The usage of laccase enzyme for degradation of ligno-cellulosic feedstock for bio-ethanol production

Introduction:

Due to a very fast transport and industry development, humanity faced a rapid increase of GHG (greenhouse gases) effect. GHG effect has a great impact on the climate. We can observe an increase of seas and oceans levels, which in turn result in formation of tornados or tsunamis. Due to such tremendous climate change, people all over the world got aware of necessity of hampering the GHG problem. A lot is being done in order to prevent it. There is a European directive on a consisting of 8 gases GHG basket. It sets a limit or a cap on the amount of pollutant that can be emitted by each factory. The firms that need to increase their CO2 emission buy permits from those who need fewer permits, it is called emissions trading. There are also fuel consumption limitations and CO2 emission limitations for new vehicles. However, the best solution for transport are bio-fuels – biodiesel and bio-ethanol. A lot of effort is done in order to produce more and more bio-fuels, which in the future are supposed to be the main if not the only transportation fuels. Except for ecological aspect, bio-fuels provide energy supply security, reduction of oil import and support agricultural industries. Up till now there are two main bio-fuels used for transportation: biodiesel and bio-ethanol. Biodiesel is produced from oil seeds. In Poland there are biodiesel blends: B20 (20% biodiesel and 80% ON or B80 (80% biodiesel and 20% ON), and biodiesel as the only fuel - B100 (100% biodiesel). Bio-ethanol is dehydrated ethanol, mainly used as a blend with petrol – E10 (10% bio-ethanol and 90% petrol). 10% is the amount of bio-ethanol that can be used in regular engines without any modifications. For the usage of blends with a higher percentage of bio-ethanol the engine adjustment is required. In the process of bio-diesel combustion the whole emitted CO2 is absorbed by plants in photosynthesis process and in the same way it doesn't influence the GHG effect. That is the main advantage of bio-ethanol usage as transportation fuel [4].

Due to its properties, bio-ethanol is considered to be the main fuel of the future. Combustion gases of bio-ethanol blend contain less CO, hydrocarbons, and sulphur compounds [17]. According to the research, the biggest amount of bio-ethanol counted for the land area, is produced from sugar-beets and sweet corn. In Poland bio-ethanol is mainly produced from sweet corn. However, bio-fuel obtained from corn competes with food producing industry for raw material and lands. Additionally, due to high prices of edible plants, lingo-cellulose feedstock is considered to be much better material for bio-ethanol production. Bio-ethanol can be classified as 1st and 2nd generation bio-ethanol, according to the way of production. 1st generation bio-ethanol is produced wit the usage of food crops as a raw material. In this process starch is the main source of sugars. 2nd generation bio-ethanol is produced from ligno-cellulosic feedstock, such as wood chips or energy crops, here, cellulose is the sugar source. Energy grasses like miscanthus, sorghum, sida hermaphrodita, or salix viminalis can be grown on poor lands or even wastelands. That makes these plants an ideal raw material for bio-ethanol production [19].
However, lignin content makes the bio-ethanol production much more difficult than the production process of 1st generation bio-ethanol. Lignin is a complex structure which hasn't been completely examined yet. It is very difficult to degrade, mainly due to its heterogeneous structure. Lignin is build of phenylopropane units connected with different types of bonds. Phenylopropanic ring may have two or three constituents. The role of lignin – non fibrous structure in the plant wall is to protect the plant. That is why lignin ideally blocks the access to cellulose, the main source of sugars. Lignin degradation and removal is the crucial step in bio-ethanol production process. Without lignin degradation it is impossible to get to cellulose and hydrolyze it into simple sugars. Lignin degradation or its partial degradation takes place during pretreatment processes. There are

many types of pretreatment processes that can be proceeded. These are mainly H2SO4 hydrolysis, NaOH hydrolysis, cutting, steaming, microwaves treatment, or enzymatic hydrolysis [19]. Ezymatic hydrolysis may be a good alternative for acid or base hydrolysis. However, enzyme research should be carried out and its affinity to different feedstocks should be examined. What's more, if the enzymatic hydrolysis would be efficient, its profitability must be examined in order to use it in the industry. There are many bacteria and fungi which are able to degrade lignin. However, a big amount of lignin types makes degradation process much more complicated than expected. In the following research laccase enzyme will be examined in order to be used as a potential enzyme used in hydrolysis during a pretreatment process in bio-ethanol production . Analyzed laccase is produced by Cerrena unicolor – Basidiomycetes class fungi. Delignifying properties of laccase are known. However, it is important and necessary to examine those ability on chosen raw material. The experiment concentrates on energy grasses which are probably the best raw material used for bio-ethanol production. Sorghum, sida and miscanthus will be the feedstock. The main point of the research is to check the possibility of growth of Cerrena unicolor on energy grasses and its ability to produce laccase. If it is possible to grow Cerrena unicolor on sorghum, miscanthus or sida, then the next step will be to examine the ability to degrade lignin in grasses with the usage of laccase from Cerrena unicolor and to check the degree of delignification. This kind of research is necessary to check the possibility of usage of laccase produced by this fungus in the pretreatment process of bio-ethanol production from energy grasses.

Materials and methods:

As a substrate energy crops representatives were used – miscanthus, sorghum and sida. The plants were mechanically pretreated. They were cut or milled. For the diversity of measurements and in order to get wider range of data, some of the material was only cut and some was cut and milled. There were samples of cut sida, cut sorghum and cut miscanthus and the samples with milled grasses of each type. In order to obtain better results and get a better overview, the mixtures of cut and milled material of one type was also done (cut and milled miscanthus and the sample of cut and milled sorghum). Plants of different types were not mixed in the samples. The experiment was carried out in round, flat bottom flasks, 500 ml of total volume each. 10g of raw material was inserted into each flask and 100ml of distilled water was added to each flask. In the samples with mixed feedstock, 5g of cut material and 5g of milled material was inserted. The samples were sterilized in autoclave in 121oC for 15 minutes. Cerrena unicolor mycelium grown on a Petri glasses (2 Petri glasses) was homogenized with 150 ml of physiological salt solution. After cooling the sterilized samples, each of them was inoculated with 5ml of the solution of Cerrena unicolor homogenized in physiological salt. 8 out of 10 flasks were cultivated in an incubator at 28oC in stationary conditions and 2 were cultivated in an incubator at 28oC in shaken flasks. Milled miscanthus and milled sorghum were cultivated in shaken flasks. These two flasks were chosen in order to compare the results in shaken conditions to the results obtained in stationary conditions. Regular laccase production on synthetic substratum is carried on in static conditions. The biggest laccase activity produced in regular Cerrena unicolor cultivation is obtained after 10 days of incubation. The first activity measurement of the laccase produced on energy grasses was performed after 12 days. The following measurements were done after 17, 21, 29 and 52 days. In order to measure laccase activity a sample of 2 ml was taken from each flask. That was performed in sterile conditions. Then the samples were centrifuged at 5000 rpm for 20 min. Laccase activity was measured according to Leonowicz i Grzywnowicz (1981). The laccase activity is expressed in . An error of one sample in three measurements was not bigger than 5%.

Results and discussion

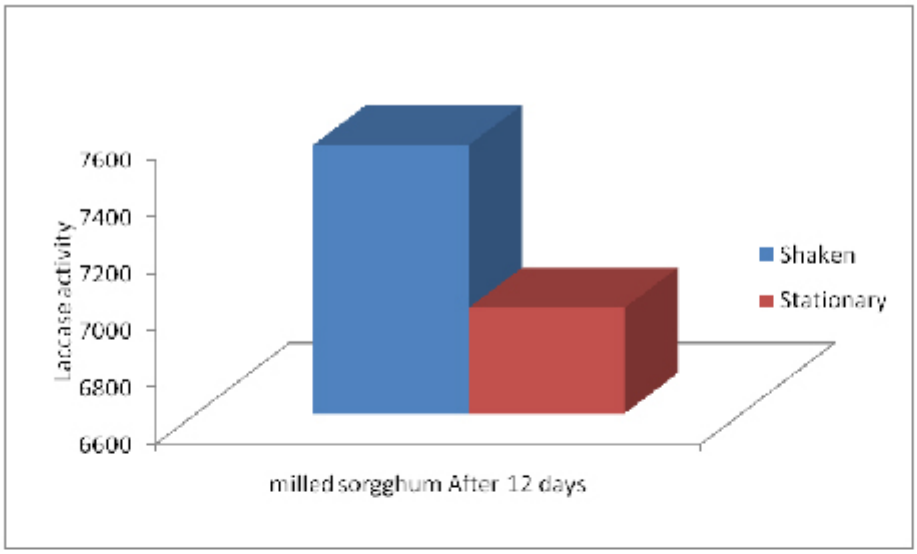
Table 1 presents the laccase activity measurements in 5 measurement points (after 12, 17, 21, 29 and 52 days). Plot 1 depicts those results. The aim of the experiment was to check the possibility of Cerrena unicolor growth on such energy grasses like: miscanthus, sida and sorghum, and to measure

laccase activity if the enzyme is produced. We could observe the growth of Cerrena unicolor on all the grasses. In each sample laccase was produced and its activity was measured. The highest activity in the whole time interval of the experiment, can be observed for laccase produced on sorghum. The best results were obtained for milled and shaken material. As far as miscanthus and sida are concerned, no significant difference can be observed, but still we can observe better results for milled material if both samples were in stationary conditions (sida) and better results for shaken conditions, although in the samples with cut miscanthus in stationary and shaken conditions no significant difference can be observed.

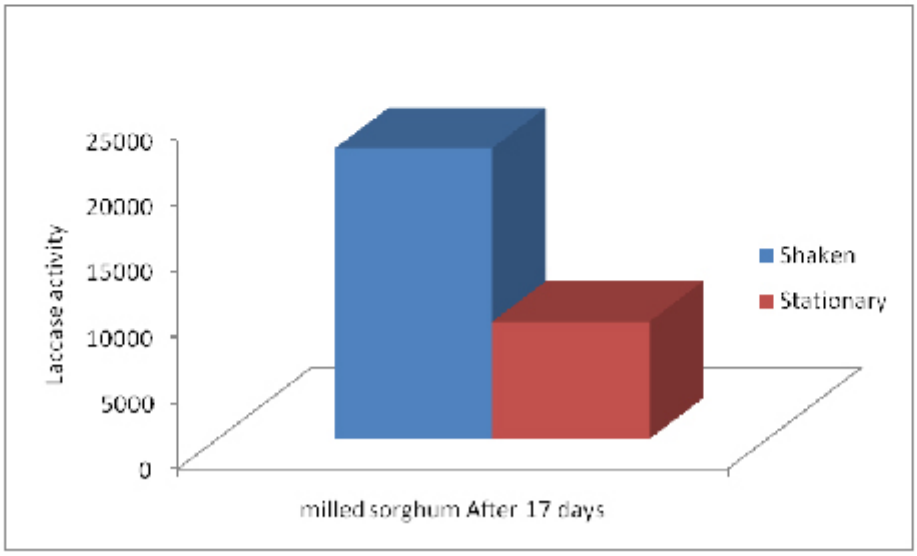
Table 1. Laccase activity measured for examined plants.

	after 12 days [nkat*l^{-1}]	**after 17 days** [nkat*l^{-1}]	**after 21 days** [nkat*l^{-1}]	**after 29 days** [nkat*l^{-1}]	**after 52 days** [nkat*l^{-1}]
milled sorghum Shaken	7543,887	22111,936	44716,160	31588,480	-
milled sorghum Stationary	6971,697	8912,464	5425,424	18296,704	-
milled miscanthus Stationary	784,584	425,624	799,968	989,704	628,180
milled miscanthus Shaken	389,728	415,368	1035,856	1661,472	466,648
cut sorghum Stationary	7490,873	9045,792	16491,648	16368,576	-
cut sorghum + milled sorghum Stationary	-	9497,056	16327,552	25537,440	-
cut miscanthus Stationary	-	899,414	1207,644	1346,100	1628,140
cut miscanthus + milled miscanthus Stationary	623,052	564,080	912,784	1633,268	1171,748
cut sida Stationary	-	1187,132	1758,904	1666,600	2292,216
milled sida Stationary	-	1869,156	3251, 152	2799,888	2997,316

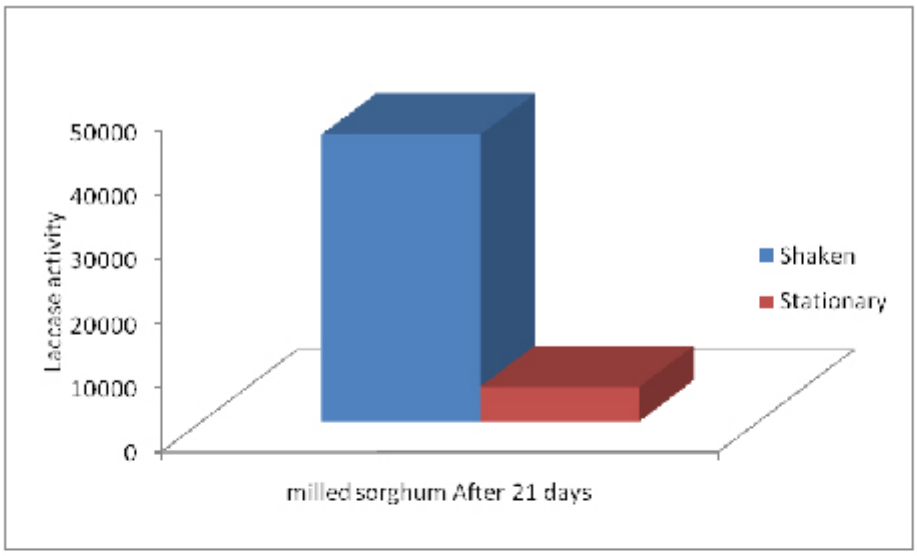
Below the best results are presented as a comparison of two different conditions - shaking and stationary, for one type of material – milled sorghum. A huge difference between laccase activity in shaken and stationary conditions can be observed. First we observe increase till the 21 day for shaken conditions and till 17 days for stationary conditions. Then we observe a slight decrease of laccase activity in shaken conditions and quite remarkable increase in stationary conditions. However, in the case of stationary sample a measurement error should be also taken into consideration, although it is unlikely to occur.



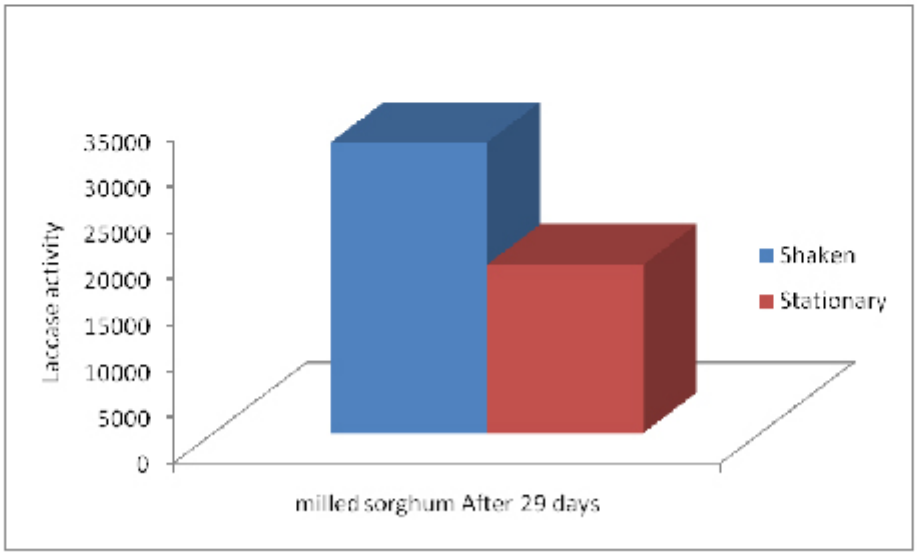
Plot 1. Milled sorghum. Laccase activity measurement after 12 days.



Plot 2. Milled sorghum. Laccase activity measurement after 17 days.



Plot 3. Milled sorghum. Laccase activity measurement after 21 days.



Plot 4. Milled sorghum. Laccase activity measurement after 29 days.

Conclusions:

The fact that we could observe a high laccase activity for nearly two months may be the proof of lignin decomposition pursued by Cerrena unicolor. However, more experiments must be carried out in order to check if this presumption is true and if yes the degree of delignification in each of examined plants must be determined. We could observe the growth of Cerrena unicolor on all types of raw material and in most cases we could observe the increase of laccase activity with the experiment time. In most cases the highest activity could be observed on the 21 or 29 day of the experiment.

The research shows the best fungi growth and the biggest laccase activity for sorghum – 45000 at the highest whereas miscanthus and sida around 2000 , 3000 for milled sida. Better results for milled material confirm the necessity of mechanical pretreatment. We also observe an impact of shake conditions on the process, in the shaken samples the laccase activity was usually bigger. In a few cases twice bigger or even more (milled sorghum after 21 days), depending on time when the measurement was done and on the plant type. The best results were obtained for sorghum which

may be the evidence of a higher kinship, the smaller amount of lignin content or the higher amount of sugars.

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