Microbiology, Vol. 74, No. 4, 2005, pp. 415–419. Translated from Mikrobiologiya, Vol. 74, No. 4, 2005, pp. 483–488.

EXPERIMENTAL ARTICLES

Conditions Favoring Differentiation and Stabilization of the Life Cycle of the Yeast *Pachysolen tannophilus*

O. I. Bolotnikova*,[1], N. P. Mikhailova, M. V. Shabalina**, E. N. Bodunova**, and A. I. Ginak****

**Petrozavodsk State University, Lenina 33, Petrozavodsk, 185640 Russia*
***St. Petersburg State Technological Institute (Technical University), Moskovskii pr. 26, St. Petersburg, 198013 Russia*

Received July 26, 2004

Abstract—Conditions favoring differentiation and stabilization of the life cycle of the yeast *Pachysolen tannophilus* have been studied. When concentrations of the carbon source in the medium were lower than 100 g/l, it was found to be favorable to the mating of vegetative cells, both haploid and diploid. The addition of nitrogen and sulfur sources to the medium influenced the life phases of haploid cells and partially stabilized the vegetative growth of diploid cells. Enrichment of the nutrient medium with potassium, vitamins, and microelements was shown to be necessary for the formation and maturation of conjugated ascospores. Microelements, vitamins, and phosphorus in excessive amounts activated conjugation but did not provide for the distinct phases of formation of unconjugated asci and spores in the diploid cells. Possible reasons for the unstable diplophase in the yeast *P. tannophilus* have been discussed.

Key words: the yeast *Pachysolen tannophilus*, vegetative culture, unconjugated and conjugated asci and spores.

The xylose-assimilating spore-forming yeast *Pachysolen tannophilus* is a suitable model for studying the regulatory mechanisms involved in the conversion of D-xylose [1]. This organism can also be used to design industrially important producers of xylitol and ethanol [2–4]. It should be noted, however, that the genetic construction of eukaryotic producers is impossible without obtaining stable haploid and hybrid diploid strains, which provide for the conservation of individual genotypic traits in the process of vegetative growth (i.e., during fermentation, culture maintenance, etc.). Unfortunately, the regeneration stage of diplophase in the yeast *P. tannophilus* is short and unstable. Moreover, the absence of a definite type of mating in this yeast gives rise to heterogeneous cultures composed of cells with different ploidy [5], which considerably hinders the application of routine genetic methods such as hybridization and tetrad analysis.

The aim of this work was to study the causes of instability in the vegetative and sexual phases of the yeast *P. tannophilus* and to investigate nutrient media components that favor vegetative growth and provide for the regulatory induction of the sexual phase. We believe that such knowledge may be of great practical importance.

[1] Corresponding author; e-mail: bolot@onego.ru

MATERIALS AND METHODS

Experiments were carried out with strain *Pachysolen tannophilus* Y-1532, which was obtained from the State Institute for Genetics and Selection of Industrial Microorganisms (GosNIIGenetika) in Moscow. The strain was grown on standard media [6–11] as well as those modified with respect to the concentration of carbon, nitrogen, sulfur, phosphorus, potassium, microelements, and vitamins (Table 1). The yeast was cultivated on agar plates at 30 ± 2°C and stored at 6 ± 1°C. The yeast colonies and cells were examined in a Jenamed Variant microscope (Germany).

Haploid cells were obtained as described by Zakharov *et al.* [6]. The derivation of diploid cells is described in the Results section. The conditions that favor vegetative growth, sexual process, and sporulation in the yeast cells were studied by transferring them from one plate to a series of different media [6]. The intensities of vegetative growth, mating, and sporulation were evaluated within the first 13 days of cultivation. The sexual process was analyzed in terms of the following three parameters: the presence of conjugated cells, formation of ascophores, and maturation of asci. The results were processed according to the handbook by Glotov *et al.* [12].

0026-2617/05/7404-0415

Table 1. Composition of the nutrient media used for the cultivation of *P. tannophilus*

Medium no.	Concentration of ingredients, g/l	Ref.
1	D-glucose, 100; peptone, 3; yeast extract, 8; agar, 20 (*)	[7]
2	$(NH_4)_2SO_4$, 17.5; other ingredients as in medium 1	Modified by us
3	D-xylose, 20; peptone, 20; yeast extract, 5; agar, 20	Modified by us
4	D-glucose, 20; peptone, 20; yeast extract, 5; agar, 20	[8]
5	D-glucose, 20; peptone, 20; yeast extract, 5; agar, 20	[9]
6	CH_3COONa, 10; KCl, 5; agar, 25	[6]
7	D-glucose, 100; peptone, 3; yeast extract, 8; agar, 20 (**)	[7]
8	As medium 7 but with agarose, 20, instead of agar (***)	Modified by us
9	As medium 8 but without a carbon source	Modified by us
10	D-glucose, 20; KH_2PO_4, 0.1; KH_2HPO_4, 0.9; $(NH_4)_2SO_4$, 3.5; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 5; agar, 20; vitamins and microelements [10] (μg/ml): biotin, 20; folic acid, 2.0; inositol, 10; *p*-aminobenzoic acid, 200; pyridoxine hydrochloride, 400; thiamine hydrochloride, 400; riboflavin, 200; nicotinic acid, 400; pantothenate Ca, 2; H_3BO_3, 500; $CuSO_4$, 40; KJ, 100; $FeCl_3$, 200; $MnSO_4$, 400; Na_2MoO_4, 200; $ZnSO_4$, 400 (****)	Modified by us
11	K_2HPO_4, 5; $(NH_4)_2SO_4$, 7; other ingredients as in medium 10	Modified by us
12	D-glucose, 10; yeast extract, 10; agarose, 20; other ingredients as in medium 10	Modified by us
13	D-glucose, 30; KH_2PO_4, 7; yeast extract, 7; agar, 20 (*****)	[7]
14	$(NH_4)_2SO_4$, 7; other ingredients as in medium 13	Modified by us
15	D-glucose, 4; yeast extract, 4; malt extract, 10; agarose, 20	[11]

Note: Five basal media are marked by one to five asterisks. For modified media, only supplementary compounds are indicated.

RESULTS

Obtaining haploid and diploid cell populations. The collection strains of *P. tannophilus* are characterized by intrapopulation heterogeneity, which is due to the presence of cells occurring at different stages of their life cycle [5] (Fig. 1). Such strains are not suitable for genetic studies and selection work. With this in mind, we isolated haploid and diploid cells from a heterogeneous *P. tannophilus* population.

The haploid strain (designated 22-Y-1532) was obtained by eliminating vegetative cells from a sporulating *P. tannophilus* culture with diethyl ether. The haploidy of the 22-Y-1532 cells was confirmed by the appearance of white lustrous convex colonies composed of small cells in the course of the vegetative growth of strain 22-Y1532, as well as by a specific morphological feature (the presence of conjugated asci) of its sporulating culture, which could be observed both with the naked eye and microscopically (Fig. 1)

Diploid cells were isolated as follows: One of the colonies of haploid strain *P. tannophilus* 22-Y-2531, after incubation on a YEPD medium for 1.5 months [9], was found to contain a dull sector, whereas the rest of the colony was white and lustrous. Microscopic examination of this colony sector showed that it contained larger cells than the rest of the colony. These cells were found to be unconjugated ascophores and asci (Fig. 1). According to the accepted description of the life cycle of *P. tannophilus* [5], the cells that were isolated from the dull sector of the colony were identified as a diploid strain isogenic to haploid strain 22-Y-1532. The diploid strain was designated 1D-22-Y-1532.

Vegetative growth and sporulation in the haploid and diploid *P. tannophilus* strains. In order to study the origin of dissociation in the haploid and diploid strains of *P. tannophilus*, we investigated the effect of various nutrient media components on the life cycle of this yeast with respect to the occurrence and duration times of particular stages of its vegetative and reproductive processes. A comparative analysis of the haploid and diploid yeast strains on 15 different nutrient media (Table 1) allowed us to reveal some regularities (Table 2).

The haploid strain. The carbon source concentration in the medium greatly influenced the life cycle of haploid strain *P. tannophilus* 22-Y-1532, which agrees well with the data provided in [13]. The vegetative growth of this strain (without the formation of conjugated cells) was observed within the first two weeks of cultivation on media 1 and 2 at a concentration of D-glucose equal to 100 g/l. Storage of the haploid strain on these media for 1 month did not lead to its dissociation.

At carbon concentrations equal to or less than 36.5 g/l, haploid cultures more than 3 days old were found to contain conjugated cells, which then copulated. The number of such cells increased with the incubation time. The intensity of the mating process (Fig. 2a)

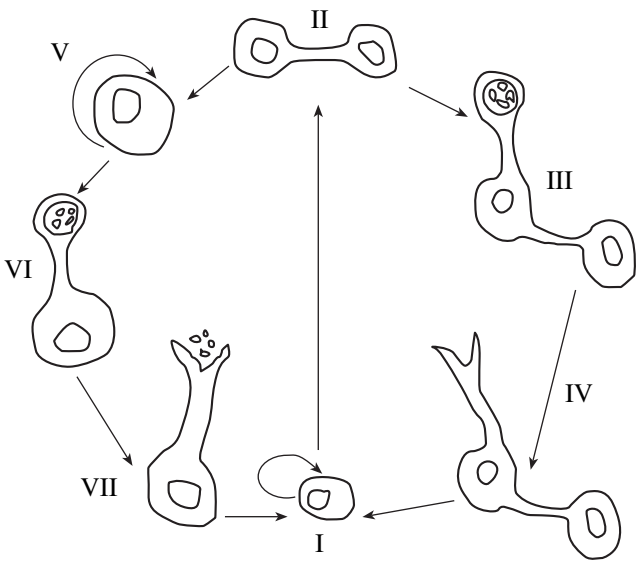


Fig. 1. The life cycle of the yeast *P. tannophilus*: (I) vegetative haploid culture, (II) copulation of haploid cells, (III) formation of conjugated asci, (IV) release of spores from conjugated asci, (V) vegetative diploid culture, (VI) formation of unconjugated asci, and (VII) release of spores from unconjugated asci.

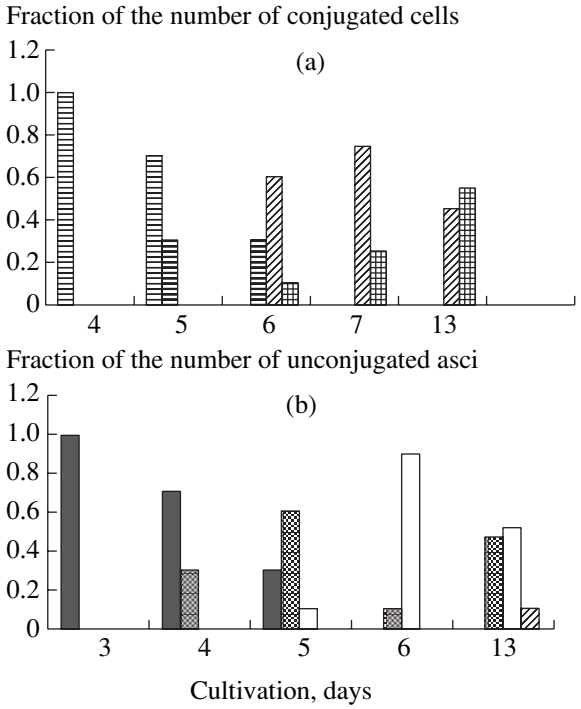
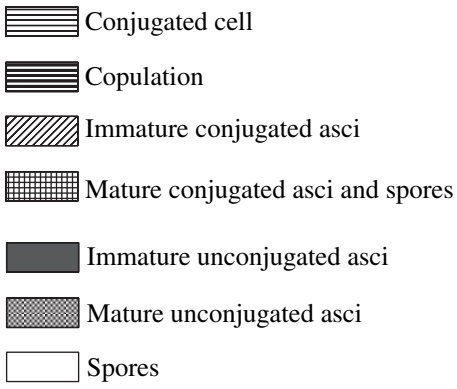


Fig. 2. Dynamics of the life cycle of (a) haploid strain 22-Y-1532 and (b) diploid strain 1D-22-Y-1532 of *P. tannophilus*. The relative error of experimental points did not exceed 5%.



depended on several factors. For instance, the addition of pentose and hexose sugars (medium 3) or the use of D-xylose as a carbon source (medium 4) lengthened the vegetative phase and retarded the formation of conjugated cells and their copulation. Carbon concentrations lower than 4 g/l (media 6 and 9) were inhibitory to the vegetative growth of the haploid strain and the sexual process.

It is known that yeast growth and cytokinesis are impossible in the absence of nitrogen and sulfur sources, which are metabolized by yeast cells more easily in the form of organic compounds with thio and amino groups than in the form of inorganic salts, for example, $(NH_4)_2SO_4$ [13]. This fact explains why the addition of peptone or amino acids to the cultivation medium (media 4 and 5) lengthened the vegetative phase of the haploid strain whereas media 10 and 12, which contained 3.5 g/l ($(NH_4)_2SO_4$ and standard concentrations of microelements and vitamins [10], favored the formation of conjugated cells and their copulation (Table 2). Phosphorus sources (K_2HPO_4 and KH_2PO_4) and inorganic nitrogen ($(NH_4)_2SO_4$, when used in excessive amounts (media 11, 13, and 14), retarded the conjugation of haploid cells but promoted spore maturation after asci had been formed (Table 2, Fig. 2a). Nitrogen deficiency (medium 15) inhibited the conjugation of haploid cells and their sporulation.

The diploid strain. Like the haploid strain, the diploid strain of *P. tannophilus* grew vegetatively for 13 days on media containing 100 g/l D-glucose (Table 2). At lower carbon concentrations, the formation of unconjugated asci began on the third day of cultivation, giving rise to unconjugated ascospores on the fifth and sixth days of cultivation (Figs. 1, 2b). An excess of $((NH_4)_2SO_4$ and phosphorus sources (medium 14) enhanced this process, whereas pentose and hexose sugars (medium 3) partially suppressed the formation of unconjugated asci. Enrichment with vitamins and microelements (medium 10) stimulated the maturation of unconjugated ascospores and the formation of spores (Table 2). On days 10 to 13 of cultivation, most of the cultures contained not only asci and spores, which are typical of diploid cells, but also conjugated asci (Fig. 2b). Therefore, these cultures were heterogeneous and contained both haploid and diploid cells. The population heterogeneity was especially profound in the case of medium 8. The addition of peptone (medium 5) and D-xylose (medium 4) as carbon sources, as well as the use of nutritionally deficient medium 15 or media lack-

Table 2. Nutrients that influence the life cycle of *P. tannophilus*

Nutrients			Cultivation time, days				
Sugar, g/l	Other nutrients		3	4	5	7	13
100.0	Yeast extract	Excess of peptone or $(NH_4)_2SO_4$					
20.0		Microelements and vitamins, excess of $(NH_4)_2SO_4$ and K_2HPO_4				#	
		Microelements, vitamins, $(NH_4)_2SO_4$			*		#
30.0		Excess of $(NH_4)_2SO_4$ and KH_2PO_4	*		*		#
4.0		Malt extract, agarose			*	*	

Notes: The complete composition of the media is described in Table 1. The media contained D-glucose as the carbon source, and agar as the solidifying agent, unless stated otherwise.

Vegetative haploid culture

Vegetative diploid culture

Conjugated cells

Conjugated asci

Mature conjugated asci and spores

Unconjugated asci

Mature unconjugated asci and spores

The asterisk and hash indicate an occurrence frequency of conjugated cells and conjugated and unconjugated asci and spores higher than 5% of the total number of cells and 10^{-4} to 10^{-3} per cell, respectively. In all other cases, this frequency was between 10^{-3} and 10^{-2}.

ing carbon sources (media 6 and 9), inhibited the dissociation of the diploid strain.

DISCUSSION

Thus, our experiments showed that the life cycle of *P. tannophilus* can be regulated by modifying the composition of the nutrient medium of this yeast. The transition of vegetative cells, irrespective of the degree of their ploidy, to the mating process was found to be induced by low concentrations of carbon sources in the cultivation medium. The effect of nitrogen and sulfur sources, which control the change of the life phases in the haploid strain but only partially stabilize the diplophase, depends on their particular forms. In contrast, an excess of microelements, vitamins, potassium, and phosphorus activates the sexual stage of the yeast. We failed to completely stabilize the sexual process in *P. tannophilus* and separate the phases of formation of unconjugated asci and the release of unconjugated ascospores (Fig. 2b). This problem was probably due to the specific natural habitat of this yeast species. Indeed, *P. tannophilus* is the only representative of the genus *Pachysolen* that has no phylogenetic relation to the other ascomycete taxa [9]. The biotope of *P. tannophilus* is limited to the bark of *Castanea vesca* and *Acacia mollissima* trees [14]. In addition to a low content of easily metabolizable hexoses, the natural substrates of *P. tannophilus* are characterized by a high content of tannins (esters of phenolcarbonic acids and polyatomic alcohols), which possess protein-denaturing properties. It can be suggested that the complex life cycle of *P. tannophilus* is due to its adaptation to a nutritionally deficient and toxic natural habitat. For comparison, yeasts of the genera *Saccharomyces* and *Shizosaccharomyces*, which are favorite objects for genetic and biotechnological studies, inhabit nontoxic sugar-rich substrates and have simple life cycles. The prevalence of the haploid cell state, which requires low amounts of macro- and microelements, as well the ability of *P. tannophilus* to rapidly change its ploidy, may provide for the high competitiveness of this yeast in its specific econiche and explain why it is difficult to obtain stable diploid cultures of *P. tannophilus.* The development of approaches yielding an increased frequency of hybrid formation and enhanced viability of spores liberated from conjugated asci [11] are necessary stages of genetic and selection studies of the yeast *P. tannophilus.*

ACKNOWLEDGMENTS

This work was supported by the Russian federal program “Integration of Science and Higher Education in Russia in 2002–2006.”

REFERENCES

1. Yablochkova, E.N., Bolotnikova, O.I., Mikhailova, N.P., *et al.*, Particular Characteristics of D-Xylose and D-Glucose Fermentation by Xylose-Assimilating Yeasts, *Prikl. Biokhim. Mikrobiol.*, 2003, vol. 39, no. 3, pp. 303–306.
2. Yablochkova, E.N., Bolotnikova, O.I., Mikhailova, N.P., *et al.*, The Activity of Xylose Reductase and Xylitol Dehydrogenase in Yeasts, *Mikrobiologiya*, 2003, vol. 72, no. 4, pp. 466–469.
3. Converti, A., Prego, P., and Dominguez, J.M., Xylitol Production from Hardwood Hemicellulose Hydrolyzates by *Pachysolen tannophilus, Debariomyces hansenii*, and *Candida guillermondii, Appl. Biochem. Biotechnol.*, 1999, vol. 88, no. 2, pp. 141–151.
4. Ergun, M. and Guneluz, U., Ethanol Production from D-Xylose by *Pachysolen tannophilus, Turk. J. Eng. Environ. Sci.*, 2001, vol. 25, no. 1, pp. 11–17.
5. Yablochkova, E.N., Shabalina, M.V., Ogorodnikova, T.E., *et al.*, Morphological Heterogeneity and Particular Characteristics of the Life Cycle of the Yeast *Pachysolen tannophilus, Mikrobiologiya*, 1994, vol. 63, no. 6, pp. 1058–1063.
6. Zakharov, I.A., Kozhin, S.A., *et al.*, *Sbornik metodik po genetike drozhzhei-sakharomitsetov* (Methods of the Genetics of Saccharomycetes), Leningrad: Nauka, 1984.
7. Inge-Vechtomov, S.G., New Genetic Lines of the Yeast *Saccharomyces cerevisiae, Vestn. Leningr. Univ., Ser. Biol.*, 1963, vol. 21, no. 4, pp. 117–129.
8. Ogorodnikova, T.E., Mikhailova, N.P., Yablochkova, E.N., *et al.*, Growth Parameters of the Xylose-Assimilating Yeasts *Pachysolen tannophilus* and *Candida shehatae, Mikrobiologiya*, 1995, vol. 64, no. 1, pp. 13–17.
9. *The Yeasts: A Taxonomic Study*, 3rd ed., Kreger-Van Rij, N.J.W., Ed., Amsterdam: Elsevier, 1984.
10. Burkholder, P.R., Vitamin Deficients in Yeast, *Am. J. Bot.*, 1943, vol. 30, p. 206.
11. James, A.P. and Zahab, D.M., The Construction and Genetic Analysis of Polyploids and Aneuploids of the Pentose-Fermenting Yeast *Pachysolen tannophilus, J. Gen. Microbiol.*, 1983, vol. 129, pp. 2489–2494.
12. Glotov, N.V., Zhivotovsky, L.A., Khovanov, N.V., and Khromov-Borisov, N.N., *Biometriya* (Biometry), Leningrad: Leningr. Gos. Univ., 1982.
13. Berry, J., *Biologiya drozhzhei* (The Biology of Yeasts), Moscow: Mir, 1985.
14. Boidin, J. and Adzet, J., Deux curieuses levuses isolees d'extraits tannants d'origine vegetale: *Pachysolen* (nov. gen.) *tannophilus* nov. sp. et *P. pelliculatus* nov. sp., *Bull. Soc. Mycol. France*, 1957, vol. 73, pp. 331–342.