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Preliminary taxonomic studies on yeast strains isolated from dairy products

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Abstract

Our paper deals with, morpho-physiological characterization and taxonomical classification of three yeast strains named BF, LC and Ri. The preliminary examinations involved macroscopic appearances of colonies on solid media and microscopic features of the cells. Physiological characterization was performed by assessing the ability to use organic compounds as sole carbon source under semianaerobic or aerobic conditions, diazonium blue B reaction, urea hydrolysis, growth at high concentrations of D-glucose and at non-optimal temperatures. All data obtained were statistically analysed using UPGA (Unweighted pair group average) method for an appropriate identification of the strains. Molecular approach was used to identify the presence of the 2 microns/2 microns-like plasmid DNA. The results allowed us to identify the BF and Ri strains as belonging to Saccharomyces cerevisiae and LC to Candida parapsilosis.

Keywords**:** yeasts, dairy products, isolation, taxonomic studies

Introduction

Nowadays the impact of yeasts in foods is beyond the original and popular notions of bread, beer and wine fermentations by *Saccharomyces cerevisiae.* There is an increasing interest in using yeasts as new sources for improvement of food properties such as: flavor, color, vitamins content, and as agents for the control of food spoilage by their anti-fungal activity [1].

The probiotic activity of some yeasts represents a rather new investigated characteristic which increased the interest for the research of these microorganisms in the past few years. The probiotics are defined as "living microbial preparation, which when consumed, alleviates, suppresses or cures a health disorder, and which acts by altering the microbial balance in gastro-intestinal tract, and consequently changing microbial metabolism, and the interactions with order microorganisms" [2].

In this concern it is believed that dairy products are ideal for delivering the probiotics being a significant nutritional benefit when consuming the whole food rather than the ingestion of the probiotic pills. The probiotics products can contribute to the general state of health, for example by enhancing lactose digestion in lactase-deficient individuals or by preventing and treating diarrhea [3,4].

The efficacy of probiotics may be enhanced by the following methods: gene manipulation; the selection of a more efficient strains of microorganisms; the combination of

probiotics with synergistically acting components (for example: fructo –oligosaccharides); the combination of a number a strains of microorganisms [5].

Our study develops the general preoccupation to isolate from dairy products new yeast strains with biotechnological value. Although yeasts isolated from dairy products are diverse, the most frequently described strains belong to the genera: *Saccharomyces, Kluyveromyces, Debaryomyces, Issatchenkia* and *Yarrowia* [6,7,8].

The isolated yeast strains were characterized with classical methods, and identified to species level by morphological and physiological standard methods as recommended by Kurtzman et al.[9].

Materials and Methods

Yeast strains

Yeast strains named *BF, Ri* and *LC* were previously isolated from dairy products such as cheese, milk and yogurt. All strains were subcultured on YPGA medium (Yeast Peptone Glucose Agar - contained: –g/L yeast extract 5; D-glucose 20; peptone 10; agar 20; pH 6.0) medium and preserved according to deep-freeze method

As reference strains we used *Saccharomyces cerevisiae X208* (wild type), *S. cerevisiae D649 (MATa/MATα MAL2/mal2 trp1/TRP1 pet6/PET6 ade2/ADE2 ADE1/ade1 lys2/LYS2 HIS4/his4 LEU2/leu2 THR4/thr4), Rhodotorula minuta,* strains from the Microbial Collection of the Laboratory of Microbial Genetics and Biotechnology from the Faculty of Biology, University of Bucharest and *Rh. glutinis ICCF* and *Rh. rubra ICCF* (National Institute for Chemical and Pharmaceutical Research and Development, Bucharest)

Preliminary identification on the basis of morphological and physiological properties

Morphological observation

Yeasts from fresh growing culture were cultivated on YPGA in Petri dishes, and the surface, color, margin and elevation of the colonies were observed. The yeasts were also inoculated in liquid YPG medium for determination of their cultural characteristics (pellicle, sediment or ring formation). The microscopic appearance of the cells grown in the same liquid media was also observed [10].

Physiological characterization

In order to distinguish between yeast species on the basis of their physiological abilities, we used the methods described by Barnet et. al. [11,12]

Assessing the ability to use certain sugars semianaerobically

The ability to use some carbohydrates for semianaerobic assimilation (fermentation) was determined by using Durham technique on YE medium (g/L-yeast extract 0.5) containing 50mM of test sugar (D-glucose, D-galactose, maltose, sucrose, D(+)- trehalose, melibiose, lactose, cellobiose, raffinose).

Negative control tubes contained no sugar. Each tube was inoculated from a fresh yeast culture and incubated at 28°C for about one week. The ability to use anaerobically these compounds, was assessed by looking for the formation of gas (CO_2). As positive control we used tubes inoculated with *S. cerevisiae X208* strain.

Assessing the ability to use organic compounds as sole source of carbon for aerobic growth

Yeast nitrogen base (YNB) medium (g/L Nitrogen Base Difco 6.7; pH= 5,6) containing 3% inoculum was used for testing the assimilation of different carbon sources by yeasts. A total of 15 tests were done: assimilation of D-glucose, galactose, sorbose, D-ribose, D-xylose, L (+)-arabinose, sucrose, D- arabinose, maltose, D(+)-trehalose, D(+)-cellobiose, salicin, lactose, raffinose, D-mannitol) [13]. The results were registered after $2^{th,}$ 4^{th} and the 6^{th} day at the corresponding optimal growth temperature (28°C), according with two different controls: a positive one (YNB medium supplied with D-glucose) and a negative one (YNB without source of carbon).

DBB test

A culture 13 days old, on YM medium (g/L – malt extract 3, peptone 5, D-glucose 10, agar 20) was incubated at 55°C for 4h. The Petri dishes were then flooded with ice-cold DBB reagent (0.1MTris-HCl; Diazonium Blue B Salt 1 mg/mL) [14].

If the culture turned red within 2 min at room temperature, the result was estimated as being positive one (basidiomycetous yeast). Strains of *Rh. rubra, Rh. minuta, Rh. glutinis* were used as positive control while *S. cerevisiae X208* strain was used as negative control.

Additional tests

In order to obtain a complete physiological characterization of the studied strains, a number of other tests were performed: the *urease test for hydrolisation activity, growth on media containing 50% or 60% glucose, growth at non-optimal temperatures (37℃, 42℃)* [11, 15].

Data analysis

All the results obtained from morpho-physiological examination, were used as data for the final statistic analysis. For this approach we chose the *Unweighed pair group average method (UPGA)* which is often used in numerical taxonomy. Using the UPGA method we obtained diagrams in which no connecting branch between two strains indicates 100% similarity, - identical strains appeared to be placed on OX axis [16].

Molecular approach

2 microns plasmid DNA isolation and purification

Plasmid DNA was isolated from overnight cultures (28°C in liquid YPG) using the method described by Vassu et al. with a series of optimisations [17]. Protoplasts were obtained by resuspending the harvested cells in 1mL TE-1 (0,1M Tris; 0.005M EDTA, pH= 9,3), 20μL β-mercaptoetanol, 1 mL CFEM (citric acid ; 0,1M Na_2HPO_4; 0,1M EDTA; 1M Manitol*)* and lyticase (3mg/mL) to a final concentration of 1 mg/mL. The obtained protoplasts were resuspended in TEG solution (25mM Tris; 10mM EDTA; 50mM Glucoza; pH = 8,0) for 15 min on ice. A lysis solution (1%SDS; 0.2 N NaOH; pH = 12.5) was added followed by incubation on ice for 15 min. Plasmid DNA was renaturated using 300μL potasium acetate solution (v/v: 11.5% acetic acid, 60% 5M potassium acetate, 28.5% water; pH=4.8). The samples were kept on ice and then the precipitated chromosomal DNA, proteins, cell walls, were removed by centrifugation. The supernatant was extracted with an equal volume of chloroform: isoamyl alcohol (24:1). Plasmidial DNA was precipitated with cold ethanol 100% and the pellet was resuspended in TE (Tris 10mM; EDTA 1mM; pH 8,0). The samples were stored at 4°C.

Results and Discussions

Three yeast strains named *BF*, *LC* and *Ri* were taxonomically characterized. Their *cell morphology* and *culture characteristics* are presented in **Table 1**. All strains possessed oval cells which reproduced by multipolar budding on narrow base. Beside that *LC* strain develop pseudohyphae formed by budding and elongation (**Figure 2**). None of the strains produced pigments (**Figure 1**).

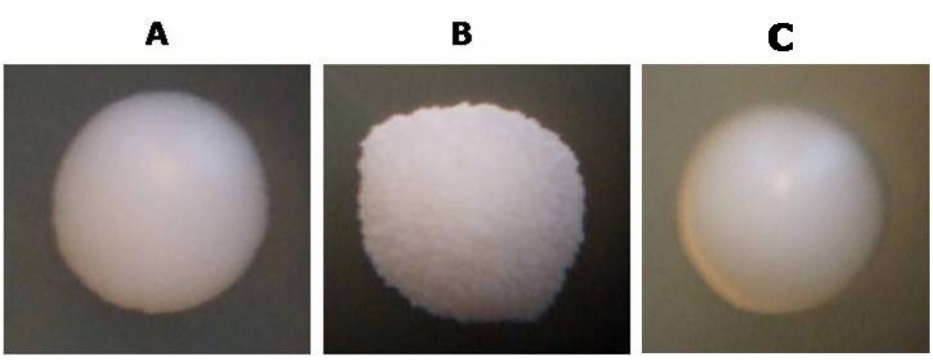

Figure 1 Macroscopical appearance of *BF* and *LC* and *Ri* strains

Table 1. Morphological and cultural characteristics of *BF, LC* and *Ri* strains

Morphological characteristics	***BF strain***	***LC strain***	***Ri strain***
Culture characteristics on YPGA			
Surface	smooth	rough, granular	smooth
Margin	entire	crispulate	entire
Color	white	white	white
Elevation	convex	convex	convex
Culture characteristics on YPG broth	sediment deposited on the bottom no pellicle	sediment deposited on the bottom pellicle on the surface	sediment deposited on the bottom no pellicle
Cells	oval cells with multilateral budding	large oval cells with multilateral budding; simple pseudohyphae	oval cells with multilateral budding

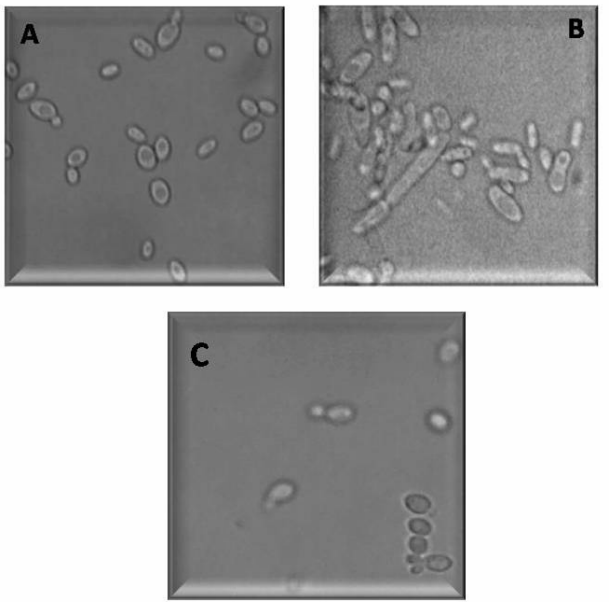

Figure 2. Cell morphology of (*A*) - *BF* and (B) - *LC* and (C) - *Ri* strains

The microscopical and macroscopical observations of *BF, Ri* and *LC* strains revealed some similarities with yeast species already characterized in the literature as: *Saccharomyces cerevisiae* and respectively *Candida parapsilosis.* Further *physiological analysis* were performed for a preliminary identification of the strains.

The *fermentation* tests showed that *BF* and *Ri* strains could catabolize galactose and sucrose by fermentation while *LC* strain could use no sugar.

Table 2. Results of the fermentation tests for *BF, LC* and *Saccharomyces cerevisiae X208* strains

	YNB medium supplied with									
Strains	**D(+)Glucose (*Positive control*)**	**No carbon source**	**D(+)-Galactose**	**Maltose**	**Sucrose**	**D(+)-Trehalose**	**Melibiose**	**Lactose**	**D(+)-Cellobiose**	**Raffinose**
BF	+	-	+	-	+	-	-	-	-	-
LC	+	-	-	-	-	-	-	-	-	-
Ri	+	-	+	-	+	-	-	-	-	-
S. cerevisiae X208	+	-	+	+	+	-	-	-	-	-

Regarding the *assimilation* ability we observed obvious differences between strains (**Table 3**). Strain *LC* assimilated galactose, sorbose, sucrose, maltose while strain *BF* could consume only xylose. The obvious disparity between *BF* and *S.cerevisiae X208* strains it is explained by the fact that various *S. cerevisiae* strains present variability in their ability to grow on some carbon sources in aerobiosis (galactose, sucrose, maltose) [11].

Table 3. Results of the assimilation tests for BF, LC, Ri and Saccharomyces cerevisiae X208 strains

	YNB medium supplied with															
Strains	**D(+)Glucose (*Positive control*)**	**No carbon source (*Negative control*)**	**D(+)-Galactose**	**L(+)-Sorbose**	**D-Ribose**	**D-Xylose**	**L(+)-Arabinose**	**Sucrose**	**D-Arabinose**	**Maltose**	**D(+)-Cellobiose**	**D(+)-Trehalose**	**Salicin**	**Lactose**	**Raffinose**	**D-Mannitol**
BF	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
LC	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-
Ri	+	-	+	-	-	-	-	+	-	+	-	-	-	-	+	-
S. cerevisiae X208	+	-	+	-	-	-	-	+	-	+	-	+/ weak	-	-	-	-

The *degree of urea hydrolysation* is proportional to the urease amount within the yeast cells. Urease hydrolyses urea to CO_2 and NH_3, and the color of the growth medium turns from yellow to red **(Figure 3)**. Our results indicated a low urease activity in *LC* cells.

Figure 3. Urease activity for (1) *Ri* (2) *BF* and *LC* strains

DBB test allowed us to identify yeast species relying on their positive or negative reaction with DBB reagent, confirming the ipothesis that our strains are ascomycetous yeasts.

The test for *resistance to high concentrations of glucose* was used because certain yeast species are able to grow on media with high concentrations of sugar. Our study showed that strains *BF, Ri* and *LC* grew on YPGA medium containing 50% and 60% D-glucose.

Concerning the *growth temperature requirements*, all studied strains except *BF*, were thermotolerant, being able to grow at non-optimal temperatures (37°C and 42°C).

Classical taxonomy analysis showed a great similarity between *BF, Ri* and *Saccharomyces cerevisiae* respectively between *LC* strains and *Candida parapsilosis,* suggesting a possible affiliation of our strains to these species.

Data analysis

UPGA is a method from "Tree clustering" category in which the distance between two clusters is calculated as the average distance between all pairs of objects in the two considered clusters.

For performing UPGA test, we converted the results from all morpho-physiological tests into 34 variables used in statistic analysis (for example: ability to use a certain sugar). A numerical value (1 for positive, 2 for negative) was assigned to each possible variant of a considered characteristic **(Table 4)**.

For *S. cerevisiae* and *C. parapsilosis* we created a data base, in which we characterized hypothetical reference strain by random combination of their possible characteristics. Data obtained for our *BF, Ri* and *LC* strains were compared to variants from the data base.

Table 4. Exemplification of the assignment of numerical values to some characteristics employed in the UPGA method

	PHYSIOLOGICAL TRAITS Ability to use certain sugars aerobically							
	D(+) Galactose	**L (+) Sorbose**	**D – Ribose**	**D – Xilose**	**L - Arabinose**	**D - Arabinose**	**Sucrose**	**Maltose**
Characteristic no.	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
Variants	**1- + 2- -**	**1- + 2- -**	**1- + 2- -**	**1- + 2- -**	**1- + 2- -**	**1- + 2- -**	**1- + 2- -**	**1- + 2- -**
Candida parapsilosis 1	1	1	1	1	1	2	1	1
Candida parapsilosis 2	1	1	1	1	1	2	1	1
Candida parapsilosis 3	1	1	2	1	1	2	1	1
Candida parapsilosis 4	1	1	2	1	1	2	1	1
LC	2	1	2	2	2	2	2	1

According to UPGA analysis *BF* and *Ri* strains were identified as *Saccharomyces cerevisiae* exhibiting zero linkage distance from the reference *strain S. cerevisiae – 105* respectively *S. cerevisiae 34* (randomly generated pattern*)* (**Figure 4, 5**)

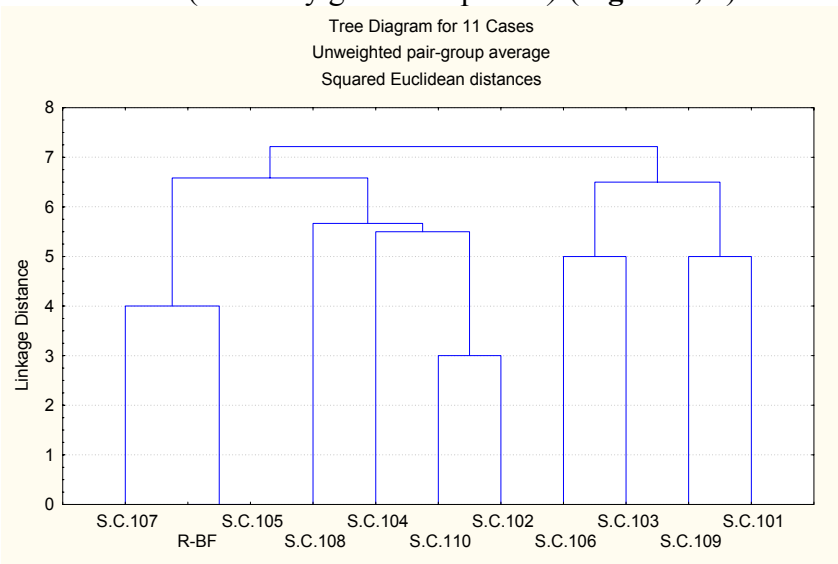

Figure 4. Identification of the *BF* strain as member of the *Saccharomyces cerevisiae* species by the UPGA method

From UPGA analysis, strain *LC* was found to belong to the genus *Candida,* clustering to the species *Candida parapsilosis* (**Figure 6**). Nevertheless, since a slight linkage distance is observed, more analysis at molecular level should be performed for a clear identification.

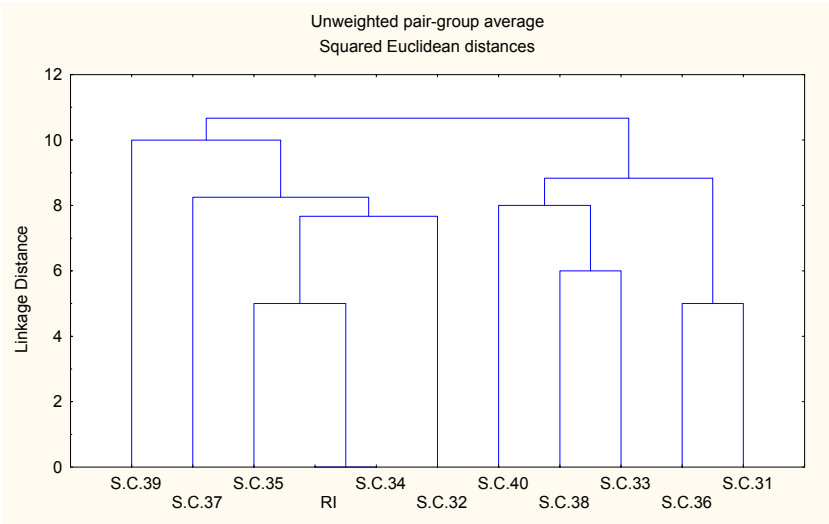

Figure 5. Identification of the *Ri* strain as member of the *Saccharomyces cerevisiae* species by the UPGA method

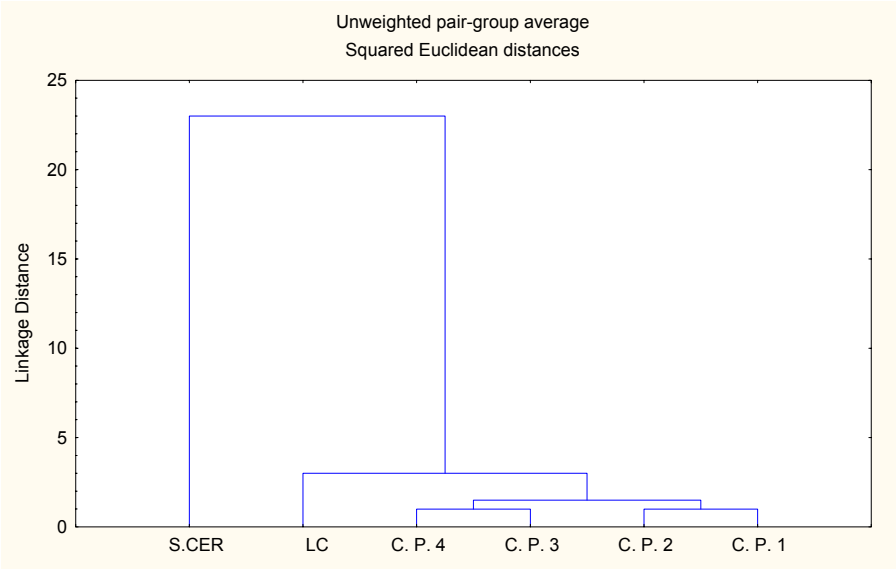

Figure 6. Identification of the *LC* strain as member of the *Candida parapsilosis* species by the UPGA method

Molecular analysis

For an accurate identification of the studied strains, further molecular analysis is necessary to be done. A first step in this approach was the isolation of 2 microns plasmid DNA. In case of the reference strain *S. cerevisiae D649*, we were able to isolate both the monomeric and dimeric forms of the 2 microns plasmid (**Figure 6).** For all the samples traces of chromosomal DNA were present. No differences between our three studied strains could be display by this analysis, all of them lacking 2 μm plasmid.

The absence of the 2 microns plasmid DNA is a characteristic for *C. parapsilosis* suggesting that LC strain could belong to this specie. Also, their absence in BF and Ri strains,

is not unusual, since 2 microns plasmids are strain-specific, being present only in some *S. cerevisiae* strains [18].

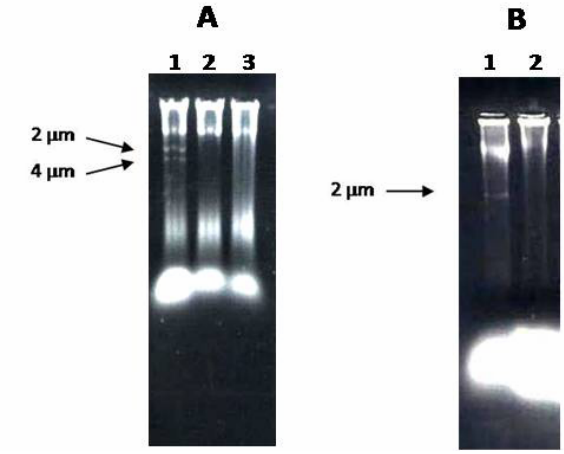

Figure 7. Electrophoresis of the 2 microns plasmid DNA isolated from *(*A*)*1- *Saccharomyces cerevisiae D649, 2-BF, 3-Ri* ; (*B*)1-*Saccharomyces cerevisiae D649, 2- LC;*

Conclusions

Classical taxonomy analysis showed a great similarity between *BF, Ri* strains and *Saccharomyces cerevisiae* as well as for *LC* strain and respectively *Candida parapsilosis,* suggesting a possible belonging of our strains to these species. This preliminary identification was confirmed by statistical analysis of the obtained data. Molecular analysis indicated the absence of the 2 microns DNA in our strains, suggesting that other molecular analysis should be performed for a more accurate identification of the strains.

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