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Gleditsia triacanthos L. biomass as a substrate for bioethanol production

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Highlights
- *Gleditsia triacanthos* biomass is a suitable feedstock for bioethanol production.
- Pretreatment improve the accessibility of fermentable sugars in the biomass.
- Utilising *G. triacanthos* improving the circular bioeconomy and reducing waste.

Abstract: The increasing demand for renewable energy sources has intensified interest in exploring biomass for bioenergy production. Selection of suitable feedstock is significant for the economic viability and ecological impact. Lignocellulosic biomass, derived from non-food plants materials, has emerged as an attractive substrate with low cost and no competition to food crops. *Gleditsia triacanthos* offers a promising alternative due to its widespread availability, adaptability to diverse climates and soil conditions, fast growth, and high biomass yield. This study investigates the potential of *G. triacanthos* biomass as a viable substrate for bioethanol production through a combination of pretreatment method, microbiological hydrolysis, and fermentation processes. The biological pretreatment method to enhance cellulose accessibility was analysed. Fermentation trials were carried out using *Saccharomyces cerevisiae* to assess ethanol yield. Eleven strains with potential cellulolytic activity to the analysed biomass were isolated. The activity index for these strains ranged from 1.09 to 4.86. Results demonstrated that *G. triacanthos* biomass using selected strains could be converted to fermentable sugars. The highest amount of distillate (83.7 cm^3) was obtained after pretreatment and hydrolysis with the BS5 strain (36.3% v/v). These findings indicate that *G. triacanthos* biomass is a viable and sustainable resource for second-generation bioethanol production, contributing to the development of renewable energy technologies and the mitigation of fossil fuel dependency.

Keywords: bioethanol, biomass, biological pretreatment, fermentation, *Gleditsia triacanthos* L., lignocellulose, microbial hydrolysis

INTRODUCTION

Global energy consumption is growing continuously. A significant part of this demand is for energy required by transportation, i.e., fuels. Currently, it is mostly covered by fossil fuels (Holechek *et al.*, 2022). Biofuels have also been used in transport for several decades. They are used as pure fuel or as an additive to conventional fuels (Ye *et al.*, 2023; Rimkus *et al.*, 2024). Biofuels are produced from biomass. Depending on the type of biomass used for production, biofuels are divided into several generations. In the case of first-generation (1G) biofuels, the substrates for their production are components that can be used as food or as feed

(Mohr and Raman, 2013; Rulli *et al*., 2016). This results in fuel-food competition, so it is necessary to diversify available biomass sources and use alternative substrates for biofuel production. Substrates for second-generation (2G) biofuels include lignocellulosic waste (Srivastava *et al*., 2023) and non-food plants (Khater *et al*., 2024). Third generation (3G) biofuels are produced from algal biomass or components contained in their cells (Hawrot-Paw *et al*., 2021; Ratomski *et al*., 2023).

Second generation biofuels are primarily biodiesel derived from, among other things, used waste oils, including used vegetable oils (Suzihaque *et al*., 2022), and cellulosic ethanol (Liu *et al*., 2019). Unlike 1G fuel, 2G bioethanol production technology is more complicated, requiring more investment, especially for lignocellulosic materials. The main sources of waste lignocellulosic biomass are agricultural residues such as straw (Shukla *et al*., 2023), forest residues (Cheng *et al*., 2015), but also energy crop biomass (Kim *et al*., 2014). These substrates require appropriate processing. This is mainly pretreatment, the purpose of which is to change the structure and properties of the material to enable the hydrolysis process to yield the sugars required for the ethanol fermentation process. The conversion efficiency, as well as the ethanol yield, varies significantly according to the source and character of the lignocellulosic biomass. The two main polysaccharides that make up lignocellulosic biomass (BLC), cellulose and hemicellulose, bond strongly to lignin and form a complicated structure that is very strong and resistant to depolymerisation (Zabed *et al*., 2016). Pretreatment can include physical, chemical, and biological methods (Vasco-Correa, Ge and Li, 2016; Chen *et al*., 2017). It is advantageous to use a combination of methods. In general, the first stage of pretreatment is physical processes involving primarily milling, which does not change the chemical properties of the substrate, but significantly improves the efficiency of subsequent stages of bioethanol production (Arce and Kratky, 2022). Chemical processes use dillute acid, mild alkali, ozonolysis, organosolv, ionic liquids, and deep eutectic solvents, among others (Kumar and Sharma, 2017). A disadvantage of some chemical methods is the need for detoxification, as the compounds used during treatment can have a negative impact on the yeast used in the ethanol fermentation process (Jönsson, Alriksson and Nilvebrant, 2013). An interesting alternative solution is the use of biological methods. A number of microorganisms, mainly bacteria and fungi, have the ability to produce lignocellulolytic egzoenzymes to degrade lignocellulosic biomass (Wu *et al*., 2022; Sharma *et al*., 2023).

Lignocellulosic biomass consists mainly of cellulose, hemicellulose, and lignin (Chundawat *et al*. 2011, Hernández-Beltrán *et al*. 2019). For the biofuel production process, the cellulose content is the most important. For *Gleditsia triacanthos*, it is high, ranging from 43–45%, with a relatively low (about 13%) lignin content (Ibañez, Romero and Camargo, 2022).

MATERIALS AND METHODS

Gleditsia BIOMASS CHARACTERISTIC

In the study, the biomass of *Gleditsia triacanthos* L. was used. They are trees belonging to the family *Fabaceae* (=*Leguminosae*), a subfamily of *Caesalpinioideae* growing in natural sites in the eastern and central regions of the USA. Characteristic are the double-pinnate or pinnate leaves, thorns, and polygamous-double flowers with radial symmetry. Honey locust is characterised by a high degree of genetic variability in terms of, among other things, fruit-pod production and thorny and thornless traits (Schnabel and Hamrick, 1990; Smolik and Kubus, 2009). This tree, which is resistant to urban xeriscaping and air and soil pollution, has a very wide range of uses in various economic sectors, among others in green areas, sylvopastoral crops, wood technology, as a fuel material (Gold and Hanover, 1993). As substrate for bioethanol production, the pods, which are the fruits of this plant, were used (Photo 1). The biomass of *G. triacanthos* in the form of mature pods came from five approximately 100-year-old trees of this species growing on a roadside site in Szczecin (Waryńskiego Street). The trees reached heights of 15 to 18 m and trunk circumference of 178 cm to 198 cm (measured at 130 cm AGL).

Photo 1. Ripening pods of *Gleditsia* (phot.: *M. Kubus)*

EXPERIMENTAL SETUP

The study was carried out in three stages. In the first stage, microorganisms involved in the pretreatment and hydrolysis of lignocellulosic materials were screened. Then their enzymatic potential was evaluated. The activity of the strains was analysed by measuring the content of reducing sugars produced in the enriched culture. The final step was to evaluate the yield of bioethanol produced from plant material during alcoholic fermentation preceded by physical and biological pretreatment and enzymatic hydrolysis by isolated bacteria.

SCREENING OF MICROORGANISMS

The screening of strains was carried out using the enriched culture method. The experiment was conducted in conical Erlenmeyer flasks of 250 cm^3 capacity. The flasks contained 100 cm^3 of liquid Mandels medium (Shah and Madamwar, 2005). Into five flasks, after sterilising the medium at 121°C, 1 g each of plant middlings with a particle size on the order of 1 mm and 1 g each of soil, which was the source for obtaining isolates, were introduced. The flasks were incubated for 14 days at 28°C on a shaker at 150 rpm. At the end of incubation, 10 cm^3 of solution was taken from each flask into subsequent flasks containing liquid culture medium and 1 g of carboxymethylcellulose (CMC). The flasks were again placed in a shaker and incubated for another

7 days. Based on this scheme, two more such passages were prepared on the following days, and then culture was performed on solid medium. Petri cell-culture dishes with isolates were incubated at 28°C for 14 days.

CELLULOLYTIC ACTIVITY OF THE ISOLATES

The enzymatic activity of each isolate was determined in Petri dishes with solid medium containing 1% carboxymethylcellulose addition. Three replicates were prepared for each strain. After seven days of incubation at 28°C, a 1% aqueous solution of Congo Red was applied to the surface of the medium in the dishes. After 15 min, the dye was removed, and 1M NaCl was introduced into the dishes to visualise the clearances around the colonies indicative of enzymatic activity. The size of the hydrolysis zone measured in mm was used to calculate the activity index (*IA*), according to the equation given below (Florencio, Couri and Farinas, 2012):

$$IA = \frac{D_{hz}}{D_c} \quad (1)$$

where: *IA* = activity index, D_{hz} = diameter of the hydrolysis zone (mm), D_c = diameter of the colony (mm).

Selected strains with the highest activity were multiplied on agar slants to provide enough material for the next stage of the study.

DETERMINATION OF FERMENTABLE SUGARS

The 10 g of plant middlings were added to 250 cm^3 Erlenmayer conical flasks containing 100 cm^3 of the liquid medium described earlier. The flasks with the contents were sterilised for 20 min at 121°C, and then 5 cm^3 of bacterial suspension was introduced into each flask. The suspension was obtained after washing off the slants with a 7-day culture of selected bacterial strains. A sterile 0.85% NaCl solution was used to wash off the slants. The flasks were placed in a thermostat at 25°C, 30°C, or 35°C, respectively. The experiment was carried out for 28 days. The presence of reducing sugars was determined using the Benedict test. Benedict's reagent was added to samples of solutions taken from individual culture flasks; the whole mixture was carefully mixed, and then the tubes were placed in a boiling water bath for 15 min. The presence of sugars and their content was evaluated after the samples cooled based on the colour change of the solution and the possible presence of a precipitate. Analyses were conducted in three replicates at the beginning of the experiment and then after 7, 14, 21, and 28 days of cultivation.

PRODUCTION OF CELLULOSIC ETHANOL

The plant material was air-dried and then ground with a quern mill to particles of about 1 mm in size. The resulting 500 g middlings were thermally processed. The substrate was introduced in small portions into a steel tank containing 2 dm^3 of water at 80°C. The set temperature was maintained for another 30 min and then reduced to a temperature of about 30°C. The material was transferred to 3 dm^3 fermentation containers. Bacterial inoculates (30 cm^3) were introduced into individual containers, which were obtained after washing off the agar slants. As a control (object C), containers that contained substrate after mechanical and thermal pretreatment and without inoculations were prepared. Three replicates were prepared for each variant of the experiment.

Bioethanol production was carried out by separate hydrolysis and fermentation (SHF). Containers with bacterial inoculations and control containers were incubated at 35°C with stirring. After 14 days, the contents of the containers were sterilised at 121°C for 20 min, and after cooling, 5 g each of *Saccharomyces cerevisiae* yeast was added to the individual containers. The containers were tightly closed, providing anaerobic conditions for ethanol fermentation, and the incubation temperature was set at 30°C. After seven days, the contents of the containers were filtered, and the obtained solutions were distilled using a deflegmator at 78.3 ±0.5°C. The ethanol content of the distillate was determined using an alcohol meter, with temperature correction for measured values. The concentration of ethanol in the sample was presented as a percentage by volume (% v/v).

STATISTICAL ANALYSIS OF RESULTS

The results of enzymatic activity of bacterial isolates and ethanol production efficiency were statistically analysed. A one-way analysis of variance was applied using the computer program Statistica ver. 13.3. The significance of the results using a post hoc test according to Tukey was evaluated at $p \leq 0.05$.

RESULTS AND DISCUSSION

ENZYMATIC ACTIVITY OF THE STRAINS

Seventeen bacterial strains were isolated from preliminary cultures. Eleven strains differing in colony morphology from each other were selected for the study and designated from BS1 to BS11, respectively. Different microbial strains have varying cellulolytic activities based on their genetics and the type, and amount of cellulase enzymes they produce (Behera *et al*., 2017). This is confirmed by the enzymatic activity results of the different strains shown in Table 1. The average activity index ranged from 1.09 for strain BS11 to a maximum of 4.86 for strain BS6. Compounds like lignin, hemicellulose, and other inhibitors can reduce the cellulolytic activity of particular strains by hindering enzyme access to cellulose or by directly inhibiting enzymes (Zhai, Hu and Jin, 2022). In earlier studies (Hawrot-Paw and Stańczuk, 2022) using lignocellulosic materials (brewing spent grain, barley straw, oak shavings) and a species of the fungus *Trichoderma viride*, the activity index (*IA*) values were lower and varied from 1.18 to 1.38. Enzyme activity therefore depends not only on the type of lignocellulosic substrate, but also on the type of microorganisms used to treat it.

Strains with high cellulolytic activity are critical in converting lignocellulosic biomass into bioethanol. Based on the obtained results, two bacterial strains designated as BS5 and BS6 were selected for the next stage of the study.

HYDROLYTIC POTENTIAL OF ISOLATED BACTERIAL STRAINS

Biological pretreatment of lignocellulosic biomass increases yield of fermentable sugars (Su *et al*., 2018; Swain *et al*., 2018). Factors such as pH, oxygen levels, substrate availability, and temperature

Table. 1. Index of cellulolytic activity (*IA*) of investigated bacterial strains

Bacterial strain	Average diameter of the hydrolysis zone	Average diameter of the colony zone	*IA*
	mm		
BS1	26.67	15.67	1.70 ±0.09^{bc}
BS2	24.33	12.33	1.97 ±0.12^{c}
BS3	18.00	10.33	1.74 ±0.13^{c}
BS4	17.33	12.00	1.44 ±0.13^{abc}
BS5	37.33	11.33	3.29 ±0.21^{d}
BS6	45.33	9.33	4.86 ±0.41^{e}
BS7	20.00	13.67	1.46 ±0.04^{abc}
BS8	21.00	13.00	1.62 ±0.33^{abc}
BS9	17.67	15.33	1.15 ±0.09^{ab}
BS10	23.00	13.33	1.73 ±0.03^{c}
BS11	16.00	14.67	1.09 ±0.10^{a}

Explanations: means marked with the same letters in each column do not differ significantly at $p < 0.05$, according to Tukey's test. Data are presented as mean ±*SD*.
Source: own study.

can significantly affect the cellulolytic activity of a strain (Li *et al.*, 2023). In the presented study, the amount of fermentable sugars in solution varied depending on the bacterial strain used to pretreat lignocellulosic biomass and the temperature of the process (Tab. 2). The content of fermentable sugars in solution after hydrolysis using the BS5 strain was higher than the values determined for BS6, ranging from more than 230% at 25°C to about 150% at 35°C. Bacterial cellulolytic enzymes have hydrolytic activity in the temperature range of 25–50°C (Jones *et al.*, 2018). In the present study, for both strains, the highest sugar content in the hydrolysates was recorded at 35°C.

Table. 2. Average index content of fermentable sugars in hydrolysates.

Bacterial strain	Approximate sugar content (g·(100 cm^3)$^{-1}$) at		
	25°C	30°C	35°C
BS5	1.0	1.5	2.00
	1.0	1.0	2.00
	1.0	1.0	2.00
Mean ±*SD*	1.00 ±0.00^{a}	1.17 ±0.2^{a}	2.00 ±0.00^{b}
BS6	0.5	0.5	1.5
	0.5	0.5	1.0
	0.3	1.0	1.5
Mean ±*SD*	0.43 ±0.12^{a}	0.83 ±0.29^{a}	1.33 ±0.29^{b}

Explanations as in Tab. 1.
Source: own study.

ETHANOL YIELD FROM *Gleditsia triacanthos* BIOMASS

After a single distillation, 32.0 to 83.7 cm^3 of distillate was obtained, depending on the experimental variant (Fig. 1). A significant difference in the amount of distillate was noted after the process using the BS5 strain. The ethanol content of each distillate ranged from 2.3% (v/v) to over 36% (v/v). The most advantageous results were obtained from biomass pretreated and hydrolysed with the BS5 strain. The effect of the pretreatment method on ethanol yield is also confirmed by other authors. Saka and Afolabi (2015) used dilute acid to treat sugar cane pulp and obtained 15.5% ethanol. Unrean and Ketsub (2018) used acid to treat sugarcane bagasse and obtained 56.1 g·dm^{-3} ethanol, while Irfan, Nadeem and Syed (2014) for the same type of substrate after using mixed H_2O_2 + NaOH obtained 66 g·dm^{-3} ethanol. During the production of ethanol, various modifications can be used that affect the efficiency of the process. Mendez *et al.* (2021) carried out an SHF process for sugarcane bagasse and obtained a total ethanol concentration of 6.5% (v/v). Dahnum *et al.* (2015) optimising bioethanol production from an empty fruit bunch, and they obtained 4.74% ethanol by the SHF process and a higher ethanol yield (6.05%) by the simultaneous saccharification and fermentation process (SSF).

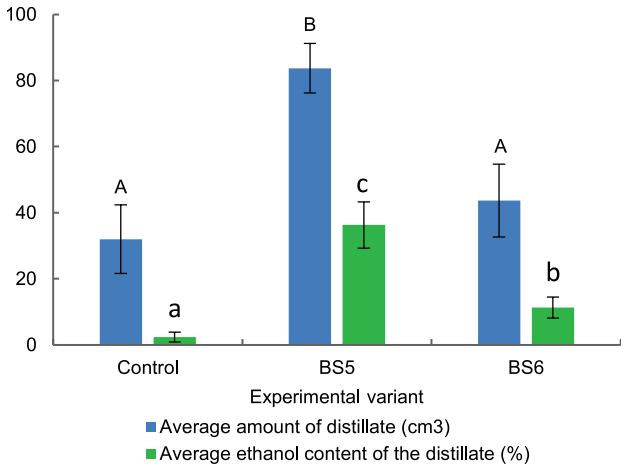

Fig. 1. Distillate amount and yield of cellulosic bioethanol; source: own study

CONCLUSIONS

Gleditsia biomass holds promise as a sustainable and efficient feedstock for biofuel production. The carried out research confirmed the possibility of bioethanol production by fermentation of middlings obtained from ground pods of *Gleditsia triacanthos*. The applied method of biological pretreatment and hydrolysis of this substrate was effective and significantly increased the ethanol content of the distillate compared to the control object. The cellulolytic activity of the isolated strains varied depending on the thermal conditions. The most favourable results were observed for a temperature of 35°C. The ethanol yield depended on the type of strain used in the biological conversion of cellulose to fermentable sugars. The maximum amount of distillate obtained was 83.7 cm^3 for the BS5 strain. The ethanol content of the distillate obtained after pretreatment and hydrolysis using strain BS5 amounted to 36.3% (v/v).

CONFLICT OF INTERESTS

All authors declare that they have no conflict of interests.

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