การคัดเลือกและวิเคราะห์ชนิดเชื้อราเซลลูโลไลติกเพื่อการผลิตเอทานอล

Selection and Identification of Cellulolytic Fungi for Ethanol Production

ยงยุทธ์ ศรีศุภภักดี,[1] จิรศักดิ์ คงเกียรติขจร[2*]
Yongyuth Srisupapakdee,[1] Jirasak Kongkiattikajorn[2*]

บทคัดย่อ

เซลลูเลสเป็นเอนไซม์ที่มีศักยภาพในการย่อยสลายชีวมวลเซลลูโลสซึ่งสามารถใช้เป็นแหล่งผลิตเอทานอล วัตถุประสงค์ของการศึกษานี้เพื่อแยกและคัดเลือกเชื้อราที่สามารถผลิตเซลลูเลสเพื่อใช้ในกระบวนการผลิตเอทานอล เชื้อราคัดแยกได้จากแหล่งต่าง ๆได้แก่เศษพืช ท่อนไม้ ดิน และอาหารหมักดองเช่น เทมเป้ และเต้าเจี้ยว กิจกรรมเอนไซม์เซลลูเลสวัดจากการทำปฏิกิริยากับ CMC (carboxymethyl cellulose) ที่พีเอช 5 การผลิตเอนไซม์เซลลูเลสของเชื้อราทดสอบโดยเพาะเลี้ยงในอาหารเลี้ยงเชื้อแข็ง CBM ที่ใช้ CMC เป็นแหล่งคาร์บอน การผลิตเอนไซม์เซลลูเลสตรวจวัดจากวงใสรอบโคโลนีและอัตราส่วนระหว่างเส้นผ่านศูนย์กลางของวงใสและโคโลนีหลังจากย้อมสีด้วยสารละลายไอโอดีน พบว่ามี 19 สายพันธุ์ที่สามารถเจริญและผลิตเซลลูเลสในอัตราส่วนต่าง ๆกัน หลังจากนี้ได้ทำการเพาะเลี้ยงในที่จำกัดออกซิเจนโดยใช้กลูโคสเป็นแหล่งคาร์บอนสำหรับการตรวจหาความสามารถในการผลิตเอทานอล พบว่ามี 8 สายพันธุ์ที่สามารถผลิตเอทานอลได้ โดยเฉพาะสายพันธุ์ F08, F09 และ M03 สามารถผลิตเอทานอลในปริมาณสูงคิดเป็น ร้อยละ 62.25, 87.10 และ 75.41 ทางทฤษฏี ทั้งสามสายพันธุ์จึงเป็นสายพันธุ์ที่สามารถผลิตเอนไซม์เซลลูเลสและเอทานอลได้และได้ทำการตรวจวิเคราะห์โครงสร้างเชื้อราทางกล้องจุลทรรศน์และลำดับเบสของ Rdna บริเวณ internal transcribed spacer (ITS) พบว่ามีลำดับยีนที่เหมือนกันคิดเป็นร้อยละ 100, 94 และ 99 กับสายพันธุ์ *Aspergillus oryzae*, *Rhizopus microsporus* และ *Flavodon flavus* ตามลำดับ

คำสำคัญ: เชื้อรา เซลลูเลส เอทานอล

Abstract

Cellulase is a potential enzyme used for hydrolysis of cellulosic biomass which can be used as a source for ethanol production. The objective of this study is to isolate and screen fungi for the cellulase production for ethanol production process. Fungi were screened sampled from different

[1]นักศึกษา, คณะทรัพยากรชีวภาพและเทคโนโลยี มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี กรุงเทพมหานคร 10150

[2]อาจารย์, คณะทรัพยากรชีวภาพและเทคโนโลยี มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี กรุงเทพมหานคร 10150

[1]Student, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand.

[2]Lecturer, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand.

*Corresponding author: Jirasak Kongkiattikajorn, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand.

sources including plant litter, logs, soil and fermented food such as tempeh and fermented soybean. The initial cellulase activity was measured as the CMC (carboxymethyl cellulose) index, which was found to be at pH 5. The fungi were tested for cellulase production in CBM agar using CMC as a carbon source. Cellulase production was assayed by measuring the clear zone around the colony and the ratio between the diameter of clear zone width and colony after iodine solution staining. The 19 isolated could grow and were able to produce cellulase with different ratios. Afterwards, it was cultured in oxygen-limiting conditions using glucose as the sole C-source for determining the potential of ethanol production. Eight isolated were able to produce ethanol, especially isolated F08, F09 and M03 which the high theoretical ethanol yield of 62.25, 87.10 and 75.41, respectively. The 3 isolated were defined as cellulase and ethanol producing fungi and they were microscopically studied and its regions of rDNA gene were sequenced and compared to GenBank database using BLAST program. The results showed that the partial sequences of 18S, 5.8S and 28S rDNA genes and ITS1 and ITS2 of the isolated F08, F09 and M03 shared 100%, 94% and 99% sequence similarity with *Aspergillus oryzae*, *Rhizopus microsporus* and *Flavodon flavus*, respectively.

Keywords: fungi, ethanol, cellulase

Introduction

Today, development of sustainable energy has become an important topic due to the increasing of energy consumption and depletion of petroleum based-fuel. Biomass-based fuels are an interesting alternative source of energy for several reasons such as feedstock variety and availability, fuel diversity, renewability, as well as many benefits to the environment. Bioethanol is one of the most widely used liquid biofuels. Mostly it is produced by fermentation of glucose directly from crops like sugarcane or sugar beets, or indirectly through starch from corn, wheat, potatoes or cassava. Consequently, the cost of bioethanol is too high and needs to reduce dependency on the price of feedstocks that are in competition with human foods and animal feeds.[1,2] Alternatively, lignocellulosic materials (e.g. stover, straw, wood) are abundant, renewable and have low cost. The carbohydrate polymer mainly found in lignocelluloses are cellulose (homopolymer of D-glucose linked together by β-(1$\longrightarrow$4)-glycosidic bonds) and hemicelluloses (branched heteropolymer and contain mainly of D-xylose, D-glucose, D-galactose, L-arabinose, D-mannose and D-glucuronic acid).[3,4] The carbohydrate polymer can be converted to a simpler sugars by hydrolysis with acid or enzymes and the sugars are subsequent fermented to bioethanol or other bioproducts.

However, the low cost technology for cellulosic ethanol is required to improve. The main cost of cellulosic ethanol are the cost of enzymes, pretreatment and the operation of bioreactors. Cellulases have attracted interest because of the diversity of their applications and also for facilitating the understanding of mechanisms of enzymatic hydrolysis of plant carbohydrate polymers. Cellulose may be hydrolyzed using enzymes to produce glucose, which can be used for production of ethanol and other chemicals. Cellulase is expensive and contributes about 50% to the overall cost of hydrolysis due to the low specific activity. It provides a key opportunity for achieving tremendous benefits of biomass utilization. There are many microorganism such as *Clostridium*, *Cellulomonas* and many fungi have ability to produce cellulase. Among these, only fungi has great potential for cellulase production due to the ability to secrete large amounts of extracellular protein and many strains are most suited for production of higher levels of extracellular cellulases.[5,6] So that, microorganism possess both cellulose degradation and ethanol production properties is a key challenge for application of ethanol. Ethanol producing fungi such as *Aspergillus niger*, *A. terreus*, *A. oryzae*, *Rhizopus jabanicus*, *R. oryzae*, *Trichoderma harzianum*, *Fusarium oxysporum*, *Peniophora cinera*, *Tremetes hirsute*, *Fomitopsis palustris* and *Neolentinus lepideus* have been discoverd.[7-14] Therefore, the fungal combination of cellulase and ethanol production capability can reduce the cost of ethanol production from lignocelluloses. The present study is aimed to isolate fungi from different samples that produce cellulase enzyme from a selective medium after enrichment and to identify the fungal isolates based on molecular characterization by sequencing the 18S rRNA coding gene.

Materials and Methods

Microorganisms

Fungi were separated and purified from plant litter, logs, soil and fermented food such as tempeh and soy bean paste and cultured on potato dextrose agar medium (PDA) (HIMEDIA) supplemented with ampicillin (final concentration of 100 μg/ml) and incubated at 28°C for 48 hours. Different colonies appearing on the plate were purified by repeated sub-culturing on the same medium. The purified isolates were stored at 4°C.

Cellulase production test

Fungi were tested for cellulase producing strains by culturing in cellulosis basal medium (CBM) agar in sterile petri dish consisting of 5 g/l $C_4H_{12}N_2O_6$, 1g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.001 g/l $CaCl_2{\cdot}2H_2O$, 0.1 g/l yeast extract and 2g/l carboxymethyl cellulose (CMC).[15] Fungi was inoculated on a CMC agar plate and incubated at room temperature at 28°C for 48 hours. After that cellulase producing strains were detected by growth ability in CBM and cellulase production efficiency was measured flooded plate after Gram's iodine solution (2.0 g KI and Iodine in 300 ml distilled water)[16] and the efficiency of cellulase producing isolated wasevaluated by measuring the ratio between the diameter of clear zone and colony width.

Ethanol production condition

The cellulase producing fungi or fungal strain was grown on PDA for 7 days. After that, fungal mycelia were grown by inoculating four discs of 0.8 cm diameter fungal PDA pieces in MYG medium consisting of 10 g/l malt extract, 4g/l yeast extract and 4 g/l glucose, which were then cultivated for 7 days. The mycelia were harvested by filtering and were transferred aseptically to a 100 ml vial bottle containing 50 ml T medium consisting of 20 g/l glucose, 10 g/l yeast extract, 10 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$. The bottle was covered with a rubber septum and the remaining air was vented with 50 ml syringe with a 23 gauge hypodermic needle. The sample was incubated for 14 days and it was collected every day for determining the ethanol concentration and the medium was set as blank.

Verification of strain identity

Cellulase and ethanol producing isolated was verified and identified by microscopic morphology, colony morphology and DNA sequencing test. The microscopic test was done by slide culture technique, the colony morphology was observed in a PDA plate and DNA sequence identification was carried out for sequencing of partial 18S 5.8S and 28S rDNA and internal transcribed spacer (ITS) regions in ITS1 ITS2 DNA sequences.

DNA isolation from fungal cultures

Fungal cells were directly sampled from a fresh colony, washed in sterile water, incubated in 100 µl lyticase solution (15 mg/ml water) at 30°C for 1 hour. Then 20 µl proteinase K (Sigma–Aldrich, 20 mg/ml water) were added and the suspension was incubated at 55°C for 90 min. After incubation, the suspension was boiled for 8 min. 10 µl these samples were used for amplification reactions.

PCR reactions, DNA digestions and fragment analysis

PCR reaction and digestion of amplified fragments were performed according to the methods described by Esteve-Zarzoso et al.[17] For the 100 µl PCR reactions, the primers ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used.[18] The PCR reaction mixture contained 0.5 µM of each primer, 10 µM deoxynucleotides, 1.5 mM $MgCl_2$ and 1 x buffer (Promega). The PCR reaction mixture was incubated at 95°C for 15 min in a PE 2400 (Perkin Elmer) thermocycler. One unit of the Taq Polymerase was then added to each tube. PCR conditions were as follows: 35 cycles of denaturing at 94°C for 1 min; annealing at 55.5°C for 2 min and extension at 72°C for 2 min; and final extension at 72°for 10 min. PCR products (10µl) were digested without further purification with the restriction endonucleases CfoI, HaeIII and HinfI. PCR products and their restriction fragments were separated on agarose gels, stained with ethidium bromide and visualized under UV light. Sizes were determined by comparison against the DNA length standard (100 bp ladder) using LabWorks 3.0.2. software (UVP).

Analytical methods

Ethanol concentration in the culture filtrates were determined by gas chromatography (Shimadzu Co. Ltd., Kyoto, Japan) using capillary column diameter of 0.32 mm and column length of 30metres with

Flame ionization as a detector. Column oven temperature was 150 °C, detector temperature was 250°C and Injector port temperature was 200 °C. Helium was used as mobile phase with a flux of 1.5 ml/min and ethanol used an internal standard. The theoretical yield of ethanol was determined to be 0.51 g of ethanol per gram of glucose (2 mol of ethanol per 1 mol of glucose). Sugar concentration was determined by dinitrosalicylic acid (DNS) method.[19]

Statistical analysis

Data was analysed by one-way ANOVA and differences among treatment means were determined by Duncan's new multiple-range test.

Results and Discussion

Isolation of cellulase producing fungi

To isolated the fungi possesses cellulase and ethanol ability, the cellulase producing fungi were grown in cellulosic basal medium agar using carboxymethyl cellulose as a substrate. CMC is a cellulose derivative composed of glucose monomers that make up the cellulose backbone and carboxymethyl groups bound to hydroxyl groups of the glucose monomers. Glucose is a simpler sugar and is often used as an energy source by the microorganisms. Microorganisms use CMC as a carbon source by producing cellulase enzymes such as endoglucanase, exoglucanase and β-glucosidase that synergistically hydrolyse CMC to celloligosaccharide and subsequently to glucose. To observe the cellulase producing strain, plates were iodine stained representing an intensely yellow-brown colour of cellulose/iodine complex and halo zones were observed in the colored background after washing. The clear zone around the mold presented the cellulase producing capacity of the fungi.[20] In this study, 67 pure fungal isolated from different sources were tested for cellulase producing strains and 19 isolated were found from 67 that can grow in CBM agar and efficiency of cellulase producing strains was measured as a ratio of the clear zone and the colony width as shown in Table 1.

Identification of ethanol and cellulase producing fungi

Glucose is a subunit of cellulose connected through the β-(1,4)-glycosidic bond. Hydrolysis of cellulose by cellulase enzyme produced several sizes of oligosaccharide and glucose as products. In natural conditions, fungi hydrolyzed cellulose to glucose in which it can be energy source. Sugars can be used as energy by either metabolism (with oxygen) and anaerobic metabolism (without oxygen). Most fungi require oxygen for aerobic respiration and some fungi (such as brewer's yeast *Saccharomyces cerevisiae*) use alcohol fermentation when oxygen becomes limited by alcohol dehydrogenase activity. It has been discovered that some species of fungi produce alcohol dehydrogenase and produce ethanol such as *A. niger, A. terreus, A. oryzae, R. jabanicus, R. oryzae, Trichoderma harzianum, Fusarium oxysporum, Peniophora cinera, Tremetes hirsute, Fomitopsis palustris* and *Neolentinus lepideus.*[7-14,21]

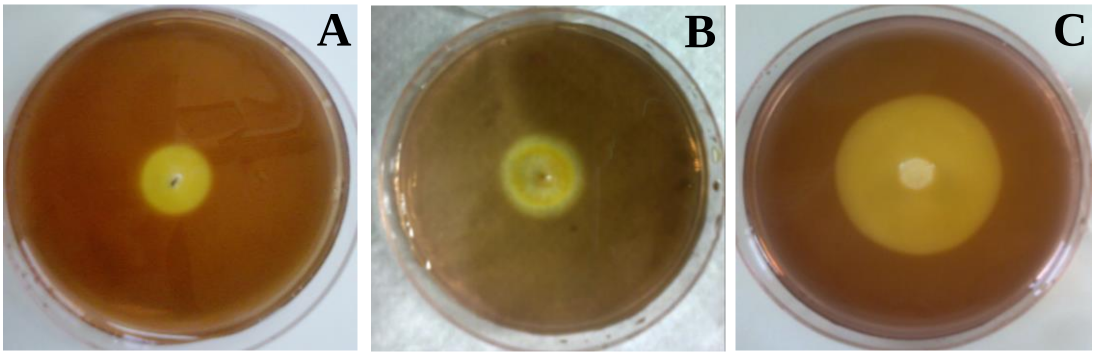

Figure 1 Cellulase producing capability of isolated F08 (A), F09 (B) and M03 (C) indicated by the clear zone around the fungal colony in CMC agar.

Table 1 Statistical characteristics of cellulase production activity by halo clear zone around the colony in CMC agar plate and ethanol production.

Isolated	clear zone diameter* (cm)	colony diameter* (cm)	clear zone/colony diameter ratio*	Ethanol production* (% theoretical yield)
F01	1.60 ± 0.12a	1.70 ± 0.02a	0.94 ± 0.10a	-
F02	4.75 ± 0.04b	4.85 ± 0.11b	0.98 ± 0.07a	-
F03	3.23 ± 0.16c	3.06 ± 0.06c	1.06 ± 0.02a	19.40 ± 1.14a
F04	5.35 ± 0.11d	5.35 ± 0.14d	1.00 ± 0.06a	-
F05	5.36 ± 0.06d	5.36 ± 0.17d	1.00 ± 0.02a	-
F06	2.30 ± 0.14e	2.10 ± 0.10e	1.10 ± 0.04a	-
F07	3.20 ± 0.11c	3.13 ± 0.16c	1.02 ± 0.06a	27.60 ± 1.28b
F08	2.10 ± 0.16e	1.60 ± 0.07a	1.31 ± 0.02b	62.25 ± 3.27c
F09	1.70 ± 0.11a	1.50 ± 0.14a	1.13 ± 0.06a	87.10 ± 2.32d
F10	2.25 ± 0.10e	2.20 ± 0.08e	1.02 ± 0.02a	-
F11	5.30 ± 0.07d	5.20 ± 0.12d	1.02 ± 0.04a	27.62 ± 1.44b
M01	1.70 ± 0.04a	1.20 ± 0.06f	1.42 ± 0.07b	8.97 ± 1.22e
M02	3.30 ± 0.05c	2.40 ± 0.17e	1.38 ± 0.06b	-
M03	6.80 ± 0.14f	6.63 ± 0.14g	1.03 ± 0.02a	75.41 ± 3.61f
M04	3.00 ± 0.12c	2.35 ± 0.16e	1.28 ± 0.04c	-
M05	2.27 ± 0.11e	1.20 ± 0.05f	1.89 ± 0.10d	14.87 ± 2.43g
M06	2.76 ± 0.14g	2.17 ± 0.10e	1.27 ± 0.12c	-
M07	2.15 ± 0.16e	1.50 ± 0.14	1.43 ± 0.04b	-
M08	3.66 ± 0.07h	2.03 ± 0.12	1.80 ± 0.06d	-

*Each value is expressed as mean ± SE (n = 3). Means with different small letters within a column are significantly different (P < 0.05).

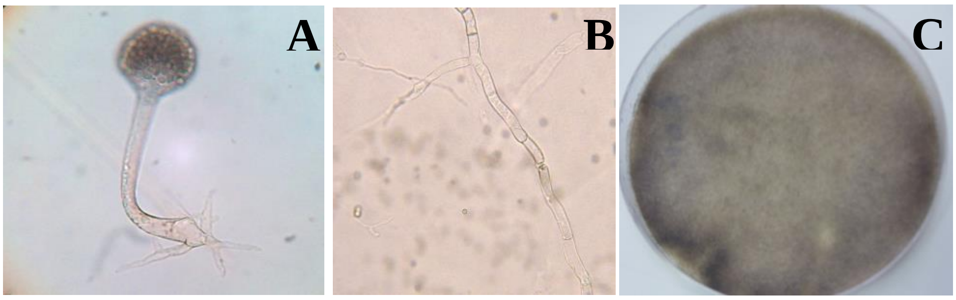

Figure 2 Microscopic morphology and colony observation of F08. (A) and (B) are microscopic morphological tests that show asexual spores called sporangium and septate hyphae. (C) is colony of F08 on PDA as cotton likej mycelium with grayish black sporangia on the top.

Table 2 Sequences producing significant alignments using BLAST–ITS1 Query length 1157 (http://ncbi.nlm.nih.gov.) of isolate F08.

blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus oryzae strain NSK internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA	1349	2197	84%	0.0	94%	JN381021.2
Aspergillus oryzae strain FH4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA	1157	1772	84%	0.0	94%	EU409806.1
Aspergillus flavus internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and ir	1151	1424	61%	0.0	96%	JQ763433.1
Aspergillus sp. AP02 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8	1146	1146	75%	0.0	90%	HQ219672.1
Aspergillus nomius strain SGE19 internal transcribed spacer 1, partial sequence; 5.8S ribosomal R	1114	1843	84%	0.0	96%	JN709035.1
Aspergillus flavus isolate GGV_BT03 internal transcribed spacer 1, partial sequence; 5.8S ribosom	1098	1259	57%	0.0	97%	KC907367.1
Aspergillus flavus isolate UPM A8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal	1094	1094	57%	0.0	96%	GU172440.1
Aspergillus sp. PRIST10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1079	1079	75%	0.0	89%	JX204747.1
Aspergillus flavus strain M1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g	1038	1038	57%	0.0	95%	JX514875.1
Aspergillus flavus strain MSSRF-IS2 18S ribosomal RNA gene, partial sequence; internal transcrib	1009	1444	84%	0.0	99%	HQ010119.1
Aspergillus oryzae strain 13/6 18S ribosomal RNA gene, partial sequence; internal transcribed spa	1005	1440	84%	0.0	98%	KF154415.1

Table 3 Sequences producing significant alignments using BLAST–ITS4 Query length 575 (http://ncbi.nlm.nih.gov.) of isolate F08.

blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_223586240

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus oryzae strain SV/09-09 18S ribosomal RNA gene, partial sequence; internal transcribe	1002	1002	96%	0.0	99%	FJ654483.1
Aspergillus nomius strain SGE19 internal transcribed spacer 1, partial sequence; 5.8S ribosomal R	1000	1327	98%	0.0	98%	JN709035.1
Aspergillus flavus strain 36 18S ribosomal RNA gene, partial sequence; internal transcribed space	1000	1000	95%	0.0	99%	HM016884.1
Aspergillus oryzae genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete se	1000	1000	95%	0.0	99%	AB470911.1
Aspergillus flavus strain CICC 2219 18S ribosomal RNA gene, partial sequence; internal transcribe	998	998	95%	0.0	99%	EF121332.1
Aspergillus sp. CeR1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.	996	996	94%	0.0	99%	KF358717.1
Aspergillus sp. OXL4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.	996	996	94%	0.0	99%	KF358714.1
Aspergillus flavus strain SX15 18S ribosomal RNA gene, partial sequence; internal transcribed spa	996	996	95%	0.0	99%	KC329628.1
Aspergillus oryzae isolate MD21_17 18S ribosomal RNA gene, partial sequence; internal transcrib	996	996	96%	0.0	99%	JQ697553.1
Aspergillus sp. QHL15 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5	996	996	95%	0.0	99%	JQ860230.1

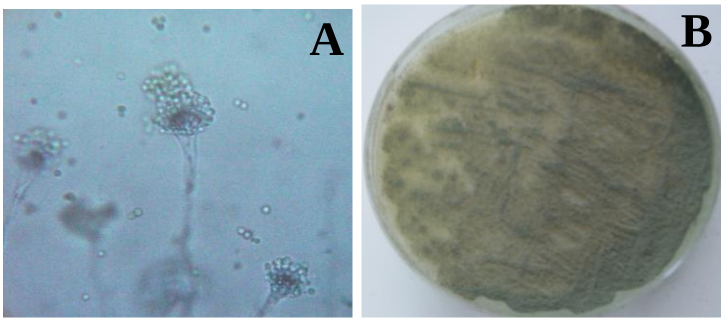

Figure 3 Microscopic morphology and colony observation of F09. (A) is the microscopic morphological test of F09 that showed asexual spores called conidiophore. (B) is the colony of F09 on PDA as green colour of conidiospore.

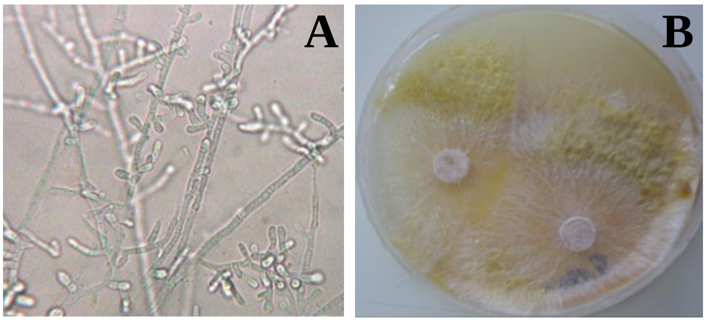

Figure 4 Microscopic morphology and colony observation of M03. (A) is the microscopic morphology that showed septate hyphae of M03 (B) is the colony of M03 on PDA which showed younger white and older yellow color cotton like mycelia

Table 4 Sequences producing significant alignments using BLAST–ITS4 Query length 949 (http://ncbi.nlm.nih.gov.) of isolate F09.

blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhizopus microsporus isolate F2-02 18S ribosomal RNA gene, partial sequence; internal transcrit	950	950	68%	0.0	93%	JN561253.1
Rhizopus microsporus strain P3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal R	950	950	68%	0.0	93%	FJ854338.1
Rhizopus microsporus var. rhizopodiformis strain R-49 18S ribosomal RNA gene, partial sequence	950	950	68%	0.0	93%	DQ641306.1
Rhizopus microsporus var. chinensis strain CBS 537.80 18S ribosomal RNA gene, partial sequen	948	948	68%	0.0	93%	AY243957.1
Rhizopus microsporus var. rhizopodiformis strain NRBC 32997 18S ribosomal RNA gene, partial s	948	948	68%	0.0	93%	AY803935.1
Rhizopus microsporus var. rhizopodiformis strain ATCC 200758 18S ribosomal RNA gene, partial	948	948	68%	0.0	93%	AY803934.1
Rhizopus microsporus var. chinensis strain CBS 631.82 18S ribosomal RNA gene, partial sequen	948	948	68%	0.0	93%	DQ119009.1
Rhizopus azygosporus strain CBS 357.93 18S ribosomal RNA gene, partial sequence; internal tra	948	948	68%	0.0	93%	DQ119008.1
Rhizopus microsporus strain 158 18S ribosomal RNA, partial sequence; internal transcribed spac	944	944	68%	0.0	93%	JX661044.1
Rhizopus microsporus strain JJ-A3 18S ribosomal RNA gene, partial sequence; internal transcribe	944	944	68%	0.0	93%	HQ285720.1
Rhizopus microsporus isolate F17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal	942	942	68%	0.0	93%	EF151442.1

Table 5 Sequences producing significant alignments using BLAST–ITS4 Query length 1052 (http://ncbi.nlm.nih.gov.) of isolate F09.

blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhizopus microsporus strain 158 18S ribosomal RNA, partial sequence; internal transcribed spac	1038	1038	62%	0.0	95%	JX661044.1
Rhizopus microsporus isolate F2-02 18S ribosomal RNA gene, partial sequence; internal transcrit	1037	1037	62%	0.0	95%	JN561253.1
Rhizopus microsporus isolate F17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal	1037	1037	62%	0.0	95%	EF151442.1
Rhizopus microsporus var. rhizopodiformis strain NRBC 32997 18S ribosomal RNA gene, partial s	1037	1037	62%	0.0	95%	AY803935.1
Rhizopus microsporus var. rhizopodiformis strain ATCC 200758 18S ribosomal RNA gene, partial	1037	1037	62%	0.0	95%	AY803934.1
Rhizopus microsporus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and compl	1037	1037	62%	0.0	95%	AB097381.1
Rhizopus microsporus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and compl	1037	1037	62%	0.0	95%	AB097380.1
Rhizopus microsporus strain D4-1 isolate 2 18S ribosomal RNA gene, partial sequence; internal t	1035	1035	62%	0.0	95%	GQ502279.1
Rhizopus microsporus var. chinensis strain CBS 631.82 18S ribosomal RNA gene, partial sequen	1035	1035	62%	0.0	95%	DQ119009.1
Rhizopus azygosporus strain CBS 357.93 18S ribosomal RNA gene, partial sequence; internal tra	1035	1035	62%	0.0	95%	DQ119008.1
Rhizopus microsporus strain JJ-A3 18S ribosomal RNA gene, partial sequence; internal transcribe	1033	1033	62%	0.0	95%	HQ285720.1

Table 6 Sequences producing significant alignments using BLAST–ITS1 Query length 1209 (http://ncbi.nlm.nih.gov.) of isolate M03.

blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Flavodon flavus isolate 10219 18S ribosomal RNA gene, partial sequence; internal transcribed spa	1116	1116	51%	0.0	99%	JN710543.1
Ceriporia sp. MA201 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8	1112	1112	50%	0.0	99%	HM012818.1
Basidiomycota sp. UD 100/08 18S ribosomal RNA gene, partial sequence; internal transcribed spa	1098	1098	50%	0.0	99%	GQ249873.1
Flavodon flavus isolate CrP11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN	1079	1079	49%	0.0	99%	KC920837.1
Trichaptum sp. E7082 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene,	1009	1009	50%	0.0	97%	AJ536658.1
Ceriporia lacerata isolate UD 98/08 internal transcribed spacer 1, partial sequence; 5.8S ribosoma	1000	1000	46%	0.0	99%	GQ249891.1
Trichaptum sp. E7083 ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial), specimen vouch	959	959	47%	0.0	97%	AJ536657.1
Trichaptum sp. E7074 ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial), specimen vouch	952	952	46%	0.0	97%	AJ536656.1
Irpex lacteus isolate UAS009 18S ribosomal RNA gene, partial sequence; internal transcribed spac	808	808	51%	0.0	90%	FJ158126.1
Ceriporia lacerata voucher Yuan5876 internal transcribed spacer 1, partial sequence; 5.8S ribosor	804	804	50%	0.0	91%	JX623924.1

Table 7 Sequences producing significant alignments using BLAST–ITS4 Query length 1309 (http://ncbi.nlm.nih.gov.) of isolate M03.

blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Flavodon flavus isolate 10219 18S ribosomal RNA gene, partial sequence; internal transcribed spa	1103	1103	46%	0.0	99%	JN710543.1
Ceriporia sp. MA201 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8	1101	1101	45%	0.0	99%	HM012818.1
Basidiomycota sp. UD 100/08 18S ribosomal RNA gene, partial sequence; internal transcribed spa	1090	1090	46%	0.0	99%	GQ249873.1
Flavodon flavus isolate CrP11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA	1033	1033	44%	0.0	99%	KC920837.1
Ceriporia lacerata isolate UD 98/08 internal transcribed spacer 1, partial sequence; 5.8S ribosoma	1009	1009	44%	0.0	98%	GQ249891.1
Trichaptum sp. E7082 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene,	994	994	46%	0.0	97%	AJ536658.1
Trichaptum sp. E7083 ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial), specimen vouch	950	950	44%	0.0	97%	AJ536657.1
Trichaptum sp. E7074 ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial), specimen vouch	948	948	43%	0.0	97%	AJ536656.1
Ceriporia lacerata strain KUC8715 18S ribosomal RNA gene, partial sequence; internal transcribe	791	791	45%	0.0	91%	HM008936.1
Ceriporia lacerata strain ATCC 42010 18S ribosomal RNA gene, partial sequence; internal transcri	789	789	46%	0.0	91%	KC881071.1

In this research, we tested 19 cellulase producing fungi for ethanol production using glucose as a substrate in oxygen limited conditions. The results showed the 8 isolated were able to produce ethanol in 7 days. Isolated F08, F09 and M03 could produce high theoretical ethanol yield of 62.25, 87.10 and 75.41, respectively (as shown in Table 1). The F08 and F09 were isolated from fermented food such as tempeh and fermented soybean, respectively. In this case the fungi had grown under facultatively anaerobic condition and they might have gotten energy from glucose to ethanol where pyruvate serves as the final electron acceptor.

Morphology was the basic method for fungal identification. However, biomolecular techniques are convenient and quick to classify fungi and more specifically provide better identification. In this study, F08, F09 and M03; which possessed cellulase and ethanol ability; were microscopically, colony morphology studied and the DNA sequence identified. The microscopic result of F08 showed septate mycelium, a root-like hyphae called rhizoid and a structure containing asexual spores such as sporangium that is important evidence of genus identification of genus *Rhizopus*. Besides, the colonies on PDA plate was grown as cotton like mycelium with grayish black sporangia on the top as shown in figure 2. Next, the partial 18S 5.8S and 28S rDNA and internal transcribed spacer (ITS) regions in ITS1 ITS2 were sequenced and the results showed that the sequence of F08 shared 100 % identity with *R. microsporus* (Table 2-3). In the case of F09, the microscopic test showed an asexual reproduction structure called conidiophore and the colony observed in PDA showed a colony with green colour of conidiospore on the top as shown in Figure 3. The DNA sequence was shown to have 94 % similarity with *A. oryzae* (Table 4-5). Last, M03 showed septate hyphae by microscopic test and the younger white and older yellow color cotton like mycelia in PDA plate as shown in Figure 4. The DNA sequence of M03 shared 99 % identity with *F. flavus* (Table 6-7).

Conclusions

In this study, fungi was isolated and tested for cellulase and ethanol production. There are 8 isolated that could produce cellulase and ethanol. Especcially, 3 isolated F08, F09 and M03 have the ability to produce ethanol higher than other isolated at 62.25, 87.10 and 75.41, respectively. The microscopic morphology, colony observation and DNA sequencing techniques showed that F08, F09 and M03 were similar to *A. oryzae*, *R. microsporus* and *F. flavus*, respectively. *A. oryzae* F08 is the best cellulase producing strain followed by *R. microsporus* F09 and *F. flavus* M03. Besides, *R. microsporus* F09 is the best for ethanol production followed by *F. flavus* M03 and *A. oryzae* F08. These fungal isolated have potential cellulase producing capability that could be applied to produce cellulosic ethanol.

References

1. Carere C, Sparling R, Cicek N, Levin D. Third generation biofuels via direct cellulose fermentation. Int J Mol Sci 2008;9(7):1342–60.
2. Amore A, Faraco V. Potential of fungi as category I consolidated bioprocessing organisms for cellulosic ethanol production. Renew Sust Energ Rev 2012;16(5):3286–301.
3. Rowell RM, Pettersen R, Han JS, Rowell JS, Tshabalala MA. Cell Wall Chemistry. In Handbook of Wood Chemistry and Wood Composites, Rowell RM. (Ed.)(Eds.), 2005. CRC PR ESS, Boca Raton London New York Washington, DC.
4. Meno V, Rao M. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. Prog Energ Combust 2012;38(4):522–50.
5. Sukumaran RK, Singhania RR, Pandey A. Microbial cellulases—production, applications and challenges. J Sci Ind Res 2005;64(11):832–44.
6. Xu Q, Singh A, Himmel ME. "Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. Curr Opin Biotech 2009;20(3):364–71.
7. Millati R, Edebo L, Taherzadeh MJ. Performance of rhizopus, rhizomucor, and mucor in ethanol production from glucose, xylose, and wood hydrolyzates. Enzyme Microb Technol 2005;36(2–3):294–300.
8. Skory CD, Freer SN, Bothast RJ. Screening for ethanol-producing filamentous fungi. Biotechnol Lett 1997;19(3):203–6.
9. Stevenson DM, Weimer PJ. Isolation and characterization of a *Trichoderma* strain capable of fermenting cellulose to ethanol. App Microbiol Biotechnol 2002;59(6): 721–6.
10. Xiros C, Christakopoulos P. Enhanced ethanol production from brewer's spent grain by a *Fusarium oxysporum*

consolidated system. Biotechnol Biofuels 2009;2:4.

11. Buaban B, Inoue H, Yano S, Tanapongpipat S, Ruanglek V, Champreda V, Pichyangkura R, Rengpipat S, Eurwilaichitr L. Bioethanol production from ball milled bagasse using an on-site produced fungal enzyme cocktail and xylose-fermenting *Pichia stipitis*. J Biosci Bioeng 2010;110(1):18–25.

12. Okamoto K, Imashiro K, Akizawa Y, Onimura A, Yoneda M, Nitta Y, Maekawa N, Yanase H. Production of ethanol by the white-rot basidiomycetes *Peniophora cinerea* and *Trametes suaveolens*. Biotechnol Lett 2010;32(7):909–13.

13. Okamoto K, Sugita Y, Nishikori N, Nitta Y, Yanase H. Characterization of two acidic □-glucosidases and ethanol fermentation in the brown rot fungus *Fomitopsis palustris*. Enzyme Microb Technol 2011;48(4–5):359–64.

14. Okamoto K, Kanawaku R, Masumoto M, Yanase H. Efficient xylose fermentation by the brown rot fungus *Neolentinus lepideus*. Enzyme Microb Technol 2012;50(2):96–100.

15. Pointing SB. Qualitative methods for determining lignocellulolytic enzyme production by tropical fungi. Fungal Diversity 1999;2):17–33.

16. Kasana RC, Salwan R, Dhar H, Dutt S, Gulati, A. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr Microbiol 2008;57(5):503–7.

17. Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int J Syst Bacteriol 1999;49(1):329-37.

18. White TJ, Bruns T, Lee S, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ. (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., New York, N.Y. 1990. P.315–322.

19. Miller G L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31(3):426–8.

20. Percival Zhang YH, Himmel ME, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 2006; 24(5):452–81.

21. Okamoto K, Nitta Y, Maekawa N, Yanase H. Direct ethanol production from starch, wheat bran and rice straw by the white rot fungus *Trametes hirsuta*. Enzyme Microb Technol 2011;48(3):273–7.