PROCESS OF OBTAINING BIOETHANOL FROM SORGHUM BIOMASS USING GENOME SHUFFLING

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One of the materials to be used for the production of bioethanol might be sorghum, an alternative plant for bioenergy production. In the process of obtaining biofuels, the bioprocess technology is as important as the plant material. The production of bioethanol from lignocellulosic materials requires degrading cell walls to specific polymers and the hydrolysis of carbohydrates to monomer sugars.
The aim of the study was to evaluate the chemical and enzymatic treatment of sorghum biomass during the preparation of the material for the production of ethanol. Biomass processing consists in the disintegration of the solid phase and breaking down of compact lignocellulose. The highest content of glucose was obtained using enzymatic hydrolysis with cellulolytic enzyme, *i.e.* Flashzyme Plus 200. After genome shuffling, the distillery yeast demonstrated an increased fermentation activity and resistance to environmental stress factors formed during the process of ethanol production.

Keywords: bioethanol, sorghum biomass, pretreatment, genome shuffling

INTRODUCTION

Biofuels constitute a favourable choice for fuel consumption due to their renewability, biodegradability and generation of acceptable quality exhaust gases.[1] Bioethanol produced from organic raw materials is a renewable and clean resource for energy production, which is used as fuel, as well as in the chemical, cosmetic and pharmaceutical industries. Some plants that can be used for bioenergy production are, for example, sorghum and miscanthus, as their high calorific value of combustion and high yield of dry biomass make them suitable materials for the production of 2nd generation biofuels.

Sorghum is an annual plant that can reach the height of 4 m. It is tolerant to drought, contains high amounts of monosaccharides (approx. 10%) and provides high yields of dry mass (28 t·ha^{-1}) at the so-called milk-wax phase of the seed.[2,3] The energetic value of combustion of sorghum is 15 MJ·kg^{-1}.

Lignocellulosic biomass is characterized by the complexity of its chemical composition, as its structure contains a polymeric complex called lignocellulose, which is relatively difficult to biodegrade. Lignin, structurally crosslinked by ester and carbon bonds, is an effective obstacle in the production of bioethanol from plant biomass. Lignin and its derivatives have a negative effect on the hydrolysis of biomass, as they physically hinder the access of cellulases to the microfibrils of crystalline cellulose and also bind cellulases and lead to their deactivation.[4-9]

The production of biofuel from lignocellulosic material requires the deconstruction of the cell wall into individual polymers and the hydrolysis of carbohydrates into monomeric sugars. This requires subjecting the biomass to pretreatment, which affects significantly the course of the further stages of bioethanol production, *i.e.* the enzymatic hydrolysis and fermentation process, as well as decides on the final efficiency of the process.[10-11] In order to disintegrate the biomass and remove lignin, several pretreatment methods have been tested – physical, chemical and biological ones.[12-15] The physical pretreatment methods of lignocellulosic biomass, which aim to reduce the size of the substrate, as well as to facilitate the access of bioactive substances to the surface, reducing polymerization and the crystallization degree of lignocellulose, include: milling, an extrusion method and an ultrasonic pretreatment.[16-17] The chemical processes include acid (sulfuric, hydrochloric acid), alkali (sodium hydroxide, calcium carbonate, ammonia) and neutral (ionic liquids) treatments, the organosolv process, the ammonia fibre explosion and ozonolysis.[18-19] Depending on the method used, different changes occur within the lignocellulosic complex. The alkali pretreatment has mainly the function of delignification, while the acid pretreatment process dissolves most hemicelluloses. The non-specificity

of the acidic treatment leads to the formation of complex sugars and compounds that inhibit the activity of the microorganisms utilized for ethanol production.[20] An effective pretreatment process should possess the following advantages: it should preserve and decrystallize the celluloses, depolymerize hemicelluloses, restrict the formation of inhibitors, require low energy input, allow recovering value-added products, such as lignin, and, finally, it should be cost-effective.[21]

Next, it is important that the simple sugars obtained in the enzymatic hydrolysis be available to the distillery yeast in the fermentation process. The synergistic action of enzymes in the process of hydrolysis involves the attack on the cellulose by bonding with cellulose fibres in amorphous places, the cleavage of cellulosic chains, cutting off their considerable fragments, and then degrading them until the glucose polymer is obtained.

Furthermore, the *Saccharomyces cerevisiae* distillery yeast is most popular for the production of biofuels from lignocellulosic biomass. However, the economic aspect is challenged by the decreased fermentation performance of the yeast in the presence of various environmental stress factors, such as high temperature or toxins. Most distillery yeasts used in bioethanol production have the optimum temperature of 30-35 °C.[22-23] Raw lignocellulosic materials are often pretreated at about 200 °C, during which various toxins are formed.[24] The commercially available cellulases and hemicellulases carry out the hydrolysis efficiently at temperatures in the range of 45-50 °C. Therefore, improving the resistance of distillery yeast to environmental stress factors is critical for achieving an efficient and economically viable bioconversion of cellulose to biofuels.[25]

Currently, genome shuffling is one of the most important tools of improving the industrial properties of microorganisms. This method involves combining several parental strains, each of which has at least one beneficial technological trait, into a hybrid. These traits can be accumulated by using several rounds.[26] The first step in the construction of new hybrids is to create a parental library, using random mutagenesis and rapid screening.[27] This way, a parental library with strong resistance properties is created. These strains are then subjected to the protoplastization and further combined in the protoplast fusion. After each round, it is necessary to conduct a fusant screening to select the best strains.[26,28]

EXPERIMENTAL

Materials and methods

The raw material used in the study was Sucrosorgo 506 biomass from the Experimental Farm of INF&MP in Sielec Stary, Poland. The raw material was subjected to preliminary crushing to particles of 2-4 cm in size and then dried at 50-55 °C for 24 h. Next, the material was disintegrated in a knife mill (Retsch SM-200, Germany) with a sieve of 4 mm mesh size.

To optimize the enzymatic hydrolysis, the Flashzyme Plus 200 enzymatic preparation from AB Enzymes was used, which consists of endoglucanase, cellobiohydrolase, cellobiase, xylanase and mannanase. The cellulolytic activity of Flashzyme determined according to the method of Adney and Baker (2008)[29] was 123 $FPU \cdot mL^{-1}$ and the xylanolytic activity according to the Osaka University procedure was 2666 $XU \cdot mL^{-1}$.[30]

Saccharomyces cerevisiae yeast (commercial strain Ethanol Red) was obtained from Lessafre Fermentis, France. The microorganisms were stored on Yeast extract Peptone Dextrose (YPD) medium with the addition of 1% yeast extract (*w/v*), 2% peptone (*w/v*), 2% glucose (*w/v*) and 2% (*w/v*) agar-agar kept at the temperature of 4-8 °C.

The effect of the chemical processing of sorghum biomass was determined for sulfuric acid after a 10-minute treatment with 2% acid and after autoclaving at 121 °C for 1 h, while for sodium hydroxide after 5 h treatment with 1.5% alkali at 90 °C.

The effect of sulfuric acid and sodium hydroxide on the content of the released reducing sugars was evaluated by Miller's method (1959)[31] with 3,5-dinitrosalicylic acid (DNS) in the enzymatic test. The test was performed with the use of the Celluclast 1.5L (Novozymes) enzymatic preparation in the dose of 10 $FPU \cdot g^{-1}$. The raw material was incubated at 55 °C in 0.05 M citrate buffer of pH 4.8 for 24 h. Then, the absorbance measurement was carried out against the reference sample at the wavelength of 530 nm (UV-VIS Spectrophotometer, Jasco V-630, Germany).

The optimization of the enzymatic hydrolysis of sorghum biomass was carried out according to the Response Surface Methodology (RSM), using the following parameters: biomass content 5-10%, temperature 50-70 °C, time 24-72 h, pH 4.2-5.4, dose of Flashzyme 10-30 $FPU \cdot g^{-1}$.

The content of glucose was determined by High Performance Liquid Chromatography (HPLC, Agilent Technologies 1200, Germany).

To construct the parental library for genome shuffling, chemical mutagenesis using ethylmethanesulfonate (EMS) was carried out. The obtained mutants were subjected to the protoplastization process with 2% (*w/v*) Glucanex (Sigma-Aldrich). The protoplastization of the yeast mutant was carried out on 1 g for 75 min at 30 °C in a thermomixer (Eppendorf), with continuous stirring. The protoplast fusion was performed using 40% (*w/v*) polyethylene glycol (PEG). The protoplasts were then regenerated (0.6 M KCL and 0.01 M $CaCl_2$) and screened for fermentation activity, thermotolerance, osmotolerance and resistance to acetic acid. The best strains after the mutagenesis (M) and screening were selected to be the parental strains for the protoplast fusion (F). The new strains were again regenerated and screened for the fermentation activity, thermotolerance, osmotolerance and resistance to acetic acid.

The sorghum biomass after the pretreatment and enzymatic hydrolysis was sterilized by autoclaving at 121 °C for 15 min and was used as fermentation medium. The medium was inoculated with *S. cerevisiae* cells from the inoculum culture (10% *v/v*). The fermentation process was carried out in a 250 mL Erlenmeyer flask incubated in a rotary shaker at 140 rpm for 72 h at 37 °C and pH 4.8. Additionally, a fermentation process using the yeast improved by the genome shuffling method was carried out. This process was performed in a 250 mL Erlenmeyer flask incubated in a rotary shaker at 140 rpm for 72 h at 37 °C, with the addition 0.1% (*v/v*) acetic acid at pH 4.8. After the fermentation process, the concentration of ethanol was determined by HPLC.

Analytical methods

The chemical components of sorghum biomass were evaluated as follows: cellulose according to TAPPI T17 m-55, hemicellulose as the difference between holocellulose (according to TAPPI T9 m-54) and cellulose, and lignin according to TAPPI T13 m-54.[32-34] The chemical composition was also determined in the solid fraction formed after the chemical treatment, while for the liquid phase after acidic treatment, toxins (furfural, hydroxymethylfurfural, acetic acid) and pentoses (xylose, arabinose) were determined using an Agilent Technologies 1200 HPLC Chromatograph, with a DAD detector for toxins and an RID detector for pentoses.

The analysis of FTIR spectra was conducted on the sorghum biomass before and after the chemical treatment, using a Fourier Transform Infrared Spectrometer (FTIR, Bruker ISS 66v/S, Germany) at infrared wavelengths of 400-4000 cm^{-1}.

The images of sorghum biomass before the process, after the chemical treatment and enzymatic hydrolysis were acquired by using a Scanning Electron Microscope (SEM, S-3400N, Hitachi, JPN) in high vacuum conditions. The samples were sputtered with gold.

The random amplified polymorphic DNA (RAPD) analysis was carried out to detect the genetic difference between the parental strains and the fusants after genome shuffling using random prime RAPD_21(GCTCGTCGCT). The DNA was isolated from the strains using Genomic Mini AX Yeast (A&A Biotechnology, Gdynia, Poland) by a procedure presented by the manufacturer. The Polymerase Chain Reaction (PCR) method was performed in a buffer (25 µL) in a Veriti Thermal Cycler (Applied Biosystems) with the following temperature profiles: the initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, the primer annealing at 36 °C for 45 s, the extension at 72 °C for 1.5 min and the final cycle of extension in 72 °C for 3 min, then the reaction was held at 4 °C. The PCR products were resolved by electrophoresis on 1% agarose gel containing ethidium bromide, visualized under a UV illuminator. GeneRuler Express (Thermo Fisher Scientific) was used as a marker.

RESULTS AND DISCUSSION

The success of using renewable biomass for ethanol production depends on the physical and chemical properties of the biomass, the pretreatment methods, efficient microorganisms and the optimization of the processing conditions. The primary goal of the chemical pretreatment is to improve the cellulose biodegradability by removing lignin and/or hemicelluloses, and, to a lesser extent, to reduce the polymerization degree (PD) and crystallinity of the cellulose component. An efficient pretreatment method is required for the enzymatic hydrolysis to yield maximum sugar productivity.

Two types of pretreatment were compared, *i.e.* acidic treatment with 2% sulfuric acid (10 min) and autoclaving process (121 °C, 1 h), and alkaline treatment with 1.5% sodium hydroxide (90 °C, 5 h), which were conducted on sorghum biomass ground on a knife mill and sieved on a mesh size of 4 mm. In order to determine the efficiency of chemical processing of the sorghum biomass, the content of reducing sugar was measured by Miller's method in the enzymatic test. The results allow concluding that the sodium hydroxide pretreatment is a more efficient method for sorghum biomass, compared to the pretreatment with sulfuric acid. The content of reducing sugar was 577 $mg \cdot g^{-1}$ and 212 $mg \cdot g^{-1}$, respectively.

The action mode of dilute acid is to solubilize hemicellulose and leave lignin and cellulose intact, so that the enzymatic digestibility of cellulose is enhanced. The alkaline pretreatment involves basically a delignification process, in which a significant amount of hemicellulose is also solubilized. In comparison with other pretreatment technologies, the alkali pretreatment usually needs lower temperatures and pressures. The pretreatment time, however, lasts a few hours, which is much longer than the time required for other pretreatment processes.[35]

Table 1 presents the chemical composition (cellulose, hemicellulose, lignin) of the sorghum biomass before and after the chemical pretreatment (in the solid fraction) with sulfuric acid and sodium hydroxide. The chemical composition confirmed that the alkaline treatment consists mainly in delignification, whereas the acid treatment dissolves most hemicelluloses.

The acidic treatment offers good performance, in terms of recovering hemicellulose sugars (especially pentoses in the liquid phase), but it also has some drawbacks. The hemicellulose sugars might be further degraded to furfural and hydroxymethylfurfural (HMF), strong inhibitors of microbial fermentation. The content of pentoses and toxins in the liquid phase, after the acidic treatment of sorghum biomass, was for xylose 9.53 $g \cdot L^{-1}$, arabinose 1.08 $g \cdot L^{-1}$, furfural 0.18 $g \cdot L^{-1}$, HMF 0.28 $g \cdot L^{-1}$, and acetic acid 1.44 $g \cdot L^{-1}$. In the case of the alkaline treatment, due to its mild conditions, the degradation of sugars to furfural, HMF and organic acids is limited. An ideal pretreatment technique should be able to maximize the recovery of available carbohydrates, such as cellulose and hemicellulose, while minimizing the degradation of sugars and the generation of possible inhibitors.

The next stage of converting biomass to bioethanol is the enzymatic hydrolysis process that determines what amount of simple sugars can be metabolized by the yeast in the ethanol fermentation process. After the alkaline pretreatment of sorghum biomass with sodium hydroxide, the enzymatic hydrolysis process was performed. The highest content of glucose released according to the response surface method (Fig. 1) was obtained using the following parameters: 50 °C, pH 4.2, 72 h, for 10% sorghum biomass and 30 $FPU \cdot g^{-1}$ Flashzyme.

Table 1
Chemical composition of sorghum biomass

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
BP	29.87 ± 0.51	29.03 ± 0.29	21.74 ± 0.39
ACP	59.18 ± 1.92	8.21 ± 0.06	28.62 ± 0.35
ALP	47.34 ± 0.22	28.40 ± 0.12	9.19 ± 0.44

BP: before pretreatment; ACP: acidic pretreatment; ALP: alkaline pretreatment

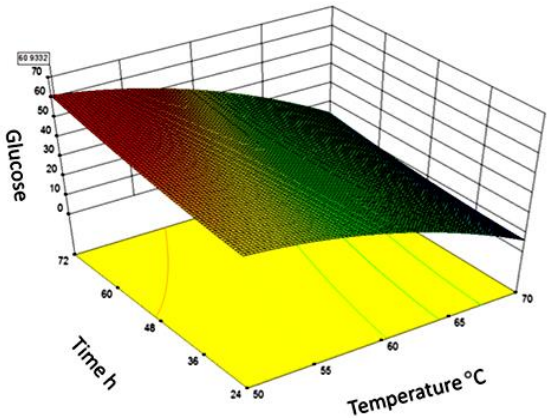

Figure 1: Enzymatic hydrolysis process of sorghum biomass (RSM)

Table 2
Characteristic infrared absorption bands

Band	Compound type	Band range (cm^{-1})
C-H stretching vibrations	aromatic rings	2800-3000
C-H stretching vibrations	aldehydes and ketones	2720
C-H bending vibrations (in-plane)	aromatic rings	1000-1100
C-H bending vibrations (off-plane)	aromatic rings	675-870
C-H bending vibrations	methyl group $-CH_3$	1430-1470, 1375
	methylene group $-CH_2-$	1430-1470
C-C stretching vibrations	aromatic rings	1500-1600
C-C stretching vibrations	guaiacyl ring	1270
C-C stretching vibrations	syringyl ring	1330
C-O stretching vibrations	phenols	1140-1230
	carboxylic acids	1250
C=O stretching vibrations	aldehydes and ketones	1675-1725
	carboxylic acids	1680-1725
O-H stretching vibrations	phenols	3200-3600
	carboxylic acids	2500-3000
O-H bending vibrations	carboxylic acids	1400, 920

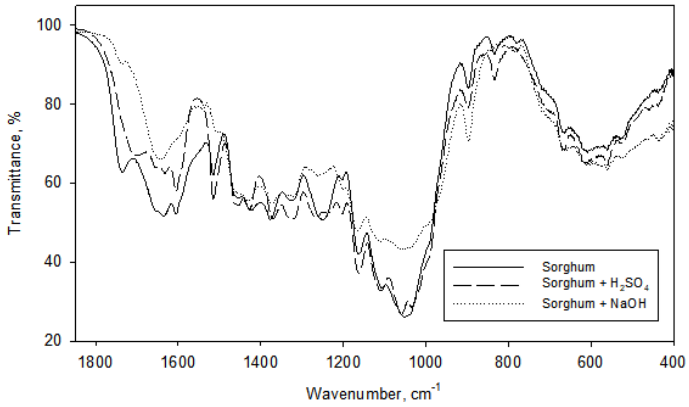

Figure 2: FTIR spectra of sorghum biomass before and after chemical treatment

The measurements of light absorption within the infrared spectrum are of high importance in studying the structure of chemical substances, as they help determine the functional groups and the location as well as the types of bonding. The absorption spectrum is characteristic of a given compound and, therefore, can be used for identification. Specific atom groups cause the formation of characteristic absorption bands, *i.e.* they absorb the radiation of precise frequency, to a large extent irrespective of the type of compound that they are found in. The absorption of radiation triggers vibrations within molecules. The frequency of the vibrations of several groups found in organic compounds and lignin are shown in Table 2.[36-38]

The spectra of untreated sorghum biomass are presented in Figure 2, along with those of the biomass pretreated with sulfuric acid and sodium hydroxide.[39] Comparing the FTIR spectra of the untreated and pretreated biomass, as regards the transmittance at 1730 cm^{-1} (C=O of carbonyl group), 1510 cm^{-1} (C-C of aromatic ring) and 1270 cm^{-1} (C-O of guaiacyl ring), it may be concluded that the spectra indicate the delignification of sorghum biomass, especially after the treatment with sodium hydroxide. It may be also observed that there exists an increase in the band intensity at 898 cm^{-1} and a decrease at 1427 cm^{-1}, which indicates lower crystallinity and an increase of the amorphous form of cellulose as a result of chemical pretreatments, in particular, of the alkaline treatment.[40]

The morphological features of sorghum biomass before and after the pretreatment and enzymatic hydrolysis are shown in Figure 3. The untreated sorghum biomass was observed to have a sedimentary layer on the surface area.[41] This layer can include waxes, hemicellulose, lignin or other bonding materials.[42] The SEM images of the biomass after the chemical pretreatment showed prominent roughness and looser structure, as compared to the untreated sample, making the fibres more

vulnerable to the enzymatic hydrolysis.[41] The surface layer was removed during the pretreatment, resulting in the exposure of the internal structure and the fibres. During the enzymatic hydrolysis, the external surface of sorghum biomass was damaged and further removal of the external surface, exposing the internal structure, can be observed.

Ten mutants with improved resistance to high temperature and toxins were obtained from populations generated by chemical mutagenesis using EMS. On the basis of the results of the screening tests, a parental library consisting of 4 mutants was created (M1, M2, M3, M4). The stage of constructing the parental strains library using the mutagenesis process is extremely important, as it allows the generation of a larger number of genotypes, which, in turn, influences the final effect of genome shuffling.[23,43] These yeast strains were then subjected to recursive protoplast fusion. After two rounds of genome shuffling, the best performing fusant (MF3) was obtained. It is characterized by better growth and about 40% higher ethanol productivity than the initial strains (Fig. 4). This is an excellent achievement, because according to other authors, after two rounds of genome shuffling, it was possible to receive a strain characterized by over 20% higher ethanol productivity.[44-46]

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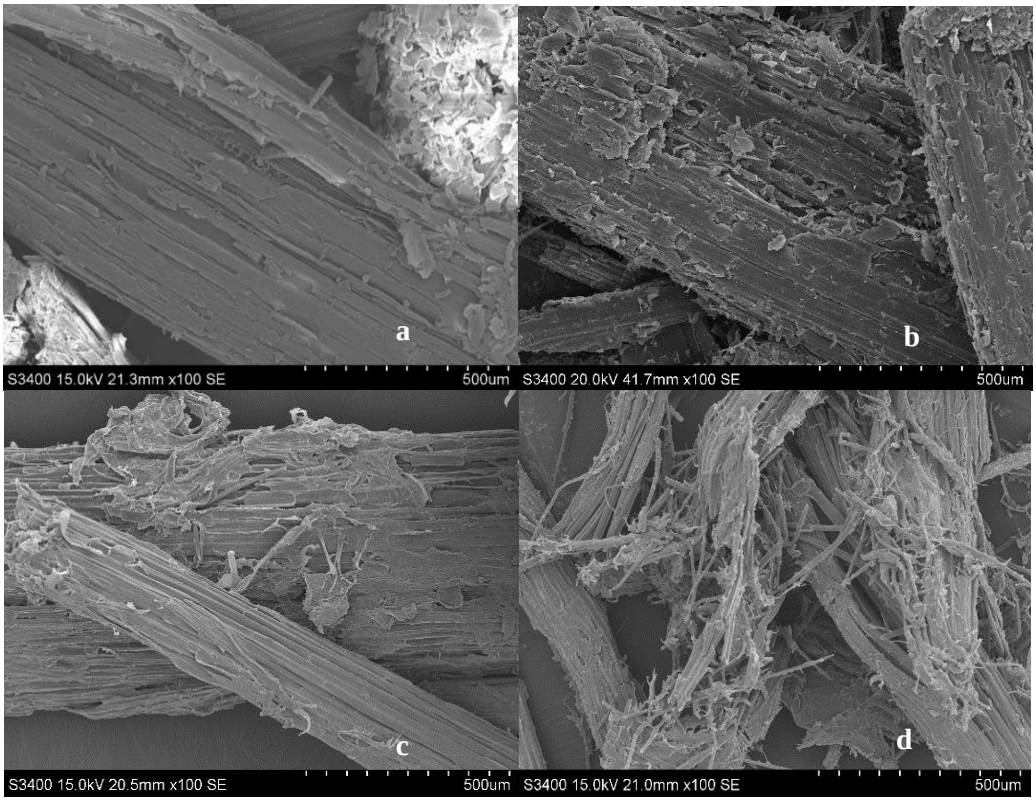

Figure 3: SEM analysis of sorghum biomass (a) before pretreatment, (b) after acidic pretreatment, (c) after alkaline pretreatment, (d) after enzymatic hydrolysis process

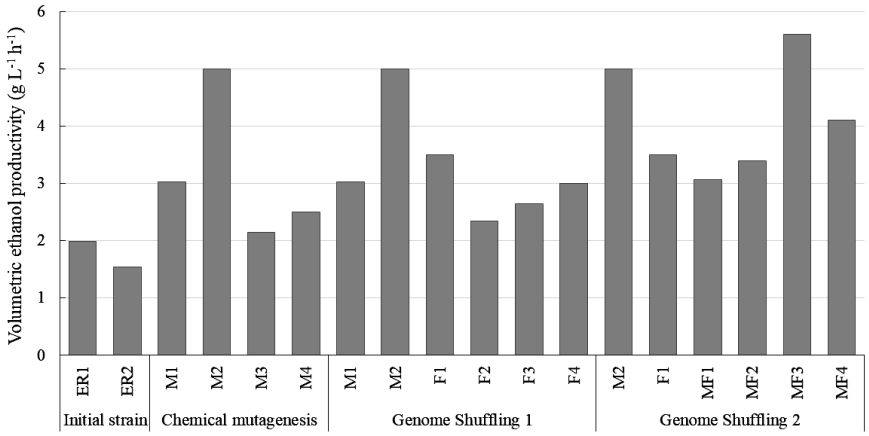

Figure 4: Ethanol productivity before and after genome shuffling (ER: initial strains; M: mutants; F: fusants after first round; MF: fusants after second round)

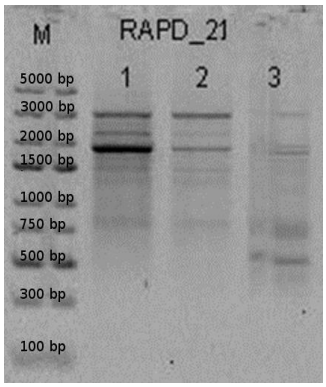

Figure 5: Polymorphism of genomic population by RAPD analysis (1, 2: parental strains; 3: fusant)

After the fermentation process with *Saccharomyces cerevisiae*, a 30% higher concentration of ethanol was observed for the samples inoculated with the yeast improved by the genome shuffling method. The maximum value of ethanol concentration was achieved after 72 h for both initial strains (19.02 $g \cdot L^{-1}$) and fusant MF3 (49.88 $g \cdot L^{-1}$). It can therefore be inferred that improved fusants are characterized by increased tolerance to temperature and toxins that occur during the pretreatment of lignocellulosic biomass.[47] These strains are also important for the industry, as they can contribute to lowering the costs of bioethanol production.[48,49]

RAPD-PCR using the primer (Fig. 5) resulted in profiles different for the parental strains and the fusant. The separation of the profiles of DNA fragments primer allowed to conclude that at the length of 500 bp PCR products appeared, while the parental strains did not show bands at this level. In turn, between 3000 and 2000 bp in the fusant, no band was seen that was present in the profiles of the parental strains. When analysing the electrophoretic separation, it was observed that the selected fusants have genomic DNA fragments typical of both parental strains, which indicates that their genomes are shuffled.

The random amplification polymorphic DNA (RAPD) is a technique involving the use of any primers that attach to genomic DNA. This method detects genetic polymorphism, and, what is important, it does not require prior knowledge of sequences that are specific to a given species of strains.[50] Therefore, the RAPD-PCR technique is often used to confirm the effectiveness of genome shuffling.[51]

CONCLUSION

It is suggested that sorghum biomass is a valuable feedstock for the production of ethanol due to its easy cultivation, favourable properties and high amounts of monosaccharides. The enzymatic hydrolysis of sorghum biomass can be significantly improved after a pretreatment with sodium hydroxide or sulfuric acid. However, the use of sodium hydroxide is a more efficient pretreatment method of sorghum biomass. The genome shuffling technique improves the phenotypic traits of

Saccharomyces cerevisiae yeast, *i.e.* an increased fermentation activity, resistance to temperature, acidic and osmotic stress, making it possible to increase the efficiency of the production of bioethanol.

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