Genetic characterization of two new *Metschnikowia* strains with antifungal activity

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Abstract

Two yeasts strains, named SG1 (L_{29}) and SG2 (L_{28}), were isolated from surface of white grapes from the region Ialomiţa, Romania. The strains showed antifungal activity against post-harvest spoilage fungi such as Penicillium spp. Morpho-physiological analysis and biochemical tests, revealed great similarity between SG1, SG2 and Metschnikowia pulcherrima. Molecular biology studies were performed for a more accurate taxonomical identification. Electrokaryotyping was done by field inversion gel electrophoresis. The conditions for cell wall lysis and plug deproteinisation were optimized. After setting up the electrical parameters, two chromosomes were separated: I – 1.8 Mb and II – 2.0 Mb. Mitochondrial DNA was isolated, purified and digested with five restriction endonucleases: Alu I, Cfo I, Eco RV, Hae III and Hinf I. The restriction profiles obtained with Alu I, Eco RV and Hinf I, were identical for SG1 and SG2. Both strains formed the same number of fragments by restriction with Cfo I and Hae III, the fragments for SG1 being approximately 100 bp smaller than those for SG2, due to possible point mutations within its mitochondrial DNA. The results obtained in our study were compared with data from literature, confirming the classification of our strains in Metschnikowia pulcherrima species.

Keywords: *Metschnikowia pulcherrima*, morpho-physiological studies, electrokaryotyping, mitochondrial DNA restriction profile

Introduction

Some yeast species, belonging to *Metschnikowia, Candida, Criptococcus* and *Zygosaccharomyces* genera, have antifungal activity against various phytopathogens. Development of new methods to inhibit fungal growth implies a deep knowledge of the yeast physiology, as well as an accurate taxonomical characterization and identification of the studied species or strains. The first step of the process is represented by preliminary observations of culture characteristics (colony colour, shapes and texture), cell size, form and type of reproduction: sexual (multipolar or unipolar budding, shape and size of ascospores/ basidiospores) or asexual (formation of hyphae, pseudohyphae). Then, biochemical analyses are performed: assimilation and fermentation tests, urea hydrolysis, growth at non-permissive temperatures, cycloheximide resistance, a.s.o. Finally, the observations are completed using molecular biology methods in order to determine the structure of the nuclear genome, such as chromosome number and dimension [6], guanine+cytosine percentage, and to characterize the extranuclear genome, especially the mitochondrial DNA [11; 14], 2 mm-like plasmids, and the killer system.

The ascomycetous yeast genus *Metschnikowia* (anamorph *Candida*) presents vegetative cells with multilateral budding and elongated asci with one or two needle-shaped ascospores. Species isolated from terrestrial habitats are typically associated with flowers or fruits [4]. These strains present antifungal activity against post-harvest spoilage fungi such as *Penicillium* spp [3; 7].

The aim of our present research was the characterization and preliminary identification of two new yeasts strains, named *L_{28} (SG2)* and *L_{29} (SG1)*, using macroscopic and microscopic observations, the colorimetric sugar assimilation test *Auxacolor®2 kit* and molecular techniques (electrokaryotyping and mitochondrial DNA analysis).

Material and Methods

***Yeast strains and media*:**

Studies were performed on two yeasts strains named *SG1 (L_{29})* and SG2 (L_{28}), isolated from surface of white grapes in the region Ialomita, Romania.

Standard yeast strains were used as reference: *S. cerevisiae* YPH 80 (Sigma), *S. cerevisiae* L5366, *C. parapsilosis* CBS604.

Yeasts were maintained at -70°C on yeast peptone glucose (YPG) medium (yeast extract 5 g L^{-1}, peptone 10 g L^{-1}, glucose 20 g L^{-1}) supplemented with 20% glycerol. Previous to taxonomical tests, yeast were cultured on YPG slants, and grown for 18 hours, at 30°C.

Morphological characterisation

Yeasts from fresh growing culture were cultivated in Petri dishes, on YPGA medium (YPG medium supplemented with agar 20 g L^{-1}), for observing the macroscopic appearance of the colony type. From same growing culture yeasts were inoculated in flasks with YPG medium, and the appearance of the cells was examined microscopically.

Physiological and biochemical studies

Biochemical analyses were performed according to BARNETT [1] and LODDER [8].

The ability of yeast strains to use different compounds (glucose, galactose, sucrose, maltose, trehalose, glycerol, mannitol, citrat, cellobiose, lactose, raffinose and starch) as sole carbon source for aerobic and semi-anaerobic growth was tested using the auxanogram technique and, respectively, fermentation tests.

Growth at non-permissive temperature was determined at 25°C, 30°C, 37°C and 42°C.

Also, there were performed tests for determination of resistance to various cycloheximide concentrations (0.01% and 0.1%), starch formation, acetic acid production, urea hydrolysis, response to diazonium blue B (DBB) reaction, and for assessing growth in high concentration of D-glucose (50%).

Additionally, a rapid identification of the yeast strains was performed using the **Auxacolor®2 kit** (BioRad). The R_2 medium was inoculated with a sufficient quantity (four identical colonies) of a 24 hours yeast culture grown on Sabouraud medium. The obtained suspension, with an opacity equivalent to the control supplied with the kit, was homogenized by vortexing, and 100 µl was distributed to each well on the R_1 microplate, containing media with different carbon sources (glucose-positive control, maltose, sucrose, galactose, lactose, raffinose, inositol, cellobiose, trehalose, adonitol, melezitose, xylose and arabinose). The microplate was covered with an adhesive film and incubated 24 to 48 hours at 28°C.

Electrokaryotyping by FIGE

1. *Preparation of chromosomal DNA molecules*

The chromosomal DNA molecules were prepared using a technique described by BIRREN [2] and optimized by VASSU et al. [13]. Yeast cell wall lysis was done using a zymolyase 20T (PROMEGA) solution in final concentration of 1 mg mL^{-1}. For deproteinisation the plugs were incubated for 48 hours, at 50°C, with sarkosyl 1% and proteinase K (final concentration 1mg mL^{-1}).

2. *Pulsed field electrophoresis*

Chromosome separation was performed by FIGE (Field Inversion Gel Electrophoresis), using a home-made apparatus, homologated in Romania. We used 1% Pulse Field Running Gel Agarose (Sigma), and TBE 0.5X buffer. The electrical parameters for sample migration were established: Initial forward time (IFw) = 50 sec.; Final forward time (FFw) = 200 sec.; Reverse time = 1/3 from IFw; Pause forward = Pause reverse = 10%; Total migration time = 28 hours; Voltage = 54V.

Mitochondrial DNA isolation and purification

The yeast culture was grown overnight at 28°C, and 25mL was centrifuged 5 min. at 3000 rpm. The pellet was washed in 20 mL water, centrifuged again and washed in 10 mL sorbitol 1M. After a new centrifugation, the pellet was resuspended in 5 mL solution A (0.5M sorbitol, 10mM EDTA, 50mM TrisHCl), 100 mL b-mercaptoethanol and lyticase. After incubation at 37°C, the obtained protoplasts were separated by spinning 10 min. at 1500 rpm. The supernatant containing mitochondria was transferred in three 1.5 mL Eppendorf tubes, and centrifuged for 15 min at 13200 rpm. The pellets were resuspended in 300 mL MitoWashing Buffer (0.5M sorbitol, 1mM EDTA, 50mM TrisHCl) and then mixed in a single Eppendorf tube. After spinning 5 min at 13200 rpm, the sediment was washed in 600 mL MitoWashing Buffer. The step was repeted three times. Finally, the sediment was mixed with 300 mL MitoLysis Buffer (0.15M NaCl, 5mM EDTA, 10mM TrisHCl, 1% sarkosyl) and proteinase K, and incubated for 30 min. at 37°C. Deproteinisation was done using phenol:cloroform:isoamilic alcohol (25:24:1 v/v). The supernatant was transferred in another tube and mitochondrial DNA was precipitated with 1 mL cold pure ethanol. After 10 min at 13200 rpm, the sediment represented by mitochondrial DNA, was washed with ethanol (70%), spinned again shortly at 14000 rpm, resuspended gently in 40 mL solution TE-RNase (10mM TrisHCl, 1mM EDTA, RNase in final concentration 20mg mL^{-1}).

The purity and integrity of mitochondrial DNA isolates were verified by electrophoresis, using 0.8% agarose gel and TBE 1X buffer (0.089M Tris, 0.089M boric acid, 0.002M EDTA, pH 8.0) [9]. Sample concentration was determined by reading the OD at 260nm, using a spectrophotometer ULTROSPEC 3000.

Mitochondrial DNA restriction profiles

Mitochondrial DNA obtained previously was digested with *Alu* I, *Cfo* I, *Eco* RV, *Hinf* I and *Hae* III (10U mL^{-1}, PROMEGA).

Electrophoretic analysis of restriction profiles was performed by agarose gel electrophoresis using 0.9% agarose gel and TBE 0.5X. The electric field voltage was 2.5V cm^{-1}, and DNA bands were stained with ethidium bromide 0.5 mg mL^{-1}.

Results and Discussions

Morphological and physiological characterization

Culture characteristics The yeast colonies were cream and smooth. On the surface of the medium, beneath the colonies, persistent red spots were observed.

Microscopic observations The cells were examined microscopically after incubation 24 - 48 hours at 28°C in YPG broth. The isolates presented sexual reproduction by budding.

The two strains had the ability to form ascospores on special medium with $CaCO_3$ 10% after eight weeks.

Physiological and biochemical analysis: Assessing the ability to use organic compounds as sole carbon source for aerobic growth was done by auxanogram technique (**Figure 1**). Strains *SG1* and *SG2* grew on glucose, galactose, sucrose, maltose, trehalose, glycerol, mannitol, citrat, cellobiose but did not grow on lactose, raffinose and starch.

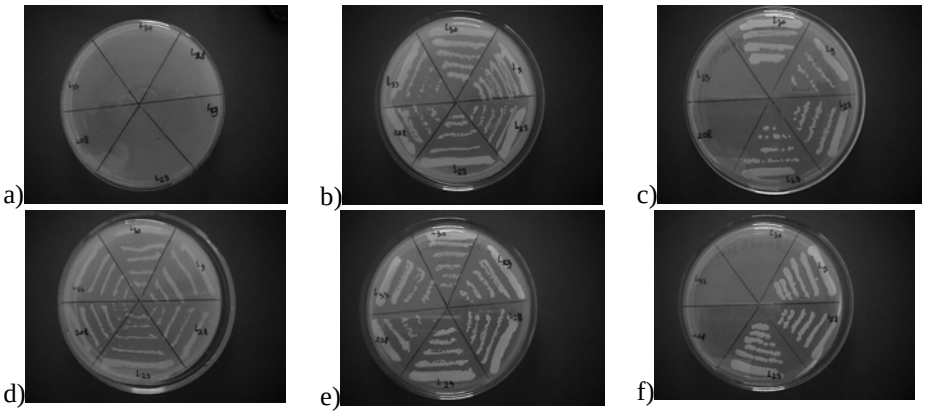


Figure 1. Auxanograms for *SG1* and *SG2*: a) YNB without carbon source; b) YNB with sucrose; c) YNB with glycerol; d) YNB with galactose; e) YNB with mannitol; f) YNB with cellobiose

The ability to use certain sugars anaerobically was assessed by fermentation. Both strains fermented only glucose. Temperature studies revealed that our strains grew at 25°C, 30°C, but could not grow at 37°C and 42°C. The studied strains presented resistance at cycloheximide and grew in medium with high concentration of D-glucose (50%). On the contrary, both strains did not show ability of starch formation did not produce acetic acid and hydrolyze urea. Also, the diazonium blue B (DBB) reaction was negative.

The results obtained were compared with those from literature [1; 5] and we observed an important similarity between strains *SG1*, *SG2* and the morpho-physiological characteristics of *Metschnikowia pulcherrima*. Moreover, the responses recorded for the 13 reactions of the ***Auxacolor®2 kit*** confirmed this observation.

Therefore, the two yeast strains were preliminary classified as belonging to *Metschnikowia pulcherrima*.

Electrokaryotyping by FIGE

Intact chromosomal DNA molecules were obtained by optimizing the conditions for cell wall lysis, using zymolyase 20T in final concentration of 1 mg mL^{-1}. Also, deproteinisation was more active than in the

original technique, due to plug incubation with a mixture of sarkosyl and proteinase K, for 48 hours at 50°C. Thus, the total duration of the experiment was shorter, five days instead of seven.

For determination of the size of *SG1* and *SG2* chromosomes, we used as molecular markers two *S. cerevisiae* strains: YPH 80 (Sigma) and L5366 with chromosomes ranging between 220 kb and 2.2 Mb, and a *C. parapsilosis* CBS604 strain with chromosomes from 945 kb to 2.2 Mb.

Analyzing the gel (**Figure 2**), we observed two chromosomes of 1.8 Mb (**I**) and 2.0 Mb (**II**). Our preliminary conventional studies, allowed us to classify *SG1* and *SG2* as belonging to *Metschnikowia pulcherrima*, which represents the sexuate state of *Candida pulcherrima.* From the literature [14], we know that, in general, *Candida* species have chromosomes larger than 1.7 Mb. Therefore, these results confirm our previous conventional taxonomy data.

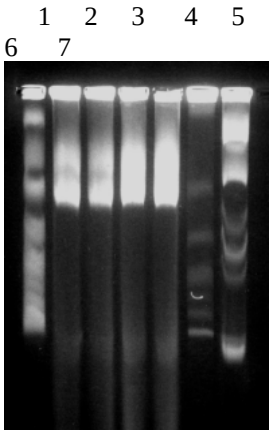


Figure 2. Electrokaryotypes obtained by FIGE for: 1– *S. cerevisiae* YPH 80; 2, 3 – *SG1*; 4, 5 – *SG2*; 6 – *S. cerevisiae* L5366; 7 – *C. parapsilosis* CBS 604.

Since FIGE allows accurate separation of DNA molecules up to 2 Mb, for the isolation of larger chromosomes of our two yeast strains, further experiments will be performed using another range of electric parameters for pulsed field migration.

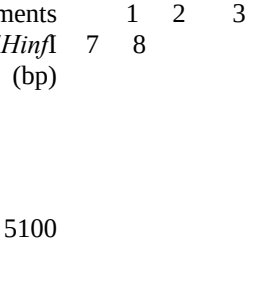
Mitochondrial DNA restriction profiles

Mitochondrial DNA (mtDNA) analysis represents an important step in the identification of yeast strains and species.

First, we established the lyticase concentration needed for cell wall lysis - 5mg mL^{-1}, in order to obtain a high concentration of mitochondrial DNA. The electrophoretic analysis showed that for all the strains, the DNA did not undergo any fragmentation during the isolation and purification procedures, since we obtained compact clear spots. Also, no RNA contamination was observed (*data not shown*).

Further, the isolates were subject to enzymatic digestion, using *Hinf* I, *Alu* I, *Hae* III, *Cfo* I and *Eco* RV. There are two reasons for which these enzymes were selected for obtaining mitochondrial DNA restriction patterns. First, they recognize only few restriction sites in mitochondrial DNA, resulting in a low number of rather large fragments, easy to isolate by conventional agarose gel electrophoresis. Second, the five endonucleases were used in similar experiments in other laboratories, and were described as providing clear data for the identification of *Metschnikowia pulcherrima* strains [10; 12].

The digestion conditions were established: incubation for 3 hours at 37°C, and the optimal ratio: 0.05 U enzyme / mL mitochondrial DNA.



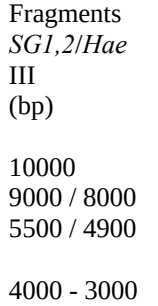
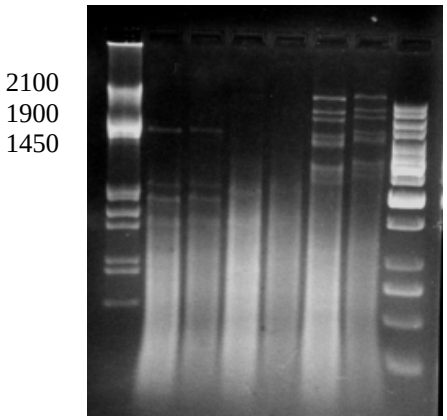


Figure 3. MitochondrialDNA restriction profiles for *SG1* si *SG2* strains, with *Hinf* I, *Alu* I and *Hae* III (lanes: 1 – DNA phage lambda/ *Eco*RI *Hind* III; 2 – mtDNA *SG1*/ *Hinf*I; 3 - mtDNA *SG2*/ *Hinf*I; 4 - mtDNA *SG1*/ *Alu*I; 5 - mtDNA *SG2*/ *Alu*I; 6 - mtDNA *SG1*/ *Hae*III; 7 - mtDNA *SG2*/ *Hae*III; 8 – 1kb Ladder SIGMA).



The two strains showed the same pattern for *Hinf* I, with fragments ranging from 5100kb to 1450 kb (**Fig. 3 – lanes 2 and 3**). For *Alu* I digestion, we obtained small size fragments (**Figure 3 – lanes 4 and 5**). This could be due to a large number of restriction sites for *Alu* I in the mitochondrial DNA of *SG1* and *SG2*, or to the presence of too much enzyme in the reaction mix. For elucidating this problem, further experiments need to be done, with the variation of enzyme/DNA ratio.

An interesting case was observed when *Hae* III was used. The restriction profiles were analyzed using the 1 kb Ladder as molecular marker, and four series of fragments with close size, were obtained: (i) larger than 10.000 bp, (ii) 9000 bp – 8000 bp, (iii) 5500 bp - 4900 bp, and (iv) 4000 bp to 3000 bp (**Figure 3 – lanes 6 and 7**). It is interesting to notice that *SG1* formed by digestion fragments approximately 100 bp smaller than *SG2*. We believe that this would be caused, most probably, by a point mutation in the mitochondrial DNA, leading to the modification of the restriction site for *Hae* III in *SG1*.

Similar observation was made for *Cfo* I (**Figure 4 – lanes 2 and 3**), the two strains showing slightly different profiles, with fragments between 21000 bp and 7000 bp, at 4500 bp and 2500 bp.

When *Eco* RV was used, *SG1* and *SG2* showed similar patterns (**Figure 4 – lanes 4 and 5**): five fragments ranging from 21000 bp to 4700 bp, and five other from 2500 bp to 1200 bp.

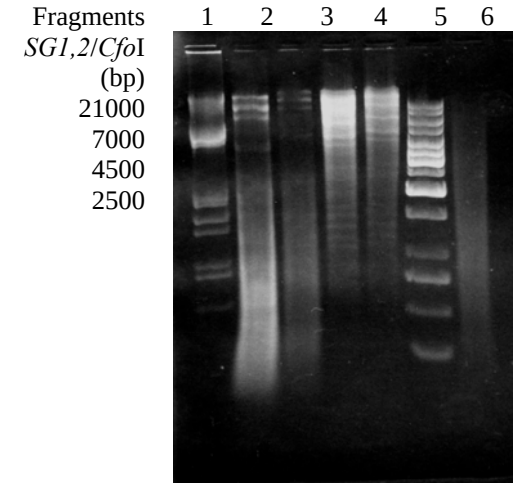


Figure 4. MitochondrialDNA restriction profiles for *SG1* si *SG2* strains with *Cfo* I and *Eco* RV (lanes: 1 – DNA phage lambda/ *Eco*RI *Hind* III; 2 – mtDNA *SG1*/ *Cfo*I; 3 - mtDNA *SG2*/ *Cfo*I; 4 - mtDNA *SG1*/ *EcoRV*; 5 - mtDNA *SG2*/ *EcoRV*; 6 - 1kb Ladder SIGMA).

Comparative analysis of restriction profiles with data from literature, confirmed the identification of *SG1* and *SG2* as *Metschnikowia pulcherrima* strains.

Conclusions

Our research aimed the characterization and preliminary identification of two new yeasts strains with antifungal activity, *SG2 (L_{28})* and *SG1 (L_{29})*, using morpho-physiological observations, biochemical analysis and molecular biology techniques.

The morphological observations and the results obtained from biochemical tests, including the *Auxacolor®2 kit*, revealed a great similarity between our strains and *Metschnikowia pulcherrima.*

Electrokaryotyping of *SG1* and *SG2* was done using FIGE technique. The method for preparation of chromosomal DNA molecules, was optimized by using zymolyase 20T (1 mg mL^{-1}) and incubating the plugs for 48 hours at 50^{o}C, with sarkosyl 1% and proteinase K (1mg mL^{-1}).

The electrical conditions for chromosome migration were also established. Two chromosomes were separated: *chromosome I* – 1.8 Mb and *chromosome II* - 2.0 Mb.

For the separation of larger chromosomes further experiments will be performed using another range of electric parameters for pulsed field migration.

The results obtained by FIGE confirmed the preliminary classification of the two strains.

Specific conditions for mitochondrial DNA isolation and purification were established, using a solution of lyticase (5 mg mL^{-1}) for cell wall lysis.

Mitochondrial DNA isolates were digested with *Alu* I, *Cfo* I, *Eco* RV, *Hae* III and *Hinf* I, in an optimal ratio of 0.05 U enzyme / mL mitochondrial DNA, by incubation three hours at 37^{o}C.

The mitochondrial DNA restriction profiles obtained with *Alu* I, *Eco* RV and *Hinf* I, were identical for *SG1* and *SG2*.

Digestion experiments with *Cfo* I and *Hae* III, resulted in the same number of fragments for both strains, the fragments for *SG1* being approximately 100 bp smaller than those for *SG2*, due to possible point mutations within its mitochondrial DNA.

The results obtained by our conventional and molecular biology studies, along with a comparative analysis of the data from literature, confirmed the identification of *SG1* and *SG2* as *Metschnikowia pulcherrima* strains.

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