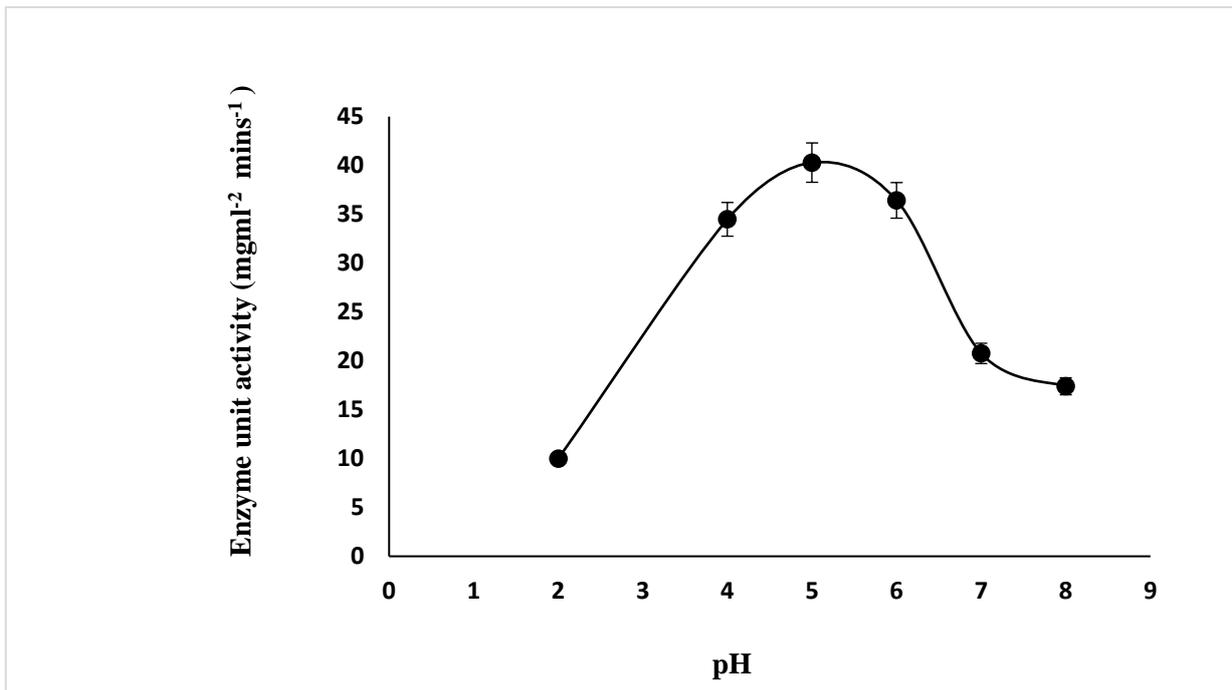


Fig. 3: Effect of different pH values on the activity of crude cellulase enzyme from the fungal strain *Aspergillus unguis* UCSC324 isolated from decaying coconut wood

Effect of substrate concentration

When the substrate concentration was increased from 0.0164 moldm⁻³, 0.0205 moldm⁻³, 0.02463 moldm⁻³, 0.0287 moldm⁻³, 0.0328 moldm⁻³, 0.0369 moldm⁻³, 0.0410 moldm⁻³ at pH 5.0 and the activity of cellulase increased up to 0.02463 moldm⁻³ and significantly higher velocity was reached at 0.0369 moldm⁻³ (Fig.

4). The Michaelis constant and V_{max} for the crude cellulase enzyme to soluble cellulose by Lineweaver-Burk plot were 4.545 × 10⁻² moldm⁻³ and 26.66 mgml²mins⁻¹ respectively at pH 5.0 and at 50°C (Fig. 5). Therefore it was decided to use 1g/100mL substrate concentration for further analysis.

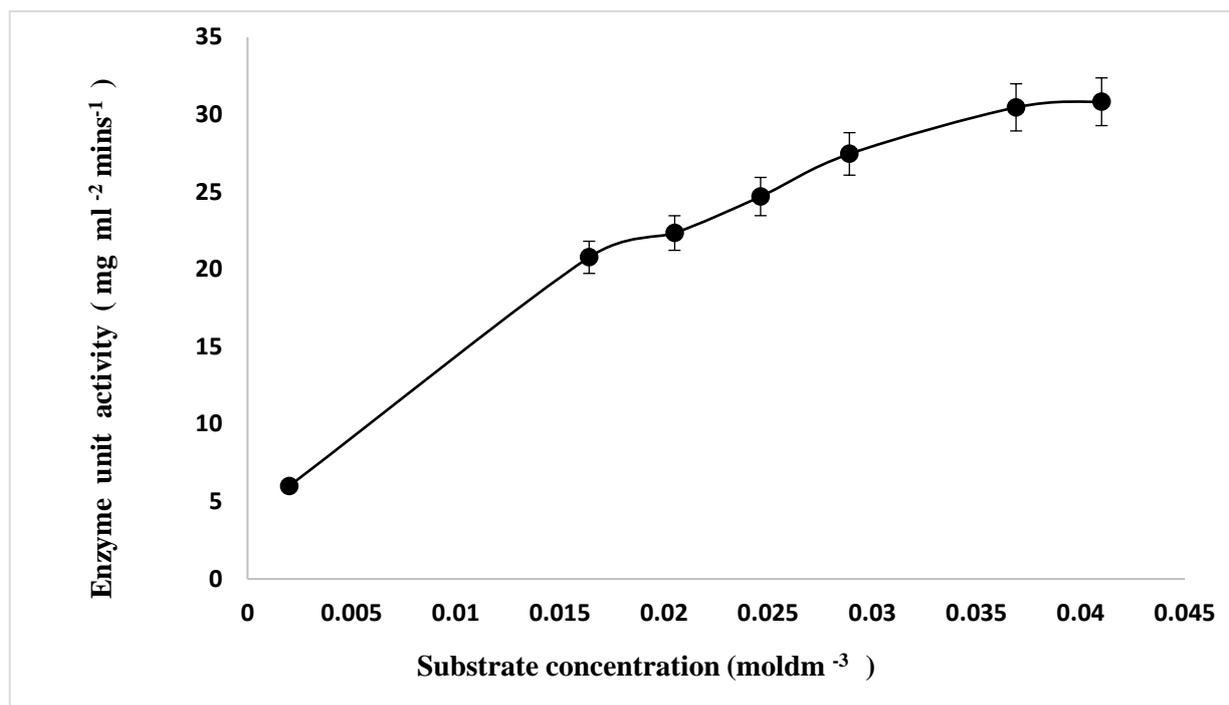


Fig. 4: Effect of cellulose substrate concentration on the crude cellulase enzyme obtained from the fungal strain *Aspergillus unguis* UCSC324 isolated from decaying coconut wood

The cellobiase activity of a *Trichoderma reesei* cellulase preparation resulted in 1.1 mM, and 16 IU mL⁻¹ as the K_m and V_{max} values respectively (José et al., 1999). When extracellular cellulases produced by *Aspergillus fumigatus* on several cellulosic substrates, the K_m value was 0.075 mM (Eduardo et al., 1996). The K_m and V_{max} of two cellulases produced by *Aspergillus fumigatus* Z5 were 37.8 mg/mL and 437.3 μmol/min/mg; 51.8 mg/mL and 652.7 μmol/min/mg,

respectively (Liu et al., 2011). The K_m and V_{max} of cellulase produced by *A. niger* BCRC31494 were found to be 134 mg/mL and 4.6 U/min/mg respectively (Li et al., 2012) while K_m and V_{max} of *Penicillium pinophilum* were 4.8 mg/mL and 72.5 U/mg, respectively (Pol et al., 2012). The K_m and V_{max} of *Penicillium purpurogenum* KJS506 were 1.15 mg/mL and 220 U/mg, respectively (Lee et al., 2011).

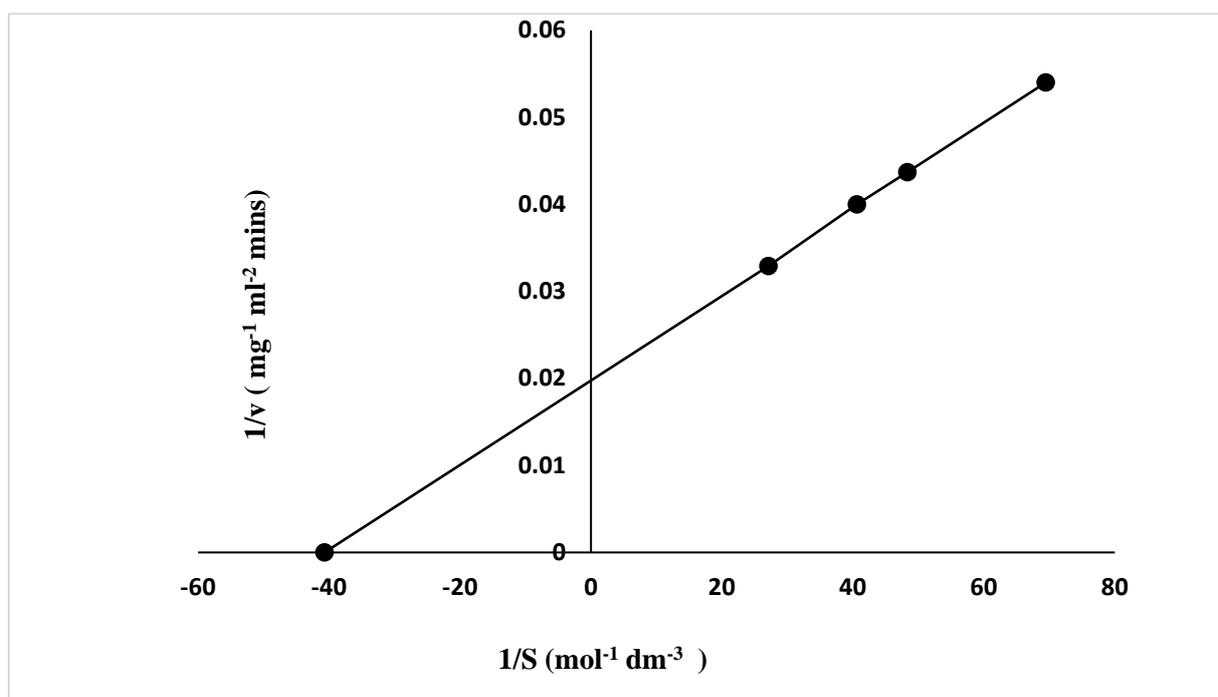


Fig. 5: Lineweaver-Burk plot of the crude cellulase from fungal strain *Aspergillus unguis* UCSC324 isolated from coconut wood, at pH 5.0 and 50°C with different concentrations of cellulose substrate

Temperature stability on crude cellulase

When the crude cellulase solution, at pH 5.0 was pre-incubated at 50°C, the enzyme activity lasted for more than an hour (Fig. 6). At 50°C and pH 5.0, the enzyme activity showed a decreasing trend after 40 minutes. Cellulase activity values obtained before 80 minutes at pH 5.0 and 50°C were significantly higher than activity values obtained after 80 minutes of incubation. Therefore, it could be suggested that this enzyme is thermostable and it has the capacity to be active for at least 80 minutes at 50°C and pH 5.0. It is also important to note that the enzyme showed optimum activity at 50°C and pH 5.0. A *Trichoderma viride* strain produced 0.5 IU/mL activity of CMCase when treated with glucose, sucrose, xylose and CMC. The CMC would act as an efficient inducer of FPase activity (Nathan et al., 2014). The pH and

temperature stability of cellulases and xylanase crude enzymes were studied and these extracellular enzymes retained their activity at 80°C for 60 minutes (Abo-State et al., 2013).

The dominance and higher cellulase production of the *Aspergillus* species may be due to its tolerance to survive under several abiotic stress including high temperature, pH and high salt concentrations and the capacity to degrade the decaying wood. Numerous species of *Aspergillus* have the ability to resist diverse stress conditions such as drought, temperature, pH, salinity and mineral ion concentrations and to produce various high efficiency lignocellulases for the bioconversion of lignocellulosic materials (Xiao et al., 2011).

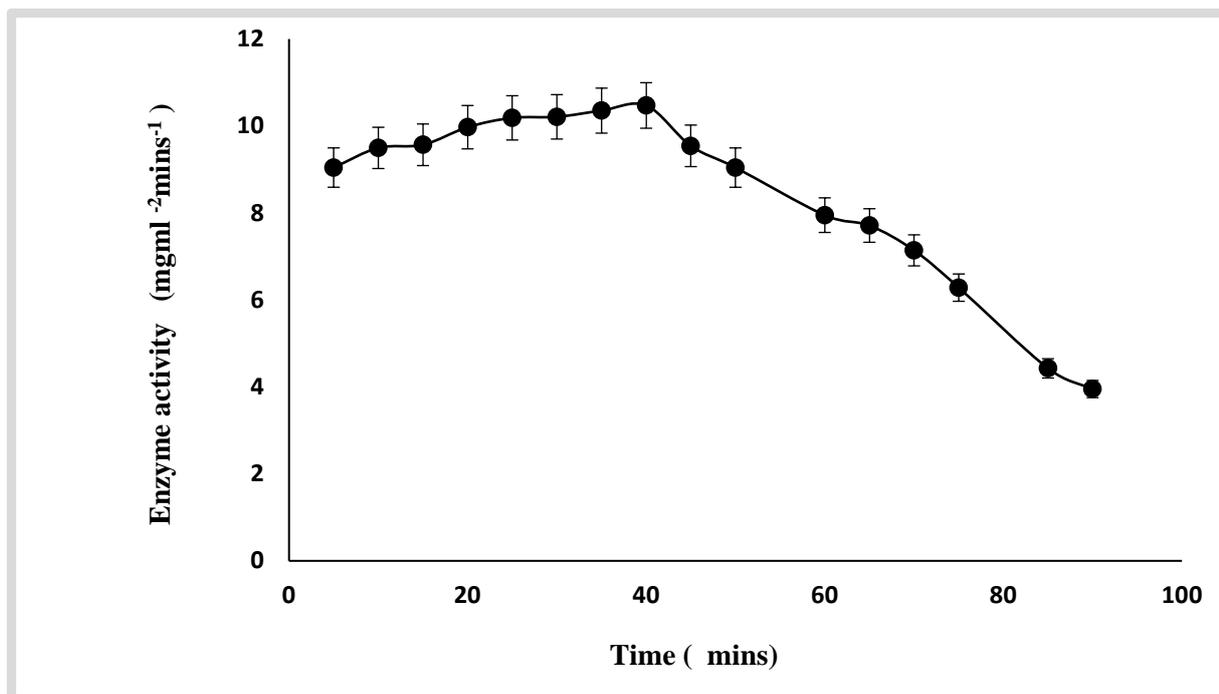


Fig. 6: Thermal stability of crude cellulase (at 50°C) produced from fungal strain *Aspergillus unguis* UCSC324 isolated from decaying coconut wood. pH of the crude mix was maintained at 5.0 throughout the experiment.

Conclusion

Among the cellulase producing fungal strains isolated from diverse sources, the best cellulase producer was from decaying coconut wood and identified as *Aspergillus unguis* UCSC324 based on the morphological, biochemical and molecular biological analysis. Crude cellulase showed zero order kinetics for 5 minutes. The optimum pH for the crude cellulase was 5.0 and the optimum temperature was 50°C at pH 5.0. Michaelis constant and V_{max} for the crude cellulase to soluble cellulose by Lineweaver-Burk plot were $4.545 \times 10^{-2} \text{ moldm}^{-3}$ and $26.66 \text{ mgml}^{-2} \text{ mins}^{-1}$ respectively, at pH 5.0 and at 50°C. The cellulase enzyme produced from *Aspergillus unguis* UCSC324 showed better stability at 50°C than any other temperatures.

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