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Study of transhydrogenase systems features in the mutants of the yeast pachysolen tannophilus for the production of ethanol and xylitol from agricultural wastes

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Abstract. The key catabolic enzymes of D-xylose, an important structural component of different agricultural wastes, were studied in cells of mutant strains of the xylose-assimilating yeast *Pachysolen tannophilus*. The evaluation of catalytic activity and cofactor specificity of xylose reductase (EC 1.1.1.307) and xylitol dehydrogenase (EC 1.1.1.9) confirmed the dependence of intracellular catabolic pathway for D-xy lose on the NAD×H/NADP×H ratio, formed under microaerobic conditions. The study of total activity of some NAD^+/NAP×H-dependent dehydrogenases revealed the metabolic characteristics of the yeast cells, which could ensure selective ethanol or xylitol production. Thus, the efficient involvement of D-xylose into the Embden–Meyerhof–Parnas pathway provided not only the high activities of xylose reductase and xylitol dehydrogenase, but also of 1-glycerophosphate dehydrogenase (EC 1.1.1.8) and lactate dehydrogenase (EC 1.1.1.27), respectively. The inhibition of activity of these enzymes led to selective production of xylitol from D-xylose. On the base of the experimental results, the principles of metabolic engineering of xylose-assimilating yeasts were formulated. The possibility of bioethanol and xylitol production from different agricultural wastes using xylose-assimilating yeasts are discussed.

Key words: agricultural wastes, D-xylose, mutants of the yeast *P. tannophilus*, xylose reductase, xylitol dehydrogenase, 1-glycerophosphate dehydrogenase, lactate dehydrogenase, ethanol, xylitol.

INTRODUCTION

Development of the global food industry has close connections with an increasing the number of agro-industrial wastes, represented mostly by heteropolysaccharides (Sadh et al., 2018). Its hydrolysis leads to the formation of hexoses (D-glucose, D-mannose, D-galactose) and pentoses (D-xylose, L-arabinose) mixtures. Innovations in bioethanol production based on such substrates are the top-most priority in EU's environmental policies (Bedoić et al., 2019). However, microbial strains suitable for this technologies must actively utilize different hexoses and pentoses.

It is known that the yeast *Saccharomyces cerevisiae* is characterized by efficiently ethanol fermentation of hexoses, the principles underlying its regulation are well studied (Siti et al., 2017). The key enzymes, encoding NAD×H/NADP×H-dependent xylose reductase (XR, EC 1.1.1.21) and NAD^+-dependent xylitol dehydrogenase (XD, EC 1.1.1.9), are necessary to utilize D-xylose, the main structural component of hemicellulose (Kwak & Jin, 2017). After phosphorylation, D-xylulose is involved in the non-oxidative phase of the pentose phosphate pathway and the Embden-Meyerhof-Parnas pathway. However, genetically modified strains of *S. cerevisiae* produce xylitol under microaerobic conditions, which activate the formation of ethanol from D-xylose (Kwak & Jin, 2017). This fact indicates the important role of transhydrogenase systems that regulate the ratio of $NAD(P)^+$/NAD(P)×H during the catabolism of D-xylose, minor sugar for yeast cells. Its analysis will be an prerequisite, necessary for the construction of *S. cerevisiae* strains capable to efficient production of xylitol and ethanol from D-xylose.

The 'respiratory' type of D-xylose catabolism, characteristic of yeasts Kreger van Rij (1984), declares the paramount importance of mitochondria for regulating the ratio of $NADP^+/NAD^+$. However, the functions of 1-glycerophosphate dehydrogenase (GPD, EC 1.1.1.8) and NAD^+-dependent malate dehydrogenase (MD, EC 1.1.1.37), which provide transport of reduced equivalents from cytosol to mitochondria, as well as cytochrome c oxidase (CO, EC 1.9.3.1), the main enzymatic complex of the electron transfer chain, has not yet been studied for xylose-assimilating yeast. Its adaptation to acidic media Kreger van Rij (1984) does not exclude the participation of lactate dehydrogenase (LD, EC 1.1.1.27) in the regulation of the ratio NAD×H /NAD^+, when the oxygen concentration in the medium decreases significantly. The effect of this enzyme on the production of ethanol from D-xylose was also not detected. *Pachysolen tannophilus* has previously been shown to be a convenient experimental object for identifying factors that regulate the catabolism of D-xylose in yeast cells (Yablochkova et al., 2004). Therefore, the goal was to study the activity and specificity of XR and XD, as well as the general activities of GPD, total MD, CO and LD in *P. tannophilus* mutants producing xylitol or ethanol from D-xylose.

MATERIALS AND METHODS

It has been experimentally proved that naturally occurring xylose-assimilating yeasts *P. tannophilus* have the unique ability to produce comparable amounts of xylitol and ethanol during microaerobic fermentation of D-xylose (Yablochkova et al., 2003). This practical result makes possible studying the conditions conducive to the formation of each target product on the example of the same model object. However, highly pronounced homotallism, population heterogeneity, instability of diplophase significantly complicate the using of genetic engineering techniques for constructing *P. tannophilus* strains, that can selectively produce xylitol or ethanol (Kreger van Rij, 1984). Nonetheless, methods for isolation of *P. tannophilus* mutants which are characterized by various defects of D-xylose catabolism, directly affected on ethanol and xylitol levels are well known (James et al., 1989). Therefore, based on the *P. tannophilus* strain -Y-1532 (Museum of Microorganisms, All-Russian Research Institute of Hydrolysis, St. Petersburg), the haploid *P. tannophilus* 22-Y-1532 was isolated (Bolotnikova et al., 2007). Two-day-old *P. tannophilus* 22-Y-1532 cells were exposed to 1-methyl-3-nitro-1'-nitrosoguanidine under experimental conditions (Bolotnikova et

al., 2007). Changes in the level of production of xylitol and ethanol were detected by analyzing the growth rate of mutants on selective media with D-glucose, D-xylose, xylitol or ethanol as the sole carbon source. In all cases, the growth of haploid *P. tannophilus* 22-Y-1532 served as a positive control. Thus, out of 51 mutants, only 3 strains were selected that produced xylitol (No. 664) or ethanol (No. 390, No. 442) from D-xylose. Evaluation of the morphological characteristics (diameter, color, shape and texture of the colonies, as well as the size, type of budding and the nature of sporulation of cells) in accordance with the criteria developed previously Bolotnikova et al. (2005), confirmed the haploidy of these mutants. These mutant strains of *P. tannophilus* were used for microaerobic fermentation of D-xylose.

The inoculum for fermentation was obtained by growing yeast in 500 mL round bottom flasks containing 100 mL of YEPD medium with 2% D-glucose, on a shaker (230 rpm) at 30 ± 20 °C. After 18–24 hours, 10 mL of yeast suspension from these the flasks were transferred to 500 mL flasks containing 100 mL YAPX medium with 2% D-xylose, which were incubated under similar conditions. The yeast biomass concentration for the fermentation of D-xylose was 6.0 ± 0.5 g d w L^{-1}. All fermentations were conducted at 250 mL round-bottom flasks containing 100 mL of the medium with 2.0% D-xylose, 2.0% peptone and 0.5% yeast extract on a shaker (100 rpm) at +30 ± 2 °C for 24 h. During this time, the parent haploid strain *P. tannophilus* 22-Y-1532 used 97% D-xylose and produced a maximum xylitol, although the ethanol yield did not reach its maximum (Bolotnikova et al., 2012; Bolotnikova et al., 2013). This allowed us to reduce the toxic effect of ethanol on the metabolic activity of mutant strains of *P. tannophilus*. The concentration of yeast biomass after fermentation was analyzed spectrophotometrically (Bazarnova et al., 2018). The concentration of reducing substances (RB) was determined with Fehling's reagent. Ethanol and xylitol were determined by gas chromatography (Vista 600, 'Varian', USA) according to the recommendations (Bolotnikova et al., 2019).

Cell-free yeast extracts were obtained in accordance with (Bolotnikova et al., 2015). The concentration of total protein was determined by the Lowry method using bovine serum albumin as a standard. The total and specific activities of XR and XD were evaluated by monitoring the concentrations of NAD(P)×H/NAD(P)$^+$ redox pairs at 340 nm under the conditions described in (Zverlov et al., 1990). The unit of enzymes' activity was defined as the amount of the enzyme that capable to oxidize mMol of NAD(P)×H per min. A similar monitoring of the concentrations of the NAD×H/NAD$^+$ pair was used for spectrophotometric determination of LD activity, total MD, and GDP (Kochetov, 1980). The activity of CO was determined after its recovery with ascorbic acid according to the procedure (Smith, 1955). The statistical analysis of experimental results was carried out using Student's T-Test for a significance level of 0.05 with MS Excel computer program.

RESULTS AND DISCUSSION

Microaerobic fermentation of D-xylose by mutant strains of *P. tannophilus*

A comparative analysis of the yields of xylitol, ethanol, and biomass by mutant *P. tannophilus* strains during microaerobic fermentation of D-xylose is presented in Fig. 1. Experimental results indicate that mutant No. 664 selectively produced xylitol with an efficiency of 2.3 times exceeding a similar control characteristic. At the same

time, its biomass level was significantly lower than the value obtained for the original parent strain. Mutants No. 390 and No. 442 without xylitol accumulation produced ethanol 19.0% and 27.0% better than *P. tannophilus* 22-Y-1532. The biomass yield of these strains was also lower than that of *P. tannophilus* 22-Y-1532 (Fig. 1). After fermentation, yeast biomass was used to determine the activity of XR, XD, GPD, total MD, CO, and LD involved in various stages of D-xylose catabolism.

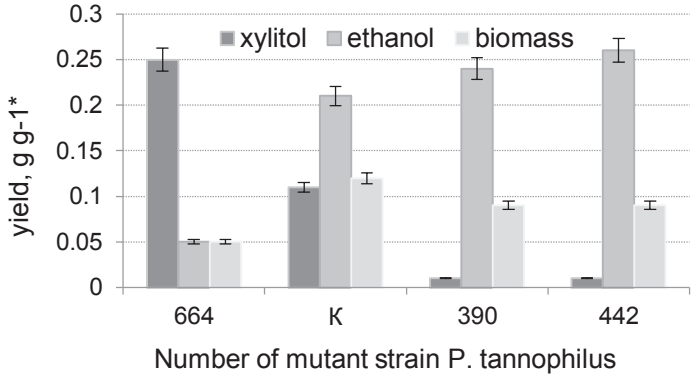

Figure 1. The yield of xylitol, ethanol and biomass by the mutants of the yeast *P. tannophilus* 22-Y-1532.

Notes: K – the parent strain of *P. tannophilus* 22-Y-1532; * – Yield for per gram of D-xylose.

Activity and coenzyme specificity of xylose reductase and xylitol dehydrogenase. The enzymes of the initial steps of D-xylose catabolism, XR and XD, have a strong effect on the level of xylitol and ethanol in yeast cells (Kwak & Jin, 2017). The specific acceptors and donors of pairs of electrons and protons in such reactions are derivatives of the $NAD(P)^+$ and NAD(P)×H dinucleotides (Zverlov et al., 1990). Therefore, the rate of these coenzymes oxidation (reduction) was used to evaluate the activity of XR and XD in the mutants of *P. tannophilus* 22-Y-1532, producing xylitol and ethanol.

As expected, the activity of the both enzymes in mutant cells turned out to be lower than the control values (Fig. 2). The lowest XR and XD activity was observed in mutant No. 664, which showed a low degree (56.0%) and a rate of utilization of D-xylose (0.078 g g^{-1} h^{-1}) during microaerobic fermentation. The XR of xylitol producing mutant had a predominant affinity for NADP×H (Fig. 3). However, 95.0% of XD activity of that yeast strain was coupled with coenzyme NAD^+.

The highest experimental values of XR and XD activity were found in ethanol-producing mutants that used 86.0% (strain No. 390) or 78.6% (strain No. 442). D-xylose. The rate of its consumption under microaerobic conditions increased to 0.119 g g^{-1} h^{-1} and 0.109 g g^{-1} h^{-1}, respectively. The pronounced double NAD×H/NADP×H specificity of XR was a common feature of these mutant strains. Moreover, the largest contribution of the NAD×H-dependent form to the total XR activity of was noted for mutant No. 390 (Fig. 3). The XD of the both yeast strains producing ethanol from D-xylose retained metabolic dependency from NAD^+ (Fig. 2).

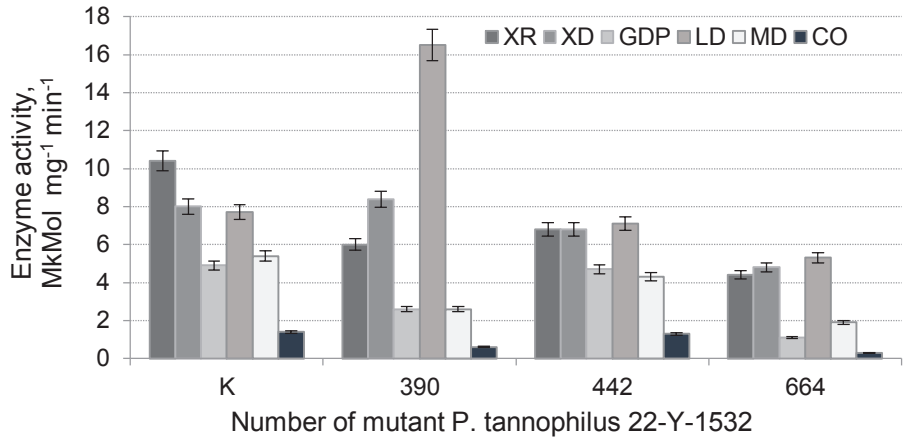

Figure 2. Activity of enzymes involved in the catabolism of D-xylose in mutants of the yeast *P. tannophilus* 22-Y-1532.

Notes: K – the parent strain of *P. tannophilus* 22-Y-1532; XR – xylose reductase; MD – malate dehydrogenase; XD – xylitol dehydrogenase; GPD – glycerol-3-phosphate dehydrogenase; LD – lactate dehydrogenase; CO – cytochrome *c* oxidase.

The activity of NAD^+/NAD×H dehydrogenases of common catabolic pathways

It is known that transformation of D-xylulose-5-phosphate in the reactions of non oxidative phase of pentose phosphate pathway is necessary for the formation of fructose-6-phosphate and glyceraldehyde-3-phosphate, intermediates of the Embden-Meyerhof-Parnas pathway. The direction of their further metabolism in the yeast cell depends on the aeration degree in the fermentation medium (Liu et al., 2018). However, microaerobiosis favoring the production of ethanol from D-xylose also stimulate the use of pyruvate in Krebs cycle. Therefore, the NAD×H regeneration points necessary for XD functioning can be located into the general sugar-catabolic pathways, which located in different cell compartments.

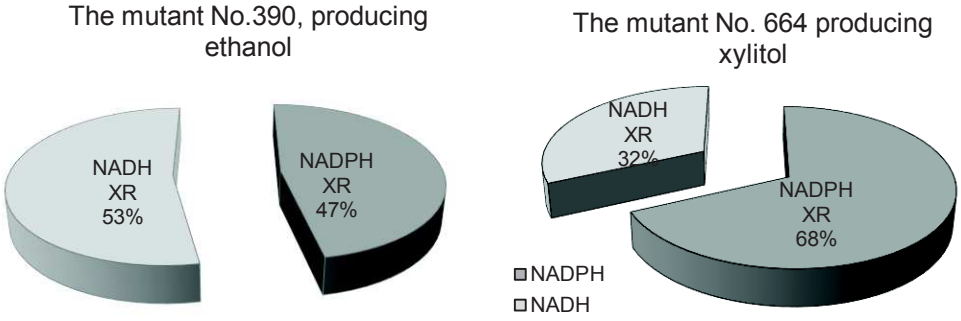

Figure 3. Contribution of various specific activities in total xylose reductase activity (XR) for mutants of *P. tannophilus*, producing ethanol and xylitol.

The activities of a number of NAD^+/NAD×H-dependent dehydrogenases (GPD, total MD, CO and LD) of the mutants *P. tannophilus* 22-Y-1532 were studied. In most

cases, their values were lower than the control values (Fig. 2). The NAD^+/NAD×H dehydrogenases activities of the ethanol-producing mutants *P. tannophilus* were significantly different. Thus, the mutant strain No. 390 was characterized by a more than two-fold increase of LD activity against the background of significant decrease of GPD, total MD and CO activities. On the other side, the mutant No. 442 had activities of all these enzymes, comparable with the dates for the parent strain *P. tannophilus* 22-Y-1532.

The correlation of various HAD /HAD×H-dehydrogenases activity in the biochemical mutants of the yeast *P. tannophilus*

To identify the features of NAD×H regeneration under microaerobic conditions favoring the selective production of xylitol and ethanol from D-xylose, the ratios of activity the enzymes capable to oxidizing NAD×H were calculated to the activity of NAD^+- dependent xylitol dehydrogenase (Table 1).

Table 1. The ratio of the activity of different HAD^+/ HADH-dehydrogenases to xylitol dehydrogenase activity in the mutant strains of *P. tannophilus* 22-Y-1532

Number of mutant strain	Main product	The ratio of enzymes activities				
		НАД×Н XR / XD	GPG / XD	LDG / XD	MDG /XD	CO / XD
664	*xylitol*	0.29	0.2	1.11	0.38	0,06
390	*ethanol*	0.38	0.3	1.96	0.36	0.06
442		0.47	0.6	1.05	0.62	0.19

Notes: XR – xylose reductase; MD – malate dehydrogenase; XD – xylitol dehydrogenase; GPD – glycerol-3-phosphate dehydrogenase; LD – lactate dehydrogenase; CO – cytochrome *c* oxidase; Colors show characteristics, similar in different mutant strains. The maximum error in all cases did not exceed 5.0%.

An analysis of the results revealed some metabolic features that determine the effectiveness of xylitol incorporation to reactions of the common sugar-catabolic pathways. For the mutant No. 664 the lowest ratios of activity of NAD×H-dependent XR, GPD, total MD and CO were noted compared to XD activity. This indirectly indicates the insignificant role of Embden-Meyerhof-Parnas pathway and the mitochondrial respiratory chain in retention of NAD^+/NAD×H balance, necessary for D-xylulose formation.

A common feature in the ethanol-produsing mutant strains was the significant contribution of XR to the NAD×H oxidation. However, other metabolic characteristics of these strains of *P. tannophilus* varied. So, NAD×H regeneration was ensured by a rather high LD activity in the cells of the mutant No. 390. The oxidation of xylitol to D-xylulose was supported by the functioning of the respiratory chains of mitochondria (fairly high ratios of activity of total MD and CO to XD activity) in mutant No. 442.

At the same time, features of D-xylose metabolism, common to different type mutants of *P. tannophilus* 22-Y-1532 were marked. The ratios of total MD and CO activities to XD activity did not differ in principle for the strains No. 390 and No. 664. The mutant No. 664, producing xylitol, and mutant No. 442, which formed the highest amounts of ethanol, were characterized by a similar participation of LD activity in the regeneration of NAD^+ coenzyme. Thus, assessment of the metabolic characteristics of *P. tannophilus* mutant strains indirectly confirmed the functioning of various mechanisms for the NAD^+/NAD×H balance regulation under microaerobic fermentation of D-xylose.

CONCLUSION

The dynamic development of the world economy is impossible without the search for cheap renewable energy sources, alternative to oil and coal. Ethanol, in comparison with other forms of bioenergy, is favorably distinguished by low cost and environmental toxicity (Sadh et al., 2018; Bedoić et la., 2019). A promising stock for its production are different agricultural wastes, which, after hydrolysis, turn into multicomponent mixtures of hexoses and pentoses, consisting of 20–92% of D-xylose. Utilization of such substrates by yeast has a number of technological advantages (Spalvins et al., 2018). However, attempts at the genetic construction of industrial strains of *S. cerevisiae*, effectively producing bioethanol from D-xylose, has not yet been successful (Cristobal-Sarramian & Atzmüller, 2018).

The catabolism of D-xylose, functioning with the participation of the non-oxidizing phase of the pentose phosphate pathway, the Embden-Meyerhof-Parnassus pathway and the Krebs cycle, leads to the formation of intermediates similar to catabolism of hexoses (fructose-6-phosphate, glyceraldehyde-3-phosphate, pyruvate, ethanol). However, a living cell is a complexly organized whole system. Often, even its genetic and biochemical assessment *in vitro* ignores aspects that fundamentally affect the course of the entire process of bioconversion of an organic substrate. Thus, evidence of the key role of enzymes in the initial stages of D-xylose catabolism has not yet fully revealed the causes of xylitol formation in the aeration, favoring ethanol production. Xylitol is widely used as a substitute for sucrose in the food and medical industry (Ur-Rehman et al., 2015). The microbiological method for producing xylitol is characterized by environmental friendliness, as well as the ease of isolation and purification of this alcohol (Cristobal-Sarramian & Atzmüller, 2015). Given this, an analysis of the metabolic characteristics of yeast that contribute to the production of xylitol and ethanol have great importance.

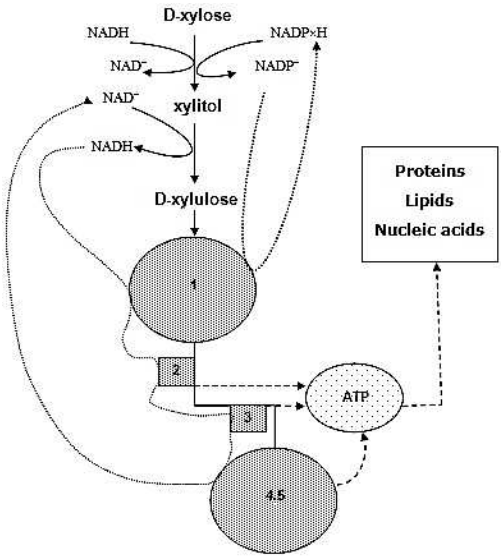

Figure 4. Regeneration of coenzymes NAD(P)×H in yeast during microaerobic fermentation of D-xylose.

Notes: 1 – Warburg-Dickens pathway; 2 – Embden–Meyerhof–Parnas pathway; 3 – The oxidative decarboxylation of pyruvate; 4 – Krebs cycle; 5 – Biological oxidation and oxidative phosphorylation. Dotted lines indicate regeneration points for NADP×H and NAD×H.

An experimental analysis of the mutants of *P. tannophilus* showed that the production of xylitol and ethanol from D-xylose represents as the rearrangement of whole metabolism of carbohydrates in oxygen deficiency - the final electron and proton acceptor by NAD×H. The increased ratio of $NADP^+$/NAD×H in microaerobiosis is not only the difference in the coenzyme

specificity of XR and XD, but also the inhibition of NAD×H/NAD^+dehydrogenases of common sugars catabolism pathways (Fig. 4). Therefore, high production of xylitol may be a manifestation of 'the Pasteur effect' for D-xylose, a reserved carbon source in yeast cells.

The production of ethanol from D-xylose is impossible without flexible regulation of $NADP^+$/NAD×H balance in oxygen deficiency. In addition to the respiratory chains of mitochondria, the xylose-assimilating yeast has at least three to four NAD×H regeneration points. One of them is reduction of D-xylose to xylitol, although the degree of its influence on the balance of $NADP^+$/NAD×H directly depends on the activity and specificity of XR (Yablochkova et al., 2004; Kwak & Jin, 2017; Siti et al., 2017; Cristobal-Sarramian & Atzmüller, 2018; Liu et al., 2018). The participation of pyruvate and dioxiacetonphosphate, as well as the mitochondrial respiratory chains in the regeneration of NAD^+, clearly illustrates the close relationship of ethanol formation from D-xylose with yeast growth. A likely explanation for this phenomenon is the greater sensitivity of the xylose-assimilating yeast to ethanol in compared to *S. cerevisiae* (Bolotnikova et al., 2012; Siti et al., 2017). The prevalence of *P. tannophilus* growth over the ethanol-forming activity may be genetically determined. Therefore, studying the genetic system of this yeast becomes an important prerequisite for determining the biochemical steps that restrict the production of ethanol from D-xylose. It will help determine the strategy for genetic construction of *S. cerevisiae* strains with high yield of ethanol and xylitol on the base of variety agricultural wastes.

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