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ORIGINAL PAPER

Plate ethanol-screening assay for selection of the *Pichia stipitis* and *Hansenula polymorpha* yeast mutants with altered capability for xylose alcoholic fermentation

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Abstract A new method for the selection of *Pichia stipitis* and *Hansenula polymorpha* yeast mutants with altered capability to ferment xylose to ethanol was developed. The method is based on the ability of *P. stipitis* and *H. polymorpha* colonies to grow and produce ethanol on agar plates with xylose as the sole carbon and energy source. Secreted ethanol, in contrast to xylose, supports growth of cells of the indicator xylose-negative strains (the wild-type strain of *Saccharomyces cerevisiae* or *Δxyl1* mutant of *H. polymorpha*) mixed with agar medium. The size of the tester culture-growth zone around xylose-grown colonies appeared to be dependent on the amount of secreted ethanol. Mutants with altered (decreased or elevated) ethanol production in xylose medium have been isolated using this method. The mutants exhibited pleiotropic alterations in enzymatic activities of the intermediary xylose metabolism.

Keywords Alcoholic xylose fermentation · Plate ethanol screening · *Hansenula polymorpha* · *Pichia stipitis*

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Introduction

Xylose is an abundant sugar in nature, being a major component of plant hemicellulose polymers. To develop the feasible process of fuel ethanol production from lignocellulose hydrolyzates, it is important to construct microbial strains capable of active xylose fermentation. Several bacterial and yeast strains, which ferment xylose, were isolated and engineered [1, 9, 29]. Several species of non-conventional yeasts capable of fermenting xylose to ethanol were found, including *Pichia stipitis* [19] and thermotolerant methylotrophic yeast *Hansenula polymorpha* [21]. This besides, recombinant strains of *Saccharomyces*-fermenting xylose were constructed [8, 11]. However, all the known xylose-fermenting organisms possess some drawbacks, among them low ethanol-production rate and low yield are the most important parameters [7, 9].

Many attempts were undertaken to improve the characteristics of xylose fermentation. In yeasts, most of them involved introduction of foreign genes (from other yeasts, fungi or bacteria), deletion of some host genes, combination of both approaches and subsequent selection of the engineered strains for the improved growth on xylose [8, 10, 13, 22, 24, 27]. The corresponding gene-engineering manipulations often are conducted based on our current understanding of the xylose-metabolic flux. At the same time, the direct selection for ethanol overproducing mutants has never been used. Isolation of such mutants and their comparison with the wild-type strains, in addition to their possible biotechnological importance, would

permit identification of the limiting enzymatic steps for the overall xylose-fermentation pathway. Isolation of the mutants producing decreased amounts of ethanol would also be important from the same point of view. However, up to now the methods for selection of the mutants producing altered (elevated or decreased) amounts of ethanol from xylose have not been described (the same is true for other sugar substrates).

We describe here a novel plate-screening method for selection of the mutants of the yeasts *P. stipitis* and *H. polymorpha*, which produce altered (elevated or decreased), relative to the wild-type strain, amounts of ethanol during utilization of xylose as sole source of carbon and energy. The isolated mutants exhibited alterations in enzymatic activities of the intermediary xylose metabolism.

Materials and methods

Strains and media

The following strains of yeasts were used: *P. stipitis* CBS 5774 (kindly provided by Dr. T. Jeffries, USDA Forest Service, Madison, WI, USA), *H. polymorpha* CBS 4732 *leu2–2* [14], *Δxyl1* #4 mutant (it is a derivative of the CBS 4732 *leu2–2*) [27] and *S. cerevisiae* SP4 (kindly provided by Dr. T. Bilinski, Rzeszów University, Poland). Yeasts were cultivated in semisynthetic yeast nitrogen-base medium supplemented with carbon source (2%) and yeast extract (0.05%) as described [21] at 30°C *(P. stipitis, S. cerevisiae)* or 37°C *(H. polymorpha)*. Liquid-medium cultivations were conducted in 50 ml of the medium in 125-ml Erlenmeyer shake flasks at a shaker. Oxygen-limited conditions were provided by agitating at 100 rpm. Cultivations for the estimation of ethanol production were conducted according to Shi et al. [22]. The cells for enzyme assays were taken from the mid-exponential growth phase.

For mutant isolation, cells of the strains *P. stipitis* CBS 5774 and *H. polymorpha* CBS 4732 *leu2-2* were cultivated in liquid medium with xylose (2%) until the mid-exponential growth phase, washed with distilled water, diluted to 10^6 cell/ml and irradiated with UV light with a dose which provides ~ 10% -cell survival. Then the cells were diluted and plated on the solid YNB medium containing xylose (2%) and suspension (10^6 cells/ml) of the cells of *S. cerevisiae* or *Δxyl1* mutant of *H. polymorpha*, which are unable to grow in xylose as a sole carbon and energy source but can grow in the medium with ethanol.

Assays

Cell biomass was determined turbidimetrically at 600 nm; dry weight was calculated according to the corresponding calibration curves. Ethanol was measured using alcohol oxidase/peroxidase-based enzymatic kit "Alcotest" [6]. The cells were disrupted with glass beads (diameter, 40 μM) using a mechanical blender. Protein concentration was determined with Folin reagent [15].

The following enzymatic activities were assayed: xylose reductase (EC 1.1.1.21) [26], xylitol dehydrogenase (EC 1.1.1.9) [16], xylulose kinase (EC 2.7.1.17) [23], glucose-6-phosphate dehydrogenase (EC 1.1.1.49) [2], transaldolase (EC 2.2.1.2) [25], glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) [5], hexokinase (EC 2.7.1.1) [17], alcohol dehydrogenase (EC 1.1.1.1) [3]. Enzyme activities were expressed in micromoles × mg protein^{-1} × min^{-1} (U/mg protein).

All experiments were repeated at least twice.

Results and discussion

To select mutants with decreased or elevated ethanol production compared with the parental strains, it is important to develop a simple and reliable method for qualitative determination of ethanol produced by the growing colonies. We checked several different chemical and enzymatic methods for direct determination of ethanol on the plate but all of them appeared to be unsatisfactory. For example, we tried to determine ethanol on plates using "Alcotest" enzymatic kit containing alcohol oxidase, peroxidase and chromogen [6], an enzymatic kit containing NAD-dependent alcohol dehydrogenase, phenazine metosulfate and nitroblue tetrazolium [12], however, for unknown reasons, these approaches were unsuccessful. Therefore, we decided to develop the plate ethanol-assaying method and use it for subsequent isolation of the mutants with altered ability of ethanol production in the medium with xylose. Our rationale was based on the fact that *P. stipitis* and *H. polymorpha* grow on xylose as the sole carbon and energy source and produce ethanol from this sugar. At the same time, *S. cerevisiae* cannot utilize xylose but it efficiently utilizes ethanol as the sole carbon and energy source at aerobic conditions. So, if colonies of xylose-fermenting yeasts grow on the surface of xylose-containing agar medium mixed with viable suspension of baker's yeast, the latter species would be able to grow around ethanol-producing colonies. The more ethanol is produced, the greater should be the size of the *S. cerevisiae* growth zone (halo)

around the colony of the ethanol producer. Inversely, no growth of *S. cerevisiae* zone should appear around mutant colonies unable to ferment xylose.

We conducted the model experiments to test this assumption. Cells of *S. cerevisiae* were added to agar medium with xylose (2%) at a density of 10^6 cell/ml just before agar solidification. Then the cells of the xylose-utilizing yeast *P. stipitis* were plated on the agar surface (approximately 40–70 colonies per plate). After 5–7 days of cultivation of *P. stipitis*, growth zones (haloes) of the baker's yeast appeared around each colony (Fig. 1 a). We hypothesized that mutant colonies with increased ethanol production would be surrounded by larger haloes whereas those, which are unable to synthesize ethanol in xylose medium, would not produce haloes at all. To test this hypothesis, the cells of *P. stipitis* were UV mutagenized and plated onto the xylose medium containing cells of baker's yeast. After 5–7 days, the size of *S. cerevisiae* growth zone around each *P. stipitis* colony was evaluated. The colonies with a significantly larger or smaller growth zones (haloes) compared to those of wild-type and majority of mutagenized colonies were picked up. After analysis of 30,000 colonies, 11 colonies, which did not show any growth zone and 25 colonies displaying larger growth zones were picked up. The selected colonies were tested for ethanol production in the liquid medium with xylose. Among 11 strains that did not display growth zone, 2 strains produced similar amounts of ethanol to the wild-type strain, 1 strain accumulated 40% less ethanol, 2 strains 70% less, whereas the rest six strains did not accumulate ethanol at all (data not shown). Among the 25 *P. stipitis* colonies showing the larger zones, only three strains accumulated more ethanol in xylose medium compared to that of the wild-type strain. Thus, the frequencies for the appearance of mutants, which produce altered (elevated or decreased) amounts of ethanol in liquid xylose medium were near 1×10^{-4} and 3×10^{-4}, respectively. This is close to frequencies for selection of auxotrophic mutants of non-conventional yeasts [28].

Our first attempts to use the developed method for isolation of *H. polymorpha* mutants able to overproduce ethanol from xylose were unsuccessful. The reason is apparently the difference in optimal growth temperature between *H. polymorpha* (37°C) and *S. cerevisiae* (30°C). It is known that at 30°C, *H. polymorpha* produces very little (if all) ethanol from xylose [21] whereas *S. cerevisiae* grows poor at 37°C. We tried to achieve feeding of *S. cerevisiae* by ethanol excreting by *H. polymorpha* xylose-grown colonies at 33 and 34°C; however, such attempts were also unsuccessful. Therefore, we decided to use the recently isolated

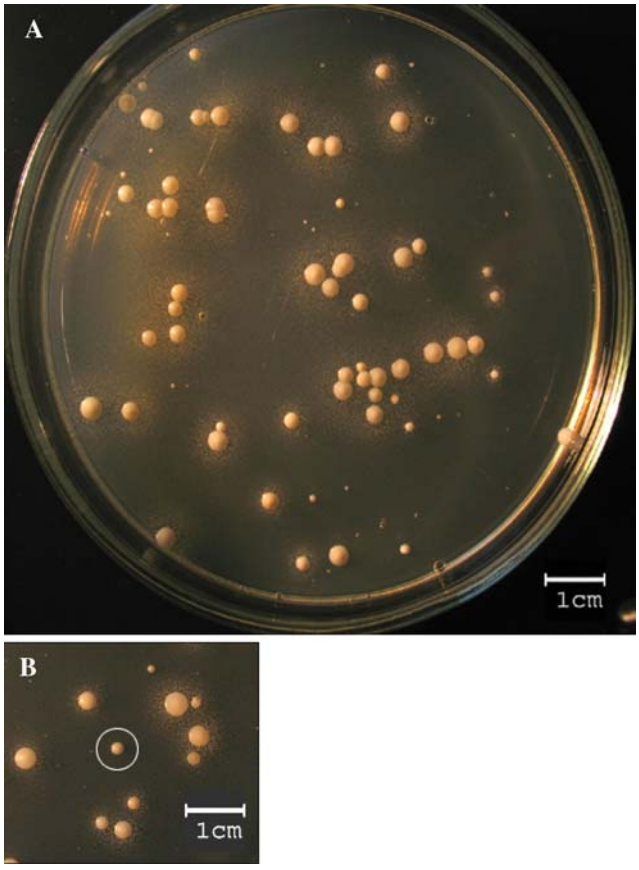

Fig. 1 Pictures showing the growth zones (*haloes*) around the *P. stipitis* colonies growing on the solid medium with xylose mixed with cells of baker's yeast. **a** Picture of the Petri dish containing solid medium with xylose (2%) mixed with cells of baker's yeast (10^6 cells/ml). After agar solidification, the cells of *P. stipitis* were plated on the medium. Haloes are seen around *P. stipitis* colonies. **b** Section of the plate containing *P. stipitis* colony (*encircled*), which did not produce any growth zone (*halo*)

mutant *Δxyl1* #4 of *H. polymorpha* [27] as a tester strain. Indeed, in such a case, we were able to determine a number of *H. polymorpha* colonies obtained after UV-irradiation of CBS 4732 *leu2-2*, which displayed larger or smaller growth zones of *Δxyl1* #4 strain compared to that of the growth zones around the colonies of the wild-type strain. In total, after investigation of more than 22,000 colonies of UV-irradiated *H. polymorpha* CBS 4732, 21 colonies were picked up, which had larger growth zones of tester *Δxyl1* #4 strain (mutants of *H. polymorpha* CBS 4732 producing smaller or no growth zones, were not analyzed). After testing these colonies in the xylose-containing liquid medium, nine mutants producing increased amounts of ethanol were selected (Fig. 2). Thus, the plate ethanol-screening method can be applied for different yeast species.

In further experiments, we studied in more detail, several isolated mutants of *P. stipitis* (the three strains

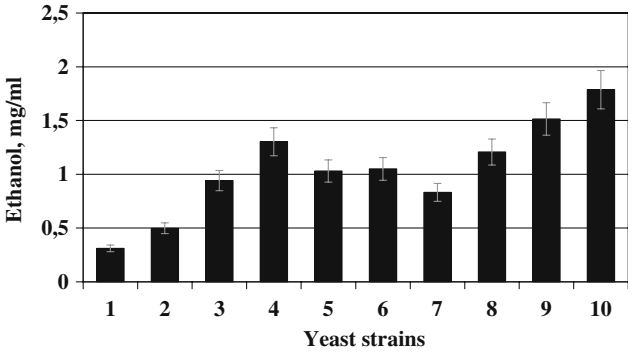

Fig. 2 Maximal ethanol production in the medium with xylose (2%) of the *H. polymorpha* wild-type strain and the mutants producing elevated amounts of ethanol within 60 h. of incubation. *1* CBS4732 wild type; *2* S1; *3* S2; *4* S3; *5* S4, *6* S5; *7* S6; *8* S7, *9* S8; *10* S9

Z15, Z36 and Z39 with elevated ethanol production as well as the two strains BZ6, BZ7 with decreased ethanol production). Kinetics of growth and ethanol synthesis by mentioned strains in xylose medium clearly showed a significant decrease (strains of BZ series) or increase (strains of Z series) in ethanol production (Fig. 3a–d). Thus, the proposed method showed its efficiency for isolation of the mutants, which produced altered (elevated or decreased) amounts of ethanol from xylose, as compared with that of the wild-type strain. Mutants accumulating less of ethanol, did not differ from the parental strain by the growth rate, although they were characterized by more prolonged lag period (Fig. 3a). In glucose medium, the mutants did not differ from the wild-type strain by the growth kinetics and accumulated three times less of ethanol (Fig. 4a, b) so being capable to ferment glucose to ethanol. Mutants Z15 and Z39 accumulated slightly less amount of ethanol whereas mutant Z36 accumulated slightly more ethanol in glucose medium comparing to the parental strain (Fig. 4c, d).

In further experiments, the preliminary biochemical characterization of the isolated mutants of *P. stipitis* and *H. polymorpha* was done. Enzymes of initial

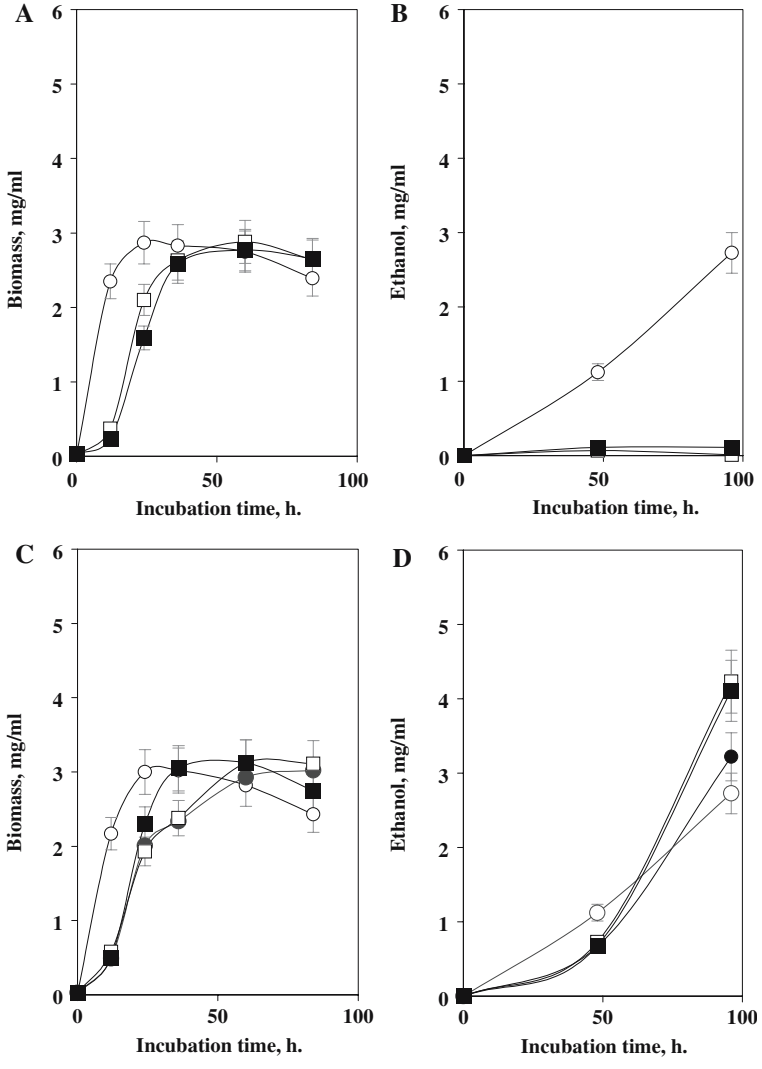

Fig. 3 Growth (**a**, **c**) and ethanol production (**b**, **d**) in the medium with xylose (2%) of the *P. stipitis* wild-type strain and the mutants accumulating decreased (**a**, **b**) or elevated (**c**, **d**) amounts of ethanol. *empty circle* wild type, *filled circle* strain Z15 (**c**, **d**), *empty square* strains BZ6 (**a**, **b**) and Z36 (**c**, **d**), *filled square* strains BZ7 (**a**, **b**) and Z39 (**c**, **d**)

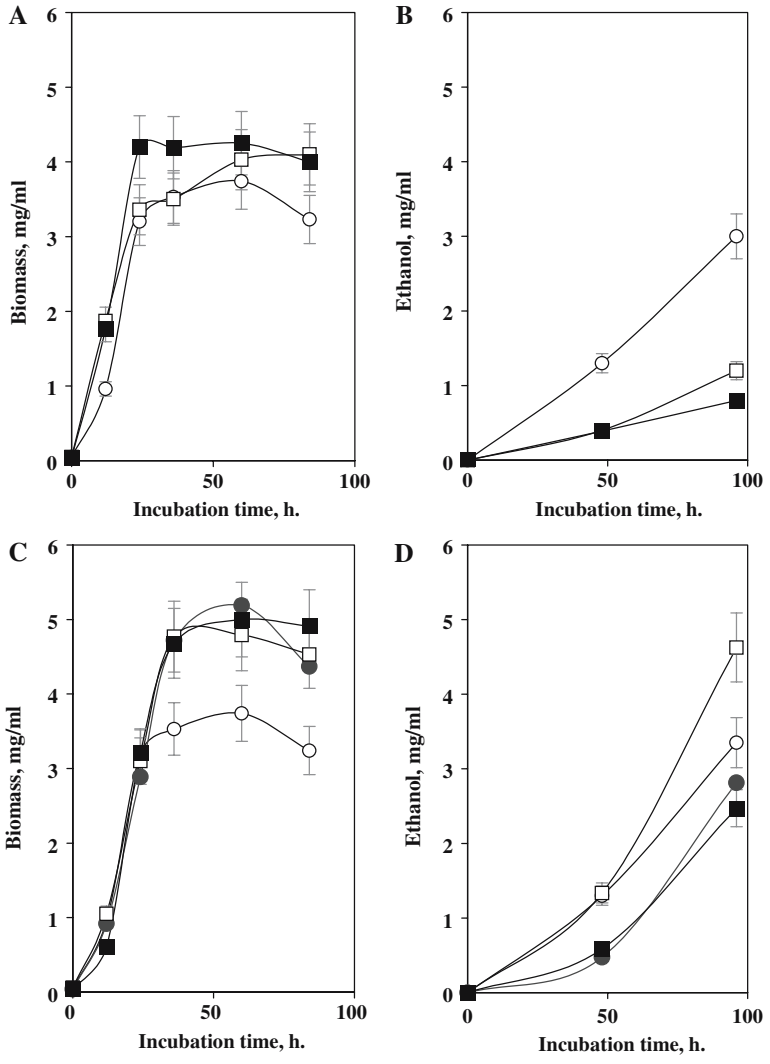

Fig. 4 Growth (**a**, **c**) and ethanol production (**b**, **d**) in the medium with glucose (2%) of the *P. stipitis* wild-type strain and the mutants accumulating decreased (**a**, **b**) or elevated (**c**, **d**) amounts of ethanol in xylose medium. *empty circle* wild type, *filled circle* strain Z15 (**c**, **d**), *empty square* strains BZ6 (**a**, **b**) and Z36 (**c**, **d**), *filled square* strains BZ7 (**a**, **b**) and Z39 (**c**, **d**)

pathway of xylose utilization (xylose reductase, xylitol dehydrogenase, xylulose kinase), pentose phosphate pathway (glucose-6-phosphate dehydrogenase, transladolase) and glycolysis/alcoholic fermentation (hexokinase, glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase) were analyzed in *P. stipitis* wild-type strain and six mutants cultivated in xylose medium. We hoped that this analysis could provide us with a clue for understanding the reasons of the observed changes in ethanol production.

It was demonstrated that the strong decrease in ethanol synthesis by mutants BZ6 and BZ7 was accompanied with a drop in the activities of all the three enzymes involved in xylose conversion to xylulose-5-phosphate and of glucose-6-phosphate dehydrogenase. At the same time, the mutants showed an increase in activity of hexokinase (Table 1). This can suggest the coordinated regulation of the corresponding enzymes in *P. stipitis*. Activities of glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase in BZ6 and BZ7 mutants were only slightly different from that in the wild-type strain.

Mutants Z15 and Z39 accumulating elevated amounts of ethanol in xylose medium did not differ significantly from the wild-type strain regarding the activities of the most assayed enzymes (exception is 24% increase of xylulose kinase activity in strain Z15). Most efficient ethanol-overproducing strain Z36 displayed pleiotropic increase in activities of xylose reductase (54%), xylitol dehydrogenase (18%), xylulokinase (44%) and glucose-6-phosphate dehydrogenase (25%). Mutants Z15 and Z39 accumulated slightly less ethanol in glucose medium compared to that of the wild-type strain. The most interesting one appeared to be the mutant Z36, which produced more ethanol in the media with both carbon sources, xylose and glucose (Fig. 3, 4).

Enzymatic pattern of one *H. polymorpha* ethanol-overproducing mutant, S1 was compared to that of the wild-type strain. Unfortunately, most of the isolated

Table 1 Enzyme activities in the cell-free extracts of the *Pichia stipitis* wild-type and the mutant strains cultivated in the medium with xylose (2%) until the mid-exponential growth phase; activity units: U/mg protein (*U* unit: μmoles × mg protein $^{-1}$ × min $^{-1}$)

Enzyme	Wild type CBS6054	Increased ethanol			Decreased ethanol	
		Z15	Z36	Z39	BZ6	BZ7
Xylose reductase (EC 1.1.1.21)	0.630 ± 0.165	0.651 ± 0.065	0.975 ± 0.118	0.631 ± 0.134	0.342 ± 0.042	0.378 ± 0.123
Xylitol dehydrogenase (EC 1.1.1.9)	1.051 ± 0.355	0.969 ± 0.098	1.236 ± 0.035	0.845 ± 0.061	0.419 ± 0.038	0.370 ± 0.069
Xylulokinase (EC 2.7.1.17)	0.331 ± 0.086	0.412 ± 0.045	0.476 ± 0.131	0.305 ± 0.050	0.244 ± 0.086	0.151 ± 0.044
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	1.081 ± 0.146	0.884 ± 0.111	1.349 ± 0.230	1.071 ± 0.015	0.421 ± 0.091	0.432 ± 0.101
Hexokinase (EC 2.7.1.1)	0.013 ± 0.003	0.019 ± 0.003	0.021 ± 0.002	0.015 ± 0.005	0.088 ± 0.028	0.086 ± 0.031
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	0.136 ± 0.051	0.121 ± 0.014	0.113 ± 0.01	0.097 ± 0.055	0.106 ± 0.031	0.099 ± 0.028
Alcohol dehydrogenase (towards ethanol) (EC 1.1.1.1)	0.259 ± 0.134	0.229 ± 0.027	0.271 ± 0.031	0.256 ± 0.042	0.278 ± 0.052	0.389 ± 0.078

mutants appeared to be unstable and reverted to the wild-type phenotype. No significant difference between the S1 mutant and the wild-type strain was observed for most of assayed enzymes, including xylose reductase (NADH). Unexpectedly, we observed that the mutant has very low activity of xylose reductase using NADPH as co-factor (Figure 5). One may assume that mutation in S1 mutant decreased the affinity of the xylose reductase toward NADPH in such a way as to decrease the cofactor disbalance (see: 18), resulting in the production of excess ethanol. Further experiments are necessary to study the nature of the S1 mutant in detail.

Summarizing, the plate-screening method for the selection of the yeast mutants producing elevated or diminished amounts of ethanol from xylose was developed. The mutants of *P. stipitis* and *H. polymorpha* were isolated. Availability of the mutants accumulating decreased or elevated amounts of ethanol in xylose medium with pleiotropic decrease or increase in several enzyme activities, respectively (Table 1), allows hypothesizing on the existence of the putative regulatory gene involved in the synthesis of several enzymes for the intermediary metabolism of xylose utilization. To test this hypothesis, it would be necessary to clone and mutate this hypothetic gene. However, as the corresponding mutants do not have any clear-cut growth phenotype, it is difficult to do so by using the conventional approach in transforming the mutant with *P. stipitis* gene library. It would be much easier to isolate the corresponding gene(s) using a method of insertional mutagenesis. As such a method is not developed yet for *P. stipitis*, this is the task for the future research. Contrary to *P. stipitis*, the method for insertional mutagenesis was described for *H. polymorpha* [4], so it could not be a problem to clone the corresponding regulatory genes in this thermotolerant organism. As two *P. stipitis* ethanol-overexpressing mutants, Z15 and Z36, showed increase in xylulokinase activity, one may assume that this enzyme catalyzes the limiting reaction of xylose fermentation. We plan to test this suggestion by cloning and overexpressing this gene in the nearest future.

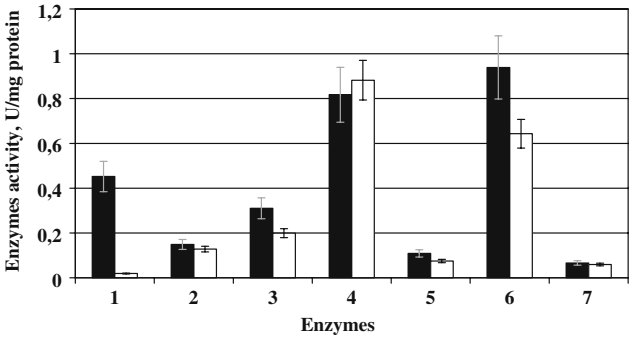

Fig. 5 Enzyme activities in the cell-free extracts of the *H. polymorpha* wild-type and the mutant strain S1 cultivated in the medium with xylose (2%) until the mid-exponential growth phase; activity units: U/mg protein (*U* unit: μmoles × mg protein $^{-1}$ × min $^{-1}$). *filled square* wild-type strain CBS4732; *empty square* mutant S1. *Enzymes*: *1* xylose reductase (with NADPH); *2* xylose reductase (with NADH); *3* xylitol dehydrogenase; *4* xylulose kinase; *5* glucoso-6-phosphate dehydrogenase; *6* alcohol dehydrogenase; *7* transaldolase

The described plate ethanol-screening method together with the methods of insertional mutagenesis of *P. stipitis* and *H. polymorpha* can be useful for studying the molecular mechanisms of regulation of xylose fermentation in parallel with the method based on DNA microarray technique, which will be possible after releasing *P. stipitis* complete genome sequence (*H. polymorpha* genome database is available for several years, see 20). Besides, our method can be used for further improvement of the available strains isolated by approaches of metabolic engineering [1, 9, 29].

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References

1. Aristidou A, Penttila M (2000) Metabolic engineering applications to renewable resource utilization. Curr Opin Biotechnol 11:187–198
2. Banerjee S, Fraenkel DG (1972) Glucose-6-phosphate dehydrogenase from *Escherichia coli* and from a "high-level" mutant. J Bacteriol 110:156–160
3. Bergmeyer H-U, Gawehn K, Grassl M (1974) Enzymes as biochemical reagents. In: Bergmeyer H-U (eds) Methods of enzymatic analysis. Academic, New York 1:428–429
4. van Dijk R, Faber KN, Hammond AT, Glick BS, Veenhuis M, Kiel JA (2001) Tagging *Hansenula polymorpha* genes by random integration of linear DNA fragments (RALF). Mol Genet Genomics 266:646–656
5. Engel M, Seifert M, Theisinger B, Welter C, Seyfert U (1998) Glyceraldehyde-3-phosphate dehydrogenase and Nm23-H1/nucleoside diphosphate kinase A. Two old enzymes combine for the novel Nm23 protein phosphotransferase function. J Biol Chem 273:20058–20065
6. Gonchar MV, Maidan MM, Sibirny AA (2001) A new oxidase-peroxidase kit "Alcotest" for ethanol assays in alcoholic beverages. Food Technol Biotechnol 39:37–42
7. Hahn-Hagerdal B, Wahlbom CF, Gardonyi M, van Zyl WH, Cordero Otero RR, Jonsson LJ (2001) Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. Adv Biochem Eng Biotechnol 73:53–84
8. Ho NW, Chen Z, Brainard AP, Sedlak M (1999) Successful design and development of genetically engineered *Saccharomyces* yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol. Adv Biochem Eng Biotechnol 65:163–192
9. Jeffries TW, Jin YS (2004) Metabolic engineering for an improved fermentation of pentoses by yeasts. Appl Microbiol Biotechnol 63:495–509
10. Jeppsson M, Johansson B, Hahn-Hagerdal B, Gorwa-Grauslund MF (2002) Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. Appl Environ Microbiol 68:1604–1609
11. Kötter P, Ciriacy M (1993) Xylose fermentation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 38:776–783
12. Kramarenko T, Karp H, Jarviste A, Alamae T (2000) Sugar repression in the methylotrophic yeast *Hansenula polymorpha* studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants. Folia Microbiol 45:521–529
13. Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT (2005) Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res 5:925–934
14. Lahtchev KL, Semenova VD, Tolstorukov II, van der Klei I, Veenhuis M (2002) Isolation and properties of genetically defined strains of the methylotrophic yeast *Hansenula polymorpha* CBS 4732. Arch Microbiol 177:150–158
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
16. Morimoto S, Matsuo M, Azuma K, Sinskey AJ (1986) Purification and properties of D-xylulose reductase from *Pachysolen tannophilus*. J Ferment Biotechnol 64:219–225
17. Peters BA, Neet KE (1978) Yeast hexokinase PII. Conformation changes induced by substrates and substrate analogues. J Biol Chem 253:6826–6831
18. Petschacher B, Leitgeb S, Kavanagh KL, Wilson DK, Nidetzky B (2005) The coenzyme specificity of *Candida tenuis* xylose reductase (AKR2B5) explored by site-directed mutagenesis and X-ray crystallography. Biochem J 385:75–83
19. du Preez JC, Bosch M, Prior BA (1986) Xylose fermentation by *Candida shehatae* and *Pichia stipitis*—effects of pH, temperature and substrate concentration. Enzyme Microb Technol 8:360–364
20. Ramezani-Rad M, Hollenberg CP, Lauber J, Wedler H, Griess E, Wagner C, Albermann K, Hani J, Piontek M, Dahlems U, Gellissen G (2003) The *Hansenula polymorpha* (strain CBS4732) genome sequencing and analysis. FEMS Yeast Res 4:207–215
21. Ryabova OB, Chmil OM, Sibirny AA (2003) Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 4:157–164
22. Shi NQ, Davis B, Sherman F, Cruz J, Jeffries TW (1999) Disruption of the cytochrome c gene in xylose-utilizing yeast *Pichia stipitis* leads to higher ethanol production. Yeast 15:1021–1030
23. Simpson FJ (1966) D-xylulokinase. Meth Enzymol 9:454–458
24. Traff KL, Otero Cordero RR, van Zyl WH, Hahn-Hagerdal B (2001) Deletion of the *GRE3* aldose reductase gene and its influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expressing the *xylA* and *XKS1* genes. Appl Environ Microbiol 67:5668–5674
25. Tsolas O, Joris L (1975) Transaldolase. Meth Enzymol 42:290–297
26. Verduyn C, Van Kleef R, Frank J, Schreuder H, Van Dijken JP, Scheffers WA (1985) Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*. Biochem J 226:669–677
27. Voronovsky AY, Ryabova OB, Verba OV, Ishchuk OP, Dmytruk KV, Sibirny AA (2005) Expression of *xylA* genes encoding xylose isomerases from *Escherichia coli* and *Streptomyces coelicolor* in the methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 5:1055–1062
28. Wolf K (1996) Nonconventional yeasts in biotechnology. Springer, Berlin, Heidelberg New York
29. Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34