Comparative morpho-physiological analysis and mutagenesis of some methylotrophic yeast strains of biotechnological interest

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Abstract

The yeasts examined in this study were assigned to certain species on the basis of their overall physiological and morphological properties.

The aim of this study was a comparative analysis regarding some basic morpho-physiological properties of several strains of methylotrophic yeasts from different culture collections or newly isolated. belonging to various species of biotechnological interest: Hansenula polymorpha, Candida boidinii, Pichia pastoris, Pichia (Candida) guilliermondii,

The morphological traits were detected by direct analysis of cultures on liquid and solid media and by examination of the microscopical appearance. Physiological tests were performed for assessing the ability to use various organic compounds as sole carbon source under aerobic conditions, to grow at non-permissive temperatures and on media with different methanol concentrations.

The morpho-physiological traits revealed the newly isolated yeast species as belonging to Hansenula polymorpha.

H. polymorpha NOR 10 was subjected to mutagenesis in order to obtain mutants with modified cellular wall and there were obtained 10 mutants: 5 diploid types and 5 haploid types.

Keywords: methylotrophic yeasts, morpho-physiological analysis, N-methyl- N'-nitro-N– nitrosoguanidine

Introduction

Yeasts are the world's premier industrial microorganism. They participate to the obtainment of foods, beverages, pharmaceuticals and in many other biotechnologies. Therefore it is essential to determine and understand the main aspects of their physiology.

Only a few yeast species are able to use methanol as sole energy and carbon source as they present a specific methanol degrading pathway. They belong to four genera: *Candida, Hansenula, Pichia* and *Torulopsis.* Following induction with methanol, key enzymes of this pathway, including methanol oxidases and formate dehydrogenase, are produced at high levels. [3].

The first enzyme involved in yeast methanol metabolism, alcohol oxidase (AOD), is localized in peroxisomes. AOD oxidizes methanol to formaldehyde, and the formaldehyde is subsequently fixed to xylulose 5-monophosphate through the intervention of peroxisomal dihydroxyacetone synthase or subjected to direct formaldehyde oxidation in the cytosol. Formaldehyde seems to be oxidized to form in a glutathione-dependent or glutathione-independent manner. The last reaction involved in the methanol dissimilation pathway is catalyzed by NAD 1-dependent formate dehydrogenase (FDH) [2; 6; 11].

The methylotrophic yeast *Hansenula polymorpha (H. polymorpha), Pichia pastoris (P.pastoris)* and *Candida boidinii (C. boidinii*) were developed as production systems for recombinant proteins. The particularly advantageous characteristics of this species generated a large number of biotechnological applications. *H. polymorpha* and *P. pastoris* especially are the systems of choice for heterologous gene expression in yeast. Recent advances in the development of these yeasts as heterologous proteins producing strains offer a multitude of applications, and methods.

The first methylotrophic yeast was reported in 1969, and since then those yeasts were subject to the constant attention of the biotechnological industry, for different reasons, one of them being the production of "single-cell proteins" [5;10]. The interest in the production of single cell protein (SCP) from methanol intensified the studies of those specific strains in order to explore their possible commercial applications and to study the specific cell compartments namely peroxisomes. *P. pastoris* for example is a very efficient system for the easy production of many recombinant proteins, due to the presence of a promoter derived from the alcohol oxidase gene perfect for the controlled expression of exogenes, to the similarity of techniques for the genetic manipulation of *P. pastoris* and *Saccharomyces cerevisiae*, to the preference of *P. pastoris* for respiratory growth, a key physiological facilitating its culturing at high-cell densities [5; 7; 8].

Pharmaceuticals produced employing methylotrophic hosts are expected to enter the market in the near future, directly competing with the traditional *S. cerevisiae*. The special features of *H. polymorpha* led to new applications by introduction of several genes of a pathway in a fixed genes dosage ratio which generates strains able to co-produce enzymes at elective stoichiometric levels. [3; 8]

The aim of this work resides in investigating the most important morpho-physiological characteristics of new indigenous methylotrophic yeast strains and also in mutagenesis of some of the new isolates performed in order to obtain cell wall mutants suitable for an easy extraction of DNA and various genetic engineering applications intended.

Materials and methods

The yeast strains used for the present study were: *Hansenula polymorpha NCYC 495, Hansenula polymorpha NOR 9, Hansenula polymorpha NOR 10, Hansenula polymorpha NOR 11, Hansenula polymorha NOR 12, Candida boidinii ICCF, Candida boidinii SM 78, Candida boidinii M57, Pichia pastoris, Pichia guilliermondii.* The yeasts were stored at –70^{o}C. The protective media was YPG supplemented with 20% glycerol, pH = 5.5- 6 (Yeast Peptone Glucose – yeast extract- 5 g/L, peptone10 g/L, glucose 20 g/L, for solid medium plus 20g agar agar) to which it was added methanol 1%, sterilized through millipore filters, according to the experimental design. The methylotrophic yeast strains were isolated from fermenting apple pulp. The sample was inoculated in 5mL minimal liquid medium supplemented with 0.5% methanol and incubated at room temperature for 10 days. From the resulting culture 50µL were transferred on the same medium and incubated at 28°C in a shaker. Single colonies were isolated and purified on minimal agar medium supplemented with methanol. After 4 days of incubation at 28°C four colonies were chosen to be investigated.

It is known that yeast strains isolated from different ecological bays present resistance of the cell wall at lytic enzyme thus obtaining some mutants with modified cellular wall allow an easier isolation of genomic DNA from the cell for molecular analysis. For obtaining mutants with modified cellular wall we used the *H. polymorpha NOR 10* strain both in diploid and haploid condition.

N-methyl- N$^{'}$-nitro –N- nitrosoguanidine (NTG- SIGMA) was used as mutagen, 1mg/mL in buffer phosphate solution (Na_2HPO_4 x $2H_2O$ – 11.876 g/L; KH_2PO_4 – 9.078 g/L, pH = 7.4).

Morphological and microscopical characteristics

The macroscopical appearance of the colony was observed after cultivating the yeast strains in Petri dishes with solid medium (YPGA - Yeast Peptone Glucose Agar 20 g/L), with 48 hours incubation at 28°C and 37°C. From the same growing culture yeasts were inoculated in flasks with YPG medium, and the morphology of the cells was examined microscopically.

Physiological characterization

Biochemical analyses were performed according to Barnett [1].

1. Assimilation tests

In order to test the ability to use various compounds as sole carbon source for aerobical growth (assimilation), the auxanograme method was used. Yeast strains were cultivated on solid YNB medium without amino acids (Yeast Nitrogen Base DIFCO) on which D-glucose, sucrose, D-ribose, rhamnose, maltose, trehalose, D- glucose amine were spotted. Petri dishes were incubated at 28°C and 37°C for 6 days. The results were recorded by observing the growth comparatively to a positive control (YNB with glucose) and a negative control (YNB with no carbon source). The yeast (inoculum) were cultivated in YPG liquid medium at 28°C and 37°C on a shaker at 200rpm for 18-20h .The inoculum represented 3.5% from the final volume of YNB medium [4;9].

2. Ability to grow on media with different methanol concentrations

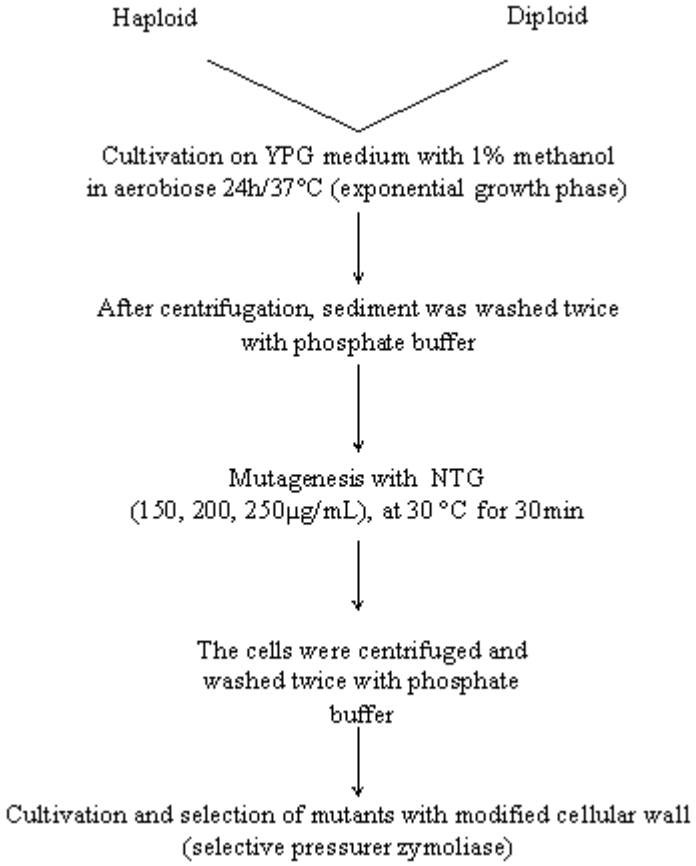
The yeast strains were cultivated on a special methanol medium (H_3BO_3- 50mg, $CuSO_4$ H_2O- 4mg, IK- 10mg, $FeCl_3$ x $6H_2O$ - 20mg, $MnSO_4$ x H_2O – 40 mg, $ZnSO_4$ x $7H_2O$ – 40mg, $(NH_4)_6MoO_7O_{24}$ x $4H_2O$ - 20mg, glucose - 1g, $(NH_4)_2SO_4$ - 5g, KH_2PO_4 – 2g, Na_2HPO_4 - 1g, $MgSO_4$ x $7H_2O$ – 0.5g, $CaCl_2$ – 0.1g, yeast extract – 4g, agar - 20g, for 1000 mL total volume, pH 6). Methanol was added in the following concentrations: 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%. Petri dishes were incubated at 28°C and 37°C for 6 days.

3. Ability to grow at non-permissive temperatures

Growth at non-permissive temperatures was examined at 25^{o}C, 42^{o}C, comparatively with the same strain cultured at its optimal growth temperature (28^{o}C/ 37^{o}C).

Mutagenesis

Mutagenesis was performed according to the following experimental design:



Mutant of *H. polymorpha* was prepared by using N-methyl-N'-nitro-N-nitrosoguanidine as mutagen. A culture of wild type was grown on YPG until the middle on the exponential growth phase. NTG was then added to a final concentration 150-200-250µL and the culture was further incubated at 37°C for 30min. The cells were then centrifuged, washed twice with buffer solution and resuspended in the same solution. Samples of appropriately diluted suspension were plated on YPG. After the plates have been incubated for 2-5 days at 37°C, the most suitable ones were chosen for replica-plating on YPG with zymoliase (100-400µg/mL). This replica plates were incubated for 3-4 days at 37°C and then compared. Presumptive mutant were selected and streaked on fresh for further examination and single- colony isolation.

Results and discussions

Morphological and microscopical characteristics

The observations made on the microscopical appearance and macroscopical aspects (Figure.1) were made on *H. polymorpha NOR 9, H. polymorpha NOR 10, H. polymorpha NOR 11, H. polymorpha NOR 12* comparatively with yeast reference strains (*H. polymorpha NCYC 495*).

H. polymorpha NCYC 495 formed white or cream white small, and round colonies; cells exhibited multilateral budding; the yeast formed evanescent asci, containing 1 to 4 hat-shaped ascospores (Fig 1a). *H. polymorpha NOR 9* was almost similar to *H. polymorpha NCYC 495;* it developed evanescent asci, containing 1 to 4 hat-shaped ascospores (Fig 1b). *H. polymorpha NOR 10, H. polymorpha NOR 11* and *H. polymorha NOR 12* were similar to *H. polymorpha NOR 9*; both had cells with multilateral budding (Fig 1c, 1d, 1e). *C. boidinii ICCF* formed white or cream white small colonies, its cells showed multilateral budding, and formed simple or elaborate pseudohyphae (Fig 1f). *C. boidinii, SM 78* and *C. boidinii M 57* were almost similar to *C. boidinii ICCF* cells, with multilateral budding, simple or elaborate pseudohyphae (Fig. 1g, 1h). *P. pastoris* formed white or cream white small colonies, had cells with multilateral budding, no filaments, and evanescent asci, containing 1 to 4 hat-shaped ascospores (Fig 1i). *P. guilliermondii* formed white or cream white small and round colonies, cells exhibited multilateral budding, it had no filaments or elaborate pseudohyphae, and developed evanescent asci, containing 1 to 4 hat-shaped ascospores (Fig 1j).

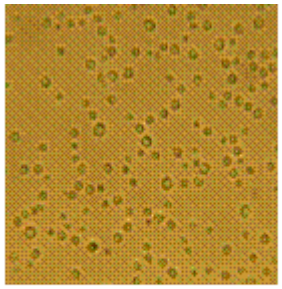


Fig .1a
Hansenula polymorpha NCYC 495

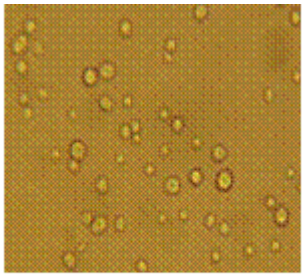


Fig .1b
Hansenula polymorpha NOR 9

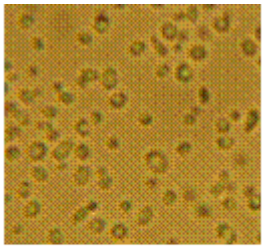


Fig .1c
Hansenula polymorpha NOR 10

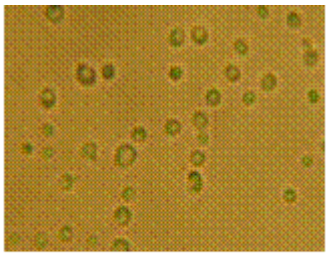


Fig.1d
Hansenula polymorpha NOR 11

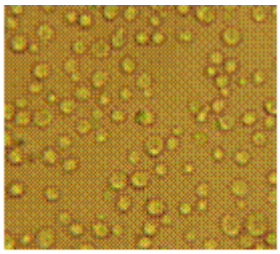


Fig. 1e
Hansenula polymorpha NOR12

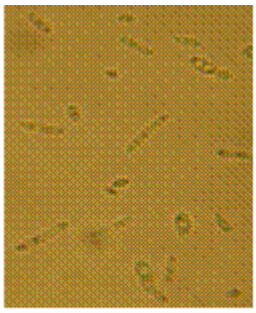


Fig. 1f
Candida boidinii ICCF

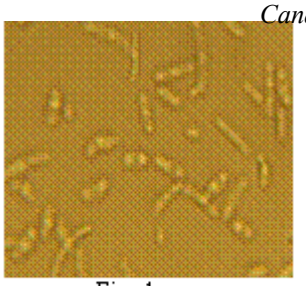


Fig. 1h
Candida boidinii M 57

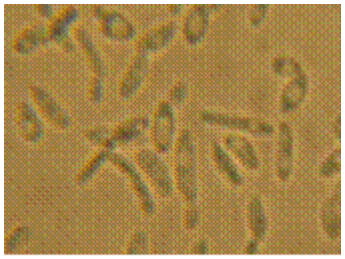


Fig. 1g
Candida boidinii SM 78

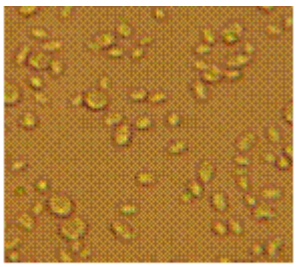


Fig. 1i
Pichia pastoris

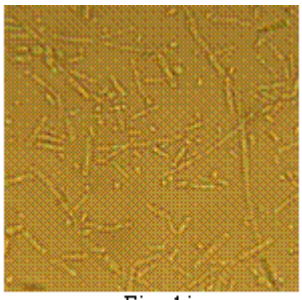


Fig. 1j.
Pichia guilliermondii

Figure 1. Microscopical appearance of yeast strains (wet mount preparation; 40 objectiv)

Physiological characterization

Assimilation tests

The ability to use different carbon sources for aerobic growth (Table 1) led to the following results: *H. polymorpha NCYC 495*, *H. polymorpha NOR 9, H. polymorpha NOR 11* and *H. polymorpha NOR 12* showed great similarity among each other, assimilating D-glucose, sucrose, D-ribose, maltose, trehalose, while *H. polymorpha NOR 10* did not grow on trehalose. *C. boidini ICCF*, *Candida boidinii SM 78, Candida boidinii M57* assimilated D-glucose and D-ribose. *P. guilliermondii* grew on almost all media, but least on D-glucose. On the contrary, *P. pastoris*, assimilated only D-rhamnose and showed a variable response on trehalose.

Table 1 Results of the assimilation tests for yeast strains

Strain	D-glucose	Sucrose	D-Ribose	D-Rhamnose	Maltose	Trehalose	D- Glucose amine
Hansenula polymorpha	+	+	+	V	+	+	–

NCYC 495							
Hansenula polymorpha NOR 9	+	+	+	V	+	+	_
Hansenula polymorpha NOR 10	+	+	+	- (V)	+	_	_
Hansenula polymorpha NOR 11	+	+	+	+ (V)	+	+	-
Hansenula polymorh NOR 12	+	+	+	V	+	+	_
Candida boidinii ICCF	+	-	+	-	-	-	V
Candida boidinii SM 78	+	-	+	-	-	-	V
Candida boidinii M57	+	-	+	-	-	-	V
Pichia pastoris	-	-	-	+	-	V	-
Pichia guilliermondii	-	+	+	V	+	+	+

+ presence of growth; - absence of growth; V – variable response

Ability to grow on media with different concentrations of methanol

We tested the ability of the yeasts strains to grow at different methanol concentrations: from 1.5% to 5.0%. All strains grew equally well on all of the tested methanol solid media.

Ability to grow at non-permissive temperatures

All five *H. polymorpha* strains were able to grow at 42°C, but no growth was recorded at 25^{o}C; while for *C. boidinii* and *P. pastoris* no growth was recorded at 25^{o}C. *P guilliermondii* grew very weakly at 42°C, and not at all at 25^{o}C.

Mutagenesis

The first step of the mutagenesis process consisted in verifying the natural variability of the parental strain *H. polymorpha NOR 10*. Adequate dilutions from cultures in stationary growth phase were subcultured on YPG medium in Petri dishes for obtaining isolated colonies. After 2 days of incubation at 37° C and 3 days at room temperature 2 types of colonies were observed - small colonies of 1.5-2mm diameter typical for the haploid form and big colonies of 5 mm diameter, typical for the diploid form.

There were examined 662 colonies: haploid, those were round, cream white, convex and glossy and diploid pinkish-cream colonies, with grooves and concentric, peripheral circles. One typical haploid and one typical diploid colony were used for mutagenesis.

As fig. 1 shows, both forms of *H. polymorpha NOR 10* present 90% lethality at 150µg/mL NTG; at 200, and 250 µg/mL respectively the viability remained unchanged (Fig.2).

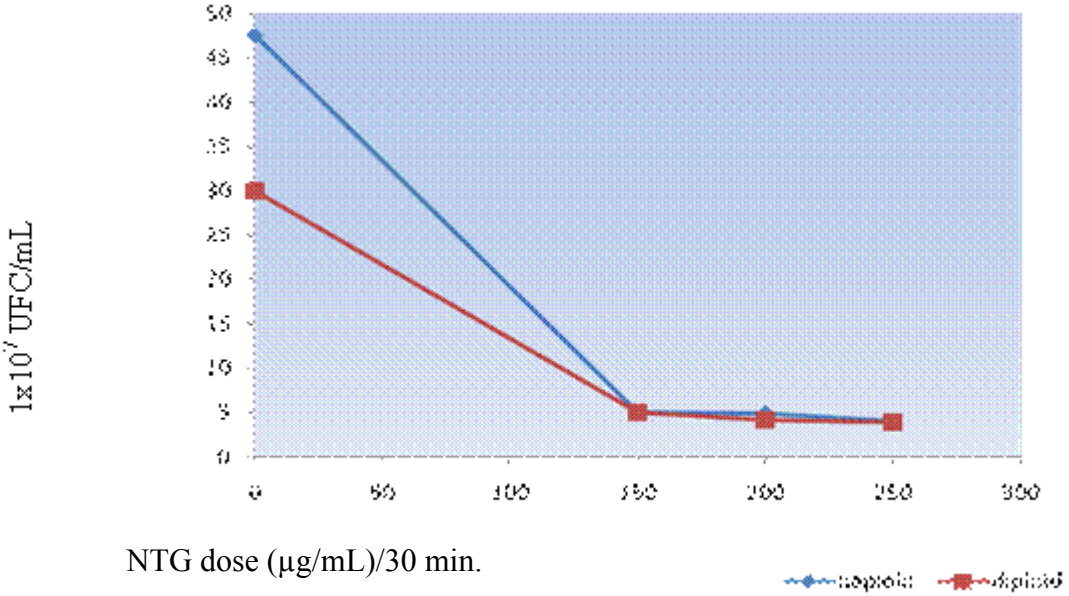


Figure 2. Mutagenesis with variable doses of NTG (*H. polymorpha* haploid and diploid forms)

The results indicate that among the haploid type surviving colonies 1.8-3.5% were mutants, while for the diploid type the mutants percentage was between 1.8 and 6.38%.) (table 2).

Table 2 Mutants of *H. polymorpha NOR 10* (150,200,250 µg NTG/mL)

NTG dose (µg/mL)	Haploid			Diploid		
	No. of checked colonies	No. of isolated mutants	Percent of mutants	No. of checked colonies	No. of isolated mutants	Percent of mutants
150	63	2	3.17	63	1	1.58
200	56	2	3.57	44	1	2.27
250	53	1	1.88	47	3	6.38

Following mutagenesis three types of colonies were obtained: colonies characteristic for the haploid type, colonies characteristic for the diploid type and also *petite colonies*. Through replica platting between 17-37 colonies for each dose were analyzed and it was isolated a variable number of mutants (table 3).

Table 3 Selection of clones with modified cellular wall using zymoliase (100-400 µg/mL)

Zymoliase µg/mL	Haploid form			Diploid form		
	150 µg/mL NG	200 µg/mL NG	250 µg/mL NG	150 µg/mL NG	200 µg/mL NG	250 µg/mL NG
400	32	32	29	26	17	19
100	31	24	24	37	277	28

In the next stage we passed to the selection of clones with modified cellular wall using zymoliase as selective pressurer in 100-400 µg/mL concentration. We isolated 10 mutants: 5 haploid and 5 diploid.

Conclusions

Identification of the strains isolated from fermenting apple pulp was achieved through morpho-physiological studies. The isolates belong to the *Hansenula polymorpha* species.

In order to obtain *H. polymorpha NOR 10* mutants, haploid and diploid colonies with modified cellular wall were mutagenised with N-methyl- N'-nitro – N-nitrosoguanidine (in concentrations of 150 µg/mL, 200 µg/mL, 250 µg/mL, acting at 37°C, for 30 min). There were obtained 10 mutants: 5 diploid and 5 haploid, using zymoliase as selective agent.

Morpho-physiological and biochemical studies will be followed by molecular tests allowing an accurate genetic characterization of the studied strains aiming to the isolation of a parental strain with an increased ability to produce formaldehyde dehydrogenase.

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