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Isolation and characterization of *Saccharomyces cerevisiae* strains of winery interest

Thais M. Guimarães[1], Danilo G. Moriel[1], Iara P. Machado[1], Cyntia M. T. Fadel Picheth[2], Tania M. B. Bonfim[1]*

[1]Laboratório de Enzimologia e Tecnologia das Fermentações, [2]Laboratório de Bacteriologia, Departamento de Farmácia, Universidade Federal do Paraná

Despite the availability of several Saccharomyces cerevisiae *commercial strains intended for wine production, strains isolated from winery regions are usually more adapted to their own climatic conditions, grapes and also partially responsible for particular characteristics that frequently identify specific wines and regions. Thus the microbiota of an important winery region (Colombo) was studied in order to isolate and characterize* S. cerevisiae *strains that could be used on wine production. From 61 yeasts isolated, 14 were identified as* S. cerevisiae. *Some of them showed fermentative characteristics even better than commercial strains indicating that they could be applied on wine production in order to increase the quality and assure the particular wine characteristics of that region.*

Uniterms
- *Saccharomyces cerevisiae*
- Wine
- RAPD-PCR

***Correspondence:**
T. M. B. Bonfim
Laboratório de Enzimologia e
Tecnologia das Fermentações
Universidade Federal do Paraná
Rua Lothário Meissner, 3400
80210-170 - Curitiba, PR - Brasil
E-mail: tbonfim@ufpr.br

INTRODUCTION

Colombo, State of Paraná, is one of the most important winery regions in Brazil producing 800,000 liters of artisan wine annually. However, the absence of standardized methods for wine production has led to a loss on its wine identity and difficulty on market insertion especially associated to the great variability of the wine produced each year.

The utilization of isolated strains of *S. cerevisiae* is an important strategy for keeping the quality and assuring the reproducibility of wine features. The utilization of strains isolated from specific regions could be even more interesting because of their high adaptation to their own climatic conditions and grapes. Even more, these strains are usually associated to particular wine characteristics that frequently identify specific wines and regions.

Thus the aims of this work are isolate and characterize *S. cerevisiae* strains from Colombo region that could be used as a tool for improving the wine quality and reproducibility of the wines obtained, allowing the creation of a strong identity that could facilitate their market insertion.

MATERIAL AND METHODS

Microorganisms

The commercial strains *S. cerevisiae cerevisiae* (CK) and *S. cerevisiae bayanus* (BC) were provided by Danster Ferment AD, Switzerland. The strains *Schyzosaccharomyces pombe, Kluyveromyces marxianus* and *Phaffia rhodozyma* were obtained from the American Type Culture Collection.

Media

The culture media used in this work were YP (10 g/L yeast extract, 10 g/L peptone, 20 g/L agar) supplemented

with different carbon sources at the concentration of 20 g/L (glucose, sucrose, galactose, maltose, raffinose, mannitol, lactose, cellobiose, xylose) or 200 g/L (glucose and sucrose), with or without the addition of 80 mL/L ethanol. The medium YP supplemented with 20 g/L glucose (YPG) were also supplemented with 6 g/L tartaric acid, 30 mg/mL erythromycin or 30 mg/mL chloramphenicol for strain isolation. The culture media Yeast Nitrogen Base (YNB) and Yeast Carbon Base (YCB) were provided by DIFCO Laboratories. YCB was prepared using the same carbon sources as for YP. YNB were prepared using potassium nitrate, sodium nitrite and lysine as nitrogen sources. The medium LA were prepared using 40 g/L glucose, 5 g/L yeast extract, 3 g/L peptone, 0.2 g/L ammonium sulfate, 1 g/L lead acetate and 20 g/L agar.

Sample collection and isolation

The yeasts were isolated from grapes, grape juice, different phases of the wine fermentation processes and bottled wine produced in 2003. The samples were plated on YPG and incubated at 30 °C for 72 hours. After incubation, the colonies were plated on YPG medium supplemented with 30 mg/mL erythromycin and incubated at the same conditions. Then the colonies were plated on YPG medium supplemented with 6 g/L tartaric acid and incubated. The grown colonies were plated on YPG medium supplemented with 30 mg/mL chloramphenicol and incubated at 30 °C for 72 hours. The colonies isolated from samples were grown and conserved on YPG tubes at 4 °C.

Inoculum growth

The isolated yeasts and the commercial strain CK were inoculated in YPG and incubated at 30 °C for 12 hours. After incubation a suspension were prepared and adjusted to an optical density of 0.100 at 650 nm (0.200 for molecular characterization). An aliquot of 200 mL was plated on specific media used for stress exclusion test, carbon and nitrogen source assimilation, temperature tolerance test and hydrogen sulfide production test. For the fermentative capacity test, flocculation and ethanol tolerance test, the volume of suspension used was that able to provide and final absorbance between 0.01 and 0.02 at 650 nm.

Taxonomic identification

The isolates belonging to *S. cerevisiae* species were identified by the carbon and nitrogen assimilation tests and fermentative capacity test as described by Vaughan-Martin and Martin (1993) and Sanni and Loner (1993).

Stress exclusion test

The isolates were plated on YPG medium at 37 °C for 72 hours. The grown colonies were plated on YPG supplemented with 80 mL/L ethanol and incubated at 30 °C for 72 hours. The colonies were then plated on YP supplemented with 200 g/L glucose and grown at the same conditions. After incubation the colonies were plated in medium YP supplemented with 200 g/L sucrose and 80 ml/L ethanol and incubated at same conditions.

Ethanol tolerance test

The isolates were inoculated in 10 mL of liquid YPG supplemented or not with 100, 130 and 150 ml/L ethanol and incubated at 30 °C for 72 hours.

Temperature tolerance test

The isolates were plated in YPG and incubated at 25, 30, 37 and 45 °C for 72 hours.

Flocculation test

The isolates were inoculated in 10 mL of liquid YPG and incubated at 30 °C for 72 hours. After incubation, the tubes were agitated for the visualization of flocculation.

Hydrogen sulfide production test

The isolates were plated in LA and also in YPG and incubated 30 °C for 10 days (ONO *et al*., 1991).

Molecular assays

The DNA extraction was performed according to Ausubel et. al. (1999). PCR for *S. cerevisiae* identification was done using a par primers specie-specific SC1 and SC2 according to Sabaté *et al*., (2000). RAPD-PCR was done according Xufre *et al*., (2000), using the following primers: OPB-01, OPB-10, OPB-12, OPB-14, OPX-01, OPX-03, OPX-06, OPX-07 (Echeverrigaray *et al*., 2000), ABI-15 e P-20 (Xufre et al. 2000). The sequence of the primers used are showed in Table I. The products of the PCR reactions were analyzed by electrophoresis on 1,8% agarose gels stained with ethidium bromide and visualized under UV. A dendogram of similarity was constructed using the program NTSYSpc (Rohlf, 1999) based on UPGMA cluster analysis of Jaccard coefficients.

TABLE I – Sequence of the primers used for the PCR and RAPD-PCR assays

PRIMER	SEQUENCE
SC1	5' – AACGGTGAGAGATTTCTGTGC – 3'
SC2	5' – AGCTGGCAGTATTCCCACAG – 3'
OPB – 01	5' – GTTTCGCTCC – 3'
OPB – 10	5' – CTGCTGGGAC – 3'
OPB – 12	5' – CCTTGACGCA – 3'
OPB – 14	5' – TCCGCTCTGG – 3'
OPX – 01	5' – CTGGGCACGA – 3'
OPX – 03	5' – TGGCGCAGTG – 3'
OPX – 06	5' – ACGCCAGAGG – 3'
OPX – 07	5' – GAGCGAGGCT – 3'
AB1 – 15	5' – GGAGGGTGTT – 3'
P – 20	5' – AGGAGAACGG – 3'

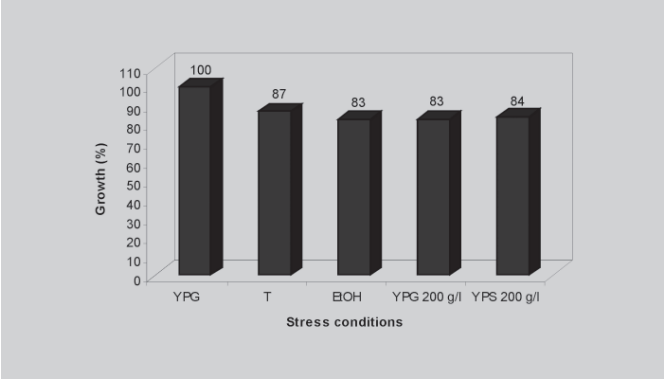

FIGURE 1 - Yeasts isolated from stress exclusion tests. Negative control (YPG); temperature stress test (T); ethanol stress test (ETOH); osmotic stress test with glucose (YPG 200 g/L); osmotic stress test with sucrose (YPS 200 g/L).

RESULTS AND DISCUSSION

The species that predominates on fermentative process for wine production is *S. cerevisiae* (Ivorra *et al*., 1999). During the fermentation the yeast produces ethanol, carbon dioxide and other secondary products important for flavor, taste and quality of wine serving as a reference on strain isolation (Lilly *et al*., 2000).

In order to isolate *S. cerevisiae* strains from bacterial contamination the samples were submitted to a set of sequential media supplemented with antibacterial substances. It allowed the isolation of 61 colonies with morphological features that could correspond to *S. cerevisiae* species.

During the fermentation the yeast usually does not find an environment of optimal conditions, being continuously exposed to several stress conditions, especially osmotic and ethanol stress (Querol *et al*., 2003). Most of strains isolated from artisan fermentative processes, including *S. cerevisiae*, are physiologically adapted to extremely conditions as growth at 37 ^{o}C, at 500 g/L glucose and 80 ml/l ethanol Pataro *et al*., 2000). Using strains that are not adapted to stress conditions could be a mistake that could compromise an entire fermentative process. Thus we submitted the 61 colonies isolated and also the commercial strain CK to stress conditions for select strains adapted for industrial processes.

Figure 1 show the results obtained on stress exclusion tests. As observed 87% (n=61) of total yeasts grew at 37 ^{o}C. When these colonies were plated on YP supplemented with 80 ml/L ethanol, only 83% (n=53) were able to grow. All the strains resistant to ethanol stress was also resistant to osmotic stress with glucose (83%, n=53). The commercial strain CK was able to grow in all conditions tested. The resistance to stress conditions could be related to gene expression and usually leads to problems during the fermentative process (Zuzuarregui *et al*., 2004). Glucose is the carbon source of first choice for *S. cerevisiae* but is also able to repress genes that code for metabolic enzymes as invertase (Gancedo, 1998). The resistance to glucose repression could be interesting for wine production as well as a high invertase activity (Pataro *et al*., 1998). Among the isolates studied 84% (n=53) showed to be resistant to osmotic stress with sucrose. This resistance could be an important indication of high

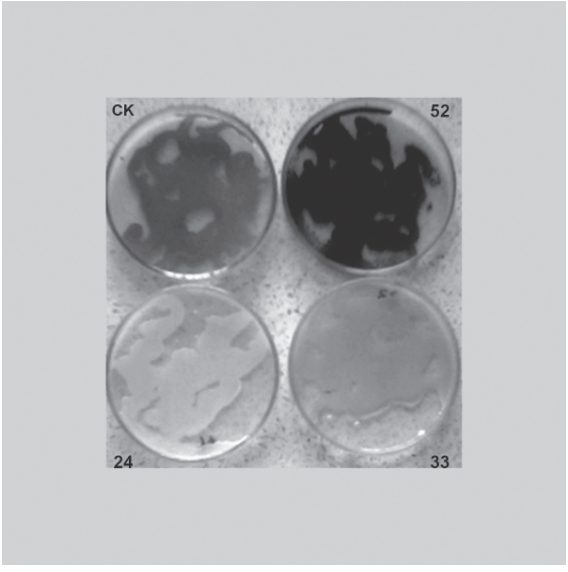

FIGURE 2 – Hydrogen sulfide production. Commercial strain (CK) and isolated yeasts (24, 33 and 52). High intensity colours indicate the production of hydrogen sulfide.

invertase activity and an important feature for strains used on wine production (Pataro *et al*., 1998). Thus from 61 colonies isolated only 37 passed on stress exclusion tests and were submitted to taxonomic identification tests.

Table II illustrates the results obtained on the taxonomic identification. From 37 colonies studied, 22 colonies showed all features of *S. cerevisiae* species.

When tested about the nitrogen source assimilation, all 22 colonies did not grow using potassium nitrate, sodium nitrite and lysine as expected (Vaughan-Martini, Martini, 1993; Sanni, Lonner, 1993).

When tested about the fermentative capacity, from the 22 colonies studied only 15 followed the pattern and were identified as *S. cerevisiae* strains (Table III).

Other tests were used for the selection of the yeasts with special features for wine production, as example of ethanol and temperatures stress. The ethanol stress is probably one of the most interesting conditions to be analyzed due to high amount of this substance during the wine fermentation process (Chi, Ameborg, 2000). Temperature affects the fermentation cinetic and the metabolism of the yeasts, which defines the wine

TABLE II – Taxonomic Identification tests

CARBON	*S.*	CK	ISOLATES																		
SOURCE	*cerevisiae*		02	03	05	06	08	11	13	14	20	21	22	23	24	25	27	28	29	31	33
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	+
Maltose	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	+
Galactose	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-	+	+	+	-	+
Raffinose	+	+	+	+	+	+	+	-	-	-	/	/	+	/	+	/	+	+	+	/	+
Mannitol	-	-	+	-	-	-	+	+	-	-	/	/	-	/	-	/	-	-	-	/	-
Lactose	-	-	/	/	-	-	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	/	/	-	-	/	/	/	/	/	/	-	/	-	/	-	-	-	/	-
Xylose	-	-	/	/	-	-	/	/	/	/	/	/	-	/	-	/	-	-	-	/	-

			34	35	37	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+	+	/	+	/	/	+	+	/	+
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	/	-	/	/	-	-	/	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	/	-	/	/	-	-	/	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	/	-	/	/	-	-	/	-

assimilate carbon source (+); do not assimilate carbon source (-); not studied (/); positive control (CK)

TABLE III – Fermentative capacity test results

CARBON	*S.*	CK	ISOLATES																				
SOURCE	*cerevisiae*		05	06	22	24	27	28	29	33	34	35	37	47	48	49	50	51	52	53	55	58	59	61
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+
Galactose	+	+	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+
Raffinose	+	+	/	/	/	+	+	/	+	+	+	/	+	+	+	+	+	+	+	+	/	+	+	-
Lactose	-	-	-	-	/	-	-	/	-	-	-	/	-	-	-	-	-	-	-	-	/	-	-	/

assimilate carbon source (+); do not assimilate carbon source (-); not studied (/); positive control (CK)

TABLE IV – Effect of ethanol and temperature on *S. cerevisiae* growth

YEAST	ETHANOL CONCENTRATION (g/L)			TEMPERATURE (ºC)		
	100	130	150	25	37	45
24	+++	++	+	+++	+++	-
27	+++	+++	+++	+++	+++	-
29	+++	++	+	+++	+++	-
33	+++	-	-	+++	+++	-
34	+++	+	-	+++	+++	-
37	+++	-	-	+++	+++	-
47	+++	-	-	+++	+++	-
48	+++	-	-	+++	+++	-
49	+++	+	-	+++	+++	-
50	+++	-	-	+++	+++	-
51	+++	-	-	+++	+++	-
52	+++	++	-	+++	+++	+
53	+++	++	-	+++	+++	-
58	+++	-	-	+++	+++	-
59	+++	+	-	+++	++	-
CK	+++	++	-	+++	+++	+

intensive growth (+++); moderate growth (++); low growth (+); no growth (-); positive control (CK)

TABLE V – Production of hydrogen sulfide and flocculation capacity of *S. cerevisiae* isolates

YEAST	FLOCULATION	HYDROGEN SULFIDE
24	-	-
27	+	+
29	-	++
33	-	+
34	-	++
37	-	-
47	-	-
48	-	-
49	-	++
50	-	+
51	-	++
52	-	+++
53	-	-
58	-	-
59	-	-
CK	-	++

intensive hydrogen sulfide production (+++); moderate hydrogen sulfide production (++); low flocculation/low hydrogen sulfide production (+); no flocculation/ hydrogen sulfide production (-).

chemistry composition and consequently its quality. Table IV shows the effects of different ethanol concentrations and temperatures on growth of the selected colonies.

The yeasts were also tested for their flocculation abilities. The flocculation is an important characteristic that allows an easy separation of the final product at the end of the fermentation without additional filtration/ centrifugation steps and also allows the utilization of immobilized yeasts on fermentation processes (Stratford, 1992). Only one isolate showed the flocculation ability as observed on Table V.

Yeasts with a elevate production of hydrogen sulfide are undesirable for wine production because confer flavor and taste that compromise the quality of the wine obtained (Ribeiro, Horii, 1999). Thus the yeasts were tested for hydrogen sulfide production (Table V and Figure 2). The yeast 52 showed a high production of hydrogen sulfide and excluded from the selected isolates.

Thus the isolates 24, 27, 29, 33, 34, 37, 47, 48, 49, 50, 51, 53, 58 and 59 (n=14) were compatible with the taxonomic identification and were in accordance to the features expected from wine production strains and then submitted to molecular identification.

In order to complement the taxonomic identification, PCR assays were done using specie-specific primers (SC1 and SC2) for *S. cerevisiae* (Sabaté *et al*., 2000). These primers permit the amplification of a 1170 bp DNA fragment located between ITS -1 region and

the LSU gene of *S. cerevisiae* strains. To confirm the specificity of these primers PCR assays were done using the commercial strains CK and BC as positive control, and *Schyzosaccharomyces pombe*, *Kluyveromyces marxianus*, *Phaffia rhodozyma* and the isolated yeast 31 as negative control. The PCR results are shown on Figure 3. While a band of 1170pb was observed for *S. saccharomyces* strains, no amplification was observed for *S. pombe*, *K. marxianus, P. rhodozyma*, and isolate 31, showing the specificity of the primers.

Then the same was done for the yeasts previously selected in order to confirm their identity. The 1170pb DNA fragment was observed for all the isolates which were then considered as *S. cerevisiae* (Figure 4).

RAPD-PCR has frequently been used for discrimination of *S. saccharomyces* strains used on wine production identifying variations at DNA level among the yeasts (Ratón, 2004). Ten primers were used for testing the strains. Except for OBP-14, all the others primers used were able to generate amplification products on the *S.*

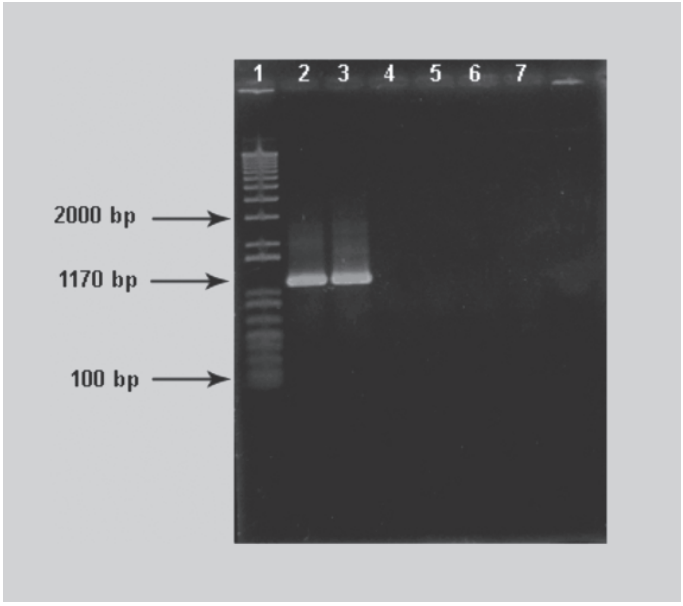

FIGURE 3 – Products of PCR for different yeasts using primers SC1 and SC2. 1 Kb Plus DNA Ladder (Invitrogen); CK (2); BC (3); yeast 31 (4); *K. marxianus* (5); *S. pombe* (6); *P. rhodozyma* (7).

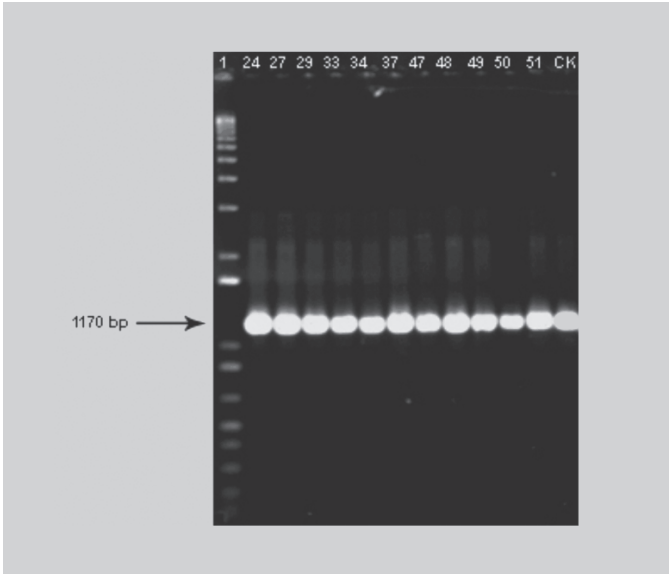

FIGURE 4 – PCR for isolated yeasts using primers SC1 and SC2. 1 Kb Plus DNA Ladder (Invitrogen); isolated strains (24-51); positive control (CK).

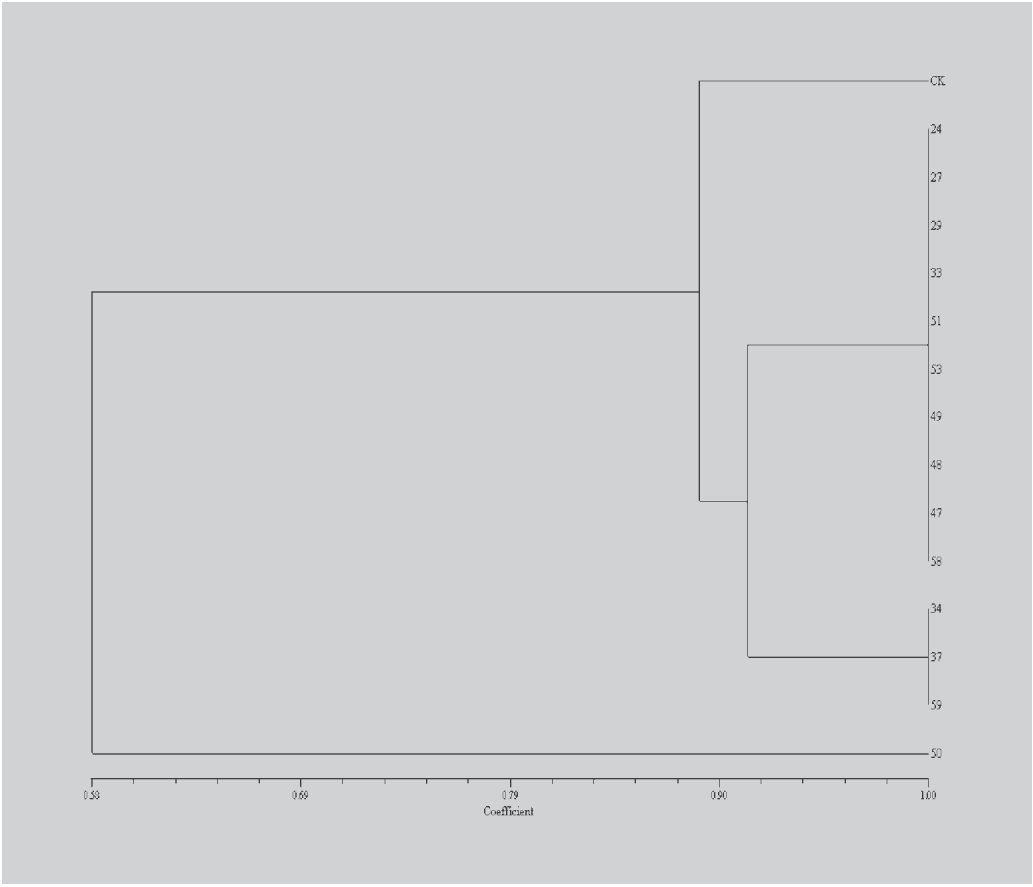

FIGURE 5 – Dendogram based on UPGMA cluster analysis of Jaccard coefficients for the selected yeasts studied.

saccharomyces strains isolated. Amplification with primers OPB-1, OPB-12, OPX-01, OPX-03, OPX-07 and P-20 indicated the presence of polymorphisms among them allowing the identification of different profiles among the isolates.

The results of RAPD-PCR allowed the construction of a dendogram for the *S. saccharomyces* strains isolated. Figure 5 shows that yeasts isolated were grouped in three clusters, while strain CK used as a control was classified in a distinct group.

CONCLUSIONS

The present study allowed the isolation and characterization of 14 *S. cerevisiae* isolates with interesting features for wine production. These isolates could contribute for the improvement of the wine quality and also could be used to create an identity for the wine produced in Colombo, facilitating its insertion in the market. Vinification tests must be still performed to complement the results obtained and confirm the ability of these isolates on the production of high quality wines.

RESUMO

Isolamento e caracterização de cepas de *Saccharomyces cerevisiae* de interesse em produção de vinho

Despite the availability of several Saccharomyces cerevisiae *commercial strains intended for wine production, strains isolated from winery regions are usually more adapted to their own climatic conditions, grapes and also partially responsible for particular characteristics that frequently identify specific wines and regions. Thus the microbiota of an important winery region (Colombo) was studied in order to isolate and characterize* S. cerevisiae *strains that could be used on wine production. From 61 yeasts isolated, 14 were identified as* S. cerevisiae*. Some of them showed fermentative characteristics even better than commercial strains indicating that they could be applied on wine production in order to increase the quality and assure the particular wine characteristics of that region.*

UNITERMS: Saccharomyces cerevisiae*. Wine. RAPD-PCR*

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