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Production of high cellulase yields from polypore fungi in solid-state fermentation using green tea waste

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Abstract

Polypores are diverse macrofungi that have been extensively studied for their enzyme production capabilities. Presently, these enzymes are being used for many industrial purposes. However, the high-cost associated with their production is the main barrier to their broader application. This work aimed to study the optimal medium and conditions by using solid state fermentation. Seven polypore strains were used for cellulase activity screening. The fermentation experiments were carried out in 250 mL Erlenmeyer flasks with green tea waste as a substrate. Notably, *Microporus* sp. KA038 showed the best level of activity of 81.8 IU/gds. Various parameters such as temperature on growth, moisture content, nitrogen source, initial pH value, inoculum size and incubation time were considered to determine the optimal conditions for cellulase production. The optimal medium consisted of green tea leaves as a carbon source, beef extract as an organic nitrogen source, and $NH_4H_2PO_4$ as an inorganic nitrogen source, while pH 7.0 and an incubation temperature of 30°C for 4 days resulted in a high enzyme yield with *Microporus* sp. KA038.

Keywords – Agicultural wastes – cellulolytic enzymes – white rot fungi

Introduction

Lignocellulosic biomass is mainly formed of three polymers; cellulose, hemicellulose, lignin along with other components (Isikgor & Becer 2015). Despite the usefulness of these materials in many sectors of the economy, a great amount of the lignocellulosic biomass ends up being discarded and becomes a pollutant (Vintila et al. 2009). On the other hand, lignocellulose biomass is a source material that has been used for previous decades in the production of bioethanol, organic acids, enzymes and biodegradable plastics in the form of cheap carbohydrates (Rajeev & Amit 2016). Lignocellulosic material is promising as an energy source because of its potential for low cost fermentation (Sherief et al. 2010). Green tea is considered one of the most popular drinks in the world and the custom of drinking tea has spread quickly and widely throughout the world (Kondo et al. 2004). However, a substantial amount of tea waste is left after the tea extraction process. Thus, looking elsewhere to minimize or eliminate tea waste has been a difficult task in the tea processing industry (Yang et al. 2015). Hence, at present the elimination of tea waste in the environment in an economically sound and efficient manner has become of significant interest among scientists.

In contrast with submerged fermentation (SmF), solid state fermentation (SSF) is a procedure wherein the substrate is fermented and barely appears in water. The low moisture content in the fermentation of microorganisms is limited primarily to yeasts, fungi and bacteria (Acharya et al. 2010). One of the most adaptive organisms to SSF is fungi because the fugal hyphae features are spread across the surface and easily penetrate into the inter particle spaces, thereby their colonization is typically more efficient than with other organisms in solid substrates (Sukumaran et al. 2005).

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Polypores (basidiomycetes) are a group of wood decaying fungi that are diverse in ecological specificity and morphological characteristics. Most polypores are capable of breaking down lignocellulose, and consequently they play a mainstay role in nutrient recycling in forest ecosystems. Although some polypores can cause tree diseases such as *Onnia tomentosa, Phaeolus schweinitzii*, and *Phellinidium weirii* (Ginns 2017), others have medicinal properties (Jayachandran et al. 2017). Notably, the ability of polypores to produce enzymes has attracted the attention of scientists. In recent years, researchers have become interested in the screening and production of cellulase from polypores (Table 1), and the resulting research has shown positive results. Zecarias and co-workers (2016) screened ligninocellulolytic enzymes by the dye decolorization plate test, with 49 of 61 fungal strains showing decolorizing activity on Phenol red, Azure B and RBBR agar. The exoglucanase, β-glucosidase production were induced using the co-culture between *Trichoderma viride* and *Ganoderma lucidum* in SSF (Afzal et al. 2014). Cellulase is an enzyme that converts cellulose into simple sugars (glucose) (Chinedu et al. 2005). Many studies have reported that microorganisms and animals are able to hydrolyze β -1,4 linkages in cellulose (Henrissat 1991). The production rate of cellulase obtained from fungi is higher than from other microorganisms and this can be advantageous (Rana & Kaur 2012). Generally, complete cellulose hydrolysis is responded to by three main types of cellulase combinations including endoglucanases, exoglucanases, and β –glucosidase (Zhang & Lynd 2006). These enzymes are widely used in numerous application areas including the beverage, paper, textile industries, in agriculture and in detergents and animal feeds, as well as to serve as an important alternative source for energy generation. Some organic compounds are inducers for cellulase such as disaccharides, spent ammonium sulphite liquor (Han et al. 2017), and glycerol (Delabona et al. 2016). This research study aims to explore the isolation of polypore fungi and the production of cellulase enzyme.

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Table 1 Recent research studies on cellulase polypore fungi

Polypores	Cellulase activity (IU/gds)	References
Coriolus versicolor	78.75	Montoya et al. 2015
Melanoporia sp.	7.50	Oliveira 2016
Pycnoporus coccineus	14.812±0.360	Chuwech et al. 2016
Pycnoporus sanguineus	16.32±2.65	Onofre et al. 2015

Materials & Methods

Morphological observation

Basidiomata of re-supinate fungi were collected from the dead stumps on Suthep mountain in Chiang Mai, Thailand from December 2016 to July 2017. The basidiomata was isolated on PDA (Potato dextrose agar) and then sub-cultured every 7 days. The dried specimens were then deposited in the herbarium at the Center of Excellence in Microbial Diversity and Sustainable Utilization, Chiang Mai University.

Macro-morphological characteristics of the hymeneal surface were observed using fresh specimens.

Micro-morphological characterization of the selected strain was studied for species confirmation. The dried specimens were mounted with 5% KOH, stained with Melzer's solution and Congo-red under light microscopy on glass slides. Microscopic structures including spores and hyphae were measured for size using an ocular micrometre under a light microscope.

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DNA extraction, amplification & sequencing

Mycelia of each fungal sample were grown on PDA and harvested after 7 days of inoculation. The ITS region was amplified using the ITS1F/ ITS4, ITS5 primers as represented by White et al. (1990). Polymerase chain reaction products were purified using the NucleoSpin Gel and PCR Clean-up kit (Machery Nagel, Germany). Sequencing reactions were performed on an ABI PRISM 3100 Genetic Analyzer. Newly obtained sequences were compared with nucleotide entries in the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/) using the Blast search tool to confirm genus identity.

Substrate and chemical preparation

Green tea waste was procured from Amazing Tea Chiang Mai Company, Chiang Mai, Thailand. It was ground using a blender. Citrate buffer was prepared by mixing sodium citrate and citric acid and the pH was adjusted to 5.0. All chemicals were of the highest reagent grade commercially available.

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Screening of cellulolytic ability

First screening: Plate screening using Carboxymethylcellulose (CMC) as carbon source:

Fungal colonies (0.5 cm diameter) from one week old PDA plates were cut by pasture pipette and then inoculated into the middle of the CMC agar plates. The plates were then incubated at ambient temperatures (28 ± 2 °C) for three days. The plates were colored with 0.1% (w/v) Congo-red for 0.5-1 hour and discolored for 15- 20 min with 1 M NaCl solution. Finally, 1 M NaCl was discarded and the plates were washed with water at a pH of 2.0. The staining of the plates was analyzed by noticing the formation of clear or yellowish zones around the fungal colonies. Cellulolytic production ability was revealed by the size of a clear halo that was considered to be indicative of the zone of hydrolysis. This area was measured for enzymatic index (EI) calculation using the following formula (Beguin & Aubert 1994):

$$EI = \frac{\text{Diameter of hydrolysis zone}}{\text{Diameter of colony}}$$

Secondary screening: Screening of cellulolytic ability in liquid medium

Cellulolytic ability of seven fungal strains was checked using the Dinitrosalicylic acid (DNS) method. Inoculum was prepared by inoculating 0.25 mL solution from seven-day-old fungal culture in PDB at 30°C with 180 rpm shaking. Supernatant was collected after 3 days of incubation and centrifuged at 10,000 rpm for 10 minutes at 4 °C. The reaction mixture containing 0.5 mL of the sample, 0.5 mL of sodium citrate buffer and 0.5 mL of 1%(w/v) CMC was then incubated at 37ºC for 60 minutes. The reaction was ended by adding 1 mL of DNS reagent. The color was then increased by boiling for 5 minutes. The measurement of optical desity was performed at 540 nm (Miller, 1959).

Temperature on fungal growth

To investigate the effect of temperature on growth, the plates were incubated at different temperatures (25, 30, 37 and 45°C) in various incubators. The optimal temperature was recorded by the high diameter of the colonies every day over 7 days.

Upscale screening on substrate by solid state fermentation

Cellulase production was studied in flasks containing 5 grams of green tea waste as a substrate containing 20 mL of distilled water with moisture content at 75%. The flasks were then sterilized at 121°C for 15 min. The strains were grown on PDA plates for 7 days and added to fermentation medium as an inoculum. The flasks were incubated at 30ºC for 7 days. The fermented substrate of each flask was extracted using 50 mL of 50 mM citrate buffer (pH 5.0) and filtered through filter paper in an ice bucket. The crude extract was centrifuged at 8000 rpm for 5 min, the

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supernatant was collected and used as a crude enzyme for cellulase assay using the DNS method (Miller, 1959).

Effect of moisture content on cellulase production

The moisture content optimization was determined. Three flasks containing 5.0 g of dried green tea were adjusted to different moisture contents of 40, 50, 60, 70, 75, and 80% by adding water at8, 10, 13, 15, 20 and 25 mL, respectively. The before and after values of dehydration and weight were calculated to determine the moisture content using the relevant equation (1). The flasks were then inoculated at 30ºC for 7 days. The cellulase activity was measured using the DNS method.

$$\text{Moisture content (\%)} = \left(\frac{\text{Wet sample - Dry sample}}{\text{Wet sample}}\right) \text{x}100 \qquad (1)$$

Effect of nitrogen source on cellulase production

Organic nitrogen sources (peptone, beef extract, yeast extract, malt extract and tryptone) and inorganic nitrogen sources (Urea, $NaNO_3$, $(NH_4)_2SO_4$, NH_4NO_3 and $NH_4H_2PO_4$) at 0.1% (w/v) concentration were added into 125 mL Erlenmeyer flasks contained 5.0 g of dried green tea and adjusted to optimal moisture content (75%). Five discs of fungal strains were inoculated. The flasks were incubated for 7 days at 30ºC and enzyme assay was then performed using the DNS method.

Effect of pH value on cellulase production

The optimized pH value was performed by adjusting the pH to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11 and 12 of the green tea waste containing fermentation media by using 0.1N HCl or 0.1N NaOH. After inoculation with 5 discs of 0.5 mm polypore colonies size, the flasks were incubated in an incubator at 30ºC for 7 days.

Effect of inoculum size on cellulase production

The inoculum size was optimized by preparing the inoculum on PDA plates containing polypores using a sterile pasture pipette of 0.5 mm in diameter. Green tea waste containing fermentation media was inoculated with 1, 3, 5, 7, 10, 12 and 15 disc(s) of the strain aseptically. After inoculation, the flasks were incubated at 30ºC for 7 days. At regular intervals, the enzyme assay was performed.

Effect of incubation time on cellulase production

To determine the effect of incubation time on cellulase enzyme production, the flasks were inoculated with the selected strain and incubated for different numbers of days (4, 10, 14, 18 and 24 days). The Optical Density (O.D) was taken at 540 nm using the DNS method.

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Results

Collection and identification

In this study, the polypore samples were collected based on their distinct morphology. A total of seven strains were selected from dead logs (Fig. 1).

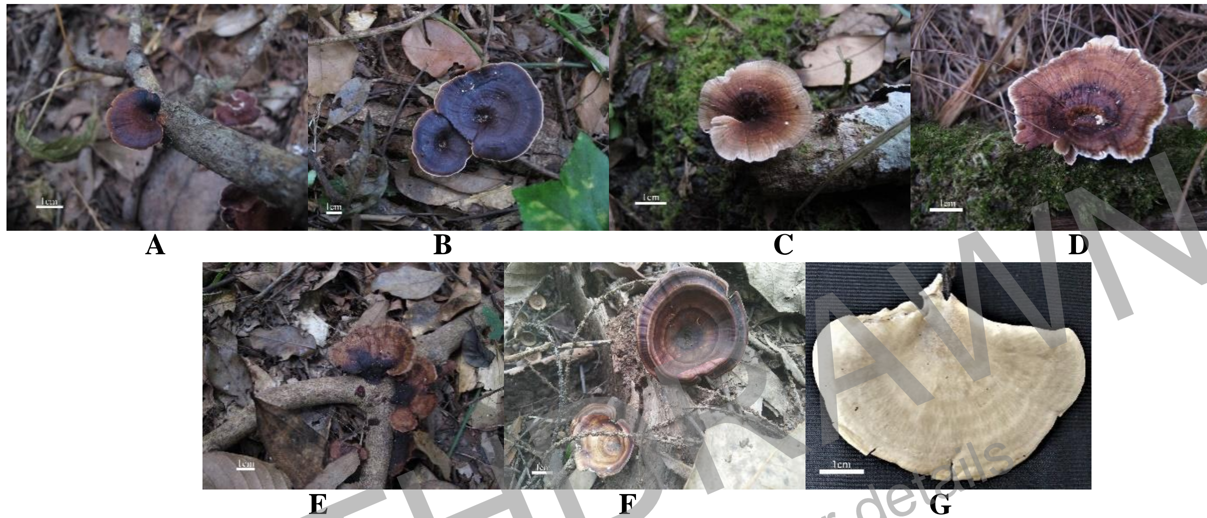

Figure 1. Fruiting body of **A.** KA007 (*Microporus* sp.), **B.** KA009 (*Microporus* sp.), **C.** KA012 (*Microporus* sp.), **D.** KA016 (*Microporus* sp.), **E.** KA018 (*Hexagonia* sp.), **F.** KA038 (*Microporus* sp.), **G.** KA053 (*Favolus* sp.) (Scale bars = 1 cm)

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Phylogenetic analyses

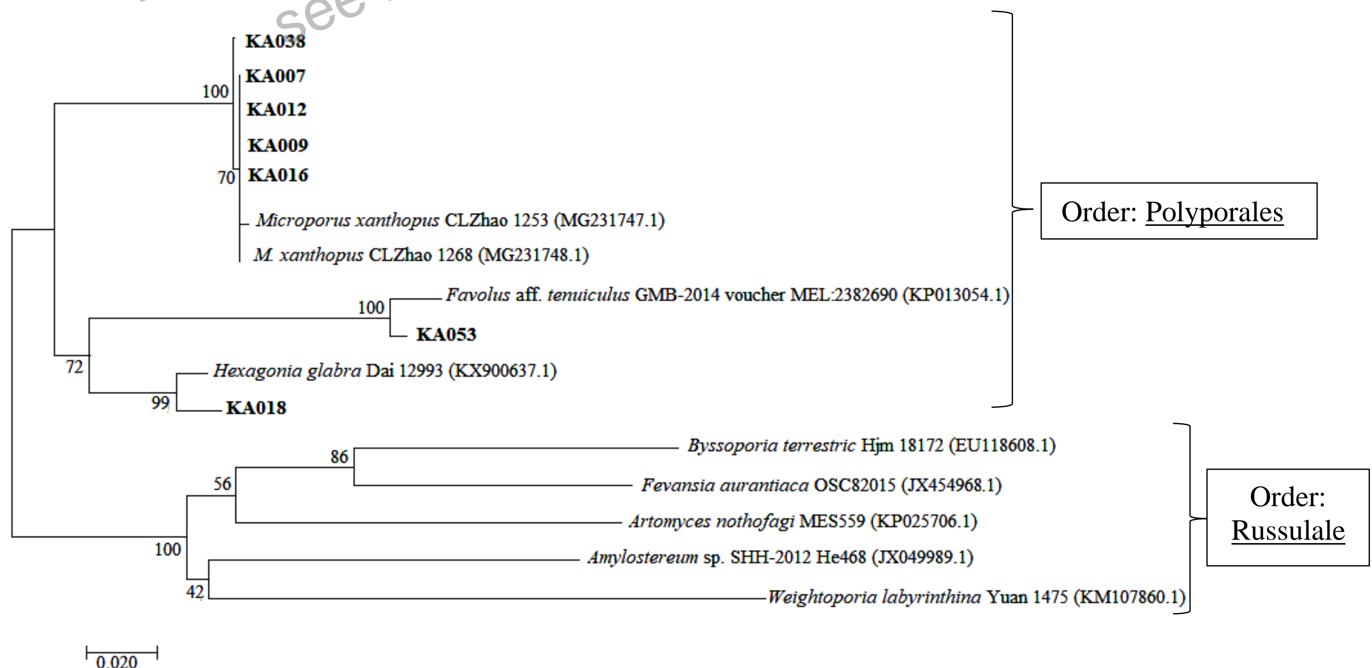

Figure 2. Phylogenetic tree of the polypore strains and related species based on ITS sequences obtained from GenBank generated by the maximum parsimony method. Order Rusulales is the outgroup.

The raw trace files were inspected and edited with MEGA version 7 software (Kumar et al. 2016). Generated sequences were searched in BLAST (https://blast.ncbi.nlm.nih.gov) and then compared with available sequences in the National Center for Biotechnology Information (NCBI) GenBank nucleotide database. The evolutionary history was inferred using the Neighbor Joining (NJ)

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method (Saitou and Nei, 1987). The replicate tree percentages in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Sequences of accession of *Byssoporia terrestris* (EU118608.1), *Fevansia aurantiaca* (JX454968), *Artomyces nothofagi* (KP025706), *Amylostereum* sp. (JX49989.1), *Wrightoporia labyrinthine* (KM107860) from the order Russulales were included as outgroups to root the trees. The phylogenetic analyses showed that all the selected strains clustered in the order Polyporales with high support.

Screening of cellulolytic ability

First screening: Plate screening using CMC as carbon source

The first screening of the 7 selected fungal strains was performed using the Congo red test. This test was based on the measurement of colony growth and the hydrolysis halos were used for the enzymatic index (EI) calculation. The production of the halos by cellulose hydrolysis was directly related to the region of action of cellulolytic enzymes, as the stain only remains attached to β-1,4-D-glucanohydrolase bonds (Lamb and Loy 2005). The results showed that the strain KA016 showed the highest degree of enzymatic index at 1.5 (Table 2).

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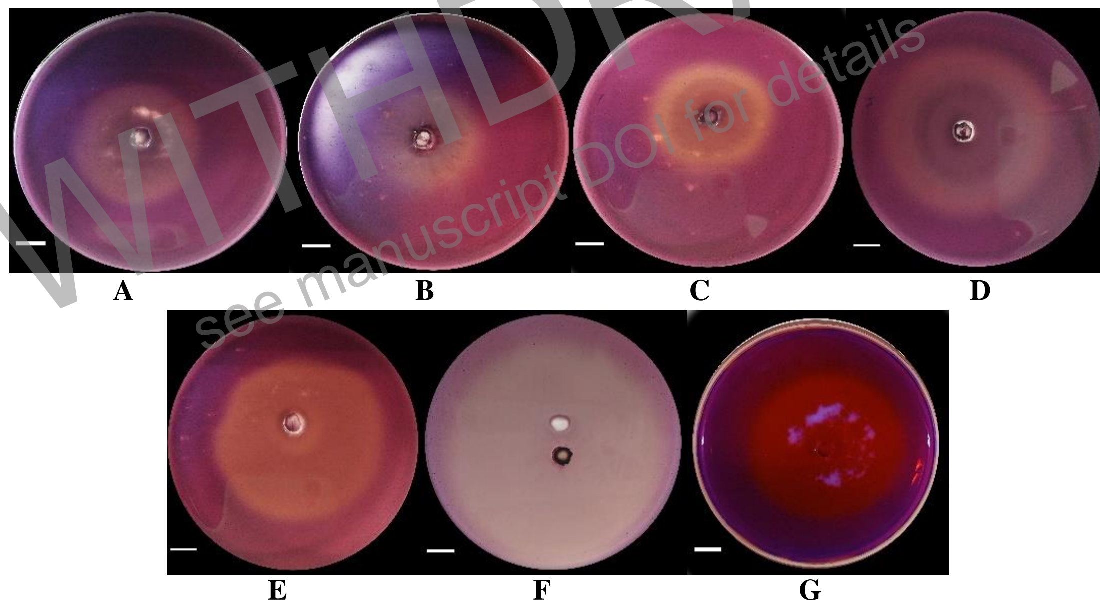

Figure 3. Observation of the clear zone around a colony of polypores using Congo red dye (Scale bar is 1 cm)

Table 2. Diameter of hydrolysis zone, diameter of colonies and enzymatic index of selected strains

Strain	KA007 (A)	KA009 (B)	KA012 (C)	KA016 (D)	KA018 (E)	KA038 (F)	KA053 (G)
Diameter of hydrolysis zone	4.2	3.7	4.1	5.8	5.7	7.5	4
Diameter of colonies	3.2	3.3	3	3.9	4.8	6.5	3
Enzymatic index (EI)	1.4	1.12	1.4	1.5	1.18	1.15	1.3

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Secondary screening: Screening of cellulolytic ability in liquid medium

All strains were positive for cellulase and able to grow in CMC agar media (Fig. 3), but the strains differed in their ability to produce cellulose degrading enzymes (Table 1 and Fig. 4). The strain KA016 revealed the highest EI value (1.5), the strain KA053 revealed the highest cellulase activity (0.65 U/mL), whereas KA009 indicated the least clear zone diameter (EI 1.12) and the lowest cellulase activity (0.01 U/mL). As a result, only strain KA009 was not considered for further studies.

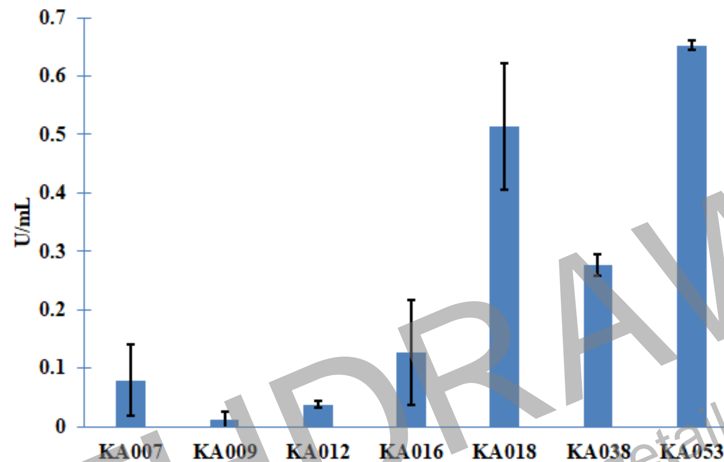

Figure 4. Screening and evaluation of potential cellulase activity producing polypore strains by DNS assay

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Temperature on fungal growth

The results showed that the best temperature for fungal growth was 30°C, as the growth of faster colonies was recorded at 30ºC. The increase or decrease in incubation temperature did not increase the degree of growth. No growth was observed at 45°C for either strain and very poor growth was reported at 37 °C for most strains. Thus, the best temperature of colony production was observed at 30°C (Fig. 4).

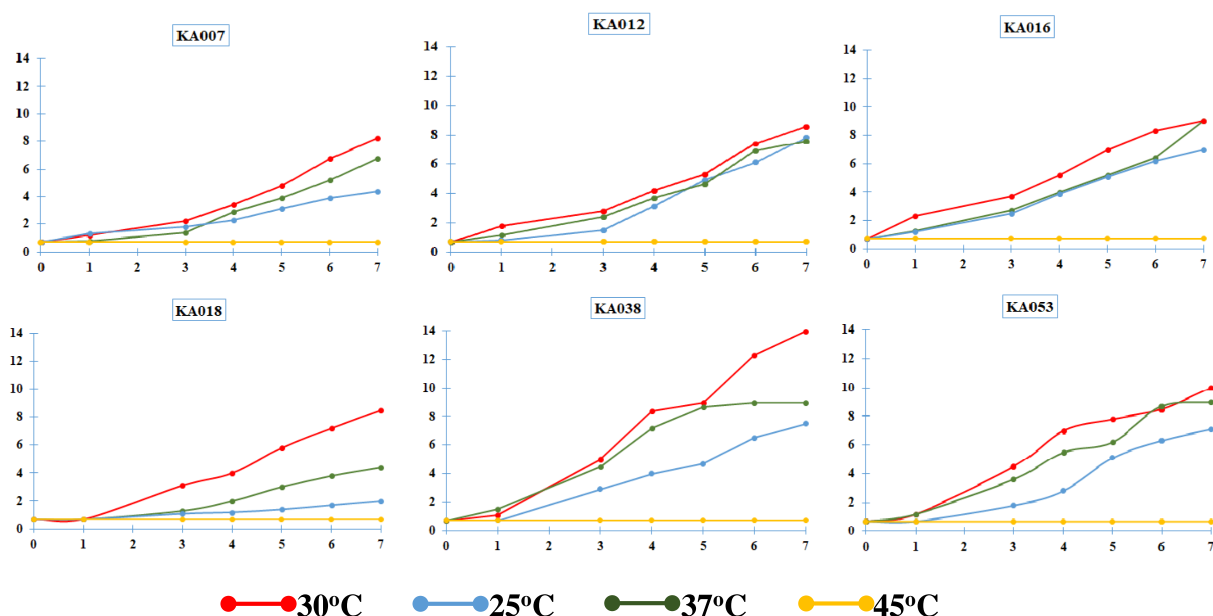

Figure 5. Fungal growth at various temperatures (X axis = Diameter of colonies (cm) and Y axis = Time (days))

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Upscale screening on substrate by solid state fermentation

The different degrees of potential to produce cellulase of various strains was studied by testing the activity of enzyme production by useing green tea as substrate. The highest enzyme activitiy was observed in strain KA038 (38.62 IU/gds) (Fig. 6). Consequently, only KA038 was selected for further experiments.

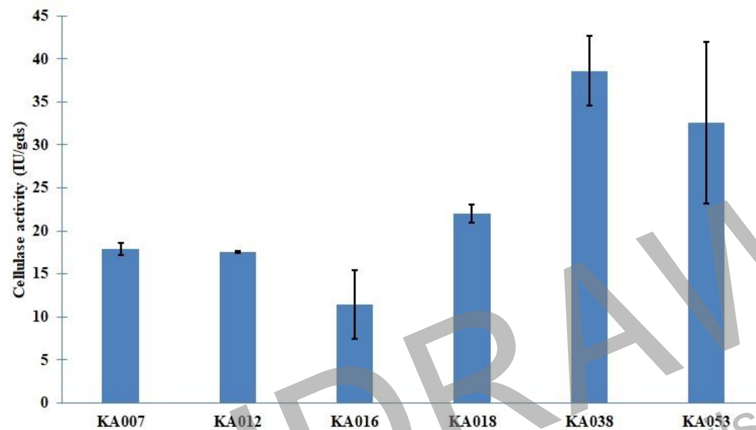

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Figure 6. Cellulase activity by polypore on green tea waste substrates expressed as units per gram dry substrate (IU/gds)

Molecular identification of selected strain KA038

The ITS region of rDNA sequence can be obtained and aligned with known nucleotide sequences in GenBank databases using the BLAST search option to calculate the percentage of identify. The percentages of similarity of the rDNA gene sequences between the fungal strain KA038 and the closely related white-rot fungi are shown in Table 2. The results revealed that polypore strain KA038 was closely related with *Microporus* sp. BPSM33, *Microporus xanthopus* voucher LE232511 and analysis of the ITS region sequence indicated that the selected strain KA038 could be a member of the genus *Microporus* with over 99% similarity.

Table 3. Percentages of similarity of the ITS gene sequences between fungal strain KA038 and closely related white-rot fungi

Fungal strain	Size (bp)	Similarity (%)	Accession number
KA038	552	-	This study
Microporus sp. BPSM33	491	100	KM985670.1
Microporus sp. BAB-4084	675	99.64	KJ670303.1
Microporus xanthopus voucher LE232511	584	99.09	KC503506.1
Microporus luteus voucher LE289450	584	98.55	KC503504.1
Microporus luteus voucher LE231601	584	98.37	KC503505.1

To investigate the inter- and intra-relationships of the members in some part of the genus *Microporus,* the obtained sequences were aligned with the sequence of the type strains retrived from the GenBank databases. A phylogenetic tree was constructed using the NJ method. The sequence of *Favolus pseudoemerici* was used as an outgroup to root the tree. As can be seen in Fig. 7, the final Neighbor-joining phylogenetic tree indicated that strain KA038 was positioned between the group of *M. xanthopus* and the undescribed species of *Microporus* genus.

The numbers at the branching points are the percentages of occurrence in 1000 bootstrapped trees. The bar indicates 1 nucleaotide substitution per 100 nucleotides.

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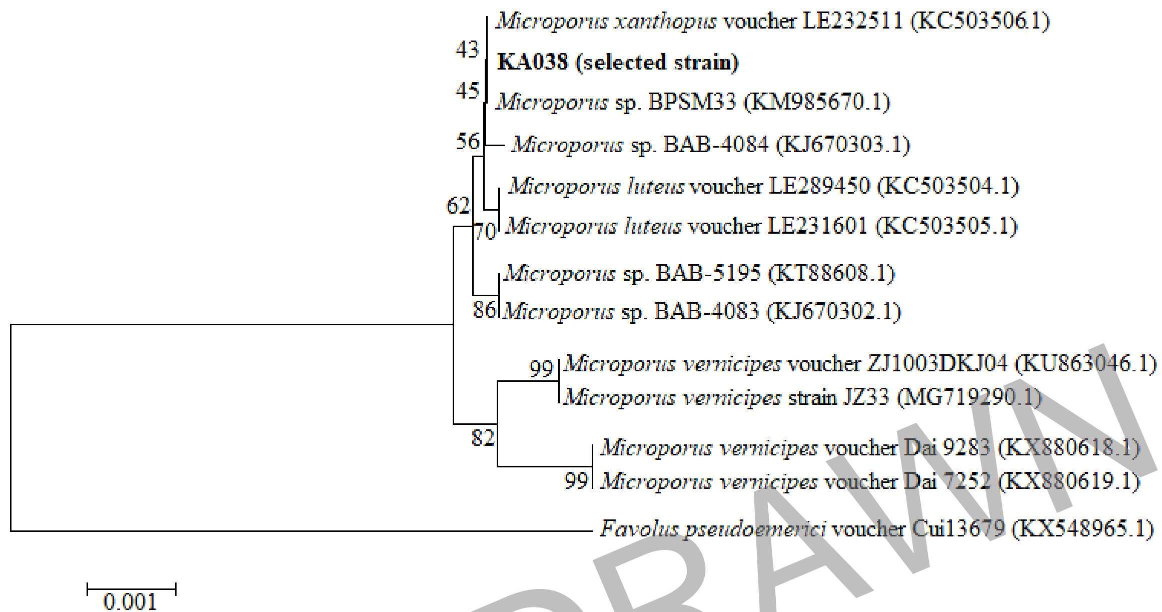

Figure 7. Neighbor-joining phylogenetic tree showing the position of selected strain *Microporus* sp. KA038 based on sequences of ITS region

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Taxonomic charcterization of selected strain KA038

White rot fungus strain KA038 was identified to *Microporus* sp., which belongs to the Basidiomycetes, Family Polyporaceae, Genus *Microporus*. The characterization of the strain is as follows. Basidiocarp centrally stipitate, margin undulated with narow sterile zone below, stipe cylinderical, 0.5−1.5 cm long, pale yellow. The phenotype of the young to old fresh sample was stipitate form, with brownish-yellow to cinanmon-brown color at the upside, white pores-surface at the downside, various basidiocarp sizes 2.5×3.6 to 3.5×5 cm (Fig. 8A). The fruiting-body was common on the dead trunks (Fig. 8B). The pores of the immature fruiting-body were very tiny, about 8 pores per milimetre for the mature fruiting-body (Fig. 8C).

Figure 8. Habitat (**A**), mature basidiocarp (**B**) and the undercarp pores (**C**) of the selected strain

Mycelial colonies of *Microporus* sp. KA038 on PDA medium were off-white, showing high density, a velvety texture, and abundant aerial hyphae (Fig. 9A). The growth rate of *Microporus* sp. KA038 completely colonized in a Petri dish over 4 days at 30ºC. The light micrograph produced under a compound microscope is shown in Fig. 9B, the fungus were generative hyphae 2-3 µm thick, colorless, binding hyphae thick-walled 1−1.5µm (Fig 10C), and 4−5µm wide (Fig. 10B). Cystidia absent. No clamp connection. Spores of the selected strain were elliptical in shape 5.5−7×2−2.5 µm, smooth, in-amyloid and sparse (Fig. 10A).

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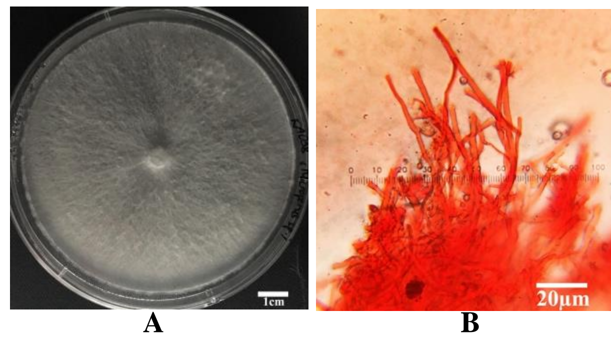

Figure 9. *Microporus* sp. KA038, colony morphology (**A**) and light micrograph without clamp connection (**B**) grown on PDA for 7 days at 30°C

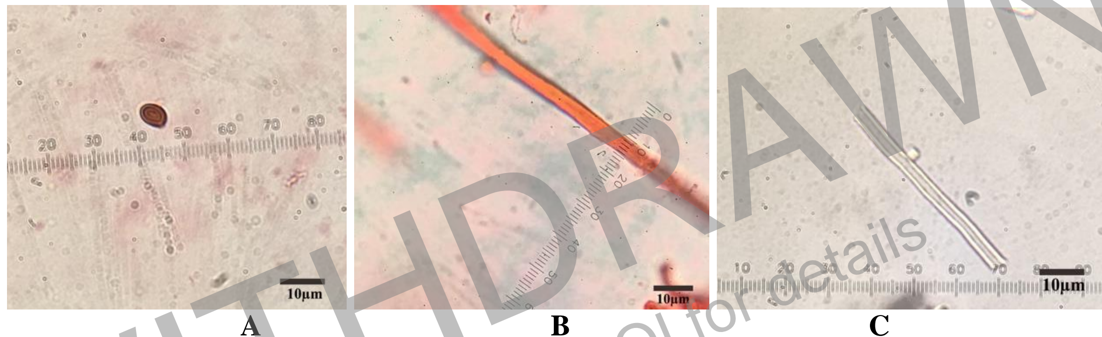

Figure 10. Spores (A), hyphae (**B**) and the thick-walled of hyphae (**C**) of selected strain *Microporus* sp. KA038

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Effect of moisture content on cellulase production

Moisture is a very essential factor in the SSF process. Consequently, this adjustment has a significant influence on growth, microbe biosynthesis and secretion of metabolites such as enzymes. For the strain *Microporus.* sp. KA038, the optimal moisture content was 75% (control) and activity was recorded at 38.62 IU/gds (Fig.11). It was reported that the production of enzymes was negatively affected in the instances of increased or decreased moisture. Ahmed (2008) and Sodhi et al. (2005) claimed that the crucial factor influencing the outcome of SSF is the moisture content of the substrate, and that the reduction of nutrients provided to the organisms resulted in lower moisture content. On the other hand, high moisture caused a reduction in enzyme production that may have been due to the substrate porosity reduction, the structure of substrate particles change, the gas volume decrease and microbial growth reduction (Baysal et al. 2003). This result was achieved in upscale screening on the substrate by solid state fermentation, and a better result was observed than with other moisture contents. The optimal moisture of 75% was used for further experiments.

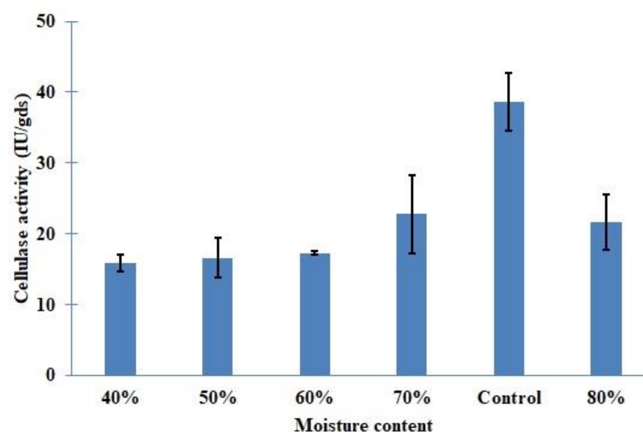

Figure 11. Effect of moisture content on cellulase production by polypore fungi

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Effect of nitrogen source on cellulase production

Nitrogen source is important for industrial fermentation medium that is designed to meet maximum enzyme production. The result of the effect of various nitrogen sources on cellulase production is illustrated in Figure 12. Among the different nitrogen sources, the highest degrees of cellulase production were 43.5 IU/gds and 46 IU/gds, when beef extract was added as an organic nitrogen source and $NH_4H_2PO_4$ was added as a source of inorganic nitrogen, respectively.

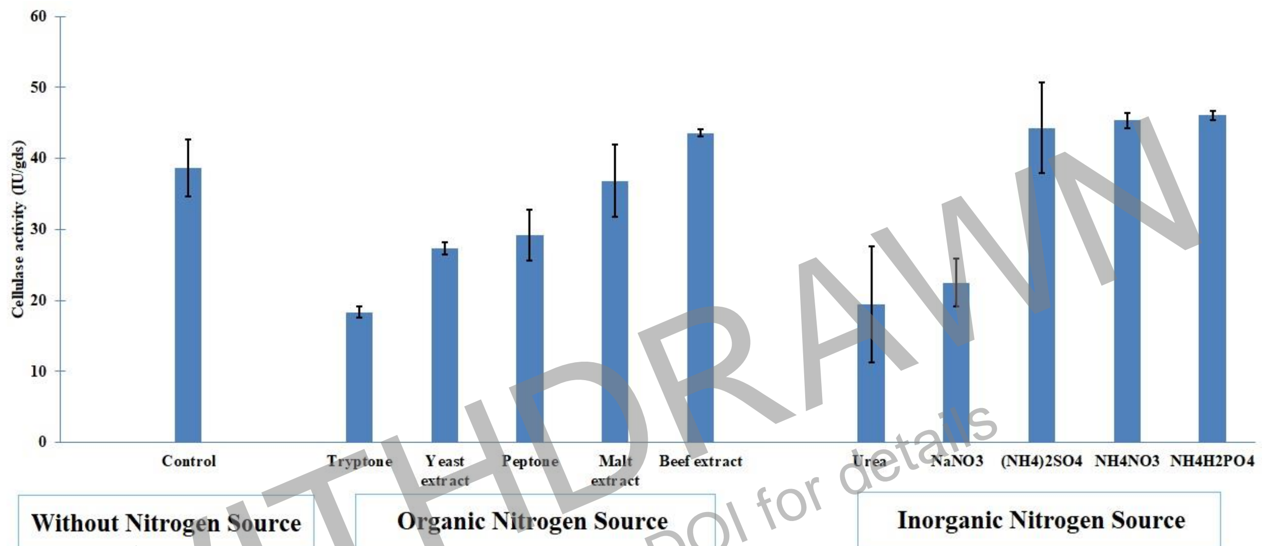

Figure 12. Optimization of nitrogen source for cellulase production

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Effect of pH value on cellulase production

As is shown in Figure 13, the effect of pH on cellulase production was determined at pH values ranging from 4.0 to 12.0. Green tea waste revealed a maximum cellulase activity of 49.9 IU/gds at pH 7.0. The enzyme was still active over a wide pH range and it was observed that the cellulase activity had a broad pH range of between 4.0 and 12.0. This was due to the fact that cultivation of fungi at an unfavorable pH value may result in reduced enzyme activity by reducing accessibility of the substrate (Bakri et al. 2008). In the SSF process, for optimizing the initial pH, precautionary measures were taken as a result of the fact that only at a particular pH, extracellular enzymes were stable and there may be rapid denaturation at lower or higher values. Notably, the metabolic activities of the microorganism are very sensitive to changes in pH because pH affects the cellulase production of fungi (Kalra and Sandhu, 1986).

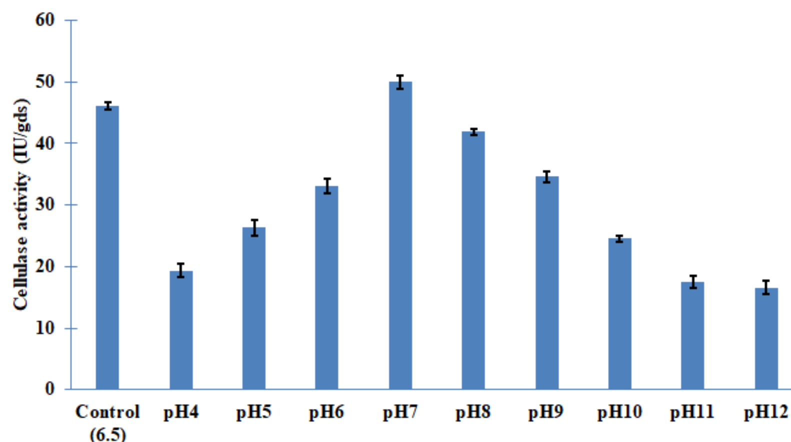

Figure 13. Optimization of pH for cellulase production

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Effect of inoculum size on cellulase production

Maximum activity was noted with 10 discs of inoculum size of the inoculated strain (Fig. 14). By increasing the inoculum size, an increase in enzyme activity was recorded. The maximum cellulase activity recorded was 51.27 IU/gds with 10 discs of inoculum (Figure 14).

Notably, there is an indispensable factor influencing the optimal inoculum size in SSF because smaller inoculations can be short of biomass and allow the unexpected organism growth, whereas a high inoculation may tend to be full of biomass and deplete the necessary nutrients (Pandey et al. 2003). Irrespective of SSF or SmF, inoculum size can greatly influence the yield of the final product (Prakasham et al. 2006).

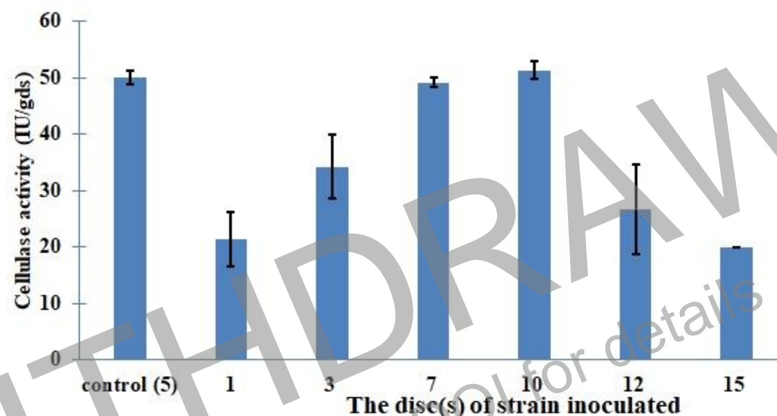

Figure 14. Optimization of inoculum size for cellulase production

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Effect of incubation time on cellulase production

The time of fermentation has an important impact on product formation. Enzyme production is related to the time of incubation (Gautam et al. 2011). Time course for the production of cellulase enzyme was investigated and fermentation for a period of 4 to 24 days was carried out. The results revealed that the maximum cellulase activity (81.8 IU/gds) was observed at an incubation time of 4 days for *Microporus* sp. The enzyme activity gradually increased with a decrease in incubation time up to the optimum level, followed by a gradual fall in activity (Fig. 15). This trend of decreased enzyme activity may have been due to the depletion of macro and micronutrients in the fermentation medium with a lapse in time, which stressed the fungal physiology resulting in the inactivation of the secretary machinery of the enzyme (Rajendran et al. 2015).

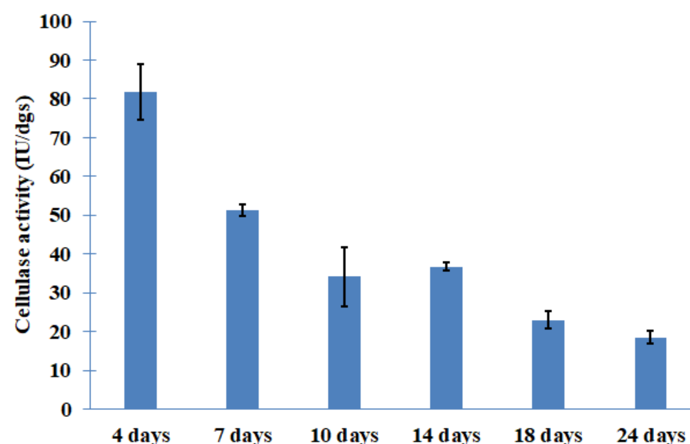

Figure 15. Optimization of incubation time for cellulase production

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Conclusions

The potential of utilizing green tea waste as a substrate for cellulase production under SSF by isolated polypore fungi was identified and studied. Under optimum SSF conditions of 5 grams of substrate for *Microporus* sp. strain KA038, 30ºC of incubation; moisture content, nitrogen source and optimum pH were found to be 75%, beef extract and $NH_4H_2PO_4$, pH 7.0. The cellulase activity increased gradually from 1 to 10 disc(s) of inoculum and then fell at 12 discs of inoculum onward. The shortest incubation time of 4 days produced the best cellulase activity that increased from 51.27 IU/gds to 81.8 IU/gds. In this study, cellulase activity increased by 1.6 fold after one factor at-a-time optimization procedure was employed using *Microporus* sp. strain KA038.

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