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Application of plant biomass in biotechnology

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List of abbreviations

1G – 1st generation bioethanol

2,5-FDCA - 2,5-furandicarboxylic acid

2G – 2nd generation bioethanol

3-HPA – 3-hydroxypropionic acid

4-HB – 4-hydroxybenzaldehyde

4-HBA – 4-hydroxybenzoic acid

5-HMF – 5-hydroxymethylfurfural

ABE – acetone-butanol-ethanol fermentation

ABXU – activity corresponding to the amount of enzyme releasing 1 μmol of xylose equivalent in 1 min

ADF – acid detergent fibre

ADL – acid detergent lignin

AFM – atomic force microscope

ALE – adaptive laboratory evolution

CBD – cellulose-binding domain

CBP – consolidated bioprocessing

CD – catalytic domain

CFU – colony-forming unit

CMC – carboxy methyl cellulose

CMCU – activity corresponding to the amount of enzyme releasing 1 μmol of glucose equivalent in 1 min

d.m. – dry matter

DNS – 3,5-dinitrosalicylic acid

FID – flame ionization detector

FPU – activity corresponding to the amount of enzyme that releases 2 mg of reducing sugars from 50 mg of filter paper in 1 hr, filter paper unit

LHW – liquid hot water

LPMO – lytic polysaccharide monooxygenase

MSW – municipial solid waste

NDF – neutral detergent fibre

PEF – pulsed electric field

PHA – polyhydroxyalkanoates

pNPGU – activity corresponding to the amount of enzyme releasing 1 μmol of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside in 1 min

psi – pound-force per square inch (lbf/in^2)

RES – renewable energy sources

RPM – revolutions per minute

$scCO_2$ – supercritical carbon dioxide

SCO – single cell oil

SEM – scanning electron microscope

SHF – separate hydrolysis and fermentation

SPORL – sulfite pretreatment to overcome recalcitrance of lignocellulose

SSCF – simultaneous saccharification and co-fermentation

SSF – simultaneous saccharification and fermentation

Abstract

The paper presents a review of the literature on the application of plant biomass in biotechnology, as well as the results of experiments aimed at enhancing the enzymatic hydrolysis step of this biomass by using recycling of enzymes adsorbed on the solid fraction of lignocellulosic material, improving the pretreatment process of plant biomass by using vacuum expansion of biomass or microwave radiation, and using lignocellulosic hydrolysates for the synthesis of carotenoids by yeasts of the genus *Rhodotorula*.

The results of the study confirmed that the use of enzyme recycling allows increasing the final efficiency of enzymatic hydrolysis of giant miscanthus and sugar miscanthus biomass, and the efficiency of biomass hydrolysis increased with increasing cellulase activity in the range of 18 - 58 FPU. The results of the study also confirmed that vacuum expansion allows increasing the efficiency of enzymatic hydrolysis of sugar beet biomass compared to mechanical grinding. The most effective process option for processing sugar beet biomass is to use vacuum expansion followed by enzymatic hydrolysis at 50°C, using enzyme compositions: Rapidase® and Accellerase 1500 with complex of supporting enzymes (Accellerase: XY, XC, BG). On the medium prepared in this way, it was possible to reuse the enzymes in the form of immobilized fractions on undecomposed lignocellulose. The highest average ethanol concentration in the post-reaction medium was obtained after sequential fermentation of hydrolysates obtained from mechanically crushed and vacuum-exploded beet biomass by *Saccharomyces cerevisiae* 7, yielding 50.5 and 53.6 $g \cdot dm^{-3}$, respectively. After using the simultaneous process, the attenuation level was lower than that obtained in the SHF system, and the alcohol content was lower by 19% (when shredded biomass was used) and by 15% (after vacuum expansion). The *Mucor indicus* filamentous fungus was found to be unsuitable for ethanol fermentation of sugar beet biomass.

In experiments using microwave-assisted pretreatment of giant miscanthus biomass, the highest hydrolysis efficiency occurred in samples with sulfuric acid (VI) at 90 psi (58.3%), but fermentation of these hydrolysates yielded only 13.1 mg of ethanol per gram of dry biomass. The opposite relationship occurred in samples treated in an acidic environment at 30 psi. At the relatively low hydrolysis efficiency of these samples (30.1%), 23.6 mg of ethanol per gram of dry biomass was obtained. Microwave processing parameters should thus be chosen as a trade-off between high enzymatic hydrolysis efficiency and ethanol fermentation productivity.

The results of the experiments demonstrated that adapting yeasts of the genus *Rhodotorula* to the use of lignocellulosic hydrolysates from refined purple willow bark for growth and multiplication will result in more efficient carotenoids biosynthesis. Laboratory adaptation made it possible to obtain *Rhodotorula* strains with increased growth capacity on medium with the addition of lignocellulosic hydrolysate. The obtained modified strains (*R. glutinis* 50H, *R. rubra* KKP350 50H and *R. glutinis* KKP347 50H) showed more efficient synthesis of carotenoids compared to the corresponding test strains. The use of lignocellulosic hydrolysate as a substrate additive increased the amount of carotenoids obtained, even in native strains. After biomass extraction of *R. glutinis* strain KKP347 50H after culture on medium with lignocellulosic hydrolysate, the highest concentration of carotenoids (130 $\mu g \cdot g^{-1}$ d.m.) was obtained.

Streszczenie

Praca przedstawia przegląd literatury dotyczącej zastosowania biomasy roślinnej w biotechnologii, a także przebieg i wyniki doświadczeń mających na celu doskonalenie etapu hydrolizy enzymatycznej tej biomasy poprzez zastosowanie recyklingu enzymów zaadsorbowanych na frakcji stałej materiału lignocelulozowego, doskonalenie procesu obróbki wstępnej biomasy roślinnej poprzez zastosowanie próżniowego rozprężania próżniowego biomasy lub promieniowania mikrofalowego, oraz wykorzystanie hydrolizatów lignocelulozowych do syntezy karotenoidów przez drożdże z rodzaju *Rhodotorula*.

Wyniki badań potwierdziły, że zastosowanie recyklingu enzymów pozwala na zwiększenie końcowej wydajności hydrolizy enzymatycznej biomasy miskanta olbrzymiego i miskanta cukrowego, a efektywność hydrolizy biomasy wzrastała wraz ze wzrostem aktywności celulaz w zakresie 18 - 58 FPU. Wyniki badań potwierdziły również, że rozprężanie próżniowe pozwala na zwiększenie efektywności hydrolizy enzymatycznej biomasy buraka cukrowego w porównaniu do rozdrabniania mechanicznego. Najskuteczniejszym wariantem procesu w zakresie obróbki biomasy buraka cukrowego jest zastosowanie eksplozji próżniowej i poddanie otrzymanego materiału hydrolizie enzymatycznej w temperaturze 50°C przy użyciu kompozycji enzymatycznych: Rapidase® i Accellerase 1500. Na tak przygotowanej pożywce możliwe było ponowne wykorzystanie enzymów w postaci frakcji immobilizowanej na nierozłożonej lignocelulozie. Największe średnie stężenie etanolu w medium poreakcyjnym uzyskano po fermentacji prowadzonej w systemie sekwencyjnym przez *Saccharomyces cerevisiae* 7, uzyskując 50.5 i 53.6 $g \cdot dm^{-3}$, odpowiednio po fermentacji hydrolizatów uzyskanych z biomasy buraka rozdrobnionego mechanicznie i poddanego rozprężeniu próżniowemu. Po zastosowaniu procesu symultanicznego, poziom odfermentowania był mniejszy niż uzyskany w systemie SHF, a zawartość alkoholu była mniejsza o 19% (w przypadku zastosowania rozdrobnionej biomasy) i o 15% (po rozprężeniu próżniowym). Stwierdzono brak przydatności grzyba strzępkowego *Mucor indicus* w procesie fermentacji etanolowej biomasy buraka cukrowego.

W doświadczeniach wykorzystujących mikrofalową obróbkę wstępną biomasy miskanta olbrzymiego, największa wydajność hydrolizy wystąpiła w próbkach poddanych działaniu kwasu siarkowego (VI), przy ciśnieniu 90 psi (58.3%), jednak fermentacja tych hydrolizatów pozwoliła otrzymać jedynie 13.1 mg etanolu na gram

suchej biomasy. Odwrotna zależność wystąpiła w próbkach poddanych obróbce w środowisku kwaśnym przy 30 psi. Przy stosunkowo niskiej wydajności hydrolizy tych próbek (30.1%) uzyskano 23.6 mg etanolu na gram suchej biomasy. Dobór parametrów obróbki mikrofalowej należy zatem rozważać jako kompromis pomiędzy wysoką wydajnością hydrolizy enzymatycznej i produktywnością fermentacji etanolowej.

Uzyskane wyniki doświadczeń dowiodły, że zastosowanie adaptacji drożdży z rodzaju *Rhodotorula* do użytkowania hydrolizatów lignocelulozowych z rafinatu kory wierzby purpurowej do ich wzrostu i namnażania spowoduje wydajniejszą biosyntezę karotenoidów. Adaptacja laboratoryjna umożliwiła uzyskanie szczepów *Rhodotorula* o zwiększonej zdolności wzrostu na pożywce z dodatkiem hydrolizatu lignocelulozowego. Uzyskane zmodyfikowane szczepy (*R. glutinis* 50H, *R. rubra* KKP350 50H i *R. glutinis* KKP347 50H) wykazywały wydajniejszą syntezę karotenoidów w porównaniu z odpowiednimi szczepami testowymi. Zastosowanie hydrolizatu lignocelulozowego jako dodatku do podłoża zwiększyło ilość uzyskanych karotenoidów, również u szczepów natywnych. Największe stężenie karotenoidów (130 $\mu g \cdot g^{-1}$ s.s.) uzyskano po ekstrakcji biomasy szczepu *R. glutinis* KKP347 50H po hodowli na pożywce z hydrolizatem lignocelulozowym.

Additional information on presented dissertation

Some of the scientific material contained in the presented work has already been published. There are two works:

Kordala N, Walter M, Brzozowski B, Lewandowska M (2022) 2G-biofuel ethanol: an overview of crucial operations, advances and limitations. *Biomass Conversion and Biorefinery*. Biomass Conversion and Biorefinery, 1-24. http://dx.doi.org/10.1007/s13399-022-02861-y

Walter M, Adamczak M, Brzozowski B (2021a) Recykling enzymów w procesie hydrolizy biomasy miskanta olbrzymiego lub cukrowego (Recycling of enzymes in the hydrolysis of biomass of *Miscanthus giganteus* and *Miscanthus sacchariflorus*). *Przemysł Chemiczny* 100(6): 576-580. http://dx.doi.org/10.15199/62.2021.6.7

The results of the first review paper were used in the literature review (mainly in tables 3 and 4), while the results obtained in paper 2 are presented in the paragraph 5.1. Both papers constitute appendices 1 and 2 to the dissertation, while appendix 3 presents the statements of the co-authors, indicating the scope of their participation in the preparation of the publication.

1. Introduction

Plant biomass is regarded as one of the world's most abundant and sustainable carbon sources. The main sources of plant biomass are wood and wood waste, waste from the production and processing of agricultural raw materials, and crops grown for energy (Saravanan et al. 2022). Waste from agriculture and forestry is produced annually in huge quantities (Ashokkumar et al. 2022), and without proper disposal can cause environmental pollution (Adejumo and Adebiyi 2020). Disposal of organic waste is normally done by combustion or by hydrolysis and subsequent fermentation of the hydrolysate (Sadh et al. 2018). This waste is a potential raw material for the production of unique biochemicals and bioenergy (Isikgor and Becer 2015) and animal feed (Nguyen et al. 2010).

As early as the beginning of the 19th century, Henry Ford suggested the implementation of a bioeconomy, or biotechnology-based economy, as a logical and necessary choice for the development of any civilization (Lucia 2008). Unfortunately, the price of oil in the last century has been significantly lower than that of any other energy resource (Vansteenkiste 2011), which has resulted in the explosion and dominance of this resource in the world market (Mohaddes and Raissi 2019). In recent decades, however, the competitive price advantage of fossil fuels gradually disappeared (Lucia 2008). Unfortunately, the recent energy crisis is related to the war in Ukraine and the global geopolitical situation.

Global energy demand is being "driven" by rising living standards in developed countries and continued population growth in developing countries. The global population reached 8 billion in 2022 (Adam 2022) and is expected to reach 10 billion by 2055, potentially leading to resource depletion (Heleniak 2020). As a result, in order to meet energy demand, the consumption of fossil fuels is increasing, resulting in ozone depletion, climate change, environmental problems, and increased health risks for living creatures (Hussain et al. 2017). Sustainable and pollution-free renewable energy technologies have been introduced to ensure the safety of the planet and counter potential environmental threats. Renewable energy is one of the solutions to mitigate climate change, and its development is leading to significant reductions in carbon emissions (Baležentis et al. 2019). For example, the transportation sector alone, which consumed 31.8% of the oil produced in 2017, accounted for 41.5% of global carbon dioxide emissions (IEA 2022). Thus, replacing gasoline and diesel with environmentally friendly

fuels is a promising way to reduce environmental impacts and become less reliant on oil (Rosales-Calderon and Arantes 2019).

Renewable energy sources (RES) currently provide about 23.7% of the total global energy demand, which is a significant improvement compared to the 2% share of renewable energy in 1998 (Hussain et al. 2017). The use of conventional fuels for the development of RES, including solar, wind, geothermal, hydro, and biofuel production, must be limited. Biofuels made from bio-based raw materials are one of the most important renewable fuel sources (Soltanian et al. 2020; Aghbashlo et al. 2018).

The oldest type of biofuel known since prehistoric times is primary biofuel - produced directly from the combustion of wood or cellulose from plant materials and dry animal waste. The first, second, third, and fourth generation biofuels are distinguished by the biomass source used, the benefits and drawbacks of their use, and the manufacturing process (Rodionova et al. 2022; Rodionova et al. 2017). First-generation biofuels are bioethanol and biogas produced from food crops (cereals and sugar beets) and biodiesel obtained from edible oilseeds and waste animal fats. The second generation of biofuels is bioethanol and biogas produced from biomass of non-food crops, energy crops, agricultural residues or waste, e.g. municipal waste and biodiesel obtained from the seeds of inedible oil plants. Third-generation biofuels are made from cyanobacteria, algae, and seaweed (Soltanian et al. 2020; Rodionova et al. 2017). Genetically modified microorganisms such as microalgae and cyanobacteria are used to create fourth generation biofuels. Developments in this area continue to pique the interest of synthetic biology researchers. They are expected to result in a major breakthrough in biofuels (Mat Aron et al. 2020; Cameron et al. 2014).

Lignocellulosic biomass, which is a waste product from the forestry, paper, and agricultural industries, or a product from energy crops intended for biofuel production, deserves special attention as a raw material for the production of biofuels and bioproducts. According to studies, the world produces approximately 181 billion tonnes of lignocellulosic biomass each year, of which approximately 8.2 billion tonnes are used; approximately 7 billion tonnes come from the wood industry and energy crops, and the remaining approximately 1.2 billion tonnes are agricultural waste. Traditionally, biomass has been used for combustion, building materials, and the paper industry, but more recently, the emphasis has been on the production of renewable biofuels and other value-

added bio-based products (Baruah et al. 2018; Preethi et al. 2021; Dahmen et al. 2019; Ashokkumar et al. 2022; Paul and Dutta 2018).

This paper provides an overview of the current literature on the use of specific types of plant biomass in biotechnology for energy purposes, as well as the course and outcomes of experiments aimed at:

a) reusing enzymes adsorbed on the solid fraction of lignocellulosic material to improve the stage of enzymatic hydrolysis of plant biomass,

b) improving the process of pretreatment of plant biomass through vacuum expansion or microwave radiation,

c) the use of lignocellulosic hydrolysates as a substrate for carotenoids synthesis by *Rhodotorula* yeast.

2. Review of the literature

2.1. Potential for the use of plant biomass

Plant biomass, as the only renewable carbon source, is being considered a potential substitute for fossil fuels. It is distinguished by its ease of accessibility, high organic content, sustainability, low cost and low greenhouse gas emissions (Qiu et al. 2022). Plant biomass can be converted into liquid and gaseous biofuels, as well as value-added chemicals. It is subjected to physical, thermochemical, and biological processes, among others, in order to be used (Hoang et al. 2021). Around the world, research is being conducted to convert plant biomass into biofuels and other potentially value-added products such as organic acids, polyhydroxyalkanoates (PHAs), biochemicals, bioplastics, and so on (Usmani et al. 2021). First-generation bioethanol (1G) is a sugar/starch derivative, but its main disadvantage is the high price of sugar/starch feedstocks (40 to 70% of the total cost) and public controversy over the fact that edible plants are consumed for bioprocessing purposes. In this context, lignocellulosic biomass is seen as more promising due to its availability, low cost, and lack of competition with food production. As defined, bioethanol produced from lignocellulose is called second-generation (2G) bioethanol or cellulosic ethanol (Rosales-Calderon and Arantes 2019; Rastogi and Shrivastava 2017). Because cellulose accounts for roughly half of the organic carbon in the biosphere, its conversion into fuels and valuable chemicals is regarded as a renewable energy source with carbon-neutral potential.

The majority of lignocellulosic biomass used for energy comes from three sources. These are: (a) agricultural waste, (b) forestry waste, and (c) industrial waste (Das et al. 2021). Depending on its chemical composition, it can be divided into six basic groups: (1) agricultural production residues (e.g., sugarcane bagasse, corn, wheat, rice and barley straw, cereal hulls, fruit pomace, sorghum), (2) hardwood (e.g., aspen, poplar), (3) softwood (e.g., pine, spruce), 4) cellulosic waste (e.g., newsprint, office waste), 5) green plants (e.g., knapweed millet, finger cynodon, willow, miscanthus), and 6) municipal solid waste; MSW) (Sánchez and Cardona 2008; Mood et al. 2013).

Because lignocellulosic biomass contains more oxygen than crude oil, more product classes can be obtained in lignocellulosic biorefineries. From lignocellulosic biomass, more than 200 value-added compounds have already been produced (Isikgor and Becer 2015; Ashokkumar et al. 2022). Unfortunately, different biomass sources have different proportions of cellulose, hemicellulose, and lignin, and as a result, most

bioproduct technologies are still in the pre-commercial stage (Cherubini and Strømman 2011; Isikgor and Becer 2015).

Global waste generation is currently estimated to be around 2 billion tonnes per year, with a projected increase to around 3.4 billion tonnes by 2050, of which at least 33% is not properly managed (Kaza et al. 2018). Almost 63% of this mass is lignocellulosic waste (food and green plants, wood, paper, and cardboard), which can be a valuable resource for the biorefinery industry, not only reducing negative environmental impacts but also fostering the development of a closed-loop bioeconomy (Usmani et al. 2021). The EU market for biorefinery products is expected to grow at a 4% annual rate, reaching nearly €50 billion by 2030. This expansion is being driven by a global shift toward green, naturally sourced, and sustainable products with little or no environmental impact. This will increase lignocellulosic biomass's share of the renewable feedstock market to around 25% by 2030 (Usmani et al. 2021).

2.2. Limitations related to the use of plant biomass

Although lignocellulosic biomass is a significant source of renewable energy, its potential for biofuel production is underutilised due to low bioethanol, biomethane, and biohydrogen yields (Bundhoo 2018). The complex structure of lignocellulosic biomass causes limited availability of fermentable sugars, which is a problem when using it (Figure 1).

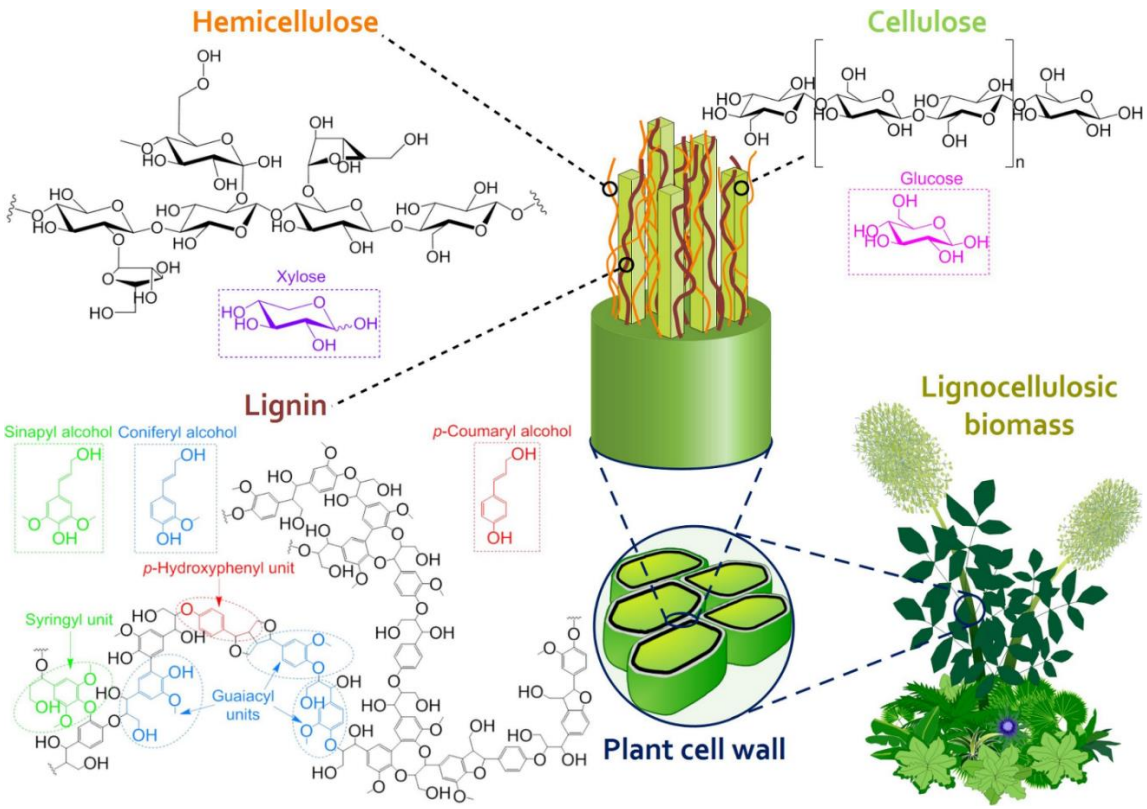


Fig. 1. Scheme of lignocellulose structure (Soltanian et al. 2020)

Biomass is made up of easily biodegradable compounds (fats and proteins) as well as compounds that are difficult to degrade: cellulose (20-50%), hemicellulose (15-35%), and lignin (10-35%) (Saravanan et al. 2022; Kan et al. others 2017). Lignocellulose is a complex matrix made up of spatially expanded lignin and less cross-linked hemicellulose wrapped around linear cellulose particles to form a complex spatial structure. Because cellulose as a main source of glucose is "hidden" inside the complex structure of lignocellulose, this structure explains the low efficiency of biofuel production. This frequently leads to incomplete hydrolysis of cellulose molecules and a lack of sugars for fermentation (Zheng et al. 2014). In addition to the content of lignin and hemicellulose, factors impeding the hydrolysis of cellulose molecules include cellulose crystallinity and degree of polymerization (Maurya et al. 2015).

Cellulose is the main component of the plant cell wall. This molecule is a highly crystalline, linear polysaccharide consisting of cellobiose units, containing from 3,000 to 14,000 D-glucose residues linked by β-1,4-glycosidic bonds. At temperatures ranging from 250 to 350°C, this bond undergoes pyrolysis, resulting in cellulose decomposition and depolymerization. Because of its strong inter- and intramolecular hydrogen bonds,

cellulose is insoluble in most common solvents, including water, and is highly resistant to lytic enzymes (Kumar and Sharma 2017; Mathews et al. 2016, Galbe and Zacchi 2012).

Hemicellulose is the second major component of the plant cell wall. It is a heterogeneous polymer whose dominant building element is mannose (conifers) or xylose (deciduous trees and agricultural waste) (Van Dyk and Pletschke 2012). Hemicellulose also contains galactose, glucose, arabinose, as well as small amounts of rhamnose, glucuronic acid and galacturonic acid. Unlike cellulose, whose structure is crystalline and compact, it has a random, amorphous and branched structure with less resistance to hydrolysis. The bonds within the hemicellulose molecule are β-1,4-glycosidic bonds (Chen et al. 2017; Zhou et al. 2017, Soltanian et al. 2020). This component of lignocellulosic biomass has a polymerization degree of 100 - 200 sugar residues, which is much lower than that of cellulose (Qaseem et al. 2021).

Lignin is a highly complex phenolic heteropolymer, composed of three different monomers of hydroxycinnamic alcohols (i.e., monolignols), including p-coumaryl, coniferyl and sinapyl alcohols. Monolignols are linked to each other by various bonds, mainly ether bonds (e.g. α-O-4, β-O-4 and 4-O-5) and C-C bonds (e.g. β-β, β-5 and 5-5). Bond energies between C-C units are higher than those between C-O bonds, making them more difficult to break (Guadix-Montero & Sankar 2018). The most abundant type of interconnection in the lignin structure is the β-O-4 aryl ether, which is responsible for the highly branched aromatic structure (Soltanian et al. 2020). Lignin is responsible for the hydrophobicity and structural rigidity of the lignocellulose molecule. It is the most resistant to chemical and enzymatic degradation component of plant cell walls, providing plant tissue with compressive strength and resistance to biotic stress. It is the primary barrier to cellulose by forming a protective layer (Zoghlami and Paes 2019; Isikgor and Becer 2015; Upton and Kasko 2016). Table 1 compares the properties of the lignocellulose components discussed and shows the main products of their complete degradation.

Table 1. Comparison of the structural properties of cellulose, hemicellulose, and lignin, as well as the products of their total hydrolysis (Ashokkumar et al. 2022; Jönsson and Martín 2016)

	Cellulose (20 - 50%)	Hemicellulose (15 - 35%)	Lignin (10 - 35%)
Characteristics	- $(C_6H_{10}O_5)_n$ - linear structure - high crystallinity - repetitive glucose molecules with β-(1,4)-glycosidic bonds - ratio of atoms O/C: 0.6 - 0.83 - ratio of atoms H/C: 0.8 - 1.67 - thermal decomposition temperature: 315 - 400°C	- $(C_5H_8O_4)_m$ - branching structure - contains pentoses, hexoses, uronic acids and acetyl groups - ratio of atoms O/C: 0.6 - 0.8 - ratio of atoms H/C: 0.8 - 1.6 - thermal decomposition temperature: 220 - 315°C	- $(C_9H_{10}O_3(OCH_3)_{0.9\text{-}1.7})_x$ - aromatic compound - three-dimensional structure - contains phenylpropane, methoxyphenol compounds and non-carbohydrate polyphenolic substances - ratio of atoms O/C: 0.36 - 0.47 - ratio of atoms H/C: 1.19 - 1.53 - thermal decomposition temperature: 160 - 900°C
Degradation products	- fermentable sugars	- pentoses and hexoses - furfural - 5-hydroxymethylfurfural - acetic acid - formic acid - levulinic acid - aliphatic aldehydes - uronic acids	- vanillin - 4-hydroxybenzaldehyde (4-HB) - 4-hydroxybenzoic acid (4-HBA) - coniferyl aldehyde - syringaldehyde - ferulic acid

In many lignocellulosic raw materials (e.g., sugar beet pulp, apple pomace, citrus pomace, etc.), pectin is additionally present as another polysaccharide that is part of dietary fiber (Zannini et al. 2021). Pectin is a type of structural fiber found in the primary cell wall and intracellular layer of plant cells. It is a polymer with a linear structure in which several hundred to several thousand galacturonic acid monomer units are linked

by an α-(1-4)-glycosidic bond. The individual residues of galacturonic acid are largely methylated. The average molecular weight of pectin is 50 - 150 kDa. The backbone of the pectin molecule is substituted in certain regions with α-(1-2)-rhamnopyranose units, from which side chains are formed. Mannose, glucose and xylose can also occur in the pectin structure (Mudgil 2017).

2.3. Bioproducts from lignocellulosic biomass

In rural areas, lignocellulosic biomass is currently used to generate thermal energy via direct combustion. The use of chemical, thermal, and biological processing methods results in the indirect conversion of lignocellulosic materials into value-added products (Zamani 2015; Menon and Rao 2012). The physicochemical properties of the individual components of these materials are taken into account when producing bioproducts. High-strength fibres and textiles, for example, are made from the cellulosic fraction of lignocellulosic materials (Garver and Liu 2014).

However, in the future, lignocellulosic biomass could be one of the most important sources of energy and value-added products (Ma et al. 2012, Zamani 2015). Growing interest in biofuels and biochemicals has resulted in the development of numerous processes for converting lignocellulose into high-value chemicals and materials (Dietrich et al. 2019). Unfortunately, only a few integrated commercialised biorefineries use lignocellulosic feedstock in processes other than gasification or pyrolysis (van Ree and van Zeeland 2014). An alternative solution for processing lignocellulosic biomass is called torrefaction (i.e., roasting). This is a preliminary thermal processing of biomass into solid fuels that involves dehydrating the biomass and bringing its properties closer to those of fuel coal. The process occurs at 200-300°C, near atmospheric pressure, and without oxygen. The unit temperature rise in the process is usually less than 50°C/min. Storified biomass is widely used to produce high-quality, low-carbon pellets for industrial and domestic use (Sarker et al. 2021).

Van Ree and van Zeeland (2014) define the term "biorefinery" as "a sustainable approach to the biotransformation of raw materials, e.g. biomass into energy and a broader spectrum of everyday goods, in an economical and environmentally friendly manner." Scientific knowledge and the public's growing environmental awareness emphasise concepts such as renewability, recyclability, and sustainability. In this context,

the transition from non-renewable materials (petroleum-based resources, for example) to renewable materials (biomass-based resources, for example) is becoming a focus of global research (Bilal et al. 2017; Vassilev et al.2015; Arevalo-Gallegos et al. 2017).

Biorefineries use two main pathways for processing plant biomass (Figure 2): biochemical conversion to sugar via fermentation of sugars obtained from biomass, and thermochemical synthesis (Liu et al. 2020 Zhang et al. 2020 Saravanan et al. 2022).

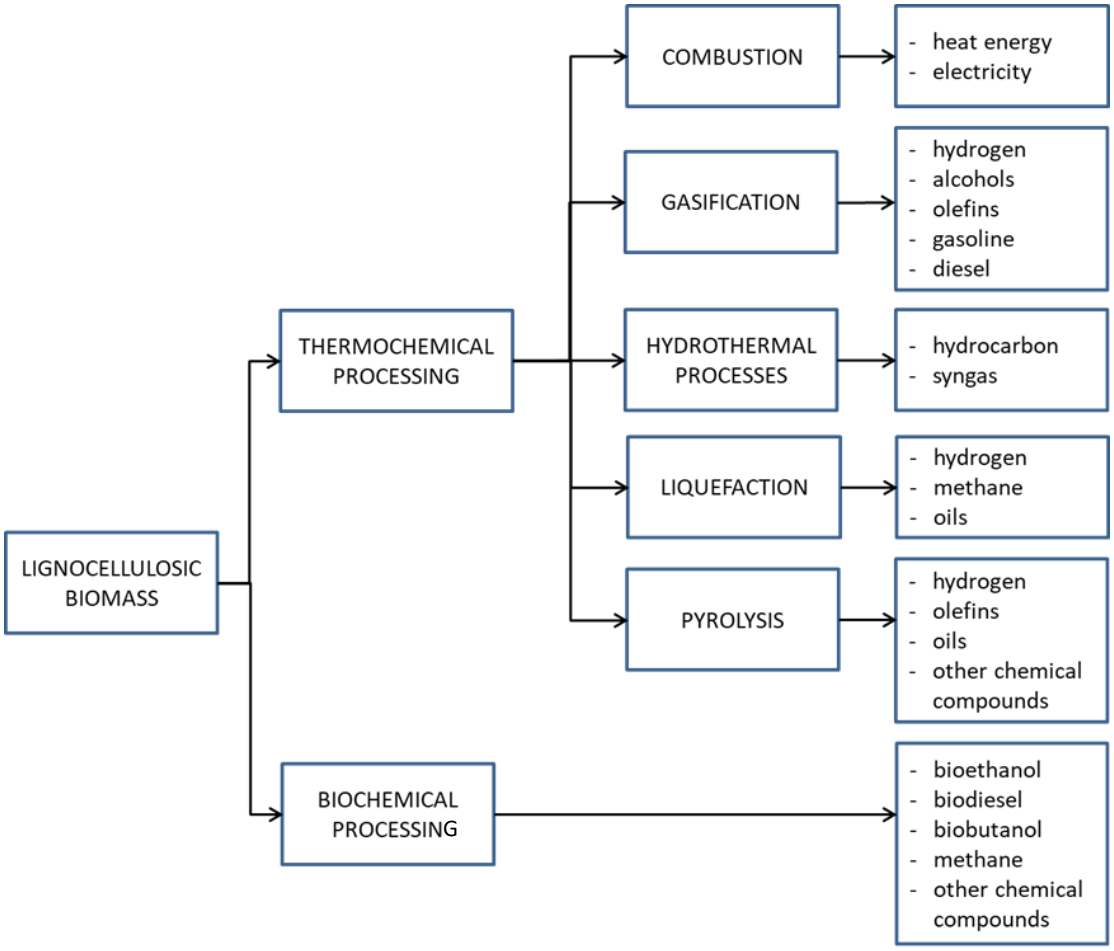


Fig. 2. Thermochemical and biochemical processing of lignocellulosic biomass (own elaboration based on Menon and Rao 2012; Güleç et al. 2021)

Biochemical processing transforms lignocellulosic components primarily into biofuels and other chemical components, whereas thermochemical conversion transforms biomass into useful substrates for further processing. Many studies have been conducted to investigate the possibility of producing valuable chemicals from biomass. Werpy and Petersen (2004) identified 12 main platform compounds (platform chemicals) that can be produced from lignocellulose sugars via simple biological or chemical conversion:

- 1,4-diacids (succinic, fumaric, malic),
- 2,5-furanodicarboxylic acid,
- 3-hydroxypropionic acid,
- aspartic acid,
- glucaric acid,
- glutamic acid,
- itaconic acid,
- levulinic acid,
- 3-hydroxybutyrolactone,
- glycerol,
- sorbitol,
- xylitol/arabinitol.

These compounds can then be transformed into a variety of valuable derivatives. In addition to the aforementioned compounds, Gallezot (2012) also reviewed the synthesis of chemicals through conversions obtained by depolymerization and fermentation of lignocellulose building blocks, adding 5-hydroxymethylfurfural (5-HMF) to the list of most desirable compounds, as its derivatives (including 2,5-FDCA - 2,5-furandicarboxylic acid) have been identified as promising chemical intermediates. Isikgor and Becer (2015) presented an extensive analysis of the potential of using lignocellulosic biomass to produce value-added compounds in their publication. They listed over 200 compounds obtained through various pretreatment methods, as well as detailed descriptions of the polymers that can be produced using these compounds. In their review paper, the authors expanded the list of platform compounds to include ethanol and butanol, among others, because these alcohols are promising precursors for the corresponding olefins. Acetone was also considered as a platform substance because its conversion products contain important monomeric chemicals. Acetone, butanol, and ethanol (ABE) fermentation is widely studied in the scientific and industrial communities. Their conversion products, such as ethylene, ethylene glycol, butadiene, propene, and vinyl chloride, have a significant impact on the polymer industry. Lactic acid was not initially identified as a platform compound, but as the authors point out, it is the most abundant carboxylic acid in nature and can be converted into a variety of products such as acetaldehyde, acrylic acid, propanoic acid, 2,3-pentanedione, and dilactide due to its biofunctionality.

Lignin conversion has also shown promise. Lignin's distinct structure and chemical properties enable the production of a diverse range of chemicals, particularly aromatic compounds. As a result, lignin can be regarded as a significant aromatic resource for the bioeconomy (Zakzeski et al. 2010).

Cherubini and Strømman (2011) discussed the prospects and potential of lignocellulosic feedstocks in biorefineries. Using the individual building blocks of the lignocellulosic structure, a diverse range of reactive platform chemicals (Figure 3) can potentially be produced, which can be classified based on the number of carbon atoms in their structure:

- C2: ethanol,

- C3: acetone, lactic acid, 3-HPA,

- C4: organic acids (succinic acid, fumaric acid, etc.),

- C5: furfural, itaconic acid, xylitol, levulinic acid,

- C6: sorbitol, 5-HMF, 2,5-FDCA and gluconic acid.

Depolymerization and deoxygenation of the biomass stream for biofuel production are required. Depolymerization can occur during gasification or pyrolysis, resulting in the complete breakdown of primary carbon chains into C1 units. It can also be done under milder conditions (for example, biochemical conversion) while preserving the key primary structure (Cherubini and Strømman 2011). Deoxygenation increases the energy content of the biofuel, and the presence of higher polarity oxygen-containing compounds limits their solubility in the hydrocarbon mixture. Other biomass applications may necessitate much less deoxygenation, as the presence of oxygen frequently confers valuable physical and chemical properties on the product. According to the cited authors, the basic reactions identified in the bioconversion of pentoses, and hexoses include hydrolysis, fermentation, reduction, oxidation, dehydrogenation, dehydration, and condensation.

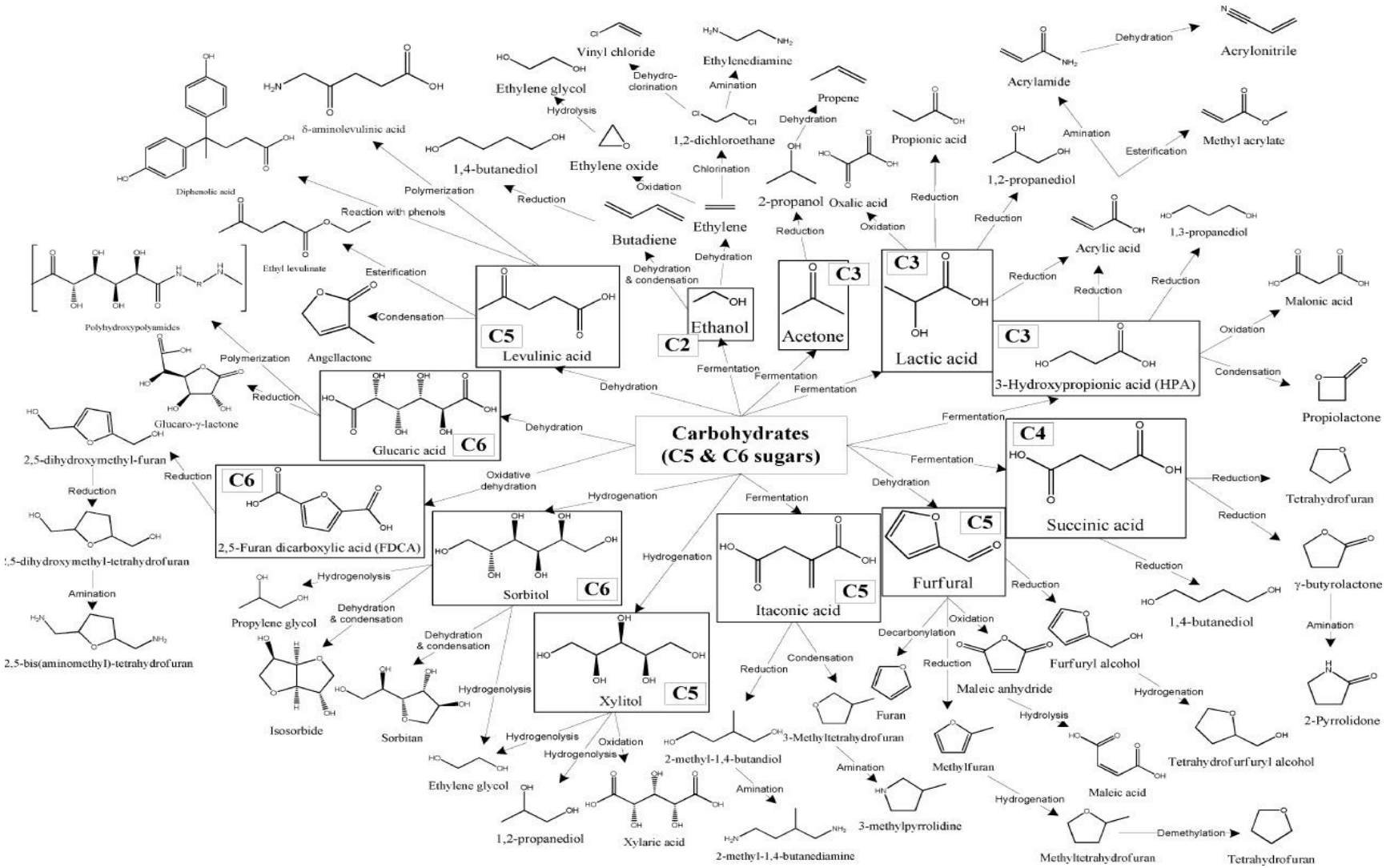


Fig. 3. Schematic representation of selected bioproducts derived from lignocellulose and its major derivatives (Cherubini and Strømman 2011)

2.4. Biomass as a renewable source of biofuels

The term "biofuels" refers to a wide range of solid, gaseous, and liquid fuels, such as alcohols (ethanol, methanol, butanol), biodiesel, Fischer-Tropsch gasoline/diesel, bio-oil, petroleum, hydrogen, methane and biocarbon.

Bioethanol (C_2H_5OH) is the most abundantly produced biofuel in the world. It is considered a substitute for gasoline or as a precursor to other value-added chemicals. First-generation ethanol production was developed, on an industrial scale, using starch products and sugarcane and corn, among others. This type of biofuel has been criticized for reasons related to the debate over the use of edible feedstocks for biofuel production (Limayem and Ricke 2012; Okolie et al. 2021). The use of lignocellulosic feedstocks in the production of 2G biofuels is not controversial. The combustion of 2G biofuels emits no additional carbon dioxide into the atmosphere, and the feedstock used in their production is either a widely available waste product or a crop grown specifically for this purpose (Dahnum et al. 2015; Ashokkumar et al. 2022). Bioethanol can be produced through thermochemical or biological processes. The refractory structure of lignocellulose is broken down into simple molecules and then converted into ethanol in both methods. The Fischer-Tropsch catalysis method is used to generate synthesis gas (CO and H_2) and convert it into ethanol, hydrocarbon fuels, butanol, and green diesel (Limayem and Ricke 2012).

Cellulases and hemicellulases are enzymes that hydrolyse the respective components of lignocellulose to monosaccharides, which are then fermented with yeast in ethanol. Despite the promising applications of bioethanol, the economic and technical feasibility of the bioconversion process, as well as the environmental impact, are some of the issues that need to be addressed further. The production of bioethanol from lignocellulose typically involves four main steps: 1) pretreatment to increase the availability of polysaccharides; 2) enzymatic hydrolysis to break down the polysaccharides into simple sugars; 3) fermentation, during which microorganisms ferment the sugars into ethanol; and 4) separation of ethanol from the fermentation medium (Rosales-Calderon and Arantes 2019). The difficulty stems from the requirement for costly and complex biomass pretreatment methods involving acids, alkalis, enzymes, chemicals, microwaves, ultrasound, and other physicochemical and mechanical factors (Ashokkumar et al 2022; Okolie et al 2021). The first commercial-scale plant designed to

produce 75 million litres of ethanol per year from agricultural waste - e.g., rice straw, wheat straw and *Arundo donax* (reed canary grass/arundo canary grass) - was built in 2013 by Chemtex (M&G Group) in Crescentino, Italy (dos Santos et al. 2016). It is estimated that 442 billion litres of bioethanol can be produced per year from lignocellulosic biomass, and that the total amount of crop residues and agricultural wastes can produce 491 billion litres of bioethanol per year, which is approximately 16 times the current global bioethanol production (Kim and Dale 2004). Bioethanol is currently available for use as a biofuel in approximately thirty countries worldwide. The United States and Brazil are the largest contributors, accounting for 62% of global bioethanol production. China, Japan, Canada, and the European Union are promoting the expansion of bioethanol production strategies and the use of bioethanol (Zhang et al. 2021; Gong et al. 2022).

Biodiesel is the world's second most produced biofuel. It is manufactured as methyl or ethyl esters of long-chain fatty acids for use as a drop-in fuel (with the same chemical composition as current conventional fuels) or as a conventional blend with diesel fuel. Unfortunately, edible oils account for approximately 95% of biodiesel production (Phimsen et al. 2016). The production of biodiesel from lignocellulosic feedstocks has the potential to provide significant environmental benefits. This, however, necessitates first converting the lignocellulosic feedstock into useful lipid products like single cell oil (SCO). The fatty acid compositions of lipids produced from lignocellulosic biomass by microorganisms capable of efficiently accumulating them (e.g., bacteria, microalgae, and yeast) are similar to those of vegetable oils (Yousuf 2012; Okolie et al. 2021). Non-edible oils (e.g., from jatropha seeds) are also used for biodiesel production.

Biogas is another important renewable energy source (RES) that can be used in place of traditional gaseous fuels. The term refers to a mixture of gases produced by anaerobic digestion or biomethanation processes from organic matter. Biogas is primarily composed of methane and carbon dioxide, with trace amounts of siloxanes and hydrogen sulphide (Adekunle et al. 2015). The anaerobic digestion process for biogas production entails a series of biochemical processes that convert organic biodegradable compounds into biogas through the use of microorganisms classified into three types based on temperature sensitivity: psychrophiles (15 - 20°C), mesophiles (35 - 40°C) and thermophiles (55 - 60°C). Due to its refractory nature, lignocellulosic feedstock is difficult to use in anaerobic digestion, resulting in reduced fermentation efficiency and

poor biodegradation. Co-fermentation and various pretreatment methods are being used to improve the efficiency of using lignocellulosic biomass in biogas production (Okolie et al. 2021).

Butanol (butyl alcohol, C_4H_9OH) is an alcohol used primarily as an important precursor for the production of value-added chemicals (e.g., acrylic acid, glycol ethers and butyl acetate), polymers, adhesives, and plastics. Most of the synthetic butanol available today is produced from petrochemicals through the oxo-synthesis of propylene (MacLean and Lave 2003). Biobutanol (biobutanol) derived from biomass is gaining popularity as an advanced biofuel to replace gasoline in internal combustion engines. In contrast to bioethanol, which requires up to 85% blending with conventional fuel, butanol can be used as a drop-in fuel or blended in a variety of proportions. Biobutanol has several advantages, including a high octane number, low toxicity, hazard, and hygroscopicity values. Biobutanol's fuel properties are comparable to those of gasoline (Okolie et al. 2021). Biobutanol can be produced from starch biomass by ABE fermentation using *Clostridium* sp. The use of lignocellulosic biomass necessitates dilute acid pretreatment as well as enzymatic hydrolysis. Butanol's partial solubility in water makes subsequent processing and extraction from the fermentation medium difficult. Some of the challenges that limit its large-scale production are issues with further butanol recovery, low synthesis yields, and butanol's toxicity to microorganisms (Nanda et al. 2017a; Okolie et al. 2021).

Hydrogen is one of the most abundant elements on the planet. Because it can be burned as a fuel, it is seen as a promising alternative to both renewable and non-renewable carbon-based fuels. The calorific value of hydrogen is very high at 120 MJ/kg (for comparison, coal has a calorific value of 25 MJ/kg and gasoline has a calorific value of 47 MJ/kg) (Wiącek 2011; Nanda et al. 2017b). Hydrogen can be produced from petrochemical resources (such as natural gas, fossil fuels, and coal), which account for approximately 96% of the hydrogen in use today, as well as renewable resources (biomass, water). Specific thermochemical, biological, electrocatalytic, electrolytic, and photocatalytic conversion pathways are used to obtain hydrogen (Okolie et al. 2021).

One important factor in favour of biofuel production is that the carbon dioxide emitted during combustion is naturally biogenic and thus has no additional negative environmental impact (Soltanian et al. 2020). Because of the high demand for food, it is also necessary to discontinue the use of food raw materials in biofuel production.

2.4.1. Lignocellulosic raw materials in the production of bioethanol and value-added biochemicals

Many studies have been conducted over the past decades on the use of lignocellulosic biomass as a feedstock for the production of biofuels and biochemicals. Unlike starch-derived bioethanol, lignocellulosic biomass is not used as a food source (Gong et al. 2022). The most commonly used feedstock was rice straw, but wheat straw, corn bagasse, and sugarcane bagasse were also of interest to researchers (Sarkar et al. 2012). Bioethanol and other biochemicals are produced using agricultural residues, wood waste, and energy crops from targeted crops such as giant miscanthus and sugar miscanthus, willow, poplar, black locust, prairie spartin, switchgrass millet, Virginia mallow, and Jerusalem artichoke (ETIP Bioenergy 2021; Singh et al. 2022; Stolarski 2021).

As previously stated, lignocellulose is a polymer complex with varying amounts of cellulose, hemicellulose, and lignin depending on the species, tissue type, and maturity of the plant cell wall (Table 2).

Table 2: Chemical composition of selected lignocellulosic raw materials (based on: Hassan et al. 2018, Rajendran et al. 2017, Paul and Dutta 2018, Krzyzaniak et al. 2014)

Raw material	Cellulose	Hemicellulose	Lignin
	Based on dry matter concentration (%)		
rice straw	28 - 36	23 - 28	12 - 14
wheat straw	33 - 38	26 - 32	17 - 19
barley straw	31 - 45	27 - 38	14 - 19
rape straw	35 - 37	24 - 26	17 - 19
corn pomace	28 - 40	24 - 26	7 - 19
sugar cane bagasse	42 - 48	19 - 25	20 - 42
sorghum pomace	34 - 45	18 - 27	14 - 21
poplar	43 - 46	20 - 23	22 - 25
acacia	47 - 49	13 - 15	31 - 34
willow	42 - 47	29 - 33	24 - 26
pine	25 - 44	26 - 32	28 - 48
spruce	28 - 43	20 - 30	28 - 35

switchgrass millet	36 - 45	28 - 30	12 - 26
miscanthus	28 - 48	19 - 30	12 - 25
rice husk	25 - 35	18 - 21	26 - 31
municipal solid waste	33 - 49	9 - 16	10 - 14

2.4.2. Pretreatment of lignocellulosic raw materials

Although lignocellulosic biomass has been recognised as a valuable feedstock for bioethanol production in recent years, one issue with its use is the cost-intensity of its processing (Singh et al. 2022).

The complex structure of lignocellulose must be destroyed before such feedstock can be used for biofuel or biochemical production (Lamichhane et al. 2021). There are numerous methods and variants for pretreating feedstock (Yamashita et al. 2010; Chen et al. 2017; Kucharska et al. 2018), but many of them require the use of environmentally hazardous concentrated acids or alkalis, or large amounts of energy. As a result, intensive work is being done, among other things, to improve 2G bioethanol production technology by developing an efficient and environmentally friendly biomass pretreatment method (Mikulski and Kłosowski 2020). The purpose of pretreatment is to separate lignin from the other polysaccharides of the lignocellulosic complex, reduce the crystallinity of the cellulose and increase the porosity of the material so that enzymes can access the cellulose in subsequent hydrolysis steps (Sánchez, Cardona 2008; Lamichhane et al. 2021). An effective pretreatment should not result in hemicellulose or cellulose losses, and it should not contribute to the formation of potential inhibitors of hydrolytic enzymes and microorganisms (Kumar et al. 2016). The economic goals of the pretreatment must include minimising energy requirements, lowering the cost of grinding lignocellulosic raw materials, lowering residues generated (solid fraction, process water, chemicals), and using few or no chemicals. At the same time, keeping all of the aforementioned criteria in mind, the overarching goal of pretreatment should be to maximise the efficiency of the bioconversion process (Taherzadeh and Karimi 2008; Cheng and Timilsina 2011). Pretreatment methods are divided into different categories, such as mechanical, chemical, physicochemical and biological methods, or various combinations thereof (Barakat et al. 2013). Researchers use various pretreatment methods to fractionate, solubilize, hydrolyze and separate the various components of lignocellulose. Milling, irradiation, microwaves,

steam explosion, fibre explosion using ammonia (AFEX), supercritical CO_2 and its explosion, SO_2, alkaline hydrolysis, liquid hot water, "organosolv" solvents, wet oxidation, ozonolysis, hydrolysis with concentrated acid, and biological pretreatments are among the methods used. These methods all have the same goal: to reduce the particle size of biomass and open up its physical structure (Soltanian et al. 2020).

The pretreatment step is responsible for breaking down the physical lignin barrier and exposing the cellulose and hemicellulose for further processing. This is critical to the commercial viability of 2G ethanol production. Variables such as sugar yield, concentration, and release rate are critical in determining the best pretreatment method for each type of feedstock (Corrêa et al. 2022).

The ideal pretreatment method for lignocellulosic raw materials results in:

- increasing accessibility and surface area for enzyme activity,
- breaking down the lignin-hemicellulose structure hindering enzymatic activity,
- reducing the crystallinity of cellulose,
- partial depolymerization of cellulose and hemicellulose,
- increased solubility of hemicellulose and lignin,
- changing the structure of lignin,
- promoting enzymatic digestibility,
- reducing the loss of sugars,
- preserving the pentose fraction of hemicellulose,
- minimizing the formation of inhibitors that negatively affect the activity of enzymes and fermentation microorganisms,
- reducing energy requirements - reducing costs

(Soltanian et al. 2020; Maurya et al. 2015; Kumar and Sharma 2017).

Pretreatment processes for lignocellulosic biomass can be divided into three main groups (Figure 4): biological (using microorganisms), non-biological (physical, chemical, and physicochemical) and combined (Kumar and Sharma 2017).

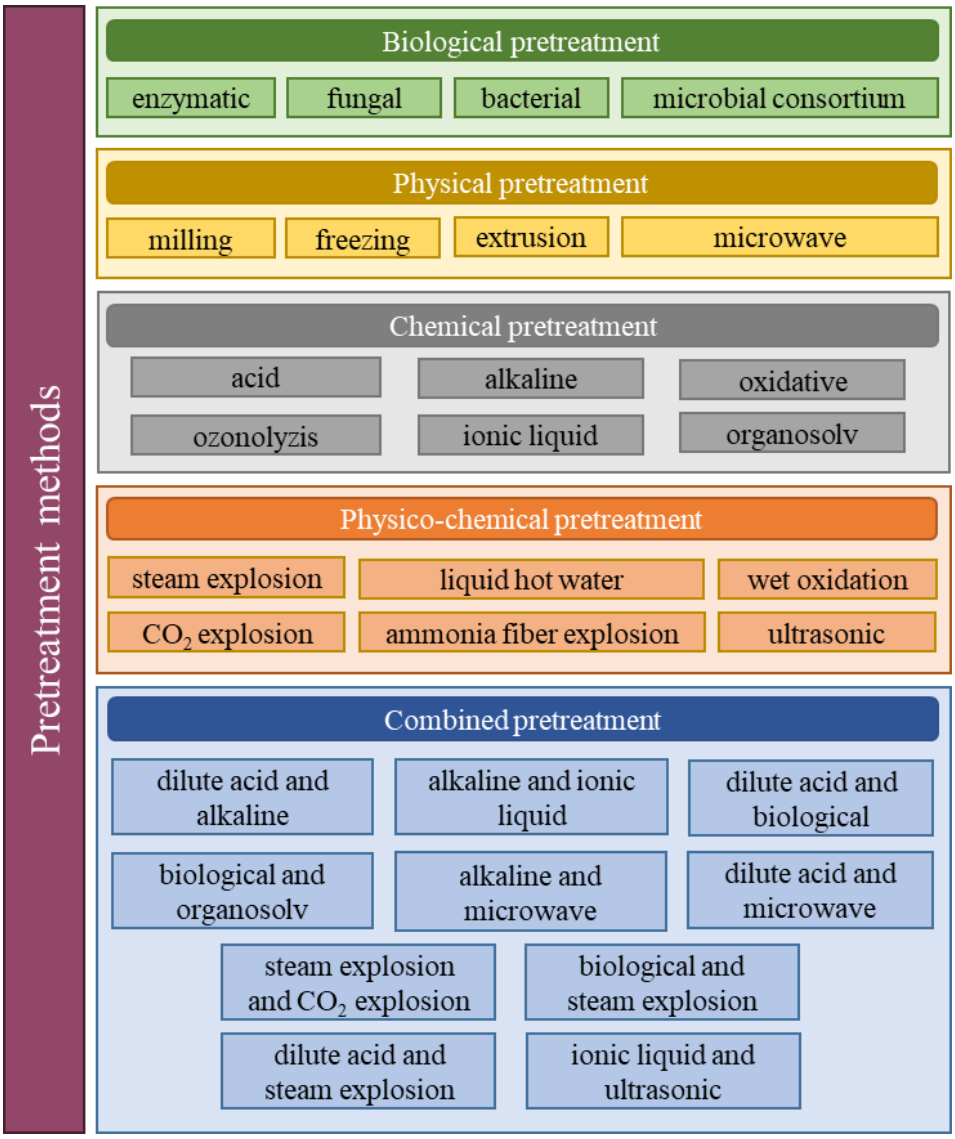


Fig. 4. Different types of pretreatment methods of lignocellulosic biomass (own elaboration based on Soltanian et al. 2020)

The use of different pretreatment methods yields different results, and each method has its own advantages and disadvantages (Table 3). Some of the methods mentioned have found industrial-scale application, but many methods require excessive energy or the use of environmentally hazardous substances. There is still an urgent need for green technological solutions for this process (Hassan et al. 2018).

Table 3. Advantages and disadvantages of selected pretreatment methods for lignocellulosic raw materials

Pretreatment method	Course and effects	Advantages	Disadvantages	Reference
Biological methods				
Microbial degradation	Use of white, brown and gray rot fungi; degradation of lignin and hemicellulose	Environmentally friendly, selective degradation of lignin and hemicellulose, low level of inhibitor formation	Very long pretreatment time (several weeks), low yield, no cellulose degradation, need to control conditions for microbial growth	Sánchez and Cardona 2008, Hassan et al. 2018
Physical methods				
Milling	Reduce particle size and crystallinity of cellulose, increase specific surface area of raw material	Increase availability of biomass structure for enzymes, no inhibitors formed	High energy consumption, no lignin removal/degradation	Kordala et al. 2022
Extrusion	Degradation of lignocellulose fibers, decrystallization of cellulose	Significant increase in yield of sugars from biomass, short process time, low formation of inhibitors	Energy-intensive process, need for high temperatures, limited effect when no additional chemicals are used	Hassan et al. 2018, Duque et al. 207
Microwave radiation	Use of microwave radiation (300 - 700 W/m^3) to conduct processing at 130 - 200°C; leads	Increased availability and reactivity of cellulose, energy efficiency, increased efficiency of enzymatic	High investment outlay	Kordala et al. 2022

	to delignification and release of fermentable sugars	hydrolysis, combinability with chemical methods, short process time		
Pyrolysis	Temperature >300°C, followed by cooling and condensation	Relatively short process time, exposure of cellulose fraction	High selectivity in biomass deconstruction	Kumar and Sharma 2017, Kordala et al. 2022
Sonification (ultrasounds)	Ultrasound in the 10 - 100 kHz range; from tens of seconds to minutes.	Disruption of cellulose and hemicellulose fractions, increased availability for lytic enzymes	High cost, difficulty of application in industrial conditions	Kumar and Sharma 2017
Pulsed-electric field (PEF)	High-voltage electrical discharge (5 - 20 kV/cm) in nanoseconds to milliseconds, under ambient conditions; breakdown of lignocellulose structure	Low energy demand, very short processing time, simple reactor design	Electrochemical reactions can lead to the formation of inhibitors or other products, wear and tear of electrodes	Kumar and Sharma 2017
Chemical methods				
Acid treatment	0.75 - 5% sulfuric acid, hydrochloric acid, nitric acid or others, approx. 1 MPa, >180°C,	High conversion rate of cellulose to glucose, possibility of skipping the enzymatic hydrolysis step	High cost, need for acid recovery, toxicity of chemicals, corrosion-resistant reactors required, large amount of	Kumar and Sharma 2017, Taherzadeh and Karimi 2008

	1 - 5 min. or <120°C, 30 - 90 min.; lignin precipitation		inhibitors formed, gypsum as a by-product of pH neutralization	
Alkaline treatment	Use of sodium hydroxide or calcium hydroxide at 20 - 120°C (tens of minutes to hours), possible combination with hydrogen peroxide (0.5 - 2.15% by volume) using lower temperature (35°C); delignification, solubilization of hemicellulose	Effective removal of lignin, reduction of polymerization level and crystallinity of cellulose	High cost, difficulty of industrial-scale adaptation, formation of hydrolysis and fermentation inhibitors, need for pH neutralization	Sánchez and Cardona 2008; Kordala et al. 2022
Ozonolysis	Ozone treatment at ambient temperature and atmospheric pressure; effective delignification	No inhibitor formation, lignin degradation, no negative environmental impact	The efficiency of the method depends on the moisture content of the raw material and the size of its particles, as well as the concentration of ozone in the gas stream, the high cost of ozone	Taherzadeh and Karimi 2008
Oxidative delignification	Use of peroxidase and 2% hydrogen peroxide, 20°C, >8h; delignification and almost	Low by-product content after the process, further cellulose conversion of about 95%.	High cost	Sánchez and Cardona 2008

	complete dissolution of hemicellulose			
Solvent fractionation	Organic solvents (methanol, ethanol, acetone, ethylene glycol) or their combination with 1% sulfuric acid or hydrochloric acid, 186 - 198°C, 30 - 60 min, pH = 2.0 - 3.4	High level of delignification	Need for solvent recovery and recycling, cellulose remains in solid phase	Kordala et al. 2022
Ionic liquids	Solid to ionic liquid ratio 1:10, 100 - 150°C; reduction of cellulose crystallinity, partial removal of hemicellulose and lignin	Suitable solvents with low vapor pressure, mild reaction conditions, dissolution of cellulose in the liquid phase	High cost of ionic liquids, complexity of synthesis and purification, toxicity, poor biodegradability and inhibitory effect on enzyme activity	Hassan et al. 2018, Kordala et al. 2022
Organosolv	Addition of aqueous organic solvents (e.g., ethanol, methanol, ethylene glycol, acetone) to biomass under specified conditions of temperature and pressure; removal of lignin and fractionation of hemicellulose	Low final lignin content, solvent and enzyme reusability	High cost of solvents, need for acid, base or salt catalysts, formation of inhibitors	Sánchez and Cardona 2008

Deep eutectic solvents	Lignin removal and hemicellulose fractionation	Environmentally friendly, biodegradable solvents	Poor stability at higher temperatures	Hassan et al. 2018
Physicochemical methods				
Steam explosion	Steam at a pressure of 6 - 34 bar, from a few seconds to a few minutes; softening of lignin, dissolution of hemicellulose, reduction of particle size	Facilitate access to cellulose through solubilization of hemicellulose; relatively low cost, low negative environmental impact	Generation of toxic compounds, low efficiency in coniferous woods	Sánchez and Cardona 2008
Liquid hot water (LHW)	Temperatures in the range of 170 - 230°C, pressure >5MPa; degradation of hemicellulose and partially lignin, increase in lignin availability	Does not require the addition of catalysts and chemicals, minimizing the formation of inhibitors at pH 4 - 7	High energy and water demand, formation of toxic compounds	Kordala et al. 2022
Wet oxidation	Oxygen pressure = 1.2 MPa, 195°C, 15 min, addition of water and small amounts of calcium carbonate or sulfuric acid	High degree of solubilization of hemicellulose and lignin	High cost of oxygen and alkaline catalyst, formation of hydrolysis and fermentation inhibitors	Kordala et al. 2022, Taherzadeh and Karimi 2008
Explosion with supercritical CO_2	4 kg CO_2/kg of fiber, pressure of 5.6 MPa, temp. up to 200°C, disruption of lignocellulose	Low cost of CO_2 and recoverability, low temperature requirements,	Very high pressure requirements, method difficult to use on an industrial scale	Kordala et al. 2022

	structure increasing its surface area	relatively low inhibitor formation, environmentally friendly method		
Ammonia recycled percolation (ARP)	Elevated pressure and temperature (160 - 180°C), ammonia concentration 5 - 15% (w/w), percolation reactor; lignin degradation and hemicellulose solubilization	Almost complete delignification, increase in enzymatic hydrolysis efficiency	Need for ammonia recovery, energy intensity	Kumar and Sharma 2017
Ammonia fiber explosion (AFEX)	1 - 2 kg ammonia/kg of d.m., 90°C, 30 min, p = 1.12 - 1.36 MPa; delignification, increasing the efficiency of enzymatic hydrolysis of cellulose and hemicellulose	High efficiency and selectivity in reaction with lignin, low amount of inhibitors formed	High cost of the process, negative environmental impact, need for ammonia recovery, low efficiency for coniferous woods	Kordala et al. 2022
Sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL)	Biomass treatment with calcium or magnesium sulfite (160 - 200°C, 30 min) combined with grinding using a disc mill; delignification	Energy saving, high efficiency, high conversion rate of cellulose to glucose, easy industrial scale application	High water consumption, high cost of chemical recovery	Hassan et al. 2018

2.4.3. Chemical and enzymatic hydrolysis

The hydrolysis of lignocellulose is a critical step in the process of obtaining bioproducts from lignocellulosic raw materials in terms of productivity and profitability. Acids (concentrated or diluted) or enzymes can be used in the hydrolysis process. The use of concentrated acids (e.g., 30 - 70% H_2SO_4) makes it possible to obtain high glucose yields (90%) in a relatively short period of time (10 - 12 h); however, high acid concentrations and extreme conditions make this process unfavorable for environmental and economic reasons (Zhou et al. 2021). To hydrolyze crystalline cellulose using dilute acids (H_2SO_4 or HCl), a concentration of about 1.5% is required, and the reaction temperature should be in the range of 200 - 240°C. Under such conditions, the conversion of xylose to furfural and glucose to 5-HMF and its derivatives, which are inhibitors of hydrolysis as well as further fermentation of the hydrolysate, takes place (Kordala et al. 2022). Alkaline pretreatment with sodium, potassium and calcium hydroxides or ammonates (6 - 20% solutions), prior to hydrolysis with dilute acids, improves hydrolysis efficiency by increasing lignin degradation (Loow et al. 2016).

In comparison to chemical hydrolysis methods, the use of lytic enzymes requires less energy, is more gentle on the environment, and has no negative environmental impact. Enzymes (cellulases and hemicellulases) depolymerize cellulose and hemicellulose into hexoses (mannose, glucose, and galactose) and pentoses (xylose and arabinose) during enzymatic hydrolysis (Rosales-Calderon and Arantes 2019). The three main groups of cellulases involved in the hydrolysis reaction are:

1) endoglucanases - endo-1,4-β-glucanases (EC 3.2.1.4), which randomly attack regions of cellulose with low crystallinity, forming free chain ends. They are monomeric enzymes with a molecular weight of 22 to 45 kDa, optimal activity in an environment of pH 4 - 5 and a temperature of 50 - 70°C;

2) exoglucanases - glucan-1,4-β-glucanases (EC 3.2.1.74) and exo-1,4-β-cellobiosidases (cellobiohydrolases) (EC 3.2.1.91), acting on the outer fragments of the cellulose molecule, release glucose or cellobiose. There is a distinction between cellobiohydrolase I (CBI), specific to the non-reducing end of the polysaccharide, and cellobiohydrolase II (CBII), which detaches saccharides from the reducing end of the chain. These enzymes are monomeric proteins with a molecular weight of 50 - 65 kDa, and their optimal catalysis conditions are pH 4 - 5, 37 - 60°C, depending on the enzyme and substrate combination;

3) β-glucosidases (EC 3.2.1.21), which degrade cellobiose and oligodextrins to glucose. These enzymes have a molecular weight of 35 - 640 kDa and can be monomeric or exist as homo-oligomers. They are active in an environment with a pH of 3.5 - 5.5 and a temperature of 45 - 75°C (Juturu and Wu 2014; Behera et al. 2016; Rosales-Calderon et al. 2016).

Cellulases are synthesized by fungi, bacteria, protozoa, plants, and animals (Zhang and Zhang 2013). Fungi, due to their versatile substrate utilization and ability to penetrate biomass, are their main industrial producer, with fungi of the *Trichoderma* genus (mainly *T. reesei*, *T. viride* and *T. longibrachiatum*) being particularly attractive. Microorganisms with a complete cellulase system, i.e. a multi-enzymatic system containing a number of catalytic modules, substrate binding sites, and/or active sites, are preferred for efficient cellulase synthesis (Taylor et al. 2006). Unfortunately, no single species has all elements of the cellulase system required for efficient lignocellulose hydrolysis. Cellulases are commercially produced by combining fungal strains to obtain the three main enzymes of the cellulase system: exo- and endoglucanase, as well as β-glucosidase. Numerous studies have found that combining *T. reseei* and *Aspergillus niger* strains produces a beneficial combination of enzymes. The former produces a large pool of endo- and exoglucanases, as well as small amounts of β-glucosidase, whereas *A. niger* produces large amounts of β-glucosidase and other cellulases to a lesser extent. Some commercial companies are now producing cellulases from genetically modified organisms, but only on a laboratory scale (Srivastava et al. 2018). The concentration of extracellular cellulases synthesized by *Trichoderma* species often exceeds 100 $g \cdot dm^{-3}$, which is now a critical value for industrial production. *Trichoderma* synthesizes at least two types of exoglucanases (cellobiohydrolases I and II, EC 3.2.1.91), five endoglucanases (endoglucanases I-V, 3.2.1.4) and two types of β-glucosidases (β-glucosidase I and II, EC 3.2.1.21) (Zhang and Lynd 2004). Structurally, fungal cellulases are simpler compared to bacterial cellulases (Quiroz-Castañeda and Folch-Mallol 2013).

Today, cellulases are among the three most commonly industrially produced enzyme preparations due to their widespread use in cotton processing and recycling, and as additives in detergents and animal feed. Commercial enzyme preparations developed over the past few years include: Spezyme® CP, Accellerase™ 1000, Accellerase™ 1500 and Accellerase™ DUET produced by Genencor, and Celluclast® 1.5L, Novozyme 188, Cellic® CTec2 i Cellic® CTec3 produced by Novozymes (Rosales-Calderon i Arantes 2019).

Hemicelluloses accompanying cellulose are usually xylans and mannans, which are broken down by several enzymes to oligomers and monosaccharides (xylose and mannose), requiring the synergistic action of xylanases and mannanases (Taherzadeh and Karimi 2008). Endoxylanase - endo-β-(1,4)-xylanase (EC 3.2.1.8) and endomannanase - endo-β-(1,4)-D-mannanase (EC 3.2.1.78) are enzymes that degrade the main polysaccharide chain to xylan and glucomannan, respectively. Xylanases are hydrolases that differ in their catalytic properties. Mannanases - β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), acetylmannanesterase (EC 3.1.1.6), β-glucosidase (EC 3.2.1.21) and α-galactosidase (EC 3.2.1.22) are heterogeneous in their properties, and their role is to break down the main and side chains of mannan. The breakdown of hemicellulose into short oligomeric compounds is influenced by enzymes such as β-xylosidase (EC 3.2.1.37), β-mannosidase (EC 3.1.1.25) and β-glucosidase (EC 3.2.1.21). Side groups are attached to the oligomers, which are removed by α-glucuronidase (EC 3.2.1.139), α-arabinosidase (EC 3.2.1.55) and α-D-glucosidase (EC 3.2.1.22). Acetyl substituents are sequentially removed by esterases (Kordala et al. 2022; Dawood and Ma 2020; Juturu and Wu 2014).

Proteins with hydrolytic activity, such as cellulases and hemicellulases, have a complex molecular architecture made up of independently folded and structurally and functionally distinct units called domains: a catalytic domain (CD) and one or more cellulose-binding domains (CBD), which is linked by a short poly-linker region to the catalytic domain at the N-terminus. The domains are linked to each other by unstructured linker sequences, and the linker region is rich in serine and threonine. The CD comprises more than 70% of the protein sequence and is responsible for cleaving the glycosidic bond. CBD contains from 30 to about 200 amino acids and occurs in the protein as a single, double, or triple domain. CBD's role is to bind the enzyme to the cellulose so that CD sticks closer to the substrate, and also gives CD time to move the chain to its active site before the enzyme diffuses from the cellulose. It is still unclear whether CBD can also modify cellulose or otherwise promote cellulose hydrolysis by the catalytic domain (Quiroz-Castañeda and Folch-Mallol 2013).

A mixture of free cellulases degrades crystalline cellulose synergistically with an efficiency up to fifteen times greater than any single cellulase, implying that synergistic cooperation between cellulases is required for efficient cellulose degradation, though the molecular mechanism of this interaction is not fully understood (Jalak et al. 2012). The mechanisms of multi-enzymatic cellulose hydrolysis identified so far are:

1) endo-exo synergy, occurring between endo- and exoglucanases, where the action of endoglucanases provides free ends of the cellulose chain for further digestion by exoglucanases;

2) exo-exo synergy, exoglucanases gradually act on reducing and non-reducing ends of cellulose chain;

3) synergy between exoglucanases and β-glucosidases, the final process that results in the formation of cellobiose molecules (Quiroz-Castañeda and Folch-Mallol 2013).

Intramolecular synergy of cellulases between the catalytic domain and the CBD domain has also been observed (Jalak et al. 2012). Cellulases, along with other enzymes such as xylanases, must work together to degrade plant cell wall polysaccharides (Tabka et al. 2006).

2.4.4. Hydrolysis and fermentation inhibitors

In addition to improving lignocellulose structure breakdown, the pretreatment process can result in the formation of compounds that inhibit the bioethanol production process. The type of lignocellulosic raw materials treated, and the type of pretreatment used, including chemical reagents and process parameters such as temperature, pressure, catalyst addition, redox conditions, pH, or time, among others, influence the composition and concentration of inhibitors. The toxicity of inhibitors is determined by their concentration in relation to the microorganism species used for fermentation, as well as the culture conditions and methods used (e.g., pH, temperature, oxygen content) (Rajendran et al. 2018; Kordala et al. 2021). The majority of lignocellulosic complex inhibitors are formed through the solubilization and degradation of hemicellulose and/or lignin. Inhibitors are organic acids (acetic acid, formic acid, and levulinic acid), sugar degradation products such as furfural (from xylose) and 5-HMF (from hexoses) and lignin degradation products such as vanillin, syringaldehyde, and 4-HB (Mahboubi et al. 2017; Kordala et al. 2021; Preethi et al. 2021). Depending on the pretreatment method used, the amount and type of inhibitors formed vary. Acetic acid, furfural, and 5-HMF are the most common by-products of dilute acid treatment, but they are not produced by AFEX treatment (da Silva et al. 2016). Coumaric acid, formic acid, and acetic acid are primarily produced following alkaline pretreatment, whereas treatment with concentrated acids results in the formation of furfural (van der Pol et al. 2016). They have inhibitory effects on enzymes and microorganisms depending on the type and concentration of inhibitors in the environment.

Pectin, xylooligosaccharides, xylose and vanillin have strong inhibitory effects on cellulose hydrolysis. Syringaldehyde and trans-cinnamic, tannic and gallic acids, on the other hand, have moderate inhibitory effects, while 4-HBA has the least negative effect on enzymatic hydrolysis efficiency (Ximenes et al. 2010). Inhibitors disrupt the fermentation process and reduce ethanol synthesis efficiency. Productivity decreases as acetic, formic, and levulinic acids concentrations rise. Furfural and 5-HMF reduce the volumetric productivity of ethanol synthesis but have no effect on the process's final yield (Larsson et al. 1999, Rosales-Calderon and Arantes 2019).

To prevent the formation of hydrolysis and fermentation inhibitors and to mitigate their negative effects, various methods are used, such as:

- detoxification,
- selection and genetic engineering of lignocellulosic raw materials,
- bioamortization (bioabatement),
- selection of microorganisms,
- application of metabolic and evolutionary engineering methods on microorganisms

(Jönsson and Martín 2016; Rajendran et al. 2018).

2.4.5. Detoxification

The most widely used method of reducing the negative effects of inhibitors on the fermentation process is detoxification. The negative effects of inhibitors can be reduced by employing various detoxification methods, such as physical, chemical, or biological methods, which can be used immediately after pretreatment and/or enzymatic hydrolysis. These methods result in the conversion of inhibitors to inert substances or the complete removal of inhibitors (Zha et al. 2012; Kordala et al. 2021). The most common and widely used physical detoxification method is rinsing materials after pretreatment with water to remove unwanted liquid-phase solutes. This improves the efficiency of enzymatic hydrolysis and hydrolysate ethanol fermentation (Xie et al. 2018). The addition of activated carbon, which causes efficient adsorption of toxic compounds, is a popular method for physicochemical detoxification of lignocellulosic hydrolysates (Kordala et al. 2021).

Popular chemical detoxification methods have traditionally involved saturating acid-treated material with calcium hydroxide, resulting in the precipitation of calcium sulfate, which adsorbs toxic compounds. This method has the disadvantage of being expensive and time-consuming (De Bari et al. 2014). Other alkaline compounds, such as ammonia water or sodium hydroxide, are also used, but alkaline detoxification degrades the saccharide structure, which can lead to a reduction in ethanol synthesis yield in addition to having negative environmental effects. The possibility of performing the procedure in situ in a bioreactor using reducing agents such as sulfur oxyanions or sulfhydryl reagents is a novel approach in chemical detoxification. Reducing agents eliminate the need for an additional detoxification step (Jönsson et al. 2013).

2.4.6. Ethanol fermentation

Microorganisms convert the sugars produced by enzymatic hydrolysis into ethanol during fermentation. The main components of detoxified lignocellulosic hydrolysate are hexoses (glucose, mannose, galactose) and pentoses (xylose and arabinose) (Kordala et al. 2022). The sugar profile in the hydrolysate is determined by the type of substrate and the pretreatment method used, which also influences the selection of appropriate microorganisms. Microorganisms used in the production of bioethanol from lignocellulosic materials should be able to convert both sugar groups to ethanol and be resistant to potential inhibitors. Additionally, the preferred characteristics are:

- resistance to ethanol concentrations above 40 $g \cdot dm^{-3}$,
- ability to produce ethanol quickly and efficiently (> 90% of theoretical yield),
- resistance to high temperature and low pH,
- ability to produce by-products in minimal quantities,
- lack of pathogenic properties,
- low nutritional requirements (Kordala et al. 2022, Balat 2011).

Beer yeast *Saccharomyces cerevisiae* is commonly used in this process due to its natural adaptation to ethanol production and resistance to high alcohol concentrations in the fermentation medium. However, this yeast species lacks the enzymatic apparatus to assimilate pentoses, hence it does not utilize the entire range of polysaccharide decomposition derivatives of the lignocellulosic substrate in the fermentation process (Lewandowska et al. 2016; Baeyens et al. 2015). Efficient conversion of biomass to ethanol necessitates optimal utilisation of both

cellulosic and hemicellulosic sugars, but no microorganism has been identified that efficiently converts all hexoses and pentoses to ethanol. Both types of sugars can be fermented by either co-cultivating microorganisms predisposed to fermenting hexoses and pentoses or fermenting them separately. Species with natural potential for pentose fermentation include: *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehatae* and *Klebsiella oxytoca*, but the use of these yeast species in commercial bioethanol production is limited, mainly due to low fermentation rates, the need for careful regulation of oxygen requirements, and lack of tolerance to inhibitors and high ethanol content (Asachi and Karimi 2013; Converti et al. 2001). Lindén and Hahn-Hägerdal (1989) conducted experiments on fermentation of hexoses and pentoses using standard distillers' yeast (*S. cerevisiae*) supported by xylose isomerase, as well as cofermentation with a strain of *Pachysolen tannophilus*. However, in coculture, the optimal conditions for yeast growth are not the same, resulting in lower productivity and fermentation efficiency. Recombinant yeast or bacteria that meet the requirements for microorganisms used in cellulosic ethanol production have recently been developed (Lamichhane et al. 2021). The modification of microorganisms by genetic engineering methods involves two lines of research: one concerns the modification of microorganisms capable of ethanol fermentation, i.e. *S. cerevisiae* or *Zymomonas mobilis*, in terms of their ability to utilize pentoses; the other is based on altering the metabolic pathways of microorganisms that naturally utilize pentoses for efficient ethanol production (Robak and Balcerek 2018). The advantages and disadvantages of selected microbial species for fermentation of lignocellulosic hydrolysates are shown in Table 4.

Various methods of integrating hydrolysis and fermentation processes have been tested to increase the efficiency of bioethanol production. These processes can be carried out separately (SHF) or simultaneously (SSF). The SHF process is a two-step process. Pre-processed lignocellulosic biomass is broken down by enzymes into fermentable sugars, which are then fermented into ethanol. The main advantage of this variant is that both stages can be performed in optimal conditions. One of the disadvantages of the SHF process is that accumulated sugars inhibit enzyme activity, resulting in lower ethanol productivity (Rastogi and Shrivastava 2017). The SSF process combines saccharification of biomass with simultaneous fermentation of released sugars, in a single reactor. The sugars produced are converted to ethanol, which reduces the inhibitory effect of sugar accumulation in the nutrient solution. Furthermore, the presence of ethanol reduces the susceptibility of the medium to

infection. The process's disadvantage is the difficulty in optimising the parameters of the enzymes, whose temperature optimum is around 50°C, and the microorganisms, which primarily ferment at temperatures ranging from 28 to 37°C (Hasunuma and Kondo 2012). The SSF strategy can be modified by performing simultaneous saccharification and co-fermentation of released hexoses and pentoses (SSCF). This bioconversion method is intended for processing lignocellulosic feedstocks with a high hemicellulose content and necessitates the use of microorganisms that ferment glucose and xylose into ethanol (Yasuda et al. 2014).

Table 4: Advantages and disadvantages of organisms potentially capable of ethanol fermentation of lignocellulosic hydrolysates (based on Limayem and Ricke 2012, Kordala et al. 2022)

Species	Characteristics	Advantages	Disadvantages
Saccharomyces cerevisiae	Facultative anaerobic yeast	- natural adaptation to ethanol fermentation - high efficiency of ethanol synthesis (>90% of theoretical yield) - high tolerance to ethanol (up to 10% by volume) and inhibitors - susceptibility to genetic modification	- lack of ability to ferment pentoses - low tolerance to high temperatures
Candida shehatae	Microaerophilic yeast	- xylose fermentation	- low tolerance to ethanol - low efficiency of ethanol synthesis - microaerophilic conditions required - low tolerance to low pH
Zymomonas mobilis	Ethanologenic Gram-negative bacteria	- ethanol synthesis efficiency exceeds *S. cerevisiae* (97% of theoretical efficiency) - high ethanol tolerance (up to 14% by volume) - high ethanol productivity - susceptibility to genetic modification	- lack of ability to ferment pentoses - low tolerance to inhibitors - neutral pH range
Pichia stipitis	Facultative anaerobic yeast	- efficient fermentation of pentoses - efficiency of ethanol synthesis about 82%	- low tolerance to ethanol - low performance in acidic environments - sensitivity to inhibitors

		- ability to ferment most sugars from cellulosic materials - have cellulases that favor the SSF process	- microaerophilic conditions required
Pachysolen tannophilus	Aerobic fungi	- pentose fermentation	- low efficiency of ethanol synthesis - microaerophilic conditions required - low efficiency in acidic environments
Escherichia coli	Mesophilic Gram-negative bacteria	- possibility of using pentoses and hexoses - susceptibility to genetic modification	- catabolic repression interferes with cofermentation - low tolerance to ethanol and inhibitors - narrow pH and temperature range - organic acid production
Kluveromyces marxianus	Thermophilic yeast	- ability to grow at high temperatures (45 - 52°C) - suitable for SSF and CBP processes - wide spectrum of fermented sugars - susceptibility to genetic modification	- low tolerance to ethanol - fermentation of xylose to xylitol
Mucor indicus	Dimorphic filamentous fungus	- ability to ferment pentoses and hexoses - high resistance to ethanol and inhibitors - fermentation yield up to 92% of theoretical yield	- high substrate requirements of culture

Consolidated bioprocessing (CBP) is an alternative to these options. CBP technology combines enzymatic hydrolysis of biomass with sugar fermentation in a process in which the fermenting microorganism also helps to produce cellulolytic enzymes (Jönsson et al. 2013). This technology uses a thermotolerant yeast strain, *K. marxianus*, which has been modified to simultaneously expose endoglucanase and β-glucosidase on its cell surface. *K. marxianus* can grow at 45 - 52°C and produces ethanol from cellulosic material with a yield of 0.47 g of ethanol per gram of digested β-glucan (Rastogi and Shrivastava 2017; Vohra et al. 2014).

To obtain pure fuel ethanol, the lignocellulose must be separated and concentrated at the end of the bioconversion process. Distillation, adsorption, or filtration using an azeotropic agent, molecular sieves, or membranes can be used to recover ethanol from the digestion broth. The solid residue is typically used as a solid fuel, but it can also be processed further to produce valuable final bioproducts (Rosales-Calderon and Arantes 2019; Isikgor and Becer 2015).

2.4.7. Improving the lignocellulose conversion process

Given the benefits of using lignocellulosic biomass, such as its low carbon footprint and, in some cases, low water footprint, product versatility, and local and global availability, there is a strong incentive to develop new processes and technologies that will increase the energy and production efficiency of biorefineries (Golberg 2015).

One promising new technology is the use of pulsed electric field (PEF). PEF has applications in several key aspects of biomass processing, including (1) improvement of biorefinery feedstocks by electroporation of genes, (2) drying of biomass, (3) extraction of high value-added products from waste, lignocellulosic biomass and microalgae, (4) extraction of selected molecules from bacterial and yeast cells, and (5) biogas production (Golberg et al. 2016). When a cell is exposed to PEF, additional transmembrane tension is induced on its membrane, allowing ions and molecules to pass through. The increase in membrane permeability can be temporary/reversible or permanent. In biorefinery processes, both reversible and irreversible electroporation can be used. Over the last four decades, research on PEF systems has resulted in the development of technologies that enable the large-scale biomass processing required for industrial scale biorefineries. PEF processes have produced very positive results that will aid in the development of many

aspects of biorefineries, ranging from feedstock refinement to product dehydration and extraction to waste processing (Golberg et al. 2016). Noteworthy processes include extraction of polyphenols from wine industry waste products (pomace, skins, seeds and vine shoots) (Boussetta et al. 2012); extraction of polyphenols and flavonoids (naringin and hesperidin) from orange peel (Luengo et al. 2013) or pretreatment of lignocellulosic biomass (Kumar et al. 2011).

One of the main improvements in the enzyme mixtures used to improve the enzymatic hydrolysis of lignocellulosic raw materials is the use of auxiliary enzymes such as lytic polysaccharide monooxygenases (LPMOs). LPMOs are non-hydrolytic copper ion-dependent enzymes (metal oxidases) that, under O_2 or H_2O_2 activation and in the presence of an electron donor, can cleave cellulose chains and hydrolyze β-1,4-glycosidic bonds by oxidizing a single oxygen atom on C1 or C4 glucose units in cellulose. The ability of LPMOs to create notch points on the fibre surface to facilitate tearing and separation of fibres makes them useful in the treatment of cellulose fibres. The enzymes act on the cellulose chains on the surface of the fibres, and their oxidative mode of action causes the fibres to tear and separate (Karnaouri et al. 2022; Ladevèze et al. 2017; Wu et al. 2022). LPMOs can also interact with chitin, starch, and hemicellulose (Bernardi et al. 2021). LPMOs are an integral component of commercial enzyme mixtures for industrial applications. Supplementation of hemicellulases or pectinases by LPMOs is beneficial for cellulose degradation by removing physical blockages and/or eliminating oligosaccharides that inhibit cellulase activity (Karnaouri et al. 2022; Song et al. 2018).

Another approach to improving the enzymatic hydrolysis step for efficient production of biofuels and biochemicals is to recycle lytic enzymes after the process. Recovering active enzymes after hydrolysis reduces the need for new enzyme doses, lowering the overall enzyme cost of the hydrolysis process. Recycling the insoluble biomass fraction after enzyme pretreatment has been shown to reduce enzyme consumption by 30% while having no effect on the final hydrolysis yield (Jørgensen and Pinelo 2017; Weiss et al. 2013; Hosseini Koupaie et al. 2019).

Increase microbial tolerance to fermentation inhibitors or change microbial metabolism toward substrates derived from lignocellulosic biomass is a practical approach to facilitating the use of lignocellulosic biomass for the production of biofuels and bioproducts. Microbial strains can be improved using genetic engineering, metabolic

engineering, and rational approaches based on synthetic biology, which requires knowledge of individual strain resistance as well as the basic functions of their genes and metabolic pathways (Wang et al. 2018). Adaptive laboratory evolution (ALE) is an alternative strategy for strain improvement based on evolutionary engineering (Wu et al. 2021, Hemansi et al. 2022). This method has been used successfully to increase the resistance of various microorganisms to inhibitors or to improve bioproduct synthesis efficiency under adverse conditions. Table 5 shows examples of successful ALE applications.

Table 5. Examples of microorganisms undergoing adaptive laboratory evolution

Microorganism	Purpose	Effect	Reference
Escherichia coli	CO_2 assimilation	Total use of biomass coal	Gleizer et al. 2019
Escherichia coli	Production of 1-butanol on minimal media	Increasing the efficiency of producing 1-butanol (up to 2 $g \cdot dm^{-3}$) in minimal medium	Pontrelli et al. 2018
Rhodosporidium toruloides	Resistance to lignocellulose-derived inhibitors	Faster growth on lignocellulosic hydrolysate, better efficiency of lipids synthesis (27.9% of dry biomass) and carotenoids synthesis (14.09 $mg \cdot g^{-1}$ dry biomass)	Liu et al. 2021
Saccharomyces cerevisiae GSY1136	Carotenoids production	3-fold increase in carotenoids productivity (up to 18 $mg \cdot g^{-1}$ of dry biomass)	Reyes et al. 2014
Saccharomyces cerevisiae after deletion of pyruvate decarboxylase genes	Redirecting metabolism from ethanol fermentation to lipogenesis	Ability to synthesize extracellular fatty acids (33.4 $g \cdot dm^{-3}$)	Yu et al. 2018
Kluyveromyces marxianus JKH5	Increased tolerance to fermentation inhibitors	3.3 times higher specific growth rate, 56% lower lag phase, 80% higher fermentation efficiency	Hemansi et al. 2022
Clostridium saccharoperbutyl-acetonicum	Increased tolerance to fermentation inhibitors	Increased acetic acid and 5-HMF tolerance, increased ABE fermentation yield (22.1 $g \cdot dm^{-3}$) and butanol synthesis (16.7 $g \cdot dm^{-3}$)	Alves et al. 2021

Zymomonas mobilis	Increased tolerance to fermentation inhibitors	Tolerance of acetic acid (up to 7 $g \cdot dm^{-3}$) and furfural (up to 3 $g \cdot dm^{-3}$), increase in ethanol synthesis yield to 94.84% of theoretical efficiency	Shui et al. 2015
Synechocystis sp. PCC 6803	Increased tolerance to the presence of alcohol in the culture medium	Tolerance to increased concentration of isobutanol in the medium (up to 5 $g \cdot dm^{-3}$), increase in ethanol synthesis yield (142% compared to the yield of the control strain)	Matsusako et al. 2017
Saccharomyces cerevisiae Ethanol Red ISO12	Increased tolerance to fermentation inhibitors and temperature	Fermentation of spruce hydrolysate at 39°C with a yield of 0.38 g ethanol/1g hexose	Wallace-Salinas and Gorwa-Grauslund 2013
Spathaspora passalidarum	Increase tolerance to the presence of acetic acid in the medium	After 100 days of gradually increasing acetic acid concentration (up to 4.5 $g \cdot dm^{-3}$) 7 times higher ethanol synthesis efficiency	Morales et al. 2017
Zymomonas mobilis	Ability to ferment xylose	Up to 250% increased utilization of xylose, 80% increased concentration of synthesized ethanol	Dunn and Rao 2016; Sarkar et al. 2020

3. Purpose and scope of the work

A review of the scientific literature shows that lignocellulosic biomass is a raw material with high hopes due to the possibility of replacing traditional fossil fuels. Plant biomass can be used to produce liquid and gaseous biofuels as well as value-added chemicals. Unfortunately, the variability of the composition of the lignocellulosic matrix of plants, as well as the complexity of the stages of substrate preparation, hydrolysis, and fermentation, require optimization of the process efficiency for each new raw material.

The following research hypotheses were developed in the work presented:

a) recycling enzymes adsorbed on the solid fraction of lignocellulosic material is an effective technique for increasing the economic efficiency of enzymatic hydrolysis of lignocellulosic hydrolysates from giant miscanthus, sugar miscanthus, and sugar beet biomass,
b) vacuum expansion of sugar beet biomass and microwave treatment of giant miscanthus biomass make the biomass more susceptible to enzymatic hydrolysis and promote alcoholic fermentation,
c) the use of laboratory adaptation of *Rhodotorula* yeast for the use of lignocellulosic hydrolysates from purple willow bark raffinate favours efficient carotenoids biosynthesis.

The scope of the study included:

1. Pretreatment of biomass of giant and sugar miscanthus, sugar beet, and purple willow bark raffinate for enzymatic hydrolysis in selected ways: vacuum expansion, mechanical shredding, microwave treatment in selected solvents (0.1 M NaOH, 0.1 M H_2SO_4, H_2O, 60% EtOH), alkaline delignification, detoxification of hydrolysates.
2. Imaging of structural changes in the tested lignocellulosic matrices subjected to initial pretreatment using scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR).
3. Enzymatic hydrolysis of prepared lignocellulosic matrices subjected to pretreatment using commercial lignocellulose degradation enzyme kits. Determination of hydrolysis efficiency based on the content of released reducing sugars in hydrolysates and the mass ratio of released reducing sugars to the enzyme preparation used.

4. Ethanolic fermentation and evaluation of the efficiency of ethanol production from lignocellulosic hydrolysates.
5. Determination of carotenoid biosynthesis by yeast strains *Rhodotorula glutinis*, *R. glutinis* KKP347, and *R. rubra* KKP350 from hydrolysates of purple willow bark raffinate.

4. Materials and methods

4.1. Research material

4.1.1. Giant miscanthus and sugar miscanthus

The biomass of giant miscanthus (*Miscanthus giganteus*) and sugar miscanthus (*Miscanthus sacchariflorus*) of a dry matter (d.m.) concentration 97.3% and 93.7%, respectively, came from the Production and Experimental Station in Bałcyny of the University of Warmia and Mazury in Olsztyn, Poland.

4.1.2. Sugar beet

Sugar beet root (*Beta vulgaris* L. subsp. *vulgaris*) of 22.4% d.m. and a total sugar content 17.2% (76.8% d.m.) was obtained from Netherlands.

4.1.3. Purple willow bark raffinate

The purple willow (*Salix purpurea*) obtained from the Experimental Station in Bałdy of the University of Warmia and Mazury in Olsztyn, Poland. The experimental material was a raffinate, which was the residue from the extraction of purple willow bark using supercritical carbon dioxide ($scCO_2$). Extraction was carried out in the Institute of New Chemical Syntheses (Puławy, Poland) at 40°C and 33 MPa using the Natex pilot plant (Ternitz, Austria) in two 40 dm^3 extractors. Extraction with $scCO_2$ was carried out from two 5-kg batches of raw material. The extraction time was 9 hours and approximately 200 kg of $scCO_2$ per kg of substrate was consumed.

4.1.4. Microorganisms

Ethanolic fermentation of plant biomass hydrolysates was carried out using the following microorganisms:

a) *Saccharomyces cerevisiae*, Ethanol Red strain (Lesaffre Advanced Fermentations). Yeast was used in the form of yeast suspension ($1.25 \pm 0.12 \times 10^9$ $CFU \cdot cm^{-3}$) prepared by suspending 1g of yeast lyophilizate in 10 cm^3 of sterile 0.9% (w/v) NaCl and rehydrating for 30 min at room temperature (according to the manufacturer's instructions). Yeast suspension was added to cultivation medium in amount of 4 $cm^3 \cdot dm^{-3}$,
b) *Saccharomyces cerevisiae* 7, NRRL 978 strain (Agricultural Research Service Culture Collection). The strain was stored at 4°C on YPG medium. Fermentation of plant biomass hydrolysates was carried out after addition of yeast inoculum (5%, v/v),

c) *Saccharomyces cerevisiae* D2 from the Department of Distillery Technology and Renewable Energy Sources in Bydgoszcz, Poland. The strain was stored at 4°C on YPG medium. Fermentation of plant biomass hydrolysates was carried out after addition of yeast inoculum (5%, v/v),
d) *Mucor rouxii* 1191 (*Mucor indicus*) from the collections of the Leibniz Institute DSMZ, Germany. The strain was stored at 4°C on PDA medium. Fermentation of plant biomass hydrolysates was carried out after the addition of yeast inoculum (5%, v/v).

Carotenoid synthesis was carried out in YGC test medium with the following yeast strains:

a) *Rhodotorula glutinis* from the German Collection of Microorganism Strains and Cell Cultures,
b) *Rhodotorula glutinis* KKP347 from Institute of Agriculture and Food Biotechnology – State Research Institute – Collection of Industrial Microbial Cultures,
c) *Rhodotorula rubra* KKP350 from Institute of Agriculture and Food Biotechnology – State Research Institute – Collection of Industrial Microbial Cultures.

The synthesis of carotenoids on the experimental media was carried out at 30°C, 72h, 300 rpm, after the addition of a yeast inoculum 5% (v/v). Incubation was conducted in Innova 44 Incubator Shaker (New Brunswick Scientific, Edison, USA).

4.1.5. Microbiological media

a) The following microbiological media were used in the experiments:
b) YPG containing in 1000 cm^3: yeast extract, 10 g; glucose, 20 g; peptone, 20 g; agar, 20 g; pH 5.1±0.1,
c) PDA containing in 1000 cm^3: potato extract, 4 g; glucose, 20 g; agar, 15 g; pH 5.5±0.1,
d) YGC containing in 1000 cm^3: yeast extract, 5 g; glucose, 20 g; chloramphenicol, 0.1 g; (liquid medium) and agar, 15 g (solid medium); pH 5.8±0.1.

4.2. Procedures of lignocellulose materials pretreatment, enzymatic hydrolysis and ethanol fermentation

4.2.1. Pretreatment of plant biomass

a) Alkaline treatment in a pressure reactor

Biomass of giant miscanthus and sugar miscanthus, as well as refined purple willow bark, was ground using a cutting mill (Retsch SM100, Verder, Vleuten, Netherlands) to a particle size of 1 - 2 mm. The crushed raw material was subjected to thermal pretreatment with sodium hydroxide (120°C, 60 min., NaOH dose 0.1 $g \cdot g^{-1}$ d.m. of raw material, 1:9, w:v) (Świątek et al. 2014). Hydrolysis was carried out in a pressure reactor (SMS, ASVE model, Góra Kalwaria, Poland). The suspension obtained after biomass treatment was centrifuged (4150×g, 10 min., 5°C). The obtained supernatant was separated, and the solid fraction was detoxified by washing twice with deionized water (2000 cm^3/kg biomass) and centrifuged again. The purified feedstock was mixed with deionized water in a ratio of 1:9, w:v. The acidity of the reaction medium was set at pH 5.0±0.1 with concentrated phosphoric acid(V), then pasteurized (90°C for 20 min) and subjected to enzymatic hydrolysis after cooling.

b) Vacuum expansion

Purified (washed) sugar beet roots were mechanically crushed using a laboratory screw press (ZMM1084S, Zelmer, Warsaw, Poland), and subjected to vacuum expansion. To carry out the vacuum expansion, sugar beet roots were finely chopped to particle size of 5 - 10 mm and heated to 60 - 65°C, before being processed in a reactor (DSD BetaProcess, Choorhoekseweg, Netherlands) (Figure 5), under a vacuum of 0.2 - 0.3 MPa. The treated material in the amount of 50 g was transferred to a 300 cm^3 conical flask and 25 cm^3 of deionized water was added. The acidity of the reaction medium was set at pH 5.0±0.1 with concentrated phosphoric acid(V), then pasteurized (90°C for 20 min) and subjected to enzymatic hydrolysis after cooling.

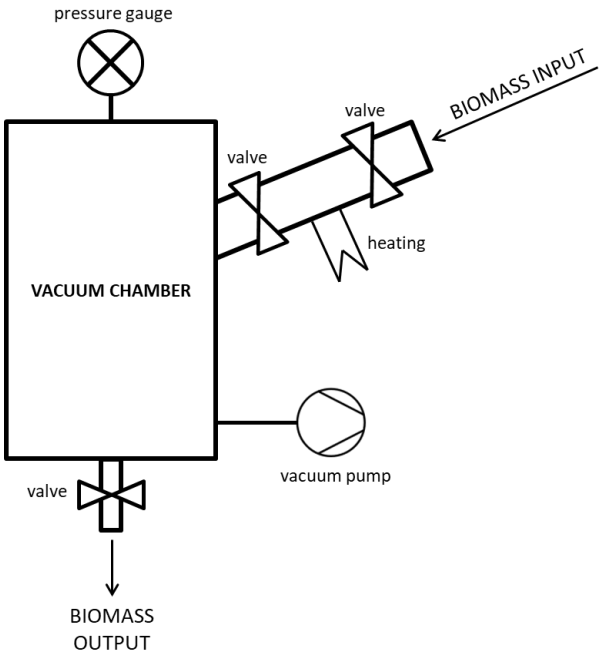


Fig. 5. Scheme of the vacuum expansion device

c) Microwave-assisted biomass pretreatment

Microwave pretreatment was carried out in a Microwave Digestion System Mars 5 (CEM Corporation, Matthews, NC, USA). The procedure began by adding raw material to solvent in a ratio of 1:10 (w:v) in a teflon tube. The microwave pretreatment was carried out according to the procedure developed by Mikulski et al. (2019), with modifications. The experiment included the use of various solvents: (0.1N NaOH, 0.1N H_2SO_4, H_2O, 60% EtOH) and pressure (30, 60, 90 psi). The power of the microwave generator was 600 W, and the pretreatment time was 60 min. After pretreatment, the suspensions were cooled to about 20°C, and the samples were centrifuged (4150×g, 10 min., 5°C). The biomass was washed twice with deionized water in 2000 cm^3/kg biomass. A 5 g of biomass with 50 cm^3 deionized water was transferred to 100 cm^3 conical flasks and adjusted to pH 5.0±0.1 using 30% NaOH or 30% HCl. The material thus prepared was pasteurized (90°C, 20 min) and subjected to enzymatic hydrolysis after cooling.

4.2.2. Enzymatic hydrolysis of substrates

For the hydrolysis of giant miscanthus, sugar miscanthus and purple willow bark raffinate, the following enzymes were used: Accellerase 1500 (a mixture of cellulases and β-glucosidase) and a mixture of enzymes supporting hydrolysis (complex of supporting

enzymes - KEW) composed of: Accellerase BG (*T. reesei*, β-glucosidase), Accellerase XC (*P. funiculosum*, endoglucanases and xylanases) and Accellerase XY (*T. reesei*, xylanase) (DuPont, USA). Enzyme doses were selected based on previous experiments (Walter et al. 2021): Accellerase 1500 (35 FPU - 0.6 $cm^3 \cdot g^{-1}$ d.m.), Accellerase BG (270 pNPGU - 0.09 $cm^3 \cdot g^{-1}$ d.m.), Accellerase XY (1250 ABXU - 0.05 $cm^3 \cdot g^{-1}$ d.m.), Accellerase XC (394 ABXU - 0.125 $cm^3 \cdot g^{-1}$ d.m.).

For the hydrolysis of sugar beet, the pectinolytic enzyme Rapidase® (DSM, the Netherlands) was also used at a dose of 0.02 $cm^3 \cdot g^{-1}$ d.m., recommended by the manufacturer.

The reducing sugars release productivity was calculated according to the formula (1):

$$P = \frac{c \times V_1 \times s}{V_2}, \frac{g}{g\ of\ DM} \quad (1)$$

where *P* is the mass of the reducing sugars released from the substrate ($g \cdot g^{-1}$ d.m.), *c* the concentration of the reducing sugars released ($g \cdot dm^{-3}$), *V1* the volume of the reaction medium (dm^3), *s* the dry substance content in the raw material (g), *V2* the hydrolysate volume (dm^3).

The efficiency of the hydrolysis of polysaccharides was calculated in relation to the concentration of cellulose and hemicellulose in the raw material according to the formula (2):

$$W_1 = \frac{c \times 0{,}9}{k} \times 100, \% \quad (2)$$

where W_1 is the efficiency of reducing sugars release (%), *c* is the concentration of released reducing sugars ($g \cdot dm^{-3}$), 0.9 is the conversion factor of simple sugars to polysaccharides, *k* is the percentage of cellulose and hemicellulose in the raw material (% d.m.).

Cellulose hydrolysis efficiency (W_2) calculated from the cellulose concentration in the biomass by the formula (3):

$$W_2 = \frac{c \times 0{,}9}{k_c} \times 100, \% \quad (3)$$

where *c* is the concentration of released glucose ($g \cdot dm^{-3}$), 0.9 is the conversion factor of simple sugars to polysaccharides, k_c is the concentration of cellulose in biomass (% d.m.).

The enzyme productivity (Pm) was calculated by the formula (4):

$$P_m = \frac{c}{m}, \ \frac{\text{mg of glucose}}{\text{mg of protein}} \quad (4)$$

where *c* is the concentration of released glucose (mg·cm^{-3}), *m* is the protein concentration in the mixture of enzyme preparations added to the hydrolysis (mg·cm^{-3}).

4.2.3. Ethanol fermentation

For the preparation of *Saccharomyces cerevisiae* 7, D2 and *Mucor indicus* inoculum, the media listed in section 4.1.6 were used. Biomass from solid media was transferred with a microbiological loop (⌀ 5mm) to the media and incubated at 30°C, in a rotary shaker at 120 rpm for 24 hours. Inoculum was added to the hydrolysates (5%, v/v) and SHF fermentation was carried out in 500 cm^3 conical flasks at 30°C under stationary conditions for 72 or 96 hours for *S. cerevisiae* 7 or *M. indicus*, respectively.

To carry out the SSF process, appropriate amounts of enzyme preparations were added to the sugar beet biomass suspension and hydrolysis was carried out at 60°C for 15 min using shaking at 250 rpm. After hydrolysis, 3.7 cm^3 (5%, v/v) inoculum of *S. cerevisiae* strain D2 was added to the media. Fermentation was carried out in 500 cm^3 conical flasks at 38°C for 96 h under stationary conditions.

Fermentation of the enzymatic hydrolysate of giant miscanthus biomass obtained after microwave treatment (section 4.2.2) was carried out using the *S. cerevisiae* Ethanol Red strain. The efficiency of enzymatic hydrolysis was determined according to the formula (5):

$$Yield\ of\ hydrolysis = \frac{C_{glu}}{1.111 \times C_{cel} \times C_{biom}} \times 100, \% \quad (5)$$

where C_{glu} is the glucose concentration (g·dm^{-3}) in enzymatic hydrolysate liquid, C_{cel} is the fraction of cellulose in the raw biomass as determined by compositional analysis (g·g^{-1} d.m.) and C_{biom} is the initial solid concentration at which the enzymatic hydrolysis was performed (g·dm^{-3}) (Rana et al. 2014).

After each fermentation, the suspension was centrifuged (4100×g, 10 min, 5°C) and the ethanol concentration and reducing sugars content were determined in the supernatant.

4.2.4. Laboratory adaptation of yeast *Rhodotorula* sp. to the use of purple willow bark raffinate as a substrate for production of carotenoids

The strains used in the experiment were: *R. glutinis*, *R glutinis* KKP347 and *R. rubra* KKP350, which were stored at 4°C in YGC medium.

Strains were transferred onto YGC solid medium and cultured for 7 days at 30°C. To adapt the strains to growth on hydrolysate from purple willow bark raffinate (H), the strains were passaged twice onto media with increasing by 5% (v/v) hydrolysate content, as shown in Fig. 6. After obtaining strains grown on solid YGC:H medium (1:1, v/v), carotenoids were synthesized in the following liquid media: (i) YGC, (ii) YGC:H (1:1, v/v). For this purpose, 50 cm^3 of culture medium, pH 5.8±0.1, was added to 100 cm^3 conical flasks. The inoculum of the respective strain (5% v/v) was added to the media and the cultivation was conducted for 7 days at 30°C, mixing the samples in an incubator (Innova 44 Incubator Shaker, New Brunswick Scientific, Edison, USA) at 300 rpm. The samples were centrifuged (4000×g, 15 min, 10°C) and the carotenoids content in biomass was determined.

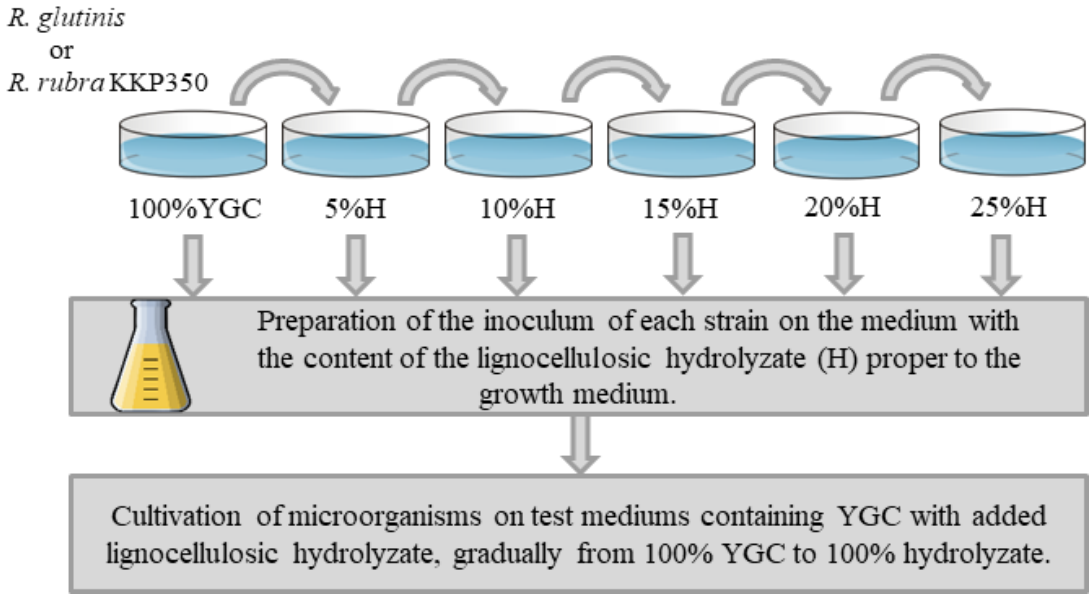


Fig. 6. Schematic of the experiment of the directed adaptation of the yeast *Rhodotorula* sp. to redirect its metabolism towards the utilization of lignocellulosic hydrolysate (own elaboration)

4.3. Analytical methods

4.3.1. Determination of dry matter content

The dry matter content in the samples was determined using a moisture analyser (Radwag WPS-30S, Radom, Poland) with operating parameters: 130°C and sampling time 20 s.

4.3.2. Determination of the composition of lignocellulosic fractions in plant biomass samples

Neutral-detergent fiber (NDF) (Van Soest et al. 1991), acid-detergent fiber (ADF) and acid-detergent lignin (ADL) (PN-EN ISO 13906:2009) were determined in the samples using a Fibertec™ 1020 (FOSS, Hilleroed, Denmark) instrument. Cellulose concentration was calculated from the difference between ADF and ADL fractions, while hemicellulose concentration was calculated from the difference between the proportion of NDF and ADF fractions.

4.3.3. Determination of pectin content

The pectin content of the samples was determined using the Morris method (Morris 1948), with modifications (Pijanowski et al. 1973). A 20.00 g of sample was weighed into a 300 cm^3 flask and 30 cm^3 of distilled water was added. The sample was shaken for 15 minutes and then filtered into a dry flask. The residue, together with a filter paper, was transferred again to the flask, water was added and shaken; the extraction was repeated three times. The resulting filtrate was transferred to a 100 cm^3 volumetric flask and made up to the mark with distilled water. A 25 cm^3 of the solution was measured into a 300 cm^3 beaker, 50 cm^3 of acetone was added and the sample was allowed to stand for 1 hour before being filtered through a previously dried and weighed filter. The filter was dried at 75°C for 30 minutes and weighed. The pectin content (%) was determined as difference between the weight of the filter with precipitate after drying and the weight of the dried filter, considering the amount of pectin solution taken for the determination and the sample weight.

4.3.4. Determination of crude fiber content

The wet mineralization method according to PN-ISO 5498:1996 was used to determine the crude fiber content.

4.3.5. Determination of crude ash content

The procedure NREL/TP-510-42622 was used to determine the crude ash content of the samples.

4.3.6. Determination of total protein content

The protein content of the samples was determined based on the nitrogen content determined by the Kjeldahl method, according to the methodology contained in the Polish Regulation of the Minister of Agriculture and Rural Development of 2 December 2004 (Journal of Laws No. 271, item 2688).

4.3.7. Determination of total sugar concentration

The determination of total sugar concentration was carried out using the anthrone method (Jacórzyński and Hryniewicz 1978). The anthrone reagent (50 mg of anthrone and 1 g of thiourea in 34 cm^3 of water with 66 cm^3 of concentrated sulphuric acid (VI). To 0.5 cm^3 of a sample containing 25-150 μg of sugar a 5 cm^3 of anthrone reagent was added. The mixture was brought to room temperature and then heated in a boiling water bath for 16 minutes. After cooling to 20°C, the absorbance of the sample was measured at λ = 620 nm (Lambda XLS spectrophotometer, PerkinElmer®, Massachusetts, USA). The concentration of total sugars in the samples was calculated using a standard curve drawn up for glucose, prepared for each analysis.

4.3.8. Determination of the reducing sugars concentration

The concentration of reducing sugars in the samples was determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959), with modifications (Yu et al. 2016). Samples were applied to a 96-well microplate. Absorbance at λ = 540nm was measured using an Infinite® F200 microplate reader (Tecan, Mannedorf, Switzerland). The concentration of reducing sugars in the samples was calculated using a standard curve drawn up for glucose, prepared for each analysis.

4.3.9. Determination of monosaccharides using high-performance liquid chromatography (HPLC)

Determination of the concentration of selected monosaccharides was performed based on peak areas related to the peak areas of standard solutions: glucose, sucrose, fructose, and arabinose (Chempur, Poland). Analysis was carried out using an Agilent 1200 kit with a RID refractometer detector (45°C) (Palo Alto, California, USA). An Animex HPX-87H, 300 mm × 7.8 mm column thermostated at 45°C was used. A 5 μl

sample was applied to the column using an autosampler, and a 10 mM H_2SO_4 solution and a flow rate of 0.6 $cm^3 \cdot min^{-1}$ was used. ChemStation software (Agilent Technologies®) was used to analyse the chromatograms.

4.3.10. Determination of ethanol concentration using gas chromatography (GC)

Ethanol concentration was determined according to the method of Gerchman et al. (2012). 600 µl of the sample was transferred to a 2 cm^3 tube and centrifuged (8000×g, 2 min, room temperature). Then 500 µl of the clear supernatant was transferred to a new tube, followed by the addition of 5 µl of *n*-butanol (as an internal standard) and the tube was vortexed for 30 s. Then 1 cm^3 of ethyl acetate was added, and the sample was vortexed for 5 min. Finally, the tubes were centrifuged to facilitate phase separation (5000×g, 2 min, ambient temperature) and the organic phase (top) was subjected to chromatographic analysis using a Clarus® 600 gas chromatograph (PerkinElmer®, Massachusetts, USA), equipped with a 60m Zebron ZB-5 column (30m × 0.25mm × 0.25µm), with autosampler and FID detector: 250°C; flow H_2: 25 $cm^3 \cdot min^{-1}$; air: 100 $cm^3 \cdot min^1$. Pressure-controlled N_2 (24 psi) was used as the carrier gas. The GC was connected to a computer running TotalChrom software version 6.3. The oven temperature (and thus the column and injection temperature) was initially set at 50°C and then raised at a rate of 7°C/min to 100°C, giving a total run time of 7 min. The injection volume was 2 µl. Each injection was repeated three times. Standard solutions of ethanol were prepared in distilled water containing 1% (v:v) of n-butanol as an internal standard, extracted and injected. Peak area ratios of the ethanol vs. n-butanol were calculated and plotted against ethanol concentration (% v:v) to afford a calibration curve which served for ethanol quantification in the fermentation samples.

4.3.11. Determination of selected carbohydrates, inhibitors, and ethanol concentration using high-performance liquid chromatography (HPLC)

The concentrations of these compounds were determined by high-performance liquid chromatography (HPLC) (Mikulski and Kłosowski, 2018). All samples analysed by HPLC were diluted 5-fold with 5 mM H_2SO_4 and then filtered through a membrane filter (PES, 0.45 µm). Chromatographic separation was carried out using an Agilent Technologies® system 1260 equipped with an RID detector (45°C). A Hi-Plex H chromatographic column (Agilent Technologies®) together with a protection column was used under isocratic conditions. The mobile phase was 5 mM H_2SO_4 at flow rate of 0.6 $cm^3 \cdot min^{-1}$. The column operating temperature was 60°C. The concentration of the

analyzed compounds, i.e., glucose, galactose and xylose, arabinose, acetic and levulinic acid, 5-HMF, furfural, vanillin, vanillic acid, syringaldehyde and ethanol, was determined by the external standard method (ESTD). The standards of the analysed compounds (>98% purity) came from Sigma-Aldrich® (Saint Louis, USA). The use of a Hi-Plex H column does not allow the separation of a mixture of xylose and galactose; therefore, the concentration of these sugars is given as the concentration of both compounds.

4.3.12. Measurement of CO_2 emission

The CO_2 emission after ethanol fermentation was determined from the difference in mass (AS 220.R2 scale, Radwag, Radom, Poland) of the fermentation flasks before and after fermentation.

4.3.13. Protein profile analysis

Sugar beet proteins were extracted according to the method described by Parpinello et al. (2004) with modifications. Analysis of the protein profile in the reaction mixtures was carried out by SDS-PAGE electrophoresis using the Mini PROTEAN II system (BioRad, Hercules, USA), according to the manufacturer's instructions (gel concentration: 10%, electrophoresis run time: 45 min., room temperature, 200V constant voltage setting, Coomassie R-250 staining, molecular weight marker: SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad, Hercules, USA).

4.3.14. Scanning electron microscope (SEM) imaging of samples

Dried samples collected after physical treatment and after hydrolysis were applied to a microscope table in a sputtering microscope (Fine Coater JFC 1200, JEOL, Nikon Metrology, Tokyo, Japan) and analysed using a scanning microscope with an image processing computer kit (Quanta 200) at 30 kV, according to the methodology of Szczęsny et al. (2012).

4.3.15. FTIR analysis of microwave treated biomass

Microwave pretreated plant biomass samples were dried at 130°C to constant weight, ground and sieved through a 150 μm sieve. A 2 mg sample was mixed with 200 mg KBr of ≥99.9% purity and ground in an agate mortar. Each sample was then subjected to 2 tons for 5 min in a hydraulic press (Specac Ltd. Kent, UK). Spectra were collected using a Nicolet iS5 spectrometer (Thermo Fisher Scientific, Waltham, USA) in the range 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}, 72 scans per sample (Hartati et al. 2021).

4.3.16. Extraction of carotenoids from Rhodotorula sp.

Extraction was performed using the method described by Kot et al. (2017) with modifications. A 200 mg of wet yeast biomass was transferred to a tube (2 cm^3) and washed twice with distilled water. The supernatant was poured off and 1 cm^3 of a mixture of acetonitrile : isopropanol : ethyl acetate (4:4:2, v:v:v) was added to the biomass and samples were shaken for several minutes. Zirconia beads were poured into an extraction tube and the biomass was transferred into the tube with the solvent. The extraction tubes were placed in a disintegrator (FastPrep-24™, MP Biomedicals, Irvine, USA) and the process was carried out twice: 5.0 $m \cdot s^{-1}$ for 30 s. Samples were centrifuged (3 min, 7400×g) and the supernatant was transferred to clean Eppendorf tubes for further analysis.

4.3.17. Spectrophotometric analysis of carotenoid content

Spectrophotometric measurement of the samples was carried out at λ = 457 nm and the carotenoid content was calculated based on a calibration curve prepared for pure β-carotene (Sigma-Aldrich®, Saint Louis, USA) (Kot et al. 2017). Standard curve equation: y = 0,0979x – 0,0049.

4.3.18. Optical density measurement

OD measurements were obtained at λ=595 nm reference wavelength (Infinite® F200 microplate reader, Tecan, Mannedorf, Switzerland), using 96-well plates, filled with 300 µl of bacterial suspension. The plates were incubated at 30°C for 168 hours and shaken before each measurement. The kinetics of the growth of microorganisms were determined after absorbance measurements, made every 6 hours.

4.4. Statistical methods

All analyses were performed in triplicate. Statistical analysis was performed using Statistica ver. 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). The experimental results obtained were statistically processed using analysis of variance (ANOVA) with Tukey's RIR test at a significance level of α = 0.05.

5. Discussion of the results

5.1. Enzyme recycling as a method to improve enzymatic hydrolysis of lignocellulosic raw materials

The technique and technology for processing lignocellulosic biomass for industrial applications must be low-cost, efficient, ensure minimal loss of sugars, and generate small amounts of inhibitors of enzymatic and fermentation processes. In biotechnological processes, the costs of enzyme preparations are significant (Ferreira et al. 2018), so it is reasonable to look for methods to reduce them. In the work presented here, it was assumed that the lytic enzymes adsorbed on the solid particles of the raffinate are still active, so that when a fresh portion of the pretreated lignocellulosic biomass is added, another hydrolysis cycle can be performed without the addition of new enzyme doses. This experiment was conducted on giant miscanthus and sugar miscanthus biomass with the profile of lignocellulosic components shown in Table 6.

Table 6. Lignocellulosic components of biomass obtained from miscanthus giganteus and miscanthus sacchariflorus

Sample	NDF [% d.m.]	ADF [% d.m.]	ADL [% d.m.]	Concentration [% d.m.]	
				Cellulose	Hemicellulose
Giant miscanthus	80.58 [a]	55.94 [a]	9.77 [b]	46.17 [a]	24.64 [a]
Sugar miscanthus	81.16 [a]	56.07 [a]	11.29 [a]	44.78 [b]	25.09 [a]

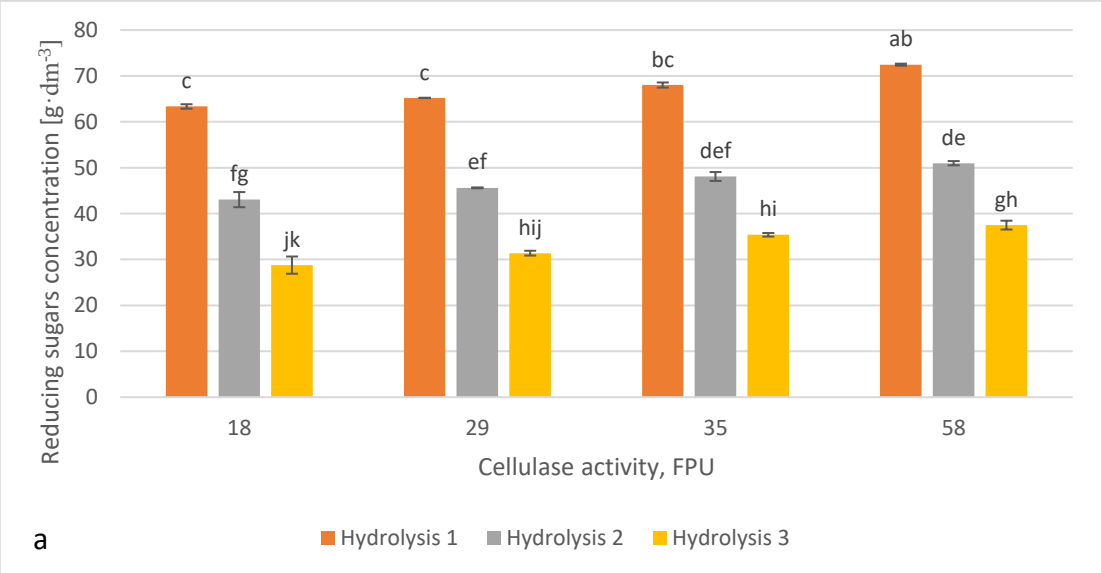
The letters (a, b) in columns indicate statistical significance at $p < 0.05$.

The biomass of giant and sugar miscanthus after grinding was subjected to alkaline pretreatment, the main task of which was the hydrolysis of lignin. Delignification is the preferred stage of plant biomass bioconversion because the highly cross-linked lignin coatings protect the cellulose and hemicellulose fibres that form the structural backbone of plant cell walls from depolymerizing enzymes (Brelowska et al. 2018; Sindhu et al. 2016). Simultaneous partial depolymerization of hemicelluloses, a decrease in cellulose crystallinity, and an increase in complex porosity make lignocellulosic biomass more susceptible to enzymatic hydrolysis (Kotarska et al. 2015).

Alkaline treatment is recognized as one of the most effective methods of preparing lignocellulosic biomass (Agbor et al. 2011). Lewandowska et al. (2016) obtained a hydrolysis efficiency of 95.0% after alkaline treatment of giant miscanthus biomass, with a concentration of reducing sugars of 51.5 g·dm^{-3}, while a hydrolysis efficiency of 99.3% after oxalic acid treatment, with a concentration of reducing sugars of only 26.3 g·dm^{-3}. After applying the alkaline pretreatment of sugar miscanthus, the polysaccharide hydrolysis efficiency was 98.6% (Cha et al. 2015). Kordala et al. (2013) obtained an 83.4% efficiency of hydrolysis of giant miscanthus polysaccharides using an alternative ammonia pretreatment, similarly to results obtained by Przybysz et al. (2018).

Following alkaline pretreatment, only a small amount of saccharides expressed as reducing sugars, i.e. 0.13 mg·g^{-1} d.m. from giant miscanthus, and 0.18 mg·g^{-1} d.m. from sugar miscanthus were found in solvent. This indicates that nearly 100% of the original cellulose and hemicellulose fraction was preserved.

Following detoxification, the solid residues with adsorbed enzymes from the first hydrolysis of the lignocellulosic biomass of both *Miscanthus* species were used to carry out two subsequent cycles of hydrolysis with fresh batches of plant matrices. Hydrolases were found in the polysaccharide hydrolysis residue, and the enzyme productivity during the first reaction was 3.0 - 4.2 mg glucose/mg enzyme protein, in the second reaction it was 1.7 - 2.5 mg/mg protein, and in the third reaction it was 1.3 - 1.9 mg/mg protein. Figures 7-10 illustrate changes in the concentration of released reducing sugars, productivity, and process efficiency.



a

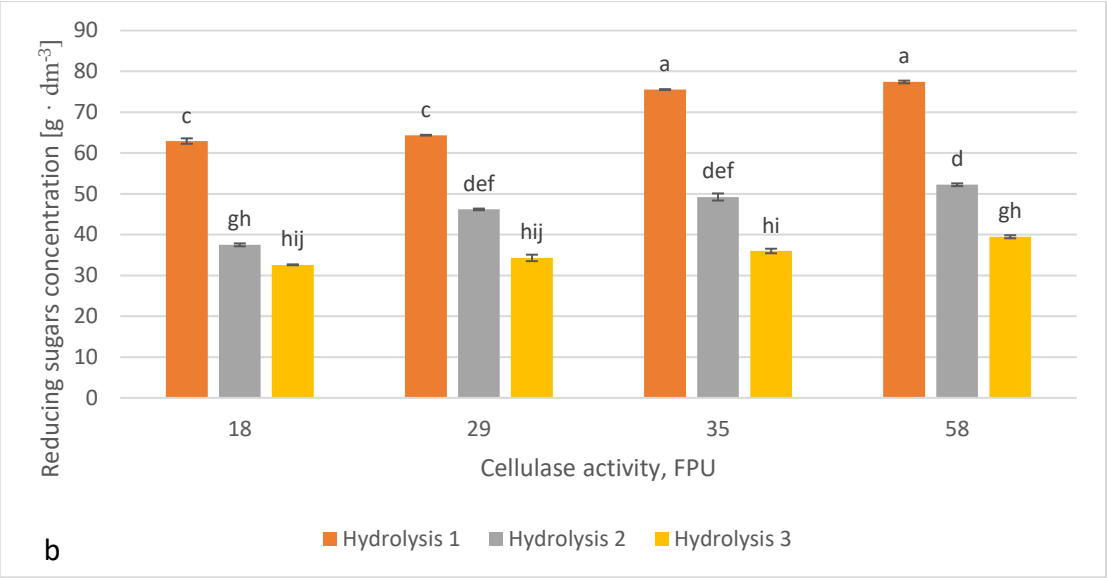


Fig. 7. Concentration of reducing sugars in biomass samples of *Miscanthus giganteus* (a) and *Miscanthus sacchariflorus* (b) after enzymatic hydrolysis (hydrolysis 1) and after recycling of the solid fraction (hydrolysis 2, 3). The letters (a, b, c, …) indicate statistical significance at $p < 0.05$.

After the first enzymatic hydrolysis, the highest concentration of reducing sugars (77.39 $g \cdot dm^{-3}$) was obtained after hydrolysis of sugar miscanthus biomass using the highest dose of Accellerase 1500 (Fig. 7b). A slightly lower concentration of reducing sugars (72.46 $g \cdot dm^{-3}$) was obtained after hydrolysis giant miscanthus biomass under the same conditions (Fig. 7a). The concentration of reducing sugars after the last hydrolysis reaction accounted for 45.4 - 51.7% and 47.6 - 53.3% of the concentration of sugars after the first hydrolysis of giant miscanthus and sugar miscanthus polysaccharides, respectively. The highest initial cellulolytic enzyme dose allowed for the highest activity associated with the residual solid fraction after hydrolysis and the highest lignocellulose hydrolysis efficiency.

The productivity of releasing reducing sugars from the biomass of giant miscanthus and sugar miscanthus was 0.61 - 0.70 $g \cdot g^{-1}$ d.m. and 0.60 - 0.73 $g \cdot g^{-1}$ d.m., respectively (Fig. 8). Statistically significant differences in productivity were shown for successive enzymatic reaction cycles ($p < 0.05$). After hydrolysis of sugar miscanthus biomass, significantly higher productivity of reducing sugars was obtained in majority of samples.

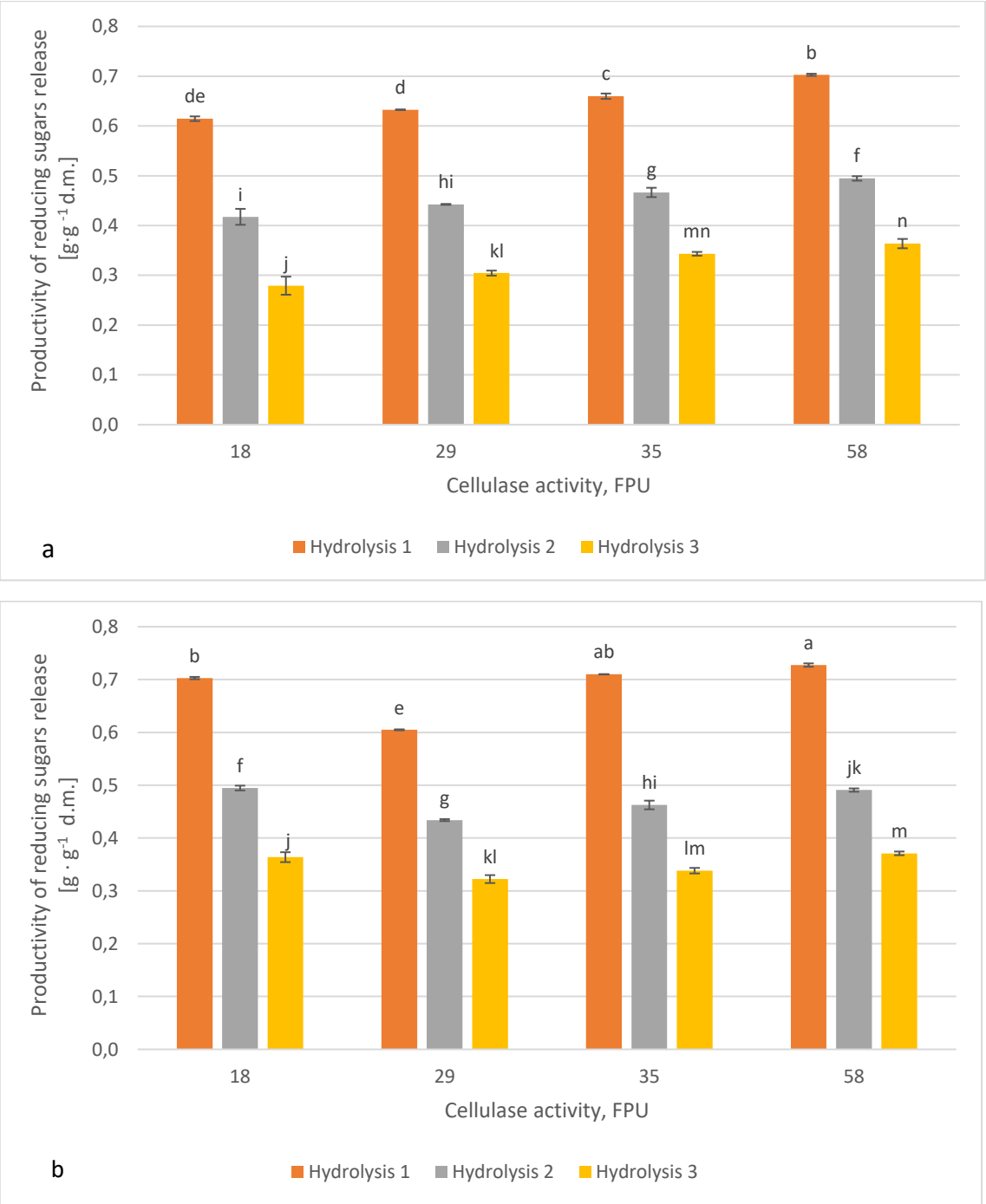


Fig. 8. Productivity of reducing sugar release from biomass obtained from *Miscanthus giganteus* (a) and *Miscanthus sacchariflorus* (b), after enzymatic hydrolysis (hydrolysis 1) and after recycling of the solid fraction. The letters (a, b, c, …) indicate statistical significance at $p < 0.05$.

Efficiency of hydrolysis of polysaccharides of giant miscanthus (31.9 - 90.1%) (Fig. 9a) and sugar miscanthus (26.9 - 96.2%) biomass (Fig. 9b) and cellulose hydrolysis in these species, respectively 22.9 - 61.6% (giant) and 10.1 - 69.8% (sugar) (Figs. 10a and

10b) were at similar levels, with a slight preference to extract saccharides from sugar miscanthus. Subsequent hydrolysis reactions showed no significant differences in enzyme stability between giant and sugar miscanthus biomass.

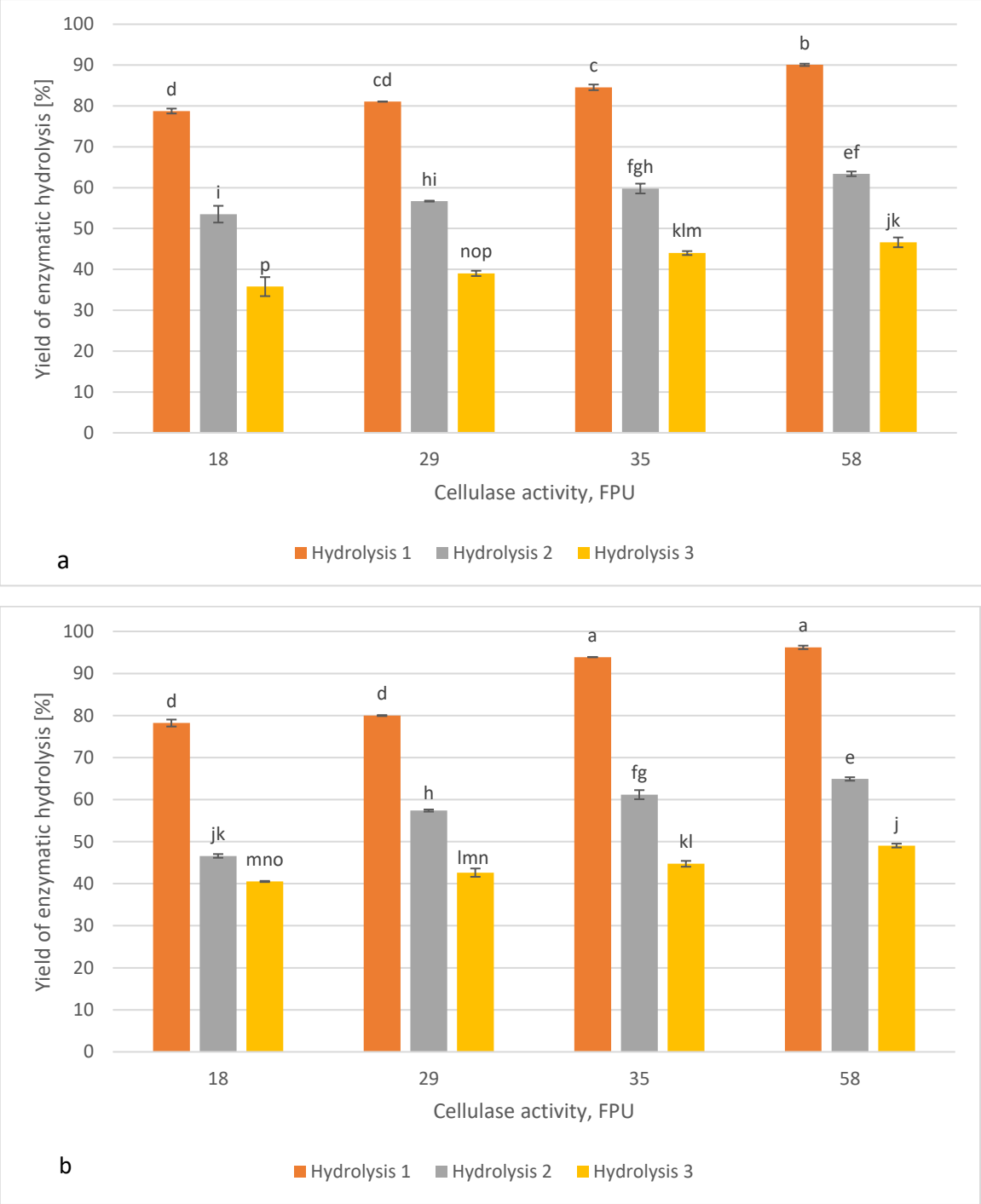


Fig. 9. Yield of enzymatic hydrolysis of polysaccharides from biomass obtained from *Miscanthus giganteus* (a) and *Miscanthus sacchariflorus* (b) after enzymatic hydrolysis (hydrolysis 1) and recycling of the solid fraction (hydrolysis 2, 3). The letters (a, b, c, …) indicate statistical significance at $p < 0.05$.

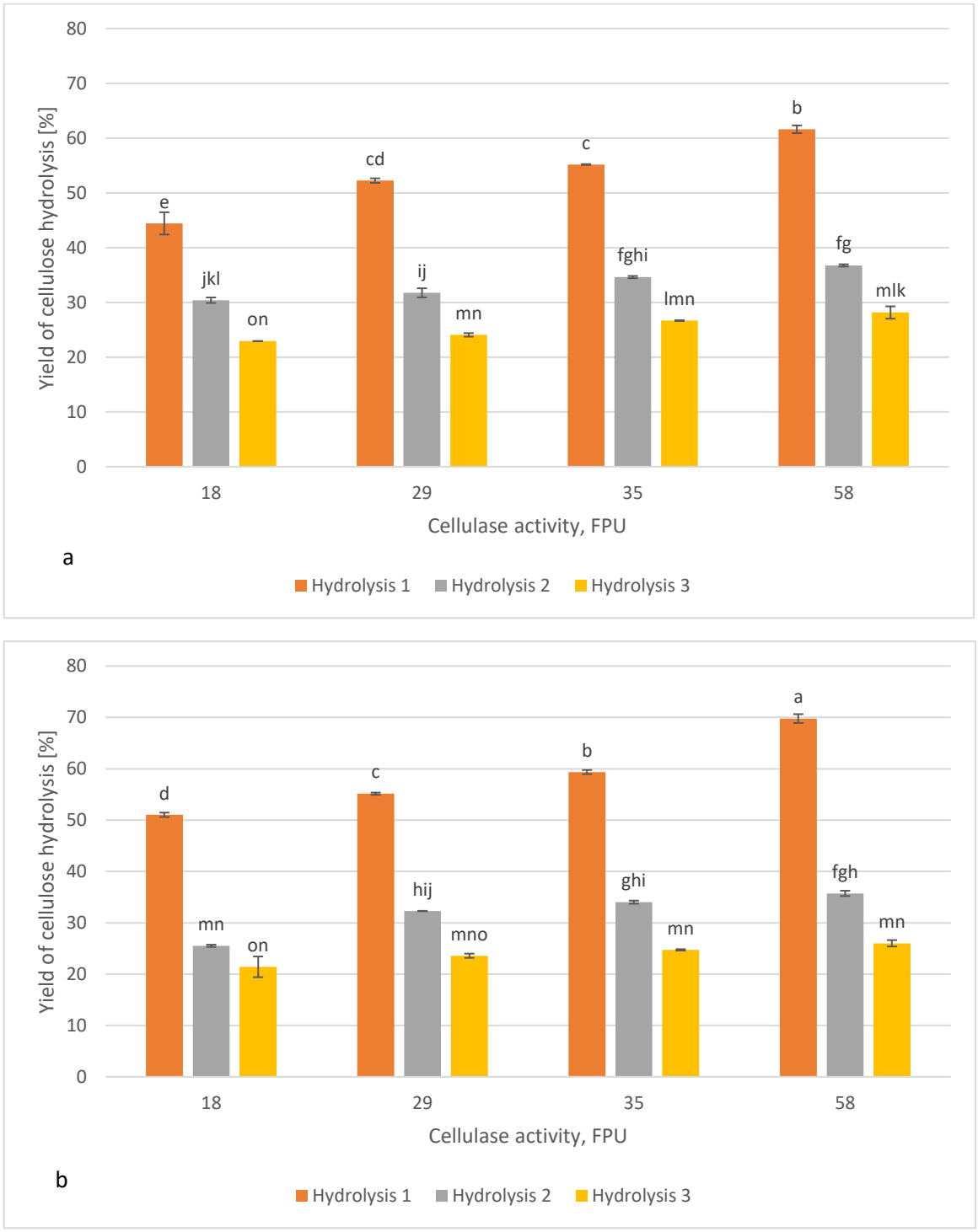


Fig. 10. Yield of enzymatic hydrolysis of cellulose from biomass obtained from *Miscanthus giganteus* (a) and *Miscanthus sacchariflorus* (b) after enzymatic hydrolysis (hydrolysis 1) and recycling of the solid fraction (hydrolysis 2, 3). The letters (a, b, c, …) indicate statistical significance at $p < 0.05$.

The economic calculation is crucial for the development of lignocellulosic biomass applications for biofuel production. According to Gomes et al. (2018), the location of the enzyme in the liquid-solid reaction mixture system, as well as the thermostability of cellulases, play a critical role in their recoverability and reusability. The cited authors recovered 80% of Celluclast activity from the liquid-solid fraction after hydrolysing sludge from a wastepaper processing plant's wastewater using ultrafiltration. On the other hand, the repeated use of cellulases for hydrolysis of spent oilseed (*Elaeis guineensis*) bunches was hampered by cellulase inhibition by glucose and non-specific enzyme binding to the solid fraction (Kim et al. 2019). When polyethylene glycol was added to the reaction mixture, the glucose release efficiency increased from 19.3% to 68.0%. After only 24 hours of hydrolysis, when hydrolysis efficiency typically decreases significantly, repeated use of biomass with enzymes resulted in beneficial increases in cost-effectiveness, saccharide release efficiency, and ethanol fermentation (Jin et al. 2012). In this regard, lytic enzyme mixture synergy is crucial (Østby et al. 2020). Weiss et al. (2013) demonstrated that using biomass recycling with enzymes allows for a 30% reduction in enzyme dose for corn straw hydrolysis. Cellulases can be used multiple times to hydrolyse lignocellulosic raw materials in a variety of ways, including using the liquid fraction obtained after hydrolysis as an enzyme source (Wang et al. 2016). Wang et al. (2016) confirmed the feasibility of up to five cycles of cellulase recycling after hydrolysis of sulphite-treated lignocellulosic pulp from hardwoods in this manner. Cellulase activity was 35.1 - 48.8% of the initial activity in the fifth hydrolysis cycle.

Before and after enzymatic hydrolysis, biomass samples from both *Miscanthus* species were examined under a scanning electron microscope (SEM). Figures 11-12 show sample images, and both native raw materials have a similar structure. They were discovered to be fibrous materials with numerous aggregated lamellar structures. After alkali pretreatment, the biomass had a partially damaged, open, macroporous fibrous structure (Fig. 11a, b; Fig. 12a, b). Łysakowski et al. (2019) observed similar changes in miscanthus biomass after alkaline and acid treatments. After enzymatic hydrolysis, further degradation of the structural elements of the biomass was observed (Fig. 11c, d; Fig. 12c, d).

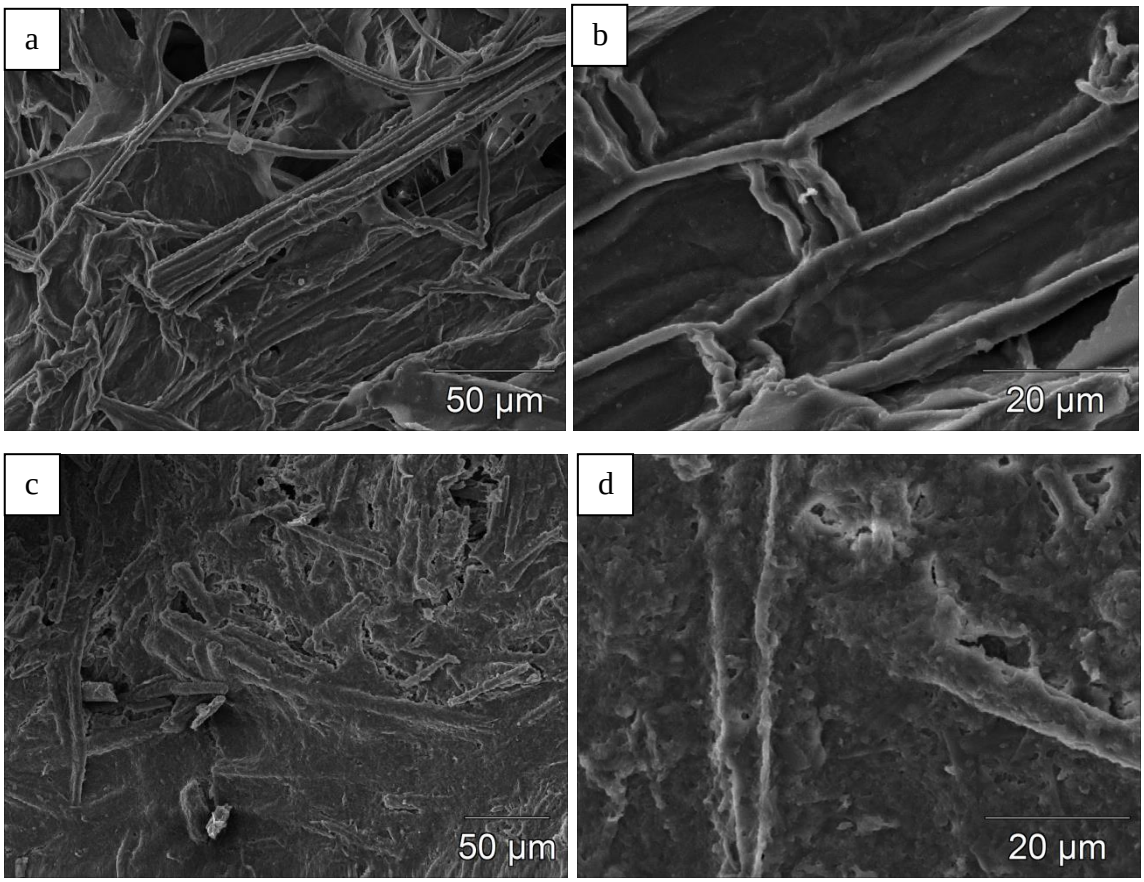


Fig. 11. Structure of native *Miscanthus giganteus* biomass (a, b) and biomass after hydrolysis with 35 FPU of Accellerase 1500 and a mixture of supporting enzymes (c, d)

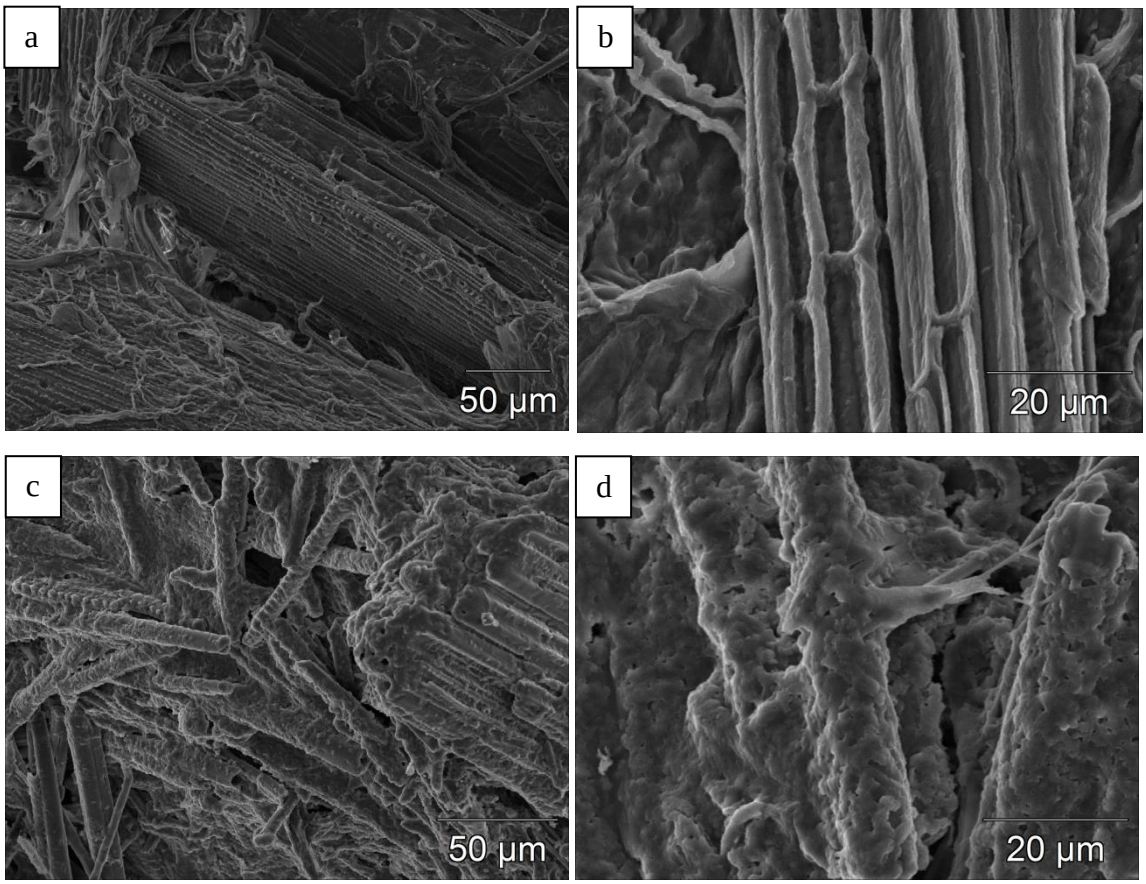


Fig. 12. Structure of native *Miscanthus sacchariflorus* biomass (a, b) and biomass after hydrolysis with 35 FPU of Accellerase 1500 and a mixture of supporting enzymes (c, d)

The obtained results validated the use of lytic enzymes in the solid fraction of the lignocellulosic raw material after hydrolysis. However, additional validation of enzyme adsorption conditions is required, such as an analysis of the impact of environmental conditions on this process. It is also necessary to make a rational assessment of the composition and properties of the solid fraction that remains after enzymatic hydrolysis. Analyses of enzyme desorption and the use of immobilised enzymes, including nanobiocatalysts, will be required.

5.2. Increasing the efficiency of sugar release from the sugar beet root

Sugar beets have high crop yields and sucrose content, making them a promising feedstock for bioethanol production. The yield of bioethanol production from this feedstock is 100 - 120 $dm^3 \cdot t^{-1}$ or about 5000 $dm^3 \cdot ha^{-1}$, based on global average yields (46 $t \cdot ha^{-1}$), but can exceed even 11000 $dm^3 \cdot ha^{-1}$ under suitable growing conditions (Alexiades et al. 2018).

Sugar beet biomass is a renewable, carbohydrate-rich feedstock that is a potential source to produce bioethanol and value-added chemicals (Cárdenas-Fernández et al. 2018). Table 7 shows the chemical composition of sugar beet root.

Table 7. Chemical composition of sugar beet root [PN-R-74458-1999]

<table>
<tr><td colspan="6">beet root
100%</td></tr>
<tr><td rowspan="6">water
72 - 75%</td><td colspan="5">dry matter
25 - 28%</td></tr>
<tr><td rowspan="5">insoluble
dry matter
4.5 - 5.5%</td><td colspan="4">soluble dry matter
20 - 23%</td></tr>
<tr><td colspan="3">non-sugars
2.5 - 3%</td><td rowspan="4">sugar
(sucrose)
17 - 20%</td></tr>
<tr><td rowspan="3">mineral
non-sugars
0.5 - 0.8%</td><td colspan="2">organic non-sugars
2 - 2.2%</td></tr>
<tr><td>nitrogenous
compounds
1.2%</td><td>bio-
nitrogenous
compounds
0.9%</td></tr>
</table>

Sucrose is the main carbohydrate in sugar beet biomass, accounting for 70-80% of dry matter (Getz 2000). Sucrose is thus the most abundant and important component of sugar beet dry matter. The by-products of sucrose extraction in the sugar plant is beet pulp, which is an untapped renewable resource. They are composed of 70-80% polysaccharides (cellulose, hemicellulose, and pectin in a 1:1:1 ratio) and other components such as lignin, protein, and residual sugar (Hemsworth et al. 2015; Zheng et

al. 2012). The main hemicelluloses are galactan and araban (24 - 32% d.m.), and lignins account for 3 - 4% d.m. (Brelowska et al. 2018). Beet pulp is typically used as an additive in animal feed or discarded as solid waste. According to some researchers, using the entire sugar beet root for biofuel production eliminates the sucrose extraction step as well as the need for any solid residue processing. The remaining stock could be used as a feed additive for animals or as a fermentation feedstock (Gumienna et al. 2016; Nahar and Pryor 2013; Liu et al. 2011). Krajnc et al. (2007) addressed the issue of increasing the efficiency of biofuel production using by-products from the sugar industry. Experiments have shown that concentrated sugar beet juice (Tan et al. 2015; Dodi et al. 2009; Li et al. 2018) and whole, cleaned beet roots (Dorado et al. 2017) are both suitable for obtaining fermentable sugars.

Sugar beet, like miscanthus, requires pretreatment to destroy the biomass's naturally ordered and rigid structure, reduce particle size, and facilitate the exposure of polysaccharide components to lytic enzymes (Li and Chen 2014). Many sugar beet pretreatment methods have been tested (Li et al. 2018; Dorado et al. 2017), but the majority of them require the use of harsh chemicals or expensive treatments, such as prolonged heating at high temperatures. It is critical to develop an environmentally friendly and cost-effective pretreatment method.

In the work, the feasibility of using whole sugar beet root for bioethanol production was investigated using various pretreatment and enzymatic hydrolysis methods. The experiments aimed to increase the amount of fermentable sugars released by using a new, simple pretreatment method for shredded sugar beet, as well as efficient enzymatic hydrolysis of the biomass by reusing enzymes adsorbed on the biomass solid fraction. Another goal was to compare the efficiency of sugar beet saccharide conversion to bioethanol using different hydrolysis and fermentation systems and to evaluate the effect of the sugar beet biomass preparation method on the efficiency of ethanol fermentation.

The basic chemical composition of the tested beet samples after the disintegration process by the methods tested is shown in Table 8.

Table 8. Chemical composition of the sugar beet biomass samples

Component	Mechanical shredding	Vacuum expansion
dry matter [%]	17.83 ± 0.17 [a]	17.19 ± 0.21 [a]
crude ash [%]	0.81 ± 0.05 [a]	0.76 ± 0.04 [a]
total protein [%]	0.89 ± 0.09 [a]	0.85 ± 0.07 [a]
crude fiber [%]	1.24 ± 0.02 [a]	1.23 ± 0.05 [a]
sucrose [%]	17.17 ± 0.03 [a]	15.65 ± 0.07 [b]
pectin [% d.m.]	14.53 ± 0.34 [a]	14.34 ± 0.22 [a]
cellulose [% d.m.]	1.07 ± 0.05 [a]	1.02 ± 0.08 [a]
hemicellulose [% d.m.]	1.04 ± 0.12 [a]	1.07 ± 0.09 [a]
lignin [% d.m]	0.43 ± 0.05 [a]	0.34±0.01 [b]

The letters (a, b) in rows indicate statistical significance at $p < 0.05$.

When compared to samples subjected to mechanical shredding, vacuum expansion had only a minor effect on changes in the content of analysed substances in sugar beet biomass. The disruption of the cell structure, on the other hand, reduced the content of lignin and sucrose (the latter component probably due to increased leakage from the cells).

Figure 13 shows microscopic images of mechanically shredded and vacuum-expansed sugar beet samples: raw, before enzymatic hydrolysis and after hydrolysis. The images show clear changes in the structure of the sugar beet tissues. Vacuum expansion caused cell walls to break down and increased the degree of tissue degradation (Figure A2) and allowed for an increase in the surface area available for enzyme action (Figure A3).

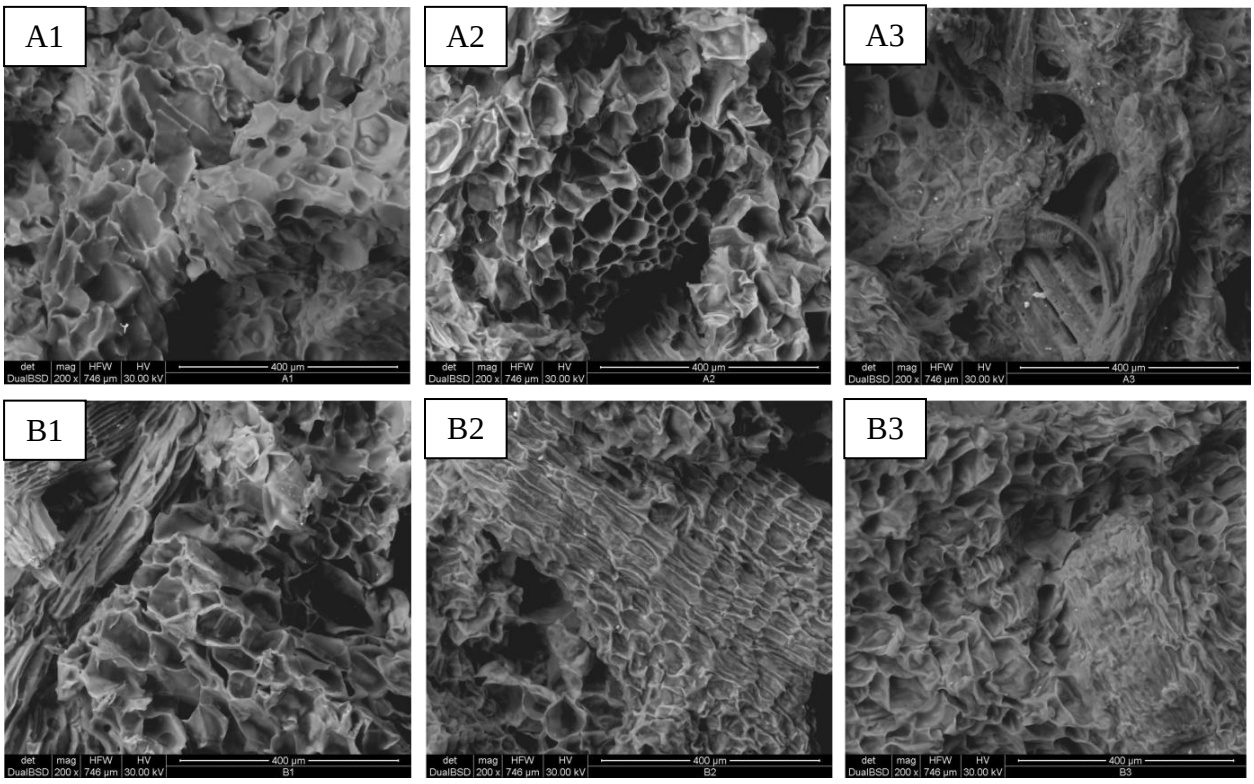


Figure 13: Image of the biomass structure obtained with a scanning microscope (SEM). Sugar beet biomass after vacuum expansion: raw (A1), before enzymatic hydrolysis (A2) and after enzymatic hydrolysis (A3), and mechanically shredded sugar beet biomass: raw (B1) before enzymatic hydrolysis (B2) and after enzymatic hydrolysis (B3)

Microscopic analysis showed that compared to mechanically shredded beet (Figure 13A), the biomass of vacuum-treated beet (Figure 13B) was characterized by more torn and frayed cellular structures. The change in hydrostatic pressure tended to result in the rupture and release of intracellular material and partial hydrolysis and dissolution of hemicellulose (Menon and Rao 2012; Yang et al. 2018).

The method used to break down the lignocellulose complex, reduce particle size, increase the available specific surface area of the feedstock (area of material per unit weight), and remove lignin is critical to achieving high efficiency in the bioconversion of plant biomass to ethanol. These structural changes in the material make it more susceptible to enzymatic hydrolysis and subsequent processes and unit operations, such as fermentation, waste treatment, and wastewater management (Alvira et al. 2010).

Pretreatment of sugar beet was done with enzyme mixtures in various combinations: (1) Rapidase® (R); (2) Accellerase 1500 (A1500); (3) Supporting enzyme

complex (BG+XC+XY); (4) Rapidase®, Accellerase 1500, Supporting enzyme complex (R+A1500+BG+XC+XY); (5) Rapidase®, Supporting enzyme complex (R+ BG+XC+XY); (6) Accellerase 1500, Supporting enzyme complex (A1500+ BG+XC+XY); (7) Rapidase®, Accellerase 1500 (R+A1500); (8) control sample without enzymes. Enzymatic hydrolysis reactions were carried out 72 h with stirring (250 rpm), simultaneously at 50°C and 60°C. After completion, the samples were centrifuged (10°C, 15 min, 4000×g) and the concentration of reducing sugars was determined in the supernatant.

The remaining solid fraction was used to hydrolyse the biomass again as a source of hydrolytic enzymes. In a 1:1 (w:w) ratio, the hydrolysed solid residue was added to a new aliquot of sugar beet biomass. Hydrolysis was carried out under the previously described conditions, and the content of reducing sugars in the resulting supernatant was determined.

Tables 9-14 summarise the results of the sugar determinations after hydrolysis of the pretreated sugar beet matrix. The use of vacuum expansion resulted in an average of 29.7% more reducing sugars than mechanical shredding of sugar beet. At 50°C, the enzymatic hydrolysis reaction of sugar beet biomass was more efficient, yielding on average of 21.3% more reducing sugars than at 60°C. These results agree with data obtained by Zanchetta et al. (2018), who confirmed optimal temperatures for the activity of cellulolytic enzymes between 45-50°C.

Table 10. Average content of reducing sugars in supernatants after enzymatic hydrolysis of sugar beet biomass

Pretreatment technique	Temperature [°C]	Enzyme composition	Yield of reducing sugars [mg·g^{-1} d.m.]	
			Enzymatic hydrolysis I	Enzymatic hydrolysis II
Vacuum expansion	60	1	52.7 ^{g}	37.1 ^{ef}
		2	54.0 ^{f}	36.6 ^{ef}
		3	84.9 ^{c}	48.1 ^{bc}
		4	77.3 ^{c}	47.9 ^{bc}
		5	76.5 ^{c}	41.0 ^{e}
		6	75.6 ^{cd}	43.5 ^{d}
		7	63.3 ^{e}	41.7 ^{d}
		control	45.3 ^{h}	32.2 ^{g}
Mechanical shredding	60	1	28.3 ^{k}	21.7 ^{j}
		2	34.6 ^{j}	19.4 ^{j}
		3	52.0 ^{g}	32.0 ^{g}
		4	47.4 ^{gh}	32.9 ^{g}
		5	42.2 ^{i}	27.0 ^{i}
		6	44.7 ^{h}	29.6 ^{h}
		7	38.3 ^{i}	25.8 ^{i}
		control	27.1 ^{k}	18.2 ^{k}
Vacuum expansion	50	1	58.5 ^{ef}	39.6 ^{e}
		2	59.6 ^{ef}	38.3 ^{e}
		3	87.5 ^{b}	50.0 ^{b}
		4	107.9 ^{a}	58.4 ^{a}
		5	92.4 ^{b}	47.3 ^{c}
		6	91.6 ^{b}	48.6 ^{b}
		7	74.3 ^{d}	42.7 ^{d}
		control	55.1 ^{f}	42.5 ^{d}
Mechanical shredding	50	1	40.9 ^{i}	26.6 ^{i}
		2	43.5 ^{h}	27.0 ^{i}
		3	69.4 ^{e}	34.0 ^{f}
		4	75.9 ^{cd}	30.5 ^{h}
		5	68.3 ^{e}	32.4 ^{g}
		6	62.6 ^{e}	34.2 ^{f}
		7	54.5 ^{f}	30.2 ^{h}
		control	39.9 ^{i}	25.8 ^{i}

1 – Rapidase; 2 – Accellerase 1500; 3 – Accellerase BG, Accellerase XC, Accellerase XY; 4 – Rapidase, Accellerase 1500, Accellerase BG, Accellerase XC, Accellerase XY; 5 – Rapidase, Accellerase BG, Accellerase XC, Accellerase XY; 6 – Accellerase 1500, Accellerase BG, Accellerase XC, Accellerase XY; 7 – Rapidase, Accellerase 1500

The letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

Table 11. Sucrose release efficiency after enzymatic hydrolysis of sugar beet biomass and hydrolysis with enzyme recycling

Pretreatment technique	Temperature [°C]	Enzyme composition	Yield of sucrose [mg·g^{-1} d.m.]	
			Enzymatic hydrolysis I	Enzymatic hydrolysis II
Vacuum expansion	60	1	42.8 ^{hi}	17.4 ^{gh}
		2	42.5 ^{hi}	17.2 ^{gh}
		3	37.2 ^{k}	14.0 ^{i}
		4	41.3 ^{i}	17.6 ^{g}
		5	43.2 ^{h}	16.1 ^{h}
		6	43.0 ^{h}	16.6 ^{h}
		7	44.0 ^{h}	22.5 ^{e}
		control	38.8 ^{j}	22.7 ^{e}
Mechanical shredding	60	1	49.7 ^{f}	22.8 ^{e}
		2	51.4 ^{e}	22.2 ^{ef}
		3	44.6 ^{h}	20.7 ^{ef}
		4	49.6 ^{f}	24.0 ^{c}
		5	50.8 ^{ef}	22.7 ^{e}
		6	49.2 ^{f}	22.5 ^{e}
		7	50.6 ^{f}	22.5 ^{e}
		control	51.5 ^{e}	22.7 ^{e}
Vacuum expansion	50	1	50.8 ^{ef}	20.1 ^{f}
		2	52.6 ^{d}	19.8 ^{f}
		3	47.2 ^{g}	16.8 ^{h}
		4	50.9 ^{ef}	13.5 ^{j}
		5	51.9 ^{e}	17.6 ^{g}
		6	52.2 ^{e}	17.7 ^{g}
		7	52.7 ^{e}	18.7 ^{fg}
		control	53.8 ^{cd}	19.0 ^{f}
Mechanical shredding	50	1	58.1 ^{bc}	25.0 ^{b}
		2	59.4 ^{b}	24.3 ^{c}
		3	56.1 ^{c}	23.5 ^{d}
		4	59.8 ^{b}	24.9 ^{c}
		5	58.5 ^{b}	27.7 ^{a}
		6	59.4 ^{b}	25.4 ^{b}
		7	60.4 ^{ab}	26.7 ^{a}
		control	61.1 ^{a}	25.5 ^{b}

The letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

Table 12. Concentration of saccharides released from sugar beet biomass after vacuum expansion or mechanical shredding, enzymatically hydrolysed at 50°C

Sample		Enzymatic hydrolysis I				Enzymatic hydrolysis II			
		sucrose	glucose	fructose	arabinose	sucrose	glucose	fructose	arabinose
		[mg·g^{-1} d.m.]							
Vacuum expansion	1	147.6 ^{c}	67.8 ^{e}	24.4 ^{b}	0.0 ^{d}	58.3 ^{c}	33.5 ^{c}	19.9 ^{b}	0.0 ^{c}
	2	152.8 ^{b}	75.7 ^{cd}	25.8 ^{b}	0.0 ^{d}	57.4 ^{c}	33.5 ^{c}	17.8 ^{b}	0.0 ^{c}
	3	137.3 ^{d}	80.9 ^{c}	33.5 ^{a}	2.9 ^{c}	48.9 ^{d}	34.4 ^{b}	22.2 ^{ab}	1.1 ^{b}
	4	147.9 ^{c}	94.7 ^{a}	28.9 ^{ab}	6.0 ^{a}	39.2 ^{e}	33.6 ^{c}	26.7 ^{a}	1.3 ^{ab}
	5	150.8 ^{b}	83.9 ^{b}	27.4 ^{b}	5.2 ^{b}	51.3 ^{c}	33.2 ^{c}	19.2 ^{b}	1.5 ^{ab}
	6	151.9 ^{b}	91.6 ^{ab}	26.6 ^{b}	5.5 ^{b}	51.4 ^{c}	34.4 ^{b}	18.4 ^{b}	1.6 ^{ab}
	7	153.3 ^{b}	84.6 ^{b}	28.5 ^{ab}	0.0 ^{d}	54.4 ^{c}	33.7 ^{c}	19.2 ^{b}	0.0 ^{c}
	control	156.5 ^{ab}	74.5 ^{cd}	26.8 ^{b}	0.0 ^{d}	55.2 ^{c}	30.9 ^{d}	17.9 ^{b}	0.0 ^{c}
Mechanical shredding	1	163.2 ^{a}	72.4 ^{d}	15.1 ^{cd}	0.0 ^{d}	70.2 ^{b}	34.8 ^{b}	9.7 ^{d}	0.0 ^{c}
	2	167.0 ^{a}	76.7 ^{cd}	15.9 ^{cd}	0.0 ^{d}	68.2 ^{b}	35.0 ^{b}	8.7 ^{d}	0.0 ^{c}
	3	157.7 ^{ab}	83.3 ^{b}	24.6 ^{b}	3.2 ^{c}	66.0 ^{bc}	38.9 ^{ab}	13.3 ^{c}	1.1 ^{b}
	4	167.9 ^{a}	91.8 ^{ab}	17.8 ^{c}	5.6 ^{b}	69.9 ^{b}	37.3 ^{ab}	8.6 ^{d}	1.8 ^{a}

	5	164.5 [a]	85.2 [b]	17.2 [c]	4.5 [bc]	77.7 [a]	40.2 [a]	11.1 [c]	1.9 [a]
	6	166.9 [a]	86.3 [b]	17.1 [c]	5.0 [b]	71.4 [b]	40.2 [g]	9.3 [d]	2.1 [m]
	7	169.5 [a]	79.3 [c]	17.5 [c]	0.0 [d]	75.0 [ab]	39.3 [g]	10.4 [c]	0.0 [n]
	control	171.7 [a]	72.8 [d]	14.4 [d]	0.0 [d]	71.7 [b]	35.1 [g]	8.3 [d]	0.0 [n]

The letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

Table 13. Concentration of saccharides released from sugar beet biomass after vacuum expansion or mechanical shredding, enzymatically hydrolyzed at 60°C

Sample		Enzymatic hydrolysis I				Enzymatic hydrolysis II			
		sucrose	glucose	fructose	arabinose	sucrose	glucose	fructose	arabinose
		[mg·g^{-1} d.m.]							
Vacuum expansion	1	124.4 [b]	57.5 [c]	22.7 [ab]	0.0 [c]	50.7 [b]	25.7 [c]	13.2 [b]	0.0 [c]
	2	123.4 [b]	57.8 [c]	21.1 [ab]	0.0 [c]	50.1 [b]	25.3 [c]	13.0 [b]	0.0 [c]
	3	108.1 [c]	65.3 [a]	28.5 [a]	3.8 [a]	40.7 [c]	26.9 [c]	15.7 [a]	0.7 [ab]
	4	120.0 [b]	67.9 [a]	0.6 [e]	3.0 [ab]	51.3 [b]	30.9 [b]	14.5 [ab]	0.8 [ab]
	5	125.5 [b]	66.7 [a]	25.4 [a]	2.9 [ab]	46.7 [bc]	25.9 [c]	14.2 [ab]	0.7 [ab]
	6	125.1 [b]	65.4 [a]	23.3 [a]	2.5 [b]	48.2 [bc]	27.7 [c]	13.6 [b]	0.8 [ab]

	7	127.8 ^{b}	61.9 ^{b}	24.9 ^{a}	0.0 ^{c}	65.3 ^{a}	29.2 ^{b}	7.6 ^{cd}	0.0 ^{c}
	control	112.9 ^{c}	47.8 ^{d}	15.6 ^{b}	0.0 ^{c}	65.9 ^{ab}	25.9 ^{c}	4.7 ^{e}	0.0 ^{c}
Mechanical shredding	1	139.5 ^{a}	54.6 ^{c}	10.3 ^{c}	0.0 ^{c}	64.1 ^{ab}	27.3 ^{c}	6.2 ^{d}	0.0 ^{c}
	2	144.4 ^{a}	58.3 ^{b}	10.8 ^{c}	0.0 ^{c}	62.3 ^{ab}	27.2 ^{c}	5.7 ^{d}	0.0 ^{c}
	3	125.2 ^{b}	60.1 ^{b}	15.6 ^{b}	2.1 ^{b}	58.2 ^{b}	31.6 ^{b}	10.7 ^{c}	0.0 ^{c}
	4	139.4 ^{a}	66.1 ^{a}	14.0 ^{b}	2.4 ^{b}	67.3 ^{a}	35.1 ^{a}	9.8 ^{c}	0.9 ^{ab}
	5	142.8 ^{a}	64.5 ^{ab}	12.6 ^{bc}	2.6 ^{b}	63.8 ^{ab}	30.7 ^{b}	7.1 ^{cd}	1.0 ^{a}
	6	138.2 ^{a}	64.0 ^{ab}	10.8 ^{c}	2.4 ^{b}	63.2 ^{ab}	30.3 ^{b}	7.4 ^{cd}	1.1 ^{a}
	7	142.0 ^{a}	58.2 ^{b}	11.6 ^{bc}	2.5 ^{b}	63.1 ^{ab}	28.2 ^{c}	7.4 ^{cd}	1.0 ^{a}
	control	144.7 ^{a}	51.2 ^{c}	7.2 ^{d}	0.0 ^{c}	63.6 ^{ab}	25.1 ^{c}	4.6 ^{e}	0.0 ^{c}

The letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

Table 14. Enzymatic hydrolysis efficiency of sugar beet biomass

Pretreatment technique	Temperature [°C]	Enzyme composition	Efficiency of hydrolysis [%]	
			Enzymatic hydrolysis I	Enzymatic hydrolysis II
Vacuum expansion	60	1	48.8 ^{i}	24.2 ^{e}
		2	48.9 ^{i}	23.9 ^{e}
		3	53.2 ^{g}	24.5 ^{e}
		4	54.3 ^{fg}	27.3 ^{d}
		5	55.5 ^{f}	24.2 ^{e}
		6	55.3 ^{f}	25.3 ^{e}
		7	52.6 ^{h}	29.5 ^{c}
		control	43.6 ^{j}	27.0 ^{d}
Mechanical shredding	60	1	50.1 ^{h}	25.6 ^{e}
		2	53.5 ^{g}	24.4 ^{e}
		3	53.0 ^{g}	27.0 ^{d}
		4	55.9 ^{f}	30.0 ^{c}
		5	55.3 ^{f}	27.1 ^{d}
		6	54.7 ^{fg}	27.8 ^{d}
		7	53.9 ^{g}	26.6 ^{de}
		control	51.4 ^{h}	24.5 ^{e}
Vacuum expansion	50	1	56.8 ^{f}	27.0 ^{d}
		2	58.5 ^{e}	26.4 ^{de}
		3	61.9 ^{de}	27.3 ^{d}
		4	70.5 ^{b}	26.9 ^{d}
		5	67.0 ^{c}	27.2 ^{d}
		6	67.1 ^{c}	27.5 ^{d}
		7	62.7 ^{d}	26.7 ^{de}
		control	58.3 ^{e}	26.9 ^{d}
Mechanical shredding	50	1	61.0 ^{e}	28.9 ^{c}
		2	62.9 ^{d}	28.4 ^{cd}
		3	67.9 ^{b}	29.9 ^{c}
		4	72.9 ^{a}	30.0 ^{c}
		5	69.6 ^{b}	32.9 ^{a}
		6	68.6 ^{b}	31.6 ^{b}
		7	67.0 ^{c}	31.4 ^{b}
		control	63.3 ^{d}	29.2 ^{c}

The letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

The above findings allowed to determine the best conditions for enzymatic hydrolysis of the sugar beet root. The most effective variant of the process was vacuum expansion followed by enzymatic hydrolysis at 50°C with enzyme compositions Rapidase® and Accellerase 1500, along with a complex of supporting enzymes. This made it possible to obtain an average of 107.9 $mg \cdot g^{-1}$ d.m. of reducing sugars and 147.9 $mg \cdot g^{-1}$ d.m. of sucrose. Enzyme reuse yielded an average of 58.4 $mg \cdot g^{-1}$ d.m. of reducing sugars and 13.5 $mg \cdot g^{-1}$ d.m. of sucrose. Enzyme recycling in samples subjected to enzymatic hydrolysis at 50°C allowed an increase in hydrolysis yield by 32.9%.

Sugar beet biomass contains three main types of polysaccharides, as demonstrated by Hemsworth et al. (2015): cellulose, hemicellulose, and pectin. The use of a cellulase-pectinase mixture to hydrolyze sugar beet biomass resulted in significantly higher saccharification efficiency than cellulase alone. Pectinase is most commonly used in the ethanol fermentation of sugar beet biomass, along with cellulase and β-glucosidase, to break down sugar beet polymers into galacturonic acid and monosaccharides (Zheng et al. 2012b).

Enzymatic hydrolysis with cellulolytic enzymes does not allow for lignin degradation (Taherzadeh and Karimi 2008). Cellulases binding to lignin was described by Saini et al. (2016). According to the authors, the adsorption of enzymatic proteins on lignin renders them ineffective, and a solution to this phenomenon should be found. Wang et al. (2016) and Gao et al. (2014) investigated protein adsorption on the surface of biomass during enzymatic hydrolysis, confirming the feasibility of up to five cycles of cellulase recycling after hydrolysis of sulphite-treated hardwood lignocellulosic pulp.

The results presented in Figure 14 show that after enzymatic hydrolysis, enzyme proteins are present in the sugar beet biomass (Figure 14 G-J), with enzyme proteins adsorbed in the biomass being greater after hydrolysis at 50°C (Figure 14 G-H) than after hydrolysis at 60°C (Figure 14 I-J).

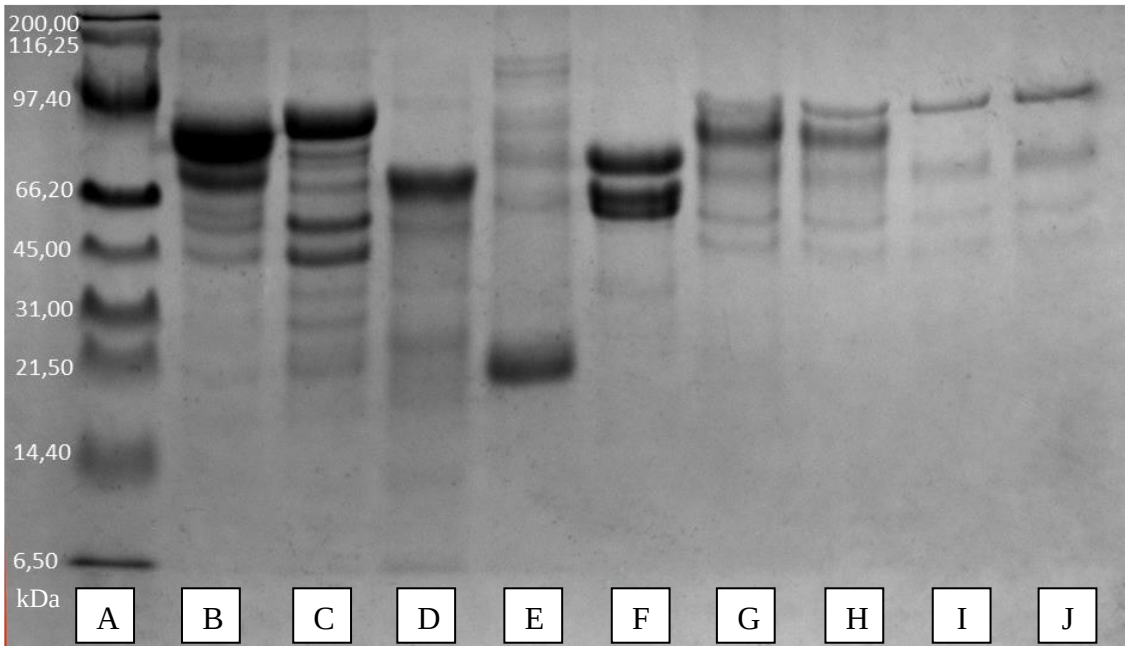


Fig. 14. Electrophoretic separation (SDS-PAGE) of enzyme solution samples and beet samples after enzymatic hydrolysis. Molecular mass marker (A), Accellerase 1500 (B), Accellerase BG (C), Accellerase XC (D), Accellerase XY (E), Rapidase (F) and sugar beet samples subjected to enzymatic hydrolysis with Rapidase, Accellerase 1500, a supporting enzyme complex: after vacuum expansion, 50°C (G), mechanically shredded, 50°C (H), after vacuum expansion, 60°C (I), mechanically shredded, 60°C (J).

After fermentation of sugar beet hydrolysates subjected to vacuum expansion conducted with distillers' yeast in the SHF and SSF systems, ethanol concentrations were higher by 6% and 10%, respectively, compared to fermentation of sugar beet hydrolysates after mechanical shredding (Table 15).

Table 15. Comparison of ethanol concentrations in sugar beet hydrolysates and fermentation efficiency depending on pretreatment, process conditions, and microorganism

Hydrolysis and ethanolic fermentation strategies	Pretreatment of raw material	Microorganism	Ethanol concentration		Efficiency
			[% v:v]	[g·dm^{-3}]	[dm^3 A_{100} · 100 kg^{-1} saccharose]
SHF	mechanical shredding	*S. cerevisiae* 7	6.40 ± 0.09 ^{a}	50.5 ± 0.7 ^{a}	71.0 ± 1.0 ^{b}
		M. indicus	0.49 ± 0.04 ^{c}	3.9 ± 0.4 ^{c}	5.5 ± 0.4 ^{d}
	vacuum expansion	*S. cerevisiae* 7	6.79 ± 0.43 ^{a}	53.6 ± 3.4 ^{a}	78.5 ± 5.0 ^{a}
		M. indicus	0.58 ± 0.14 ^{c}	4.6 ± 1.1 ^{c}	6.7 ± 1.6 ^{d}
SSF	mechanical shredding	*S. cerevisiae* D2	5.21 ± 0.00 ^{b}	41.1 ± 0.0 ^{b}	56.4 ± 0.0 ^{c}
	vacuum expansion	*S. cerevisiae* D2	5.79 ± 0.17 ^{b}	45.7 ± 1.4 ^{b}	50.7 ± 1.5 ^{c}

dm^3 A_{100} · 100 kg^{-1} saccharose (dm^3 of 100% ethanol per 100 kg of saccharose)

The letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

The highest average ethanol concentration in the post-reaction medium was obtained after fermentation carried out in the sequential system by S. cerevisiae 7, yielding 50.5 and 53.6 g·dm-3, respectively, after fermentation of hydrolysates obtained from mechanically shredded and vacuum-exploded sugar beet biomass. After using the simultaneous process, the attenuation level was lower than that obtained in the SHF system, and the alcohol content was 19% lower (when shredded biomass was used) and 15% lower (after vacuum expansion). Analysis of variance showed statistically significant differences in the amount of alcohol produced after fermentation of sugar beet hydrolysates depending on the pretreatment method used for the raw material ($p<0.05$) and the microorganism used ($p<0.05$). The interaction of the factors analyzed, i.e., pretreatment × microorganism, was found to be statistically insignificant ($p>0.05$). The effect of the hydrolysis and fermentation management system on the conversion efficiency of sugar beet saccharides to ethanol was also statistically confirmed (data not presented in the work).

Fermentation of sugar beet hydrolysates with *M. indicus* allowed ethanol concentrations of 0.49 and 0.58% (v/v) to be obtained in the substrate from mechanically shredded and vacuum-expansed sugar beet, respectively. Fermentation yields considering the amount of available sugars in the substrate in both variants did not exceed 7 dm^3 A100 · 100 kg^{-1} sucrose. *M. indicus* requires a much longer fermentation time to utilize the glucose present in the fermentation medium (up to 8 days) (Sues et al. 2005). Considering the above results, it was found that the filamentous fungus *M. indicus* is not useful in the process of ethanol fermentation of sugar beet biomass. An ethanol synthesis yield of 92% of the theoretical yield (41.1 $g \cdot dm^{-3}$) was achieved by Nahar and Pryor (2013) during a 48h fermentation in an SSF system of sugar beet roots with *S. cerevisiae*, assisted by enzymatic hydrolysis using cellulase and pectinase preparations at a dose of, respectively: 0.51 $FPU \cdot g^{-1}$ d.m. and 51 $U \cdot g^{-1}$ d.m. The authors showed that an increase in alcohol production occurs only when a mixture of cellulases and pectinases is used. An additional benefit of using enzyme preparations was a reduction in the amount of digestate (by 63-74%), an increase in the susceptibility of the beet to subsequent processing and, as a result, a reduction in the cost of its bioconversion process.

Dorado et al. (2017) used a steam explosion (3.45 bar, 2 min) to pretreat beet biomass. The raw material thus prepared was subjected to 24h of enzymatic hydrolysis with pectinase, cellulase and β-galactosidase preparations, followed by ethanol fermentation with *S. cerevisiae* yeast. After 48 h of fermentation, the ethanol

concentration ranged from 3.31 to 6.72 (%, w:v) depending on where the raw material was grown and when it was harvested. The efficiency of sugar beet saccharide conversion to ethanol is determined by factors other than sucrose content. The metabolic activity of microorganisms has a significant impact on the process. The type of beet used, as well as the content of dry matter components such as lignin and hemicellulose, are important. Casey et al. (2013) showed that ammonium salts applied during fertilization can be inhibitors of *S. cerevisiae* yeast growth, reducing the bioconversion of glucose to ethanol. The authors also found that the presence of sodium chloride at a concentration of 0.5 M resulted in a 64.5% reduction in xylose consumption rate and greater glycerol synthesis compared to a sample free of this salt. Gumienna et al. (2016) presented similar findings, claiming that the fermentation efficiency of sugar beet biomass depends not only on the sucrose content, but also on its concentration relative to the other dry matter components. Contamination with elements such as calcium, sodium, and nitrogen reduces ethanol synthesis efficiency.

5.3. Characterization and optimization of microwave pretreatment of giant miscanthus lignocellulose for bioconversion to ethanol

In this part of the work, experiments were carried out using a novel microwave pretreatment approach for giant miscanthus biomass. The effects of various microwave pretreatment solvents and pressures on the efficiency of enzymatic hydrolysis and ethanol fermentation, as well as the formation of hydrolysis and fermentation inhibitors, were compared. Changes in the content of the main components of the lignocellulosic matrix of giant miscanthus after microwave treatment in the environment of selected solvents are shown in Table 16.

Table 16. Lignocellulose fraction content of microwave-treated giant miscanthus samples after different solvents and pressure conditions pretreatments

Sample description		Cellulose content [% d.m.]	Hemicellulose content [% d.m.]	Lignin content [% d.m.]
0.1M NaOH	30 psi	54.30 ± 0.21 ^{c}	24.39 ± 1.24 ^{bc}	10.21 ± 0.15 ^{e}
	60 psi	58.68 ± 0.15 ^{bc}	20.93 ± 1.02 ^{c}	10.42 ± 0.15 ^{e}
	90 psi	61.36 ± 0.29 ^{b}	7.73 ± 1.02 ^{d}	15.47 ± 0.15 ^{c}
0.1M H_2SO_4	30 psi	64.42 ± 0.19 ^{ab}	5.14 ± 0.02 ^{e}	15.51 ± 0.23 ^{c}
	60 psi	67.39 ± 0.36 ^{a}	0.26 ± 0.07 ^{f}	18.97 ± 0.00 ^{b}
	90 psi	67.08 ± 0.36 ^{a}	0.10 ± 0.00 ^{f}	20.06 ± 0.07 ^{a}
H_2O	30 psi	48.67 ± 0.44 ^{d}	28.46 ± 0.88 ^{b}	12.48 ± 0.00 ^{d}
	60 psi	53.26 ± 1.53 ^{c}	22.38 ± 0.15 ^{c}	13.77 ± 0.66 ^{cd}
	90 psi	59.35 ± 0.36 ^{b}	8.92 ± 0.07 ^{d}	14.02 ± 0.29 ^{c}
60% EtOH	30 psi	48.16 ± 0.58 ^{d}	33.57 ± 0.80 ^{a}	8.71 ± 0.07 ^{f}
	60 psi	49.91 ± 0.15 ^{d}	32.79 ± 1.02 ^{a}	9.18 ± 0.44 ^{ef}
	90 psi	48.73 ± 0.51 ^{d}	32.43 ± 0.22 ^{a}	10.93 ± 0.15 ^{e}

The letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

Microwave treatment is typically used in conjunction with chemical pretreatment techniques involving acids, bases, ionic liquids, oxidizing agents, and high boiling point liquids for lignocellulosic materials (Bundhoo 2018). It has been reported that acid pretreatment with hydrochloric acid or sulfuric acid (VI) aids in the dissolution of hemicellulose, increasing the availability of cellulose for enzymatic hydrolysis (Maurya et al. 2015; Bundhoo 2018). The present experiment determined the effect of low environment-impact solvents on changes in the composition of individual lignocellulose fractions. Each of the solvents used had a significant effect ($p<0.05$) on the content of the individual lignocellulose fractions. The pressures used had a significant effect ($p<0.05$) on the content of hemicellulose and lignin in the microwave-treated samples. The various pretreatment methods degraded the biomass structure to varying degrees. The solvent with the highest cellulose content was sulphuric acid, while the solvent with the highest level of delignification was 'organosolv,' in this case - 60% ethanol. The treated samples were subjected to SEM microscopic analysis (Figs. 15-18), which confirm the gradual degradation of the native structure of giant miscanthus biomass.

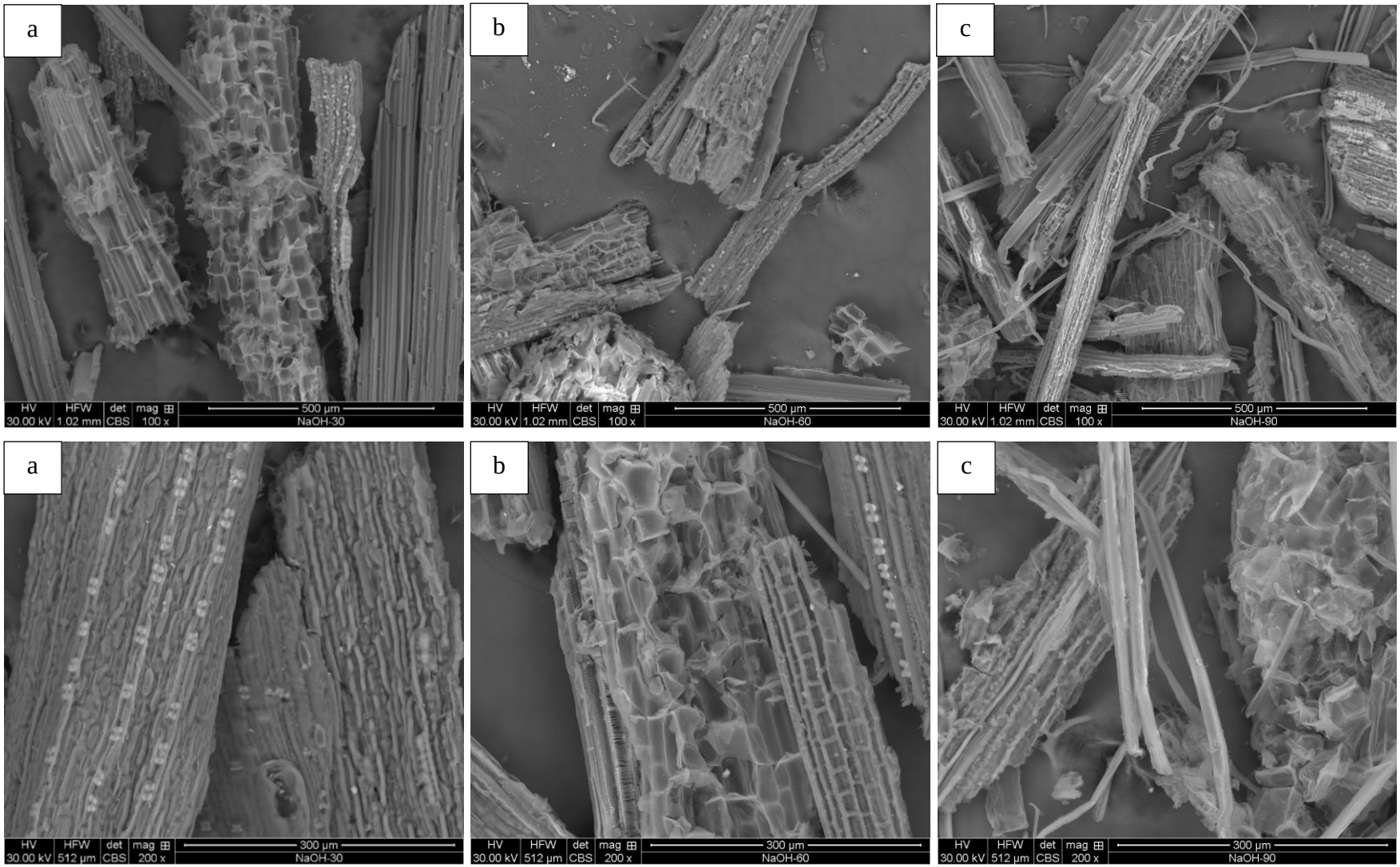


Figure 15: Image of the structure of giant miscanthus biomass obtained by scanning electron microscopy (SEM). Microwave pretreated biomass in alkaline medium at 30 psi (a), 60 psi (b) and 90 psi (c)

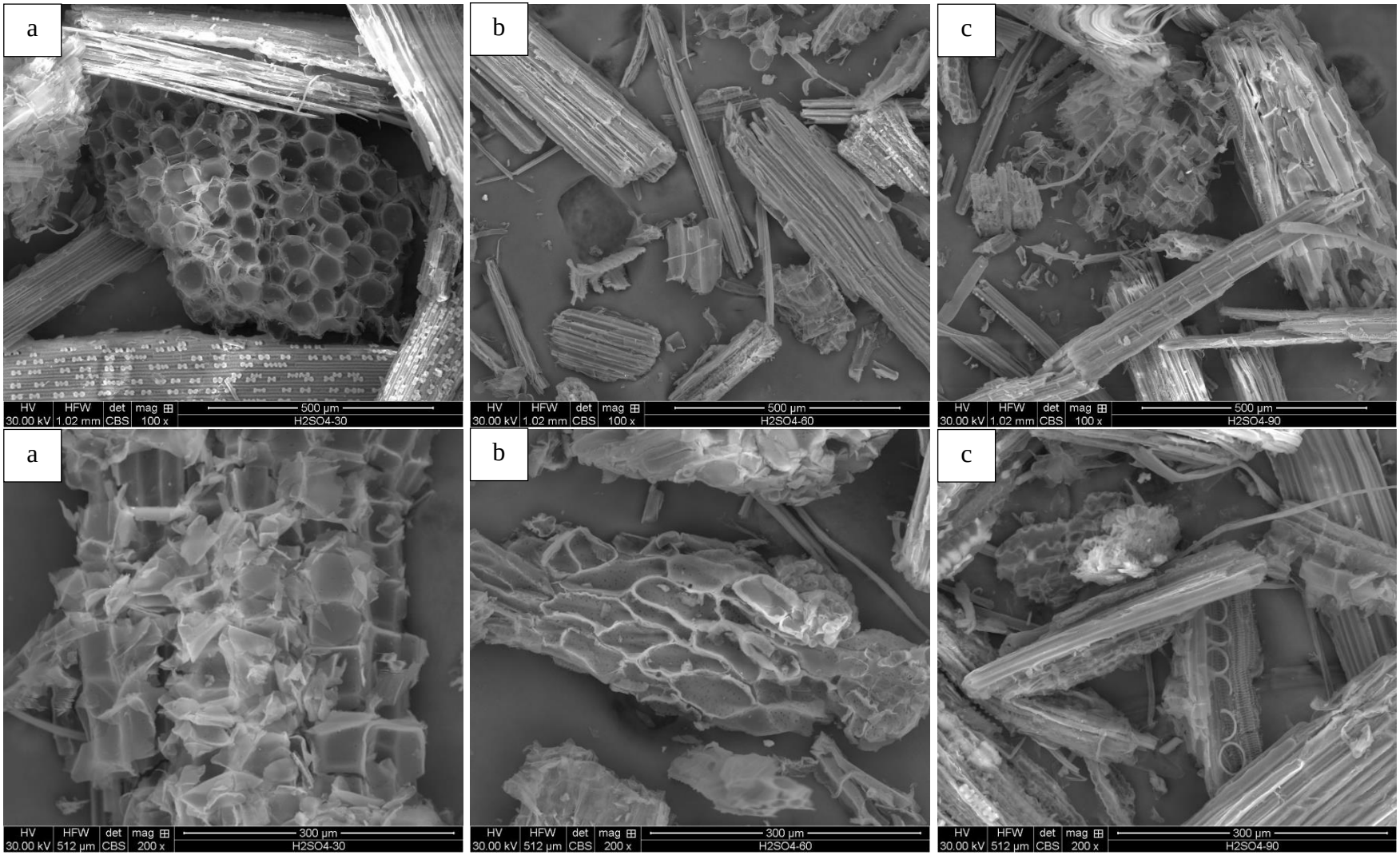


Figure 16: Image of the structure of giant miscanthus biomass obtained by scanning electron microscopy (SEM). Biomass microwave pretreated in an acidic environment at 30 psi (a), 60 psi (b) and 90 psi (c)

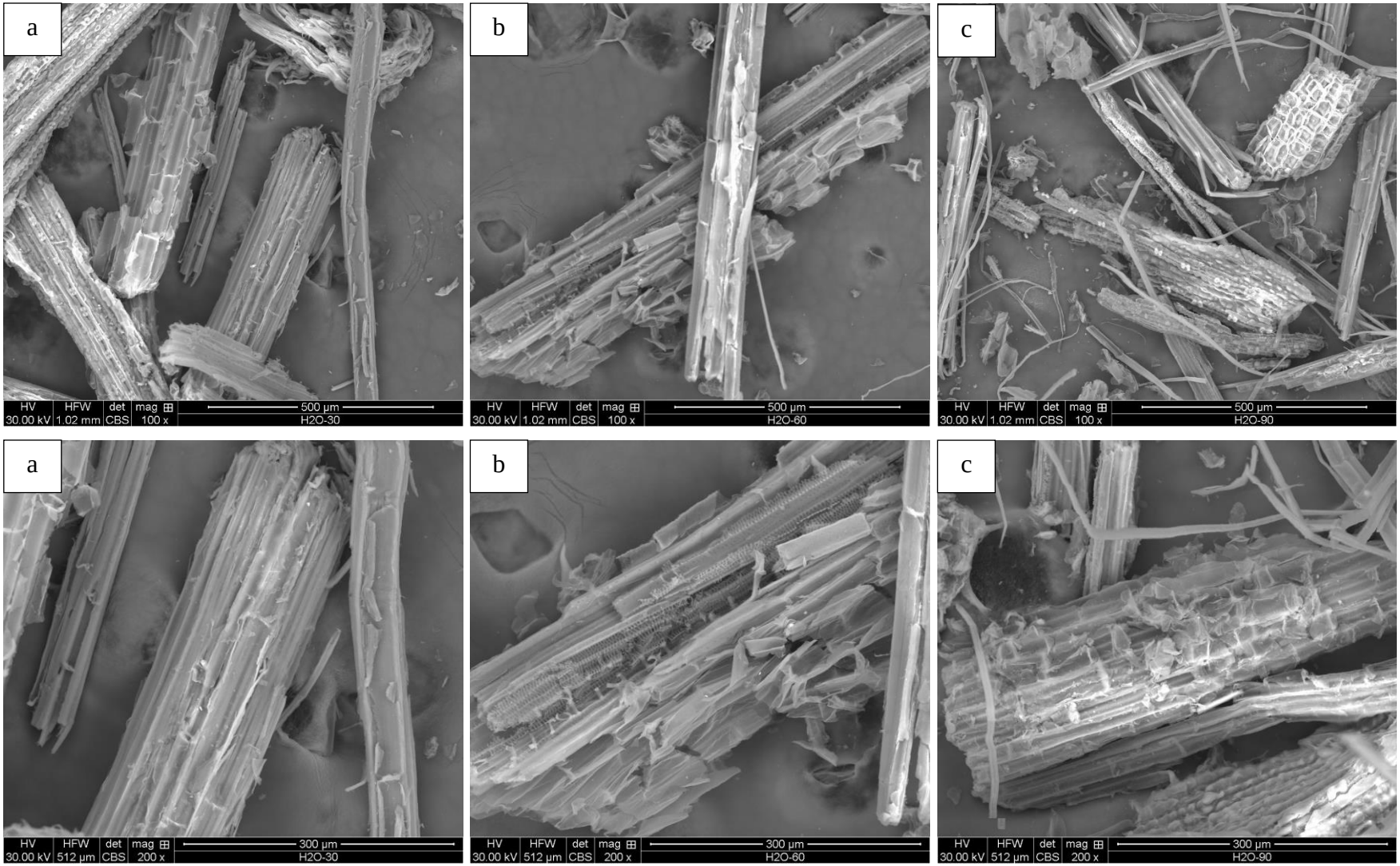


Figure 17. Image of giant miscanthus biomass structure obtained by scanning electron microscopy (SEM). Biomass microwave pretreated in water at 30 psi (a), 60 psi (b) and 90 psi (c)

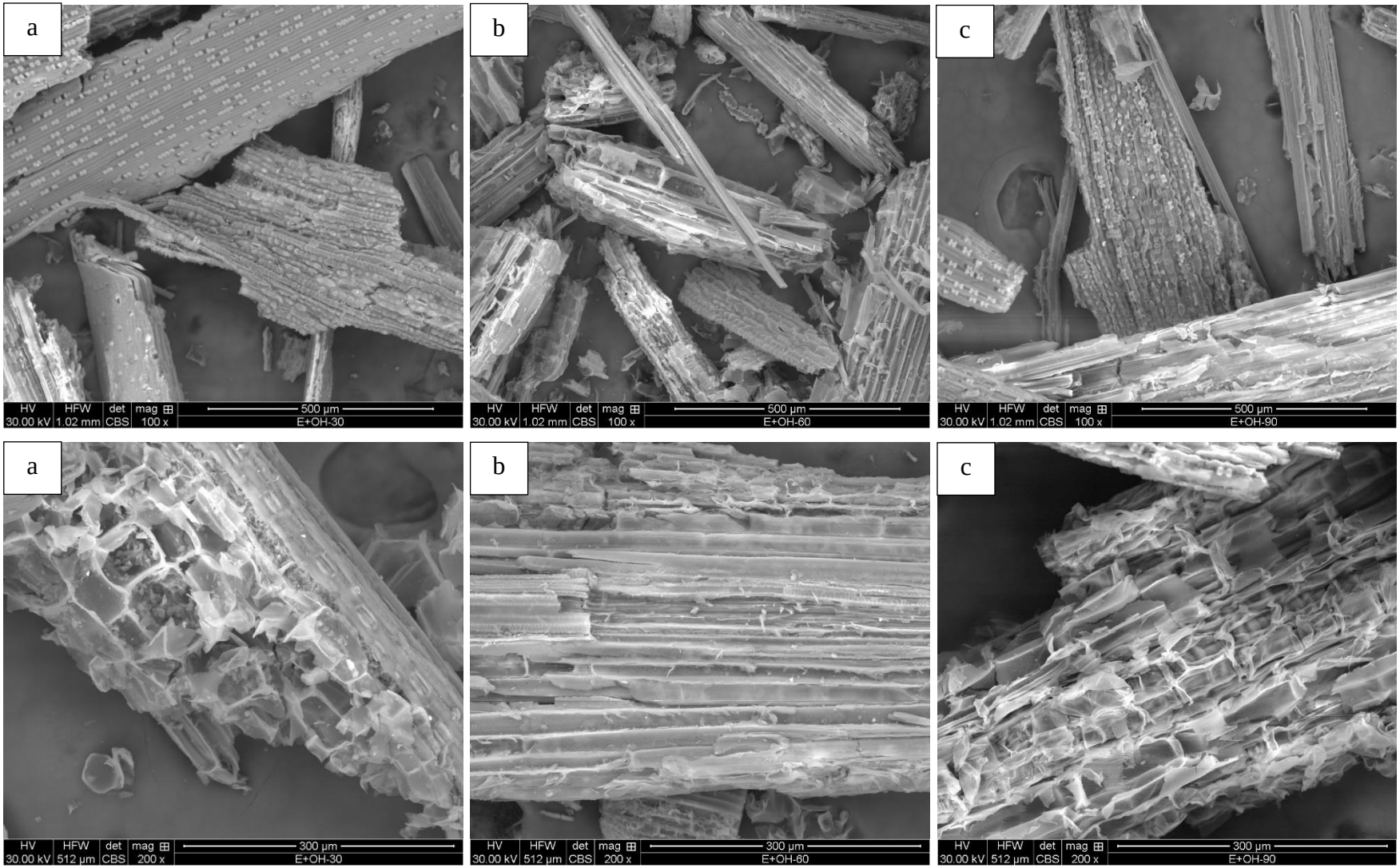
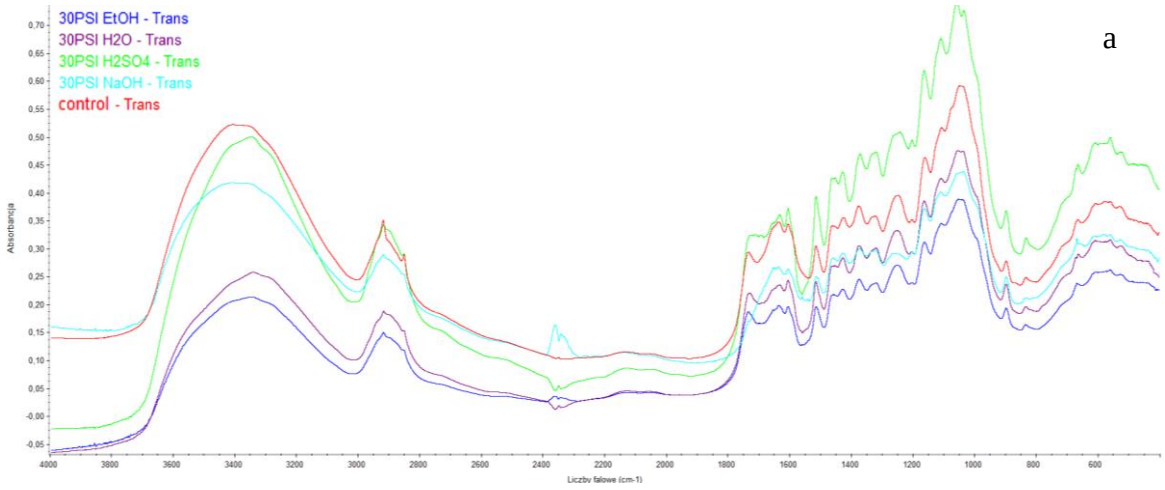


Figure 18: Image of the structure of giant miscanthus biomass obtained by scanning electron microscopy (SEM). Biomass microwave pretreated in 60% ethanol at 30 psi (a), 60 psi (b) and 90 psi (c)

The use of microwave treatment in an acidic environment resulted in almost complete hydrolysis of the hemicellulose. This relationship is confirmed by previous publications, including Jönsson and Martín (2016) and Solarte-Toro et al. (2019).

Figure 19 depicts the FTIR spectra of microwave-treated giant miscanthus biomass samples in various solvents at pressures ranging from 30-90 psi. The most noticeable changes in the FTIR transmission spectrum are seen between 3700 and 3000 cm^{-1}. This is a crystalline structure of cellulose region that represents the sum of the vibrations of the valence bands of the OH group hydrogen bonds as well as the intra- and intermolecular hydrogen bonds (Hinterstoisser and Salmén 1999). Regardless of the applied pressure, the changes in this region were similar. The type of solvent used influences the change in crystallinity of cellulose. The degree of crystallinity of cellulose was reduced by ethanol, water, and sulphuric acid (VI). A similar relationship can be seen in the 2918 cm^{-1} peak and 2850 cm^{-1} peak regions, representing the asymmetric stretching of CH_2 and CH groups in cellulose (Cherian et al. 2010).



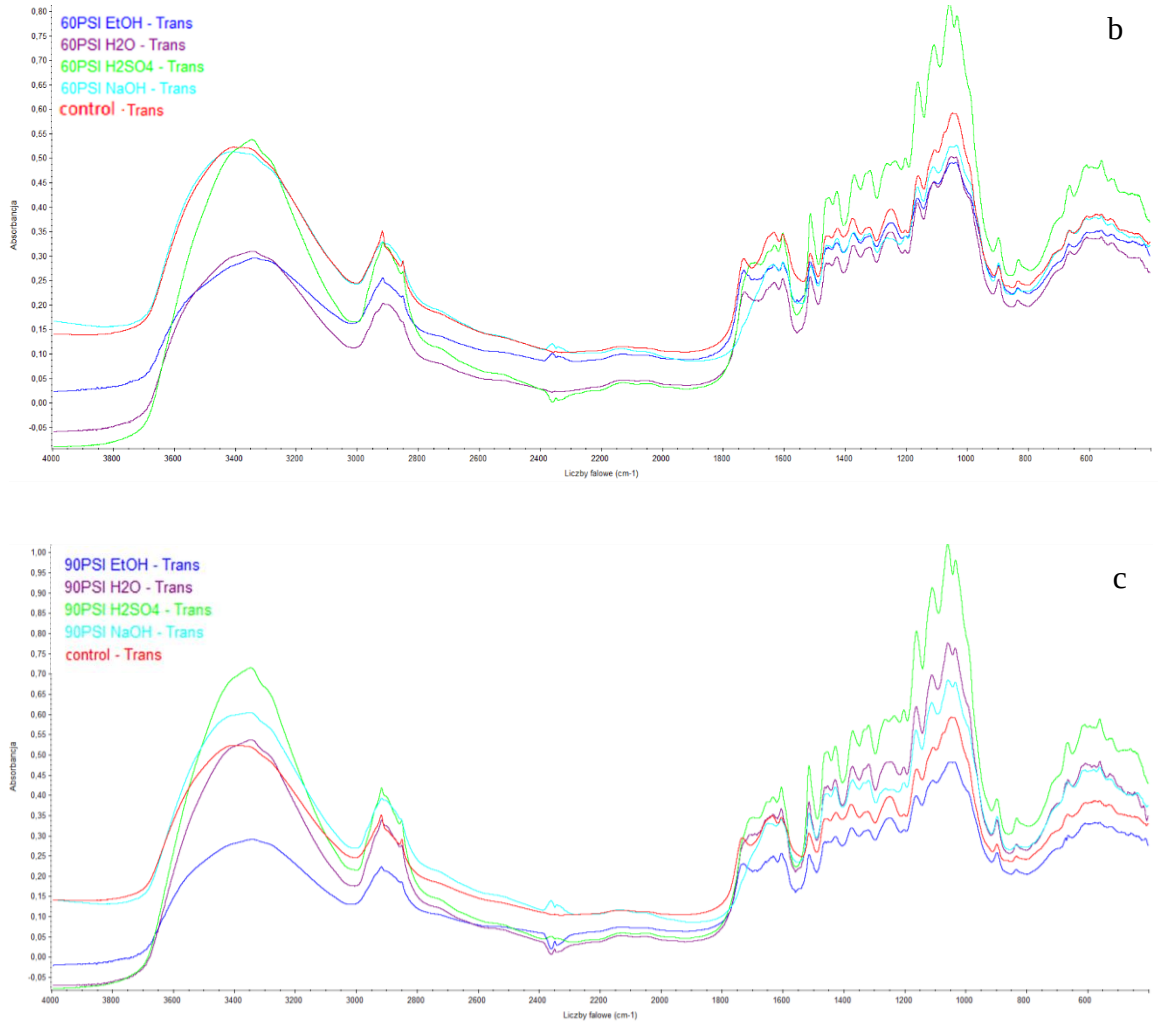


Fig. 19. FTIR spectrum of microwave treated Miscanthus biomass samples in different solvents at 30 psi (a), 60 psi (b), 90 psi (c)

Microwave treatment in an alkaline environment affected lignin degradation, as seen in the 2360 cm^{-1} peak region, which is characteristic of C-H bond stretching in lignin (Sharma et al. 2016), and uncoupled hemicellulose C-O bond stretching, as seen in the 1733 cm^{-1} peak region, which is characteristic of this group (Cherian et al. 2010).

An indication of the substitution pattern of phenol can be observed by out-of-plane vibrations of the aromatic C-H in the region from 900 to 650 cm^{-1} (Zhang et al. 2006). Peaks in the areas of 744 cm^{-1} (adjacent four hydrogen atoms on the benzene ring), 810 cm^{-1} (adjacent two hydrogen atoms) and 879 cm^{-1} (single hydrogen atom on the benzene ring) suggest the presence of p-substitution, o-substitution and di- and/or tri-substitution of phenol, respectively. The peak at 700 cm^{-1}, indicates the presence of unreacted phenol in the samples (Li et al. 2015). The FTIR spectra indicate that the biomass components

were decomposed, phenolated and carbonized during microwave treatment in an acidic environment.

The pretreatment step allowed the release of trace amounts of fermentable sugars (maximum 0.5 $mg \cdot g^{-1}$ d.m.). The samples were subjected to enzymatic hydrolysis. The results of the analyses of the released sugars are shown in Table 17 and Figure 20.

Table 17. Average concentration of fermentable sugars released from giant miscanthus biomass after enzymatic hydrolysis of microwave-treated biomass

Sample pretreatment		Glucose concentration	Galactose/ xylose concentration	Arabinose concentration
		[$mg \cdot g^{-1}$ d.m.]		
0.1 M NaOH	30 psi	14.0 ± 4.1 ^{c}	15.5 ± 0.5 ^{b}	2.5 ± 0.3 ^{a}
	60 psi	19.8 ± 0.8 ^{c}	18.5 ± 0.3 ^{a}	2.5 ± 0.2 ^{a}
	90 psi	36.9 ± 2.6 ^{b}	15.1 ± 0.5 ^{b}	1.3 ± 0.1 ^{b}
0.1 M H_2SO_4	30 psi	26.6 ± 0.4 ^{bc}	5.0 ± 0.1 ^{d}	0.0 ± 0.0 ^{e}
	60 psi	39.7 ± 1.5 ^{b}	4.0 ± 0.2 ^{d}	0.0 ± 0.0 ^{e}
	90 psi	49.6 ± 2.3 ^{a}	3.3 ± 0.3 ^{d}	0.0 ± 0.0 ^{e}
H_2O	30 psi	18.4 ± 0.0 ^{c}	6.5 ± 0.2 ^{d}	0.8 ± 0.1 ^{c}
	60 psi	21.3 ± 1.2 ^{bc}	9.1 ± 0.5 ^{c}	0.6 ± 0.1 ^{c}
	90 psi	35.6 ± 1.7 ^{b}	8.4 ± 0.6 ^{c}	0.1 ± 0.0 ^{d}
60% EtOH	30 psi	15.1 ± 4.3 ^{c}	3.9 ± 0.9 ^{d}	0.8 ± 0.1 ^{c}
	60 psi	15.2 ± 6.1 ^{c}	5.4 ± 0.8 ^{d}	0.8 ± 0.4 ^{c}
	90 psi	23.0 ± 0.1 ^{bc}	7.4 ± 0.1 ^{c}	1.3 ± 0.1 ^{b}

The various letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

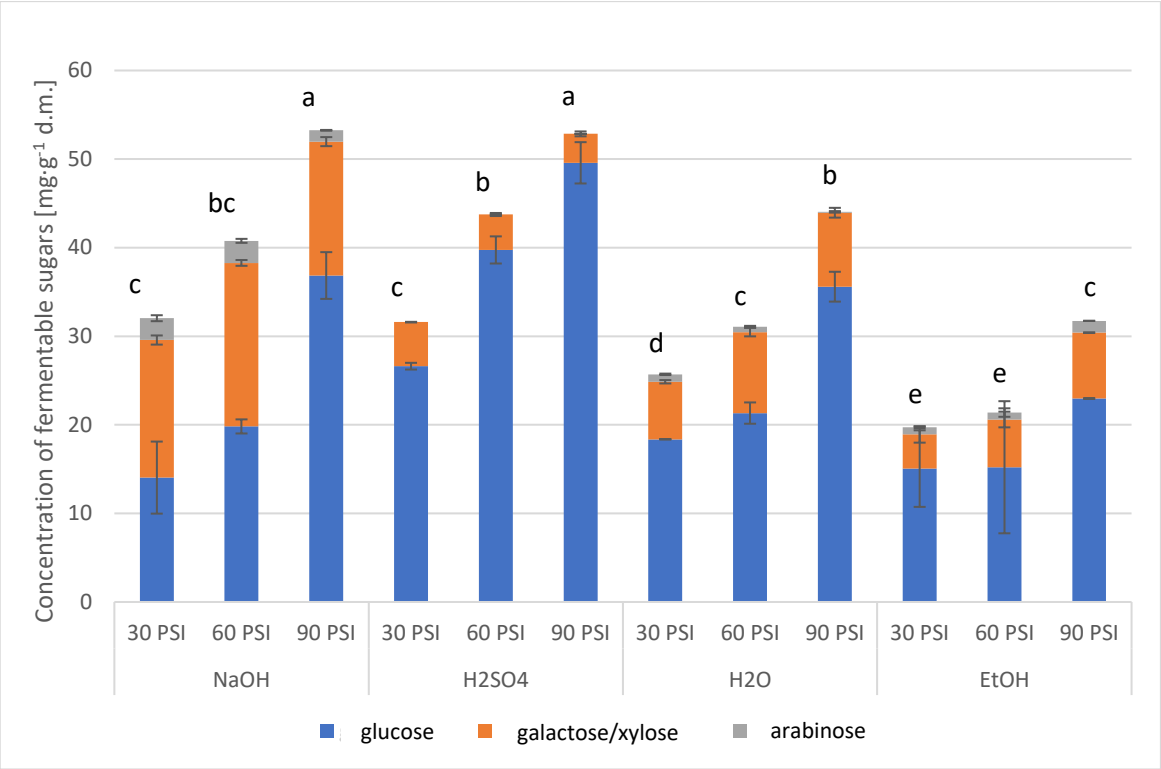


Fig. 20. Concentration of fermentable sugars released from biomass after enzymatic hydrolysis of microwave-treated giant miscanthus biomass

The various letters (a, b, c, …) indicate statistical significance at $p < 0.05$.

Increased concentrations of pretreatment by-products in lignocellulosic hydrolysates have a negative impact on yeast metabolism and fermentation activity (Tian et al. 2009). The statistical analysis of the results (Fig. 21) revealed that, enzymatic hydrolysis was not significantly affected by vanillin ($p=0.13$) or syringaldehyde ($p=0.61$). Similarly, syringaldehyde concentration in the samples analyzed did not significantly affect ethanol concentration ($p=0.60$). In contrast, the final ethanol concentration in the post-fermentation samples was significantly affected by the concentration of 5-HMF ($p=0.00$), vanillin ($p=0.01$) and vanillic acid ($p=0.00$). The most important microwave pretreatment parameters are the temperature (and corresponding pressure) and the exposure time of the biomass to microwave radiation (Mikulski et al. 2019).

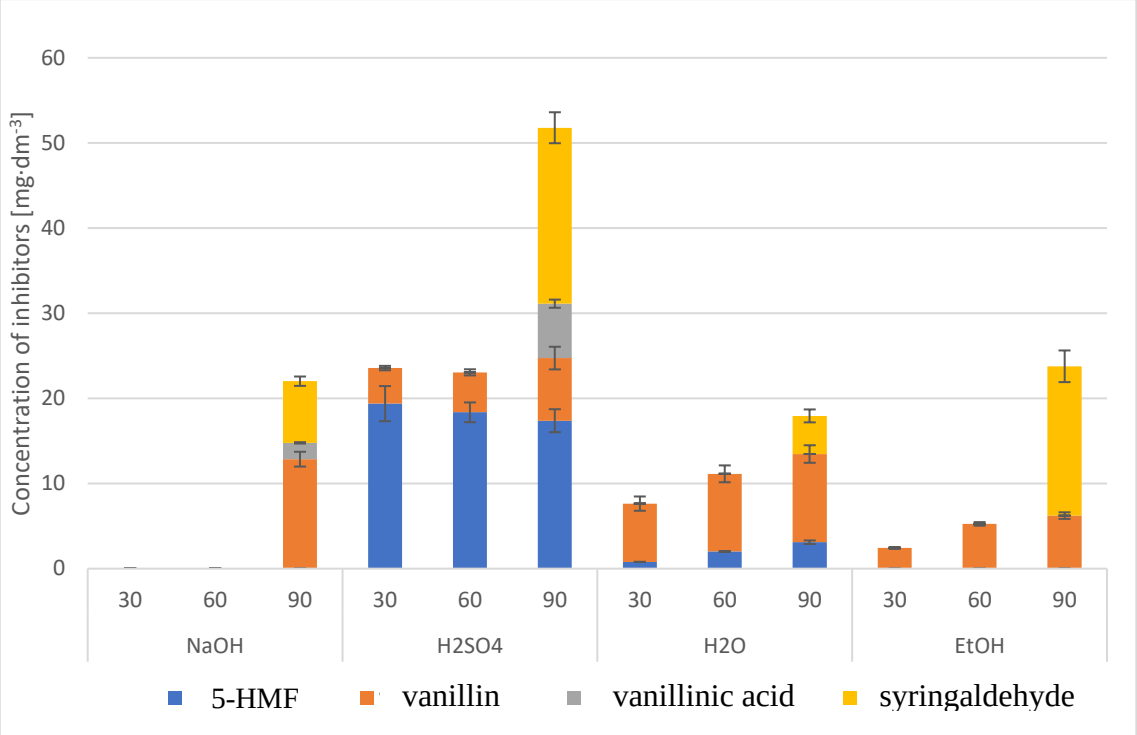


Fig. 21. Concentration of inhibitors formed as a result of pre-treatment of lignocellulosic biomass of giant miscanthus

Rana et al. (2014) discovered a relationship similar to the observed in this experiment. In their study, higher pressure and temperature pretreatment of corn straw increased the concentration of fermentation inhibitors, such as furfural and HMF, while decreasing the concentration of sugars, including xylose. Koo et al. (2011) in an experiment on pretreatment of American tulip tree (*Liriodendron tulipifera*) by the "organosolv" process with sodium hydroxide showed that an increase in pretreatment temperature increased enzymatic hydrolysis efficiency.

The hydrolysis efficiency of giant miscanthus biomass after chemical and microwave pretreatment is shown in Table 18 and Figure 22. It was found that achieving high enzymatic hydrolysis yields does not imply the best fermentation conditions for microorganisms, as shown by the results obtained after fermentation of microwave-treated samples in an acidic environment. The highest hydrolysis efficiency occurred in samples with sulphuric acid (VI) at 90 psi (58.3%), but fermentation of these hydrolysates yielded only 13.1 mg of ethanol per gram of dry biomass. An inverse correlation occurred in samples treated in an acidic environment at 30 psi. At the relatively low hydrolysis efficiency of these samples (30.1%), 23.6 mg of ethanol per gram of dry biomass was obtained. The amount of fermentation inhibitors formed during microwave treatment has

a negative impact on ethanol fermentation productivity (Hou et al. 2019, Bundhoo 2018). The trade-off between high enzymatic hydrolysis efficiency and ethanol fermentation productivity should be considered when selecting microwave treatment parameters. Alternative detoxification methods should also be considered (Ludwig et al. 2013).

The loss of CO_2 (Table 18) after fermentation was greater as the applied pressure increased in all samples, except for samples in which distilled water was used as a solvent. In this case, the higher the applied pressure during microwave treatment, the lower the CO_2 loss was.

Table 18. Summary of enzymatic hydrolysis efficiency and ethanol concentration and CO_2 loss after fermentation of lignocellulosic biomass of giant miscanthus

Sample pretreatment		Hydrolysis efficiency [%]	Ethanol concentration [mg·g^{-1} d.m.]	CO_2 loss [g]
0.1M NaOH	30 psi	18.0 ± 5.2 ^{f}	7.8 ± 0.8 ^{f}	0.102 ± 0.015 ^{e}
	60 psi	23.6 ± 0.9 ^{e}	10.2 ± 0.3 ^{e}	0.116 ± 0.001 ^{de}
	90 psi	46.1 ± 3.3 ^{b}	19.0 ± 0.2 ^{b}	0.186 ± 0.004 ^{a}
0.1M H_2SO_4	30 psi	30.8 ± 0.4 ^{cd}	23.6 ± 0.9 ^{a}	0.084 ± 0.004 ^{f}
	60 psi	45.3 ± 1.7 ^{b}	19.6 ± 1.0 ^{b}	0.110 ± 0.006 ^{e}
	90 psi	58.3 ± 2.7 ^{a}	13.1 ± 0.2 ^{c}	0.143 ± 0.040 ^{c}
H_2O	30 psi	22.3 ± 0.0 ^{e}	9.7 ± 0.6 ^{e}	0.150 ± 0.008 ^{b}
	60 psi	27.5 ± 1.5 ^{d}	11.1 ± 0.1 ^{d}	0.142 ± 0.049 ^{c}
	90 psi	44.1 ± 2.1 ^{b}	19.0 ± 1.4 ^{b}	0.128 ± 0.006 ^{d}
60% EtOH	30 psi	20.5 ± 5.8 ^{ef}	12.7 ± 1.3 ^{c}	0.104 ± 0.016 ^{e}
	60 psi	26.7 ± 1.3 ^{d}	11.4 ± 0.1 ^{d}	0.123 ± 0.006 ^{d}
	90 psi	34.7 ± 0.1 ^{c}	13.0 ± 2.3 ^{c}	0.159 ± 0.027 ^{b}

The various letters (a, b, c, …) in columns indicate statistical significance at $p < 0.05$.

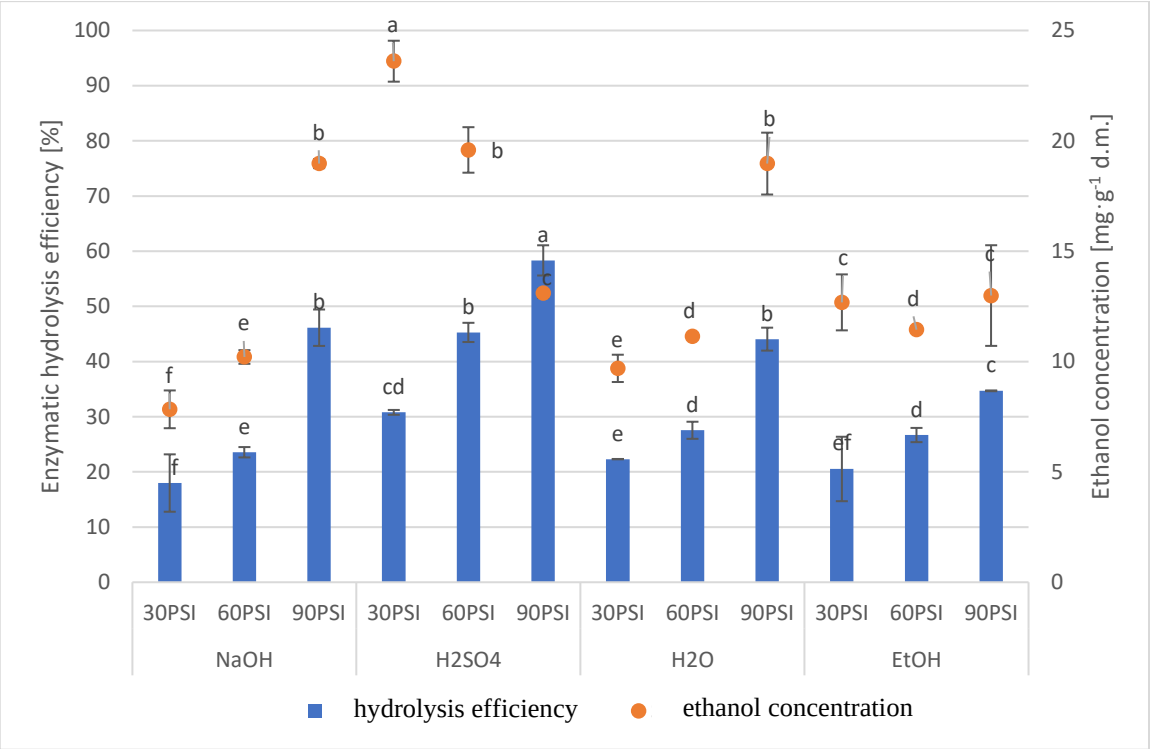


Fig. 22. Enzymatic hydrolysis efficiency and ethanol concentrations after fermentation of lignocellulosic biomass of giant miscanthus

The various letters (a, b, c, …) above the columns and above the dots indicate statistical significance at $p < 0.05$.

5.4. Directed adaptation of the yeast *Rhodotorula* sp. to use lignocellulosic hydrolysates as raw material for carotenoid synthesis

Rhodotorula sp. are unicellular yeasts that can accumulate a high content of lipids, carotenoids, and enzymes intracellularly, making them valuable bioproducts due to their natural biological activity (Pi et al. 2018). Carotenoids have numerous applications in the pharmaceutical, cosmetic, chemical, and food industries. They function as pigments and vitamin A precursors, as well as having antioxidant properties, tumor suppressor activity, and tumor-induced immune response (Garcia-Cortes et al. 2021). *Rhodotorula* synthesizes three kinds of carotenoids: β-carotene, torulene, and torularhodin (Petrik et al. 2013a). The high cost of microbial biosynthesis of lipids and carotenoids by yeast currently precludes their use on an industrial scale. One possible solution to this problem is to use waste products as ingredients in culture media (Kot et al. 2019, Kurcz et al. 2016).

The purple willow (*Salix purpurea*) bark raffinate used in this experiment is a waste product and a potential substrate for producing bioproducts in biotechnological processes (Walter et al. 2021b). Table 19 shows the composition of the purple willow bark raffinate after supercritical CO_2 extraction.

Table 19. Contribution of lignocellulosic components of raffinate biomass of purple willow bark after supercritical CO_2 extraction

	NDF [% d.m.]	ADF [% d.m.]	ADL [% d.m.]	Concentration [% d.m.]	
				Cellulose	Hemicellulose
purple willow bark refiner after supercritical CO_2 extraction	51.79 ± 0.17	51.47 ± 0.33	21.50 ± 0.35	29.97	0.32

The experiment's goal was to direct the yeast *Rhodotorula* sp. metabolism toward the use of lignocellulosic raffinate hydrolysate from purple willow bark after $scCO_2$ extraction as a component of the culture medium. Initially, the biomass of the raffinate (10%, w:v) was subjected to alkaline pretreatment and enzymatic hydrolysis. Hydrolysate was used as a component of the culture medium. *Rhodotorula* sp. strains (*R. glutinis* and *R. rubra* KKP350) were passaged twice from solid YGC culture medium to media with gradually increasing lignocellulosic hydrolysate content, until 25% (v/v) hydrolysate in the medium was reached (Figure 6 in experimental part), in order to adapt the strain to growth under unfavorable conditions.

The strains with potentially enhanced growth capacity on lignocellulosic substrate were cultured for one week on test media containing liquid YGC microbial medium with lignocellulosic hydrolysate, ranging from 100% YGC to 100% hydrolysate. Changes in the optical density of the culture were used to assess microbial growth (Figure 23; Table 20).

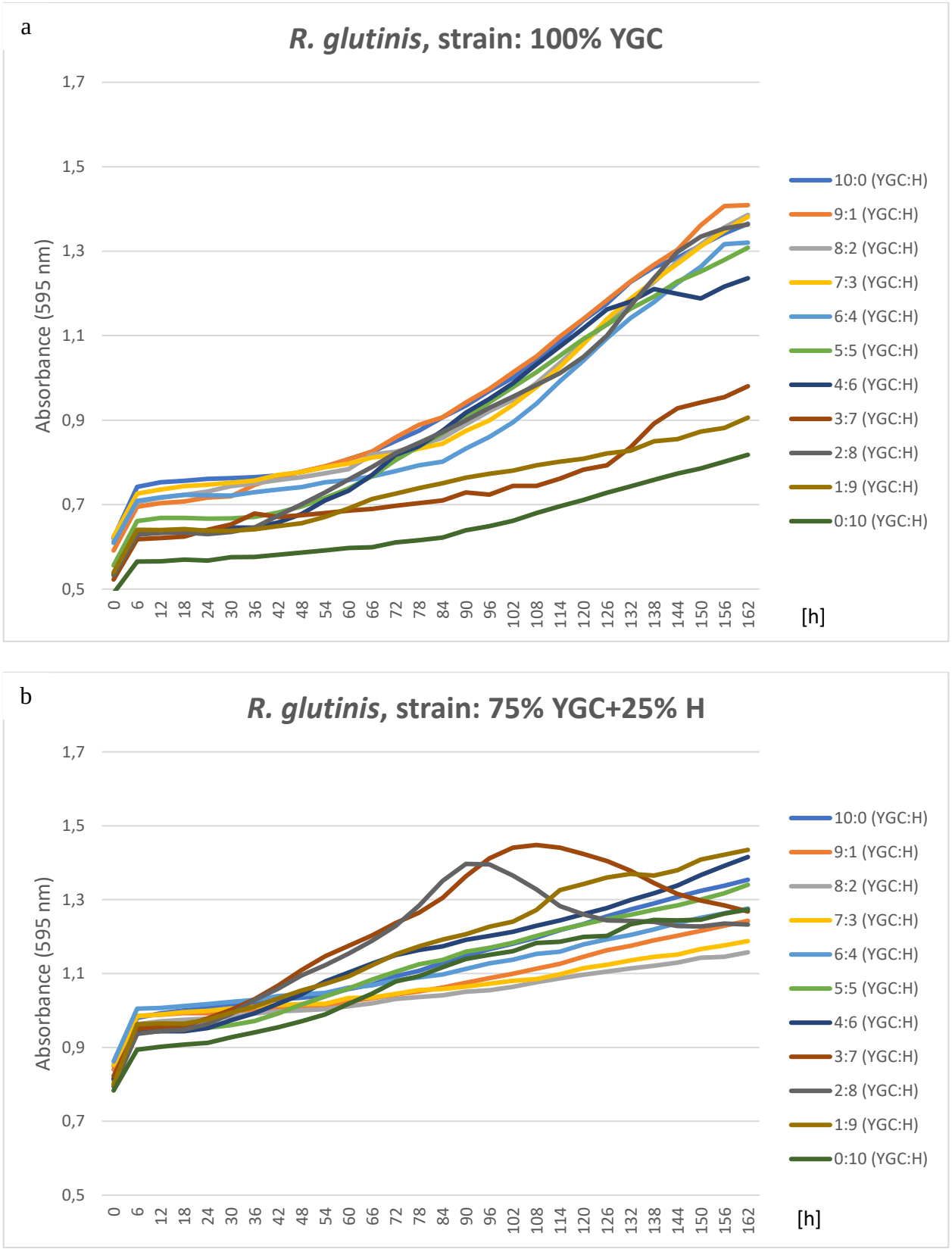


Fig. 23. Example growth kinetics of *R. glutinis* strains: native (a) and subjected to adaptation (b)

Table 20. Changes in optical density (absorbance measured at 595 nm) of *Rodotorula* cultures grown on mediums with increased share of hydrolysate from purple willow bark

YGC : hydrolyzate ratio	*R. rubra* KKP350 culture optical density change	*R. glutinis* culture optical density change
10:0	- 11.1%	- 0.8%
9:1	- 18.4%	- 11.8%
8:2	- 21.8%	- 16.5%
7:3	- 18.7%	- 14.0%
6:4	- 21.4%	- 3.4%
5:5	- 12.0%	+ **2.4%**
4:6	- 14.3%	+ **14.5%**
3:7	+ **23.2%**	+ **29.4%**
2:8	+ **51.2%**	+ **9.6%**
1:9	+ **23.7%**	+ **58.4%**
0:10	+ **45.0%**	+ **80.0%**

The comparison of the growth capacities of the modified strains and the native strains (Table 20) demonstrated that the guided adaptation strategy was successful. Strains subjected to guided adaptation to the use of lignocellulosic hydrolysate, cultured in media containing 70% (v/v) hydrolysate, demonstrated increased growth ability in lignocellulosic hydrolysate-containing media. When compared to the native strain, the modified *R. glutinis* strain grew 80% faster in the liquid lignocellulosic hydrolysate.

It was considered possible that strains with increased growth capacity on medium supplemented with lignocellulosic hydrolysate would be able to synthesize carotenoids more efficiently. Obtained modified strains (*R. glutinis* 50H, *R. rubra* KKP350 50H and *R. glutinis* KKP347 50H) and native strains were used to synthesize carotenoids in liquid YGC medium with the addition of hydrolysate (YGC:H) in a ratio of 1:1. After 7 days of culture, extraction of carotenoids from yeast biomass and their quantitative analysis was performed. Each of the modified strains showed a more efficient synthesis of carotenoids compared to the corresponding test strain (Figure 24, Table 21).

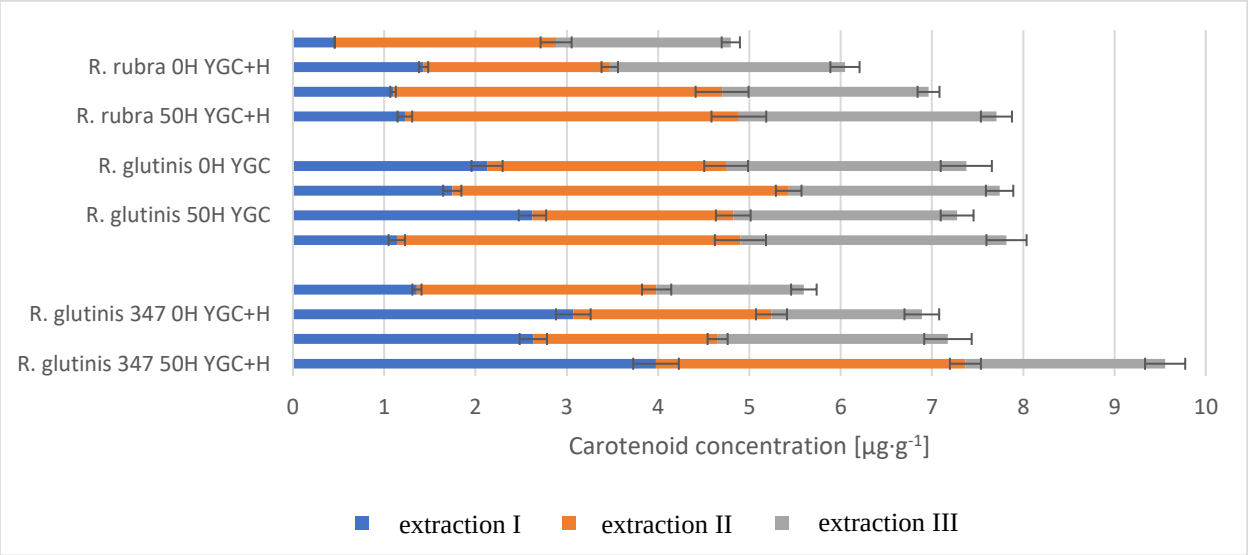


Fig. 24. Carotenoid concentrations obtained by three-fold extraction of carotenoids from native (0H) and adapted (50H) yeast biomass after culture in YGC or YGC media with lignocellulosic hydrolysate (YGC+H)

The use of lignocellulosic hydrolysate as a substrate additive increased the amount of carotenoids obtained, even in native strains. After biomass extraction of *R. glutinis* KKP347 50H strain and culture on medium with lignocellulosic hydrolysate, the highest concentration of carotenoids (130 $\mu g \cdot g^{-1}$ d.m.) was obtained.

Table 21. Carotenoid concentrations in the biomass of native and adapted yeast to use lignocellulosic hydrolysate (50H) after culture in YGC or YGC+H media.

		YGC			YGC with added lignocellulosic hydrolysate (1:1)		
	strain	[μg·g^{-1} wet biomass]	[μg·g^{-1} d.m.]	[mg·dm^{-3} of culture]	[μg·g^{-1} wet biomass]	[μg·g^{-1} d.m.]	[mg·dm^{-3} of culture]
R. rubra KKP350	native	6.96 ± 0.31 ^{a}	71.43 ± 3.10 ^{a}	0.50 ± 0.02 ^{a}	7.71 ± 0.40 ^{b}	84.17 ± 4.36 ^{c}	0.58 ± 0.03 ^{b}
	50H	6.05 ± 0.35 ^{b}	59.21 ± 3.41 ^{b}	0.28 ± 0.01 ^{c}	4.80 ± 0.27 ^{d}	59.15 ± 3.36 ^{d}	0.30 ± 0.02 ^{c}
R. glutinis	native	7.38 ± 0.28 ^{a}	62.58 ± 2.27 ^{b}	0.39 ± 0.01 ^{b}	7.74 ± 0.34 ^{b}	83.85 ± 3.72 ^{c}	0.58 ± 0.03 ^{b}
	50H	7.27 ± 0.35 ^{a}	69.53 ± 3.37 ^{a}	0.37 ± 0.02 ^{b}	7.82 ± 0.28 ^{b}	87.77 ± 3.16 ^{c}	0.62 ± 0.02 ^{a}
R. glutinis KKP347	native	5.60 ± 0.26 ^{b}	53.27 ± 2.48 ^{c}	0.48 ± 0.02 ^{a}	6.89 ± 0.16 ^{c}	104.86 ± 2.42 ^{b}	0.58 ± 0.01 ^{b}
	50H	7.18 ± 0.42 ^{a}	72.29 ± 4.42 ^{a}	0.46 ± 0.03 ^{a}	9.56 ± 0.11 ^{a}	130.22 ± 1.46 ^{a}	0.59 ± 0.00 ^{ab}

The various letters (a, b, c, …) indicate statistical significance at $p < 0.05$.

Under controlled conditions, adaptive laboratory evolution is successfully used to obtain a specific microbe population. Natural selection thus targets a specific environment with desired conditions for the population to become better adapted to it (Mavrommati et al. 2022). Because of their utility, microorganisms are commonly used in adaptive laboratory evolution experiments. These include short generation times (20 minutes to 10 hours), large population sizes, and an environment that is easily applicable and reproducible (Buckling et al. 2009). During microbial adaptive evolution, a selected microorganism is cultured for an extended period of time (weeks to years) under specific conditions that exert pressure on it toward a desired trait, using natural evolution principles, until a new trait emerges. In this way, a new strain is created that is enriched with advantageous mutations (Sandberg et al. 2019). In this experiment, cells of the desired microorganism are inoculated into a suitable medium, cultured with the selection agent, and then transplanted into the medium with increasing concentrations of the selection agent. The process is repeated for the desired number of generations. The selection pressure either remains constant or increases during the experiment (Zhu et al. 2018).

Only the preliminary results of the *Rhodotorula* yeast growth propagation stage were used in the dissertation experiment to use substrates derived from the hydrolysis of lignocellulose from purple willow bark raffinate. Due to its high yielding potential, rapid regrowth of annually cut shoots, and the unique chemical composition of bark and wood, purple willow biomass has a wide range of industrial applications (Sulima et al. 2021). Advanced genetic studies are required to confirm that the obtained new *Rhodotorula* yeast acquired new skills in the metabolism of substrates from the lignocellulosic matrix.

In addition to saccharides and micronutrients, biomass hydrolysates typically contain a mixture of inhibitory compounds, and their synergistic effect can strongly inhibit microbial growth (Mussatto and Roberto 2004). The yeast directed adaptation strategy used in this study was designed to improve the growth capacity of strains in medium containing purple willow bark raffinate hydrolysate.

After optimizing the conditions for carotenoid synthesis by the yeast *R. mucilaginosa* on glycerol medium, Allahkarami and team (2021) determined that under optimal conditions the average maximum carotenoid content could be 223.5 $\mu g \cdot g^{-1}$ d.m.

In their experiments, they discovered that irradiating the culture with white LED light at 395-530 nm had a positive effect.

Petrik et al. (2013b) conducted similar experiments with *R. glutinis,* among other strains. They involved the use of hydrolysate of wheat straw acid pretreated after ethanol fermentation by *S. cerevisiae* Ethanol Red as a culture medium to obtain bioproducts such as ergosterol and carotenoids. The experiments used a native strain that did not prefer the presence of hydrolysate in the medium and a strain that had been pre-adapted to the presence of hydrolysate in the medium (50:50 addition of hydrolysate to inoculum). Depending on the temperature used during pretreatment of wheat straw, the *R. glutinis* strain produced between 9.7 and 37.5 μg of carotenoids per gram of biomass. Despite lower biomass gain efficiency, strains pretreated with the presence of hydrolysate in the substrate showed more efficient synthesis of carotenoids using hydrolysate of straw treated at 190 and 195°C. Similar experiments were carried out by Liu et al. (2021), who adapted *Rhodoporidium toruloides* to grow in a wheat straw hydrolysate-based medium. The modified strains performed better on the test medium, but biomass growth was higher after nitrogen supplementation of the medium with ammonium chloride in their experiment.

The yeast *Rhodotorula* sp. can use a variety of carbon sources, including glucose, xylose, cellobiose, sucrose, glycerol, sorbitol, and others (Parajó et al. 1998), making lignocellulosic waste products a low-cost substrate for their cultivation. Many factors influence the efficiency of growth and bioproduct synthesis by the yeast *Rhodotorula* sp., including medium composition and acidity, the presence of a nitrogen source, temperature, culture time, oxygenation, and the degree of exposure of the culture (Allahkarami et al. 2021).

6. Conclusions and observations

The following hypotheses were put forward and verified in this study:

Hypothesis 1:

The use of recycling of enzymes adsorbed on the solid fraction of lignocellulosic material is an effective technique for improving the economic efficiency of lignocellulosic hydrolysis of miscanthus species and sugar beet biomass.

The results obtained confirmed that:

- The hydrolysis efficiency of the lignocellulosic biomass, measured by the amount of reducing sugars released relative to the primary content of these sugars obtained in the hydrolysates from cycle I (using commercial enzyme preparations), is about 70% in cycle II (in both miscanthus and sugar beet samples) and about 50% for both miscanthus species in cycle III,
- The efficiency of biomass hydrolysis of both miscanthus species using enzyme recycling increases with increasing cellulase activity in the range 18-58 FPU,
- The recycling of enzymes in sugar beet samples is mostly effective in the variants using vacuum expansion, particularly for samples obtained at 50°C,
- The highest yield of reducing sugars (58.4 $mg \cdot g^{-1}$ d.m.) in the second cycle of sugar beet enzymatic hydrolysis is obtained in the vacuum-exploded variant after treatment with the Rapidase® and Accellerase 1500 enzyme combination with a supporting enzyme complex (Accellerase BG + Accellerase XC+ Accellerase XY).

Hypothesis 2:

The use of vacuum expansion of sugar beet biomass and microwave treatment of giant miscanthus biomass increases the susceptibility of biomass to enzymatic hydrolysis and promotes alcoholic fermentation.

The results obtained confirmed that:

- Vacuum expansion allows an increase in enzymatic hydrolysis capacity of giant miscanthus and sugar miscanthus biomass by 16-32 $mg \cdot g^{-1}$ d.m. and 11-28 $mg \cdot g^{-1}$

d.m. of reducing sugars in hydrolysis cycle I and II, respectively, compared to mechanical shredding,

- The most effective process option for treating giant miscanthus and sugar miscanthus biomass is the use of vacuum expansion and subjecting the resulting material to enzymatic hydrolysis at 50°C using enzyme compositions: Rapidase® and Accellerase 1500, with the addition of a supporting enzyme complex. On the medium prepared in this way, it was possible to reapply the enzymes as an immobilized fraction on undecomposed lignocellulose. This made it possible to obtain an average of 58.4 $mg \cdot g^{-1}$ d.m. of reducing sugars and 13.5 $mg \cdot g^{-1}$ d.m. of sucrose,
- The highest average ethanol concentration in the post-reaction medium is obtained after fermentation carried out in the sequential system by *S. cerevisiae* 7, yielding 50.5 and 53.6 $g \cdot dm^{-3}$, respectively, after fermentation of hydrolysates obtained from mechanically shredded and vacuum-exploded beet biomass. After using the simultaneous process, the attenuation level is lower than that obtained in the SHF system, and the alcohol content was 19% lower (when shredded biomass was used) and 15% lower (after vacuum expansion). The lack of suitability of the filamentous fungus *M. indicus* in the ethanol fermentation of sugar beet biomass is found,
- The highest hydrolysis efficiency occurs in samples with sulphuric acid (VI) at 90 psi (58.3%), but fermentation of these hydrolysates yielded only 13.1 mg of ethanol per gram of dry biomass. The opposite relationship occurs in samples treated in an acidic environment at 30 psi. At the relatively low hydrolysis efficiency of these samples (30.1%), 23.6 mg of ethanol per gram of dry biomass is obtained,
- The choice of microwave treatment parameters should be considered with a trade-off between high enzymatic hydrolysis efficiency and ethanol fermentation productivity.

Hypothesis 3:

The use of a directed adaptation of the yeast *Rhodotorula* predisposed to carotenoid synthesis will result in more efficient bioconversion of lignocellulosic hydrolysates from purple willow bark raffinate to carotenoids.

The results obtained confirmed that:

- Guided laboratory adaptation produces *Rhodotorula* strains with enhanced growth capacity on medium supplemented with lignocellulosic hydrolysate,

- The obtained modified strains (*R. glutinis* 50H, *R. rubra* KKP350 50H and *R. glutinis* KKP347 50H) show a more efficient synthesis of carotenoids compared to the corresponding test strain. The amount of carotenoids obtained is positively influenced by the use of lignocellulosic hydrolysate as a substrate additive, also in the case of the native strains. The highest carotenoid concentration (130 $\mu g \cdot g^{-1}$ d.m.) is obtained after biomass extraction of the *R. glutinis* KKP347 50H strain, following culture on medium with lignocellulosic hydrolysate.

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8. Appendices

Biomass Conversion and Biorefinery
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REVIEW ARTICLE

2G-biofuel ethanol: an overview of crucial operations, advances and limitations

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Abstract
From year to year, mankind is struggling with an increasing climate crisis. Constant climate change is related to human activities. The global dependence on non-renewable fossil fuels to meet our current energy needs cannot be sustained longer in the face of depleting fuel reserves. Particular attention should be paid to biofuels produced from lignocellulosic biomass, which is a waste product from the forestry, paper and agricultural industries, or a product derived from energy crops, intended for biofuel purposes. A problem that arises when using lignocellulosic biomass is the limited availability of fermentable sugars due to its complicated structure. For this reason, various methods of pretreatment of the raw material are used. The aim of the pretreatment is to increase the availability of cellulose for hydrolytic enzymes and to separate the main fractions of the lignocellulosic material, mainly the lignin molecule. After pretreatment and hydrolysis, the monomeric sugars are further processed into ethanol through the fermentation process. The last stages in biotechnological lignocellulose conversion are the distillation and dehydration process to obtain pure biofuel. This work is a review of the literature on second-generation biofuels produced from lignocellulosic biomass. Issues ranging from various types of raw materials, methods of its pretreatment, enzymatic hydrolysis, fermentation with the participation of various microorganisms, to various methods of dehydration of the obtained bioethanol were discussed.

Keywords Bioethanol · Bioconversion · Lignocellulosic biomass · Pretreatment · Fermentation · Hydrolysis

1 Introduction

The need to develop alternative solutions for energy acquisition is a consequence of limited resources of fossil fuels, increasing oil prices and environmental protection [1]. New types of fuels are being sought all over the world to replace partially or completely conventional fuels derived from crude oil processing and pose little threat to the environment [2]. Bioethanol, along with biodiesel, is currently one of the most popular biofuels produced for energy purposes [3]. Lignocellulosic biomass is an abundant and cost-effective renewable bioresource that can be used to produce second-generation bioethanol [4]. Moreover, lignocellulosic materials are renewable, which means that the carbon released to the atmosphere as carbon dioxide in the process of liquid fuel combustion is returned and assimilated by new plants [5].

Pilot second-generation bioethanol biorefineries have been operating in Denmark, Spain and Canada. The annual global production of lignocellulosic biomass is estimated at 10–50 bn tonnes of dry mass. According to statistics, lignocellulosic biomass from agricultural residues can be used to produce 491 bn litres of bioethanol a year [6, 7]. Rice straw is one of the lignocellulosic raw materials, produced at about 731 million tonnes a year. According to Kim and Dale [8], this amount of biomass can be used to produce 205 bn litres of ethanol a year. For comparison, wheat straw can be used to produce 104 bn litres of ethanol. The growing importance of bioethanol from lignocellulose arises not only from the economic aspects (seeking a cheap replacement for oil) and increasing demand for energy, but also from the benefits that its use brings for the environment. As an

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additive to fuels used in transport, it increases their octane number. It also helps to achieve greater power and more complete combustion, and as a result, it reduces the amount of smoke produced by engines fuelled with ethanol [9]. With this solution, the greenhouse gas emission can be reduced by approx. 31% [10]. It is also noticeable that bioethanol is soluble in water and biodegradable, which determines its low environmental toxicity and minimises the risk of environmental disasters should a leak occur [11].

Cellulosic ethanol is dehydrated ethyl alcohol produced by the alcohol fermentation of saccharides obtained from plant raw materials. It can be used directly as fuel or as an additive to fossil fuels, at 5–20% of the mixture (E5–E20)—in Europe, it is usually 5% or 10% [11]. It can potentially replace petrol on the market of fuels for transport, but the cost of its production is still greater compared to traditional fuels [7]. Second-generation bioethanol has a high flash point (150 °C), which makes it safe, and good lubricating properties that have a positive effect on engine life. In addition, its use may allow biomass to be used for non-food purposes, which will enable the use of fallow and chemically contaminated land, as well as to maintain economic sovereignty by countries that do not have large reserves of conventional fuels [12].

Given the specific structure of lignocellulosic materials, their conversion to ethanol is much more difficult than of materials rich in sucrose or starch. Despite the environmental benefits brought by cellulosic ethanol production, its cost-effectiveness remains a problem. Lignocellulose conversion to ethanol and other products of a higher added value requires multi-stage processing, which includes pretreatment (mechanical, chemical, biological), polysaccharide hydrolysis to monosaccharides, fermentation of hexoses and/or pentoses to ethanol and ethanol isolation from the fermentation mixture [8, 13, 14].

The aim of this article was to review the literature on the production of second-generation bioethanol, detailing the leading methods of pretreatment and its role for the effective and efficient conduct of subsequent bioconversion steps—hydrolysis and alcoholic fermentation. Attention was also drawn to the limitations in the commercialisation of second-generation bioethanol, and the directions for further research in this area were presented.

2 Preparing lignocellulosic raw materials for fermentation

Lignocellulosic biomass available for energy generation is derived mainly in the form of (a) agricultural waste, (b) forest waste and (c) industrial waste [15]. Depending on the chemical composition, it can be divided into six main groups: agricultural residues (cane bagasse, maize straw, wheat straw, rice straw and barley straw, rice husks, sweet sorghum bagasse), hardwood (aspen, poplar), softwood (pine, spruce), cellulose waste (newspaper paper, office wastepaper), algae and green plants (switchgrass, coastal Bermudagrass) and municipal solid wastes (MSWs) [16, 17].

The main components of lignocellulosic biomass include carbohydrate polymers—cellulose (40–55% per dry weight), hemicellulose (24–40% per dry weight) and lignin (18–25% per dry weight), which is an aromatic heteropolymer. These polysaccharides are closely bonded to lignin by covalent and hydrogen bonds [18]. The percentage of each component in lignocellulosic raw materials varies and depends on the type of plant, its age, climate conditions and the time of harvest [19].

Due to the structural features of the lignocellulosic complex, pretreatment is a necessary stage before hydrolysis, whose aim is to release monosaccharides, subsequently used by microorganisms in fermentation. Pretreatment should not contribute to polysaccharide loss or producing by-products, which inhibit the ethanol fermentation process [16, 20, 21].

The conversion of lignocellulosic biomass to second-generation bioethanol encounters economic and technological difficulties [22, 23]. Liquid fuels can be obtained by thermochemical and biochemical process. In the first process, the biomass undergoes thermal decay and almost all organic components of the biomass are converted. In contrast, the biochemical process uses only polysaccharides from biomass, which are converted into sugars and fermented. The conversion of biomass to sugars is a major technological obstacle to the production of cost-effective liquid biofuels [2]. The production of bioethanol from lignocellulose is characterised by a complicated production process and high capital intensity. This is largely due to the high inefficiency of lignin extraction under mild pretreatment conditions, guaranteeing minimal polysaccharide losses [24]. The success of second-generation bioethanol production will therefore depend on the development of simple pretreatment technologies that ensure the effective delignification of different types of lignocellulosic biomass [25]. Reducing enzyme costs while increasing cellulose hydrolysis efficiency is another important factor in the development of appropriate pretreatment technologies [24]. Lignin, by creating a physical barrier limiting the access of cellulases to cellulose, is the main obstacle to enzymatic hydrolysis. Selective removal can minimise cellulose degradation and thus increase the efficiency of the hydrolysis process itself [2, 26]. Another challenge in developing a cost-effective method for the bioconversion of lignocellulose to bioethanol is to ensure the supply of lignocellulosic biomass from various plant sources throughout the year [24], to reduce the energy intensity of subsequent process steps and their integration, to develop fermentation technology under conditions of high constant load and to genetically modify fermenting microorganisms,

mainly in terms of their tolerance to high concentrations of ethanol and fermentation of pentoses [3, 27, 28]. The economic rationale for the introduction of second-generation bioethanol into the industrial scale must also include solutions to reduce the environmental impact of the whole process, the distribution of biofuels and public acceptance [3].

Modern pretreatment methods can account for more than 40% of the total production costs and are the most energy-intensive stage of processing lignocellulosic biomass into biofuels [2]. Most conventional pretreatment methods have drawbacks, including the release of degradation products, lack of selectivity, low sugar yield, low process efficiency, use of aggressive reagents, expensive tooling and high processing costs and negative environmental impact. Therefore, it is extremely important to search for and develop a novel and ecological pretreatment method with low strain, lower energy consumption and less waste [29].

2.1 Pretreatment

The aim of pretreatment is to separate lignin from polysaccharides of the lignocellulosic complex, to decrease the crystallinity of cellulose and to increase material porosity so that hydrolytic enzymes have access to the substrate polysaccharides in the subsequent phase of enzymatic hydrolysis [3, 16]. Effective pretreatment should not result in hemicellulose or cellulose loss or the formation of potential inhibitors of hydrolytic enzymes or microorganisms of ethanol fermentation [30]. Its economic purposes must consider minimising energy demand, reducing the cost of lignocellulose material cutting up, reducing the amount of produced residues (solid fraction, process water, chemical substances) and small consumption of chemical substances or their total elimination. At the same time, when comparing various pretreatment methods, all these criteria should be regarded as the basis for achieving the maximum bioconversion yield [13, 31].

Given the diverse chemical composition of lignocellulosic biomass, it is difficult to identify a universal method for its pretreatment. It has been observed that lignin and phenolic products of its degradation disrupt the process of enzymatic hydrolysis of the remaining components of the lignocellulose complex as a result of non-specific binding of cellulases, causing their inactivation [19, 32]. Pretreatment is a critical stage of producing ethanol from cellulose; it is responsible for 18% of the total production costs and has a significant impact on the effectiveness of subsequent unit processes, especially on the efficiency of releasing monosaccharides from polysaccharides in the material. Polysaccharide hydrolysis degree without pretreatment is lower than 20%, while pretreatment increases its level to 90% [6].

There are various known methods of lignocellulosic material pretreatment. In terms of the applied agents, they can be classified into physical, physicochemical, chemical and biological [33] (Table 1). The physical methods mainly include milling (diminution 0.2–2 mm) [34], pyrolysis [35], extrusion [36], sonication [37] and applying microwaves [38, 39] or a pulsating electric field [40]. Physicochemical methods (e.g., autohydrolysis, liquid hot water, ammonia fiber explosion) are more effective than physical ones. The main chemical methods include treatment of lignocellulosic material with acids (mainly sulphuric, hydrochloric, nitric (V) or phosphoric), alkalis (sodium or calcium hydroxide), peroxides, organic solvents and ozone [16].

2.1.1 Physicochemical pretreatment

Among the physicochemical methods, steam explosion (SE), with water as the main factor affecting lignocellulose, is the most common [43]. Particles of lignocellulosic biomass are treated briefly with steam at a high temperature (up to 260 °C) and pressure (0.69–4.85 MPa), after which they are cooled down rapidly, which results in breaking the bonds connecting lignin with polysaccharides of the complex. The process of steam explosion is accompanied by autohydrolysis reactions; as a result of which, some lignin hemicelluloses are transformed into soluble oligomers. Autohydrolysis is brought about by acetic acid released from acetyl groups in hemicelluloses at a high temperature [16, 36, 54]. Steam explosion is regarded as one of the most cost-effective methods in the pretreatment of hardwood (poplar, oak, birch, maple) and agricultural residues (maize straw, wheat straw), but it is less effective with softwood (pine, cedar) because of the lower content of acetyl groups in the hemicellulose fraction [48].

Another hydrothermal method is liquid hot water (LHW) treatment at high pressure (over 5 MPa) and at a temperature of 170–230 °C for several minutes to an hour [55, 56]. The pressure applied in the process keeps water in the liquid state at an elevated temperature and brings about changes in the lignocellulose structure. The LHW method uses the autohydrolysis process without rapid decompression. The main goal is to dissolve hemicellulose and to separate it from the solid material. By this method, it is possible to remove 80% of hemicellulose and to improve the yield of enzymatic hydrolysis of grassy materials [36, 47].

The use of microwaves to heat material immersed in a solution of chemicals (alkalis, acids, H_2O_2) accelerates the reactions that occur during the pretreatment process, compared to conventional heating. Microwaves alter the ultrastructure of cellulose by removing lignin and hemicellulose and thus increase the availability of cellulose for hydrolytic enzymes [45]. They enable homogeneous heating of biomass by inducing dipole rotation [57], and the high and uniform heating efficiency, easy handling and short processing time

Table 1 Selected methods of lignocellulosic biomass pretreatment (own study, based on [5, 41, 42])

Pretreatment method	Procedure	Comments	Refs
Physical methods			
Milling	Vibratory ball mill, hammer mill	Reduction in particle size (ultimately 0.2–2 mm or 3–6 mm) and cellulose crystallinity	[34]
Microwave radiation	300–700 W/m^3, 130–200 °C, addition of H_2SO_4 or NaOH	Reduction of the crystal structure of cellulose Simple operation, energy saving, short time	[38, 39]
Sonication (ultrasounds)	20–40 kHz, 60 °C	Breaking the hydrogen bonds in the structure of lignocellulose High cost and difficulties in carrying out in industrial conditions	[37]
Pyrolysis	Temperature > 300 °C, then cooling and condensation	Volatile products and char are formed Mild acid hydrolysis possible (1 N H_2SO_4, 2.5 h, 95 °C) to form 80–85% reducing sugars (> 50% glucose) The process can be carried out under reduced pressure (400 °C, 20 min, $p = 1$ mmHg)	[35]
Physicochemical methods			
Steam explosion	High-temperature steam (160–260 °C) and pressure (0.69–4.85 MPa), a few seconds or minutes, then decompress to atmospheric pressure	Hemicellulose hydrolysis 80–100% at 150 °C Destruction of the xylan structure Xylose recovery ~ 45–65% Dissolving lignin at a temperature of 180 °C and higher Creation of toxic compounds (including furfural, 5-HMF, acetic acid, formic acid, levulinic acid, phenolic compounds)	[43]
Liquid hot water	Hot water under pressure > 5 MPa, temperature 170–230 °C, 1–46 min, permanent load < 20%, pH = 4–7	Inhibitor formation Partial depolymerization of cellulose Lignin is not dissolved	[16, 44]
Ammonia recycled percolation	Increased pressure and temperature (160–180 °C), ammonia concentration 5–15% (w/w)	Removal of most of the lignin High cellulose content after pretreatment High energy costs and smooth charging	[45, 46]
AFEX (ammonia fiber explosion)	1–2 kg ammonia/kg d.s., 90 °C, 30 min, $p = 1.12$–1.36 MPa	Highly effective for biomass with a low lignin content No inhibitors formation Efficiency of enzymatic hydrolysis of cellulose and hemicelluloses > 90% Ammonia recovery needed Low degree of delignification (approx. 10–20% of lignin is dissolved)	[45–47]
CO_2 explosion	4 kg CO_2/kg of fiber, $p = 5.62$ MPa, temperature to 200 °C	Inhibitors are formed in a small amount Easy recovery of CO_2 after the process High pressure requirements Further cellulose conversion > 75% Low sugar degradation	[43, 45]
Wet oxidation	Oxygen pressure, $p = 1{,}2$ MPa, 195 °C, 15 min, addition of H_2O and small amounts of Na_2CO_3 or H_2SO_4	Formation of inhibitors of hydrolysis and fermentation Lignin degradation Mainly the hemicellulose fraction is dissolved	[42]
Chemical methods			
Oxidative delignification	Peroxidase and 2% H_2O_2, 20 °C, 8 h	Almost complete dissolution of hemicellulose Further cellulose conversion around 95% 50% degradation of lignin	[42]

Table 1 (continued)

Pretreatment method	Procedure	Comments	Refs
Soaking in aqueous ammonia	Temperature 20–80 °C, ammonia concentration 15–30% (w/w), time depends on the temperature used and the concentration of ammonia (12 h to 11 weeks)	Simplified bioconversion system Possibility to carry out the process at room temperature Hemicellulose remains in the solid fraction	[45, 46]
Fractionation with solvents	Organic solvents (methanol, ethanol, acetone, ethylene glycol) or a combination thereof with 1% H_2SO_4 or HCl, 186–198 °C, 30–60 min, pH = 2.0–3.4	The need for solvent recovery and recycling High xylose yield High degree of delignification and destruction of bonds between lignin and hemicellulose The cellulose remains in the solid phase	[48]
Acid treatment	0.75–5% H_2SO_4, HCl, HNO_3, $p \sim 1$ MPa, temperature > 160 °C	Gypsum as a by-product of pH neutralization Hemicellulose hydrolysis 80–100% High glucose yield Xylose yield 75–90% Precipitation of lignin High cost of acids and the need for their recovery High cost of corrosion-resistant equipment Formation of inhibitors of hydrolysis and fermentation	[15, 36]
Alkaline treatment	NaOH, $Ca(OH)_2$; can be combined with H_2O_2 (0.5–2.15%) when using a lower temperature (35 °C)	Effective lignin removal for hardwoods (24–55%), lower for softwoods Low inhibitor formation Hemicellulose hydrolysis > 50% Recovery of 60–75% of xylose	[46, 49]
Ozonolysis	Ozone, ambient temperature and pressure	The effectiveness of the method depends on the moisture content of the raw material and its particle size as well as the ozone concentration in the gas stream No inhibitor formation Lignin degradation Cellulose hydrolysis > 57%	[16]
Ionic liquids (Ils)	100–150 °C, solid to ionic liquid ratio 1:10	Dissolving cellulose in the liquid phase High cost of ionic liquids	[42, 50]
Biological methods			
White-rot fungi, brown-rot fungi and soft-rot fungi White rot *Phanerochaete chrysosporium* *Cyathus steercoreus* *Pleurotus* spp. Brown rot *Gloeophyllum trabeum* Soft rot *Ascomycota* *Deuteromycota* *Trichoderma reesei* *Chaetomium* sp. *Ceretocystis* sp		Brown rot fungi mainly degrade cellulose, while white and gray rot fungi both lignin and cellulose Mushrooms produce cellulases, hemicellulases and enzymes that break down lignin to CO_2: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase Long treatment duration (several weeks), relatively low efficiency Low energy consumption The need to control the conditions for the growth of microorganisms	[51–53]

make it an attractive method in the pretreatment of lignocellulose [58].

Ammonia solutions are used as catalysts in the next group of methods. Such solutions can be used in ammonia recycled percolation (ARP), soaking in aqueous ammonia (SAA) and ammonia fiber explosion (AFEX). The ARP process involves multiple washing of lignocellulosic biomass placed in a column reactor with an ammonia solution at a concentration of 5–15% (w/w) at an increased pressure (> 2 MPa) and temperature (160–180 °C). In these conditions, an aqueous solution of ammonia reacts mainly with lignin, causing its depolymerisation and breaking the bonds between polysaccharide residues in the complex [46, 59]. In contrast, when the AFEX method is applied, biomass is treated with anhydrous ammonia at high pressure (1.12–1.36 MPa) and a moderate temperature (60–100 °C). A pressure decreases, and ammonia decompression breaks the bonds between lignin and polysaccharides in the complex and causes partial decrystallisation of cellulose. The AFEX procedure is more effective in the pretreatment of herbal plants and agricultural residues than of woody biomass and other high-lignin materials [36, 47].

2.1.2 Chemical pretreatment

Acid and alkali pretreatments are considered desirable because of their potential to remove lignin and hemicellulose at a good extent. It can also increase the surface area by decreasing the crystallinity of cellulose [23] and provide easily accessible binding sites for enzymes [60].

One of the longest-used methods of lignocellulosic material pretreatment is their acid hydrolysis with the use of sulphuric acid (at a concentration of 0.05–5% and higher) in combination with a thermal treatment (100–200 °C). These are conditions in which hemicellulose hydrolysis takes place, with consequent degradation of bonds which stabilise the lignocellulose complex structure [15]. As a result, a considerable portion of lignin is released to the acidic environment, in which it is precipitated. These changes are accompanied by an increase in cellulose availability to hydrolytic enzymes [19, 36, 47]. Following the acidic pretreatment and before its enzymatic hydrolysis, the material requires neutralisation and detoxification (e.g., with activated carbon or calcium hydroxide). The monosaccharides produced by hydrolysis of hemicelluloses can be degraded to such products as furfural (from pentoses) and 5-hydroxymethylfurfural (HMF, from hexoses). Acetic acid is formed from acetyl groups in hemicelluloses [61, 62]. The method with sulphuric acid is a dedicated method in woody biomass pretreatment [38]. The application of sulphuric acid at high concentrations (10–30%) brings little benefit in bioethanol production because of the formation of a considerable amount of hydrolysis and fermentation inhibitors and a significant loss of sugars [54].

According to many studies, organic acids are less corrosive and are well suited to the pretreatment of agricultural waste, especially because of weak pentose degradation properties [44, 63, 64]. Kootstra et al. [63] used maleic acid in wheat straw pretreatment, and they achieved the enzymatic hydrolysis yield at 96% of the theoretical value, which was nearly identical to the experiment with sulphuric acid (98%). Moreover, the amount of furfural produced in these conditions was twofold less than when sulphuric acid was used for pretreatment.

Catalysts used in alkaline pretreatment act on acetyl groups in hemicelluloses and ester bonds connecting lignin with the other polymers and partially liquefy the lignocellulosic complex, decrystallise cellulose and remove considerable parts of lignin [45]. The main benefits of the methods based on the use of alkali use include the ability to conduct the process at room temperature, lower saccharide degradation compared to the methods employing sulphuric acid and the absence of inhibitors (mainly furfural and HMF) formed in the process. These methods are useful mainly in the treatment of agricultural residues (e.g., straw) [36, 49]. Sodium hydroxide is a commonly used catalyst in lignocellulose pretreatment. This method effectiveness depends on the parameters applied, i.e., process temperature and time, as well as the chemical agent concentration. Studies of alkaline treatment optimisation conducted by McIntoch and Vancov [65], evaluating an effect of time, concentration and temperature on the effectiveness of pretreatment of sorghum (*Sorghum bicolor*) straw, demonstrated the effectiveness of sodium hydroxide at a concentration of 2% (w/v) at 121 °C for 1 h. Cardona et al. [66] obtained similar findings in the pretreatment of grass *Pennisetum purpureum*. The authors examined the effect of physicochemical (steam explosion) and chemical (SAA, NaOH, H_2SO_4, H_2O_2) methods on enzymatic hydrolysis of this lignocellulosic material. They achieved the best results in pretreatment with 2% NaOH solution at 120 °C for 1 h, when ethanol was produced at 95% of the theoretical yield).

Calcium hydroxide is also often used for the pretreatment of various lignocellulosic materials. However, due to its poor solubility at high temperatures, this method is usually applied at relatively low temperatures (20–120 °C), extending the reaction times. Compared to sodium hydroxide, pretreatment with $Ca(OH)_2$ is cheaper and more beneficial to the environment because of easier catalyst recovery after its reaction with carbon dioxide [36, 47]. The addition of an oxidiser (oxygen, hydrogen peroxide) during the pretreatment process with these alkalis improves the effectiveness of lignin removal [67].

2.1.3 Biological pretreatment

Biological pretreatment processes can be conducted with white-rot fungi, brown-rot fungi and soft-rot fungi taking part. Brown-rot fungi cause mainly cellulose degradation, while white-rot and soft-rot fungi produce a complex of enzymes which decompose both lignin and cellulose. White-rot *Basidiomycetes* fungi are the most useful in the biological treatment of lignocellulosic materials [51, 52]. They have the unique ability to degrade lignin to carbon dioxide. The high effectiveness of the polymer decomposition by the fungi is a consequence of the strong oxidising properties and low substrate-related specificity of the oxygenases produced by them [53]. Lignin is degraded by enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. However, biological methods have significant limitations—they are characterised by a long duration with relatively low effectiveness, which excludes them from industrial applications [6, 42].

2.1.4 Advancements in recently developed pretreatment methods

It is also important to develop eco-friendly and cost-effective methods for lignocellulose fractionation [23]. Pretreatment methods such as ionic liquids [68], supercritical fluid based [69], low temperature steep delignification (LTSD) [70], deep eutectic solvent (DES) [71] and co-solvent enhanced lignocellulosic fractionation (CELF) [72], which are considered to be the most advanced methods, result in higher sugar yield with a minimum amount of byproducts generated [25, 73].

The pretreatment of lignocellulosic biomass using supercritical CO_2 is carried out at temperatures up to 200 °C, within a few minutes. In an aqueous solution of carbon dioxide, carbonic acid is formed, which promotes the hydrolysis of hemicellulose [74]. A sharp decrease in pressure combined with a CO_2 explosion causes a disruption of the cellulose and hemicellulose structure, thereby increasing the available substrate surface area for enzymes [75]. Pretreatment of biomass with supercritical fluids enables the effective removal of lignin under relatively mild conditions, resulting in high sugar yield, low production of fermentation inhibitors and high susceptibility to enzymatic hydrolysis while reducing the consumption of chemicals including solvents, reagents and catalysts [76]. The use of supercritical CO_2 has many advantages, such as low CO_2 cost, no toxicity and flammability, easy catalyst recovery and low environmental impact [36, 76]. The main limitations relate to the need to ensure high pressure and significant installation costs [77]. In addition, this method is not suitable for biomass with a low level of moisture [78], device design and process parameters need to be optimised [79], and technologies for synthesizing value-added products from biomass in one vessel have still not been developed [25].

Ionic liquids (ILs) are called "green" solvents because of their unique solvation properties [25]. IlLs are salts, usually consisting of organic cations (with a high molecular weight) and inorganic anions (with a low molecular weight), which occur in the form of liquids at relatively low temperatures, often at room temperature. They are characterised by chemical and thermal stability, non-flammability, low vapour pressure and a tendency to remain in a liquid state over a wide temperature range [80]. They selectively remove lignin and hemicellulose from biomass, increasing the available cellulose specific surface area for hydrolytic enzymes. ILs are used to pretreat different lignocellulosic biomass under mild thermal conditions, and separation of cellulose from lignin can be easily achieved by adding different precipitation solvents [81, 82]. However, lignocellulosic materials with a higher lignin content require more stringent pretreatment conditions to dissolve them in a solution of ionic liquids [47]. The advantage of pretreatment of lignocellulose with ILs is the ability to run the process continuously and with a high biomass load [83]. However, the main drawbacks are the toxicity of ILs, pH requirements, the strenuous tendency to denaturate the enzyme, the costly and complexity of the process [84]. The cost intensity of method was partially reduced by synthesizing cheaper and more environmentally friendly ILs using compounds derived from lignin and hemicellulose (vanillin, *p*-anizaldehyde and furfural) [85]. Although many studies point to the use of ionic liquids as a new potential pretreatment [86–88], their prices and the complexity of the process make them uncompetitive technologies on an industrial scale. For this reason, treatment with dilute acid or base [25] as well as hydrothermal methods, including SE [89], is most often used.

Recently developed methods for the pretreatment of lignocellulosic biomass include low-temperature steep delignification (LTSD), co-solvent enhanced lignocellulosic fractionation (CELF) and deep eutectic solvent (DES). The first of these—LTSD—was developed by the Bioprocess Innovation Company and is characterised by a low input of non-toxic chemicals (oxygen, alkalis), mild operating conditions and a high conversion rate and removal of lignin from raw materials at the level of 90% [78]. In addition, the chemical compounds used can be recycled, and the process itself is characterised by environmental sustainability. The main disadvantages are the high-cost intensity of the technology and the formation of toxic products [25].

Co-solvent enhanced lignocellulosic fractionation (CELF) is the process of fractionating lignin from lignocellulosic biomass using an organic compound, tetrahydrofuran water and dilute sulphuric acid [90]. CELF is an effective solvolysis pretreatment that effectively reduces biomass resistance by providing up to 95% of the total sugars (glucose, xylose and arabinose) after enzymatic hydrolysis of lignocellulosic materials at low doses of enzymes (e.g., 2 mg protein/g glucan) [91]. However, CELF treatment at high temperatures

may promote the formation of hydrolysis and fermentation inhibitors, including furfural, 5-hydroxymethylfurfural and levulinic acid [90].

Deeply eutectic solvent (DES) is similar to ionic liquids in many features. It is a mixture usually consisting of two or three cheap and safe ingredients that are capable of self-association [92]. They can be obtained from natural sources (choline, urea, sugars, amino acids and organic acids) [93] and, unlike ILs, are cost-effective, easier to synthesise, non-toxic, biocompatible and highly biodegradable [78]. They have been successfully used to extract high-purity lignin from agricultural residues such as rice straw [94]. Despite their high potential, the high viscosity of DESs is their biggest drawback in widespread use [78].

Not all available pretreatment methods are cost effective and efficient enough to be used in the production of bioethanol. Due to the specific structure of the lignocellulosic complex, pretreatment methods are more and more often combined, using their various combinations. Thanks to this, it is possible to improve the efficiency of lignocellulose conversion processes by up to several percent. It is important that these processes are properly selected for the specific type of lignocellulosic substrate. This affects not only the effectiveness of the pretreatment, but also its cost, and thus the profitability of further conversion of lignocellulose [41].

Combined methods combine two or more methods from the same or different groups but are not separately classified. The following combinations are used: mechanical crushing–chemical, physical or biological treatment, mechanical crushing–electronic radiation–alkali treatment, mechanical crushing–microwave–chemical processing and mechanical crushing–chemical treatment–steam explosion [95–97]. Combining ultrasound (45 min, 400 W, 100% amplitude, 24 kHz) with ammonia pretreatment of sugarcane bagasse (80 °C, 10% ammonia, 1:10 solid/liquid ratio) resulted in a cellulose recovery of 95.78%, with 58.14% delignification [98]. Parveen et al. [99] investigated the usefulness of a combined pretreatment method in the conversion of bamboo biomass. The combination of chemical ($NaOH + H_2O_2$) and biological (*L. elegans* culture) methods turned out to be the most advantageous. Under these conditions, the authors noted the highest level of delignification of the material (twofold) and an increase in cellulose content compared to the native raw material by 73% (73.6% vs 44.2%, respectively). Dimos et al. [100] searched for the most advantageous method of pretreatment of cotton stalks and found the sequential combination of organosolv and hydrothermal pretreatment the best. After 6 h of prehydrolysis, the ethanol yield reached the level of 32.3 g/L, being greater from 32 to 50% than other individually used pretreatment methods of the analysed biomass. The combined method involving the physical (milling, autoclaving—121 °C, 1 h) and alkaline (NaOH) methods for the initial preparation of *Miscanthus giganteus* biomass was also used in the research by Kordala et al. [101].

An assessment of the methods for treatment should be based on technological, economic and environmental criteria. The most important aspects, which allow for cost reduction and applying a method on an industrial scale, include achieving high efficiency of sugar release, removal of considerable amounts of lignin, reduction of inhibitor formation, low energy, water and chemical consumption and the possibility of conducting pretreatment as a continuous process. Table 2 shows a comparison of several pretreatment methods with respect to the major criteria. The toxicity of the chemicals applied and the possibility of recycling them, as well as waste management, are important environmental factors. The possibility of lignin recovery is also important, as it is a precursor of valuable chemicals, largely contributing to biorefinery profitability.

To date, no cost-effective and environmentally friendly pretreatment method has been developed to achieve complete delignification of lignocellulosic biomass. Due to the complexity of the lignocellulose complex, it is difficult to expect one method to be a universal chip for all types of raw materials. Therefore, there is a constant need for research into the reaction mechanisms of different pretreatment technologies to improve existing methods and optimise process conditions.

2.2 Lignocellulose hydrolysis

Lignocellulose pretreatment is followed by hydrolysis, which leads to cellulose and hemicellulose decomposition to fermenting sugars, mainly glucose and xylose. Hydrolysis of lignocellulosic materials can be affected with (dilute or concentrated) acids or enzymes. The application of concentrated acids (e.g., 30–70% H_2SO_4) allows achieving a high glucose yield (90%) within a relatively short time (10–12 h). However, high acid concentration and extreme conditions make the process disadvantageous for environmental and economic reasons [103]. Hydrolysis of crystalline cellulose with dilute acids (H_2SO_4 or HCl) requires a concentration of ca. 1.5%, and the reaction temperature should range from 200 to 240 °C. However, in such conditions, degradation of xylose to furfural, glucose to hydroxymethylfurfural (HMF) and other undesirable compounds becomes unavoidable. In another variant of acidic hydrolysis, very low acid concentrations and very high temperatures are applied in periodical/charge processes (autohydrolysis) [16].

Enzymatic hydrolysis is an environmentally friendly process which involves the application of cellulase and hemicellulase enzymes which decompose polysaccharides (cellulose and hemicellulose) to fermenting sugars [20]. Cellulases are highly specific enzymes. The whole process runs in three successive stages: adsorption of cellulases on the

surface of cellulose, cellulose hydrolysis to glucose and cellulase desorption from the material surface [104]. Cellulose degradation is difficult because of the presence of internal and external hydrogen bonds, which stabilise bunches of cellulose fibres [105]. Currently, cellulases are one of the three most produced enzymatic preparations because of their wide use in cotton processing and recycling and as additives to detergents and fodder for animals. Cellulase production is growing steadily—mainly because of the demand for bioethanol production from lignocellulosic substrates, intended for use as transport fuel [106]. Although cellulose is a homopolymer, several enzymes are necessary to bring about its degradation (Fig. 1). Endo-1,4-glucanases (EC 3.2.1.4) hydrolyse internal structures of amorphous cellulose; exo-1,4-glucanases (EC 3.2.1.91, also known as cellobiohydrolases) are responsible for detaching cellobiose molecules from the ends of cellulose and β-glucosidases (EC 3.2.1.21), which hydrolyse cellobiose to glucose. These enzymes break down cellulose owing to special properties of synergistic action. For most of them, the optimum conditions are a temperature of 50 °C and pH 4.0–5.0 [50]. The cellulase mechanism of action is shown in Fig. 1.

Hemicelluloses accompanying cellulose are usually xylans and mannans. These are decomposed by several enzymes to oligomers and simple sugars (xylose and mannose), which requires the synergistic action of xylanases and mannanases [31]. Endoxylanase-endo-β-(1,4)-xylanase (EC 3.2.1.8) and endomannanase-endo-β-(1,4)-D-mannanase (EC 3.2.1.78) are enzymes which degrade the main polysaccharide chain, to xylan and glucomannan, respectively. Xylanases are hydrolases which differ from one another with respect to their catalytic properties. Mannanases—β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), acetyl mannan esterase (EC 3.1.1.6), β-glucosidase (EC 3.2.1.21) and α-galactosidase (EC 3.2.1.22)—are heterogeneous with respect to their properties, and their role is to break the main and the side chains of mannan [107, 108]. Hemicellulose decomposition to short oligomeric compounds is affected by such enzymes as β-xylosidase (EC 3.2.1.37), β-mannosidase (EC 3.1.1.25) and β-glucosidase (EC 3.2.1.21). Side groups are attached to oligomers, which are removed sequentially by α-glucuronisidase (EC 3.2.1.139), α-arabinosidase (EC 3.2.1.55) and α-D-glucosidase (EC 3.2.1.22). Acetyl substituents are removed sequentially by esterases [109, 110].

Commercial preparations of cellulases and hemicellulases are obtained from filamentous fungi. Fungi of the genus *Trichoderma* are regarded as the most productive and efficient. The most representative species include *Trichoderma reesei*, *Trichoderma viride* and *Trichoderma longibrachiatum*. Commercial preparations of cellulases are based on genetically modified mutants of *T. reesei* and are produced on an industrial scale by many companies worldwide. As they have genes encoding the production of endoglucanase and cellobiohydrolase, fungi of the genus *Aspergillus* are regarded as an alternative to *T. reesei*. However, most of the research into these microorganisms has focused on obtaining β-glucosidase, xylanase and xyloglucanase [111]. There have been many literature reports on obtaining an advantageous enzyme combination from the joint culturing of strains of *T. reesei* and *A. niger*. The first of them synthesises a large amount of endo- and exo-glucanases, and a small amount of β-glucosidase. It is different with *A. niger*, which synthesises large amounts of β-glucosidase, while the other cellulases are produced in smaller amounts. Combinations of synergistically acting cellulases can also be obtained from *T. reesei* and *T. longibrachiatum* [112, 113]. Other important sources of cellulolytic enzymes include bacteria—both aerobic and anaerobic. They produce relevant nanocomplexes of enzymatic systems and special complexes of cellulases, known as cellulosomes [114]. However, bacteria cannot compete with fungi mutants because the level of protein expression in these microorganisms is much lower [111]. The effectiveness of polysaccharide hydrolysis in lignocellulosic substrates depends on their effective pretreatment and proper choice of enzyme complexes [115].

The leaders in the production of enzymatic preparations used to decompose lignocellulosic complex components

Table 2 Comparison of different pretreatment methods [89, 95, 102]

Pretreatment method	Use of chemical reagents	Investment costs	Operating costs	The scale of use
Mechanical processing	No chemical required	High	Low	Commercial
Steam explosion	Not required; possibility of using H_2SO_4, SO_2, CO_2 as a catalyst	High	High	Commercial
AFEX	NH_3, possibility of reagent recovery			Laboratory
LHW	No chemical required	High		Commercial
Diluted acids	Mainly H_2SO_4	Medium	High	Pilot
Concentrated acids	Mainly H_2SO_4	Low	Medium	Pilot
Alkaline	CaO, Ca_2, NH_3, NaOH	High		Laboratory
Organic solvents	Alcohols, esters, ketones, organic acids, phenols, ethers	Low	Low	Laboratory

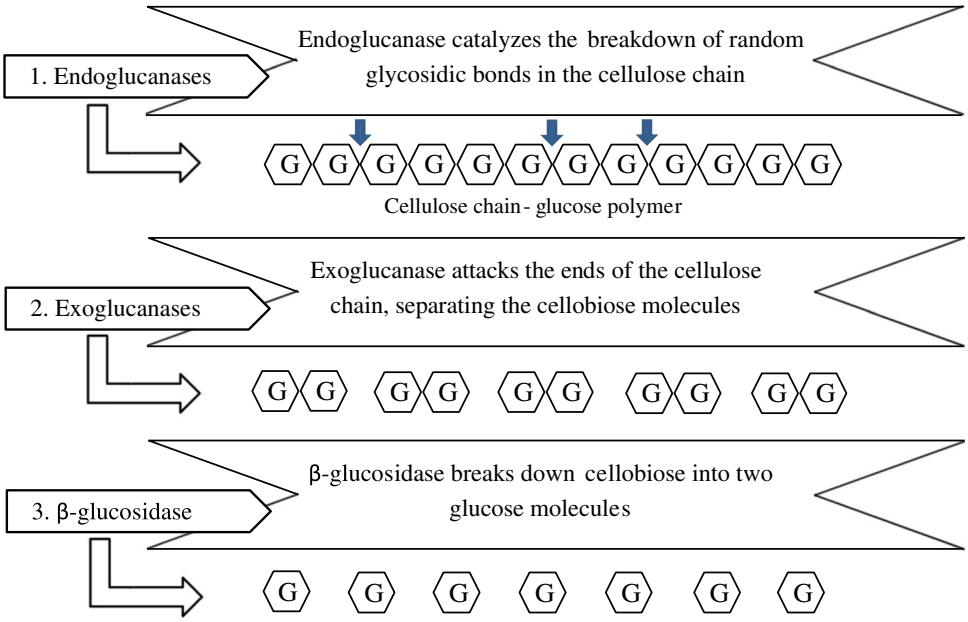


Fig. 1 Cellulose degradation mechanism (own study, based on [20, 97])

include Novozymes and DuPont Industrial Biosciences (DuPont). DuPont offers innovative solutions for industrial biorefineries processing lignocellulosic materials to produce biofuels. Commercial enzymatic preparations containing complexes of cellulases and hemicellulases include Accellerase® 1500 manufactured by DuPont: a composition of exoglucanase, endoglucanase, hemicellulase and betaglucosidase and Viscozyme L made by Novozymes, which is a mixture of betaglucanases, pectinases, hemicellulases and xylanases [116, 117]. One of the latest products offered by DuPont is an enzymatic complex Accellerase® TRIO, obtained from a genetically modified strain of *T. reesei* [118]. The preparation contains a potent combination of various enzymatic activities: exoglucanase, endoglucanase, hemicellulase and β-glucosidase, which guarantees effective hydrolysis of polysaccharides contained in lignocellulosic biomass to monosaccharides. Moreover, Accellerase® TRIO can be used for hydrolysis of a wide range of lignocellulosic substrates, including straw (maize, wheat, rice), bagasse, woodchips, wastepaper, switchgrass, following pretreatment by various methods (e.g., with alkalis, acids, by AFEX, steam explosion).

The enzymatic preparations introduced to the market by Novozymes include Cellic® CTec3 and Cellic® HTec3 [119]. The former contains a complex of cellulases and hemicellulases (with an increased activity of β-glucosidase) which improves the efficiency of the hydrolysis of lignocellulosic materials by limiting the inhibiting effect of cellobiose. The manufacturer claims that Cellic® CTec3 is 1.5 times more effective than the previous product offered by Novozymes (Cellic® CTec2). Furthermore, Cellic® HTec3 is a complex of hemicellulases, showing the activity of endoxylanase and β-xylosidase and allowing for efficient hemicellulose conversion. This preparation is very effective in hydrolysing xylooligomers and hemicellulose, both undissolved and dissolved as a result of biomass pretreatment. The next generation of the enzymatic complex from Novozymes is Customized Cellic® 1.0, developed for material and process fitting—intended for specific applications defined by the customer [120]. The manufacturer guarantees beneficial effects of comprehensive hydrolysis and fermentation in the biorefinery approach. The lack of characteristics of enzyme behaviour in mixtures is the greatest difficulty in preparing compositions of commercial enzymes. The application of such preparations requires the determination of the enzyme activities present in them and their proportion, thereby allowing their effective adaptation to the planned enzymatic reaction on a specific substrate. To this end, it can be useful to supplement the composition with purified enzymes. Table 3 shows examples of commercial enzyme preparations from the companies Novozymes and DuPont, which can facilitate the choice of a suitable composition for a given lignocellulosic substrate [121]. Hydrolysis of pretreated acacia wood with Cellic® CTec3 led to the high glucose conversion (73.8%) at 10 FPU/g of glucan, and high conversion yield to ethanol, 94.9% at 5 FPU [122]. The enzymatic hydrolysis of cassava stems (CS) after 1.0 M NaOH pretreatment at 121 °C for 15 min and cellulase addition (Accellerase® 1500, 2500 CMC U/g CS) obtained 10.94 ± 0.29 g/L reducing sugar (RS) concentration. Hydrolysis of CS with amylases (Termamyl® 120, 1.2 U/g CS and AMG 300L™ 3.5 U/g CS) provided 34.85 ± 0.75 g/L RS, and the maximum RS amount of 47.90 ± 0.39 g/L was obtained from the hydrolysis with mixed enzymes (Termamyl® 120, 1.2 U/g CS, AMG 300L™ 3.5 U/g CS followed by Accellerase® 1500, 2500 CMC U/g CS) [123]. The use of the enzymatic

cocktail Multifect CL® to hydrolysis of alkali-pretreated sugarcane bagasse after 72 h of saccharification, the glucose concentration was 21.10 g/L, and the amount of xylose released was 17.01 g/L [124].

One of the major improvements to former enzyme blends is the use of accessory enzymes, such as lytic polysaccharide monooxygenases (LPMOs). LPMOs are enzymes that oxidatively cleave the β-1,4-glycosidic bond of polysaccharides upon activation of O_2 or H_2O_2 and presence of an electron donor. Their use for treatment of cellulose fibers is related to the ability of LPMOs to create nicking points on the fiber surface, thus facilitating fibre disruption and separation. The enzymes are active on the cellulose chains located on the surface of fibres, and their oxidative mode of action leads to disruption and fibril separation, thus yielding thinner structures [127, 128]. LPMOs can also act on chitin, starch and hemicellulose [129].

The synergy between lignocellulolytic enzymes can be linked by two mechanisms: an increase in the accessibility of a substrate and the prevention of enzymes' inhibition. For the second mechanism, the supplementation of enzymes acting on oligosaccharides, sugar lactones and oligomeric phenolics can mitigate their inhibition of lignocellulolytic enzymes and therefore improve their degrading efficiency [130].

The synergistic reactions between LPMOs and cellulases on the decomposition of lignocellulosic biomass have been well documented throughout the literature over a decade now. LPMOs constitute an integral component of commercial enzyme mixtures for industrial uses, thanks to enhancing the action of hydrolytic enzymes by disrupting the tight structure of crystalline cellulose, and debranching enzymes promote the degradation of hemicelluloses by increasing their accessibility to backbone-degrading enzymes. In addition, the synergy of some cellulases is believed to come from the liberation of stalled enzymes through the action of other cellulases. Also, supplementation of hemicellulases or pectinases is beneficial for the degradation of cellulose through the removal of physical blocks and/or the elimination of oligosaccharides that inhibit cellulases' activities [129–132].

3 Fermentation

Pretreatment and hydrolysis are essential before the fermentation process. Unlike starchy materials, a lignocellulosic fermentation substrate contains the following:

- A mixture of oligosaccharides, hexoses (glucose, mannose, galactose) and pentoses (xylose, arabinose).
- Fermentation inhibitors: acids (formic, acetic and levulinic), furan derivatives (furfural and 5-hydroxymeth-

Table 3 Examples of commercial enzyme preparations from the companies Novozymes and DuPont, and the range of activities reported for these compositions. Actual activities may vary depending on the conditions under which they were tagged [114, 125, 126]

Commercial preparation	Supplier	Reported enzymatic activities (U cm^{-3}, unless otherwise stated)	
Celluclast 1.5 L FG	Novozymes	65 FPU, 12 β-glucosidase, 660 xylanase	[125]
Cellic® CTec3	Novozymes	Cellulose, hemicellulase (unreported activity)	Manufacturer's brochure
Cellic® HTec3	Novozymes	Xylanase, β-glucosidase (unreported activity)	Manufacturer's brochure
Customized Cellic® 1.0	Novozymes	(unreported activity)	Manufacturer's brochure
Novozyme 188	Novozymes	8.5 FPU, 665 β-glucosidase, 123 xylanase, 29.3 α-arabinofuranosidase, 16.6 β-xylosidase, 116 α-galactosidase, 0.6 ferulic esterase	[125]
Shearzyme	Novozymes	27 FPU, 5.0 β-glucosidase, 2293 xylanase	[125]
NS50013	Novozymes	63 FPU, 8 β-glucosidase, 1117 xylanase	[125]
NS50010	Novozymes	992 β-glucosidase, 124 xylanase	[125]
Spezyme CP	DuPont Industrial Biosciences	58.2 FPU, 128 β-glucosidase, 2622 xylanase, 22.6 α-arabinofuranosidase, 7.3 β-xylosidase, 0.39 α-galactosidase	[126]
Multifect (xylanase)	DuPont Industrial Biosciences	0.77 FPU, 35.9 β-glucosidase, 25.2 xylanase, 9.4 α-arabinofuranosidase, 22.6 β-xylosidase, 2.3 α-galactosidase, 1.3 p-coumaryl esterase	[126]
Multifect (pectinase)	DuPont Industrial Biosciences	4.18 FPU, 345.8 β-glucosidase, 1664 xylanase, 1862 α-arabinofuranosidase, 186.2 β-xylosidase, 31.9 α-galactosidase, 9.67 ferulic esterase, 21.7 *p*-coumaryl esterase	[126]
Accellerase® 1000	DuPont Industrial Biosciences	93 FPU, 1632 β-glucosidase, 849 xylanase	[126]
Accelerase® Trio	DuPont Industrial Biosciences	2600 CMC U/g, > 3000 xylanase, > 2000 β-glucosidase	[126]

ylfurfural), products of lignin degradation (vanillin and 4-hydroxybenzaldehyde) [15].

Microorganisms used in bioethanol production from lignocellulosic materials should be mainly capable of conversion of both sugar groups to ethanol and resistant to the presence of the inhibitors. The following properties could provide additional benefits:

- Resistance to an ethanol concentration of more than 40 g dm^{-3}.
- The capability of producing ethanol quicky and efficiently (> 90% of the theoretical yield).
- Resistance to high temperature and low pH.
- Ability to produce by-products in minimum amounts.
- Absence of pathogenic properties.
- Low nutritional requirements [6].

Hexoses dominate as the source of sugars in hydrolysates. Their fermentation runs easily in the presence of *Saccharomyces cerevisiae* yeasts, commonly used in the brewing industry. These yeasts do not have the enzymes necessary to effect pentose fermentation, which is why this process carried out with *S. cerevisiae* does not fully use the potential of lignocellulosic material [133].

A microorganism which can be applied in practice in lignocellulose bioconversion to ethanol is *Mucor rouxii* (*Mucor indicus*), which is capable of fermenting glucose, xylose, mannose, fructose and galactose. It is a filamentous fungus of the family *Mucoraceae* and is a dimorphous strain, which means it grows in filamentous form in aerobic conditions and transforms into the yeast-like form, with greater potential for ethanol fermentation in anaerobic conditions [134]. There have been many research reports on the potential for the use of *M. rouxii* in the fermentation of lignocellulosic hydrolysates [49, 134, 135]. This fungus can affect the fermentation of glucose, but also of xylose, and it shows greater resistance to potential inhibitors present in lignocellulosic hydrolysates, mainly furfural, hydroxymethylfurfural and acetic acid [101, 134]. Literature data show that *M. indicus* used to fermentation of wheat straw pretreated with *N*-methylmorpholine-*N*-oxide (NMMO) with the fungal extract as a nutrient-rich supplement can achieve a fermentation yield of up to 92% of the theoretical value, which is comparable to the level obtained with *S. cerevisiae*. Additionally, *M. indicus* was able to assimilate all the glucose in fermentation media in less than 24 h [134, 136, 137].

Species with natural potential for pentose fermentation include *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehatae* and *Klebsiella oxytoca* [138, 139]. Xylose conversion to xylitol runs by xylose reductase and then of xylitol to xylulose by xylitol dehydrogenase. Subsequently, xylulose is phosphorylated by xylulokinase to xylulose-5-phosphate, which is present in pentose phosphate pathway and glycolysis [140]. *P. tannophilus* was the first yeast strain with the identified potential for xylose fermentation. The specificity of the resulting product depends on oxygen availability in the environment. *P. tannophilus* synthesises both xylitol and ethanol in aerobic conditions, whereas anaerobic conditions favour the production of xylitol. It has been found in many studies that ensuring microaerophilic conditions is the best solution for effective bioethanol production by this strain [141]. *P. stipitis*, which demonstrates the ability to produce ethanol from glucose, xylose, mannose, galactose and cellobiose, is another pentose-fermenting microorganism. However, *P. stipitis* prefers glucose as a substrate in the fermentation process, and it is used at a higher rate than pentoses. Studies of *P. stipitis* and *P. tannophilus* have shown that *P. stipitis* produces smaller amounts of xylitol—a by-product formed during the fermentation of xylose to ethanol. However, the use of these yeast species in commercial bioethanol production is limited mainly due to a low fermentation rate, the need for careful oxygen demand regulation, susceptibility to inhibitors and low ethanol tolerance [142].

For this reason, the latest research has been oriented towards obtaining yeast or bacteria recombinants which meet the requirements for microorganisms used in the production of ethanol from cellulose [3]. Microorganism modification by genetic engineering involves two directions of research: modification of microorganisms capable of ethanol fermentation, i.e., *S. cerevisiae* and *Zymomonas mobilis*, with respect to the disposal of pentoses. The other is based on a change of metabolic pathways in microorganisms which naturally use pentoses for effective ethanol production [143]. *Escherichia coli* is more resistant (compared to *Z. mobilis* and *S. cerevisiae*) to furans and organic acids present in lignocellulosic materials. Introducing a plasmid (the so-called PET operon) derived from cells of *Z. mobilis* resulted in the creation of the recombinant strain *E. coli* KO11, synthesising alcohol with a high yield, close to theoretical values. An increase in ethanol production was made possible by minimising the production of by-products of hexose and pentose fermentation [144–146]. Inserting genes from *E. coli*, encoding xylose isomerase, transketolase and transaldolase, resulted in the strain *Z. mobilis* CP4, capable of producing ethanol from xylose at a yield of 86% [144]. In one of the directions of *S. cerevisiae* yeast improvement, genes responsible for xylose use are transferred from *P. stipitis*, which results in a hybrid with the morphological features of *S. cerevisiae*, with an ability to metabolise xylose like *P. stipitis* [146]. The use of recombinant strains of *S. cerevisiae*, capable of fermenting xylose, allows obtaining high ethanol yield from the available saccharides—from 70% to over 90% of the theoretical level [147, 148].

The applied strategy is essential in the processes of fermentation of lignocellulose derivatives. The separate

hydrolysis and fermentation system is the best-explored method. Enzymatic hydrolysis and fermentation run sequentially in two different bioreactors, making it possible to apply the optimum temperature conditions for each stage, 45–50 °C for hydrolysis and 30–35 °C for fermentation. The liquid in this system flows from the hydrolysis reactor to the fermentation reactor. The drawback of this strategy is the inhibition of xylanase and cellulose activity by the reaction products, i.e., glucose, xylose and cellobiose [149]. The sequential system allows for introducing an indirect operation of hydrolysate concentration—which increases the concentration of sugars available for fermentation. However, this may result in the disadvantageous concentration of substances which are present together with the fermenting sugars (e.g., oligosaccharides), which increase the osmotic pressure of the environment, thereby limiting the fermenting activity of yeast [150].

The SSF (simultaneous saccharification and fermentation) is another widely studied method for the bioconversion process. The enzymatic hydrolysis and fermentation of hexoses take place simultaneously in the same bioreactor, which makes it necessary to apply the compromise parameters—which are beneficial for both processes [151]. This usually results in a decrease in the hydrolysis temperature and, in consequence, allows for the effectiveness of the catalytic enzymes being lower than optimum. Hexoses released by polymer hydrolysis are used immediately by microorganisms in ethanol fermentation. In consequence, cellulase activity is not inhibited by hydrolysis products. This favours a more complete use of the available polysaccharides of the material [150–152] and achieving greater ethanol productivity and efficiency [153].

The process of presaccharification of simultaneous saccharification and fermentation is a modified SSF strategy. Other terms to describe this process configuration include simultaneous saccharification and fermentation with delayed addition of inoculum (dSSF), liquefying with simultaneous saccharification and fermentation (L + SSF) and non-isothermal simultaneous saccharification and fermentation (NSSF). The principle of the PSSF is to conduct the preliminary hydrolysis of lignocellulosic material at a temperature that is optimal for enzyme activity (45–50 °C), which is followed by a decrease in the process temperature to the indirect value for the hydrolysis and fermentation processes (usually optimal for ethanol-producing microorganisms) [3]. The main aim of this modification is to increase the cellulose hydrolysis-to-glucose rate at an early stage of the process and to eliminate the glucose deficit at the start of the SSF process, which results in an increasing ethanol production rate. Additionally, it allows for reducing the reaction medium viscosity before the inoculum is added, which is beneficial mainly for the processes running at a higher concentration of solids in the wort. The duration of the preliminary hydrolysis (usually from 12 to 24 h) is the factor with the greatest impact on the process yield and the final ethanol concentration [151, 152].

Simultaneous saccharification of cellulose and hemicellulose integrated with co-fermentation of the released hexoses and pentoses is referred to as SSCF (simultaneous saccharification and co-fermentation). This method of bioconversion is intended for the transformation of high-hemicellulose lignocellulosic materials (e.g., xylan, arabino(glucurono)xylan) [154]. The SSCF process requires the use of microorganisms which enable glucose and xylose fermentation to ethanol. These are usually genetically modified microorganisms, capable of metabolising hexose and pentose microorganisms or those naturally fermenting pentoses [19]. A special role in this system is played by the genetically modified yeast *S. cerevisiae* and thermotolerant *E. coli* KO11 [155].

The use of SSF or SSCF for the production of bioethanol reduces enzyme loading and the time required for processing polysaccharides during enzymatic hydrolysis. Reducing sugars obtained by enzymatic hydrolysis are fermented immediately by the yeast, counteracting enzyme inhibition. To prevent feedback inhibition, in SSF process, glucose molecules are fermented immediately by the fermentative microbes. In the production of lignocellulosic bioethanol by this route, there is a shortage of end-product feedback inhibition, because sugar monomers released during the saccharification are immediately fermented by the microorganisms. In SHF process, saccharification and fermentation take place in individual vessels. The increased number of vessels makes SHF uneconomical. It causes end-product inhibition of hydrolytic enzymes and has a negative influence on the efficiency of saccharification. End-product inhibition is caused by simple carbohydrates (such as cellobiose) and has an impact on the inhibition of the cellulolytic enzymes, for example, cellulases or celobiases. The application of enzyme cocktails with additional β-glucosidase activity plays a greater role in SHF than in SSF processes [150, 156, 157]. Tokin et al. in their experiments tested the effect of the synergistic action of cellulase and LPMO in the SHF processes. In the work, they emphasised that LPMO activity is important in increasing the conversion of cellulosic substrates by cellulases. Increased saccharification of pretreated corn stover has been reported in a study, where the addition of TaAA9A (LPMO from *Thermoascus aurantiacus*) to a cellulase mixture (or co-expression) allowed a 50% reduction in the enzyme load required to reach 90% cellulose hydrolysis [158, 159].

Furthermore, the SSFF (simultaneous saccharification, filtration and fermentation) system involves the fermentation medium circulating between the hydrolysis and fermentation tanks (Fig. 2). Following pretreatment, the lignocellulosic material is mixed with enzymes and pumped through a selective membrane and the resulting filtrate entering the fermentation tank contains only sugars released in the

hydrolysis. The process requires microorganisms capable of flocculation. Flocculation in the internal decanter present in the system enables recirculation of the post-fermentation wort free from microorganisms to the hydrolysis tank. The SSFF process is more beneficial than SHF and SSF because it eliminates enzyme activity inhibition by the hydrolysis products and enables running both hydrolysis and fermentation in the optimum conditions [160].

Consolidated bioprocessing (CBP) is an alternative to these hydrolysis and fermentation strategies. The CBP technology combines biomass enzymatic hydrolysis and sugar fermentation in one process affected in one reactor, with the use of sufficiently effective microorganisms [138, 161]. This requires microorganisms effective in the transformation of pentoses and hexoses to ethanol, while at the same time producing a complex of various cellulases and exhibiting operational stability in industrial conditions [162]. CBP offers the possibility of reducing biofuel production costs due to simple raw material processing, reduction of the energy input and lower capital costs [162]. The stages of the process of cellulosic ethanol production depending on the strategy applied are shown in Fig. 3.

Industrial ethanol production from lignocellulosic raw materials is cost-effective when the ethanol concentration in the wort is higher than 4% (v/v). For most of the types of lignocellulosic materials, it requires determination of the dry weight concentration at about 15% to achieve a sufficiently high cellulose level in the reaction medium. However, ethanol production at a high dry mass concentration creates many problems (increased inhibitor concentration, high wort viscosity) [164, 165]. Many literatures reports express support for the SSF strategy. Various solutions have been considered to increase the final ethanol concentration in the process, e.g., additional multiple raw material feed [166] or special bioreactor construction solutions [165].

4 Bioethanol dehydration—new techniques

4.1 Adsorption methods

Ethanol for consumption is produced at concentrations under 95% (v/v), which means that no operations are required to remove the residual water from the rectificate. However, if ethanol is intended for use in the production of biofuels, it must be dehydrated to contain under 0.3% (v/v) of water [1]. This implies additional actions as rectification processes yield azeotropes containing up to 95.57% (v/v) of ethanol.

Water is separated from ethanol in three-component azeotrope distillation—with benzene in the past and with cyclohexane nowadays [167]. Newer methods include adsorption on molecular sieves and membrane pervaporation [1]. Molecular sieves are synthetic porous substances—zeolites, produced from aluminium silicates, whose pore size is adapted to the size of molecules to be separated. Ethanol is dehydrated with zeolites whose pores have a diameter of ca. 3 Å, i.e., similar to the size of the water molecule (2.8 Å), and too small for the ethanol molecule (4.4 Å) [168]. Ethanol dehydration in the technological process requires the installation of a system comprising two columns with zeolite packing, operating alternately in the variable pressure adsorption cycle (pressure swing adsorption (PSA)). The adsorption stage involves passing hydrated ethanol through the column, with water molecules being retained on the "sieve" and dehydrated ethanol leaving the system. The regeneration stage begins with the column pressure reduction and water desorption, and full regeneration is affected with dehydrated alcohol vapours supplied to the system and returned in the cycle before the condenser [169–171]. The whole process runs automatically, alternately, in both columns—it is computer-controlled, for example,

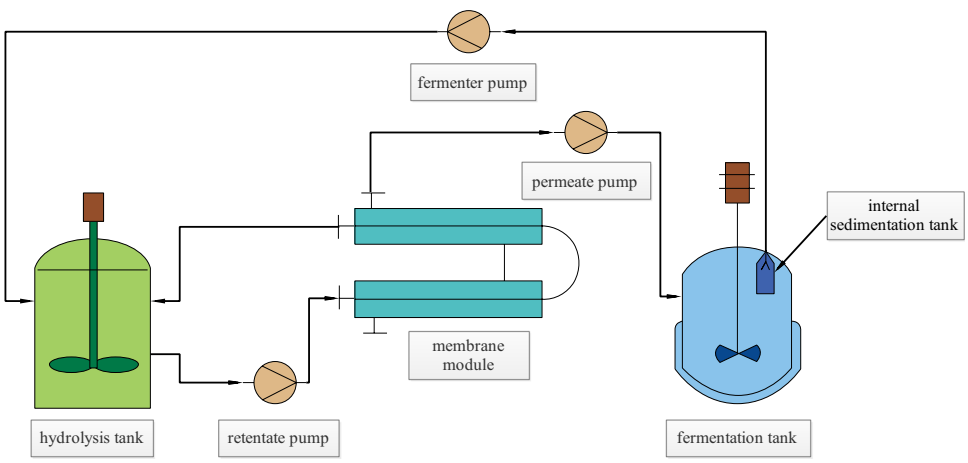


Fig. 2 SSFF process scheme (own study, based on [160])

West Technology & Trading Polska Sp. z o.o. (Poland) offers a two-column, alternate system for dehydrating 1550 dm^3 of bioethanol/h, where each cycle of the column operation lasts ca. 30 min. Such systems are usually constructed in modules, and they can be adapted to the production output required by an ethanol producer. A diagram of a two-column system operating in the PSA mode is shown in Fig. 4.

Rakoczy et al. [173] propose a method for ethanol dehydration on bioadsorbents, e.g., on maize flour. A bioadsorbent in such a process is used once in a process run under normal pressure and the used-up bed (containing the water removed from ethanol) is transferred for processing by traditional mashing and fermentation or for fodder. Since the emptied column must be packed with dry flour, it is best to use a several-column arrangement to keep the process cyclic, like in a system with molecular sieves. A flour bed moving in the counter-current to the ethanol being dehydrated can be used to make the process continuous. Ethanol containing over 99% of pure alcohol can be obtained by this method. However, the authors do not state whether it allows for standardisation of the moisture content to the level required for bioethanol to be used as fuel. Rapeseed meal as a biosorbent in the pressure swing adsorption (PSA) process for ethanol dehydration was used by Tajallipour et al. [174]. In the research conducted by Yan and Niu [175], the biosorbent was obtained from barley straw, impregnated with 5% H_3PO_4 and heated with microwaves. This biosorbent had significantly increased micropores and was characterised by high water uptake and dynamic selectivity of its adsorption, generating higher purity ethanol (from 78 to 89 wt% within 0.5 h).

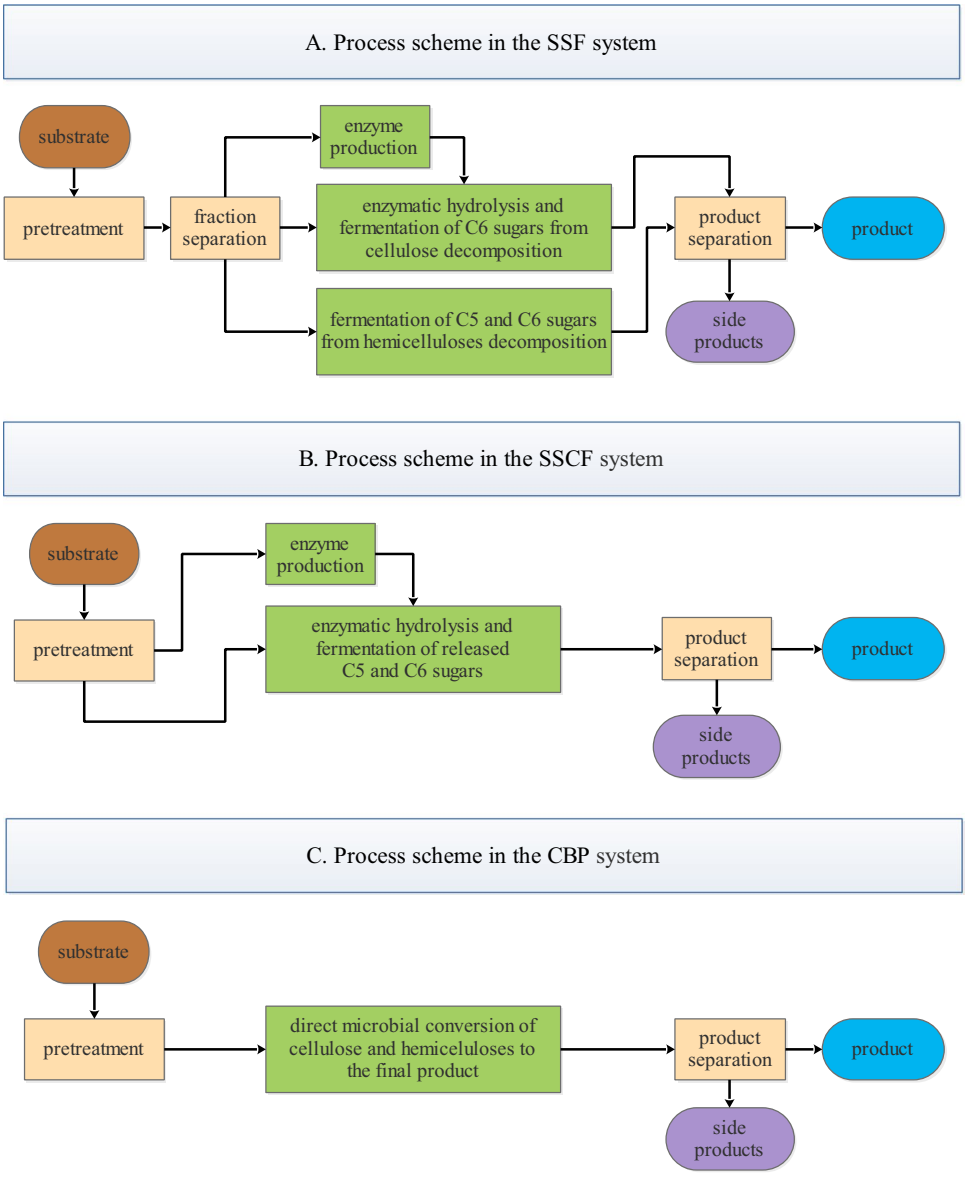


Fig. 3 Process scheme in the SSF (**A**), SSCF (**B**) and CBP (**C**) system (own study, based on [163])

4.2 Pervaporation

Membrane techniques, including pervaporation, have been competitive with conventional separation techniques for over 20 years, providing an environmentally friendly solution. It can be used in ethanol production in many stages of the production process, e.g., for continuous ethanol removal from mash (membrane distillation, pervaporation through hydrophobic membranes) to dry fusel oils and for ethanol dehydration (pervaporation with hydrophilic membranes) [176]. The principle of the pervaporation process is based on the phase transition of a liquid mixture being in contact with one side of a non-porous membrane associated with mass transport [177]. The permeate is evaporated selectively on the other side of the membrane, which is a consequence of the different solubility of the mixture components in the membrane. Separation by this method does not depend on the liquid–vapour equilibrium, so it can be applied successfully to separate azeotropes or liquid mixtures with similar boiling points [178]. The pervaporation process is driven by a difference in partial pressures of the components on both sides of the membrane, with the fed material (e.g., a mixture of water and alcohol) must be liquid and the permeate—gaseous [179]. This condition is achieved, as a rule, by reducing the pressure on the permeate side with vacuum pumps (vacuum pervaporation), by reducing temperature (thermal pervaporation), or with a flow of inert gas (pervaporation with carrier gas). Ethanol dehydration by means of pervaporation techniques was initiated in the 1980s when Deutsche Carbone AG, GFT (currently Sulzer Chemtech) introduced composite hydrophilic membranes to the market consisting of a carrier layer made of polymer needled cloth with a coating of polyvinyl alcohol. Such membranes were then used in the first industrial plants. Industrial ceramic membranes were also developed in the late twentieth century and were applied in over 100 plants worldwide [180]. The first one was produced by Mitsui Engineering and Shipbuilding Co. Ltd in 2000 in Japan. Other manufacturers of commercial ceramic membranes include i3 Nanotec (USA, India), Inocermic GmbH (currently Fraunhofer IKTS, Germany) and Pervatech BV (The Netherlands) [181].

The effectiveness of ethanol dehydration by pervaporation is closely related to the progress in membrane quality—their selectivity, flow capacity and mechanical strength. The currently available composite, polymer and zeolite membranes have different properties. Zeolite membranes are highly selective, well permeable and mechanically stable, although they are susceptible to rapid stress emergence caused by temperature changes and difficult to shape, which affects their price. Polymer membranes are highly resistant to temperatures but have poor permeability and physical strength [182].

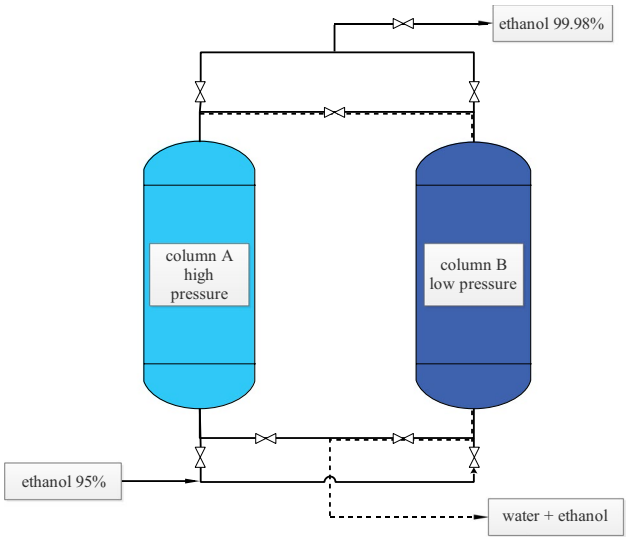


Fig. 4 Ethanol dehydration system, using molecular sieves with the variable-pressure method: column A (high pressure), column B (low pressure)-adsorption-regeneration (own study, based on [172])

Attention has been drawn to the production of composite membranes, which combine the mechanical stability of ceramic membranes with the strength of polymer membranes. A composition consisting of a polymer coating layer (predisposed to contact with the fed material at a higher temperature or highly selective at low temperatures) on a ceramic base with high permeability of the released permeate vapours. One such example is a membrane consisting of a ceramic base layer of aluminium aluminosilicate (Mullite)—developed by a team of Samei et al. [183] and widely used in the production of membranes for microfiltration, coated with a layer of polyvinyl alcohol with an addition of silica dust cross-linked with glutaraldehyde and hydrochloric acid. Noteworthy properties of the composite membrane, as described by the authors, include high permeability (up to 2.9 kg/m^2 h), high selectivity within a temperature range of 30–60 °C and low cost of production. A study conducted by Gao et al. [184] demonstrated the usability of two layers coating a polyacrylonitrile (PAN) layer: sodium alginate and hyaluronic acid, with the order of the coating application determining the membrane properties. A composite with an inner layer of AlgNa and hyaluronic acid coating proved to be a beneficial solution. A more hydrophilic material used in the coating, combined with low diffusion resistance and high selectivity of the inner layer, proved to be better suited for separating water from ethanol.

The research conducted by Meireles et al. [185] is also noteworthy, as they prepared a composite membrane from polyether sulphone (PES) coated with exopolysaccharides

from microorganisms (EPS). The coating material was obtained from *Pseudomonas oleovorans* cultured on media containing waste glycerol as a source of carbon. The researchers compared PES-EPS membranes with commercial PERVAP™ membranes in experiments involving the dehydration of ethanol solutions with various water percentages. The membrane under assessment proved to be highly selective in solutions containing 5% of water. However, it requires improvement in selectivity for its higher content in the feeding stream (10%). Its advantages also include the fact that the selective coating layer is obtained by biological methods, using cheap waste material, without the need to use aggressive chemicals.

However, when taking into consideration the industrial scale of the pervaporation processes, one of the leaders in designing, production and assembly of membrane separation systems is the Sulzer company, which—in cooperation with Mitsui Zosen Machinery (a manufacturer of zeolite ceramic membranes)—constructed an integrated system for bioethanol distillation and dehydration (the largest installation of this type in the world), which was launched in spring 2011, for the Finnish company St1 Biofuels Oy [186].

Moreover, Sulzer Chemtech is still a leading manufacturer of PERVAP™ composite membranes with a separating layer of polyvinyl alcohol with various purposes—including the first, highly permeable PERVAP™ 1210 membrane used for effective alcohol dehydration. Figure 5 shows a diagram of an integrated system for distillation with dehydration, constructed by Sulzer. The use of pervaporation as an alternative technique of ethanol dehydration is a promising solution, especially because of its lower operating costs, which is of extreme importance given the urge to reduce the price of ethanol.

5 Summary

Second-generation ethanol is set to play a significant role in the transport fuel market in the near future owing to the renewable resources of lignocellulosic biomass and continuous improvement of the methods for its bioconversion. Its use in this direction may favour the reduction of greenhouse gas emissions and limit energy dependence in many countries. Research carried out worldwide is aimed at fine-tuning an efficient and cost-effective technology for obtaining second-generation bioethanol, with a cost intensity similar to that of obtaining it from maize or, even more beneficially, from sugar cane. This requires the determination of optimal process conditions at each stage of lignocellulosic biomass conversion: pretreatment, hydrolysis, fermentation, considering the environmental and economic aspects. Microorganisms have a crucial role to play, as their activity in the acquisition of hydrolytic enzymes, their metabolic resistance to inhibitors generated during the processing and their ability to ferment five- and six-carbon derivatives of the lignocellulosic substrate determine the success of the predefined objectives.

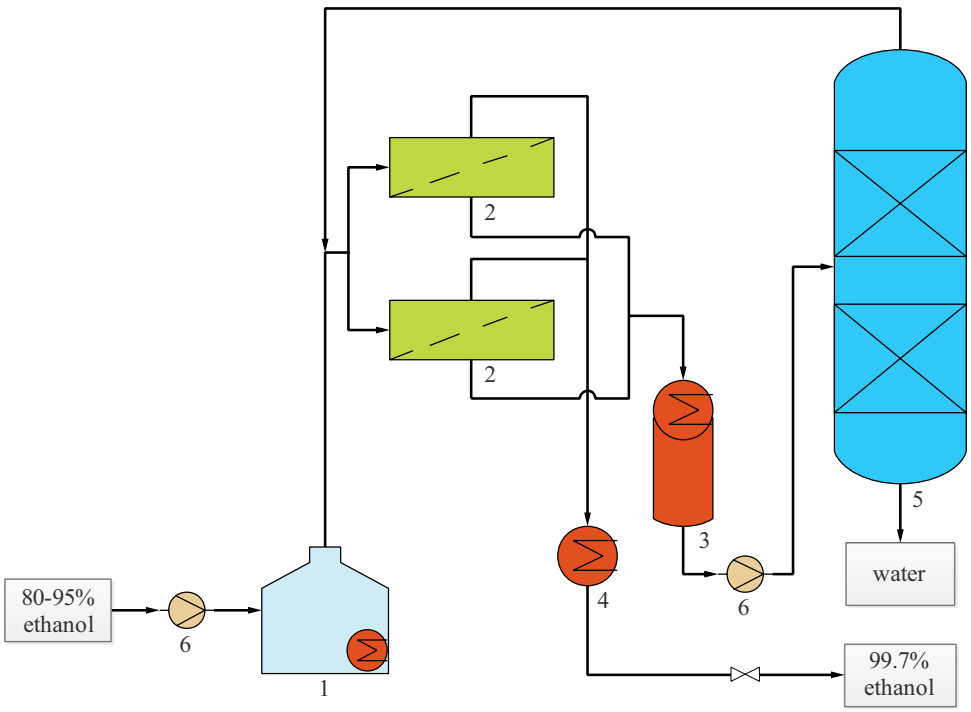


Fig. 5 Diagram of the process of pervaporative dehydration of ethanol, integrated with distillation by Sulzer: (1) feed heating tank, (2) pervaporation modules, (3) heat exchanger with a condenser, (4) heat exchanger, (5) ethanol recovery column, (6) pumps (own study, based on [186])

Author contribution All authors contributed to the idea for the article. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability All the data is present in the manuscript itself.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication The authors give their consent for publication upon acceptance of the article.

Conflict of interest The authors declare no competing interests.

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Recycling of enzymes in the hydrolysis of biomass of miscanthus giganteus and miscanthus sacchariflorus

Recykling enzymów w procesie hydrolizy biomasy miskanta olbrzymiego lub cukrowego

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The miscanthus giant and miscanthus sugar biomass were ground with a cutting mill and subjected to thermal pretreatment in an alk. medium, followed by hydrothermal treatment with a comm. cellulase prepn. and a mixt. of auxiliary enzymes. The solid fraction after the enzymatic reaction was used for enzymatic hydrolysis of fresh biomass batch. This process was carried out 3 times. The glucose productivity was 3.0–4.2, 1.7–2.5, and 1.3–1.9 mg/mg enzyme protein for the first, second and third reactions, resp. The concn. of reducing sugars after the third enzymatic hydrolysis was about half lower than after the first hydrolysis.

Przedstawiono wyniki badań doświadczalnych hydrolizy biomasy miskanta olbrzymiego i cukrowego, po hydrotermicznej obróbce alkalicznej, z użyciem preparatu celulaz (Accellerase 1500) i mieszaniny enzymów wspomagających. Ponad 50% aktywnych celulaz było związanych z frakcją stałą po hydrolizie lignocelulozy. Wykazano możliwość 2–3-krotnego wykorzystania enzymów związanych z biomasą miskanta olbrzymiego i cukrowego do hydrolizy lignocelulozy.

Ograniczenie dostępu do kopalnych nośników energii zmusza do poszukiwania odnawialnych, przyjaznych środowisku jej źródeł[1]. Gwałtownie wzrastające zapotrzebowanie na energię i wzrost jej zużycia wpływają na wzrost emisji gazów cieplarnianych. Konieczne jest również zaprzestanie wykorzystywania surowców żywnościowych do produkcji biopaliw[2].

Według danych przedstawionych w raporcie dotyczącym niezależności i bezpieczeństwa energetycznego EISA (Energy Independence and Security Act of 2007)[3] głównym nośnikiem energii otrzymywanym ze źródeł odnawialnych miał być i jest etanol pozyskiwany głównie z surowców lignocelulozowych i odnawialnych, np. z kukurydzy. Szczególną uwagę wzbudza fakt przewidywanego znacznego zwiększenia produkcji paliw z surowców celulozowych z ok. 380 mln L w 2010 r. do ok. 57 mld L w 2022 r., przy czym zakłada się, że ich produkcja spowoduje 60-proc. zmniejszenie emisji gazów cieplarnianych. Analiza produkcji alternatywnych nośników energii wymaga wieloczynnikowej analizy korzyści i problemów, ponieważ wykazano, że do produkcji 25% zakładanej objętości etanolu w USA wykorzystywanych będzie 4,2 mln akrów gruntów, w tym 3,6 mln akrów łąk (obszar prawie dwukrotnie większy niż Park Yellowstone)[4].

Najtańsza i najczęściej wykorzystywana do produkcji biopaliw jest biomasa lignocelulozowa, jednak na przestrzeni ostatnich lat zmianie uległy warunki ekonomiczne, a krytycznymi parametrami determinującymi opłacalność produkcji pozostają rodzaj surowca, warunki obróbki wstępnej oraz hydrolizy enzymatycznej i fermentacji[5]. Problemem związanym z wykorzystaniem biomasy lignocelulozowej jest mała zawartość w niej prostych cukrów fermentujących. Biomasa składa się ze związków łatwo ulegających biodegradacji, np. tłuszcze, białka, a także trudnych do biokonwersji: celulozy, hemicelulozy i ligniny, których udział wynosi przeciętnie 33−51%, 19−34% i 21−32%[6]. Celuloza, hemiceluloza i lignina tworzą ścianę komórek roślinnych o wysoce uporządkowanej strukturze krystalicznej. Lignocelulozę poddaje się obróbce wstępnej w celu degradacji struktury sieciowej ligniny i zwiększenia dostępności celulozy. Ligniny są najbardziej opornymi na działanie enzymów hydrolitycznych składnikami biomasy roślinnej, a dodatkowo są inhibitorami enzymów i wykazują działanie bakteriostatyczne[7]. Celem obróbki są zmiany strukturalne lignocelulozy, ale także konieczne jest zapewnienie minimalnej straty cukrów redukujących i ograniczenie powstawania szkodliwych produktów ubocznych, które nie sprzyjają hydrolizie i fermentacji[8]. Preferowane są tanie metody obróbki wstępnej, niewymagające stosowania reagentów zanieczyszczających środowisko[9].

Z uwagi na cenę surowców lignocelulozowych i bioetanolu rekomendowane jest stosowanie substratów odpadowych. Cenę etanolu



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determinuje wydajność hydrolizy oraz koszt i stabilność enzymów[10]. Zastosowanie rozwiązań charakterystycznych dla biorafinerii, czyli produkcji poza etanolem innych wartościowych bioproduktów, może wpłynąć na efektywność ekonomiczną tego procesu. Z biomasy lignocelulozowej otrzymywane są substytuty ropy naftowej lub inne bioprodukty, np. kwasy organiczne, furfural, 5-hydroksymetylofurfural (HMF), kwas 2,5-furanodikarboksylowy (FDCA) i γ-walerolakton (GVL)[11]. W zakresie enzymatycznej hydrolizy znaczący jest postęp dotyczący poznania warunków przygotowania surowca, złożonego mechanizmu hydrolizy lignocelulozy, weryfikacji roli układu wieloenzymatycznego w tym procesie, współdziałania i synergii, szczególnie litycznych monooksygenaz polisacharydowych (LPMO)[12]. Ważne zagadnienie dotyczy aktywności i stabilności enzymów, w tym możliwości ich wielokrotnego zastosowania po immobilizacji. Znaczny postęp obserwowany jest w zakresie magnetycznych nanocząstek, które umożliwiają immobilizację enzymów litycznych i łatwe ich wydzielanie[13]. Łatwe do realizacji i umożliwiające zmniejszenie kosztów bioprocesu są próby odzysku enzymów po procesie i ich wielokrotnego zastosowania[14]. Znane są różne sposoby recyklingu enzymów z frakcji stałej po hydrolizie surowców lignocelulozowych lub z frakcji płynnej po zastosowaniu technik membranowych[15, 16].

Celem badań było określenie możliwości zastosowania enzymów zaadsorbowanych w biomasie po hydrolizie lignocelulozy miskanta olbrzymiego lub cukrowego do katalizy kolejnych reakcji hydrolizy.

Część doświadczalna

Materiały

Biomasa miskanta olbrzymiego (*Miscanthus* × *giganteus*) oraz miskanta cukrowego (*Miscanthus sacchariflorus*), o zawartości suchej substancji odpowiednio 97,35% i 93,68%, pochodziła z Zakładu Produkcyjno-Doświadczalnego „Bałcyny" Sp. z o.o.

Metodyka badań

Biomasę rozdrabniano za pomocą młyna tnącego (Retsch SM100) i selekcjonowano z użyciem sit na cząstki o średnicy 1−2 mm.

W surowcu, za pomocą urządzenia Fibertec™ 1020 (FOSS), oznaczono udział włókna neutralno-detergentowego (NDF) wg Van Soesta[17], ligniny kwaśno-detergentowej (ADL) i włókna kwaśno-detergentowego (ADF)[18]. Na podstawie różnicy zawartości frakcji ADL i ADF obliczono stężenie celulozy, a z różnicy zawartości frakcji ADF i NDF stężenie hemicelulozy.

Biomasę roślinną poddano termicznej obróbce wstępnej w środowisku alkalicznym, zgodnie z procedurą zweryfikowaną przez Świątek i współpr.[19]. Zastosowanie tego rodzaju metody obróbki wstępnej umożliwiło m.in. uzyskanie dużej wydajności hydrolizy enzymatycznej biomasy sorgo i konopi[20].

Do 10% (mas./obj.) zawiesiny surowca w wodzie dejonizowanej dodawano NaOH do uzyskania stężenia 1% mas. Obróbkę prowadzono przez 1 h w 120°C w reaktorze ciśnieniowym. Zawiesinę po obróbce wirowano (4150*g*, 10°C, 10 min). Supernatant usuwano, a osad przemywano 2-krotnie wodą dejonizowaną w celu usunięcia inhibitorów enzymów i drobnoustrojów. Przygotowano 200 g zawiesiny o stężeniu 10% mas. otrzymanej biomasy w wodzie dejonizowanej i skorygowano jej kwasowość do wartości pH 5,0, stosując lodowaty kwas octowy. Rozwój niepożądanych drobnoustrojów hamowano, dodając do zawiesiny 2 mL 10-proc. (mas./obj.) roztworu azydku sodu.

Do zawiesiny biomasy po obróbce wstępnej dodawano preparat celulolityczny Accellerase 1500 (4750 FPU/g, DuPont), w dawkach 18, 29, 35 lub 58 FPU/g s.s. surowca (FPU, aktywność odpowiadająca ilości enzymu uwalniającej 1 µmol równoważnika glukozy w czasie 1 min). Hydrolizę lignocelulozy wspomagano zgodnie z zaleceniami producenta i na podstawie własnych wstępnych analiz takimi preparatami, jak: β-glukozydaza (Accellerase BG, 3000 pNPG/g) (pNPG, aktywność odpowiadająca ilości enzymu uwalniającej 1 µmol *p*-nitrofenolu z *p*-nitrofenylo-β-D-glukopiranozydu w czasie 1 min), mieszanina ksylanazy i celulaz (Accellerase XC, 1000 CMCU/g and 2500 ABXU/g) (CMCU i ABXU, aktywność odpowiadająca ilości enzymu uwalniającego odpowiednio 1 µmol równoważnika glukozy lub 1 µmol równoważnika ksylozy w czasie 1 min) oraz hemicelulazy (Accellerase XY, 20000 ABXU/g), dodawanymi w dawce odpowiednio: 0,090 mL/g s.s., 0,125 mL/g s.s. i 0,050 mL/g s.s. Reakcję hydrolizy prowadzono w 60°C przez 72 h w dwóch powtórzeniach, mieszając próbki w inkubatorze z szybkością 300 rpm. Po zakończeniu hydrolizy próbki wirowano (4000*g*, 10°C, 15 min), a w uzyskanym supernatancie natychmiast oznaczano stężenie cukrów redukujących z zastosowaniem metody z kwasem 3,5-dinitrosalicylowym (DNS)[21]. Produktywność uwalniania cukrów redukujących obliczono wg wzoru (1):

$$P = \frac{c \cdot V_1 \cdot s}{V_2} \quad (1)$$

w którym P oznacza masę uwolnionych cukrów redukujących z substratu, g/g s.s., c stężenie uwolnionych cukrów redukujących, g/L, V_1 objętość medium reakcyjnego, L, s zawartość suchej substancji w surowcu, g, a V_2 objętość hydrolizatu, L.

Wydajność hydrolizy polisacharydów obliczono względem stężenia celulozy i hemicelulozy w surowcu ze wzoru (2):

$$W_1 = \frac{c \cdot 0{,}9}{k} \cdot 100 \quad (2)$$

w którym W oznacza wydajność uwalniania cukrów redukujących, %, c stężenie uwolnionych cukrów redukujących, g/L, 0,9 to współczynnik przeliczeniowy cukrów prostych na polisacharydy, a k zawartość celulozy i hemicelulozy w surowcu, % s.s.

Wydajność hydrolizy celulozy (W_2) obliczano na podstawie stężenia celulozy w biomasie wg wzoru (3):

$$W_2 = \frac{c \cdot 0{,}9}{k_c} \cdot 100 \quad (3)$$

w którym c oznacza stężenie uwolnionej glukozy, g/L, 0,9 to współczynnik przeliczeniowy cukrów prostych na polisacharydy, a k_c stężenie celulozy w biomasie, % s.s.

Produktywność enzymów (P_m) obliczono wg wzoru (4):

$$P_m = \frac{c}{m}, \frac{\text{mg glukozy}}{\text{mg białka}} \quad (4)$$

w którym c oznacza stężenie uwolnionej glukozy, mg/mL, m stężenie białka w mieszaninie preparatów enzymatycznych, dodawanego do hydrolizy, mg/mL.

Frakcję stałą po reakcji enzymatycznej mieszano ze świeżą porcją biomasy i zakwaszoną lodowatym kwasem octowym wodą dejonizowaną (pH 5,0) w proporcji masowej 1:1:2, po czym próbki inkubowano w 60°C przez 72 h. Procedurę powtarzano 2-krotnie, za każdym razem oznaczając w hydrolizacie stężenie cukrów redukujących.

Próbki biomasy przed i po hydrolizie enzymatycznej analizowano, wykorzystując mikroskop skaningowy. Próbki przygotowywano za pomocą napylarki jonowej (Fine Coater JFC 1200), a obserwacje prowadzono za pomocą elektronowego mikroskopu skaningowego JSM 5310LV przy napięciu 20 kV.

Wyniki przedstawiono jako średnią z co najmniej 2 niezależnych doświadczeń, a błąd pomiarów jako odchylenie standardowe. Analizę wariancji wyników przeprowadzono za pomocą testu Tuckeya, $p < 0{,}05$.



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Omówienie wyników

W tabeli przedstawiono udział składników lignocelulozowych w biomasie miskanta olbrzymiego i cukrowego. Nie stwierdzono znaczących różnic w składzie frakcji włókna surowców. Po obróbce wstępnej zmieniła się mikro- i makrostruktura biomasy.

Table. Lignocellulosic components of biomass obtained from miscanthus giganteus and miscanthus sacchariflorus

Tabela. Udział składników lignocelulozowych w biomasie miskanta olbrzymiego i cukrowego

Gatunek miskanta	NDF, % s.s.	ADF, % s.s.	ADL, % s.s.	Stężenie, % s.s	
				celulozy	hemicelulozy
Olbrzymi	80,58	55,94	9,77	46,17	24,64
Cukrowy	81,16	56,07	11,29	44,78	25,09

Próbki biomasy miskanta olbrzymiego i cukrowego po obróbce wstępnej i detoksykacji poddano hydrolizie enzymatycznej, z zastosowaniem czterech dawek preparatu enzymów celulolitycznych (Accellerase 1500) oraz rekomendowanej dawki kompleksu enzymów wspomagających, m.in. ksylanaz, celulaz, hemicelulaz (Accellerase BG, XC, XY). Wykazano, że po obróbce hydrotermicznej do frakcji wodnej przechodziła niewielka ilość sacharydów wyrażonych jako cukry redukujące, tj. 0,13 mg/g s.s. z miskanta olbrzymiego oraz 0,18 mg/g s.s. z miskanta cukrowego.

Na rys. 1 i 2 przedstawiono przykładowe mikrofotografie SEM próbek biomasy surowców przed hydrolizą enzymatyczną oraz po hydrolizie. Zaobserwowano podobną natywną strukturę obu surowców, materiałów włóknistych o dużej liczbie zagregowanych struktur lamelarnych. Biomasa po wstępnej obróbce alkalicznej charakteryzowała się strukturą włóknistą częściowo uszkodzoną, otwartą i makroporowatą (rys. 1a i b; rys. 2a i b). Podobne zmiany zaobserwowali Łysakowski i współpr.[22] po obróbce alkalicznej i kwasowej miskanta. Po hydrolizie stwierdzono dalszą degradację elementów strukturalnych biomasy (rys. 1c i d; rys. 2c i d). Zastosowana technika i technologia wstępnej obróbki biomasy musi być tania, efektywna, zapewniać minimalne straty cukrów oraz generować małe ilości inhibitorów procesów enzymatycznych i fermentacji. Za jedną z najbardziej efektywnych metod uważana jest zastosowana w ramach przeprowadzonych doświadczeń obróbka alkaliczna[23]. Kordala i współpr.[24] stosując alternatywną obróbkę wstępną z użyciem amoniaku, uzyskali wydajność hydrolizy polisacharydów miskanta olbrzymiego wynoszącą 83,4%, podobnie jak Przybysz i współpr.[25]. Lewandowska i współpr.[26] po zastosowaniu obróbki alkalicznej biomasy miskanta olbrzymiego uzyskali wydajność hydrolizy wynoszącą 95,0%, a stężenie cukrów redukujących 51,5 g/L, zaś po zastosowaniu obróbki z kwasem szczawiowym wydajność hydrolizy enzymatycznej wyniosła 99,3%, jednak zawartość cukrów redukujących wynosiła tylko 26,3 g/L. Dużą wydajność hydrolizy polisacharydów miskanta cukrowego (98,6%) uzyskano również po zastosowaniu wstępnej obróbki alkalicznej[27].

Po pierwszej hydrolizie enzymatycznej wydzieloną frakcję stałą użyto jako źródło enzymów katalizujących dwie kolejne reakcje hydrolizy nowych porcji lignocelulozy. Zmiany stężenia uwolnionych cukrów redukujących, produktywność i wydajność procesów przedstawiono na rys. 3−5. W pozostałości po hydrolizie polisacharydów stwierdzono obecność hydrolaz, które uwalniały cukry redukujące, a produktywność enzymów podczas pierwszej reakcji, wyrażona zawartością glukozy, wynosiła 3,0−4,2 mg/mg białka enzymatycznego, w drugiej 1,7−2,5 mg/mg białka, a w trzeciej 1,3−1,9 mg/mg białka. Po pierwszej hydrolizie enzymatycznej największe stężenie cukrów redukujących (77,39 g/L) uzyskano po hydrolizie miskanta cukrowego z użyciem największej dawki Accellerase 1500 (rys. 3b). Nieznacznie mniejsze stężenia cukrów redukujących (72,46 g/L) uzyskano po hydrolizie w tych samych warunkach miskanta olbrzymiego (rys. 3a). Zastosowanie największej dawki enzymów celulolitycznych umożliwiało zachowanie największej aktywności związanej z frakcją stałą po hydrolizie oraz największej wydajności hydrolizy lignocelulozy. Stężenie cukrów redukujących po ostatniej reakcji hydrolizy stanowiło 45,42−51,70% i 47,64−53,29% stężenia cukrów po przeprowadzeniu pierwszej hydrolizy, odpowiednio polisacharydów miskanta olbrzymiego i miskanta cukrowego (rys. 3).

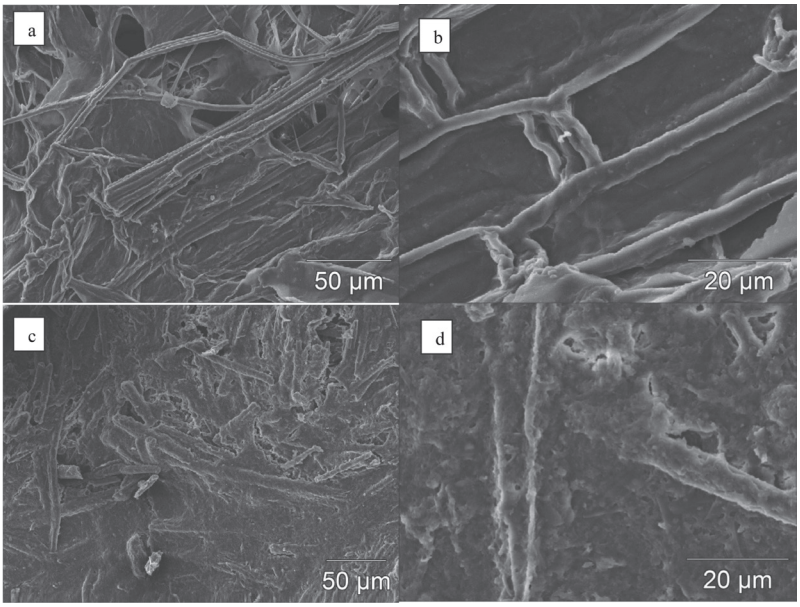


Fig. 1. Structure of miscanthus giganteus biomass after pretreatment (a, b) and hydrolysis catalyzed by Accellerase 1500 (35 FPU) and auxiliary enzymes (c, d)

Rys. 1. Struktura biomasy miskanta olbrzymiego po obróbce wstępnej (a, b) oraz biomasy po hydrolizie z użyciem 35 FPU preparatu Accellerase 1500 i mieszaniny enzymów wspomagających (c, d)

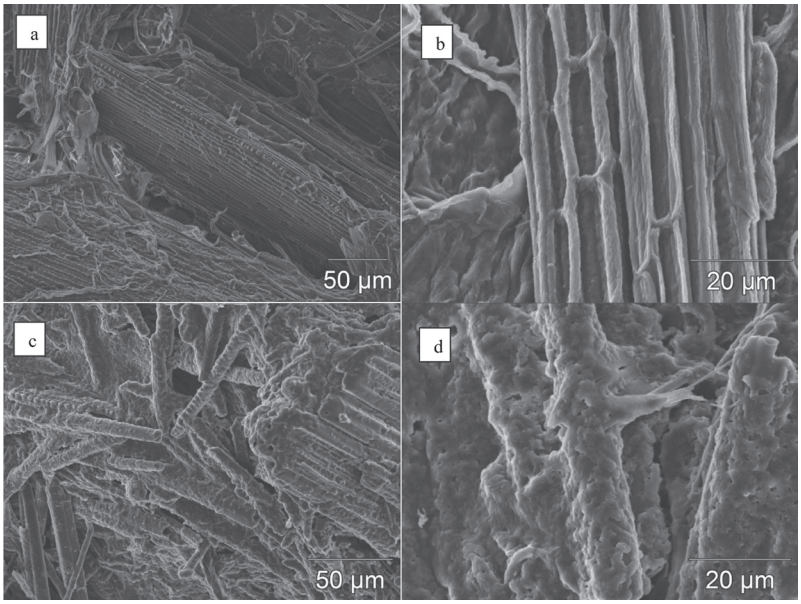


Fig. 2. Structure of miscanthus sacchariflorus biomass after pretreatment (a, b) and hydrolysis catalyzed by Accellerase 1500 (35 FPU) and auxiliary enzymes (c, d)

Rys. 2. Struktura natywnej biomasy miskanta cukrowego (a, b) oraz biomasy po hydrolizie z użyciem 35 FPU preparatu Accellerase 1500 i mieszaniny enzymów wspomagających (c, d)

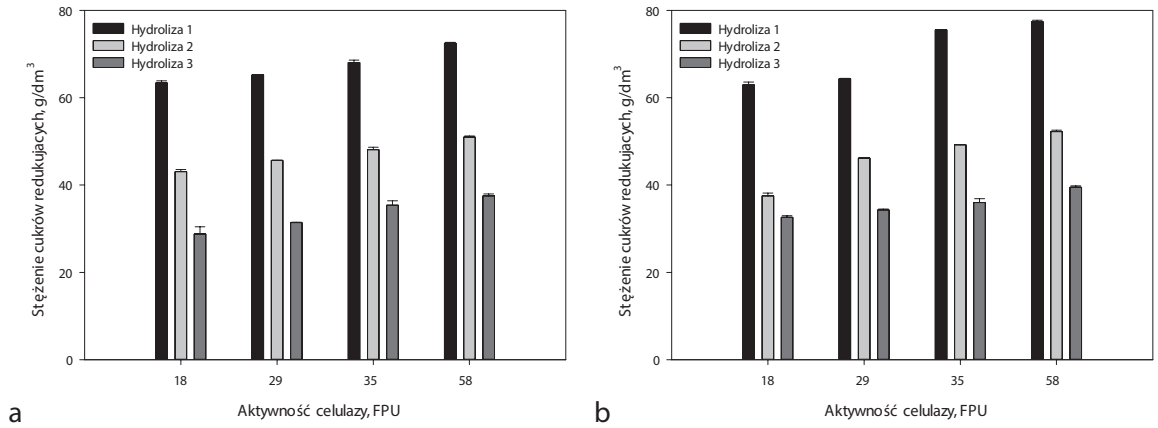


Fig. 3. Concentration of reducing sugars in biomass samples of miscanthus giganteus (a) and miscanthus sacchariflorus (b), after enzymatic hydrolysis (hydrolysis 1) and after recycling of the solid fraction (hydrolysis 2, 3)

Rys. 3. Stężenie cukrów redukujących w próbkach biomasy miskanta olbrzymiego (a) i cukrowego (b) po hydrolizie enzymatycznej (hydroliza 1) oraz recyklingu frakcji stałej (hydroliza 2, 3)

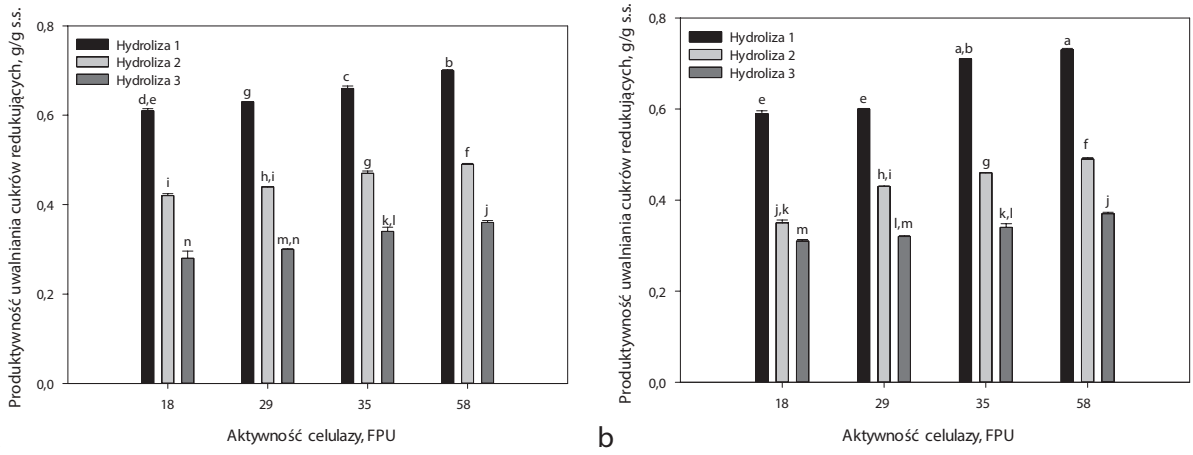


Fig. 4. Productivity of reducing sugar release from biomass obtained from miscanthus giganteus (a) and miscanthus sacchariflorus (b), after enzymatic hydrolysis (hydrolysis 1) and after recycling of the solid fraction (hydrolysis 2 and 3). The various letters (a, b, c, …) indicate statistical significance at $p < 0.05$

Rys. 4. Produktywność uwalniania cukrów redukujących z biomasy miskanta olbrzymiego (a) i cukrowego (b), po hydrolizie enzymatycznej (hydroliza 1) oraz recyklingu frakcji stałej (hydroliza 2, 3); różne litery (a, b, c, …) oznaczają istotność statystyczną różnic na poziomie $p < 0,05$

Produktywność uwalniania cukrów redukujących z biomasy miskanta olbrzymiego i miskanta cukrowego była zbliżona i wynosiła odpowiednio 0,25−0,50 g/g s.s. i 0,20−0,73 g/g s.s (rys. 4). Wykonano analizę statystyczną danych dla tego reprezentatywnego parametru opisującego efektywność hydrolizy lignocelulozy. Wykazano statystycznie istotne różnice produktywności dla kolejnych reakcji ($p < 0,05$) (rys. 4). Statystycznie istotnie większą produktywność cukrów redukujących uzyskano po hydrolizie biomasy miskanta cukrowego z użyciem największych dawek (35 i 58 FPU) preparatu Accellerase 1500. Wydajność hydrolizy polisacharydów biomasy miskanta olbrzymiego i cukrowego, wynosząca odpowiednio 31,93–90,07% (rys. 5a) i 26,88−96,20% (rys. 5b), oraz celulozy, wynosząca odpowiednio 22,94−61,61% (rys. 5c) i 10,06−69,77% (rys. 5d), była na podobnym poziomie, z niewielką preferencją do pozyskiwania sacharydów z miskanta cukrowego. Kolejne reakcje hydrolizy, z zastosowaniem recyklingu biomasy z enzymami, nie wykazały znaczących różnic sorpcji i stabilności enzymów przez biomasę miskanta olbrzymiego lub cukrowego.

Z użyciem pozostałości biomasy po hydrolizie polisacharydów zawierającej aktywne enzymy hydrolityczne przeprowadzono dwie reakcje. Produktywność enzymów po trzeciej reakcji hydrolizy zmniejszyła się o ponad 50% ich początkowej aktywności. Problemem podczas prowadzenia reakcji było nagromadzenie znacznych ilości biomasy (w tym akumulacja lignin), które znacząco oddziaływały na aktywność hydrolityczną enzymów[13]. Proces adsorpcji enzymów celulolitycznych do ligniny opisali i zbadali Rahikainen i współpr.[28].

Kluczowym elementem rozwoju produkcji biopaliw z biomasy lignocelulozowej jest rachunek ekonomiczny, a także dostępność biokatalizatorów specyficznych dla danego substratu. Gomes i współpr.[15] wskazali na zasadniczą rolę lokalizacji enzymu w układzie mieszaniny reakcyjnej ciecz-ciało stałe, na termostabilność celulaz oraz możliwość ich odzysku i wielokrotnego zastosowania. Po zastosowaniu ultrafiltracji odzyskano 80% aktywnego preparatu Celluclast z zawiesiny reakcyjnej po hydrolizie osadu ze ścieków zakładu przetwarzającego odpady celulozowe. Wielokrotne użycie celulaz do hydrolizy zużytych kiści olejowca było utrudnione z uwagi na inhibicję celulaz przez glukozę oraz niespecyficzne wiązanie enzymów z frakcją stałą[29]. Poprawę wydajności uwalania glukozy z 19,30% do 68% uzyskano po dodaniu do mieszaniny reakcyjnej glikolu polietylenowego. Korzystne zwiększenie opłacalności, wydajności uwalniania sacharydów i fermentacji etanolowej uzyskano po wielokrotnym wykorzystaniu biomasy z enzymami już po 24 h hydrolizy, kiedy wydajność hydrolizy zwykle znacznie się zmniejsza[30]. W tym zakresie ważna byłaby także analiza współdziałania i synergii mieszaniny enzymów litycznych. Weiss i współpr.[31] wykazali, że zastosowanie recyklingu biomasy z enzymami umożliwia zmniejszenie o 30% dawki enzymów do hydrolizy słomy kukurydzianej. Wielokrotne wykorzystanie celulaz do hydrolizy substratów lignocelulozowych może być realizowane w różny sposób, np. poprzez użycie jako źródła enzymów płynnej frakcji uzyskanej po hydrolizie[11]. W ten sposób do pięciu reakcji zastosowano celulazy z mieszaniny reakcyjnej po hydrolizie miazgi drzewnej pozostałej po bieleniu, a odzysk aktywnych celulaz wynosił 35,1−48,8%.

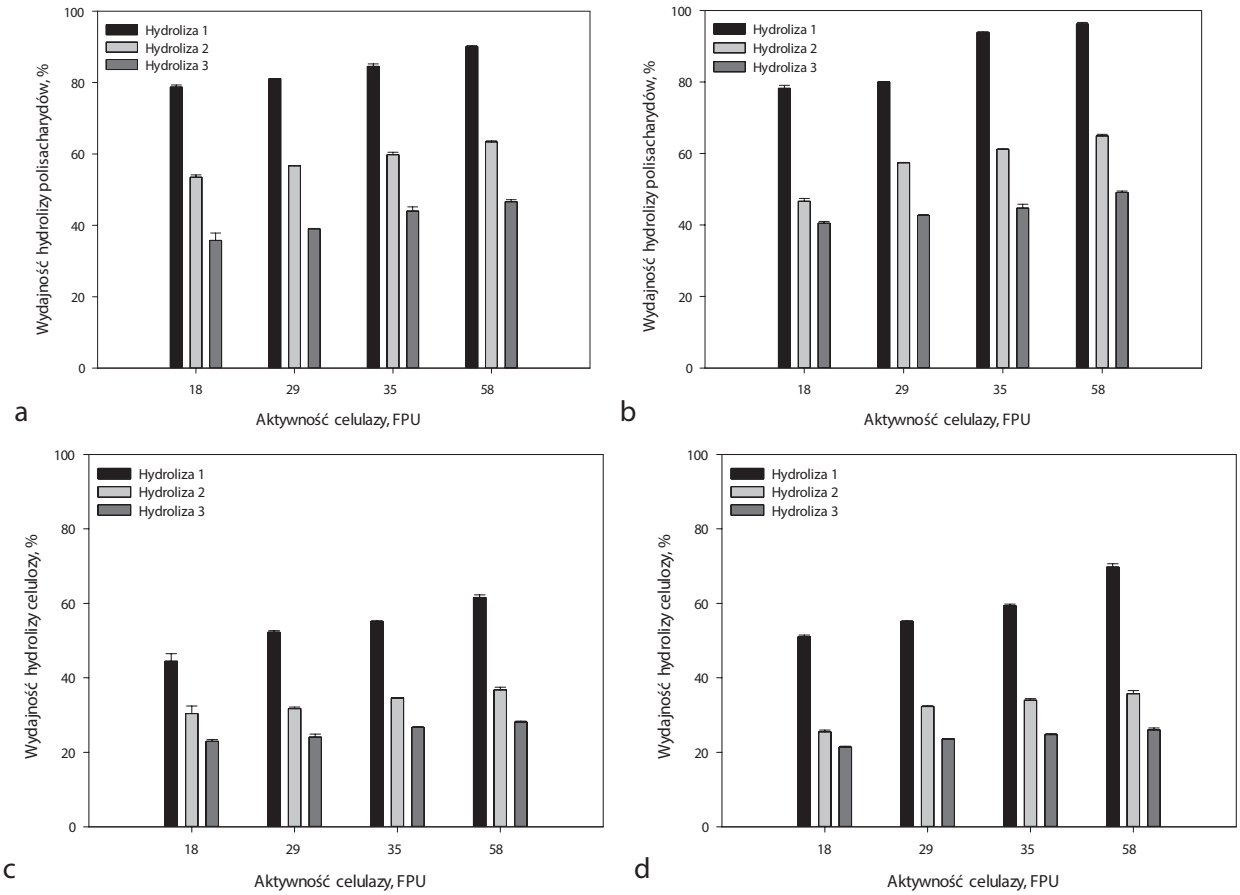


Fig. 5. Yield of enzymatic hydrolysis of polysaccharides and cellulose from biomass obtained from miscanthus giganteus (a, c) and miscanthus sacchariflorus (b, d) after enzymatic hydrolysis (hydrolysis 1) and recycling of the solid fraction (hydrolysis 2, 3)

Rys. 5. Wydajność hydrolizy polisacharydów i celulozy biomasy miskanta olbrzymiego (a, c) i miskanta cukrowego (b, d) po hydrolizie enzymatycznej (hydroliza 1) oraz recyklingu frakcji stałej (hydroliza 2, 3)

Podsumowanie

Obróbka alkaliczna umożliwiła skuteczną hydrolizę biomasy, usunięcie lignin i/lub hemicelulozy. Uzyskano zwiększenie porowatości materiału i zmniejszenie udziału celulozy krystalicznej. Otrzymany substrat był podatny na działanie hydrolaz, co zapewniało sorpcję enzymów w biomasie. Uzyskane wyniki potwierdzają możliwości zastosowania enzymów litycznych związanych z frakcją stałą surowca lignocelulozowego do jego hydrolizy. Wykazano możliwość katalizy dwóch reakcji z użyciem jednej dawki mieszaniny enzymów i zachowanie co najmniej 50% wydajności początkowej procesu.

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