SUMMARY

During last decades, inverse correlation between exhaustible reserves of fossil fuels and increasing demands for them observes. Therefore it is rationale to search for alternative energy sources especially as fuel costs are increasing steadily and the energetic security with each passing day becomes less and less. Due to this, there is increasing interest for the use of natural feedstock, including biomass, as an almost inexhaustible source of renewable fuels. However, to biomass has become a powerful source of energy, should be in the maximum way to exploit the potential of sugars that are its components. Efficient alcoholic fermentation of both pentose and hexoses by yeast is prerequisite for achievement the maximal productivity of ethanol production from lignocellulosic hydrolysates.

Plant biomass has near 75% of polysaccharides being the rich source of sugars. In total, lignocelluloses contains of three main components: cellulose, lignin and hemicelluloses. Cellulose is homopolysaccharide of glucose, more precisely, residues of β-D-glucopyranose connected with 1-4 bonds. Lignin is the aromatic heteropolymer containing mostly phenylpropanoid residues. Hemicelluloses are the branched heteropolysacharides consisting of hexoses, pentoses and uronic acids. The ratio of monosaccharides in hemicellulose hydrolysates varies depending on feedstock and procedure of hydrolysis, however, the major sugar of most of hemicelluloses is pentose, xylose. It is worth to say here that the conversion of lignocelluloses to ethanol is much more complicated relative to ethanol production from more homogenous feedstocks as sugarcane, sugarbeet, wheat or corn.

Currently, pilot plants for fuel ethanol production from lignocelluloses uses baker's yeast *Saccharomyces cerevisieae*. However it has severe defects as ferments only hexoses and do not possess ability to metabolize varieties of biomass pentoses. That's why scientists are interested in other microorganisms as bacteria (*Escherichia coli*, *Zymomonas mobilis*) and yeasts (*Pichia stipitis*, *Hansenula polymorpha*). Simultaneously, scientists working on these alternative microorganisms try to construct the strains which, in addition to fermenting varieties of lignocellulosic sugars, fulfill this process with high yield and productivity. To hydrolyze lignocelluloses to monosaccharides, most often, acid hydrolysis is used. However, near 30% of sugars are converted during this process to furfural and 5-hydroxymethylfurfural. This diminishes accumulation of ethanol for near 30% and substantial drop of fermentation process. In alternative process of enzymatic hydrolysis, monosaccharides, which are the final products of hydrolysis, inhibit cellulose and hemicellulase activities, therefore for complete hydrolysis of polysaccharides, liberated

sugars have to be directly converted by fermenting microorganisms to ethanol in the same vessel (i.e. process of simultaneous saccharification and fermentation SSF). However, this process needs to be conducted at high temperatures, optimal for cellulases and hemicelulases (near 50°C), so only thermotolerant microorganisms could tolerate such temperatures, including one of the most thermotolerant yeast *Hansenula polymorpha*. Unfortunately, the wild strains of this yeast accumulate only 0.5 g/L of ethanol from xylose.

Therefore the aim of this work was the construction of the strains of the yeast *H. polymorpha* accumulating elevated amounts of ethanol from xylose under elevated temperatures using methods of metabolic engineering and classical selection.

The first step was the overexpression of *XYL1m, XYL2, XYL3* and *PDC1* genes encoding engineered xylose reductase, xylitol dehydrogenase, xylulokinase and pyruvate decarboxylase. As the parental strain for construction, the mutant 2EthOH- which is characterized by elevated ethanol production from xylose, was used. Introduction of the mentioned modifications led to strains accumulated near 7,5 g of ethanol/L from xylose, whereas strain 2EtOH$^-$ produced only 2 g/L. it was found that the influence of *XYL3* on xylose alcoholic fermentation exceeds those of *PDC1* genes. One may assume that overexpression of *PDC1* on the background of three overexpressed genes of xylose metabolism drops the maximal level of their transcription, especially of *XYL3*, whereas activity of xylulokinase could be the limiting factor of the process, However, isolated at this stage strains still were non-competitive relative to strains of *S. cerevisie* regarding titers if accumulated ethanol.

The next step of our work was selection of the strain obtained on the medium with 3-bromopyruvate (3BrPA). This compound is well known anticancer drug characterized by inhibition of main enzymes of glycolysis. Based on this, we suggested that 3BrPA-resistant strains should possess enhanced activities of glycolytic enzymes and consequently accumulate elevated amounts of ethanol. Maximal amount of ethanol accumulated by 3-BrPA-resistant strains was 10 g/ L under 45°C. Efficiency of xylose alcoholic fermentation under elevated temperature in the resulted strains isolated by combination of the methods of metabolic engineering and classical selection was increase 25 fold relative to the wild-type strains.

We continued to search for approaches which would improve ethanol production from most abundant pentose sugar of lignocellulose. We hypothesized that knock out of *CAT8* gene, ortholog of *S. cerevisiae* global transcription regulator responsible for transcription of gluconeogenic genes, will activate xylose alcoholic fermentation. It was found that ortholog of *CAT8* gene of *H. polymorpha* shows 31% of identity and 53% similarity to *CAT8* gene of *S. cerevisiae*. We decided to delete this gene in the wild0-type strain *H. polymorpha* NCYC

495 *leu 1-1* and in the best at that moment ethanol producer from xylose isolated as 3BrPA-resistant strain. The corresponding deletants were characterized by elevated ethanol production from xylose: 0,78 g/L (for the strain NCYC/ΔCAT8) and 12,5 g/L (for the strain 2EtOH/XYL1m/XYL2/XYL3/BrPA/ΔCAT8), i.e. 40% and 30% more relative to the parental strains. We studied possible changes in genome of *H. polymorpha* deleted in *CAT8*. It is known that in *S. cerevisiae* transcriptional activator *CAT8* controls near 200 genes involved in the pathways of central metabolism and several transcription factors. Conducted experiments allowed to state that the regulation of gluconeogenesis in the yeast *H. polymorpha* differs from that in *S. cerevisiae*. It cannot be excluded that in *H. polymorpha* gene, distinct from *CAT8* plays the central; role in transcription activation. Total genome microarray could answer the question which changes bears deletion of *CAT8*, so it could be interesting development of current research.

There are still a lot of non-studied processes, regulatory pathways and their interactions which open the gate for further endeavors.