Selection of a yeast strain suitable for the production of aromatic wines with geraniol-based flavour [1]

Selezione di un ceppo di lievito adatto alla vinificazione di uve aromatiche a base geraniolo

E. Vaudano, M.D.C. Pazo Alvarez, M. Cersosimo, A.Costantini, V. Del Prete, E. Garcia-Moruno

CRA - Centro di Ricerca per l'Enologia Via P.Micca, 35 - 14100 ASTI,Italy.
Email: biologia.molecolare@isenologia.it

Summary

In the vinification process of aromatic grapes with geraniol based flavour, as Gewurtztraminer, and some italian aromatic red varieties such Brachetto, Moscato rosa and Moscato di Scanzo, the interaction between terpenic compounds and the fermenting yeast *Saccharomyces cerevisiae* plays a primary role in the perceived quality of the wines produced.

Indeed, yeasts are able to act on the geraniol with an assimilation mechanism, probably toward the steroids synthesis, and to transform it, by stereospecific reduction, in citronellol.

The final result is a dramatic reduction in geraniol concentration and noequally production of other monoterpenols, mainly citronellol. This depletion may results in a reduction of floral intensity in the flavour of wines that having in the olfactive features the most important quality parameter.

In this study, we have observed the influence of *S.cerevisiae* on these transformation carrying out a selection process for a strain, specifically indicated in the fermentation of aromatic grapes with geraniol based flavour.

At first, five yeast strains belonging to the collection of the CRA-Istituto Sperimentale per l'Enologia have been selected, showing the best oenological features. The yeasts were previously tested with a PCR technique based on a multiplex microsatellite PCR of three highly polymorphic loci an band pattern analysis to confirm their intraspecific differences.

Afterwards the strains were tested in regard to geraniol metabolism, in a fermentation trials at laboratory scale (200 mL), using a sterile neutral must added of the monoterpenol. The consumption of geraniol and the production of citronellol and the other sensorial interesting monoterpenols were observed with a GC-MS analysis.

The strain with the best characteristics has been subsequently put to comparison with commercial yeast broadly used, in a 30 L fermentation by using a must of red aromatic grape and simulating the production of a sparkling aromatic dessert wine and of an aromatic dry one. Moreover, an industrial fermentation

[1] Part of this paper was communicated at the 8TH International Symposium of Oenology, Bordeaux 25, 26, 27 June 2007.

was carried out, in vintage 2007, to evaluate the strain behaviour in cellar condition.

The semindustrial and the industrial trial results reveal the superior efficiency in regard to the geraniol metabolism, with a minor geraniol depletion and citronellol formation for the ISE strains in comparison to the commercial one.

Riassunto

Nella vinificazione di uve aromatiche ad aroma terpenico basato sul geraniolo, quali il Brachetto, la Malvasia di Casorzo, il Moscato di Scanzo ed alcune varietà aromatiche a bacca bianca quali Gewurtztraminer, l'interazione tra la componente terpenica e il lievito ha un ruolo primario nella qualità del prodotto ottenuto. Il metabolismo del lievito è in grado di agire, infatti, sul geraniolo attraverso un processo di assimilazione, probabilmente verso la sintesi steroidea, e, con un meccanismo di riduzione stereospecifica, che è in grado di trasformarlo in citronellolo. Il risultato finale è una drastica riduzione del geraniolo a fronte di una produzione di citronellolo e, in misura minore, di altri composti terpenici. Questo impoverimento può ridurre sensibilmente l'intensità aromatica di vini che hanno nell'aroma floreale il loro principale parametro di qualità.

In questo lavoro viene messo in evidenza il ruolo del ceppo di lievito in queste trasformazioni nell'ambito di un processo di selezione di un potenziale lievito selezionato indicato specificatamente per la fermentazione di uve aromatiche a base geraniolo.

Il progetto è iniziato con la selezione di 5 ceppi raccolti nella collezione dell'ISE con le migliori caratteristiche enologiche. I lieviti sono stati preventivamente saggiati attraverso l'analisi del profilo di banda generato da una PCR multiplex di 3 locus microsatellitari per confermare inequivocabilmente la loro diversità intraspecifica. Successivamente i ceppi sono stati oggetto di un saggio del loro metabolismo nei confronti del geraniolo attraverso fermentazioni in scala di laboratorio (200 mL), utilizzando un mosto neutro sterile addizionato del composto terpenico osservando il consumo di geraniolo e la produzione di citronellolo. Il ceppo con le migliori caratteristiche è stato successivamente messo a confronto con un lievito selezionato commerciale ampiamente usato in una fermentazione pilota in scala 20 litri utilizzando un mosto di uva aromatica rossa e simulando la produzione di uno spumante dolce aromatico e di un vino secco aromatico. Il ceppo è stato in seguito testato in vinificazione industriale in vinificatore da 650 qli e confrontato sempre con un lievito commerciale comunemente usato.

I risultati dell'analisi gas cromatografia mostrano la diversità del comportamento dei ceppi nella fase di selezione in scala di laboratorio. Si evidenzia in modo particolare un ceppo potenzialmente adatto. Il ceppo selezionato si è rivelato vantaggioso sia nelle prove pilota a scala 20 litri sia nella vinificazione industriale in grandi volumi dimostrando di possedere, rispetto al ceppo commerciale tradizionalmente usato, una minore necessità di consumare geraniolo

a favore di una maggiore capacità di trasformarlo in citronellolo a tutto vantaggio delle caratteristiche olfattive del vino prodotto.

Introduction

During the alcoholic fermentation of aromatic grapes, *Saccharomyces cerevisiae* interacts with some monoterpenols transforming or metabolizing them. These reactions are added to those spontaneously occurring in the acid medium, the must of grape (acid catalysis).
From a technological point of view, interesting aspects in regard to the transformation of geraniol (2-trans-3,7-dimetilocta-2,6-dien-1-ol). This molecule is the most important monoterpenol presents in aromatic red grape varieties such as Brachetto, Malvasia di Casorzo, Moscato di Scanzo and Moscato Rosa and in Gewurtztraminer white grapes; this last variety is widely cultivated in north east Italy and in other European countries. During fermentation, its concentration is drastically reduced: it is partly transformed by acid catalysis in linalool, while yeasts transform by stereospecific reduction, a large amount of geraniol to citronellol in the enantiomeric form (R)-(+). *S. cerevisiae* can also form the compounds geranil acetate and citronellol acetate. (Gramatica *et al.*, 1982; Versini et al., 1990; Di Stefano *et al.*, 1992; King *et al.*, 2000; King *et al.*, 2003).
Together with remaining geraniol, citronellol obtained gives olfactive character to wine produced.
These two geraniol transformation pathways do not completely explain its drastic reduction because, after fermentation, the total quantities of monoterpenols produced and the residual geraniol reaches, at best, 40% of the initial geraniol amount (García -Moruno *et al.*, 2002; King *et al.*, 2003).
The biosynthesis of the steroids can be responsible for the consumption of geraniol; starting with the acetyl-CoA, via mevalonate, this pathway forms sterols, which are used in membrane forming during cellular growth. In this metabolism some terpenic compounds act as intermediates, among them also geraniol in its active geranyl pyrophosphate form.
In a previous work (Vaudano *et al.*, 2004) we have highlighted that the presence of steroids and the oxygenation level can affect the geraniol metabolism, influencing the ratio between the citronellol production and the incorporation in steroid metabolism.
The influence of the yeast metabolic conditions on the geraniol fate, depending on must composition and fermentation conditions, allows us to suppose that a relation exists between geraniol metabolism and yeast strain used. The genetic diversity found in selected and wild strains could be reflected in a difference in metabolic features. Indeed, recent studies (Garcia -Moruno *et al.*, 2002) have shown that different yeast strains show different behaviours as regard to geraniol consumption and citronellol formation, probably because of metabolic differences of the pathway seen above.
In this work we have tested some yeast strains belonging to the ISE, (CRA-Istituto Sperimentale per l'Enologia di Asti, Italy) previously discriminated with

a molecular technique at strain level. This technique, based on a microsatellite analysis has allowed us to characterize five strains that have been tested in laboratory scale fermentations. The best of them have been subsequently compared with a commercial strain, in a semi-industrial fermentation of an aromatic red must simulating the production of a sparkling sweet wine and of a dry wine.

Materials And Methods

Strains

The following *Saccharomyces cerevisiae* strains were used in this study: ISE 99, ISE 88, ISE 196, ISE 4, ISE 41.The strains belong to the collection of the CRA-Istituto Sperimentale per l'Enologia (ISE). Commercial strain used in semi-industrial and industrial trial was purchased from wine yeast suppliers.

The species identification was done with the assimilation test ID32C (Biomerieux). Identification of *Saccharomyces cerevisiae* at strain level was based on multiplex PCR analysis of polymorphic microsatellite loci located in three different chromosomes, and band pattern analysis of the fragments generated, by agarose gel electrophoresis (Field e Wills, 1998; Gonzalez Techera *et al.*, 2001, Vaudano and Garcia-Moruno, 2007).

Laboratory scale fermentation trials

Sterile red grapes must without free and glycosilate monoterpenols was used (Biotta AG, Tagerwilen, Switzerland). Ammonium sulphate and ammonium phosphates, in a final concentration of 150 mg/L for each compound, were added as nitrogen supplement in fermentations. Geraniol used was prepared in a 50% hydro-alcoholic solution, filtered in sterile conditions and added to a final concentration of 1000 μg/L. The inoculation was carried out after 48 hours of premultiplication in semi-anaereobiosis at 20°C, to reach 10^6 cells/mL.

For each strain, six fermentations in 300 mL flask filled with 200 mL of must were carried out. Fermentations were conducted at 20° C and CO_2 release was checked by weighting the flasks.

The analysis were done at two stages of the fermentation process by sampling three flasks when they reached 5° % of alcohol and the other three at the end of fermentation, after 15 days.

A further trial in triplicate was implemented with a must containing geraniol without inoculation to observe the terpenol transformation not caused by yeasts.

Semi-industrial fermentation trials

The semi-industrial fermentations were carried out in 30 litres pressure tank filled with 20 L of partially fermented must from Brachetto grapes, an aromatic red variety growing in Piemonte. This is a typical base must used for sparkling sweet wine production. The analytical parameters of the Brachetto must were: alcohol 3.4 % Vol; sugar 147 g/L, pH 3.41, free SO_2 6.0 mg/L.

The trials were performed in duplicate using two yeast strains: the best one obtained from the laboratory scale fermentations and a commercial strain usu-

ally used for this
lated at 5 X 10^6 c
mentation (at 20
pling was done a
to 5.0 ATM of pr
aromatic wine. Tl
a dry aromatic w
tation (sugar <2
Discriminant
order to individu
were regarded a
In addition, ta
of floral and citru

Industrial fermen
With the aim
, a trial of vinific
Brachetto grape
tion procedure c
the partially ferm
tation in pressuri
By now the p
data reported ar
The grapes (
two 650 HL verti
strain was comp
Some characteri
two fermentation

Determination of
The extractio
C18 cartridges u
The instrume
mass detector H

Tab.1: Musts data
Tab.1: Dati analitic

Sugar
Total acidity
pH
vinification tem
maceration
pumping over

ally used for this grape. After a premultiplication phase, the yeasts were inoculated at 5 X 10^6 cell/mL and the tanks were hermetically closed. During the fermentation (at 20° C) the alcohol production was monitored and the first sampling was done at 5.0° alcoholic degree (1.6° from the inoculum) corresponding to 5.0 ATM of pressure. These parameters corresponding to a sparkling sweet aromatic wine. Then, the outlet valves were opened simulating the production of a dry aromatic wine and the second sampling was done at the end of fermentation (sugar <2g/L).

Discriminant (duo-trio) and preference sensorial test were carried out in order to individuate differences between samples using the ISE panel. Products were regarded as differently perceived for a probability higher than 95%.

In addition, tasters were asked for indicated the sample with higher intensity of floral and citrus fruit flavour.

Industrial fermentation

With the aim to verify in cellar condition the behaviour of the selected strain , a trial of vinification in the vintage 2007 was carried out in Piemonte winery. Brachetto grapes were used to produce a sparkling dessert wine. The vinification procedure consist in a first fermentation to reach 3.5-4.0° alcohol; afterward the partially fermented must is keep at low temperature until a second fermentation in pressurised tank where the wine reach 5.5-6.0 ° alcohol and 5.0 ATM.

By now the partially fermented musts are settled at low temperature and the data reported are referred to a first fermentation.

The grapes (as homogeneous as possible) were crushed and fermented in two 650 HL vertical vat. Even in this trial the fermentation with the ISE selected strain was compared with a commercial one commonly used in the winery. Some characteristics of the musts after crushing and the protocol used in the two fermentation was reported in table 1.

Determination of monoterpene compounds

The extraction of monoterpenols from musts and wines was performed with C18 cartridges using the method proposed by Di Stefano (1991).

The instrument used was a gas chromatograph HP 5890 coupled with a mass detector HP 5970 equipped with a Zebron ZB-WAX capillary column (30

Tab.1: Musts data and protocol of industrial vinification.
Tab.1: Dati analitici del mosto e protocollo di vinificazione industriale.

	COMM	ISE99
Sugar	224,2	223,9
Total acidity	5,8	5,9
pH	3,38	3,36
vinification temperature	18-16°	
maceration	3 days	
pumping over	15' every 3 hours	

m x 0.25 mm x 0.25 µm film thickness). The GC operative conditions were: injector temperature: 250°C; interface temperature: 230°C He carrier gas: 1.0 mL/min; injection: 1 l splitless; oven temperature program: 40°C ; 30°C/min up to 60°C; 2 min at 60°C, 2°C/min up to 190°C and 5°C/min up to 230°C, 15 min at 230°C; MS ion range: from m/z 29 to 300.

Results And Discussion

Identification at strain level confirms the separation, the band profile showing clear differences in band number and position (Fig. 1)

The data obtained from the GC-MS analysis of the laboratory trials (Tab. 2) reveal a strong influence of the strains on geraniol consumption and citronellol formation. After 3 days from the inoculum, when the fermentation reached 5° alcohol, the differences result significative; the strains ISE 88 e ISE 41 show to be strong geraniol consumers in front of a limited production of citronellol. At the opposite, strain ISE 99 is the most interesting one, with a less geraniol consuming metabolism and this advantages the citronellol formation pathway. The strains ISE 196 and ISE 41 are in an intermediate position. At the end of fermentation, after 15 days, a further diminution of the geraniol concentration is observed. Also at this stage ISE 99 strain appears the most advantageous among the tested strains, maintaining the largest terpenols concentration, above all citronellol.

It is interesting to note the rapidity of the geraniol consumption in the first days of fermentation, subsequently it degraded much more slowly. This can be explained considering that, in the growth phase, the necessity to support the steroid synthesis is higher than in the stationary phase. In the first days the ratio between geraniol consumption for steroid formation and citronellol formation is strongly favourable to steroid; citronellol formation probably becomes dominant in the stationary phase when the requirement of steroids is reduced. (Vaudano *et al.*, 2004)

Differences in citronellol production were found among the strains. For ISE 196 and ISE 4 the synthesis is more intense in the first days with respect to the later phases. The ISE 41 shows a slow synthesis in the first phase and the majority of citronellol is produced after this time. In the ISE 99 and ISE 88 citronellol is

Tab.2 Free monoterpenols concentration (µg L^{-1}) in laboratory trials.
Tab.2 Concentrazione in terpenoli in forma libera (µg L^{-1}) nelle prove di laboratorio.

	5° alcohol					End of fermentation					
	ISE 99	ISE 88	ISE 4	ISE 41	ISE 196	ISE 99	ISE 88	ISE 4	ISE 41	ISE 196	C-
LINALOL	22 ± 2,1	17 ± 2,8	18 ± 0,7	17 ± 1,4	20 ± 2,8	30 ± 0,5	19 ± 3,5	20 ± 0,7	24 ± 1,4	28 ± 7,8	105 ± 0,7
CITRONELLOL	78 ± 3,5	45 ± 11,3	84 ± 18,4	29 ± 1,4	106 ± 8,5	179 ± 39,6	92 ± 9,9	93 ± 38,2	114 ± 21,9	82 ± 0,7	0
GERANIOL	346 ± 9,9	87 ± 19,1	173 ± 43,8	82 ± 2,8	230 ± 33,9	103 ± 12,0	68 ± 14,1	70 ± 9,9	97 ± 4,2	72 ± 29,7	735 ± 32,5

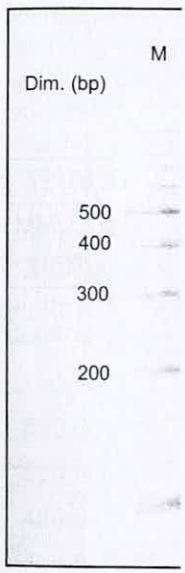

Fig.1: Band prof
Fig.1: Profilo di b

constantly proc
in efficiency of
strate, influenci
A limited pr
detected in the
is abundantly f
ed by the yeast
In the semi-i
the end of ferm
geraniol and ne
don'tdoesn't dif
tive decrease ir
don't show any
between the str
fore, to a possi
to be very limite
The simulate
ences in free m
citronellol forma
No significative
an nerol. The s
rose like flavour
The analysis
ic wine (Fig. 3

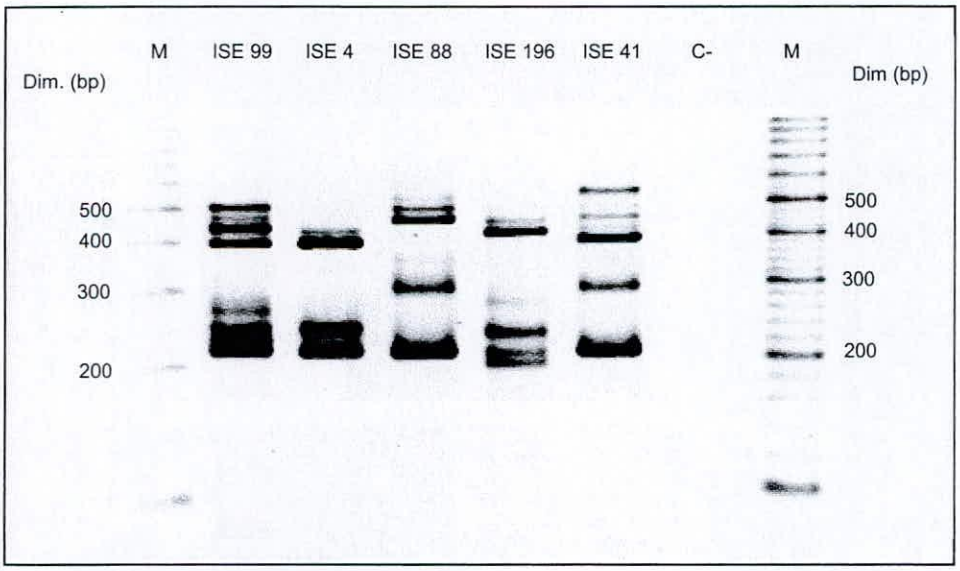

Fig.1: Band profile of the five tested strains.
Fig.1: Profilo di banda nei cinque ceppi testati.

constantly produced. These differences are probably caused by the difference in efficiency of the two antagonist reactions having geraniol as common substrate, influencing consequently the citronellol production.

A limited production of linalol, due to the acidity of the medium, is observed detected in the fermentation trials. In the trials without inoculation, this molecule is abundantly formed owing to the absence of the enzymatic reactions promoted by the yeasts, which have an higher efficiency than the acid catalysed ones.

In the semi-industrial fermentations, the analysis of bound monoterpenols at the end of fermentation shows that concentration of the most abundant ones, geraniol and nerol, doesn't change with respect to the initial amount and they don'tdoesn't differs between the two strains. It is observed instead, a significative decrease in the bound fraction of linalool citronellol and α-terpineol but they don't show any difference between strains (data not shown). The differences between the strains found in the free portion of the terpenols are not due, therefore, to a possible difference of the hydrolytic activity that however it appears to be very limited.

The simulated production of a sparkling dessert wine (Fig. 2) reveals differences in free monoterpenols concentration, with a minor geraniol depletion and citronellol formation in the ISE strains in comparison with the commercial one. No significative differences are found between strains in linalol and α-terpineol, an nerol. The sensorial analysis shows a significative major intensity of floral rose like flavour in the ISE strain.

The analysis at the fermentation end, during the production of a dry aromatic wine (Fig. 3), shows the almost total consumption of free geraniol (which

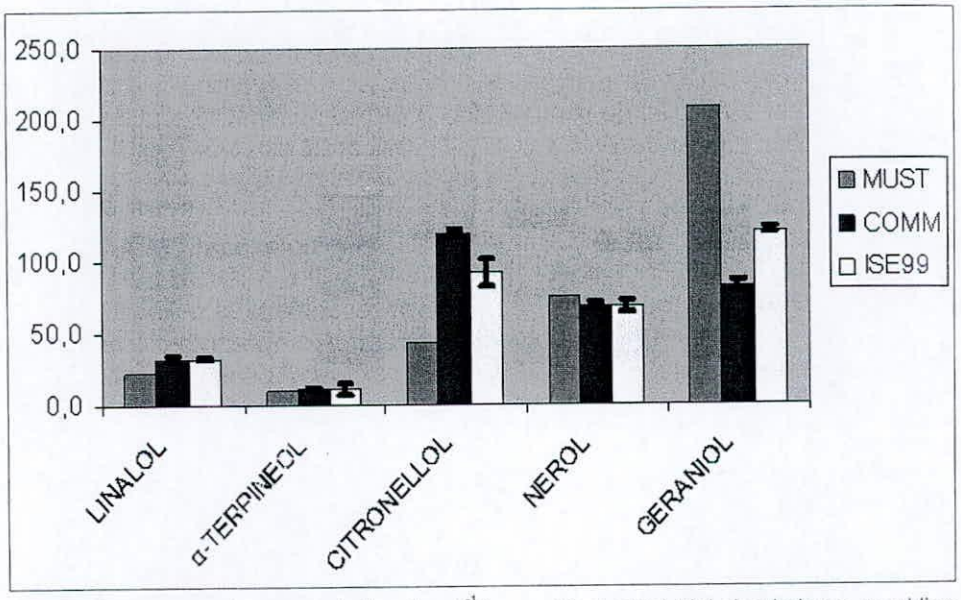

Fig. 2: Free terpenols concentration (µg L^{-1})in semi-industrial trial simulating a sparkling dessert wine production.

Fig. 2: Concentrazione di terpenoli in forma libera (µg L^{-1}) nella prova semi-industriale che simula la produzione di un vino dolce aromatico.

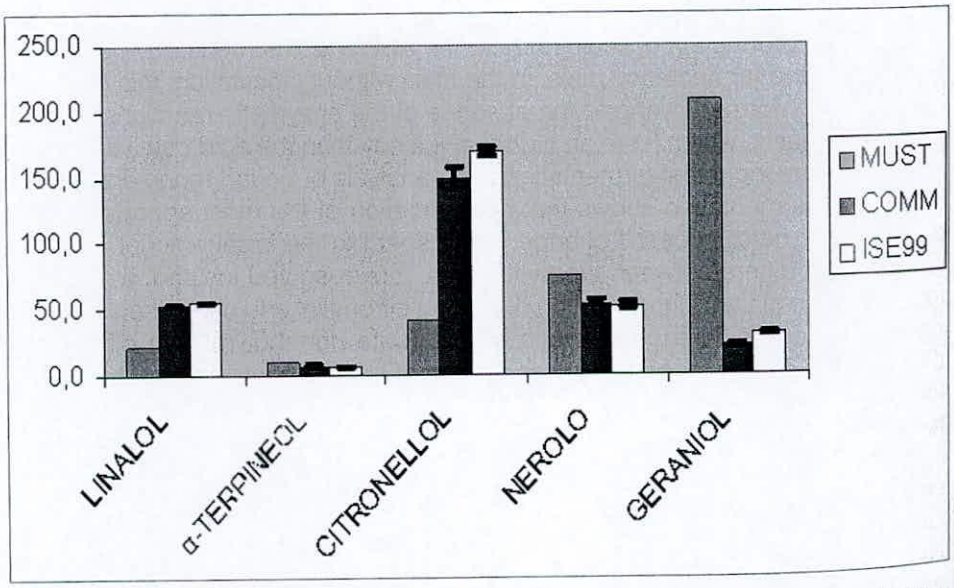

Fig. 3: Free terpenols concentration (µg L^{-1})in semi-industrial trial simulating a dry aromatic wine production.

Fig.3: Concentrazione di terpenoli in forma libera (µg L^{-1}) nella prova semi-industriale che simula la produzione di un vino secco aromatico.

results however
lol in the ISE 99
of fermentation
commercial one
analysis.

Finally, the pr
fermentation rea
the ISE selected
ISE 99 strain, sh
of the five princi
commercial stra

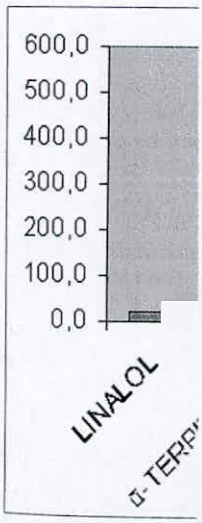

Fig. 4: Free terpen

Fig. 4: Concentraz ale

In conclusion
lism, found in *S.*
strain for the ferr
tion of wines whi
tant quality parar

References

1. DI STEFANO, R. *détermination de* (64): 7-2137, 581
2. DI STEFANO, R., *niolo indotte dai l*

results however higher in the ISE 99 trial) but a major production of free citronellol in the ISE 99 strain. This monoterpenol is the most important one at the end of fermentation and it is present in an amount about 10% greater with respect to commercial one. No significative differences have been revealed by sensorial analysis.

Finally, the preliminary results obtained from the industrial fermentation (must fermentation reaching 3.5 ° alcohol) seem to confirm the good performance of the ISE selected yeast. As illustrate in fig. 4 the wine partially fermented with the ISE 99 strain, shows higher amount of terpenols in particular citronellol. The sum of the five principal monoterpenols is 20% higher than the fermentation with the commercial strain.

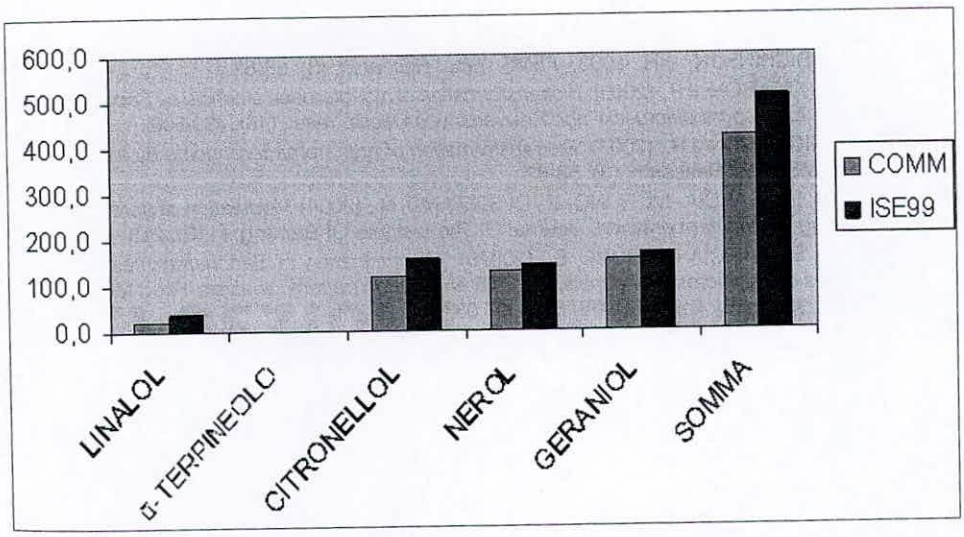

Fig. 4: Free terpenols concentration (µg L^{-1})in industrial fermentation.

Fig. 4: Concentrazione di terpenoli in forma libera (µg L^{-1}) nella fermentazione industriale

In conclusion the metabolic variability as in regard to the geraniol metabolism, found in *S.cerevisiae* strains, was applied to the selection of a specific strain for the fermentation of geraniol based aromatic grapes, in for a production of wines which havehaving in their olfactive characteristics the most important quality parameter.

References

1. DI STEFANO, R., (1991). *Proposition d'une méthode de preparation de l'echantillon pour la détermination des terpènes libres et glycosides des raisins et des vins.* Bulletin de l'. O.I.V., (64): 7-2137, 581-595.
2. DI STEFANO, R., MAGGIOROTTO, G., GIANOTTI, S., (1992). *Trasformazioni di nerolo e geraniolo indotte dai lieviti.* Riv. Vitic. Enol., (45, 1): 43-49.

3. FIELD, D., WILLS, C., (1998). *Abundant microsatellite polymorphism in Saccharomyces cerevisiae, and the different distributions of microsatellites in eight prokaryotes and S. cerevisiae, result from strong mutation pressures and a variety of selective forces.* Proceedings of the National Academy of Sciences of the United states of America (95): 1647-1652.
4. GARCIA -MORUNO, E., RIBALDONE M., DI STEFANO, R., (2000). *L'idrolisi dei glicosidi terpenici della buccia dell'uva nella preparazione del mosto base delle uve aromatiche a frutto colorato.* Riv. Vitic. Enol., (53, 1): 27-36.
5. GARCÍA GARCIA-MORUNO, E., RIBALDONE, M., DI STEFANO, R., CONTERNO, L., GANDINI, A., (2002). *Etude de cinq souches de Saccharomyces cerevisiae par rapport à leur metabolisme du géraniol.* J. Int. Sci. Vigne Vin., (36, 4): 221-225.
6. GONZALEZ TECHERA, A., JUBANY, S., CARRAU, F.M., CAGGERO, C., (2001). *Differentiation of industrial wine yeast strains using microsatellite markers.* Letters in Applied Microbiology, (33, 1): 71-75.
7. GRAMMATICA, P., MANITTO P., RANZI, B., DAL BIANCO, A., FRANCAVILLA, M., (1982). *Stereospecific reduction of geraniol to (R)-(+)-citronellol by Saccharomyces cerevisiae.* Experimentia, (38):, 775-776.
8. KING , A., DICKINSON , J.R., 2003, *FEMS Yeast Research,* (3): 53-62.
9. KING, A., DICKINSON, J.R., (2000). *Biotransformation of monoterpene alcohols by Saccharomyces cerevisiae, Torulaspora delbrueckii and Kluyveromyces lactis.* Yeast, (16): 499-506.
10. KING A., DICKINSON J.R., (2003). *Biotransformation of hop aroma terpenoids by ale and lager yeasts.* FEMS Yeast Research, (3): 53-62.
11. VAUDANO, E., GARCIA--MORUNO, E., DI STEFANO, R., (2004).*Modulation of geraniol metabolism during alcohol fermentation.* Journal Of The Institute Of Brewing, (110): 213 - 219.
12. VAUDANO E., GARCIA--MORUNO E., (20078). *Discrimination of Saccharomyces cerevisiae wine strains using microsatellite multiplex PCR and bands pattern analysis.* Food Microbiology, (25), 56-64http://dx.doi.org/10.1016/j.fm.2007.08.001
13. VERSINI D., DALLA SERRA A., SCIENZA A., BARCHETTI P., (1990). *Proceedings of the Gewurztraminer Traminer Aromatico Symposium*, Bolzano, Ed. , Assessorato all'Agricoltura della provincia autonoma di Bolzano, Bolzano: 59-71.

VI

VI