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Bioethanol Production from *Miscanthus sinensis* Cellulose by Bioconversion

Olga V. Kriger[1,*], Olga O. Babich[1], Vyacheslav F. Dolganyuk[2], Oksana V. Kozlova[2], Stanislav A. Sukhikh[1], Timothy A. Larichev[2]

[1] *Immanuel Kant Baltic Federal University, Kaliningrad, Russia*

[2] *Kemerovo State University, Kemerovo, Russia*

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**e-mail: OKriger@kantiana.ru*

Abstract.
Introduction. Cellulose-containing parts of herbs are an excellent source of alternative energy and can be used to produce biological ethanol. The present research aims at improving this fundamental and promising area of biotechnology. It introduces a new consortium of microorganisms that can saccharify while fermenting the substrate.
Study objects and methods. The research featured technical cellulose obtained from *Miscanthus sinensis* using hydrotropic delignification and oxidation with pertrifluoroacetic acid. The ethanol content in the culture liquid was determined using an Agilent 7890B gas chromatograph with a flame ionization detector. The biocompatibility of the strains was studied by growing a direct co-culture in a dense nutrient medium.
Results and discussion. The research objective was to create a new microbial consortium for the single-step production of bioethanol from *Miscanthus sinensis* cellulose. A set of biocompatibility experiments and cultivation conditions made it possible to select the optimal producers. The two developed microbial consortia required optimal compositions of culture media, which were determined by varying the ratio of components and measuring the yield of ethanol in the resulting culture liquid.
Conclusion. The best consortium for *Miscanthus sinensis* cellulose consisted of *Pichia stipites* Y7124, *Candida shehatae* NCL3501, *Kluyveromyces marxianus* Y-4290, and *Zymomonas mobilis* 113 at a ratio of 1:1:1:1. The optimal parameters of bioethanol production included: temperature = 35 ± 1°C, pH = 5.2, time = 16 ± 1 h. The most efficient culture medium had the following composition (g/l): glucose – 5.0; peptone – 5.0; yeast extract – 0.4; K_2HPO_4 – 1.5; $(NH)_2HPO_4$ – 1.5; $MgSO_4$ – 0.5.

Keywords. Bioethanol, bioconversion, consortium, Miscanthus, technical cellulose, single-stage fermentation

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Разработка способа биоконверсии целлюлозы *Miscanthus sinensis* для получения биоэтанола

О. В. Кригер[1,*], О. О. Бабич[1], В. Ф. Долганюк[2], О. В. Козлова[2], С. А. Сухих[1], Т. А. Ларичев[2]

[1] *Балтийский федеральный университет имени Иммануила Канта, Калининград, Россия*

[2] *Кемеровский государственный университет, Кемерово, Россия*

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*e-mail: OKriger@kantiana.ru

Аннотация.
Введение. Фундаментальным и перспективным направлением биотехнологии является получение биологического этанола из целлюлозосодержащего сырья травянистых растений как альтернативного источника энергии. С целью совершенствования технологии получения биоэтанола из биомассы мискатуса китайского были проведены исследования по созданию консорциума микроорганизмов, осуществляющих одновременное осахаривание – сбраживание исходного субстрата и оптимизации режимов их культивирования.
Объекты и методы исследования. Техническая целлюлоза, полученная из мискатуса китайского (*Miscanthus sinensis*) гидротропной делигнификацией в условиях окисления пертрифторуксусной кислотой. Содержание этанола в культуральной жидкости по окончании культивирования определяли, используя газовый хроматограф Agilent 7890B с пламенно-ионизационным детектором. Определение биосовместимости штаммов проводили методом прямого совместного культивирования на плотной питательной среде.
Результаты и их обсуждение. С целью создания консорциума микроорганизмов и выбора оптимальных продуцентов для одностадийного получения биоэтанола из целлюлозы мискантуса китайского проводили исследования биосовместимости изучаемых штаммов и условий их культивирования. Выбор оптимальных составов питательных сред для культивирования двух изучаемых консорциумов микроорганизмов осуществляли путем варьирования соотношения компонентов и измерения выхода этилового спирта в полученной культуральной жидкости.
Выводы. Установлены оптимальные параметры для получения этилового спирта из технической целлюлозы мискантуса китайского с использованием консорциума, состоящего из *Pichia stipites* Y7124, *Candida shehatae* NCL3501, *Kluyveromyces marxianus* Y-4290 и *Zymomonas mobilis* 113 в соотношении 1:1:1:1: температура 35 ± 1 °С, pH 5,2, продолжительность 16 ± 1 ч с применением питательной среды для культивирования консорциума следующего состава (г/л): глюкоза – 5,0; пептон – 5,0; дрожжевой экстракт – 0,4; K_2HPO_4 – 1,5; $(NH)_2 HPO_4$ – 1,5; $MgSO_4$ – 0,5.

Ключевые слова. Биоэтанол, биоконверсия, консорциум, *Miscanthus*, техническая целлюлоза, одностадийная ферментация

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Introduction

At the present stage of its economic development, Russia is going through a crisis of traditional technologies, especially in the fields of ecology and energy. The modern agenda involves new technological solutions for food processing, organic waste recycling, pollution reduction, and alternative energy.

Chinese miscanthus (*Miscanthus sinensis*) (Fig. 1) is a perennial herb in the grass family *Poaceae*, which can serve as a renewable raw material for biodiesel. One hectare can yield up to 30 tons of crops during 30 years. Plant biomass cultivation improves the environmental situation and prevents soil erosion, not to mention that biodiesel is carbon neutral [1, 2].

However, miscanthus is difficult to process because its acid hydrolysates are chemically unstable and tend to accumulate harmful substances, e.g. volatile acids, lignohumic substances, formaldehyde, furfural, oxymethylfurfural, etc. These compounds appear as a result of the combined action of chemical agents and such physical factors as temperature and pressure [3–5].

Enzymatic hydrolysis is known to occur in milder conditions than acid hydrolysis. As a rule, enzymatic hydrolysis requires 4.0–6.5 units of active acidity, while the temperature range usually depends on the type of microorganisms and varies from 30 to 60°C. This technological mode converts sugars into the target product and forms no harmful by-products [6–9].

Bioethanol production from lignocelluloses requires simultaneous fermentation and saccharification – for the following reasons:
– cellulose hydrolysis to simple sugars is a lengthy process that can take from 48 to 144 h. Combining the two steps into can significantly reduce time costs;
– the one-step method possesses a higher microbiological stability and efficiency [14, 15].

However, the one-step method is not without flaws. For instance, enzyme catalysis systems have different optimal parameters. Those that catalyze saccharification operate at 40–60°C, while those that catalyze glucose-to-bioethanol reaction operate at 26–28°C [16, 17].

Figure 1. Chinese miscanthus (*Miscanthus sinensis*)

Nevertheless, biotechnological methods make it possible to obtain strains of microorganisms with an optimal temperature mode. They are applicable both for enzyme preparations that produce simple sugars and for enzyme complexes that catalyze the glucose-to-bioethanol reaction. However, genetically engineered microorganisms have several disadvantages, such as:
– higher mutation rates;
– sensitive cultivation conditions, i.e. nutrient media, aseptic conditions, etc.;
– eventual degradation of ethanol biosynthesis.

As a result, biotechnological industries still rely on traditional strains of yeasts and bacteria that are capable of producing simple sugars and target products.

Saccharomyces cerevisiae are one of the main producers of bioethanol. However, saccharomycetes do not ferment pentoses that take up 40% of total sugars in the biomass of herbaceous plants, which limits their industrial applicability for lignocellulosic biomass conversion.

Thermotolerant yeasts are an alternative to *S. cerevisiae* in ethanol production. Thermotolerant yeasts consume glucose, xylose, cellobiose, and arabinose. Glucose, fructose, and mannose substrates have better indicators of biomass production. *Clostridium*, *Geobacillus*, and *Thermoanaerobacter* can process a wide range of compounds, including D-glucose, D-xylose, and L-arabinose, at 55–70°C, which makes them especially advantageous for bioethanol production.

The present study confirmed the available foreign and domestic research results in that saccharification followed by fermentation of miscanthus cellulose can increase bioethanol processing time, thus increasing the accumulation of by-products and microbial contamination. In this regard, simultaneous saccharification and fermentation of the initial substrate by an effective microbial consortium can improve the bioethanol production from lignocellulosic raw materials.

The research objective was to create such a microbial consortium, as well as to optimize bioethanol production by developing a one-step fermentolysis of technical cellulose obtained by delignification of miscanthus.

Study objects and methods

The research featured Chinese miscanthus (*Miscanthus sinensis*) of the grass family *Poaceae* harvested in the Northwestern Federal District of the Russian Federation in 2019. It is a perennial herb that grows as tall as 0.8–2 m and yields 10.1–15.4 t/ha. Its leaf blades are linear, hard, and rough, with a prominent mid-rib, and can reach up to 1.5 cm in width, while its ne-flowered spikelets are arranged into loose panicles with a short axis and can be up to 0.7 cm long.

Below are the characteristics of technical cellulose obtained from *Miscanthus sinensis* using hydrotropic delignification with pertrifluoroacetic acid:
– mass fraction of α-cellulose = 81.56–82.68%;
– dynamic viscosity = 22.78–24.33 mPa·s;
– average polymerization degree = 756.2–977.5;
– brightness = 72.6–82.7%;
– lignin content ≤ 0.2%.

The preparation of the plant mass of miscanthus before the delignification process was carried out according to the standard procedure described in related scientific and technical sources.

Table 1 introduces the types and strains of microorganisms that were used to create the microbial consortium for the subsequent cellulose bioconversion:

The ethanol content in the culture liquid after cultivation was determined using an Agilent 7890B gas chromatograph with a flame ionization detector. The final chromatography time was 10 min; split flow – 100:1; injected sample volume – 1 μl; helium served as the carrier gas; 2 mL of the resulting alcohol sample was extracted with diethyl ether. The sample was then poured into a 15 mL falcon together with 2 mL of diethyl ether and stirred in an orbital shaker for 30 min. After that, the samples were centrifuged at 3900 rpm for 10 min. The upper organic layer was removed into a flask to evaporate diethyl ether. Then, 1 mL of ethyl acetate was added to the concentrated sample, and the resulting solution was placed into a vial and injected into the gas chromatographer.

The biocompatibility of the strains was tested by direct co-cultivation in a solid nutrient medium as described by N.A. Glushanova. The culture was grown in a liquid nutrient medium until its optical density reached 0.10–0.11. After that, it was applied onto a solid nutrient medium with a 3 mm inoculating loop. After the drop had been absorbed, a drop of another test culture was applied to the surface of the same medium. When spreading, it covered a half of the first drop. The Petri dishes were incubated for 24 h at 27°C. In the overlapping area, the

Table 1. Microorganisms used for one-step bioethanol production from technical cellulose obtained from Chinese miscanthus

Microorganism	Medium	Cultural and morphological characteristics	Physiological and biochemical properties
Pichia stipites Y7124	Intestinal tract	ascomycete yeast	facultative anaerobic, capable of fermenting xylose, t = 28–32°C, pH = 4.0–4.5
Pachysolen tannophilus Y-3269	decaying wood	yeast	facultative anaerobic, t = 28–32°C, pH = 2.0–2.5; ferments glucose and xylose
Candida shehatae NCL3501	decaying wood	yeast	ferments both hexose and pentose sugars; t =28–30°C, pH = 3.5–4.0
Kluyveromyces marxianus Y-4290	Cultured yoghurt	round and oval cells of 4-8 μm	ferments D-glucose, D-galactose, sucrose, raffinose; t = 25–50°C; pH = 3.0–7.0
Clostridium thermocellum B-10909	plants, cow and horse manure	gram-positive spore-forming bacillus	anaerobic, thermophilic, t = 55–60°C, pH = 6.0–8.0; ferments cellulose, glucose, and xylose
Clostridium thermohydrosuluricum	plants, cow and horse manure	gram-positive spore-forming bacillus	anaerobic, thermophilic, t = 55–60°C, pH = 4.7–8.0; ferments glucose, xylose, and arabinose
Thermoanaerobacter ethanolicus JW 200	Yellowstone hot springs	non-spore-forming bacteria	thermophilic, anaerobic; ferments sugars, including xylose and arabinose; t = 37–77°C, pH = 4.4–9.9
Geobacillus stearothermophilus B-1169	bottom sediment of thermal springs, Northern Baikal region	gram-positive bacillus	aerobic, thermophilic; t = 60–65°C, pH = 6.5–7.5; converts lignocellulosic biomass into bioethanol
Bacillus stratosphericus B-11678	bottom sediments of Lake Solenoe, Novosibirsk region	gram-positive bacillus	aerobic, mesophilic; t = 30–37°C, pH = 6.5–7.5; converts lignocellulosic biomass into bioethanol
Zymomonas mobilis 113	palm wine	gram-negative, non-spore-forming, polar flagellate bacterium	facultative anaerobic, ferments glucose, sucrose, xylose; t = 30–37°C, pH = 4.0–6.5

cultures competed with each other. Two drops of one and the same culture were used as control, according to the technique described above. Inhibition of one of the strains was interpreted as antagonism; if one of the cultures surfaced, regardless of the sequence of application, it was interpreted as weak antagonism; fusion of the drops or increased growth of both strains indicated biocompatible cultures.

The results were processed using regression analysis. The dependence diagrams were compiled after the obtained data had been processed using the least squares method and described in Microsoft Excel and MatLAB 6.5. The studies were carried out in 5-fold repetition. The confidence level was 0.95; 0.99 ($P \leq 0.05$; $P \leq 0.01$).

Results and discussion

The research objective was to develop an effective microbial consortium and select the optimal producers for one-step bioethanol production from cellulose of Chinese miscanthus. The technical cellulose obtained by hydrotropic delignification was fermented with all the microbial strains.

Table 2. Mass fraction of ethanol in the test samples ($P \leq 0.05$)

Microorganism	Bioethanol yield, %	Fermentation time, h	Fermentation temperature, °C
Pichia stipites Y7124	1.20 ± 0.01	12.0 ± 0.5	30 ± 1
Pachysolen tannophilus Y-3269	0.07 ± 0.01	12.0 ± 0.5	30 ± 1
Candida shehatae NCL3501	0.45 ± 0.01	12.0 ± 0.5	30 ± 1
Kluyveromyces marxianus Y-4290	1.80 ± 0.01	12.0 ± 0.5	35 ± 1
Clostridium thermocellum B-10909	0.51 ± 0.01	12.0 ± 0.5	60 ± 1
Clostridium thermohydrosuluricum	0.46 ± 0.01	12.0 ± 0.5	60 ± 1
Thermoanaerobacter ethanolicus JW 200	2.03 ± 0.05	12.0 ± 0.5	60 ± 1
Geobacillus stearothermophilus B-1169	2.00 ± 0.05	12.0 ± 0.5	60 ± 1
Bacillus stratosphericus B-11678	0.56 ± 0.05	12.0 ± 0.5	37 ± 1
Zymomonas mobilis 113	0.31 ± 0.01	12.0 ± 0.5	30 ± 1

Table 4. Composition of nutrient media

Component	Culture medium number depending on the content of the components, g/l					
	1	2	3	4	5	6
Yeast extract	0.3	0.4	0.5	–	–	–
Glucose	1.0	5.0	10.0	20.0	25.0	30.0
Sodium chloride	–	–	–	0.5	1.0	1.5
Peptone	1.0	5.0	10.0	10.0	5.0	5.0
K_2HPO_4	1.0	1.5	2.0	1.5	1.0	1.5
$(NH_4)_2HPO_4$	1.0	1.5	1.5	1.0	1.5	1.5
KH_2P_04	–	–	–	0.5	1.0	1.5
$MgSO_4$	0.5	0.5	1.0	0.5	0.5	1.0
CaC_03	–	–	–	10.0	5.0	10.0
pH of the medium	4.5	5.2	6.0	7.0	6.8	7.0

During the enzymatic hydrolysis, the substrate sample in an acetate buffer solution (0.1 g/mL, pH 5.0) was inoculated with a suspension of microorganisms with optical density = 0.60–0.61 in the amount of 5% of the total sample volume. The enzymatic hydrolysis was carried out in an incubator shaker at 100 rpm for 12 h at the optimum temperature for each of the producers. Table 2 describes the content of ethanol in the samples.

Table 2 shows that all the microorganisms proved quite active in the cellulose fermentation. The best bioethanol yield belonged to the thermophilic bacteria *Geobacillus stearothermophilus* B-1169 (2.0%) and *Thermoanaerobacter ethanolicus* JW (2.03%) and ethermotolerant yeast *Kluyveromyces marxianus* Y-4290 (1.80%) at the optimal culture temperature.

Table 3 demonstrates the biocompatibility of the strains.

The strains were subjected to cultural, morphological, hysiological, and biochemical analyses. They also underwent an enzymatic hydrolysis of technical cellulose obtained by hydrotropic delignification and a biocompatibility assessment. The experiments resulted in the following bioethanol-producing consortia:

– Consortium I: facultative anaerobic mesophilic yeasts *Pichia stipites* Y7124, *Candida shehatae* NCL3501, and *Kluyveromyces marxianus* Y-4290 and bacteria *Zymomonas mobilis* 113 in a ratio of 1:1:1:1;

– Consortium II: thermophilic anaerobic bacteria *Clostridium thermocellum* B-10909, *Clostridium thermo-*

Table 3. Biocompatibility of bioethanol producers ($P \leq 0.05$)

Microorganism	Microorganism									
	Pichia stipites Y7124	*Pachysolen tannophilus* Y-3269	*Candida shehatae* NCL3501	*Kluyveromyces Marxianus* Y-4290	*Clostridium thermocellum* B-10909	*Clostridium thermo-hydrosuluricum*	*Thermoanaerobacter ethanolicus* JW 200	Geobacillus stearothermophilus B-1169	*Bacillus stratosphericus* B-11678	*Zymomonas mobilis* 113
Pichia stipites Y7124	–	BC	BC	BC	BIC	BIC	BIC	BIC	BIC	BC
Pachysolen tannophilus Y-3269	BIC	–	BIC	BIC	BIC	BIC	BIC	BIC	BIC	BIC
Candida shehatae NCL3501	BC	BIC	–	BC	BIC	BIC	BIC	BIC	BIC	BC
Kluyveromyces marxianus Y-4290	BC	BIC	BC	–	BIC	BIC	BIC	BIC	BIC	BC
Clostridium thermocellum B-10909	BIC	BIC	BIC	BC	–	BC	BC	BIC	BC	BIC
Clostridium thermohydrosuluricum	BIC	BIC	BIC	BIC	BC	–	BC	BIC	BC	BIC
Thermoanaerobacter ethanolicus JW 200	BIC	BIC	BIC	BIC	BC	BC	–	BC	BC	BIC
Geobacillus stearothermophilus B-1169	BIC	BIC	BIC	BIC	BIC	BIC	BIC	–	BIC	BIC
Bacillus stratosphericus B-11678	BIC	BIC	BIC	BIC	BIC	BIC	BIC	BIC	–	BIC
Zymomonas mobilis 113	BC	BC	BC	BC	BIC	BIC	BIC	BIC	BC	–

BC – biocomatibility;
BIC – bioincomatibility.

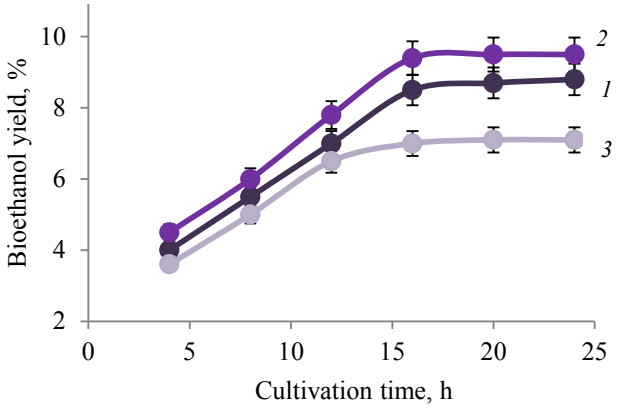

Figure 2. Effect of cultivation time for Consortium I in nutrient media 1 (curve *1*), 2 (curve *2*), and 3 (curve *3*) on bioethanol yield, $P < 0.05$

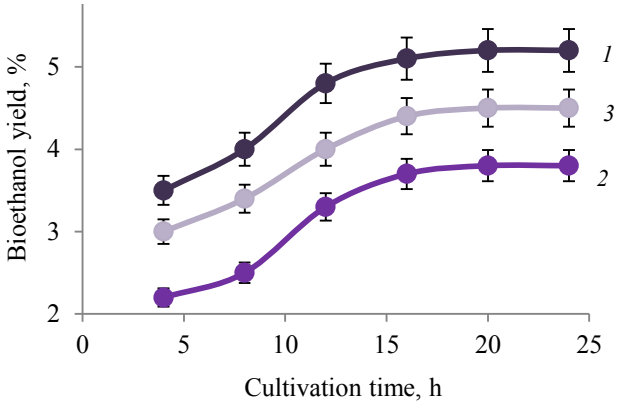

Figure 3. Effect of cultivation time for Consortium II in nutrient media 4 (curve *1*), 5 (curve *2*), and 6 (curve *3*) on bioethanol yield, $P < 0.05$

hydrosuluricum, and *Thermoanaerobacter ethanolicus* JW 200 in a ratio of 1:1:1.

The selection criteria were based on:

– specific properties of the microorganisms;

– specific properties of the metabolism of microorganisms isolated from various natural sources;

– biocompatibility of these microorganisms;

– cultivation conditions, i.e. time, temperature, pH, nutrient medium, etc.

The next step was to define the co-cultivation conditions in order to increase the yield of ethanol. The compositions of nutrient media (g/l) were taken from related scientific publications:

– for yeasts: yeast extract – 0.3; peptone – 1.0; glucose – 1.0; agar – 2;

– for *Zymomonas* bacteria: K_2HPO_4 – 1.0; $(NH_4)_2$ HPO_4 – 1.0; $MgSO_4$ – 0.5; peptone – 10.0; yeast extract – 0.5; glucose – 10.0;

– for *Clostridium* and *Thermoanaerobacter* bacteria: KH_2PO_4 – 0.5; K_2NRO_4 – 0.5; $MgSO_4$ 0.5; NaCl – 0.5; glucose – 20.0; peptone – 5.0; $CaCO_3$ – 10.0.

The optimal compositions of nutrient media were selected by varying the ratio of the components and measuring the yield of ethanol in the resulting culture liquid. Table 4 illustrates the compositions of the culture media.

Consortium I, based on the mesophilic facultative anaerobic microorganisms, was cultivated in nutrient media 1–3. These media included different concentrations of yeast extract and glucose in a weak acidic medium, the cultivation temperature being 30 ± 1°C. Consortium II consisted of thermophilic anaerobic bacteria grown in nutrient media 4–6, which contained glucose, at 60 ± 1°C and neutral pH. Figure 2–3 show the effect of the composition of the nutrient medium and cultivation time on the bioethanol yield.

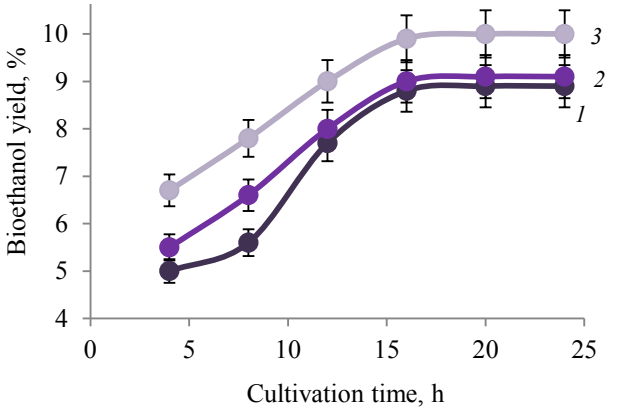

Figure 4. Effect of cultivation time on bioethanol yield for Consortium I: *1* – cultivation temperature = 25°C, *2* – cultivation temperature = 32°C, *3* – cultivation temperature = 35°C, $P < 0.05$

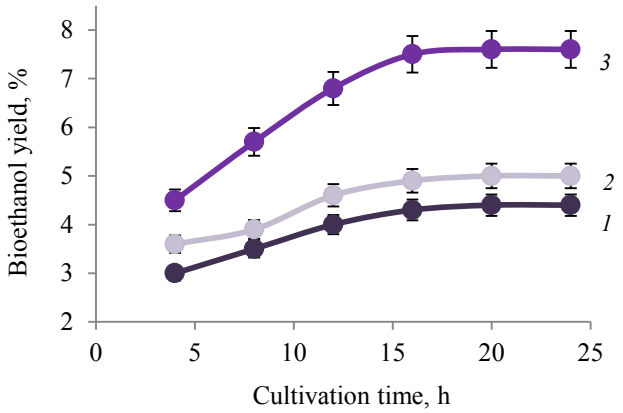

Figure 5. Effect of cultivation time on bioethanol yield for Consortium II: *1* – cultivation temperature = 50°C, *2* – cultivation temperature = 60°C, *3* – cultivation temperature = 65°C, $P < 0.05$

Figure 2–3 made it possible to select the optimal compositions of nutrient media for the cultivation of the two microbial consortia with the maximal bioethanol yield. For Consortium I, it was nutrient medium 2 (g/l): yeast extract – 0.4; glucose – 5.0; peptone – 5.0; K_2HPO_4 – 1.5; $(NH_4)_2$ HPO_4 – 1.5; $MgSO_4$ – 0.5; pH – 5.2. For Consortium II, it was nutrient medium 4 (g/l): glucose – 20.0; peptone – 10.0; K_2HPO_4 – 1.5; $(NH_4)_2$ HPO_4 – 1.0; KH_2PO_4 – 0.5; $MgSO_4$ – 0.5; $CaCO_3$ – 10.0; pH – 7.0.

In all the samples of nutrient media, the maximal accumulation of bioethanol occurred after 16 h of cultivation. It was 9.4% for Consortium I and 5.1% for Consortium II, which significantly exceeded the bioethanol yield obtained by using individual strains.

The next stage involved selecting the optimal temperature values for the cultivation process of microbial consortia that provide the maximal bioethanol yield. The consortia were cultivated in the selected nutrient media at various temperature conditions. Consortium I, which united mesophilic microorganisms, was cultivated at 28–35°C, while Consortium 2, which consisted of thermophilic microorganisms, was cultivated at 50–70°C (Fig. 4–5).

Figure 4–5 show the optimal temperature cultivation modes: for Consortium I, it was 35°C (maximal bioethanol yield – 9.8%); for Consortium II, it was 65°C (maximal bioethanol yield – 7.4%).

Therefore, microbial consortium with enzyme complexes for cellulose bioconversion and subsequent fermentation of sugars increased the bioethanol yield by more than 50%, in comparison with individual strains. The consortium that united thermotolerant yeasts of the genera *Pichia*, *Candida*, and *Kluyveromyces* with *Zymomonas* bacteria proved to be the most effective one.

Conclusion

The research produced a highly effective consortium of microorganisms for a one-step fermentation of technical cellulose obtained from *Miscanthus sinensis* by hydrotropic delignification. It also improved the conditions for the cultivation of microorganisms that provide the maximal bioethanol yield. The new consortium included mesophilic facultative anaerobic yeasts *Pichia stipites* Y7124, *Candida shehatae* NCL3501, and *Kluyveromyces marxianus* Y-4290 and bacteria *Zymomonas mobilis* 113 in a ratio of 1:1:1:1. The optimal cultivation modes were the following: temperature = 35 ± 1°C, pH = 5.2, cultivation time = 16 ± 1 h. The optimal nutrient medium had the following composition (g/l): glucose – 5.0; peptone – 5.0; yeast extract – 0.4; K_2HPO_4 – 1.5; $(NH)_2$ HPO_4 – 1.5; $MgSO_4$ – 0.5.

Contribution

All the authors equally contributed to the research development, processing, data analysis, and manuscript design.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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