ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 4, pp. 453–458. © Pleiades Publishing, Ltd., 2013.
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EXPERIMENTAL ARTICLES

Molecular Identification of the Industrially Important Strain *Ogataea parapolymorpha*

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Received August 23, 2012

Abstract—The thermotolerant strain 1-IR was isolated as a contaminant microflora of the industrial strain "Red" (the Netherlands) during the study of the baker's yeast *Saccharomyces cerevisiae*. The strain was assigned to the species *Ogataea parapolymorpha* by sequencing the 26S rDNA D1/D2 domain. The strain 1-IR was shown to be capable of efficient glucose and xylose fermentation at an elevated temperature of 45°C. In this respect, the strain 1-IR surpassed the thermotolerant yeasts *O. polymorpha* CBS 4732, NCYC 495, and *O. parapolymorpha* DL1. The prospects of using the *O. parapolymorpha* yeasts as producers of biofuel from lignocellulose wastes of agricultural and woodworking industries is discussed.

Keywords: sibling species of *Ogataea angusta, O. parapolymorpha*, and *O. polymorpha*; 26S rDNA D1/D2; thermotolerance; ethanol fuel; glucose and xylose fermentation

DOI: 10.1134/S0026261713030090

The nonconventional, industrially important yeast *Ogataea/Hansenula polymorpha* is an object of intensive genetic and molecular biology studies [1–11]. Its thermotolerance, methylotrophy, and active fermentation of glucose and xylose are attractive. The interest in the study of these yeasts as potential producers of ethanol fuel from lignocellulose wastes of agriculture and woodworking industry has grown in recent years [7, 12, 13]. Conversion of lignocellulose into ethanol usually proceeds in two steps. During saccharification, the feedstock is treated with cellulases and hemicellulases at an elevated temperature (45–50°C) optimal for the functioning of hydrolytic enzymes. The second step is microbiological fermentation of the sugars from lignocellulose hydrolysates into ethanol. The economically sound combination of the processes of hydrolysis and fermentation requires thermotolerant strains [14].

The taxonomic position of methylotrophic yeasts has been frequently revised and their genus and species status has been reconsidered. Based on the 18S and 26S rRNA gene sequencing, methylotrophic yeasts have been attributed to three novel genera: *Ogataea*, *Kuraishia*, and *Komagataella* [15, 16]. The data of genetic analysis demonstrated the heterogeneity of the *O. polymorpha* taxon combining several closely related sibling species [17–19]. The subsequent phylogenetic analysis of molecular markers confirmed the existence of this complex of biological species [20–23]. At present, three closely related species have been described: *O. polymorpha* (syn. *O. thermophila* Peter et al. [24]), *O. angusta*, and *O. parapolymorpha*. The positions of cactus isolates [25–27] showing the lower level of DNA–DNA reassociation with the type culture *O. angusta* CBS 7073 and the type strain *Candida parapolymorpha* NRRL Y-7560 (64 and 72%, respectively) is still not quite clear [28]. The latter strain was isolated from polluted river silt and water and is an anamorph of the yeast *O. parapolymorpha*. The cactus strains are genetically isolated from the species *O. angusta* and *O. polymorpha* and differ from them karyotypically and in the UP-PCR profiles [19].

The goal of the present work was molecular genetic identification of the industrially important strain *O. parapolymorpha* 1-IR. We have shown that this strain is capable of efficient fermentation of the principal sugars of plant biomass lignocellulose hydrolysates at elevated temperatures.

MATERIALS AND METHODS

Strains and media. The strains studied in this work are presented in Table 1. The species testers of the *Saccharomyces* yeasts were monospore cultures of the following strains: *S. cerevisiae* X2180-1A, *S. arboricola* CBS 10644, *S. bayanus* VKM Y-1146, *S. cariocanus* UFRJ 50816, *S. kudriavzevii* NBRC 1802, *S. mikatae* NBRC 1815, and *S. paradoxus* CBS 432. The yeasts

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Table 1. Origin of the yeast strains under study

Strain, species name		Source and site of isolation
Original strain number	Number in other collections	
Ogataea angusta		
CBS 7073 (T)	NRRL Y-2214	*Drosophila pseudoobscura*, USA
Ogataea parapolymorpha		
NRRL YB-1982 (T)	CBS 12304	Insect frass, quaking aspen, USA
DL1	NRRL Y-7560 = ATCC 26012	Polluted river silt and water, USA
1-IR		Wild microflora of the bakers strain "Red", Ukraine
Ogataea polymorpha		
CBS 4732 (T)	NRRL Y-5445	Soil, Brazil
NCYC 495	CBS 1976 = NRRL Y-1798	Spoiled orange juice, USA
Saccharomyces cerevisiae		
VKM Y-381	Race XII	Distillers yeast

Abbreviations: the All-Russian Collection of Microorganisms, Moscow (VKM); American Type Culture Collection, Manassas, United States (ATCC); Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands (CBS); National Collection of Yeast Cultures, Norwich, England (NCYC); Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA (NRRL). T is the type culture.

were cultivated on YPD (0.5% yeast extract, 1% peptone, 2% glucose) or minimal YNB (Yeast Nitrogen Base) media (Difco, United States) without amino acids (0.67%); 2% glucose, 2% xylose, or 1% methanol were used as a carbon source. The strain NCYC 495 marked with the *leu1-1* auxotrophic mutation was cultivated on the minimal medium with leucine (40 mg/L).

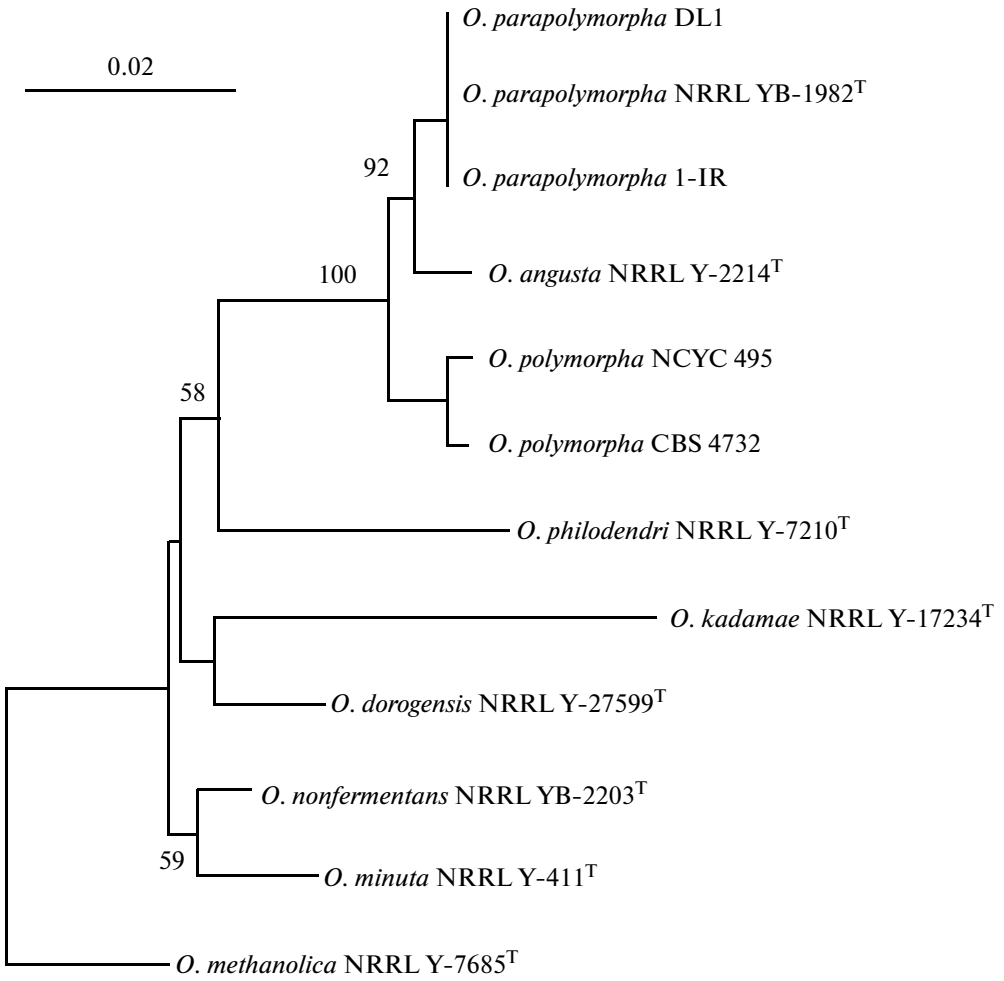
The yeast biomass for alcoholic fermentation of glucose and xylose was grown in YPD/YPX medium (1% yeast extract, 2% peptone, 4% glucose/xylose) for 1–2 days on an orbital shaker (200 rpm) at 41°C. Cell biomass (2 mg/mL) was transferred into the minimal medium with 10% glucose or 12% xylose. Alcoholic fermentation was performed on a shaker at 45°C under limited aeration conditions (140 rpm). Ethanol concentration in the medium was determined using an Alcotest kit [29]. The biomass was assayed by turbidimetric analysis on a FEK-56M photoelectrocolorimeter (3-mm cuvette, light filter no. 6) using gravimetric calibration. All experiments were performed in triplicate.

Polymerase chain reaction was performed in a Tercyc DNA amplifier (DNA-Technology, Russia) directly on the yeast cells. A small amount (on the tip of a microbiological loop) of yeast biomass was suspended in 30 μL of the buffer containing 3 mM $MgCl_2$, 0.3 mM dNTP, and 50 pmol of each primer. The resultant mixture was incubated at 95°C for 15 min for cell lysis, followed by the addition of 2.5 U *Taq* polymerase (Syntol, Russia). The 26S rDNA D1/D2 domain and the 5.8S-ITS fragment were amplified using the following pairs of primers: NL-1/NL-4 (GCATATCAATAAGCGGAGGAAAG and GGTCCGTGTTTCAAGACGG), ITS1/ITS4 (5'-TCCGTAGGTGAACCTGCGG and 5'-TCCTCCGCTTATTGATATGC). PCR (30 cycles) was performed as follows: denaturing at 94°C, 45 s; annealing of the primers at 52°C, 30 s; DNA synthesis at 72°C, 120 s. Amplification products were exposed to electrophoresis in 1% agarose gel at 60–65 V in 0.5× TBE buffer (45 mM Tris, 10 mM EDTA, 45 mM boric acid) for 1.5 h and stained with ethidium bromide.

Sequencing and phylogenetic analysis. The nucleotide sequences of the D1/D2 domain were determined by two chains using the Sanger sequencing method on a Beckman Coulter automated sequencer (United States). The homology with the known nucleotide sequences was searched with the BLAST software. Multiple nucleotide alignment for the obtained and previously known sequences was performed manually with the BioEdit software package. The phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA5 [30]. The bootstrap values for statistical reliability of grouping were determined for 1000 pseudoreplicas.

RESULTS

Isolation and identification. The analyzed strain 1-IR was isolated as a wild microflora of industrial baker's yeast *Saccharomyces cerevisiae* "Red" (the Netherlands) obtained from the Lvov yeast plant. Plating and cultivation of the "Red" strain on a complete agarized medium at 42°C showed the presence of colonies containing the cells which were smaller than the typical cells of *Saccharomyces* yeasts. The contaminant strain 1-IR was able to grow at higher temperatures (up to 48°C), as well as on the media with xylose



Phylogenetic analysis of nucleotide sequences of the 26S rDNA D1/D2 domain of some species of the genus *Ogataea*. The D1/D2 sequence of the *O. methanolica* type culture was used as an outgroup. Bootstrap values are >50%. The scale corresponds to 20 nucleotide substitutions per 1000 nucleotide positions. T is the type culture.

and methanol, which is atypical of the yeast *Saccharomyces*.

At present, the genus *Saccharomyces* comprises seven species: *S. cerevisiae*, *S. arboricola*, *S. bayanus*, *S. cariocanus, S. kudriavzevii, S. mikatae*, and *S. paradoxus* [31]. Phenotypically indistinguishable *Saccharomyces* species can be differentiated by sequencing or restriction analysis of the 5.8S-ITS region including the 5.8S rRNA gene and internal transcribed spacers ITS1 and ITS2 [32, 33]. The 5.8S-ITS fragments of strain 1-IR and seven species testers of *Saccharomyces* were amplified. The PCR products were of the same size in all species testers of *Saccharomyces*: approximately 850 bp, while the 5.8S-ITS fragment of the strain 1-IR was about 750 bp (the figure not shown). This confirmed that the latter strain was not a member of the genus *Saccharomyces*.

In the modern taxonomy of yeasts, the species affiliation of strains is determined by the 26S rDNA D1/D2 domain sequencing [34]. The GenBank database of nucleotide sequences of this rDNA region is used to define the taxonomic position of new strains. To identify the species affiliation of the strain 1-IR, we sequenced the 26S rDNA D1/D2 domain. The obtained nucleotide sequence was compared with the D1/D2 sequences from the GenBank database. According to the analysis performed, the strain 1-IR falls into the species *O. parapolymorpha*. The phylogenetic tree was constructed on the basis of comparative analysis of D1/D2 nucleotide sequences (figure). Apart from *O. polymorpha* (CBS 4732, NCYC 495), *O. angusta* (CBS 7073) and *O. parapolymorpha* (NRRL YB-1982, DL1, 1-IR), our analysis also included the type cultures of *O. philodendri*, NRRL Y-7210, *O. kadamae* NRRL Y-17234, *O. dorogensis* NRRL Y-27599, *O. nonfermentans* NRRL YB-2203, and *O. minuta* NRRL Y-411. The choice of the five latter species was determined by their closeness to the type culture of *O. parapolymorpha* on the phylogenetic tree presented in the work [22]. The type culture *O. methanolica* NRRL Y-7685 remotely related to the *O. polymorpha* complex was used as an outgroup.

Table 2. Productivity of ethanol synthesis (mg ethanol/g yeast biomass/h) by the yeast *O. paraqpolymorpha* (1-IR, DL1), *O. polymorpha* (NCYC 495, CBS 4732), and *S. cerevisiae* VKM Y-381

Strain	Ethanol synthesis (mg/g/h)	
	Xylose	Glucose
1-IR	10.9 ± 0.5	393.7 ± 21.1
DL1	7.8 ± 0.4	331.9 ± 15.5
NCYC 495	9.8 ± 0.5	268.7 ± 14.2
CBS 4732	5.6 ± 0.3	192.2 ± 10.1
VKM Y-381	0	142.7 ± 7.1

Three clusters can be seen on the phylogenetic tree (figure). The first one with a 100% reliability comprises the yeasts *O. polymorpha, O. angusta*, and *O. parapolymorpha*, including the strain 1-IR. The type culture of *O. philodendri* joins this cluster with low statistical support (58%). The type cultures of the species *O. kadamae, O. dorogensis*, *O. nonfermentans*, and *O. minuta* are grouped in pairs in the second and third cluster, respectively. It should be noted that the statistical support of the latter two clusters is extremely low: less than 50 and 59%, respectively. The data of phylogenetic analysis show the distant relatedness between the yeasts *O. philodendri, O. kadamae, O. dorogensis, O. nonfermentans, O. minuta*, and the species of the *O. polymorpha* complex.

Biochemical characteristics of the strain 1-IR. At present there are 15 known *O. parapolymorpha* strains of different ecological and geographical origin within the United States [22]; however, their industrially important physiological characteristics have not been investigated. The two strains of *Candida parapolymorpha* (ATCC 26012, ATCC 58401) that can grow at 45°C are a somewhat exceptional in this respect [21, 35].

We investigated the fermentation activity of the strain 1-IR during alcoholic fermentation of xylose and glucose. The following yeast strains were taken as the controls: *O. polymorpha* (NCYC 495 and CBS 4732), *O. parapolymorpha* DL1, and the distiller's yeast *S. cerevisiae* VKM Y-381 (Table 1). Alcoholic fermentation was performed in a liquid minimal medium with 12% xylose or 10% glucose at 45°C (Table 2). The productivity of ethanol synthesis during alcoholic fermentation of xylose was the highest in the strain 1-IR (10.9 mg/g/h) and the lowest in the strain *O. polymorpha* CBS 4732 (5.6 mg/g/h). At the same time, the strain *S. cerevisiae* VKM Y-381 did not ferment xylose. More substantial differences between the strains were revealed in the experiments on alcoholic fermentation of glucose (Table 2). The productivity of ethanol synthesis by strain 1-IR was 1.2-, 1.5- and 2-fold higher than in the control strains DL1, NCYC 495 and CBS 4732, respectively. Ethanol synthesis by the distiller's yeast *S. cerevisiae* VKM Y-381 during glucose fermentation was the lowest, probably due to the elevated fermentation temperature.

Thus, the strain 1-IR surpassed all of the control strains in the efficiency of alcoholic fermentation of xylose and glucose at 45°C.

DISCUSSION

In recent years, the interest in ethanol fuel production from renewable plant raw materials as an alternative to nonrenewable sources of energy (oil and gas) has been growing worldwide. Alcohol production from starch-containing (rye, wheat, potato, maize) and sacchariferous (sugarcane, sugar beet) raw materials is based on fermentation using the conventional yeast *S. cerevisiae*. As of today, ethanol fuel is competitively produced from sugarcane in Brazil and from starch-containing raw materials in the United States. However, the available amounts of the starting plant feedstock do not meet the growing demand for bioethanol and may lead to grain deficit and price rise. The renewable and inedible raw materials, i.e., hydrolysates of lignocellulose wastes of agriculture and woodworking industry, seem to be the most optimal and promising sources for ethanol biofuel production. The main component of lignocellulose hydrolysates (besides glucose) is xylose. The yeast *S. cerevisiae* cannot assimilate and especially ferment xylose. In a number of works, it was attempted to construct *S. cerevisiae* strains capable of alcoholic fermentation of xylose [36]. However, as yet no substantial progress has been made in this field because the productivity of constructed strains is still too low for profitable ethanol production. Moreover, *S. cerevisiae* is a mesophile. Therefore, it is relevant to search for microorganisms capable of high-temperature alcoholic fermentation of xylose and glucose.

The contaminant strain 1-IR retrieved during the biochemical analysis of the baker's yeast *S. cerevisiae* was identified as *O. parapolymorpha*. This strain was shown to be capable of effective fermentation of the principal sugars of plant biomass lignocellulose hydrolysates at elevated temperatures. Moreover, these parameters of the strain 1-IR surpass those of the yeasts *O. parapolymorpha* DL1, *O. polymorpha* CBS 4732 and NCYC 495; the latter two strains were used in construction of the yeasts capable of active xylose fermentation at elevated temperatures [7, 12, 13]. Thus, the yeast strain 1-IR is promising for genetic manipulations aimed at creating competitive strains for the technology of simultaneous saccharification and fermentation of lignocellulose.

As we have already mentioned, at present there are few known *O. parapolymorpha* strains, probably because this species has been described only recently [22]. The closely related species of the *O. polymorpha* complex are phenotypically similar and cannot be differentiated on the basis of the standard morphological

and physiological tests. It cannot be excluded that in many yeast collections the *O. parapolymorpha* strains are stored under the species names of *O. polymorpha* and/or *O. angusta*. Molecular analysis of collection yeasts of the two latter species may reveal the new strains of *O. parapolymorpha* capable of efficient fermentation of the principal sugars of plant biomass at elevated temperatures.

ACKNOWLEDGMENTS

The work was supported by the Russian–Ukrainian joint grant of the Russian Foundation for Basic Research (no. 11-04-90450) and the National Academy of Sciences of Ukraine (no. F40.4/006). The synthesis of oligonucleotide primers and the 26S rDNA D1/D2 domain sequencing were performed using the equipment of the Center for Collective Use of the State Research Institute for Genetics and Selection of Industrial Microorganisms, with partial financial support of the Ministry of Education and Science of the Russian Federation (Federal Contract no. 16.552.11.7029). The authors are grateful to V.I. Kondratieva (Moscow) and T.B. Luzhetsky (Lviv) for their attention to this work.

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Translated by E. Makeeva