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Production of second generation bioethanol from straw during simultaneous microbial saccharification and fermentation

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Abstract: The aim of the study was to evaluate the biochemical possibilities of converting waste lignocellulosic biomass to second generation bioethanol. Three substrates were used in the research: barley straw, rye straw and triticale straw. In the first stage of the research bacterial strains capable of converting waste biomass to produce sugars used to produce energy-useful ethanol were selected. Of the eight strains isolated the three with the highest potential were selected on the basis of activity index value. The raw materials were subjected to enzymatic hydrolysis using the simultaneous saccharification and fermentation method (SSF process). Based on the conducted research, it was found that the examined waste biomass is suitable for the production of cellulosic bioethanol. As a result of distillation 10% and 15% (v/v) ethanol was obtained, depending on the strain and the type of raw material. It was demonstrated that the bacterial strain had a greater impact on the effectiveness of the process than the type of straw used.

Introduction

The intensive development of new technologies, industry, and, above all, transport has caused an increasing demand for energy, including fuel. Currently, these needs are still being met primarily by means of crude oil, coal and natural gas, which belong to non-renewable energy sources, and their resources are continually declining due to intensive use. In addition, their combustion adversely affects the environment.

An alternative to fossil fuels are biofuels produced from biomass (Czop and Kajda-Szcześniak 2013), and interest in them has been systematically growing for many years (Sørensen et al. 2010). The most popular of them are biodiesel and bioethanol, which are produced mainly from plants containing sucrose or starch (Lennartsson et al. 2014), ingredients that are also both a source of food for humans and animal feed. Economic and social as well as ethical problems resulting from such competition between biofuels and food (Bezergianni 2013) have contributed to the intensification of work on the possibility of replacing the currently used substrates. In the EU Directive 2015/1513 of 9 September 2015, apart from the need to limit the production of biofuels from cereals and sugar plants, there were also provisions regarding the need to support research in the field of the so-called advanced biofuels, including second generation ethanol. Waste lignocellulosic biomass may be a raw material used for its production, and the obtained biofuel is the so-called cellulosic ethanol. The unquestionable advantage of its production is not only the possibility of managing agricultural waste, waste from the wood industry and energy crops or solid municipal waste, e.g. waste paper (Lin and Tanaka 2006, Sanchez and Montoya 2013), but also partial energetic independence from the countries that currently make up the conventional fuel market.

Production of second generation ethanol is time-consuming and can be expensive, but it does not threaten food production (Shahare et al. 2017) and, above all, it has a positive impact on the environment. The use of bioethanol produced from cellulosic biomass reduces energy consumption and greenhouse gas emissions. Replacing traditional gasoline produced from petroleum with maize or sugar cane can reduce GHG emissions in the life cycle by 19–48% and 40–62%, respectively. Even greater benefits are achieved by using, as substrate, waste lignocellulosic biomass, e.g. corn straw, 90–103% (Wang et al. 2012).

The technology of bioethanol production from lignocellulosic biomass includes pre-treatment of the material, which is hindered by the presence of lignin, hydrolysis, i.e. the breakdown of cellulose into smaller sugar units, yeast

fermentation as well as product separation/distillation (Bajpai 2013, Shahare et al. 2017). The pretreatment is used to break down the material structure and increase its degradability (Kumar et al. 2009, Fig. 1). This process has a major impact on further stages of ethanol production and involves the use of physical, chemical, physicochemical and biological methods (Galbe and Zacchi 2013), in which living organisms, e.g. bacteria or fungi are applied.

Microorganisms produce and secretion of enzymes breakdown the plant cell wall to release sugar monomers (Woo et al. 2014) that can be used by yeast as substrates for ethanolic fermentation. The commonly used microorganisms can be isolated from the soil, living plants (Vats et al. 2013) or from the enrichment culture on lignocellulose as a sole carbon source (Maki et al. 2009). Their activity can be quantified by different methods e.g. on agar plates containing easily degradable soluble cellulose derivatives like carboxymethylcellulose (Johnsen and Krause 2014). Next step could be enzyme production and determination of their activity (Ghimire et al. 2016). The hydrolysis of lignocellulose materials occurs during biological treatment (Wagner et al. 2018).

The main advantages of biological methods are relatively low energy expenditure and mild process conditions (Świątek et al. 2011), they do not require any special reagents or apparatus. In addition, these methods are environmentally friendly and non-toxic (Robak and Balcerek 2017). Microorganisms allow the selective degradation of both the lignin and hemicellulose (Talebnia et al. 2010). Biological pretreatment is a promising technology for increasing the rate of enzymatic saccharification (Sindhu et al. 2016).

The ethanol fermentation process can be carried out by various strategies (Scully and Orlygsson 2015, Fig. 2) including separate hydrolysis and fermentation – SHF method (Dahnum et al. 2015) or simultaneous saccharification and fermentation – SSF method. The problem with using the latter method, however, is finding the same optimal temperature for both processes (Olofsson et al. 2008). The SSCF (simultaneous saccharification and cofermentation) method also carries out saccharification and fermentation of hexoses and pentoses (Koppram et al. 2013). It is also possible to combine pretreatment, hydrolysis and fermentation – CBP method (Świątek et al. 2011).

The aim of the study was isolation and screening of microorganisms active in degradation of lignocellulosic materials and the possibility of bioethanol production from straw after biological treatment.

Materials and methods

As part of the research, three laboratory experiments were carried out. The aim of the first of them was to obtain strains of microorganisms active in the processes of biological decomposition of lignocellulosic materials. In the subsequent experiment, the activity index of individual strains was evaluated, while the last experiment determined the efficiency of the enzymatic hydrolysis process with the participation of selected isolates based on the bioethanol amount produced in the alcohol fermentation process.

Substrates

The material for testing in these studies was rye straw, barley straw and triticale straw. It has been reported that triticale straw contains 36% cellulose, 25% hemicellulose, and 20% lignin (Shao and Lynd 2013), rye straw contains 33–35%

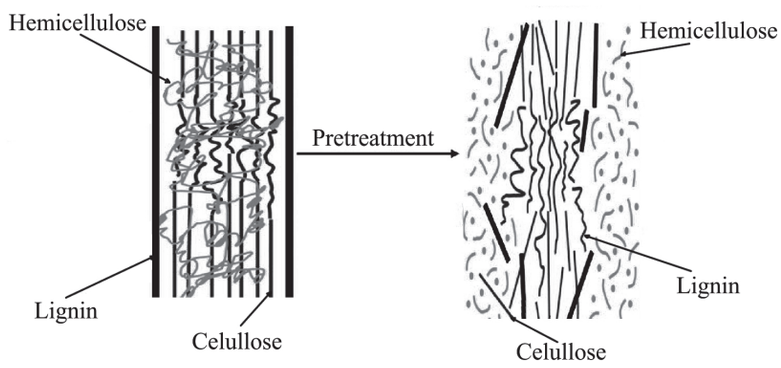

Fig. 1. Schematic of pretreatment process in the conversion of biomass (Kumar et al. 2009)

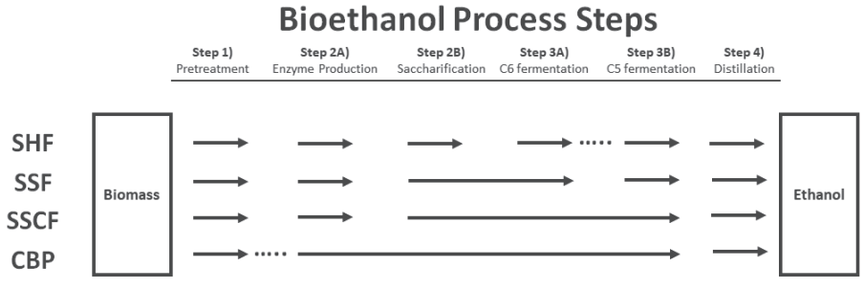

Fig. 2. Process steps of bioethanol production (Scully and Orlygsson 2015)

cellulose, 27–33% hemicellulose, 16–19% lignin (Sanchez 2009) and barley straw contains 31–45% cellulose, 27–38% hemicellulose, 14–19% lignin (Saini et al. 2015). The straw came from an agricultural holding located in the West Pomeranian province.

Isolation of cellulose degrading microorganisms and determination of their cellulolytic activity

The isolation of strains was carried out by the method of culture enriched with the Mandels medium, the composition of which is presented in Table 1. The experiment was carried out in 250 cm^3 conical flasks containing 100 cm^3 of liquid medium. 1 g of straw previously milled to the size of 1–2 mm was put into each flask, which in the medium was the only source of carbon promoting the growth of microorganisms with the properties sought. 1 g of soil was also introduced into each flask, which was to be the source of isolates. The soil (a fraction of clay sand) was collected from the Agricultural Test Station of the West Pomeranian University of Technology in Szczecin, located in Lipnik near Stargard in Poland (53°20′N, 14°58′E). Material was taken from a depth of 0–15 cm of the arable-humic horizon. Three experimental objects were created, denominated depending on the kind of lignocellulosic substrate used as TS (triticale straw), RS (rye straw) and BS (barley straw). Three replications were prepared for each variant.

The flasks were incubated for 7 days at 24±1°C on a rotary shaker at 150 rpm. After the incubation was completed, the first passage was made. From individual objects, a 10 cm^3 solution was withdrawn from the sediment to subsequent flasks containing Mandels liquid culture medium, this time with the addition of 1 g of carboxymethylcellulose (CMC). The flasks were returned to the shaker and incubated for 7 days. In the following weeks, two such passages were prepared. The last passage, in order to isolate the strains, was made on Mandels medium fixed with agar. After 7 days of incubation, to determine the activity of these isolates, they were punched out onto Mandels medium with a 1% addition of CMC and incubated for further 7 days at 30±1°C. Then, the surface of the medium in each Petri dish was flooded with 1% aqueous Congo red for 15 minutes. After removing the dye, 1M NaCl was introduced into the Petri dishes for 20 minutes (Hawrot-Paw and Izwikow 2016). The above mentioned dye was used to observe the zone of clearance produced by the activity of cellulase enzymes. The diameter of the zone of clearance indicates the ability of the bacteria to hydrolyze cellulose. The activity of the strains was assessed on the basis of colony diameter measurements and the diameter of the hydrolysis zone (Fig. 3). The clearness around the colony (hydrolysis zone) was measured in mm and the values were substituted to the formula below (Florencio et al. 2012):

$$\text{Activity index (AI)} = \frac{\text{diameter of the hydrolysis zone}}{\text{diameter of the colony}}$$

The strains for which the activity index had the highest value were considered to be potential producers of cellulases and selected for the next experiment. The material for subsequent experiment was propagated on agar slopes with Mandels medium.

Table 1. Composition of Mandels culture medium (Shah and Madamwar 2005)

Components	Amount [g×L^{-1}]
peptone	1.0
$(NH_4)_2SO_4$	1.4
KH_2PO_4	2.0
urea	0.3
$CaCl_2$	0.3
$MgSO_4x7H_2O$	0.3
Components	Amount [mg×L^{-1}]
$FeSO_4x7H_2O$	5.0
$MnSO_4xH_2O$	1.6
$ZnSO_4xH_2O$	1.4

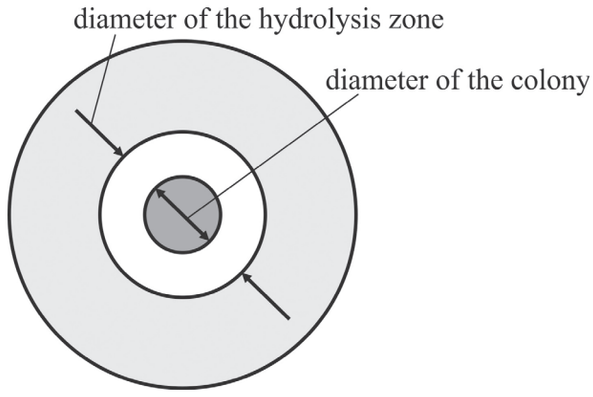

Fig. 3. Scheme of measurements carried out to determine the activity index

Experimental procedures of SSF process

In this experiment barley straw and rye straw were used. The material was subjected to physical-mechanical pre-treatment (grinding) and thermal treatment. A straw of 160 g was poured over with 2 dm^3 of water and boiled for ca. 40 minutes. The material was cooled to a temperature of approximately 30±1°C. Saccharification and fermentation process was performed in 3 dm^3 capacity containers holding 160 grams pretreated straw in 2 dm^3 suspension after thermal treatment. The pH medium was adjusted to 5.0. The mixture was supplemented with 25 cm^3 bacterial inoculum obtained after washing the culture on slopes with 0.85% NaCl, and 6 g of commercially dry active distillery yeasts of the *Saccharomyces cerevisiae* species (Turbo Pure Yeast, MAXX Johnnie Cotton). The containers were placed in a thermostat at the temperature of 30±1°C. The fermentative production of bioethanol was carried out in stationary anaerobic conditions. The ethanol content was measured after 14 day--fermentation. At the end of the fermentation, the contents of the containers were filtered to separate the liquid and solid fractions, and the obtained solution (1 dm^3) was distilled at c.a. 80°C maintained by the controller in a heating mantle. The distillation was carried out in an apparatus with a heating jacket (regulation Dz.U.03.138.1318). The ethanol content in the ethanol-water mixture was determined with an alcoholmeter in accordance with the regulation for analyzing agricultural ethyl alcohol (Dz.U.03.138.1318). The SSF process was carried out once for each substrate. Results were reported as volume percent (v/v percent).

Discussion of results

Twenty-seven strains were isolated in the first stage of the experiment and, for the next stage, the eight were selected which showed the most intense growth on culture medium with the addition of CMC, designated as BS 1.3, BS 2.3, BS 3.2, BS 3.3, RS 2.2, RS 3.3, TS 1.2, TS 2.2.

On the basis of activity index, there were significant differences in their potential in the field of biological decomposition of lignocellulosic materials. The average activity index values for the eight selected strains ranged from about 1 to almost 4. The lowest activity was found for strain BS 2.3, and the highest for strain BS 3.3 (Table 2). In a study conducted by Hawrot-Paw and Izwikow (2016) regarding the evaluation of cellulolytic activity of *Trichoderma viride* strain in the presence of three lignocellulosic substrates – wheat, barley and maize straw, under variable temperature conditions, the authors obtained the activity index of 1.82 for the same incubation temperature of barley straw (30°C) as in the presented work,. It can be concluded that the differences in activity also concern the type of microorganisms used in the biomass decomposition process.

On the basis of the activity index value, for the next stage, the simultaneous saccharification and fermentation process with *Saccharomyces cerevisiae*, three bacterial strains were selected, i.e. BS 1.3, BS 3.3 and TS 1.2. After fermentation and single-stage distillation 37 to 57 cm^3 of distillate was obtained. The ethanol content ranged from 10 to 15% (v/v) (Table 3). Biological pretreatment of lignocellulosic biomass increase yield of fermentable sugars (Su et al. 2018, Swain et al. 2018). Ali et al. (2012) also used two species of microorganisms, *Pichia stipites* and *Saccharomyces cerevisiae*, and obtained about 1.14% ethanol from wheat straw. In the presented studies, the concentration of 10% (v/v) was the minimum obtained during the experiments with triticale straw. Microorganisms have different lignocellulolytic activity which affects the efficiency of ethanol. Wilhelm et al. (2019) found high bacterial degradation of lignin, while fungi were more active in cellulose degradation. However, according to Souza (2013) the bacterial degradation of cellulolytic material is restricted to biomass containing low amounts of lignin.

The activity of isolated bacterial strains depended largely on the type of material undergoing the biochemical conversion process. When using barley straw, the concentration of ethanol

Table 2. Activity index for individual strains

Strain	Diameter of the hydrolysis zone [mm]	Diameter of the colony zone [mm]	Activity index (AI) value
BS 1.3	20	11	1.82
BS 2.3	44	43	1.02
BS 3.2	23	21	1.10
BS 3.3	82	22	3.73
RS 2.2	11	7	1.57
RS 3.3	22	20	1.10
TS 1.2	36	11	3.27
TS 2.2	27	16	1.69

Table 3. Amount of distillate and ethanol content in disti llate from SSF process

	Amount of distillate [cm^3]	Ethanol content in distillate [% v/v]
BS 1.3	37	10
BS 3.3	45	15
TS 1.2	57	10

was 50% higher and amounted to 15% (v/v). To get a high concentration of bioethanol, attention must be paid to the type of material used. In the studies by other authors, these values were lower and amounted to 1.81% for rape straw (Świątek et al. 2014), 10.22% for threshing maize residues (Cutzu and Bardi 2017), and 14.5% for sugar cane pulp (Saka and Afolabi 2015).

The lignocellulosic biomass needs to be pretreated because the original structure of the material prevents microorganisms from converting effectively. Physical methods, such as grinding or milling, reduce the size of the material, making it more available for microorganisms (Galbe and Zacchi 2013). Nikolić et al. (2011) in their research on the production of bioethanol from maize applied microwave and ultrasound treatment and obtained the maximum ethanol concentration of 11.15% and 13.40%, respectively. In the conducted tests, the biochemical processes were preceded by mechanical and thermal treatment. Based on the results, it can be concluded that the applied methods, including the use of enzymatic hydrolysis with the participation of isolated strains, more effectively reduce the negative impact on the environment and also reduce the consumption of fossil fuels.

Conclusions

Waste lignocellulosic biomass of agricultural origin can be a low cost substrate for the production of cellulosic bioethanol. Rye, barley and triticale straw was subjected to a simultaneous microbial saccharification and fermentation process, obtaining a distillate of 10 to 15% (v/v) ethanol.

The efficiency of the process depended more on the strain used for hydrolysis than on the type of waste lignocellulosic biomass. Microorganisms are producers of enzymes that decompose cellulose and hemicelluloses. Twenty-seven bacterial strains have been isolated in this study. The value of their cellulose degradation activity index ranged between 1.02 and 3.73. Some isolates showed promising results. The BS 1.3, BS 3.3 and TS 1.2 strains proved to be good candidates for lignocellulosic biomass hydrolysis process.

SSF process could be expected as a promising method for second generation bioethanol production from waste lignocellulosic biomass.

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Produkcja bioetanolu 2 generacji ze słomy podczas jednoczesnego mikrobiologicznego scukrzania i fermentacji

Streszczenie: Celem pracy była ocena możliwości biochemicznej konwersji odpadowej biomasy lignocelulozowej do bioetanolu 2 generacji.

W badaniach użyto trzech substratów: słomy jęczmiennej, żytniej oraz pszenżytniej. W pierwszym etapie badań zostały wyselekcjonowane szczepy bakterii zdolne do konwersji biomasy odpadowej z wytworzeniem cukrów wykorzystywanych do produkcji użytecznego energetycznie etanolu. Z wyizolowanych ośmiu szczepów, na podstawie wartości indeksu aktywności, wybrano trzy charakteryzujące się największym potencjałem.

Surowce poddano hydrolizie enzymatycznej stosując metodę jednoczesnego scukrzania i fermentacji (proces SSF).

Na podstawie przeprowadzonych badań stwierdzono, że badana biomasa odpadowa nadaje się do produkcji bioetanolu celulozowego. W wyniku destylacji, w zależności od szczepu i rodzaju surowca, uzyskano etanol o stężeniu 10% i 15% (v/v).

Wykazano, że większy wpływ na efektywność procesu miał szczep bakterii niż rodzaj użytego materiału słomiastego.