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From Waste Biomass to Cellulosic Ethanol by Separate Hydrolysis and Fermentation (SHF) with *Trichoderma viride*

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Abstract: Advanced biofuels can reduce fossil fuel use and the number of harmful compounds released during combustion, by reducing the use of fossil fuels. Lignocellulosic materials, especially waste biomass, are suitable substrates for the production of advanced biofuels. Among the most expensive steps in the production of ethanol is enzyme-based hydrolysis. Using microorganisms can reduce these costs. This study investigated the effectiveness of hydrolyzing three waste lignocellulosic biomass materials (barley straw, oak shavings, spent grains) into ethanol, after biological pretreatment with *Trichoderma viride* fungi. The number of fermentable sugars obtained from each substrate was subjected to preliminary study, and the correlation between the temperature and fungal activity in the decomposition of lignocellulosic materials was determined. Ethanol was produced by the separate hydrolysis and fermentation (SHF) method. It was found that not all lignocellulosic biomass is suitable to decomposition and hydrolysis in the presence of *T. viride*. Regardless of the process temperature, the average enzymatic activity of fungi (activity index) ranged from 1.25 to 1.31. 94 mL of distillate, with a 65% (*v*/*v*) ethanol concentration produced by the hydrolysis and fermentation of the sugars released from the barley straw.

Keywords: waste biomass; lignocellulose; biological treatment; *Trichoderma viride*; ethanol

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1. Introduction

The greenhouse effect, mainly caused by transport emissions, is driving the search for solutions to curb global temperature rise and environmental pollution. Conventional fuels can be substituted with biofuels. There are three main generations of biofuels, based on the substrates that are used for their production [1]. First-generation biofuels (1G) are produced from ingredients that are also intended for food and fodder; they are called conventional biofuels [2]. In Directive 2015/1513 of the European Parliament and the Council of the European Union of 9 September 2015, the share of first-generation biofuels was limited to 7%, in favor of advanced biofuels. In December 2018, the RED II Directive entered into force, according to which the target for 2030 is 14% biofuels used in transport, with first-generation biofuels no longer being included in renewable energy sources. Therefore, this favors the sector of second-generation (2G) biofuels, the production of which involves non-food energy plants, as well as waste lignocellulosic and third-generation (3G) biomass, which are produced from algae biomass. Compared to the production of first-generation fuels, the production of second-generation biofuels is more complex, but the use of residues, inter alia, from agriculture, forestry, industrial processes, and municipal management, allows for the production of biofuels with respect for the environment, under the conditions of sustainable development [3,4], without competing with food production [5].

Lignocellulosic materials require appropriate preparation; however, they are a very attractive, also for economic reasons, source of the sugars necessary for fermentation [6]. The ethanol obtained in this process, after dehydration, can be used as an additive to gasoline, but it can also be an independent fuel. The first step in the production of ethanol

is pretreatment, which separates lignin from cellulose and hemicellulose [7,8]. For this purpose, the options include physical methods (milling, microwave, mechanical extrusion, pyrolysis, pulse electric field), chemical methods (acid and alkali pretreatment, organosolv, ionic liquids, ozonolysis), physico-chemical methods (ammonia fiber expansion (AFEX), steam explosion, carbon dioxide explosion, liquid hot water (LHW), wet oxidation, SPORL treatment), or biological methods based on the enzymatic activity of bacteria and fungi [9], which have two types of exogenous enzyme systems. One of them is responsible for the degradation of polysaccharides, while the other is the ligninolytic system [10]. Fungi break down lignin more efficiently than bacteria [11]. Strains belonging to the genus *Trichoderma* have a significant role in these processes [12,13]. Biological pretreatment is an environmentally safe and low-energy method [14].

The next stage is enzymatic hydrolysis, incl. using cellulases and xylanases [15], the aim of which is to depolymerize polysaccharides isolated from biomass and release monomeric sugars [16]. These are five-carbon and six-carbon sugars, mainly glucose, used by ethanol-fermentation microorganisms [17]. Hydrolysis conditions and biomass types affect the conversion of lignocellulosic biomass to fermentable sugars [18]. In the hydrolysis process, not only commercial enzymes but also microbial cells can be used. The advantage of using microorganisms is the enzymatic activity closely adapted to the specific type of biomass [19], and the technology itself is classified as one of the future-oriented and promising methods favoring the increase in the level of saccharification [20]. The hydrolysis can be carried out separately from the fermentation process or simultaneously. Five basic methods are most often mentioned: separate hydrolysis and fermentation—SHF, simultaneous saccharification and fermentation—SSF [21], separate hydrolysis and co-fermentation—SHCF [22], simultaneous saccharification and co-fermentation—SSCF [23], and consolidated bioprocessing—CBP [24]. The hexose fermentation process can be carried out by bacteria and various types of mold fungi; however, on a commercial scale, mainly due to the efficiency of sugar conversion to ethanol, fast growth rate, efficient production of ethanol, and tolerance to its high concentration in the environment, *Saccharomyces cerevisiae* yeasts are mainly used [25].

After the fermentation process is completed, the solution is distilled and rectified, which not only increases the ethanol content but also purifies the biofuel [26]. The last stage of bioethanol production is its dehydration [27].

The economic feasibility of ethanol production from lignocellulosic feedstocks is currently a major challenge. The hydrolysis of lignocellulosic biomass applies commercial enzymes with cellulolytic and hemicellulolytic activity. The utilization of such enzymes increases production costs; therefore, it is necessary to search for simple and low-cost alternatives, especially for fuel utilization of the waste biomass. The aim of this study was to estimate the potential of *Trichoderma viride* to degrade different lignocellulosic materials, considering the optimum conditions for enzymes biosynthesis, and to estimate the efficiency of the enzymatic hydrolysis process based on ethanol yield. It was hypothesized that, under appropriate conditions, using *T. viride* for the pretreatment and hydrolysis of lignocellulosic substrates would enable simple sugars to be obtained, which are the ethanol precursors during the ethanol fermentation process. Due to the biological treatment of biomass, chemical compounds and a detoxification process are not required. Different species of *Trichoderma* have been used during the pretreatment of lignocellulosic feedstocks or as a source of the enzymes required for hydrolysis; however, to our best knowledge, this is the first time that whole *T. viride* cells have been used simultaneously for both processes.

2. Materials and Methods

2.1. Feedstock for Ethanol Fermentation

The materials that were used in the research include brewing spent grain (from the brewery), barley straw, and oak shavings (both from the university research center). The biomass was ground using a laboratory mill to a particle size of 1–2 mm and then stored at room temperature before pretreatment.

2.2. Biological Biomass Pretreatment

The possibility of obtaining fermentable sugars from biomass was determined during the enriched culture, which was carried out in Erlenmeyer conical flasks with a capacity of 250 mL. The flasks contained 100 mL of liquid medium with the following composition: (g/L) peptone 1.0 (BTL), $(NH_4)_2SO_4$ 1.4 (cz.d.a., Chempur), KH_2PO_4 2.0 (cz.d.a., Chempur), urea 0.3 (cz.d.a., Chempur), $CaCl_2$ 0.3 (cz.d.a., Chempur), $MgSO_4 \cdot 7H_2O$ 0.3 (cz.d.a., Chempur), (mg/L) $FeSO_4 \cdot 7H_2O$ 5.0 (cz.d.a., Chempur), $MnSO_4 \cdot H_2O$ 1.6 (cz.d.a., Chempur), $ZnSO_4 \cdot 7H_2O$ 1.4 (cz.d.a., Chempur), and $CoCl_2$ 2.0 (cz.d.a., Chempur). Next, 1 g of substrate was added to each of the flasks. The flasks with their contents were sterilized at a temperature of approx. 100 °C. Then, one disc (size 5 mm) cut from a 7-day *T. viride* culture was added to each flask (Figure 1). The flasks were set in a thermostat at 25 °C. The experiment was carried out for 28 days. The samples subjected to enzymatic hydrolysis were subjected to the qualitative test for monomer sugar content using Benedict's test. The solutions from each culture flask were taken into test tubes, Benedict's reagent was added, and the whole was mixed and placed for 5 min in a bath of boiling water. The color change and the presence of sediment after the samples had cooled down were used to determine the presence of sugars and their concentration. Analyses were performed in three replications after 7, 14, 21, and 28 days of culture.

Figure 1. *Trichoderma viride* colony on potato dextrose agar (PDA).

2.3. Production of Cellulase—Index Activity

The cellulolytic activity of *T. viride* was assessed on a culture medium with a 1% addition of carboxymethyl cellulose (CMC; Sigma-Aldrich, St. Louis, MI, USA). The experiment was prepared in accordance with the previously described methodology. Conical flasks were inoculated with *T. viride* culture discs and then incubated for 7 days at 25, 30, and 35 °C, respectively. After one week, 0.1 mL of the culture solution was withdrawn from each flask and transferred to a Petri dish with solidified medium. The Petri dishes were incubated at 25, 30, and 35 °C for the next 7 days, and then the activity index (I_A) was determined. A 1% aqueous solution of Congo red (Sigma-Aldrich St. Louis, MI, USA) was poured into the plates and left for 15 min. Then, the excess solution was poured off, and 1M NaCl solution was introduced to the dishes. Positive reaction was indicated by the formation of a clear zone (halo) around the colony. The decolorization was carried out over a period of 20 min. The cellulolytic activity index was determined by comparing the hydrolysis zone visible in the form of brightening around the colony (A) to the diameter of the colony (B), according to the following formula:

$$I_A = A/B$$

where A—diameter of the hydrolysis zone (mm), and B—diameter of the colony (mm).

2.4. Experimental Procedure of SHF Method

The lignocellulosic biomass was subjected to physical and mechanical pretreatment. In a laboratory mill, 200 g of the substrate was ground to 1–2 mm particle size. Then, thermal treatment was applied. The substrate was placed in 2 L of water, boiled for about 30 min,

cooled to 25 °C. and poured into a 3L polyethylene container. Ten discs were cut from a 7-day *T. viride* culture (5 mm in diameter). The experiment was prepared in three containers to obtain enough solution for the fermentation process. The containers were kept in a thermostat at 25 °C (optimum temperature for *T. viride* cellulolytic enzymes) for 21 days and then sterilized at approx. 100 °C. The contents of the containers were filtered through a mesh filter, separating the solid from the liquid fraction used for ethanol fermentation. After filtration, 3.4 L of solution were obtained; therefore, fermentation of monomeric sugars present in the hydrolysates was carried out in two fermentation containers with a capacity of 3 L, with the participation of *Saccharomyces cerevisiae* distillery yeast (Turbo Pure Yeast, MAXX Johnnie Cotton). The pH was set at 5.0 using 10 N sulfuric acid (cz.d.a, Chempur). The containers were placed in a thermostat at 30 °C (optimum temperature for *S. cerevisiae* activity) and incubated for 14 days. The wort was distilled at a temperature of 78.32 °C in an apparatus equipped with a heating mantle with a power regulator, a dephlegmator with a receiver, a cooler, and a thermometer that allowed for control of the temperature of the vapors. The ethanol content of the distillate was determined with an alcohol meter and is given as a percentage by volume (v/v). The experimental flow chart for all stages is shown in Figure 2.

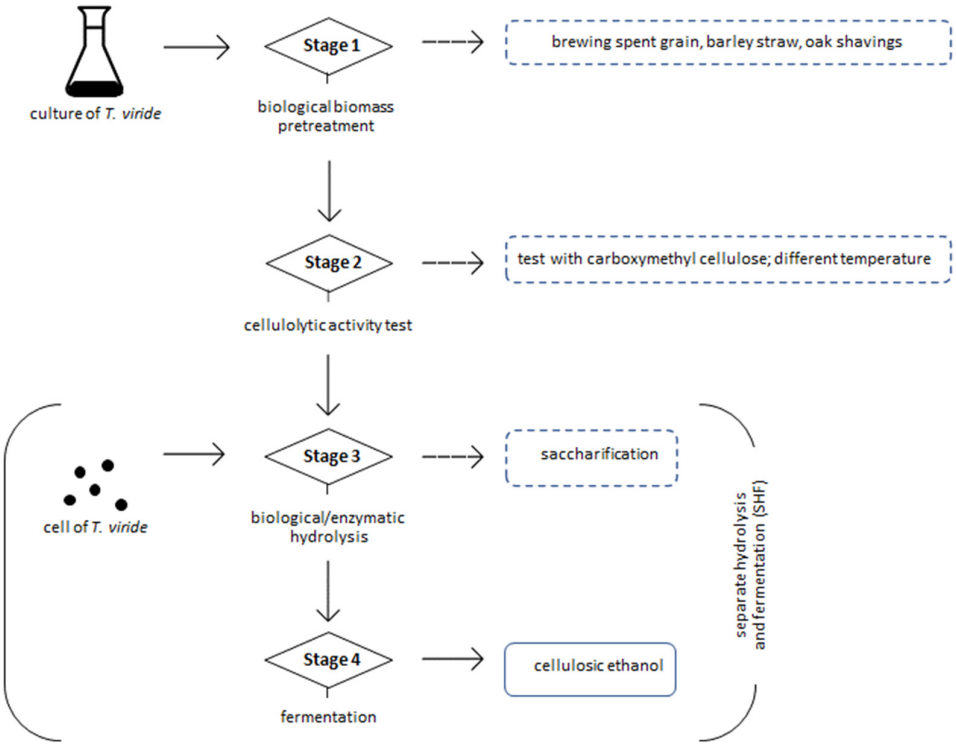

Figure 2. The experimental procedure flowchart.

2.5. Statistical Analysis

The results concerning the cellulolytic activity of the *T. viride* strain were analyzed statistically. Software for Windows (Statistica version 13.3; Dell Inc., Tulsa, OK, USA) and one-way analysis of variance were used. The standard deviation was also determined.

3. Results and Discussion

3.1. Susceptibility of Lignocellulosic Biomass to Biological Pretreatment

The production of second-generation biofuels is important both for environmental and economic reasons [28]. Lignocellulosic biomass, especially waste biomass, is not only a widely available but also a relatively cheap substrate for second-generation biofuels' production [29]. Its annual global energy potential is estimated at 100–270 EJ [30].

These substrates contain polysaccharides, which are the source of ethanol-fermentable monomeric sugars.

Different biomass types have different amounts of cellulose and hemicellulose, which may affect the efficiency of the biofuel production using them [31]. This composition may also be important in the processes of the biological treatment of lignocellulosic biomass, which is confirmed by the conducted research. Not all lignocellulose substrates could be used as a source of sugars for the alcohol fermentation obtained with the participation of enzymes secreted by *T. viride*. During the first 14 days of incubation, no reducing sugars were found in any of the cultures. After 21 days, a positive result was obtained in one of the samples of barley straw. After 28 days of incubation, the presence of fermentable sugars in the flasks containing oak sawdust was additionally noted. The lignin content in oak wood is 28% [32]. Lignin can bind to cellulolytic enzymes and reduce their effectiveness [33,34]. The efficiency of lignin degradation depends on pretreatment method. Singhvi and Kim [35], in a study of hydrogen production, treated raw maize cobs with peroxidase mimicking $CeFe_3O_4$ and noted lignin degradation at the level of 43.26%. Wi et al. [36] carried out a study on the conversion of lignocellulose to biofuel. The authors used hydrogen peroxide and acetic acid (HPAC) for pretreatment, removing up to 97.2% of lignin from the substrate. Biological methods are also effective. Su et al. [37] used the fungus *Myrothecium verrucaria* to biological treatment of corn stover and reported a 42.30% reduction in lignin content. Bari et al. [38] analyzed the effect of fungi on the degradation of beech wood and recorded a decrease in lignin content from 22.23% to 9.67% for *Pleurotus ostreatus* and 9.47% for *Trametes versicolor* after 120 days. Ghorbani et al. [39], during delignification of rice straw by Trichoderma viride, reported an increase in lignin-removal efficiency of up to 74% under optimal conditions. Ladeira Azar et al. [40], in their research on enzymatic hydrolysis of low-lignin bagasse from sugarcane, confirmed that even slight differences in lignin content can increase sugar yield. There is definitely less of it in barley straw. This straw contains 31–45% cellulose, 27–38% hemicellulose, and 14–19% lignin [41]. Earlier studies confirm the usefulness of this biomass for the production of cellulosic ethanol [19]. During simultaneous microbial saccharification and fermentation, ethanol with a concentration of 15% (*v*/*v*) was obtained.

The sugar content in the flasks was relatively low and did not exceed 0.1–0.3 g/100 mL. No reducing sugars were found in culture solutions in which the substrate was spent grains (BSG). The composition of this material is variable, which may make their valorization difficult [42]. In a study by Mishra et al. [43], BSG contained 24.5% cellulose, 9.8% hemicellulose, and 15.8% lignin. In the presented research, this substrate was not used by *T. viride* as a source of carbon and energy. Barley straw was selected for the next stage of the research.

3.2. Cellulolytic Activity of Trichoderma Viride

The temperature had no statistically significant effect on the extracellular *T. viride* hydrolase production. According to measurements of the hydrolysis zones (Figure 3), the activity index (IA) values varied from 1.18 to 1.38, as shown in Table 1.

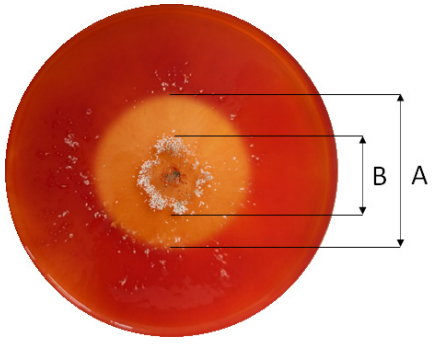

Figure 3. Scheme for determining the index activity (A—diameter of clear zone, B—diameter of fungal colony).

Table 1. Enzymatic activity index of *T. viride*.

Index Activity (I_A)	Temperature		
	25 °C	30 °C	35 °C
	1.31	1.33	1.25
	1.25	1.20	1.18
	1.38	1.23	1.38
Mean ± SD	1.31 ± 0.7	1.25 ± 0.7	1.27 ± 0.10

Bhattacharya et al. [44] investigated the relationship between the cellulase production efficiency of *T. viride* and temperature and found that the 30 °C is the optimum temperature for the production of these enzymes. This was also confirmed by the results of previous experiments on the evaluation of the potential enzymatic activity of *T. viride* for the decomposition of various types of straw [45]. For barley straw, the activity index increased from 1.45 at 25 °C to 1.82 at 30 °C. According to Malik et al. [46] an increase in temperature above 30 °C reduces the production of enzymes by *T. viride*.

3.3. Separate Hydrolysis and Fermentation

Under optimal conditions, it is possible to obtain the sufficient amount of reducing sugars necessary for the proper course of the next stage of the experiment—ethanol fermentation. Therefore, according to the results obtained, the straw material was hydrolyzed at a temperature of 25 °C. The solution after hydrolysis (3.4 L) was subjected to the ethanol fermentation process, and the result was 94 mL of distillate with an ethanol concentration of 65% (*v*/*v*). The available studies also confirmed the effectiveness of obtaining ethanol in the process of separate hydrolysis and fermentation. Mitra et al. [47] investigated ethanol production from lignocellulosic-starch biomass. Fermentation was carried out by SHF or SSF with batch feed. Substrates were pretreated with steam or dilute sulphuric acid (DSA). The highest ethanol yield (42.6 g/L) was obtained for separate hydrolysis and fermentation with DSA. Mendez et al. [48] carried out an SHF process for sugarcane bagasse and obtained a total ethanol concentration of 6.5% (*v*/*v*), including 33 g/L ethanol from the cellulose fraction and 18 g/L from the hemicellulose fraction. Different results were presented by Dahnum et al. [21]. When optimizing bioethanol production from an empty fruit bunch, they obtained 4.74% ethanol by the SHF process and 6.05% ethanol by the SSF process. This indicates that the efficiency of these processes also depends on other factors, including the type of substrate used during ethanol fermentation.

For the production of 2G ethanol, different types of waste biomass are used, e.g., rice; wheat and corn straw [49]; corn and cotton stalks and sugar cane processing waste [50]; and coffee pomace [51]. The amount of sugar obtained, precursors for the fermentation process, and high ethanol concentration depend significantly on the type of lignocellulosic biomass (Table 2).

Table 2. Example ethanol production from different types of lignocellulosic biomass.

Lignocellulosic Biomass	Pretreatment	Ethanol Yield	References
barley straw	biological	65% (*v*/*v*)	present study
barley straw	ionic liquids	18.5 g/L	Lara-Serrano et al. [52]
maize residues	-	10.22% (*v*/*v*)	Cutzu and Bardi [53]
wheat straw	deep eutectic solvent (des)	15.42 g/L	Xian et al. [54]
sugar cane pulp	dilute acid	15.5%	Saka and Afolabi [55]
wheat straw	steam explosion	21.3 g/L	Tomás-Pejó et al. [56]
corncob	alkaline	32.32 g/L	Boonchuay et al. [57]
sugarcane bagasse	acid	56.1 g/L	Unrean and Ketsub [58]
sugarcane bagasse	mixed H_2O_2 + NaOH	66 g/L	Irfan et al. [59]

Table 2. *Cont.*

Lignocellulosic Biomass	Pretreatment	Ethanol Yield	References
rapeseed straw	acid	39.9 g/L	López-Linares et al. [60]
pineapple leaves waste	hydrothermal	9 g/L	Saini et al. [61]
sugarcane leaves and tops	combination of oxidative alkali–peroxide	13.7 g/L	Dodo et al. [62]

The amount of substrate is also important. In a study by Paschos et al. [63], the concentration of ethanol increased from 39.55 to 46.62 g/L when the amount of barley straw biomass subjected to hydrolysis and the SSF process was increased from 15% to 20%. A similar relation was reported by Zhang et al. [64] for corn stover. With 15% solids, they obtained 24.7 g/L of ethanol, while 31.0 g/L, 39.3 g/L, and 40.6 g/L were obtained with 20%, 25%, and 30% solids, respectively. On the other hand, these authors observed a correlation between increasing solids content and decreasing ethanol yields, compared to theoretical values.

Conversion efficiency can be changed by using microorganisms other than yeast. Tivari et al. [65], from rice bran with *Bacillus cereus* strain McR-3, obtained a maximum of 10.50 ± 0.10%. The efficiency of bioethanol production depended on temperature and the appropriate pH. Da Silva et al. [66] used alkaline-pretreated Carnauba straw for ethanol production. During simultaneous saccharification and fermentation (SSF), they used two strains of *Saccharomyces cerevisie*; however, the highest yield above 7.5 g/L was recorded for the *Kluyveromyces marxianus* strain. Takano and Hoshino [67] carried out simultaneous saccharification and fermentation of rice straw. They used a mixture of commercial cellulases for hydrolysis and a mutant of *Mucor circinelloides* J (a xylose-fermenting fungus) for fermentation. Under aerobic conditions, 30.5 g/L ethanol was obtained after 36 h; however, the straw was also pretreated with alkaline. Waghmare et al. [68] used *Saccharomyces cerevisiae* (KCTC 7296) and *Pachysolen tannophilus* (MTCC 1077) to improve the yield from 2.113% and 1.095% for the single strains to 2.348% for the co-culture, respectively.

Our study demonstrated that biological methods could be used to produce a high concentration of ethanol from lignocellulosic material. These methods do not require chemicals, can be conducted at milder temperature conditions, and obtain strains that actively degrade and hydrolyze lignocellulosic substrates, which would enable large-scale ethanol production to be cost-effective [11].

4. Conclusions

The efficiency of the pretreatment method and the type of feedstock utilized both determine the amount of ethanol produced from lignocellulosic substrates. Pretreatment and hydrolysis with the participation of fungi do not require chemical and energy supply, as they are effective and environmentally friendly; however, not every waste biomass is a suitable material for obtaining fermentable sugars in biological processes. Among the lignocellulosic materials used in the research, the susceptibility to bioconversion with the participation of *T. viride* was demonstrated by oak chips and barley straw. The enzymatic activity of fungi did not depend on the temperature, and the determined values of the activity index ranged from 1.25 to 1.31. Ninety-four mL of distillate was obtained from barley straw, subjected to pretreatment and hydrolysis with the *T. viride* strain, and then fermented with the *Saccharomyces cerevisiae* strain. The ethanol concentration in the distillate was 65%.

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