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## Characterization of petite mutants of the basidiomycetes *Phaffia rhodozyma* CBS 5905

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**ABSTRACT** Small, "petite" colonies appeared with almost 1% frequency among the normally growing colonies in the astaxanthin-producing *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) CBS 5905 strain. These colonies fermented glucose in the presence of oxygen, were not able to grow on nonfermentable carbon sources and did not respond to inhibitors of respiration. Besides spontaneous occurrence, petite mutation proved to be inducible almost to 100% by low levels of ethidium bromide treatment. Cell hybridization experiments revealed that both the spontaneous and induced mutation was mitochondrially inherited. The RFLP pattern of the mtDNAs isolated from purified mitochondria of the grand and petite strains were similar. Consequently, unlike to the mtDNA of *Saccharomyces cerevisiae*, not large deletions were the reason for petite mutant induction. The size of mtDNA in the grand cells was calculated by restriction enzyme analysis and proved approximately 17.6 kb. In teleomorphic strains of *P. rhodozyma* (*Xanthophyllomyces dendrorhous*) spontaneous petite mutants did not arise and the phenotype could not be induced either by ethidium bromide treatment. According to our results, *Phaffia rhodozyma* is the only known petite-positive basidiomycetous yeast.

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### KEY WORDS

basidiomycetous yeast  
petite positive  
mitochondrial DNA  
*Phaffia rhodozyma*

*Phaffia rhodozyma* is an asexual, psychrophilic, fermentative yeast species which is represented by the single isolate, strain CBS 5905. Its relation to basidiomycetous yeasts was verified via cell wall ultrastructure, the mode of bud formation and diazonium B test (Miller et al. 1976). The teleomorphic form was described as *Xanthophyllomyces dendrorhous* by Golubev in 1995, although comparison of sequences of rDNA on the IGS and ITS regions, suggested that *P. rhodozyma* CBS 5905 may be a distinct species from the other examined strains (Fell and Blatt 1999). Additional results from the analysis of viral RNAs (Pfeiffer et al. 1996), electrophoretic karyotyping (Nagy et al. 1994), RAPD analysis (Palágyi et al. 2004) and the detection of DNA plasmids in the mitochondria of *Xanthophyllomyces* strains (Wilber and Proffitt 1987; Kucsera et al. 2000; Santopietro and Kula 2001) reinforced this further. The hypothesis that *P. rhodozyma* and *X. dendrorhous* may represent the same species is favoured again (Fell et al. 2007; Libkind et al. 2007). The microorganism is biotechnologically important, as the cells produce high amount of astaxanthin. The well-functioning respiratory system is an important requirement both for biomass production and high amount of astaxanthin production. Low aeration slows growth, consequently reduces the yield of yeast cells and markedly reduces the red pigment

yield (Lewis 1990). In this paper we report, that respiratory-deficient petite mutants occurred spontaneously, or could be induced by ethidium bromide (EtBr) treatment in the strain *Phaffia rhodozyma* CBS 5905. Such mutant could not be isolated either spontaneously or induced by EtBr treatment in *Xanthophyllomyces dendrorhous*. RFLP pattern of mtDNA from the wild-type and petite strains were compared in strain CBS 5905. The size of the wild type mtDNA calculated on the bases of restriction enzyme analysis proved about 17.6 kb, what is quite small genome size in yeast (Wolf and Del Giudice 1988; Pramateftaki et al. 2006). According to our results *Phaffia rhodozyma* CBS 5905 is a member of the petite positive yeast group (Bulder 1964), which is the only known case among the basidiomycetous yeasts.

### Materials and Methods

#### Strains, media and growth conditions

The yeast strains used in this study are listed in Table 1. The media used were 2xYPD: 1% yeast extract, 2% peptone, 2% glucose; YPEt: 0.05% yeast extract, 0.05% peptone, 2% ethanol; for plating media contained 2% agar. Yeast cells were grown under continuous shaking at 20°C.

#### Determination of spontaneous petite frequency

Yeast cells were grown in YPEt medium for 48 hr, then diluted and plated onto 2xYPD plates. After 5 days of incubation at

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**Table 1.** Yeast strains used in this experiment.

Strains	Markers		Origin
	Nuclear	Mitochondrial	
<i>Ph. rhodozyma</i>			
CBS 5905	prototrophic	$\rho^+$	Kindly provided by BioColours Ltd
CBS 5905 <i>pet1/1</i>	prototrophic	$\rho^-$	Spontaneously arising petite isolates from CBS 5905
<i>arg ben<sup>R</sup>2</i>	<i>arg</i> , benomyl resistant	$\rho^+$	Isolated after repeated mutagenesis from CBS 5905
<i>arg ben<sup>R</sup>2 <math>\rho^-10</math></i>	<i>arg</i> , benomyl resistant	$\rho^-$	Ethidium bromide induced petite mutant of <i>arg ben<sup>R</sup>2</i>
<i>X. dendrorhous</i>			
CBS 5908			Kindly provided by BioColours Ltd
CBS 6938			Kindly provided by BioColours Ltd
ATCC 24203			Kindly provided by BioColours Ltd
ATCC 24229			Kindly provided by BioColours Ltd
ATCC 24261			Kindly provided by BioColours Ltd

**Table 2.** Frequencies of petite mutants after 10  $\mu\text{g/ml}$  ethidium bromide treatment.

Time of treatment (hour)	Frequencies of petite colonies (%)
0	0.5
12	9.2
24	40.7
48	74.3

20°C, the small colonies were scored and tested for ability to grow on YPEt.

### Isolation of respiratory deficient mutants

The isolation of respiratory deficient mutants using ethidium bromide has been described by Rickwood et al. (1988).

### Respiration measurements

All respiration measurements were made using Clark type oxygen electrode, at 20°C, according to the method published by Rickwood et al. (1988).

### Colony assay for cytochrome c oxidase activity

Five-day-old colonies were stained by tetramethyl-*p*-phenylenediamine (TMPD) according to the method published by McEwen et al. (1985).

### Electron microscopy

Yeast cells from the stationary growth phase were fixed at 4°C for 24 h in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After fixation, the cells were washed four times in phosphate buffer, postfixed in 2%  $\text{KMnO}_4$  for 2 h, dehydrated in a graded series of ethanol, and embedded in Spurr resin. Sections were cut with a Reichert-Jung ultramicrotome, and stained with uranylacetate. Ultrathin sections were examined under a JEM 1200EX electron microscope at 60 kV (Bozzola

and Russel 1992).

### Determination of extrachromosomal inheritance in strain *pet1/1*

For hybridization of strain *pet1/1* the protoplast fusion method was used. Protoplasts were prepared from exponentially growing cultures of *arg ben<sup>R</sup>2*, *arg ben<sup>R</sup>2  $\rho^-10$*  and *pet1/1* essentially according to the method described by Maráz et al. (1978) with the following modifications: cells of the fusion partners were precultivated in 2xYPD liquid medium; after pretreatment in 1% 2-mercaptoethanol, the cell wall was digested with 1.5% snail enzyme and 0.1% NovoZym in 0.8 M KCl for 2 hours. Partners in protoplast fusion were: *pet1/1* x *argben<sup>R</sup>2  $\rho^-10$*  and *pet1/1* x *argben<sup>R</sup>2*; for hybrid selection minimal medium supplemented with 1 $\mu\text{g/ml}$  benomyl and 0.8 M KCl was used.

### Separation of nuclear and mitochondrial DNA

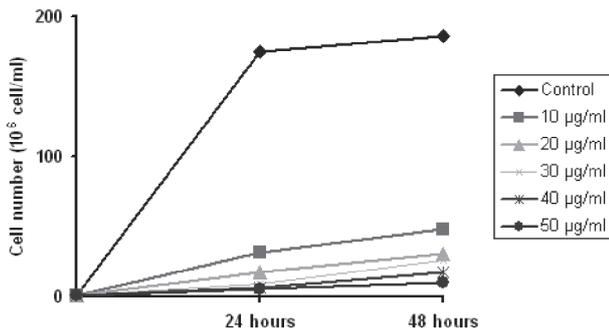
A modification of the procedure previously described for *Saccharomyces cerevisiae* was applied. Protoplasts were formed from cells of stationary phase ( $10^7$  cells/ml). After two hours incubation in the lysing enzyme the crude lysate of  $5 \times 10^9$  protoplasts were applied to separate DNAs on isopycnic CsCl/ bisbenzimidazole (Hoechst 33258) gradient according to Williamson and Fennel (1974), in Sorvall Pro80 Ultraspeed Centrifuge.

### Isolation of mitochondria

Protoplasts from about  $10^9$  cells were broken by French press at 4°C at 3000 $\psi$ . Mitochondria were isolated on sucrose gradient according to Rickwood et al. (1988).

### Restriction endonuclease digestion and electrophoresis

Restriction endonucleases were obtained from Fermentas (Vilnius, Lithuania) and the reactions were carried out ac-



**Figure 1.** Effect of different concentration of ethidium bromide on cell growth.

according to the supplier's directions.

### Pulsed field gel electrophoresis (PFGE)

DNA samples from *P. rhodozyma* protoplasts were prepared in agarose blocks and separated in a 1.0% (w/v) agarose gel using a CHEF-DRTM II System (BIORAD) with switching time 60s. All separations were performed in 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA pH 8.0) at 12°C for 14 hours.

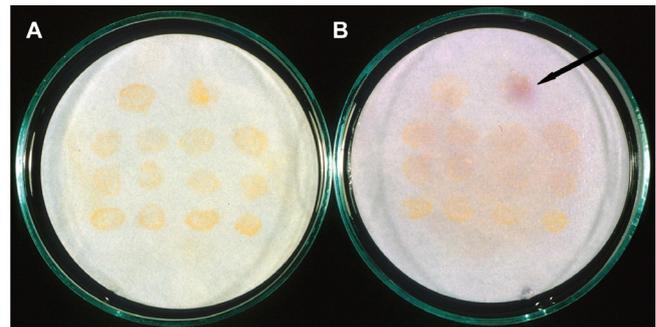
## Results and Discussion

### The occurrence of spontaneous petite mutation in *Phaffia rhodozyma*

During our preliminary study on *P. rhodozyma* CBS 5905 strain the occurrence of small colonies was revealed with about 1% frequency on complete media (Kucsera et al. 1995). Replicating colonies onto media containing nonfermentable carbon source (ethanol) the small ones failed to grow. One of these spontaneous petite mutant strains (*pet1/1*) was isolated for further experiments. No such mutants were found in the *X. dendrorhous* strains when 10<sup>4</sup> cells were plated for colonies.

### Detection of the respiration rate in isolated grand and petite mitochondria

The O<sub>2</sub> consumption of isolated mitochondria of the grand and the petite strains was measured by 'Clark-type' oxygen electrode. The grand mitochondria respired at a rate of 25 µM O<sub>2</sub>/min/mg protein in the presence of succinate as substrate and the respiration was almost totally blocked with Antimycin A. Some residual respiration which can be due to the activity of an alternative oxidase enzyme system was also detected (An and Johnson 1990). The mitochondria of petite cells neither respired nor responded to the addition of Antimycin A in the presence of the substrate.



**Figure 2.** TMPD staining of *Phaffia rhodozyma* wild type and petite mutant colonies. A: colonies without TMPD, B: colonies with TMPD, arrows show the respiratory competent wild type colonies.

### Specific induction of petite mutation by ethidium bromide

Ethidium bromide (EtBr) inhibits *de novo* synthesis of mtDNA as well as stimulates destruction of the pre-existing mtDNA molecules resulting in almost 100% conversion of *S. cerevisiae* culture into a respiratory-deficient one. Cells of *P. rhodozyma* were exposed to various concentrations of EtBr for various periods of time in complete liquid medium and the changes in the growth rate and survival rate were determined. The inhibition of cell growth is illustrated on Figure 1. Although the growth rate was gradually inhibited at concentrations higher than 10 µg/ml, the yeast cells still grew reasonably well in the presence of EtBr. Even at higher concentration they remained viable (data not shown). This behaviour of *P. rhodozyma* mtDNA suits the well-known sensitivity of fungal mtDNAs to low levels of EtBr. The conversion of cells to petite phenotype by EtBr treatment (10 µg/ml) as a function of time was also determined (Table 2). Petite mutants could be isolated almost in 80% after 48 hours treatment. These results are also similar to those of *S. cerevisiae*, where low concentration of EtBr could cause a 99% of petite induction (Dujon 1981). Treatment with EtBr did not result in petite mutation of the *X. dendrorhous* strains: 2500 surviving colonies of each strain were tested after 10-25-50 µg/ml EtBr treatments of 48 hours and respiratory deficient isolates could not be found.

### Screening of cytochrome c oxidase activity in ethidium bromide induced petite isolates

The activity of the respiratory-chain enzymes was checked by cytochrome *c* oxidase test, in more than 100 isolates of the ethidium bromide induced mutants. Grand type colonies showed purple coloration within two minutes due to intensive oxidation while all of the petites remained pink when stained by TMPD (Fig. 2). The result proved that cytochrome *c* oxidase enzyme did not function, however other enzymatic

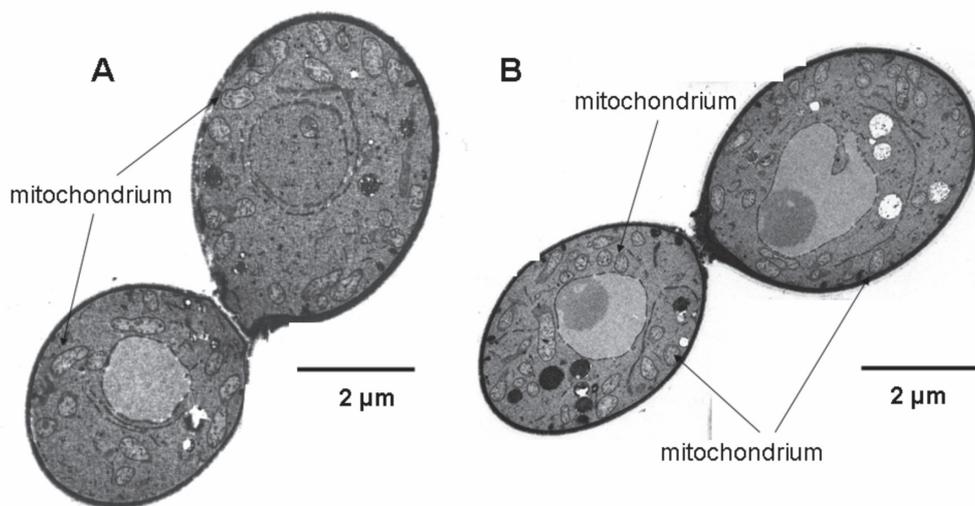


Figure 3. Ultrathin section from *Phaffia rhodozyma* wild type (A) and *pet1/1* petite cells (B).

(e. g. cytochrome b) deficiency prior to cytochrome c oxidase could not be excluded.

### Electron microscopic structure of the grand and petite cells

Studies on the morphology of mitochondria in grand cells and cytoplasmic petite mutants of *S. cerevisiae* revealed that in the later they are often smaller and spherical. The major alteration is the loss of normal cristae, and very often the mitochondria tend to aggregate in some cytoplasmic regions (Stevens 1981).

In *P. rhodozyma* the wild-type cells had very well structured grand mitochondria with developed internal lamellae (Fig. 3a). The mutant cells also contained recognizable mitochondria (Fig. 3b). Its structure appeared normal we could observe neither distinct morphological alteration nor irregular distribution of the organelles in the cytoplasm.

### Characterization of the mtDNA

Mitochondrial DNA of *P. rhodozyma* CBS 5905 migrates as a "smear" during PFGE what is peculiar for circular mitochondrial genomes. Separation of nuclear and mtDNA in isopycnic CsCl gradient showed that the buoyant density of DNA deriving from whole cell lysates was the same. Accordingly, for further experiments the mtDNA was isolated from purified mitochondria and analysed by restriction endonucleases.

Enzymes having six-base recognition site gave partial digestion, therefore endonucleases with four-base recognition sites were used to determine the size of the mitochondrial DNA (Fig. 4.). The estimated molecular weight was 17.6 kb, however, this result can alter after refining the number of the comigrated fragments. The mtDNA contained relatively

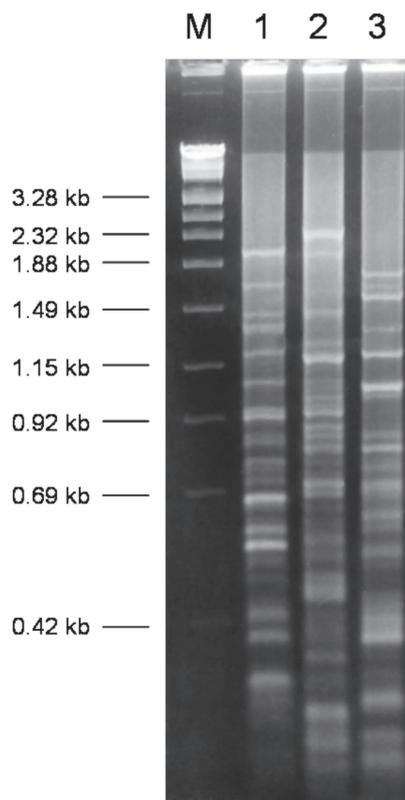


Figure 4. RFLP patterns of mtDNA of *P. rhodozyma* CBS 5905 strain. Lane M:  $\lambda$ -pUC Mix as molecular weight marker (Fermentas). Lanes 1-3: *RsaI*-, *MspI*-, and *HaeIII*-digested mtDNA, respectively.

high number of restriction sites recognized by enzyme which specifically cut in GC clusters. This is similar to the published

results by Piskur et al. (1996, 1998) in the *Saccharomyces sensu stricto* complex where the members of the species group showed variable RFLP pattern, when such enzymes were used, although there were only slight differences when enzymes specifically cutting in the coding regions were applied.

### Characterization of the mtDNA of petite mutants

The cytoplasmically inherited petite mutation in *S. cerevisiae* is due to smaller or larger deletions in mitochondrial DNA (Dujon 1981). The deleted segments may derive from any part and length of the mitochondrial genome or in extreme cases the mtDNA may be completely lost ( $\rho^0$  mutant). The non-deleted sequences may be amplified, and its amphimeric organization is thought to be general (Rayko and Goursot 1996a, b). In *P. rhodozyma* petite strain either induced by EtBr or arisen spontaneously the restriction enzyme pattern of the mtDNA generated by *Hae*III and *Rsa*I was the same as in the wild type mtDNA. The mutants possibly arose by point mutation or may be due to only very small deletions which could not be detected by using these enzymes.

### Inheritance of respiratory deficient mutation in strain *pet1/1*

As RFLP pattern of the mtDNA in spontaneous petite and in the grand strain was similar, we were wondering about the origin of the mutation (viz. mitochondrial or nuclear). As *P. rhodozyma* is not capable for sexual processes, unlike its teleomorph form *Xanthophyllomyces dendrorhous* (Kucsera et al. 1998), the protoplast fusion method was applied for hybridization. The idea was, if the mutation of the spontaneous petite strain 1/1 is on nuclear DNA, hybrid cells obtained from a fusion to *arg ben<sup>R</sup>2*  $\rho^-$  ethidium bromide induced petite should have a grand phenotype. The reason is the presence of a normal complement of nuclear genes in strain *arg ben<sup>R</sup>2*  $\rho^-$  (since EtBr has no effect on nuclear DNA) and of mitochondrion (in strain 1/1). On the other hand, if the mutation is on mtDNA, the hybrid progeny resulting from the fusion will exhibit the mutant phenotype, since the petite parent (strain 1/1), having no functional mtDNA cannot supply the wild-type copy of the mutated gene. In fusion experiment A the 1/1 petite was fused to *arg ben<sup>R</sup>2*. After selection on minimal medium containing benomyl, all of the twelve isolated colonies were grand. In fusion experiment B, the strain 1/1 was fused to *arg ben<sup>R</sup>2*  $\rho^-$  EtBr induced petite. After selection ten fusion colonies were isolated and all of them were petites. According to this result, the spontaneous petite mutation was mitochondrially inherited in strain 1/1.

In conclusion the occurrence of small colonies during the cultivation of *Phaffia rhodozyma* and the fact that they are not able to use nonfermentable carbon sources revealed that *Phaffia rhodozyma* is a petite-positive basidiomycetous

species. This was confirmed by the following results: i. the difference between the O<sub>2</sub> consumption of the grand and the petite cells and their mitochondria suggested that the petite cells do not contain functioning mitochondria; ii. ethidium bromide proved to be an effective petite inducer; iii. the petite mutants might arise by point mutation or small deletion even after ethidium bromide treatment as their RFLP profile generated by *Hae*III or *Rsa*I digestion proved the same as the RFLP pattern of the wild type.

### References

- An GH, Johnson EA (1990) Influence of light on growth and pigmentation of the yeast *Phaffia rhodozyma*. *Antonie van Leeuwenhoek* 57:191-203.
- Bozzola JJ, Russel LD (1992) *Electron Microscopy; Principle and Techniques for Biologists*. Jones and Bartlett Publisher. Inc., Boston.
- Bulder CJE (1964) Induction of petite mutation and inhibition of synthesis of respiratory enzymes in various yeasts. *Antonie van Leeuwenhoek* 30:1-9.
- Dujon B (1981) Mitochondrial genetics and functions. In Strathern, J. N., Jones, E. W. and Broach, J. R. (Eds), *The Molecular Biology of the Yeast Saccharomyces, Life Cycle and Inheritance*, Cold Spring Harbour Laboratory, pp. 505-635.
- Fell JW, Blatt GM (1999) Separation of strains of the yeasts *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma* based on rDNA IGS and ITS sequence analysis. *Ind Microbiol Biotechnol* 23:677-681.
- Fell JW, Scorzetti G, Statzell-Tallman A, Boundy-Mills K (2007) Molecular diversity and intragenomic variability in the yeast genus *Xanthophyllomyces*: the origin of *Phaffia rhodozyma*? *FEMS Yeast Research* 7:1399-1408.
- Golubev WI (1995) Perfect state of *Rhodomyces dendrorhous* (*Phaffia rhodozyma*). *Yeast* 11:101-110.
- Jadeyel DM (1986) Variation in the organization and structure of the mitochondrial DNA of species *Aspergillus*. *Ph.D. Thesis*, University of Birmingham, Birmingham, UK
- Kucsera J, Pfeiffer I, Ferenczy L (1995) Occurrence of petite mutation in *Phaffia rhodozyma*. *Acta Microbiol Immunol Hung* 42:134-135.
- Kucsera J, Pfeiffer I, Ferenczy L (1998) Homothallic life cycle in the diploid red yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). *Antonie van Leeuwenhoek* 73:163-168.
- Kucsera J, Pfeiffer I, Takeo K (2000) Biology of the red yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). *Mycoscience* 41:195-201.
- Lewis MJ (1990) *Phaffia rhodozyma*: A red yeast with potential as an animal feed. In Lyons, T. P. (ed) *Biotechnology in the Feed Industry*, Proceedings of Alltech's Sixth Annual Symposium, pp. 391-403.
- Libkind D, Ruffini A, Broock MJ, Alves L, Sampaio JP (2007) Biogeography, host specificity, and molecular phylogeny of the basidiomycetous yeast *Phaffia rhodozyma* and its sexual form *Xanthophyllomyces dendrorhous*. *Appl Environ Microbiol* 73:1120-1125.
- Maráz A, Kiss M, Ferenczy L (1978) Protoplast fusion in *Saccharomyces cerevisiae* strains of identical and opposite mating types. *FEMS Letters* 3:319-322.
- McEwen JE, Cameron VL, Poyton O (1985) Rapid method for isolation and screening of cytochrome *c* oxidase-deficient mutants of *Saccharomyces cerevisiae*. *J Bacteriol* 161:831-835.
- Miller M, Yoneyama WM, Soneda M (1976) *Phaffia*, a new yeast genus in the Deuteromycotina (Blastomycetes). *Int J Syst Bacteriol* 26:286-291.
- Nagy Á, Garamszegi N, Vágvölgyi Cs, Ferenczy L (1994) Electrophoretic karyotypes of *Phaffia rhodozyma* strains. *FEMS Microbiol Letters* 123:315-318.
- Palágyi Zs, Papp T, Takó M, Nagy Á, Pesti M, Vágvölgyi Cs (2004) Genetic variability of astaxanthin-producing yeasts: random amplified polymorphic DNA (RAPD) analysis of *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous*. *Acta Biol Szeged* 48:35-38.

- Pfeiffer I, Kucsera J, Varga J, Párducz Á, Ferenczy L (1996) Variability and inheritance of double-stranded RNA viruses in *Phaffia rhodozyma*. *Curr Genet* 30:294-297.
- Piskur J, Smole-Mozina S, Stenderup J, Pedersen MB (1996) A mitochondrial marker, *ori-rep-tra*, for differentiation of yeast species. *Appl Environ Microbiol* 51:2780-2782.
- Piskur J, Smole S, Groth C, Petersen RF, Pedersen MB (1998) Structure and genetic stability of mitochondrial genomes vary among yeasts of the genus *Saccharomyces*. *Int J Syst Bacteriol* 48:1015-24.
- Pramateftaki PV, Kouvelis VN, Lanaridis P, Typas MA (2006) The mitochondrial genome of the wine yeast *Hanseniaspora uvarum*: a unique genome organization among yeast/fungal counterparts. *FEMS Yeast Res* 6:77-90.
- Rayko E, Goursot R (1996a) Amphimeric mitochondrial genomes of petite mutants of yeast. I. Flip-flop amphimers make up the mitochondrial genomes of "palindromic" petite mutants of yeast. *Curr Genet* 30:126-134.
- Rayko E, Goursot R (1996b) Amphimeric mitochondrial genomes of petite mutants of yeast. II. A model for the amplification of amphimeric mitochondrial petite DNA. *Curr Genet* 30:135-144.
- Rickwood D, Dujon B, Darley-Usmar VM (1988) Yeast mitochondria. In Campbell I and Duffus JH eds., *Yeast - a practical approach*. IRL Press, pp. 185-254.
- Santopietro LM, Kula MR (2001) Cloning and nucleotide sequence of a linear DNA plasmid from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). *Folia Microbiol (Praha)* 46:277-88.
- Stevens B (1981) Mitochondrial structure. In Strathern JN, Jones EW and Broach JR eds., *The Molecular Biology of the Saccharomyces, Life Cycle and Inheritance*. Cold Spring Harbour Laboratory, pp. 471-504.
- Wilber KA, Proffitt JH (1987) Detection of linear plasmids in *Phaffia rhodozyma*. *UCLA Yeast Symp.* p. 106.
- Williamson DH, Fennell DJ (1974) Apparent dispersive replication of yeast mitochondrial DNA as revealed by density labelling experiments. *Mol Gen Genet* 131:193-207.
- Wolf K, Del Giudice L (1988) The variable mitochondrial genome of Ascomycetes: organization, mutational alteration, and expression. *Adv Genet* 25:185-308.