Lignocellulosic Bioethanol-Producing *Fusarium* Isolates from the Eastern Anatolia

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Abstract— Bioethanol with its unique features has been considered as one of the best alternatives to liquid fossil fuels mainly used for transportation on roads and seas. On the other hand, the use of food-based raw materials in the production of first-generation bioethanol significantly limits its use as fuel. This undesired situation forced research efforts on development of second-generation production technologies where bioethanol is also produced from sustainable feedstock but, in this case, these feedstocks are not normally used for human or animal consumption. In this regard, lignocellulosic biomass as non-food raw material and ethanol-producing fungi as fermentative agents are commonly accepted as the most promising components for development of new biofuel sources. In this frame, the present work was conducted to isolate ethanol-producing fungal strains from decaying lignocellulosic materials and identify active ones by using molecular techniques. Decaying woody materials were collected from the forests in Erzurum and aseptically transferred to the laboratory. Purification of isolates was done according to general procedures. Ethanol production capability of each isolate was determined by the cultivation in modified BMC media and ethanol levels were determined by gas chromatography method. Molecular identification of the ethanol-producing isolates was done by using PCR with universal ITS primers, sequencing of amplicons and the BLAST analysis on NCBI database. According to the results, 4 active strains (MG35, MG43, MG58 and MG62) were determined and they produced bioethanol at 5.35 g/L, 1.36 g/L, 6.92 g/L and 2.48 g/L concentrations in modified BMC media for 5 days of the fermentation process, respectively. Finally, they were identified as *Fusarium solani* (MG35), *F. oxysporum* (MG43), *F. verticillioides* (MG58) and *Fusarium* sp. (MG62). As a consequence of these results, it can be concluded that 4 wild-type bioethanol-producing *Fusarium* strains isolated from local areas of Turkey and they may be useful for the development of new approaches for the fuel industry in the near future.

Index Terms— Bioconversion, Bioethanol, Biofuel, Fungi, *Fusarium*, Lignocellulose, Renewable Energy.

1 Introduction

The current economy of the world is predominantly dependent on fossil fuels such as petroleum, coal and natural gas. These raw materials are the most basic elements required by people for the heating, fuel for motor vehicles, electricity generation and production of various industrial products [1]. On the other hand, the reserves of these non-renewable raw materials are drastically decreasing as a result of the influence of increasing the world population [2]. In addition, the overuse of these raw materials frequently causes detrimental effects on living organisms and the environment. Such negative situations have become a driving force for researchers in the matter of seeking for sustainable and eco-friendly new energy sources.

Biofuels produced from biomass-based renewable raw materials are widely accepted as the most promising sustainable and eco-friendly energy sources for the near future [3], [4]. Among the recent biofuel technologies, the production of bioethanol is the most studied and the well-understood one, which the history of ethanol use goes back to the ancient times [5].

Bioethanol is considered as an energy source having the potential to be an alternative of petroleum products used for transportation in the fuel market. Moreover, the cost of bioethanol production is comparable to that of fossil fuels; which positively affects the bioethanol fuel studies and production rates. According to the literature, world bioethanol production was recorded at 31 billion liters in 2001 and 39 billion liters in 2006 and it is expected to reach over 100 billion liters in the near future [2], [6].

Brazil and the United States of America, which are responsible for 62% of the world's total bioethanol production, are the two most important countries in this area. In Brazil, most of the bioethanol used as fuel is produced from the sucrose obtained from sugar cane; and it is mainly produced from starch obtained from corn in the United States of America [7]. On the other hand, these raw materials, which are predominantly used in bioethanol production and are important food sources for people and other living things, have been the main source of many controversies and disagreements that have prevented bioethanol from having more widespread use around the world. Similarly, the use of various grains used as animal feed in the production of bioethanol is also economically disadvantageous [6].

In solving this problem, lignocellulosic wastes, which are not associated with the food of human beings and other living things and do not provide economic value on their own, have enormous potential [8]. According to the researches, it is assumed that 491 billion liters of bioethanol can be produced annually from the waste lignocellulosic material. This value can be described as about 16 times that of current bioethanol production around the world [2], [7].

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In this context, our present work was designed for the isolation and identification of wild-type fungal strains that natively live on decaying woody materials and have a potential for bioethanol production from the lignocellulosic biomass.

2 Materials and Methods

2.1 Collection of Decaying Woody Materials

Decaying woody materials were collected from the forests in Erzurum (Table 1) and aseptically transferred to the laboratory.

TABLE 1
SAMPLING LOCATIONS OF DECAYING WOODY MATERIALS

Locations	Coordinates	Sampling Counts
Pazaryolu	40°25′44′′N - 40°50′13′′E	10
Tortum	40°18′15′′N - 41°31′56′′E	8
Olur	40°49′4′′N - 42°4′26′′E	34

2.2 Isolation of Fungal Strains

Isolation of fungal strains was done by repeated inoculations on the potato dextrose agar [agar, 15 g/L; dextrose, 20 g/L; potato extract, 4 g/L] plates till pure colonies were seen.

2.3 Determination of Bioethanol Production Capabilities

Ethanol production capability of each isolate was determined by the cultivation in modified BMC media [$(NH_4)_2SO_4$, 7.5 g/L; KH_2PO_4, 3 g/L; $MgSO_4 \bullet 7H_2O$, 0.5 g/L; yeast extract, 2.5 g/L; mannose, 27 g/L; glucose, 9.7 g/L; xylose, 10 g/L; trace metal solution 6.7 mL/L; vitamin solution, 0.7 mL/L] and ethanol levels were determined by gas chromatography (FID and DBWAX capillary carbowax column).

2.4 Molecular Identification of the Bioethanol-Producing Isolates

DNA isolation studies of the active strains were performed with the method described by Karadayı in 2016 [9]. The ITS gene regions were amplified using polymerase chain reaction for molecular identification of the isolates. In this reaction, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used as forward and reverse primers, respectively. The reaction was carried out in a 30 µl reaction mixture containing 1.2 µl of dimethyl sulfoxide (DMSO), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 25 pmoles of forward primer and reverse primer, 50 ng DNA template and 5 U Taq DNA polymerase along with reaction buffer. The reaction was performed with an initial step at 95 °C for 2 min, and 36 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C, followed by a final 5 min extension step at 72 °C, then brought down to 4 °C. Then, in the electrophoresis stage, 7 µl of the PCR products was mixed with 3 µl of 6× gel loading buffer and loaded onto an agarose gel (1.5% w/v) supplemented with ethidium bromide. Electrophoresis was done in 0.5× TBE (Trise-Borate-EDTA) buffer at 90 V for 120 min. The DNA product was detected by using the Bio Doc Image Analysis System with Uvisoft analysis package (Cambridge, UK). The amplified gene products were sequenced by Macrogen Inc. (Netherlands). The nucleotide BLAST (Basic Local Alignment Search Tool) search program of NCBI was used to determine the nucleotide sequence homology.

3 Results

According to the results, 4 active strains (MG35, MG43, MG58 and MG62) were determined as bioethanol-producing fungal strains in modified BMC media for 5 days of the fermentation process (Table 2).

TABLE 2
ETHANOL YIELDS OF ACTIVE ISOLATES IN MBMC MEDIUM

Isolate Codes	Ethanol Yields (g/L)
MG35	5.35
MG43	1.36
MG58	6.92
MG62	2.48

These isolates were identified according to the sequence similarities of their ITS gene region. The detailed data was given as Table 3.

TABLE 3
TAXONOMIC AFFILIATION OF BIOETHANOL-PRODUCING STRAINS

Isolate Codes	The Closest Relative Species	Sequenced Nucleotide Counts
MG35	*Fusarium solani*	304
MG43	*Fusarium oxysporum*	355
MG58	*Fusarium verticillioides*	377
MG62	*Fusarium* sp.	396

4 Discussion

Energy plays an important role in raising living standards; economic and social development. Sustainable development is also possible with a continuous and high quality energy supply [10]. The rapid decline of fossil fuels (e.g. coal, oil and natural gas), which is the most important energy source today, and the environmental problems created by the resources, necessitated the search for alternative energy sources and the determination of new policies [1], [10], [11], [12], [13]. In this context, it is envisaged that fossil fuels will be replaced by renewable energy sources such as wind, sun, hydrogen and biofuels [13], [14], [15], [16], [17], [18], [19]. Among these alternative energy sources, biofuels attract attention because their production is easy, feasible and technologically oriented [14], [15], [16], [20], [21], [22]. Within biofuels, bioethanol, one of the renewable energy sources in liquid form used for motor vehicles, is increasingly used in our day [11], [14], [18], [22], [23], [24], [25].

However, the use of a major part of bioethanol produced from the feedstocks with high nutritional value (e.g. corn and starch) is significantly limited for living beings due to their being edible by people and relatively their high costs [6].

In this manner, lignocellulosic materials with their non-food nature are the most promising raw materials for the future [8]. Lignocellulose is a heterogeneous substance consisting of lignin, cellulose and hemicellulose. Although the six-carbon glucose-fermenting microorganisms that make celluloses are well-known, the number of identified microorganisms that can use five-carbon sugars such as xylose and arabinose in the hemicellulosic structure is very limited [20], [26]. To address this problem, limiting the production of bioethanol from lignocellulosic material, some researchers have succeeded in producing genetically modified *Saccharomyces cerevisiae*, *Zygosaccharomyces* and *Zymomonas mobilis* microorganism species [20]. Another genetically modified microorganism capable of using five carbon sugars for the production of bioethanol is *Pichia stipites* [20], [27].

However, an important problem encountered in the use of genetically modified microorganisms in the production of bioethanol from lignocellulosic materials is that these microorganisms can not survive for a long time and return to the wild characters at the beginning after several fermentation cycles [27].

In the ongoing studies for solving this problem; it is envisaged to use newly isolated microorganisms from natural sources that can produce bioethanol from both five-carbon and six-carbon sugars [20], [26], [27].

On the other hand, the high degree of tolerance of selected natural microorganisms against metabolic product inhibition also increases the chances that these organisms can be used in industrial scale production. Literature studies in this context show that the most important groups of microorganisms with high tolerance to ethanol inhibition are mold and yeast [27], [28].

In this contex, 4 ethanol-producing fungal strain (*Fusarium solani* MG35, *F. oxysporum* MG43, *F. verticillioides* MG58 and *Fusarium* sp. (MG62) were isolated from the decaying lignocellulosic woody materials collected from the nature.

These species obtained in the context of our current work have been the main research focus of many literature works with their unique enzyme production capacities. It is noteworthy, however, that some isolates, especially *Fusarium oxysporum*, can both produce lignolytic enzymes and ferment sugars with 5 and 6 carbon to ethanol [29]. These properties make it possible to efficiently utilize *Fusarium* species in methods such as SSF (simultaneous saccharification and fermentation) and CBP (consolidated bioprocessing), which are developed to combine the steps of hydrolysis and fermentation of the ethanol production process in a single reactor and thereby reduce both final product inhibition and product cost. *Fusarium* species have great potential for the development of CBP applications aiming to produce both cellulase production and fermentation in a single microorganism (cellulolytic and fermentative microorganism), especially during ethanol production from lignocellulosic materials. Hossain et al. [30] have indicated that *Fusarium oxysporum* can be used both in the hydrolysis step and in the fermentation step in ethanol production from agricultural wastes.

In addition, in current studies, *Fusarium* species have been used alone or in combination with other ethanol-producing strains such as *S. cerevisiae* or other lignolytic enzyme producers such as *Trichoderma reesei*, and these studies have led to significant improvements in the production of lignocellulosic bioethanol [29}, [31], [32].

4 Conclusion

In conclusion, it is clear that the ethanol-producing *Fusarium* species obtained from the present study could be used for ethanol production from lignocellulosic raw materials similarly to the previous studies. It is also thought that optimization studies can contribute to an increase in the efficiency and to the development of economically acceptable new technologies.

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