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New Strains of Basidiomycetes that Produce Bioethanol from Lignocellulose Biomass[1]

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Abstract—Sixty six isolates were screened for ability of bioethanol production; dynamics of product accumulation and substrate utilization were investigated for two selected strains *Trametes hirsuta* MT-24.24 and *Trametes versicolor* IT-1. The strains' efficiency was evaluated as bioethanol production by 1 g biomass. Strain *T. versicolor* IT-1 producing over 33 g/L of the ethanol for 9 d was selected. Direct conversion of Na-carboxymethyl cellulose, microcrystalline cellulose and straw was shown with ethanol yields of 2.1, 1.6 and 1.7 g/L, respectively, for 9 d fermentation time.

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Ethanol is one of the perspective high-energy fuels, obtained from cheap and easily available plant raw materials. The usage of non-edible lignocellulose, instead of edible agricultural products, has several advantages: the absence of pressure on food market, and the promoted utilization of wood-working, agricultural and other industrial wastes [1, 2]. Basidiomycetes are potent biodestructors of lignocellulose and can be used in the plant biomass saccharification [3–7]. Furthermore, basidiomycetes can be perspective bioethanol producers from lignocellulose, as was shown in recent literature. [8–12]. Advantages of basidiomycete fungi are the following: they can be easily grown on different wastes; they are environment-friendly, implying acid-free lignocellulose processing; they do not produce the toxic wastes; and they are not human pathogens.

The important characteristic of basidiomycete ethanol producer strains is not only the product concentration, but also the strain productivity, i.e. its capability to produce maximum quantity of bioethanol per unit of biomass.

The purpose of this work is to investigate the ability of isolated and acquired from collections basidiomycete strains to produce ethanol from glucose and model lignocellulose substrates.

[1] The article was translated by the authors.

METHODS

Strains, Medium and Culture Conditions

Sixty one strain of basidiomycete fungus were isolated from natural fruit bodies, growing on the wood and wastewood, in biotechnology laboratory of Russian State- University of oil and Gas. Five strains, including *Trametes versicolor* IT-1, were obtained from the collection "Edible and biotechnology important basidiomycete fungi" of Mycology and algology department, Moscow State University.

Strains were isolated on Petri dishes with medium, containing (g/L): agar—15 ("Panreac", USA), soya peptone—3 ("CarlRoth", Germany), liquid malt—30 ("Chimmed", Russia). Cultures were incubated at the temperature 28°C. Isolated strains were transfered to culture tubes with slant malt-agar and kept at the temperature 4°C. Standard culture medium contained (g/L): glucose—20 ("Chimmed", Russia), semi-skimmed soybean flour—10 ("Soyka", Russia), $MgSO_4 \cdot 7H_2O$—0.25 ("Chimmed", Russia), $KH_{2P}O_4$—2.5 ("CarlRoth", Germany) was used for the submerged cultivation [13]. Pieces of agar medium colonized by mycelium (30–40 pieces with 3–5 mm size) were inoculated in the 750 mL shake flasks with 100 mL of medium and were cultivated on the rotary shaker at 250 rpm and 28°C. The inoculum was prepared in a liquid medium until clarification and total consumption of suspended substrate particles. For screening of basidiomycete strains for the ability to produce etha-

Table 1. Glucose conversion by isolated strains and locations of basidiomycete fungi isolation

Strain	Location	Glucose conversion, %
Trametes versicolor IT-1	Italy, Rome	92.3
Trametes hirsute MT-24.24	Vladimir region	71.8
Fomitopsis pinicola MT-5.21	"	57.3
Fomes fomentarius MT-4.05	Moscow region	42.7
Flammulina velutipes MT-3.03	"	37.3
Fomes fomentarius MT-4.23	Vladimir region	36.6
Fomes fomentarius MT-5.14	Moscow region	28.2
Fomitopsis pinicola MT-5.09	"	27.6
Fomitopsis pinicola MT-5.37	Vladimir region	12.0
Ganoderma lucidum MT-6.09	Krasnodar region	9.4
Laetiporus sulphureus MT-11.01	Moscow region	6.0
Pleurotus ostreatus MT-17.01	Vladimir region	6.0

nol liquid inoculum (10 mL) was dispensed in 750 mL shake flasks with 100 mL of medium and was cultivated during the time required for complete consumption of flour. Then 5 ml of 400 g/L sterile solution of glucose were added, and flasks were closed with sterile rubber plugs with fermentation locks. Flasks were incubated under stationary anaerobic conditions at 25°C for 7 d. In order to study dynamics of substrate utilization and ethanol accumulation by strains *T. hirsuta* MT-24.24 and *T. versicolor* IT-1, the dynamics of biomass accumulation was investigated. The four-days inoculum was used for further experiments. In order to study effect of initial glucose concentration on ethanol production by strain *T. versicolor* IT-1, the standard medium was supplemented with glucose at concentrations of 20, 60, 100, 160 and 200 g/L. The flasks were incubated for 4 d, and then in anaerobic conditions during 9 d at 25°C.

The possibility of direct conversion of lignocellulose to ethanol was investigated with model substrates, namely, Na-carboxymethyl cellulose (20 g/L, "Carbocam", Russia), microcrystalline cellulose (20 g/L) Avicel ("Fluka", Germany) and chopped rye straw with particle size up to 5 mm (30 g/L). Flasks containing substrates were inoculated with 100 ml of biomass in culture liquid, and were incubated without aeration for 9 d at 25°C.

Identification of Strains

Strains MT-24.24 and MT-5.21 were identified in Russian National Collection of Industrial Microorganisms (VKPM). Strain IT-1 was identified in Mycology and algology department, Moscow State University. Species identification/verification of fungal strains was performed by sequencing the internal transcribed spacer region (ITS) rRNA gene cluster. Amplification of ITS sequences, coding gene of low molecular 5.8S rRNA and flanking spacer sequences ITS1 and ITS2, were performed using standard ITS1 primers (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

Analytical Methods

The fermentation broth was regularly sampled in order to determine the concentration of sugars and ethanol. Samples were collected in Eppendorf tubes and were centrifuged for 10 min at 10000 *g* to precipitate the biomass. To determine the ethanol concentrations, samples were diluted with isopropyl alcohol in a ratio of 1 : 1 and again centrifuged for 20 min at 14000 *g* to precipitate the residual substrates and exopolysaccharides produced by basidiomycetes and excreted into the broth during the fermentation. Ethanol content was determined by gas chromatography (Crystal-5000.2 "Chromatec", Russia) with Chromosorb 102 column (3 m × 3 mm) under isothermal conditions at a column temperature of 200°C by FID detector. To determine the sugar content, the samples were not treated with isopropyl alcohol. Sugar content was determined with 3,5-dinitrosalicylic acid using glucose as standard [14].

Statistic Methods

Processing of the results was performed using Microsoft Excel 2010. Experimental values were obtained in triplicate; the results are given as mean values and errors as sample standard deviations.

RESULTS AND DISCUSSION

Isolation, Screening and Identification of Basidiomycetes Strains

Sixty six isolates were tested for ability of ethanol production, and it was found that twelve of them have the ability to convert more than 5% of the initial substrate to ethanol. Characteristics of these strains are

Table 2. Ethanol production and biomass yield of studied basidiomycetes strains

Strain	Cultivation time, d	Biomass yield, g/L	Ethanol yield	
			g/L	g/g biomass
Trametes versicolor IT-1	4	5.1 ± 0.1	10.8 ± 0.3	2.12 ± 0.02
Trametes hirsuta MT-24.24	4	12.0 ± 0.2	8.4 ± 0.4	0.70 ± 0.02
Fomitopsis pinicola MT-5.21	6	7.0 ± 0.1	6.7 ± 0.3	0.96 ± 0.01

given in Table 1. All isolated fungi strains were identified based on morphological features. Most promising strains having a conversion of initial substrate more than 50% were identified genetically as *Fomitopsis pinicola* MT-5.21, *Trametes hirsuta* MT-24.24, *Trametes versicolor* IT-1, and were used for further experiments.

Study on Biomass Accumulation Dynamics and Determination of Biomass-Dependent Ethanol Production

Dynamics of biomass accumulation by strains *T. versicolor* IT-1, *T. hirsuta* MT-24.24 and *F. pinicola* MT-5.21 were investigated. Maximum biomass yield was provided by strains belonged to genus *Trametes* by 4 d, and for strain *F. pinicola* by 5 d and later. Taking into account slower growth of the latter strain, it was not employed in the following experiments.

As a result of study on ethanol production it was found that there was no direct dependence between the yields of biomass and ethanol for different strains. To compare the strains' efficiency, product yield per biomass unit was evaluated (Table 2). It was found that *T. versicolor* produced larger quantity of ethanol per biomass unit (2.12 g/g), by comparison with *T. hirsuta* (0.7 g/g). The same cultivation time of *T. versicolor* and *T. hirsuta* allowed us to investigate these two strains in parallel.

Investigation of Ethanol Production Dynamics

Ethanol production and substrate (glucose) utilization dynamics were investigated by submerged cultivation of basidiomycetes strains. It was found that ethanol production by *T. versicolor* IT-1 was being already started on the first day (5.1 g/L of ethanol at 44% initial substrate conversion) and reached maximum by 9 d (11.3 g/L at 97.4% initial substrate conversion).

Ethanol production was uniform for all the fermentation time for strain *T. hirsuta* MT-24.24; the maximum ethanol content was reached after 10 d—11.0 g/L at substrate conversion of 93.2% (Fig. 1). It was noted that in stationary conditions glucose was only slightly consumed by mushrooms, and almost all was converted to ethanol. Ethanol yield for strain *T. versicolor* IT-1 depended from initial glucose content in medium (Fig. 2). It was found that ethanol content increased up to 33.4 g/L while increasing initial glucose concentration to 200 g/L, but substrate conversion was decreased to 45%.

Investigated basidiomycetes were also compared with known ethanol-producing basidiomycetes. The comparison (see Table 3) showed that ethanol production by *T. hirsuta* MT-24.24 and *T. versicolor* IT-1 was similar to that of known basidiomycetes on the basis of g consumed substrate. Biomass yield after end of cultivation was ranged from 3.5 to 3.7 g/L, irrespective of

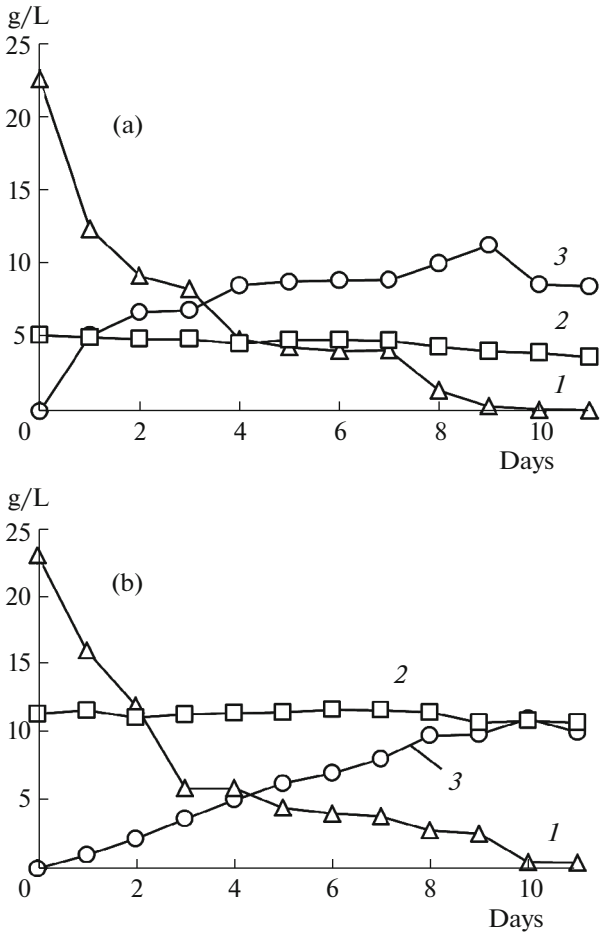

Fig. 1. Dynamics of ethanol production and glucose utilization by strains *Trametes versicolor* IT-1 (a) and *Trametes hirsuta* MT-24.24 (b). (*1*) Glucose, (*2*) biomass, (*3*) ethanol.

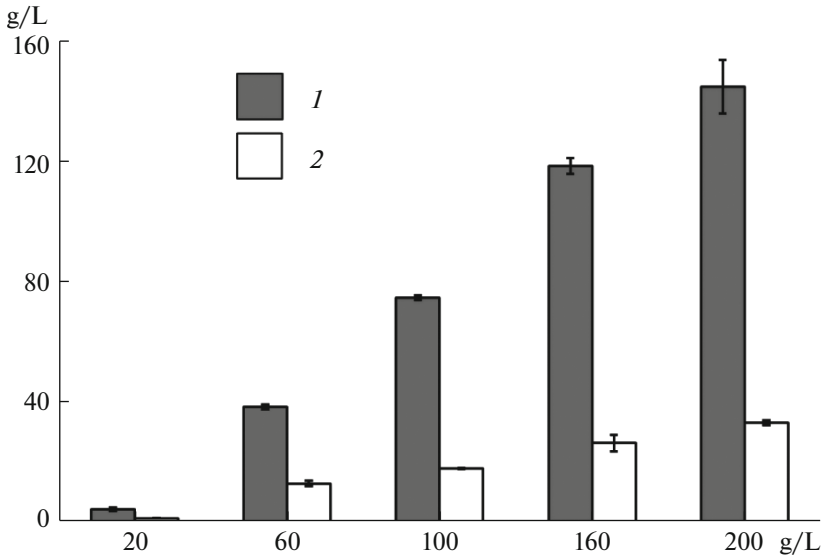

Fig. 2. Dependence of ethanol production and glucose consumption by strain *Trametes versicolor* IT-1 from initial glucose content. Initial glucose content is plotted on the horizontal axis; *1*—glucose consumption in anaerobic conditions, *2*—ethanol production.

the initial glucose concentration. Increases in the exopolysaccharide content in flasks with more than 100 g/L of glucose was observed.

Direct Conversion of Lignocellulose to Ethanol

Strain *T. versicolor* IT-1 was employed in experiments of direct conversion of lignocellulose to ethanol. The highest conversion to ethanol was observed for Na-CMC with ethanol content of 2.1 g/L for 9 d of fermentation. Apparently, Na-CMC has higher availability compared to other tested substrates. The microcrystalline cellulose and chopped straw fermentation resulted in ethanol content of 1.6 and 1.7 g/L, respectively.

The results showed that the best ethanol producers among the studied strains are *T. hirsuta* MT-24.24 and *T. versicolor* IT-1, capable of ethanol production from glucose with the yield about 0.5 g/g glucose at the initial glucose concentration of 20 g/L, providing maximum ethanol content about 33 g/L at the initial glucose concentration of 200 g/L. Furthermore, strain

Table 3. Ethanol production by basidiomycetes using glucose as carbon source

Strain	Initial glucose concentration, g/L	Ethanol yield, g/g glucose	Source
Flammulina velutipes Fv-1	150	0.34	[11]
Trametes versicolor KT9427	20	0.46	[15]
Peniophora cinerea	20	0.41	[9]
Trametes suaveolens	20	0.39	[9]
Flammulina velutipes Fv-1	10	0.45	[8]
Flammulina velutipes Fv-1	50	0.45	[8]
Trametes hirsuta	20	0.49	[10]
Trametes hirsuta MT-24.24	23.0	0.47	This study
Trametes versicolor IT-1	22.6	0.50	"
Trametes versicolor IT-1	200	0.23	"

T. versicolor IT-1 is capable of direct lignocellulose fermentation to ethanol.

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