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IMPROVEMENT OF HIGH-TEMPERATURE XYLOSE AND GLUCOSE ALCOHOLIC FERMENTATION IN METHYLOTROPHIC YEAST *OGATAEA (HANSENULA) POLYMORPHA* BY OVEREXPRESSION OF *PDC1* AND *ADH1* GENES

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Second generation bioethanol from lignocellulose is environmentally friendly alternative to first generation bioethanol obtained from traditional feedstocks(corn starch and sugarcane). Xylose is the second major fermentable sugar of lignocellulosic hydrolyzates, however only several microorganisms are able to convert this monosaccharide to ethanol. The methylotrophic yeast *Ogataea (Hansenula) polymorpha* belongs to the most thermotolerant yeast species known with ability to fermentxylose at elevated temperature up to 50 °C.However, the main drawbacks of the wild-type strains are very low ethanol yield and productivity from xylose. In this work, we describe the evaluation of *O. polymorpha* strains with increased ethanol production from xylose and glucose by overexpression of *PDC1*and *ADH1* genes, coding for pyruvate decarboxylase andalcohol dehydrogenase, respectively, that catalyzes the last reactions of ethanol fermentation pathway. Overexpression of both genes in the background of wild-type strain resulted in 4-fold activation of xylose and 1.5 times activation of glucose alcoholic fermentation in *O. polymorpha*. Therefore, optimization of *PDC1* and *ADH1* gene expression would be a promising approachfor further improvement of ethanol production during xylose and glucose fermentation in the *O. polymorpha*.

Keywords: xylose, *Ogataea (Hansenula) polymorpha*, high-temperature fermentation, metabolic engineering.

One of the most promising alternatives to conventional petroleum-based transport fuels is fuel ethanol produced from renewable feedstock. Lignocellulosic biomass, one of the most abundant renewable resources on the planet, offers a high potential as a source for the production of second-generation bioethanol [8]. Lignocellulose is a complex heteropolymer, therefore process of conversion of lignocellulosic biomass to ethanol is much more complicated than that of starch- or sucrose-based ethanol. Lignocellulosic biomass requires thermo- and / or chemical pretreatment with subsequent hydrolysis of pretreated material by cellulolytic enzymes (temperature optima around 55 °C) for liberation of monosaccharides, so-called saccharification process. By far the most successful strategy for the production of bioethanol from lignocellulosic hydrolysates has been the metabolic engineering of natural ethanologenic microorganisms to ferment sugars liberated during lignocelluloses treatment [13]. The enzymatic hydrolysis and fermentation can be performed in a combined process, known as Simultaneous Saccharification and Fermentation (SSF) [4]. However, one of the bottlenecks in the SSF process is the absence of the microbi-

al strains capable of efficient conversion of liberated sugars to ethanol at high temperatures. Besides thermotolerance, efficient alcoholic fermentation of xylose, the second abundant after glucose sugar of lignocellulosic hydrolysates, is one of the main unresolved problems [6, 10]. The methylotrophic yeast *Hansenula (Ogataea) polymorpha* belongs to the most thermotolerant yeast species able to xylose alcoholic fermentation at elevated temperatures up to 50 °C [14]. Several metabolic engineering approaches have been successfully developed to improve ethanol production from xylose in *H. polymorpha*. The positive cumulative effect for the overexpression of engineered xylose reductase (*XYL1m* gene) along with native xylitol dehydrogenase (*XYL2* gene) and xylulokinase (*XYL3* gene) on ethanol production from xylose was previously demonstrated onthe background of *O. polymorpha* wild-type strain and mutant ethanol non-utilizing strain $2EtOH^-$ [2, 11].

In yeast, alcoholic fermentation involves pyruvate decarboxylation toacetaldehyde by pyruvate decarboxylase, followed by the acetaldehyde reduction to ethanol by alcoholdehydrogenase [12, 16]. Overexpression of pyruvate decarboxylaseresulted in a 3-fold increase in ethanol production from xylose in the wild-type strain and mutant strain $2EtOH^-$ [7]. Moreover, overexpression of *PDC1* on the background of *XYL1m* and *XYL2* overexpressed strain increased ethanol production. However, additional overexpression of *PDC1* gene did not lead to further improvement of ethanol synthesis from xylose in strain expressing *XYL1m, XYL2, XYL3* genes [11].

Among alcohol dehydrogenase isozymes Adh1 seems to be primarily responsible for the production of ethanol from acetaldehyde under fermentative conditions in different yeast species, such as *Saccharomyces cerevisiae*, *Pichiastipitis*, *O. polymorpha* [1, 16]. Overexpression of *PDC1* and *ADH1* genes in *S. cerevisiae* is able to activate ethanol production from glycerol [17]. Moreover, co-expression of *ADH1* and *TAL1* genes in recombinant xylose-fermenting *S. cerevisiae* resulted in increased ethanol production from lignocellulosic hydrolysates in the presence of furfural [5]. Recently, it was reported that overexpression of *ADH1* gene in *O. polymorpha* led to the improvement of ethanol production from glucose and glycerol [9, 16].The role of alcohol dehydrogenase in xylose alcoholic fermentation in *O. polymorpha* remained unknown. In this work, we describe the construction of the *O. polymorpha* strains with simultaneous overexpression of *PDC1* and *ADH1* genes coding for pyruvate decarboxylase and alcohol dehydrogenase, respectively. Overexpression of both genes in *O. polymorpha* resulted in 4-fold and 2-fold activation of xylose alcoholic fermentationas compared to the wild-type strain and strain, expressing solely*PDC1*, respectively.

Materials and methods

Strains and growth conditions

The following strains of *O. polymorpha* were used in this study: NCYC495 *leu1-1* (wild-type strain), WT/PDC1 overexpressing *PDC1* gene [7], WT/PDC1/ADH1 overexpressiong both *PDC1* and *ADH1* genes [9]. Yeast cells were grown on YPD (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) or mineral medium (1.7 g/L YNB without amino acids, 5 g/L ammonium sulfate, 20 g/L of glucose) at 37 °C. For the NCYC495 *leu1-1* strain, leucine (40 mg/L) was added to the medium.

Ethanol production and sugar consumption assays

Alcoholic fermentation of yeast strains was fulfilled by cultivation in liquid mineral medium at oxygen-limited conditions at 37 °C and 45 °C. The cells were pre-grown in 100 mL of liquid YPX medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L xylose) in 300 mL Erlenmeyer flasks at 220 rpm till the mid-exponential growth phase. Than the cells were harvested by centrifugation, washed by water and inoculated into 40 mL of the fermentation medium to the final cell density 2 mg of dry weight/mL. 100 g/L xylose was added into the mineral medium used for

the fermentation. For glucose fermentation cells were pre-grown in YPD medium following by cultivation of harvested biomass in the mineral medium with 100 g/L glucose. The oxygen-limited conditions were provided by agitation at 140 rpm. Samples of medium for ethanol production assay were taken daily.

The biomass was determined turbidimetrically with a Helios Gamma spectrophotometer (OD, 590 nm; cuvette, 10 mm) with gravimetric calibration. Concentrations of xylose, glucose and ethanol in medium broth were analyzed by HPLC (Perkin Elmer, Series 2000, USA) with an Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, USA). A mobile phase of 4 mM H_2SO_4 was used at a flow rate 0.6 mL/min and the column temperature was 35 °C. Alternatively, the concentration of ethanol in the medium was determined using the 'Alcotest' kit [3]. Fermentation experiments were performed at least twice.

Results and discussion

Previously, strains of *O. polymorpha* overexpressing both genes involved in the final stages of alcoholic fermentation (*PDC1, ADH1*) were constructed and characterized by improvement of ethanol production of glycerol [9]. In this study, we compared the efficiency of ethanol productionduring xylose alcoholic fermentation in constructed *O. polymorpha* recombinant and wild-type strains.

The best selected transformant WT/PDC1/ADH1 accumulated higher amounts of ethanol from xylose relative to the parental strain WT/PDC1, reaching ethanol concentration 2.3 g/L at 45°C (Table 1, Figure).

Table 1

Ethanol productionat the second day of xylose and glucose alcoholic fermentation

Strain	Ethanol concentrations [g/L]			
	37 °C		45 °C	
	Xylose	Glucose	Xylose	Glucose
WT	0.48 ± 0.05	12.75 ± 0.80	0.60 ± 0.06	13.47 ± 0.76
WT/PDC1	1.10 ± 0.11	22.07 ±1.24	1.20 ± 0.14	25.96 ± 1.28
WT/PDC1/ADH1	2.11 ± 0.21	36.50 ± 1.94	2.31 ± 0.23	39.08 ± 1.51

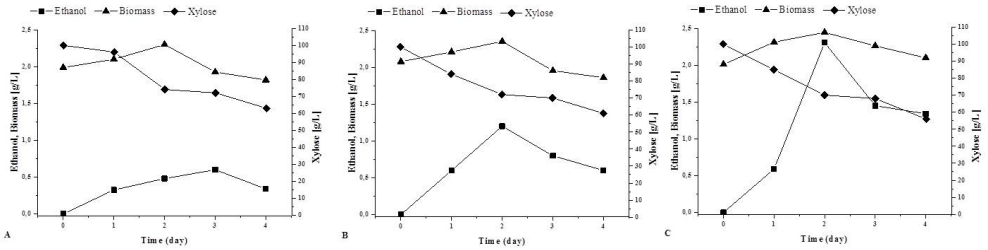
Improvement of ethanol production in *O. polymorpha* was observed under cultivation both at optimal growth temperature (37 °C) and elevated temperature (45 °C). Ethanol yield and productivity during xylose fermentation at 45 °C by mentioned strains are represented in Table 2. Strain WT/PDC1/ADH1 produced 0.062 g of ethanol per g of consumed xylose, which is 3-fold higher than produced by WT strain (0.021 g/ g of xylose) and 1.77-fold more than that by recombinant strain with *PDC1* overexpression (0.035 g/ g of xylose).

Table 2

Ethanol yield, specific production rate and productivity duringxylose alcoholic fermentation by the *O. polymorpha* strains at 45 °C under semi-anaerobic conditions

Strain	Ethanol yield (g/g consumed xylose)	Ethanol specific production rate (g/g biomass/h)	Ethanol productivity (g/L/h)
WT	0.021 ± 0.005	0.010 ± 0.001	0.020 ± 0.002
WT/PDC1	0.035 ± 0.007	0.013 ± 0.002	0.025 ± 0.001
WT/PDC1/ADH1	0.062 ± 0.011	0.018 ± 0.001	0.048 ± 0.003

Representative profiles of ethanol synthesis, biomass accumulation and xylose consumption for all studied strains under high-temperature xylose fermentation are shown in Figure.



The ethanol production, xylose consumption and biomass accumulation during xylose fermentation at 45 °C of *H. polymorpha* strains: A - WT, B - WT/PDC1, C - WT/PDC1/ADH1. The data represent means of typical single cultivation

Overexpression of *PDC1* gene on the background of the wild-type strainled to significant improvement of glucose conversion to ethanol. Strain WT/PDC1produced almost 2-fold more ethanol relative to the parental strain during fermentation at 37 °C and 45 °C (Table 1).The ethanol production during glucose fermentation was also increasedin WT/PDC1/ADH1 strain as compared to strain, expressing only *PDC1*. Overexpression of *ADH1*gene in WT/PDC1 strain resulted in about 1.5-fold increase in ethanol production during glucose fermentation at 37 °C and 45 °C (Table 1).

In the current study, the impact of simultaneous overexpression of *PDC1* and *ADH1* genes, coding for enzymes involved in the final metabolic steps of alcoholic fermentation, on xylose conversion to ethanol in *O. polymorpha* was investigated. Change of expression of the mentioned genes on the background of the wild-type strain led to significant increase in ethanol production from xylose. It is worth to be mentioned that the overexpression of *PDC1* and *ADH1* genes also led to substantial activation of glucose alcoholic fermentation.

The *O. polymorpha* is regarded as a promising yeast species for second generation ethanol production due to its thermotolerance and native ability to metabolize and ferment xylose. Using the combination of methods of metabolic engineering and classical selection the best ethanol producing strain of *O. polymorpha* was constructed accumulating up to 12.5 g/l of ethanol at 45 °C [11; Ruchala et al., under preparation]. However, to be industrially feasible, ethanol yield in *H. polymorpha* has to be further increased. Identification of rate-limiting enzymes for xylose conversion to ethanol is necessary for rational strain modification to improve the fermenting efficiency. Therefore, optimization of *PDC1* and *ADH1* gene expression is a promising approach for further improvement of ethanol production from xylose and glucose in the *O. polymorpha*.

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ПІДВИЩЕННЯ ЕФЕКТИВНОСТІ ВИСОКОТЕМПЕРАТУРНОЇ АЛКОГОЛЬНОЇ ФЕРМЕНТАЦІЇ КСИЛОЗИ ТА ГЛЮКОЗИ У МЕТИЛОТРОФНИХ ДРІЖДЖІВ *OGATAEA (HANSENULA) POLYMORPHA* ШЛЯХОМ ПОСИЛЕННЯ ЕКСПРЕСІЇ ГЕНІВ *PDC1* ТА *ADH1*

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Альтернативою етанолу першого покоління з харчової сировини (кукурудзяний крохмаль і цукрова тростина) є етанол другого покоління з нехарчової рослинної

біомаси (лігноцелюлози). Ксилоза є другим за вмістом цукром у складі гідролізатів лігноцелюлози, проте лише незначна кількість мікроорганізмів здатні зброджувати цей цукор до етанолу. Метилотрофні дріжджі *Ogataea (Hansenula) polymorpha* належать до найбільш термотолерантного виду дріжджів, що здатні зброджувати ксилозу до етанолу при підвищених температурах (до 50 °С). Проте основним недоліком природних штамів цих дріжджів є низький вихід і продуктивність етанолу при ферментації ксилози. У даній роботі визначено ефективність алкогольної ферментації ксилози рекомбінантними штамами *O. polymorpha* з посиленою експресією генів *PDC1* та *ADH1*, що кодують піруватдекарбоксилазу й альдегіддегідрогеназу, які каталізують кінцеві реакції на шляху алкогольної ферментації. Ко-експресія обох генів у геномі штаму дикого типу приводила до 4-кратного зростання продукції етанолу із ксилози та 1,5-кратного зростання утворення етанолу з глюкози у *O. polymorpha*. Отже, оптимізація рівня експресії *ADH1* та *PDC1* генів є перспективним підходом до подальшого покращення параметрів алкогольної ферментації ксилози та глюкози у *O. polymorpha*.

Ключові слова: ксилоза, *Ogataea (Hansenula) polymorpha*, високотемпературна ферментація, метаболічна інженерія.

ПОВЫШЕНИЕ ЭФФЕКТИВНОСТИ ВЫСОКОТЕМПЕРАТУРНОЙ АЛКОГОЛЬНОЙ ФЕРМЕНТАЦИИ КСИЛОЗЫ И ГЛЮКОЗЫ МЕТИЛОТРОФНИХ ДРОЖЖЕЙ *OGATAEA (HANSENULA) POLYMORPHA* ПОСРЕДСТВОМ УСИЛЕНИЯ ЭКСПРЕССИИ ГЕНОВ *ADH1* И *PDC1*

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Альтернативой этанолу первого поколения, получаемого из пищевого сырья (кукурузный крахмал и сахарный тростник), является этанол второго поколения из непищевой растительной биомассы (лигноцеллюлозы). Ксилоза является вторым по содержанию сахаром в составе гидролизатов лигноцеллюлозы, однако лишь незначительное количество микроорганизмов способны сбраживать этот сахар до этанола. Метилотрофные дрожжи *Ogataea (Hansenula) polymorpha* относятся к наиболее термотолерантному виду дрожжей, способному осуществлять алкогольную ферментацию ксилозы при повышенных температурах (до 50 °С). Однако основным недостатком природных штаммов этих дрожжей является низкий выход и производительность этанола при алкогольной ферментации ксилозы. В данной работе проведена оценка эффективности алкогольной ферментации ксилозы рекомбинантными штаммами *O. polymorpha* с усиленной экспрессией генов *PDC1* и *ADH1*, кодирующих пируватдекарбоксилазу и альдегиддегидрогеназу, которые катализируют конечные реакции спиртового брожения. Ко-экспрессия обоих генов в геноме штамма дикого типа приводила к 4-кратному увеличению продукции этанола из ксилозы и 1,5-кратного увеличения продукции этанола из глюкозы у *O. polymorpha*. Следовательно, оптимизация уровня экспрессии генов *PDC1* и *ADH1* является перспективным способом дальнейшего улучшения параметров алкогольной ферментации ксилозы и глюкозы в *O. polymorpha*.

Ключевые слова: ксилоза, *Ogataea (Hansenula) polymorpha*, высокотемпературная ферментация, метаболическая инженерия.