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Isolation and characterization of cellulolytic yeast belonging to *Moesziomyces* sp. from the gut of Grasshopper§

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메뚜기의 내장에서 분리한 Moesziomyces 속에 속하는 셀룰로오스 분해 효모의 분리 및 특성§

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An intensive interaction between yeasts and insects has highlighted their relevance for attraction to food and for the insect's development and behavior. Yeast associated in the gut of insects secretes cellulase which aided in the food digestion (cellulose degradation). Three strains of cellulose-degrading yeast were isolated from the gut of adult grasshoppers collected in Gyeonggi Province, South Korea. The strains ON22^T, G10^T, and G15^T, showed positive cellulolytic activity in the carboxymethyl cellulose (CMC)-plate assay. The phylogenetic tree based on sequence analysis of D1/D2 domains of the large subunit rRNA gene and the internal transcribed spacer (ITS) regions revealed that the strains ON22^T (100 and 98.4% sequence similarities in D1/D2 domains and ITS) and G10^T (99.8 and 99.5% in D1/D2 domain and ITS region) were most closely related to the species *Moesziomyces aphidis* JCM 10318^T; G15^T (100% in D1/D2 domains and ITS) belongs to the species *Moesziomyces antarcticus* JCM 10317^T, respectively. Morphology and biochemical test results are provided in the species description. Cellulase with its massive applicability has been used in various industrial processes such as biofuels like bioethanol productions. Therefore, this is the first report of the cellulolytic yeast strains ON22^T, G10^T, and G15^T related to the genus *Moesziomyces* in the family *Ustilaginaceae* (*Ustilaginales*), in Korea.

Keywords: *Moesziomyces* sp., D1/D2 domain, grasshopper, ITS, rRNA, yeast

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Cellulose is the most abundant and renewable organic material in the environment (Tomme *et al*., 1995). Cellulose consists of glucose monomers, which is the abundantly available source of sugar for fermentation and has a high potential for bioconversion to important byproducts such as ethanol. Inexpensive fuel can be supplied by selecting/using highly potential cellulose-degrading microorganism in the ethanol production. Cellulases are hydrolytic enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials (Lee *et al*., 2004). The best source of isolating these microbes is from the gut of insects, such as, termites, bookworm and cockroach thriving on cellulosic substances as their feed (Dillon *et al*., 2004). Therefore, the endosymbiotic microbes inside the insects are most capable of degrading cellulose. So far, *Chaetomium*, *Trichoderma*, *Fusarium*, *Myrothecium*, *Trichoderma. Penicillium* and *Aspergillus* are some of the reported fungal genera responsible for cellulosic biomass hydrolyzation (Romão-Dumaresq *et al*., 2016). Among them fungus *Trichoderma reesei* is a well-known cellulolytic organism and capable of producing a family of different cellulolytic enzymes, including endoglucanases, exo-cellobiohydrolases, and *β*-glucosidases (Ito *et al*., 2004). Endoglucanases can hydrolyze amorphous, soluble, and substituted celluloses randomly (Fujita *et al*., 2002).

The yeast species gained importance in the genetically modified production of enzymes. For example, in *Saccharomyces cerevisiae* cellulase and *β*-glucosidase genes have been over expressed to produce ethanol (Van Rensburg *et al*., 1998). Also yeast strains exhibiting various proteins on the cell surface have been developed by using genetic engineering techniques (Takahashi *et al*., 2000). Microbial enzymes have advantages over the animal and plant enzymes. They are economical and can be produced on large scale within the limited space and time. It can be easily extracted and purified. They are capable of producing a wide variety of enzymes in a wide range of environmental conditions, which shows genetic flexibility (Trevan, 1987). Thus, our preliminary study on isolating yeast species with cellulolytic activity will add importance in the field of enzyme technology for the cellulase production.

In this study, the yeast species ON22^{T}, G10^{T}, and G15^{T} belonging to the genus *Moesziomyces*, family *Ustilaginaceae* are capable of degrading cellulose in CMC-plate assay. At present this teleomorphic yeast contain five species *M. antarcticus*, *M. aphidis*, *M. bullatus*, *M. parantarcticus*, and *M. rugulosus* (Wang *et al*., 2015). Hence, it is the first report of cellulase activity in this teleomorphic *Moesziomyces* species.

Materials and Methods

Yeast isolation and maintenance

The yeast strains were isolated from grasshoppers collected from Onam-ri (37.6911° N, 127.2158° E), Gyeonggi Province, South Korea. The grasshoppers bodies were frozen separately at -20°C. During the experimental day of dissection, frozen grasshoppers taken out from the freezer and instantly rinsed with 70% ethanol. The exoskeleton of each grasshopper was then cut along the side and the digestive system was extracted. The whole guts were then stored in 1.5 ml microcentrifuge tubes, the tissue was dissected. The samples were cut into segments of 0.2~0.4 cm without surface-sterilization. Between 10 and 30 tissue segments were then evenly placed in 9 cm diameter Petri dishes containing yeast extract-malt extract (YM) agar plates (pH 3.7~5.0) (Difco) supplemented with 0.01% (w/v) chloramphenicol (Difco) and 0.02% (w/v) sodium propionate (Difco), then incubated at 25 ºC in the dark. The colonies appeared on the plates around a period of 3~5 days were transferred to YM broth for growth. The strains were then purified by repeated streaking of an isolated colony onto YM agar followed by incubation at 25ºC. Purified yeast strains were suspended in YM broth supplemented with 10% glycerol (v/v) and maintained at -80ºC.

Phenotypic characterization

Morphology, physiology, and biochemical studies were performed following the methods described by Kurtzman *et al.* (2011). Formation of pseudohyphae and true hyphae was investigated by cultivation on potato dextrose agar (PDA, Difco) in the slide culture at 25ºC for 2 weeks. The results were observed in light microscopy. Growth at various temperatures (15, 20, 25, 30, 32, 40, and 42°C) and NaCl concentrations was

determined by cultivating yeast in YM agar.

Cellulase activity in CMC plate assay

The cellulolytic degrading yeast strains were screened using CMC (Carboxymethyl cellulose) plate-based clearing assay as described by Johnsen and Krause (2014) with some modified with an antibiotic identification as given below. The Czapek-Dox-CMC medium containing KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_20$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), CMC (30 g/L), $NaNO_3$ (2 g/L), noble agar (20 g/L), and antibiotics (chloramphenicol) (0.02%) were poured into Petri dishes and was allowed to polymerize at room temperature overnight. The yeast strains were streaked onto the plates and were incubated at 27°C for 12~16 h and then hydrolysis zones were visualized by flooding of the plates/wells with Gram's iodine (2 g potassium iodide and 1 g iodine in 300 ml water) for 5 min followed by a rinse with deionized water. CMC-free plates were (non-substrate) were used as controls in all experiments. All the chemicals were purchased from Difco.

DNA sequencing and phylogenetic analysis

DNA was extracted from yeast and purified using the CTAB method (Cubero *et al*., 1998). The D1/D2 domain was amplified with the primers NL1 and NL4 as described by Kurtzman and Robnett (1998). Amplification of the D1/D2 domain was performed as follows: 94ºC for 6 min, followed by 40 cycles of 94ºC for 60 sec, 50ºC for 60 sec, and 72ºC for 60 sec; and a final extension at 72ºC for 5 min. The ITS (ITS1−5.8 S−ITS2) region of the rRNA gene was amplified with the primers ITS1F and ITS4 as described by White *et al*. (1990). Amplification of the ITS region was performed using the following conditions: 95ºC for 3 min, followed by 37 cycles of 94ºC for 30 sec, 52ºC for 30 sec, and 72ºC for 30 sec; and a final extension at 72ºC for 10 min. The related D1/D2 domain and ITS region sequences were obtained from GenBank database and edited with the BioEdit program (Hall *et al*., 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson *et al*., 1997) and the alignment was manually verified prior to the construction of phylogenetic trees. Phylogenetic trees were constructed using the MEGA 7 program (Kumar *et al*., 2016) by the neighbour-joining method (Saitou and Nei, 1987) and maximum likelihood (Fitch, 1971). During the phylogenetic analysis, evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980), and bootstrap values were calculated based on 1,000 replications (Felsenstein, 1985).

Results and Discussion

Morphology and physiology of yeast strains

The yeast strains ON22^T, G10^T, and G15^T are selected based on their cellulase activity. The cells of the strains ON22^T and G10^T were cylindrical or fusiform with 2.0−3.5 × 5.4−9.8 µm and 3.2−4.0 × 6.0−10.2 µm in size and are occurred either in single or pairs (Fig. 1A−C) after grown in YM broth at 20°C for 5 days. On YM agar, after 5 days the colonies are light brownish, butyrous and wrinkled. The margin is eroded. Budding is polar on short stalks. Growth was observed at 20, 25, 30, and 37°C. Growth was observed in 50% glucose. Cells are positive to diazonium blue B. Production of starch-like substances are absent. The type strain of *Moesziomyces aphidis* JCM 10318 showed similar pattern of results reported by Parahym *et al*.

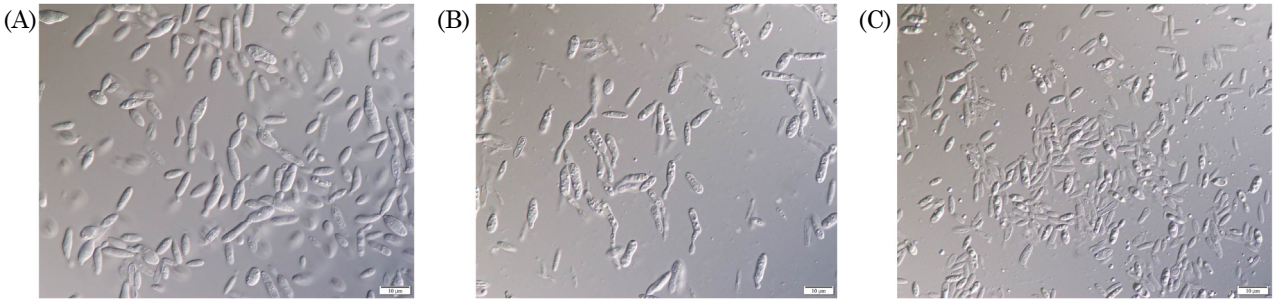

Fig. 1. Morphology of the cell on YM agar after 3 days at 20°C. (A) ON22^T (B) G10^T and (C) G15^T. Scale bar, 10 µm.

(2013).

Whereas, in YM broth at 20°C for 5 days. the strain G15^T showed cylindrical or fusiform cells with 1.3~2.0 × 4.5~5.6 μm in size and are occurred in singles. They showed polar budding. On YM agar, at 20°C after 5 days the colonies are cream color, smooth and glistening with fringed margin. Growth was observed at 20, 25, 30, and 37°C. Growth at 50% glucose was positive. Cells are negative to diazonium blue B. Starch-like substances are not produced. The type strain of *Moesziomyces antarcticus* JCM 10317^T also reported with similar pattern of results (Wei *et al*., 2005). Fermentation is negative for all the three strains. Pseudohyphae was absent. Based on the morphology and physiology characteristic the strains isolated in Korea are belonging to the species of *Moesziomyces.*

Cellulase activity in CMC plate assay

The strains ON22^T, G10^T, and G15^T showed clear zone area on the surface of the medium after staining with iodine solution (Supplementary data Fig. S1), which proved the degradation efficiency of the strains. The strain G1 is used as a negative control, which was a non-cellulolytic strain. The results were compared by using CMC free medium as well without yeast cells. These plates assay results provided advantage of observing the results visually using smaller sample volumes. The cellulolytic activity can be quantified by a variety of methods that have been summarized in papers (Zhang *et al*., 2009). The previous methods used crystalline cellulose, but the degradation rates were very slow, then most assays were adapted to use more easily degradable soluble cellulose derivatives like carboxymethylcellulose (CMC) (Yeoh *et al*., 1985). Therefore, preliminary screening for extracellular cellulase production by bacteria and fungi is often done on agar plates containing CMC as substrate (Dashtban *et al*., 2010). Following the results, the screening plate assay of the strains ON22^T, G10^T, and G15^T are selected as a candidate producer of cellulase, which showed hydrolytic activity on carboxymethyl cellulose (CMC).

Sequence analysis

The three strains ON22^T, G10^T, and G15^T recognized from morphological characterization were confirmed by the sequence analysis of D1/D2 domains and ITS regions. The sequence analysis revealed the assignment of the strains ON22^T, G10^T, and G15^T to the genus *Moesziomyces.* Therefore, strain ON22^T shared 100% and 98.4% sequence similarity (0% and 0.5% substitutions) in D1/D2 domain and ITS region with *Moesziomyces aphidis* JCM 10318^T; as well strain G10^T showed 99.8% and 99.5% (0% and 0.2% substitutions) in D1/D2 domain and ITS region with *Moesziomyces aphidis* JCM 10318^T. Strain G15^T shared 100% sequence similarities in both the D1/D2 domains and ITS with *Moesziomyces antarcticus* JCM 10317^T, respectively. Further the analysis proved the theories of Boekhout and Fell (1998) and Sugita *et al.* (1999) therefore, conspecific strains have a less than 1% nucleotide difference in their ITS regions. Recently, the taxonomic thresholds predicted to discriminate yeast species were 98.4% for ITS and 99.5% for D1/D2 domain of the LSU (Vu *et al*., 2016). The type strain of *M. antarcticus* previously described as *Pseudozyma antarctica* was isolated from lake sediment in Antarctica and was initially classified in the genus *Sporobolomyces*. However, morphologically it differed from other *Sporobolomyces* species by lacking ballistospores (Goto *et al*., 1969). More recently, the genus *Pseudozyma* is a polyphyletic anamorphic genus reclassified with species occurring in clusters together with teleomorphic species of *Moesziomyces.* (Wang *et al*., 2015).

The phylogenetic analysis using neighbor joining method on the combined sequence of D1/D2 domain of LSU rDNA and ITS regions showed the strain ON22^T and G10^T formed independent clade with *Moesziomyces aphidis* JCM 10318^T and G15^T formed independent clade with *Moesziomyces antarcticus* JCM 10317^T (Fig. 2). Thus, the phylogenetic trees added additional evidence for the strain's taxonomic positions.

Comparison of biochemical analysis between strains ON22^T, G10^T, and G15^T

The strains ON22^T, G10^T, and G15^T lack fermentation of carbon sources, which is the major property of this genus previously reported by Wei *et al.* (2005) and Wang *et al.* (2006). The major similarity in assimilation tests in between the species were given in the Table 1. The strain G15^T differed from ON22^T and G10^T by showing negative to Diazonium blue B. The strain G10^T differed from ON22^T by assimilating inulin, cellobiose, L-sorbose, D-ribose, xylitol and DL-lactate. Moreover, ON22^T and G10^T displays similar physiological charac-

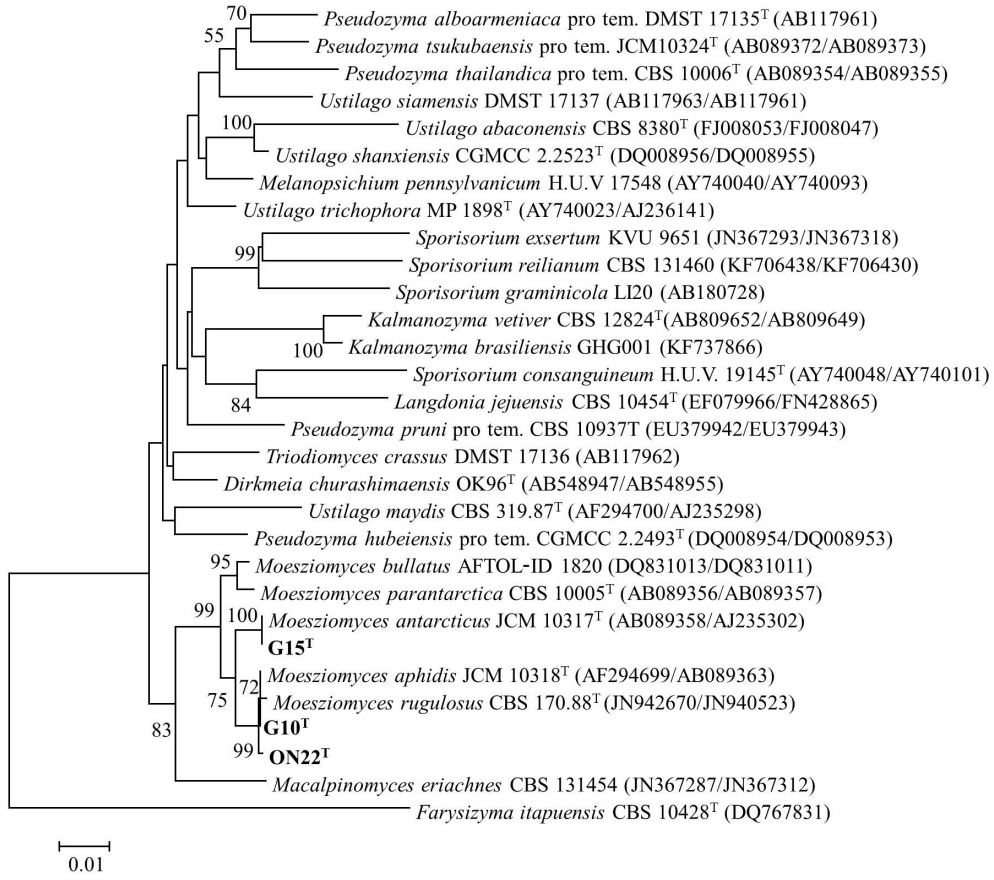

Fig. 2. Phylogenetic tree using the neighbour-joining and maximum likelihood methods based on the sequences of the ITS regions and LSU D1/D2 domain of strains ON22^T, G10^T, and G15^T with the members of closely related taxas. Bootstrap values based on 1,000 replications are shown at the branch nodes. *Farysizyma itapuensis* CBS 10428^T (DQ767831) is used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Table 1. Difference in physiological characteristics between the strains ON22^T, G10^T, and G15^T. All the data obtained in this study. Growth reactions: -, no growth; w, weak growth; +, strong growth.

Characteristic	1	2	3
Colony color	Light brown	Light brown	Cream
Diazonium blue B reaction	+	+	-
Assimilation			
α-Methyl-D-glucoside	+	+	-
Salicin	w	w	-
D-Gluconate	+	+	w
D-Ribose	+	w	-
Inulin	-	+	-

teristics in the assimilation with the identified species *M. alphidis* (Table 2). The strain G15^T assimilation results with *M. antarticus* were compared in Table 3. These strains have Q10 as their major quinone. Therefore, assimilation tests and other growth characteristics are important for the identification of *Moesziomyces* species.

Conclusion

Most of the insects that depend exclusively on nutritional restricted diets such as plant sap, and woody material, commonly possess obligate mutualistic endosymbionts (gut microbes) involved in the provision of essential nutrients or in the degradation of food materials. While studying and selecting yeasts for a specific function and classification, identification of the isolates is important. Isolation of cellulolytic yeast strains gained importance for their incredible usage in biofuel and bioethanol productions. Based on the theories on sequence analysis [Boekhout and Fell (1998), Sugita *et al.* (1999), and

Table 2. Physiological and biochemical tests capable of showing similarity between strains of ON22^T, G10^T and *Moesziomyces aphidis*

Characteristic	1	2	3
Glucose (50%)	+	+	+
Production of starch-like substance	-	-	-
Assimilation			
Cellobiose	-	+	-
Galactose	+	+	+
Glucose	+	+	+
Inulin	-	+	-
Lactose	+	+	+
Maltose	+	+	+
Melezitose	+	+	+
Melibiose	+	+	+
α-Methyl-D-glucoside	+	+	+
Raffinose	+		+
L-Rhamnose	+	+	+
D-Ribose	+	w	-
Salicin	W	w	+
Soluble Starch	-	-	+
L-Sorbose	W	+	+
Trehalose	+	+	+
Citrate	-	w	-
D-Mannitol	+	+	+
D-Gluconate	+	+	w
Glucanolactone	+	+	+
D-Glucosamine	+	+	+
D,L-Lactate	+	w	w
Galactitol	-	w	-
Erythritol	+	+	+
Ethanol	+	+	
D-Glucitol	+	+	+
Cadaverine	+	+	+
Potassium Nitrate	+	+	+
Sodium nitrate	-	-	-

Species: 1, ON22^T; 2, G10^T; 3, *Moesziomyces aphidis*
Growth reactions: -, no growth; w, weak growth; +, strong growth.
Moesziomyces aphidis - data obtained from Wang *et al*. (2006).

Table 3. Physiological and biochemical tests capable of showing similarity between strains of G15^T and *Moesziomyces antarticus*

Characteristic	1	2
Glucose (50%)	+	+
Production of starch-like substance	-	-
Assimilation		
Cellobiose	W	+
Galactose	+	+
L-Arabinose	+	+
D-Arabinose	+	w
Glucose	+	+
Inulin	-	-
Lactose	+	+
Maltose	+	+
Melezitose	+	+
Melibiose	+	+
α-Methyl-D-glucoside	-	+
L-Rhamnose	+	+
D-Ribose	-	+
Salicin	-	w
Soluble Starch	-	+
L-Sorbose	+	+
Sucrose	+	+
Trehalose	+	+
Citrate	-	w
D-Gluconate	W	s
Glucanolactone	+	+
D-Glucosamine	+	+
myo-inositol	W	+
Galactitol	-	-
Erythritol	+	s
Methanol	-	-
Ethanol	+	+
D-Glucitol	+	+
L-Lysine	+	ND
Mannitol	+	+
Ribitol	-	w
Cadaverine	+	+
Potassium Nitrate	+	ND
Sodium nitrate	-	ND

Species 1, G10^T; 2, *Moesziomyces antarticus*
Growth reactions: -, no growth; w, weak growth; +, strong growth; ND, not determined; s, slow positive.
Moesziomyces antarticus - data obtained from Wei *et al*. (2005).

Vu *et al*. (2016)] and polyphasic taxonomic approaches, the strains ON22^T and G10^T was identified as *Moesziomyces aphidis* and the strain G15^T as *Moesziomyces antarcticus*.

Nucleotide sequence accession number and culture deposition

The accession numbers of D1/D2 domain of ON22^T, G10^T, and G15^T were MK205287, MK203814, and MK212920. The accession number of ITS regions of ON22^T, G10^T, and G15^T

were MK212919, MN038047 and MK212921.

The yeast strains ON22^T, G10^T, and G15^T were deposited in Korean Collection for Type Cultures, Korea. The KCTC numbers for the strains were ON22^T (= KCTC 27804^T), G10^T (= KCTC 2780^T), and G15^T (= KCTC 27802^T).

적 요

효모와 곤충 간의 집중적인 상호 작용은 곤충의 먹이에 대한 유인과 발달 및 행동에 대한 관련성을 보였다. 곤충 내장에서 분리된 효모는 먹이의 소화를 돕는 셀룰라아제(셀룰로오스 분해)를 분비한다. 한국의 경기도에서 수집한 메뚜기의 장에서 셀룰로오스를 분해하는 효모 세 균주를 분리 하였다. 효모 균주의 cellulase 활성을 확인하기 위해, 카르복시 메틸 셀룰로즈(CMC)를 함유하는 배지로 플레이트상의 투명한 영역을 요오드 용액을 사용하여 관찰하였다. 효모 ON22^T, G10^T 및 G15^{T}균주는 CMC-플레이트 분석에서 양성 셀룰로오스 활성을 나타냈다. Large subunit rDNA 유전자와 Internal transcribed spacer (ITS) 영역의 D1/D2 영역의 서열 분석에 기초한 계통수를 통해 ON22^{T}와 G10^T 균주가 *Moesziomyces aphidis* JCM 10318 (D1/D2 영역에서 각 100%와 99.8%, ITS 영역에서 각 98.4% 및 99.5% 서열유사성)와 가장 가깝고 G15는 *Moesziomyces antarcticus* JCM 10317^T 종 (D1/D2 영역에서 100%, ITS에서 100% 서열 유사성)에 속한다는 것을 밝혔다. 셀룰라아제는 바이오 에탄올 생산과 같은 바이오 연료와 같은 다양한 산업 공정에서 사용되고 있다. 따라서, 셀룰로오스 분해 미생물의 분리 및 연구는 중요성을 갖게 되었다. 이 논문은 한국의 *Moesziomyces* 속의 셀룰로오스 분해 효모 균주인 ON22^T, G10^T, G15^{T}에 대한 첫 번째 보고이다.

Author's contributions

All authors equally contributed in this work.

Conflict of Interest

All authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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