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Abstract

The aim of this research was to genetically and technologically characterize *Candida zemplinina* strains isolated from different sources of enological interest. Phenotypic and genotypic subtyping, as well as enological characterization, were carried out on 36 *C. zemplinina* isolates collected from grapes, must and wines of different regions of Italy. RAPD-PCR fingerprinting of the isolates revealed a high genetic heterogeneity. At physiological level, yeasts were grouped into different clusters on the basis of sugar and ethanol tolerance. Common enological characteristics were examined and strains resulted to be highly fructophilic while presenting low ethanol and acetic acid production, high glycerol production, capacity to metabolize malic acid and slower fermentation kinetics when compared to *Saccharomyces cerevisiae*.

The genetic and phenotypic intraspecies biodiversity of *C. zemplinina* gave useful data to understand its potential technological role in winemaking. This research represents a first step for the selection of *C. zemplinina* strains to be used as a starter in co-culture or in sequential inoculation with *S. cerevisiae* to improve the complexity and to enhance the particular characteristic of wines.

1. Introduction

The yeast ecology of grape juice fermentation is more complex than previously thought, and involves not only the growth of a succession of non-*Saccharomyces* and *Saccharomyces* species, but also the sequential development of strains within each species (Fleet, 2003, 2008; Ciani et al., 2010). Among the non-*Saccharomyces* wine yeasts involved in grape juice fermentation, *Candida stellata* has been frequently isolated during the course of must fermentations in different countries, especially during the fermentation of botrytized wines and other wines produced by overripe grapes, in cooked musts, and in traditional balsamic vinegars (Sipiczki, 2003; Mills et al., 2002; Solieri et al., 2006). Recent taxonomic studies revealed that *C. stellata* can easily be mistaken for the closely related species *C. zemplinina* (Sipiczki, 2003, 2004; Sipiczki et al., 2005), a wine yeast firstly isolated from musts with high sugar content and in botrytized musts (Sipiczki, 2003; Mills et al., 2002; Tofalo et al., 2009). The unequivocal identification of *C. zemplinina* requires molecular techniques, such as restriction fragment length polymorphism (RFLP) analysis of the 5.8S internal transcribed spacer (ITS) and sequencing of the D1/D2 domain of the 26S rRNA gene. When 5.8S ITS RFLP analysis is applied, the most frequently used restriction endonucleases (e.g. *Hinf*I, *Cfo*I and *Hae*III) produce identical patterns for both species, whereas distinct species specific patterns can only be obtained using *Mbo*I and *Dra*I (Sipiczki, 2004). The molecular taxonomic examination of 41 strains deposited in six culture collections or described in the literature as *C. stellata*, showed that most of those isolated from grapes or wines belonged to the species *C. zemplinina* (Csoma and Sipiczki, 2008). Indeed, *C. zemplinina* is almost indistinguishable from *C. stellata*: both species have three chromosomes, and display chromosomal length polymorphism (Sipiczki, 2004). A complete mitochondrial genome sequence of *C. zemplinina* was carried out by Pramateftaki et al. (2008). The mtDNA is a circularly mapping genome of 23.114 bt, containing 35 genes and is the smallest in yeasts; however, it contains all typical genes detected as a minimum set in yeast mitochondrial genomes (Pramateftaki et al., 2006). After that, most of the recent publications about the microbiota of wines reported only the presence of *C. zemplinina*, without detecting any *C.*

stellata populations (Nisiotou et al., 2007; Lopandic et al., 2008; Tofalo et al., 2009, 2011; Urso et al., 2008; Esteve-Zarzoso et al, 2010; Andorra et al., 2010).

Candida zemplinina has a small genome and from a physiological point of view is acidogenic, highly osmotolerant, growing better than *C. stellata* in the presence of ethanol and at low temperature (Sipiczki, 2004). It is apparently favored by lower temperatures, being dominant at 4 °C and present at 10 °C, but it is not detected at 15 °C (Zott et al., 2010). As regards sugar utilization, the strains of *C. stellata*, now identified as *C. zemplinina*, were known as fructophilic yeasts of oenological importance (Ribéreau-Gayon et al., 1975; Minárik et al., 1978; Ciani and Ferraro, 1998; Soden et al., 2000).Comparative evaluation of some enological properties in wine strains of *C. stellata* and *C. zemplinina* showed that both species are fructophilic, but this characteristic is very strong in *C. zemplinina* (Magyar and Tóth, 2011). The same authors found that all the tested *C. zemplinina* strains possessed an extremely poor ethanol yield from sugar consumed, which cannot be explained by the overproduction of any other metabolic products investigated in the study. Ciani and Ferraro (1996) and Ciani et al. (2000) found that *C. stellata* DBVPG 3827 under anaerobic conditions showed a low alcohol dehydrogenase activity (3-4 fold) and higher glycerol-3-phosphate dehydrogenase activity (40 fold) than that of *Saccharomyces cerevisiae*. This tendency to form glycerol has been explained as a main cause of its low growth and fermentative rate. However, several discrepancies were obtained regarding results of the production of some secondary metabolites by *C. zemplinina*, such as acetic acid, glycerol and volatile compounds (Soden, 2000; Magyar and Tóth, 2011). So, new knowledge of such physiological and molecular characteristics could contribute to the understanding of the role of *C. zemplinina* in winemaking. *Candida zemplinina* is a common non-*Saccharomyces* wine yeast and its association with the ubiquitari apiculate yeasts (*Hanseniaspora uvarum*/*Kloeckera apiculata*) strongly influence the analytical composition of the final product (Heard and Fleet, 1985; Ciani, 1997). In winemaking, the use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces* strains can have advantages over fermentations inoculated with pure cultures of *S. cerevisiae*. This can thus

lead to the production of wines with more predictable and desirable characteristics. With the aim of elucidating the potential technological role of *C. zemplinina* in wine fermentations, a collection of 36 yeast strains isolated from grapes, must and wines of different regions of Italy were studied and analysed. Phenotypic and genotypic subtyping, as well as enological characterization were carried out either in synthetic medium or in red grape must. This research represents a first step for the selection of *C. zemplinina* strains to be used as a starter in co-culture or in sequential inoculation with *S. cerevisiae* to improve the complexity and to enhance the particular characteristic of wines.

2. Materials and Methods

2.1. *Yeast strains and growth conditions*

The 36 *C. zemplinina* isolates included in this study and their source of isolation are listed in Table 1. Isolates were obtained from previous studies (Tofalo et al., 2009; Urso et al., 2008) or they were identified in this study as described below. All isolates were routinely grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) for 18 h under aerobic conditions. Yeasts were maintained as culture stocks in YPD containing 20% (v/v) glycerol at -80 °C.

2.2. *Identification and RAPD-PCR analysis of the isolates*

Genomic DNA was extracted from yeast cultures as reported by Querol et al. (1992). For identification purposes or to confirm yeast identification, sequencing of the D1/D2 region of the 26S rRNA was carried out according to Kurtzman and Robnett (1998), using primers NL-1 and NL-4.

RAPD-PCR analysis was performed with the oligonucleotides R5 (5′-AACGCGCAAC-3′) and RF2 (5’-CGGCCCCTGT-3’) according to Martín et al. (2006) and Paffetti et al. (1995), respectively. RAPD-PCR patterns were acquired using the Gel Doc 2000 (Bio-Rad, Milan, Italy). Conversion, normalization, and further analysis of the patterns were carried out with the Fingerprinting II

Informatix software (Bio-Rad). Band similarities between RAPD-PCR patterns were analysed using Dice coefficient, and correlation coefficients were calculated by the unweighted pair group method with arithmetic averages (UPGMA).

2.3. *Sugar and ethanol tolerance*

Cells grown in YPD were collected by centrifugation at 3,000 × g for 10 min, and washed twice with potassium phosphate buffer (pH 7.4). Then, cells were suspended in the same buffer to give a concentration of about 10^6 colony forming units (cfu)/ml. A 20 µL aliquot was inoculated into *Bioscreen* C (M-Medical S.r.l., Italy) plates containing 180 µL YPD broth with 150 ppm chloramphenicol and supplemented with 0 (control), 8, 14, or 20 % (v/v) ethanol or 2, 20, 40, 60 % (w/v) glucose and incubated at 25 °C for 100 h. The increase in cell number was determined by measuring the optical density (O.D.) of cultures at 600 nm. In order to evaluate yeast growth, the values were analysed over time according to the Gompertz equation modified by Zwietering et al. (1990):

$$y = A \cdot \exp\left\{-\exp\left[\left(\frac{\mu_{\max} \cdot e}{A}\right) \cdot (\lambda - t) + 1\right]\right\}$$

where y is the O.D. value at time t (h), A represents the maximum O.D. (when t→∞), μ_{max} is the maximum specific growth rate (as h^{-1}), and λ is the lag time (h) for O.D. increase. For modelling with the Gompertz equation, means of three replicates and two repetitions were used. In all the cases, the variability coefficient of raw data (cell load as O.D.) was < 5 %. The data relative to the growth kinetics were subjected to Student's t test to identify significant differences between yeast species using a statistical package (STAT. version 8.0, StatSoft Inc. Tulsa, OK).

2.4. *Experimental fermentation*

Experiments were carried out in 130 ml Erlenmeyer-flasks using 95 ml aliquots of pasteurised red grape juice from Montepulciano d'Abruzzo cultivar, that contained 220 g/l fermentable sugars, 6.5

g/l titratable acidity, and a pH of 3.6. The must samples, after treatment at 70 °C for 30 min, were inoculated with 5 ml of a pre-culture grown for 48 h in the same must, as described by Tofalo et al. (2007). Fermentations were carried out in duplicate for each strain at a controlled temperature of 25 °C, and the weight loss as a result of CO_2 production was monitored daily. Fermentation was considered completed when the CO_2 release ceased, and the samples were refrigerated for 2 days at 4 °C, racked and stored at -20 °C until analysis. Non inoculated must was used as a negative control.

2.5. *Physico-chemical determinations*

pH, total titratable acidity (TTA), ethanol, glycerol and free sulfur dioxide (SO_2) were determined on samples taken at the end of fermentation following the official OIV methods of analysis (1990). The ability to produce hydrogen sulfide was tested with lead acetate paper. Foam production was observed qualitatively comparing all the microvinifications.

Organic acid content, glucose and fructose concentrations were determined according to Tofalo et al. (2011) and Lopez et al. (1996), respectively.

Biogenic amines production was determined according to Tofalo et al. (2007). All analyses were performed in triplicate.

2.6. *Statistical analysis*

Principal component analysis (PCA) was performed using statistical software STATISTICA for Windows (STAT. version 8.0, StatSoft Inc. Tulsa, OK).

3. Results

3.1. *Identification and typing of isolates*

By D1/D2 region sequencing, all 36 isolates included in this study were identified or confirmed in their identification to belong to the species *C. zemplinina*. All the isolates were differentiated at

strain level by RAPD-PCR assay, using the primers R5 and RF2. Indeed, RAPD-PCR has proved to be an informative method suitable for the study of a large number of strains in a short time, and has been used successfully for identification and intraspecific differentiation of *Saccharomyces* and non-*Saccharomyces* yeast species (Quesada and Cenis, 1995; Torriani et al., 1999; Bujdoso et al., 2001; Urso et al., 2008; Lopandic et al., 2008). Figure 1 shows the dendrogram based on numerical analyses of the combined PCR patterns. The reproducibility of the RAPD-PCR patterns was assessed by comparing the profiles obtained from separate cultures of the same strain. The reproducibility of the assays and running conditions was higher than 90 % (data not shown). Clusters were arbitrarily identified at a similarity level of 85 %. The majority of the isolates were grouped in 6 main clusters, however a high number of single-strain clusters were obtained. RAPD-PCR revealed a remarkably high genetic diversity within our set of strains, but low correlation with the source of isolation was found. Most of the *C. zemplinina* isolates from Vino cotto were included in cluster II and III (82 %) while, cluster V (the most numerous) included isolates from Picolit, Ramandolo and Amarone grapes.

3.2. *Sugar and ethanol tolerance*

Candida zemplinina isolates were tested for their ability to grow at various concentrations of glucose (2, 20, 40 and 60 % w/v). Data relative to O.D. of three replications for each isolate were analyzed according to the modified Gompertz equation. The predicted curves fitted well with the experimental points, and their regression coefficients ranged between 0.95 and 0.98. As shown in Fig. 2, *C. zemplinina* isolates could be distinguished into five biotypes showing different patterns of growth in response to different glucose concentrations.

The first biotype was represented by 10 strains that grew better in the medium added with 2 and 20 % glucose with a decreased growth in presence of higher sugar concentrations. These isolates originated from 4 different Italian regions (Table 1). The biotype two was represented by five strains able to grow only at 40 % and to a lesser extent at 20 %. The origin of these strains was

Picolit grapes and Amarone must. The biotype three was represented by strains growing better with 20 %, followed by 2 and 40 % of glucose added, but at the end of the growth the strains reached similar O.D. values. This biotype was represented also by strains originated from Vino cotto. Five strains from grapes and Picolit must and one from Amarone must grew better with 40% glucose than with 2 and 20 %, respectively (biotype four). The last biotype was characterized by strains growing better with 20 % than with 40 % and 2 %.

All *C. zemplinina* isolates from different Italian areas grew in the media supplemented with 2, 20, 40 % (w/v) glucose, and most of them grew faster in the medium containing 20 or 40 % glucose than in that with 2 %. Glucose at 60 % inhibited many isolates, and only those of biotype 1 showed some growth (Table 2). With an increase of glucose concentration up to 40 %, most of the isolates grew better (higher A_{max}). As regards the length of lag phase (λ), a wide diversity was found among isolates, whereas no significant differences were determined for maximum growth rate (μ_{max}). Probably, λ is strain-dependent.

Considering ethanol resistance all the strains grew only at 8 % ethanol (Fig. 3). In general ethanol affected yeast growth by increasing the lag phase (λ), but it did not influence greatly the A_{max} and μ_{max} (Table 3).

3.3. *Enological characterization*

The performances of the *C. zemplinina* isolates were determined in Montepulciano d'Abruzzo must. By comparing the levels of residual sugars (fructose and glucose) and fermentation kinetics, different phenotypes were identified among the strains.

Fig.4 shows the distribution of the strains in function of their fermentation kinetics, determined as CO_2 weight loss. In general, during the first days, the fermentation started slowly, but after 15 days, the majority of the strains (20/36) were able to release more than 9 g CO_2/100 ml of must and after 25 days several strains released more than 12 g CO_2/100 ml. Fifty % of these strains showed a

similarity of 70 % according to the RAPD analysis, whereas the strains that released CO_2 >13 g/100 ml clustered at 78 % similarity, and most of them were isolated from Vino cotto. A similarity of 50 % was also found for the strains releasing from 11 to 12 g/100 ml CO_2.

Ethanol and some secondary compounds were produced by *C. zemplinina* strains during red must fermentation and were analyzed using PCA, in order to describe the data set. Firstly, the correlation matrix was computed in order to discriminate the variables, thus selecting six parameters (ethanol, reducing sugars, volatile acidity, polyphenols, dry extract and glycerol). Two factors with eigenvalues greater than 1 were computed, accounting for 83.43 % of the variance. Fig. 5 shows the 36 *C. zemplinina* isolates on the plane defined by the two principal components. The first principal component (PC1) accounts 62.93 % of the total variance and correlates with volatile acidity, ethanol, reducing sugars and polyphenols. The second principal component explained 20.50 % of the total variance and is correlated with glycerol. Three groups were well differentiated. Most of the strains were characterized by the same metabolism with a high residual sugars content, showing an average of 63.0 g/l. In addition, all these strains produced a high quantity of glycerol ranging from 9.85 to 11.65 g/l. Wine ethanol content ranged from 8.63 to 10.08 g/l. The volatile acidity showed an average of 0.71 g/l. Three isolates (two of which belonged to the same RAPD cluster, see Fig. 2) grouped together. They were characterized by the complete consumption of fructose and very low residual sugars (6.9 g/l as average). Ethanol, glycerol and volatile acidity reached levels similar to those of the other strains, from 8.95 to 9.65 g/l, from 8.1 to 8.43 g/l and from 0.63 to 0.66 g/l, respectively.

Among the other enological characteristics studied, 10 out of 36 strains formed high levels of H_2S, and 6 high and persistent foams. Apart from strain L344, which formed about 20 mg/l of SO_2, the other strains were normal producers of this compound reaching values up to 12 mg/l.

The biogenic amines ethylamine, putrescine, and cadaverine were formed only at low level ranging from <0,4-1.2 mg/L for ethylamine, 1.9-5.0 mg/L for putrescine and <0.4 to 3.1 mg/L for

cadaverine (data not showed). All strains produced putrescine, whereas 20 and 22 out of the 36 tested isolates produced cadaverine and ethylamine, respectively.

To evaluate differences in the content of organic acids, PC analysis was carried out (Fig. 6).

It is possible to distinguish some strains with different metabolisms. In particular R1, R5 e BC60 had low citric and tartaric acid content (0.15 and 4.25 g/l as average, respectively), while PISO02 showed the highest concentration of all examined organic acids. Most of the other strains were characterized by the same content. All the strains produced wine with low volatile acidity (0.52 g/l as average value), and reduced malic acid by 40 % of the initial content.

4. Discussion

In this research, 36 *C. zemplinina* isolates originating from grapes, must and wines mainly from different regions of Italy, but also from California, Greece and France, were studied for their genotypic and physiological/enological characteristics.

RAPD-PCR was used as fingerprinting technique to obtain information on the genetic relatedness of these isolates. This method proved to be informative and suitable for the study of a large number of strains in a short time, and has been successfully used for identification and intraspecific differentiation of *Saccharomyces* and non-*Saccharomyces* yeast species (Torriani et al., 1999; Bujdoso et al., 2001; Urso et al., 2008; Tofalo et al., 2009). A good correlation between genomic relatedness and phenotypic traits was observed, therefore we validated the 85 % similarity of RAPD-PCR profiles a threshold to delineate 15 clusters.

The growth of single cultures of *C. zemplinina* at different glucose concentrations was investigated; this yeast is considered osmotolerant, together with other non-*Saccharomyces* wine yeasts, such as *C. apicola* and *Zygosaccharomyces* spp. (Tofalo et al., 2009). Sipiczki (2003) reported that isolates from sweet botrytized wines were able to grow at 50 % glucose and to show some growth even in the presence of 60 % glucose. In a previous paper (Tofalo et al., 2009) some *C. zemplinina* strains isolated from a single habitat (Vino cotto) showed best growth in medium with 20 % glucose than

with 2 %. The comparative evaluation of the *C. zemplinina* growth at different sugar concentration revealed five biotypes, two of which showed an optimal growth kinetic at 40 % glucose. It is interesting to note that the strains showing this behavior were isolated from wines produced by overripe grapes, but not from Vino cotto. Indeed, in Vino cotto a relatively reduced biodiversity in terms of yeast species and strains was found (Tofalo et al., 2009) due to the particular vinification process (must cooking), and to the selective pressure exerted by the environmental conditions, such as high osmotic pressure and high concentration of some must compounds like polyphenols. On the contrary, in wines produced by overripe or botrytized grapes, yeasts are subjected to a progressive adaptation to the increasing dry conditions and high sugar. *C. zemplinina* is normally associated with overripe and botrytized grape berries and musts obtained from botrytized grapes (Magyar and Tóth, 2011). Botrytis-affected grapes have been found to harbor a dominant population of *C. zemplinina*, yeast that was also able dominate at early and mild stages, particularly in Botrytis-affected fermentations, which is also in accordance with previous reports (Sipiczki, 2003). Considering ethanol resistance in YPD medium with 2 % glucose, two phenotypes were detected, low tolerant or tolerant up to 8 % ethanol, independently of the origin of the strain. Similar results have been reported by Sipiczki (2003) and justify the presence of *C. zemplinina* in the middle of fermentation (Fleet, 2003; Mills et al., 2002; Sipiczki, 2003; Nisiotou, 2007).

The fructophilic nature of this species was also investigated in a grape juice containing 220 g/l fermentable sugars. The majority of *C. zemplinina* strains showed a fructophilic character, in accordance with the findings of Soden et al. (2000) and Magyar and Tóth (2011). However, three strains consumed glucose almost completely. It is possible to hypothesize that this characteristic is strain-dependent. Considering sugars consumption, it has been demonstrated that during wine fermentations *C. zemplinina* strains could, like *S. cerevisiae*, co-ferment both monosaccharides or do not utilize glucose until the fructose is completely depleted (Soden et al., 2000). It is plausible that *C. zemplinina* shows different affinities for glucose and fructose. This data should be confirmed by specific and more detailed studies.

Discrepancies between glucose and fructose utilization by *S. cerevisiae* strains during wine fermentations has been studied by Berthels et al. (2004), and have been related with different hexokinase kinetic properties of the strains (Berthels et al., 2008). Even if the glucophilic character of *Saccharomyces* wine yeasts has been confirmed, at low temperatures, some *Saccharomyces* yeasts show a fructophilic character at the beginning of fermentation (Tronchoni et al., 2009). Similarly, it is possible that some *C. zemplinina* strains could have an altered expression of an hexose transporter or could be influenced by some environmental variables, such as nitrogen or ethanol content (Berthels et al., 2004). According to Magyar and Tóth (2011), all *C. zemplinina* isolates, and particularly strains FC54, BC60 and RC5, showed an extremely poor ethanol yield from sugars, which cannot be explained by the overproduction of any other metabolic products investigated in this study.

In conclusion, *C. zemplinina* strains from different origins show several common characteristics: highly fructophilic, similar tolerance to high glucose concentrations, medium ethanol tolerance, low ethanol production, high glycerol and low acetic acid production, capacity to metabolize malic acid and slower fermentation kinetics compared to *S. cerevisiae*. Some strains investigated here possess phenotypes such as glycerol production, fructophilic character, low acetic acid production that can be interesting from an oenological point of view. The characterization of intraspecific biodiversity at phenotypic level gave useful data to understand the physiological traits of a species in winemaking and its possible use, as a starter in co-culture or in sequential inoculation with *S. cerevisiae*.

The mixed cultures of selected non-*Saccharomyces* and *Saccharomyces* strains can have advantages over fermentations inoculated with pure cultures of *S. cerevisiae* so as to improve the complexity and to enhance the particular characteristics of a wine (as review see Ciani et al., 2010; Comitini et al., 2011). Different biotypes of a species, such as *C. zemplinina*, could be useful for this purpose, even if the knowledge of its genetic and metabolic regulation still needs to be improved.

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Figure legends

Fig. 1 - Dendrogram obtained from analysis of the combined RAPD-PCR profiles using the primers R5 and RF2 for the 36 isolates analysed.

Fig. 2 - Growth kinetics of *C. zemplinina* in response to different glucose concentrations. Glucose (w/v): (▲) 2 %; (■) 20%; (□) 40%; (●) 60%. Each point is expressed as the mean of three replicates and two repetitions.

Fig. 3 - Growth kinetics of *C. zemplinina* in response to different ethanol concentrations. Ethanol (v/v): (▲) 0%; (♦) 8%; (■) 14%; (●) 20%. Each point is expressed as the mean of three replicates and two repetitions

Fig. 4 - Evolution of CO_2 during the growth of *C. zemplinina* strains in grape juice.

Fig. 5 - Principal Component (PC) analysis scores for secondary compounds obtained from grape juices fermented with *C. zemplinina* isolates.

Fig. 6 - Score plot of the first and second principal components (PC) after PC analysis encompassing organic acid contents for grape juices fermented with *C. zemplinina* isolates.

Fig.1

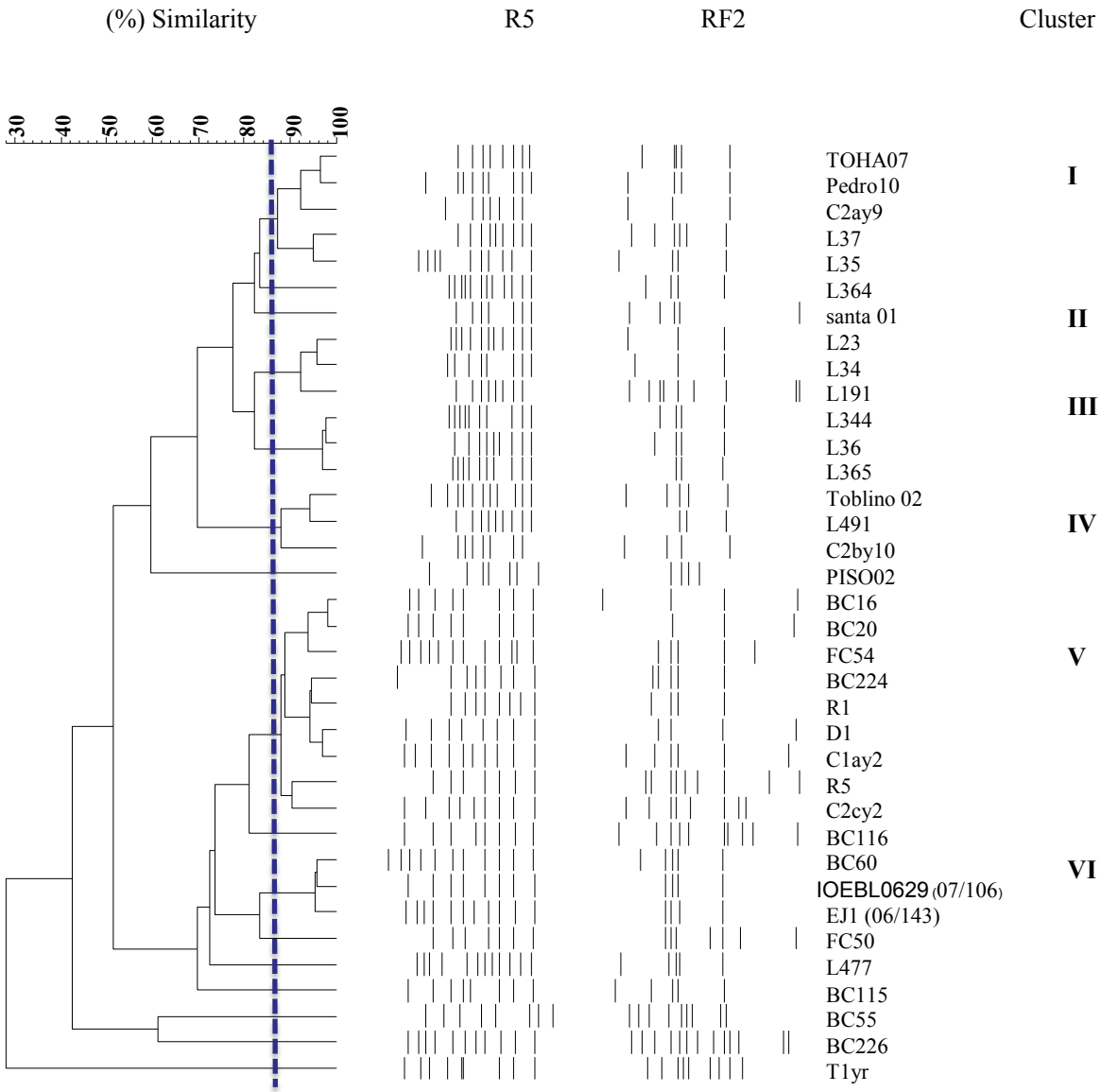
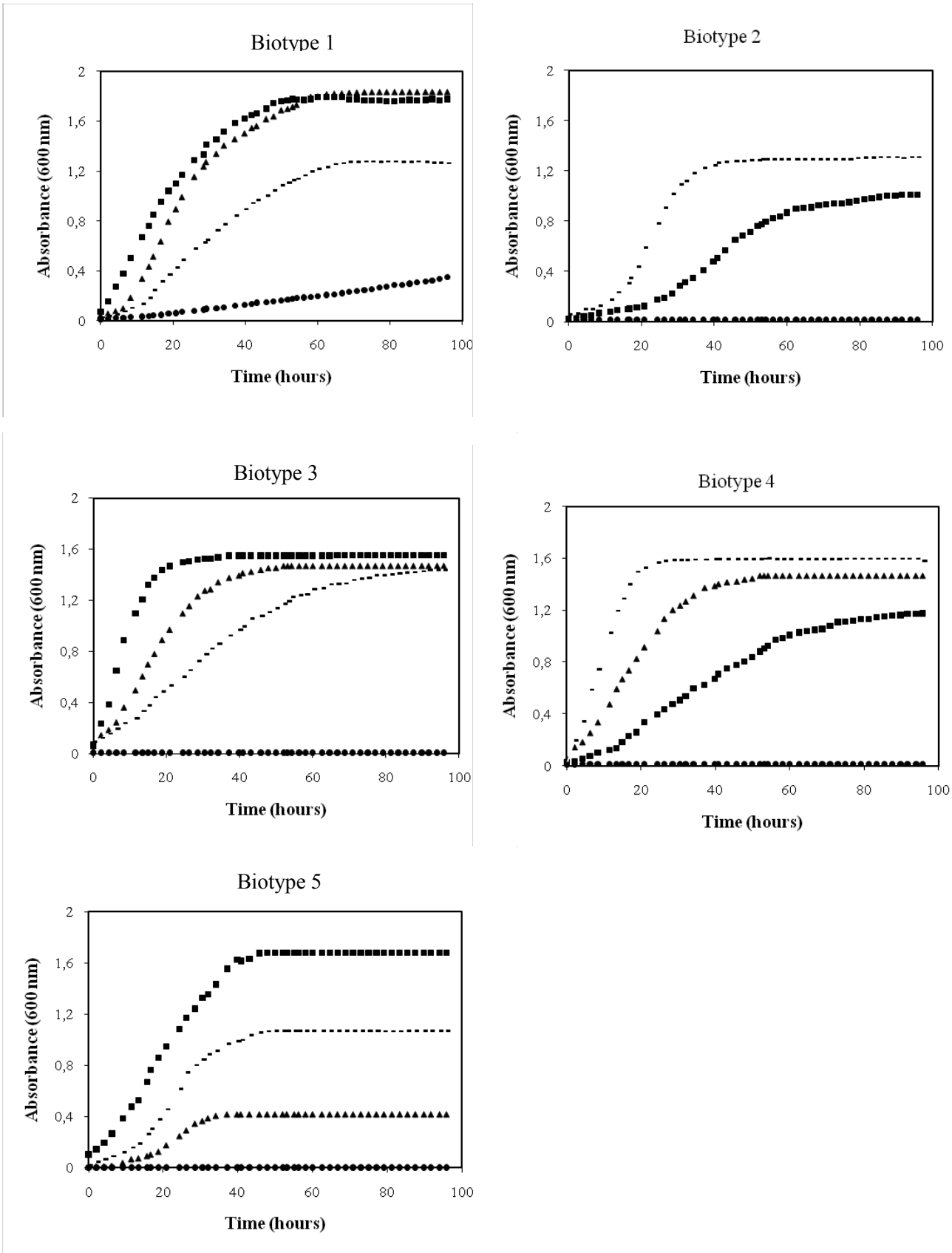

(%) Similarity
R5
RF2
Cluster
30
40
50
60
70
80
90
100
TOHA07
Pedro10
C2ay9
L37
L35
L364
santa 01
L23
L34
L191
L344
L36
L365
Toblino 02
L491
C2by10
PISO02
BC16
BC20
FC54
BC224
R1
D1
C1ay2
R5
C2cy2
BC116
BC60
IOEBL0629 (07/106)
EJ1 (06/143)
FC50
L477
BC115
BC55
BC226
T1yr
I
II
III
IV
V
VI

Fig.2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Absorbance (600 nm)
Time (hours)
Biotype 2
Absorbance (600 nm)
Time (hours)
Biotype 3
Absorbance (600 nm)
Time (hours)
Biotype 4
Absorbance (600 nm)
Time (hours)
Biotype 5
Absorbance (600 nm)
Time (hours)

Fig.3

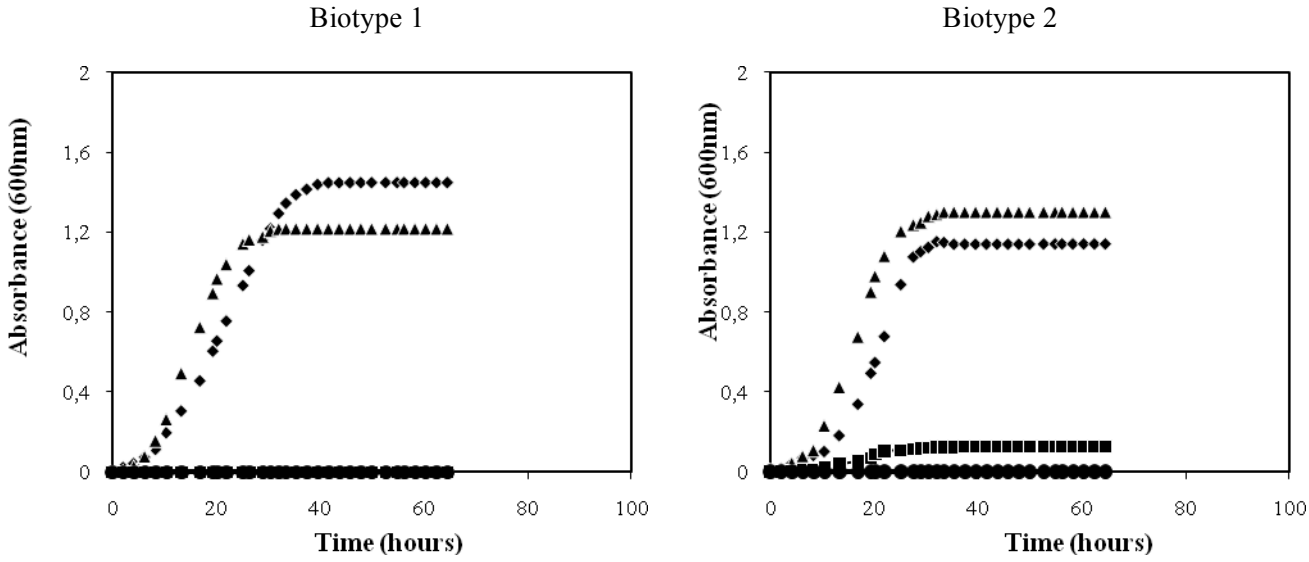
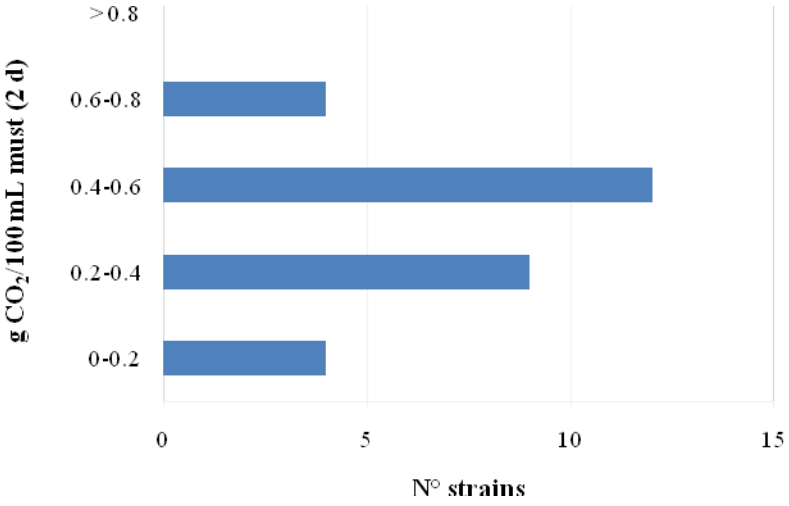
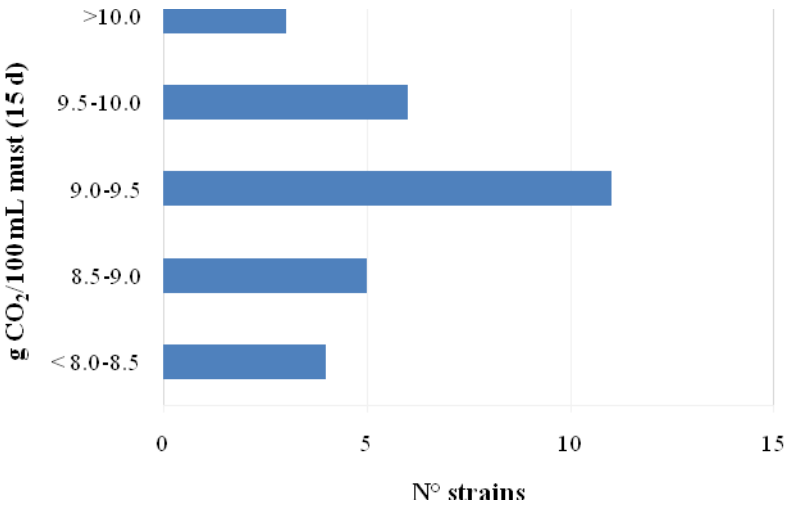

Biotype 1
Biotype 2
Absorbance (600nm)
Time (hours)
0
0,4
0,8
1,2
1,6
2
0
20
40
60
80
100

Fig. 4


>0.8
0.6-0.8
0.4-0.6
0.2-0.4
0-0.2
g CO2/100 mL must (2 d)
0
5
10
15
N° strains


>10.0
9.5-10.0
9.0-9.5
8.5-9.0
< 8.0-8.5
g CO2/100 mL must (15 d)
0
5
10
15
N° strains

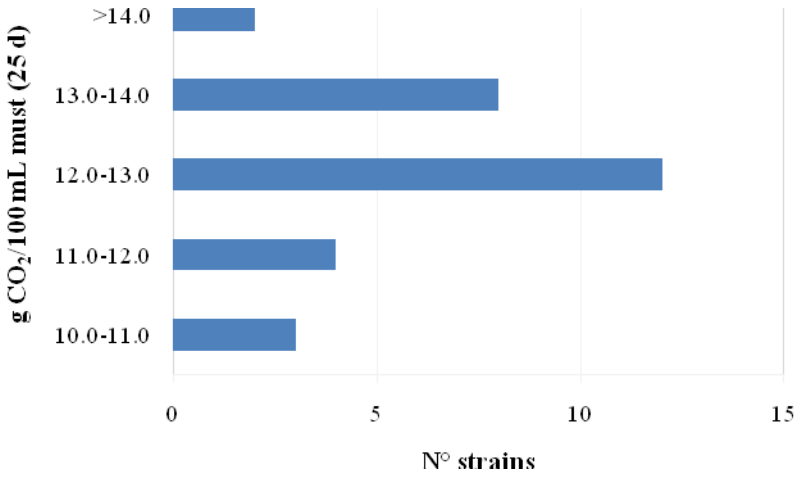
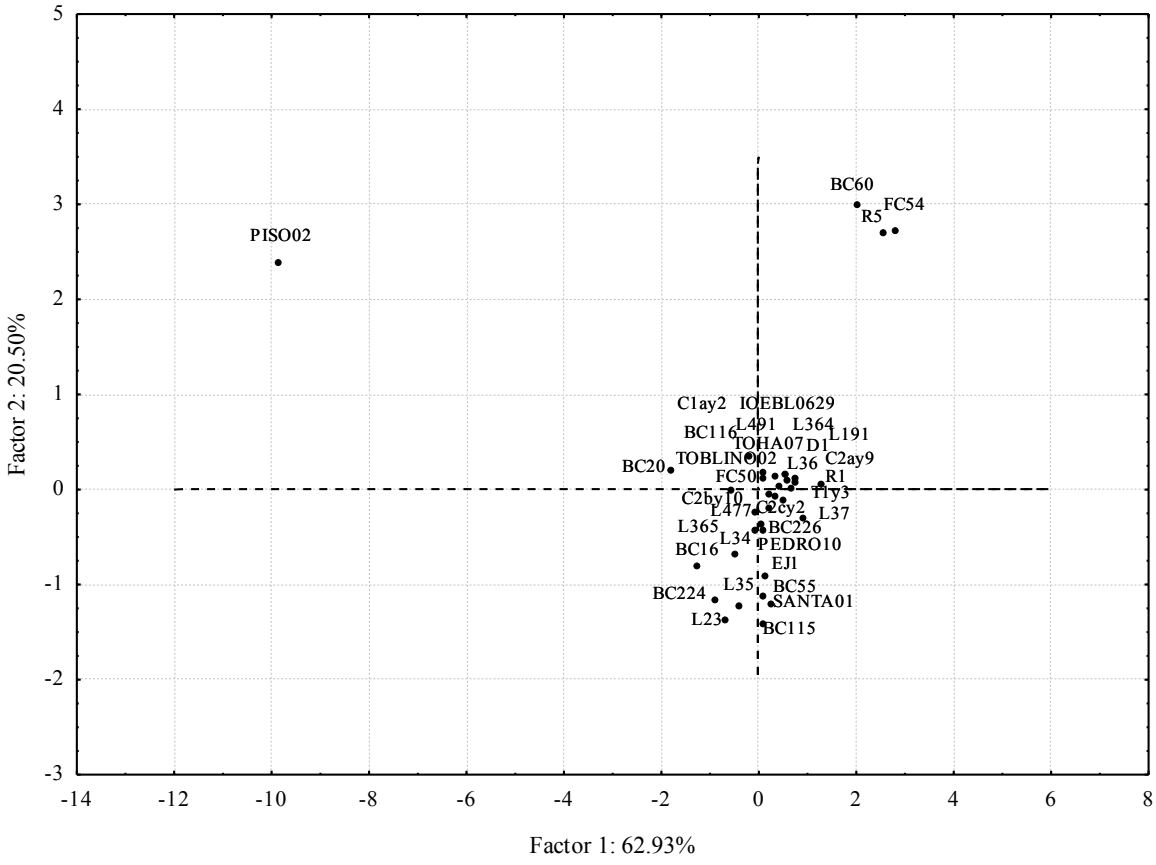

>14.0
13.0-14.0
12.0-13.0
11.0-12.0
10.0-11.0
g CO2/100 mL must (25 d)
0
5
10
15
N° strains

Fig.5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Factor 1: 62.93%
PISO02
BC60
FC54
R5
C1ay2
IOEBL0629
BC116
L491
L364
L191
IOHA07D1
BC20
TOBLIN002
L36
C2ay9
FC50
R1
T1y3
C2by10
L477
C2cy2
L37
L365
BC226
L34
PEDRO10
BC16
EJ1
L35
BC55
BC224
SANTA01
L23
BC115

Fig.6

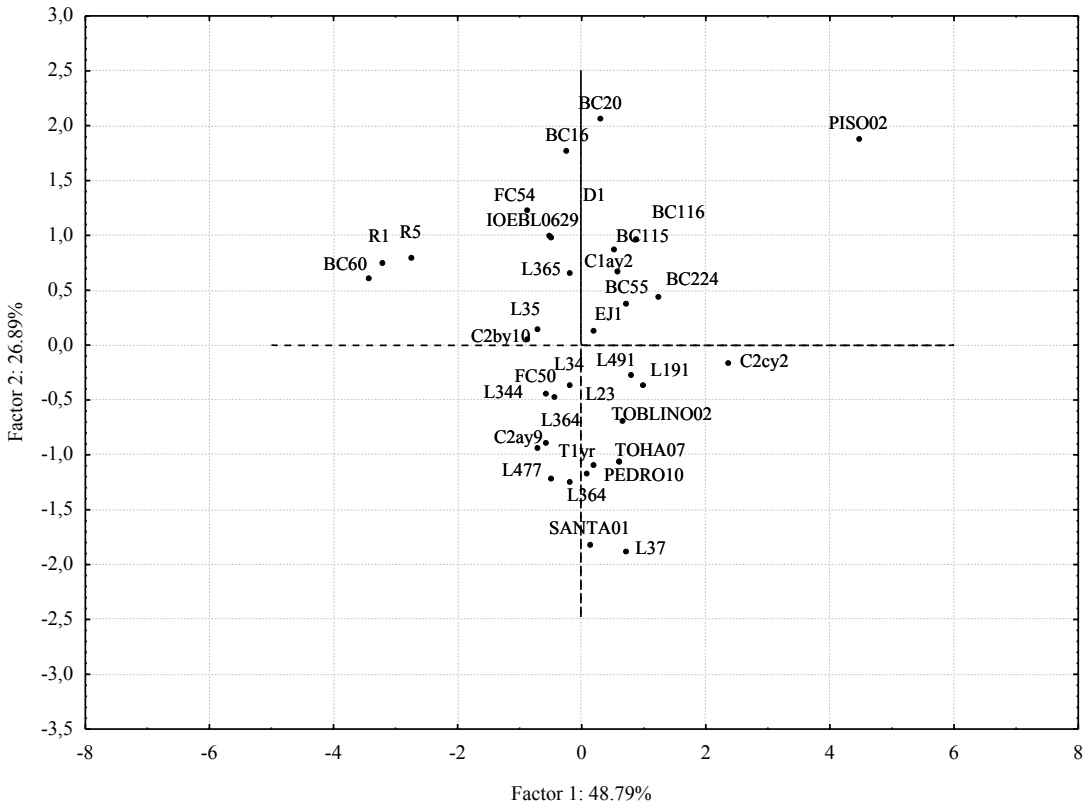
Factor 2: 26.89%
Factor 1: 48.79%
3,0
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
-3,0
-3,5
-8
-6
-4
-2
0
2
4
6
8
BC20
BC16
PISO02
FC54
D1
IOEBL0629
BC116
BC115
R1
R5
BC60
C1ay2
L365
BC55
BC224
L35
EJ1
C2by10
L34
L491
L191
C2cy2
FC50
L344
L23
TOBLINO02
L364
C2ay9
T1yr
TOHA07
L477
PEDRO10
L364
SANTA01
L37

Table 1. List of the *Candida zemplinina* strains used in this study.

Geografical origin	**Producer**	**Isolation source**	**Strain code**	**Culture Collection**
USA - California	nd	Sweet wine fermentation	EJ1	Dept.DIVAPRA, University of Torino, Italy
France	nd	Merlot fermentation	IOEBL0629	
Greece	nd	Must fermentation	D1	
Italy - Friuli Venezia Giulia region	F	Picolit grape	FC50, FC54	
	R	Ramandolo grape	R1, R5	
	B	Picolit grape	BC16, BC20	
	B	Picolit crusched grapes	BC55, BC60	
	B	Picolit fermentation (3 days)	BC115, BC116	
	B	Picolit fermentation (14 days)	BC224, BC226	
Italy - Veneto region	Ms	Amarone must	C2cy2, C1ay2, T1yr	Dept. Scienze, Tecnologie e Mercati della Vite e del Vino-University of Verona, Italy
	Ms	Amarone fermentation (7 days)	C2ay9, C2by10	
Italy - Trentino region	Po	Nosiola grape	SANTA01	
	Ps	Nosiola grape	PISO02	
	Pd	Nosiola dried grape	PEDRO10	
	To	Nosiola dried grape	TOHA07	
	Tob	Nosiola dried grape	TOBLINO02	
Italy - Abruzzo region	G	Fresh must	L37	Dept. Scienze degli Alimenti- University of Teramo, Italy
	G	Vino cotto	L491	
	G	Vino cotto	L191, L35, L23, L34, L364, L344, L36, L365, L477	

nd, not defined

Table 2. Growth parameters of *C. zemplinina* at different glucose concentrations

Glucose (% w/v)	Strain code	Growth parameters [a]		
		A_{max} (O.D)	μ_{max} (h^{-1})	λ (h)
2	BC20, FC50, FC54, BC16	1.08±0.03c	0.04±0.01a	4.68±0.03c
	R1, R5	1.44±0.02a	0.04±0.01a	3.39±0.01a
	SANTA01, PISO02, PEDRO10, TOHA07, TOBLINO02	1.20±0.01d	0.05±0.02c	8.35±0.21d
	BC55, BC60	0.71±0.01b	0.03±0.01c	3.42±0.01a
	L37	1.42±0.01a	0.04±0.02a	3.44±0.04a
	L491, L191, L35, L23, L34, L364, L344, L36, L365, L477	0.72±0.01b	0.01±0.0a	0.63±0.02b
	D1	-	-	-
	C2cy2, C1ay2, T1yr, C2ay9, C2by10	-	-	-
	EJ1	-	-	-
	BC115, BC116, BC224, BC226	1.42±0.01a	0.04±0.01a	3.41±0.02a
	IOEBL0629	1.42±0.01a	0.04±0.02a	3.43±0.02a
20	BC20, FC50, FC54, BC16	1.38±0,03c	0.02±0,0a	3.09±0.04b
	R1, R5	1.23±0.04a	0.02±0.01c	7.91±0.06f
	SANTA01, PISO02, PEDRO10, TOHA07, TOBLINO02	1.12±0.01f	0.04±0.01b	8.54±0.05g
	BC55, BC60	1.23±0.02a	0.02±0.0ac	5.25±0.04e
	L37	1.49±0.02d	0.10±0.0d	1.13±0.01a
	L491, L191, L35, L23, L34, L364, L344, L36, L365, L477	1.21±0.01a	0.03±0.0ab	3.14±0.02b
	D1	1.06±0.01b	0.04±0.01b	9.80±0.02h
	C2cy2, C1ay2, T1yr, C2ay9, C2by10	0.82±0.01e	0.03±0.0ab	4.25±0.02d
	EJ1	1.03±0.01b	0.02±0.0ac	18.39±0.03i
	BC115, BC116, BC224, BC226	1.28±0.01c	0.03±0.0ab	3.36±0.05c
	IOEBL0629	1.46±0.01d	0.10±0.0d	1.15±0.04a
40	BC20, FC50, FC54, BC16	1.39±0.06c	0.04±0.01abc	1.92±0.04a
	R1, R5	1.63±0.05e	0.13±0.03d	1.40±0.06d
	SANTA01, PISO02, PEDRO10, TOHA07, TOBLINO02	1.15±0.06d	0.07±0.01c	8.30±0.28g
	BC55, BC60	1.28±0.01a	0.03±0.0abc	7.62±0.16f
	L37	1.38±0.01bc	0.03±0.01ab	4.43±0.04b
	L491, L191, L35, L23, L34, L364, L344, L36, L365, L477	1.32±0.02abc	0.04±0.0abc	4.91±0.04c
	D1	1.63±0.05e	0.05±0.0abc	5.09±0.04c
	C2cy2, C1ay2, T1yr, C2ay9, C2by10	1.30±0.01ab	0.02±0.0a	6.69±0.04e
	EJ1	1.25±0.01a	0.07±0.01bc	11.85±0.35h
	BC115, BC116, BC224, BC226	1.53±0.02f	0.05±0.01abc	1.76±0.02a
	IOEBL0629	1.08±0.01d	0.06±0.06abc	4.42±0.02b
60	BC20, FC50, FC54, BC16	-	-	-
	R1, R5	-	-	-
	SANTA01, PISO02, PEDRO10, TOHA07, TOBLINO02	0.52±0.04a	0.03±0.02a	13.55±0.06b
	BC55, BC60	-	-	-
	L37	0.43±0.01a	0.04±0.01a	12.84±0.08a
	L491, L191, L35, L23, L34, L364, L344, L36, L365, L477	-	-	-
	D1	-	-	-
	C2cy2, C1ay2, T1yr, C2ay9, C2by10	-	-	-

	EJ1	-	-	-
	BC115, BC116, BC224, BC226	-	-	-
	IOEBL0629	-	-	-

[a]Results are means of three replicates for two repetitions; standard deviations are also indicated; A_{max} maximum abs; μ_{max}: maximum growth rate; **λ**: length of lag. -: no growth; Means within a column in the same block with different letters are significantly different ($P<0.05$).

Table 3 - Growth parameters of *C. zemplinina* isolates at different ethanol concentrations

Ethanol (% w/v)	Strain code	Growth parameters [a]		
		A_{max} (O.D)	μ_{max} (h^{-1})	λ (h)
0	BC20, FC50, FC54, BC16	1.21±0.02ab	0.08±0.01a	9.07±0.06e
	R1, R5	1.24±0.02a	0.06±0.01ab	7.29±0.04b
	SANTA01, PISO02, PEDRO10, TOHA07, TOBLINO02	1.20±0.03ab	0.04±0.0b	8.30±0.05a
	BC55, BC60	1.23±0.02a	0.06±0.04ab	7.51±0.06c
	L37	1.24±0.04a	0.07±0.0ab	8.37±0.06a
	L491, L191, L35, L23, L34, L364, L344, L36, L365, L477	1.24±0.01a	0.06±0.0ab	8.14±0.04d
	D1	-	-	-
	C2cy2, C1ay2, T1yr, C2ay9, C2by10	-	-	-
	EJ1	-	-	-
	BC115, BC116, BC224, BC226	1.16±0.02b	0.09±0.0a	13.76±0.05f
	IOEBL0629	-	-	-
8	BC20,FC50,FC54, BC16	1.42±0.04c	0.05±0.03ab	8.40±0.04b
	R1,R5	1.16±0.01a	0.05±0.0ab	9.24±0.04a
	SANTA01, PISO02, PEDRO10, TOHA07, TOBLINO02	1.17±0.01a	0.03±0.0a	9.35±0.04d
	BC55, BC60	1.51±0.02d	0.07±0.01ab	9.71±0.03e
	L37	-	-	-
	L491, L191, L35, L23, L34, L364, L344, L36, L365, L477	1.23±0.04a	0.04±0.01ab	9.24±0.04a
	D1	-	-	-
	C2cy2, C1ay2, T1yr, C2ay9, C2by10	-	-	-
	EJ1	-	-	-
	BC115, BC116, BC224, BC226	1.33±0.04b	0.08±0.02b	8.54±0.03c
	IOEBL0629	-	-	-

[a] Results are means of three replicates for two repetitions; standard deviations are also indicated; A_{max} maximum abs; μ_{max}: maximum growth rate; **λ**: length of lag. -: no growth; Means within a column in the same block with different letters are significantly different (P<0.05).