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Studies on acetate ester production by non-*Saccharomyces* wine yeasts

Virginia Rojas, José V. Gil, Francisco Piñaga, Paloma Manzanares *

Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (CSIC), P.O. Box 73, 46100 Burjassot, Valencia, Spain

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Abstract

A double coupling bioreactor system was used to fast screen yeast strains for the production of acetate esters. Eleven yeast strains were used belonging to the genera *Candida*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces* and *Zygosacharomyces*, mainly isolated from grapes and wine, and two wine *Saccharomyces cerevisiae* strains. The acetate ester forming activities of yeast strains belonging to the genera *Hanseniaspora* (*Hanseniaspora guilliermondii* and *H. uvarum*) and *Pichia* (*Pichia anomala*) showed different substrate specificities and were able to produce ethyl acetate, geranyl acetate, isoamyl acetate and 2-phenylethyl acetate. The influence of aeration culture conditions on the formation of acetate esters by non-*Saccharomyces* wine yeast and *S. cerevisiae* was examined by growing the yeasts on synthetic microbiological medium. *S. cerevisiae* produced low levels of acetate esters when the cells were cultured under highly aeration conditions, while, under the same conditions, *H. guilliermondii* 11104 and *P. anomala* 10590 were found to be strong producers of 2-phenylethyl acetate and isoamyl acetate, respectively.

Keywords: Non-*Saccharomyces* wine yeasts; Acetate ester formation; Wine aroma

1. Introduction

An important part of wine aroma arises during the alcoholic fermentation of grape sugars by the wine yeast *Saccharomyces cerevisiae*, which produces ethanol, carbon dioxide, and a number of by-products including higher alcohols and esters (Rapp and Mandery, 1986), whose formation is closely related to the particular yeast species involved (Rankine, 1967; Suomalainen, 1971; Soufleros and Bertrand, 1979). Although numerous studies underline the influence that *S. cerevisiae* has on the amount of fermentation products (Ribéreau-Gayon, 1971), in recent years the so-called non-*Saccharomyces* wine yeasts (including genera such as *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*) have been described as producers of high concentrations of some fermentation compounds, such as acetic acid, glycerol, esters and acetoin (Romano et al., 1993a,b, 1997), whose influence on the sensory quality of wine has also been discussed (Fleet et al., 1984;

* Corresponding author. Tel.: +34-96-3900022; fax: +34-96-3636301.

E-mail address: pmanz@iata.csic.es (P. Manzanares).

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Heard and Fleet, 1986; Mateo et al., 1991; Gil et al., 1996).

Acetate esters, such as ethyl acetate, hexyl acetate, isoamyl acetate and 2-phenylethyl acetate are recognised as important flavour compounds in wine and other grape-derived alcoholic beverages. It has been suggested that in *S. cerevisiae*, alcohol acetyltransferase (AATase) encoded by the *ATF1* gene is one of the most important enzymes for acetate ester formation (Fujii et al., 1994). AATase reacts with acetyl coenzyme A (acetyl-CoA) and, depending on the degree of affinity, with a variety of alcohols (Yoshioka and Hashimoto, 1981). It has also been shown that *S. cerevisiae* AATase is strongly repressed under highly aerobic conditions and by the addition of unsaturated fatty acids to the culture media (Malcorps et al., 1991; Fujii et al., 1997). Recently, it has been reported that the overexpression of the *ATF1* gene in wine yeasts significantly increases the concentration of ethyl acetate, ethyl caproate, hexyl acetate, isoamyl acetate and 2-phenylethyl acetate in the wine produced by these transgenic microorganisms (Lilly et al., 2000). With respect to non-*Saccharomyces* yeasts, a similar role of AATase in the production of isoamyl acetate in sake brewing has been described in *Hansenula mrakii* (Inoue et al., 1994, 1997).

Oda et al. (1996) developed a system based on the double coupling of glucose metabolism for the production of acetyl-CoA and microbial esterification of alcohol by using a nutrient agar plate as hydrophilic carrier and decane as a hydrophobic organic solvent. This system can be used to produce acetate esters of alcohols without adding any acetyl donors, and has been applied in the production of citronellyl acetate with AATase in *Han. saturnus*, *Pichia heedii* and *P. quercuum* (Oda and Ohta, 1997).

We have previously studied the potential of non-*Saccharomyces* wine yeasts to produce the glycosidases involved in the flavour releasing processes which are of interest in winemaking (Manzanares et al., 1999, 2000). In the present study, we report the screening of acetate ester synthesis by non-*Saccharomyces* wine yeasts using the double coupling system and the production of esters in liquid microbiological medium when the yeasts were cultured under highly aerobic or minimally aerobic conditions.

2. Materials and methods

2.1. Yeast strains

Candida dattila 10387, *Hanseniaspora guilliermondii* 11104, *H. osmophila* 11206, *H. uvarum* 1444, 11105 and 11106, *Metschnikowia pulcherrima* 10546, *P. anomala* 10590, *P. heedii* 11452, *P. membranaefaciens* 10113, *Schizosaccharomyces pombe* 1377 and *Zygosaccharomyces bailii* 11042 were obtained from the Spanish Type Culture Collection (CECT, Burjassot, Spain) and were mainly isolated from grapes and wines. Two widely used commercial wine yeast strains, *S. cerevisiae* T_{73} (CECT 1894) and Fermol Primeurs (Pascal Biotech, Paris, France), were also included in the present study.

2.2. Screening of yeasts by the double coupling system

The synthesis of the acetate esters, ethyl acetate, geranyl acetate isoamyl acetate, linalyl acetate and 2-phenylethyl acetate, was screened basically following the protocol described by Oda et al. (1996) using the double coupling system in glass petri dishes (diameter, 90 mm) containing GPYM medium [40 g glucose (Panreac, Barcelona, Spain), 5 g peptone (Oxoid, Basingstoke, UK), 3 g yeast extract (Pronadisa, Madrid, Spain), 3 g malt extract (Pronadisa), 1 g $MgSO_4 \cdot 7H_2O$ (Panreac), 1000 ml distilled water, pH 6 solidified by adding 15 g/l of agar (Pronadisa)]. Two hundred microliters from 24 h cultures on GPYM medium were spread onto the agar surface and the plates incubated at 30 °C for 24 h. After incubation, 8 ml of a 1% (v/v) isoamyl alcohol (Sigma-Aldrich, Steincheim, Germany) or 0.5% (v/v) ethanol absolute (Merck, Darmstadt, Germany), geraniol (Fluka, Buchs, Switzerland) linalool (Fluka) or 2-phenylethanol (Fluka) solution in decane (Sigma-Aldrich) was placed onto the agar plate, and incubated at 30 °C for 24 h with mild shaking. Aliquots of 1 μl of the decane solution were taken after 2, 4, 6, 8 and 24 h of incubation for analysis of the products by gas chromatography (GC). β-ionona (Fluka) was added as internal standard to the alcohol decane solution at a concentration of 1% (v/v). Analyses were carried out on an HP5890 series II

gas chromatograph (Hewlett-Packard, Waldbronn, Germany) using an MXT-1 capillary column (Restek, Bellafonte, PA, USA) (length, 30 m; inside diameter, 0.28 mm; film thickness, 0.10 μm). The injection block and detector temperatures were kept constant at 200 °C for the analysis of geranyl acetate, isoamyl acetate, linalyl acetate and 2-phenylethyl acetate, and at 200 and 250 °C, respectively, for the analysis of ethyl acetate. The oven temperature was programmed as follows: 80 (6 min) to 250 °C at 20 °C/min for the analysis of geranyl acetate, isoamyl acetate, linalyl acetate and 2-phenylethyl acetate, and 60 (3 min) to 250 °C at 20 °C/min and then kept 2 min at 250 °C for the analysis of ethyl acetate. Ester concentrations were calculated using standard solutions and are the average of three independent plate cultures.

2.3. Liquid culture conditions for acetate ester formation

To measure ester formation by yeast strains, liquid cultures were grown in 200 ml of GPYM medium (pH 6, 30 °C) in 500-ml flasks. To study the effect of aeration conditions the cultures were grown either with shaking at 120 rpm (highly aerobic) or under static (minimally aerobic) conditions (Fujii et al., 1997). Aliquots of the cultures were taken at 8, 24 and 48 h of growing and analysed by headspace solid-phase-microextraction sampling (SPME) using poly(dimethylsiloxane) (PDMS) fibers (Supelco, Sigma-Aldrich, Barcelona, Spain) and GC. Aliquots of 1.5 ml of the samples were placed in 15-ml vials and 0.3 g of NaCl and 81 μl of 0.1% (v/v) 2-octanol (Fluka) as internal standard were added. The vials were closed with screwed caps and 3-mm thick teflon septa. Solutions were stirred for 2 h at 25 °C to get the required headspace–liquid equilibrium. PDMS fibers were injected through the vial septum and exposed to the headspace for 7 min and then desorbed during 4 min in an HP 5890 series II gas chromatograph equipped with a HP Innowax column (Hewlett-Packard) (length, 15 m; inside diameter 0.25 mm; film thickness, 0.25 μm). The injection block and detector temperatures were kept constant at 220 and 280 °C, respectively. The oven temperature was programmed as follows: 35 (5 min) to 150 °C at 5 °C/min and to 250 °C at 20 °C/min and then kept at 250 °C for 2 min. Ester concentrations were calculated using standard solutions (Fluka) and are given as the average of three independent cultures.

3. Results

3.1. Production of acetate esters by using the double coupling system

The 11 non-*Saccharomyces* wine yeast strains and two wine *S. cerevisiae* strains described in Section 2 were screened for the production of ethyl acetate, geranyl acetate, isoamyl acetate, linalyl acetate and 2-phenylethyl acetate by adding the alcohol to be esterified in a decane solution onto a glucose agar plate grown for 24 h. *P. heedii* (CECT 11452) described by Oda and Ohta (1997) as having a strong double coupling activity was included as a positive control. In a first screening, ester formation was determined after 24 h of incubation with the alcohol decane solution. Six of the tested strains were able to esterify at least one of the alcohols studied although none of them were able to produce linalyl acetate. *P. anomala* 10590 and *P. heedii* 11452 were able to esterify ethanol, geraniol isoamyl alcohol and 2-phenylethanol. *H. guilliermondii* 11104 produced ethyl acetate, geranyl acetate and isoamyl acetate whereas *H. uvarum* 1444 was able to produce ethyl acetate and isoamyl acetate. *H. uvarum* 11105 only yielded ethyl acetate whereas *P. membranaefaciens* 10113 only produced isoamyl acetate. *C. dattila* 10387, *H. osmophila* 11206, *H. uvarum* 11106, *Sch. pombe* 1377, *Z. bailii* 11042 and both wine strains of *S. cerevisiae* were not able to produce any of the esters studied under these experimental conditions.

The three tested strains producing more than one acetate ester were selected to study ester formation during an incubation period with the alcohol decane solution, sampling at 2, 4, 6, 8 and 24 h. Fig. 1 shows that the three yeast strains accumulated ethyl acetate in large amounts. *H. guilliermondii* 11104 and *H. uvarum* 1444 reached a maximum in the production of ethyl acetate at 2 h of incubation (5.51 ± 0.09 and 8.09 ± 0.15 mg/ml, respectively), whereas *P. anomala* 10590 produced the greatest quantity (13.61 ± 0.12 mg/ml) after 24 h of incuba-

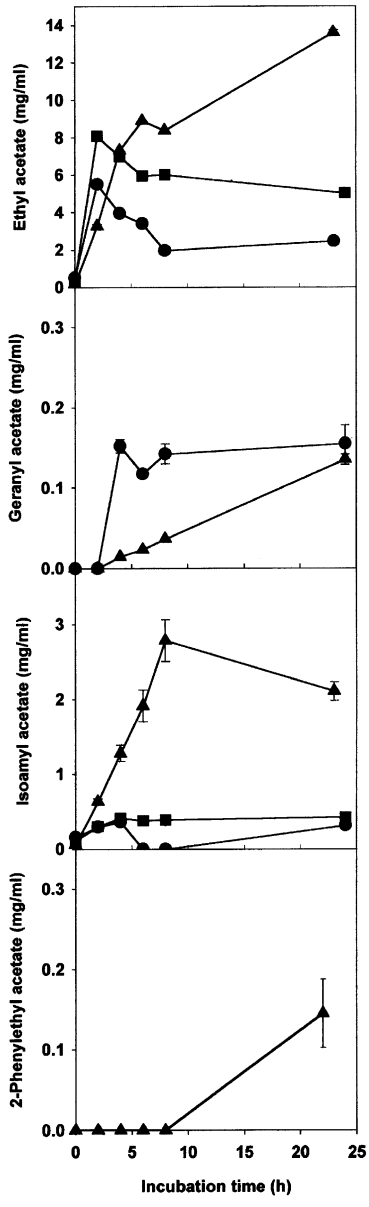

Fig. 1. Production of ethyl acetate, geranyl acetate, isoamyl acetate and 2-phenylethyl acetate via the double coupling system by *H. guilliermondii* 11104 (●), *H. uvarum* 1444 (■) and *P. anomala* 10590 (▲). Mean values and standard errors for three replicas. See Section 2 for more details.

tion. Geranyl acetate was accumulated at a low reaction rate by *P. anomala* 10590 and *H. guilliermondii* 11104, both producing less than 0.2 mg/ml of ester after 24 h of incubation. *P. anomala* 10590 was the best isoamyl acetate producer, yielding 2.11 $\pm$ 0.12 mg/ml of ester after 24 h of incubation, whereas the two species belonging to the genera *Hanseniaspora* produced less than 0.5 mg/ml of isoamyl acetate at the same time point. Low levels of 2-phenylethyl acetate (0.15 $\pm$ 0.04 mg/ml) could be detected in the case of *P. anomala* 10590 only after 24 h of incubation.

3.2. Influence of aeration conditions on the formation of acetate esters

The effect of aeration conditions in ester production by *H. guilliermondii* 11104, *H. uvarum* 1444 *P. anomala* 10590 and *S. cerevisiae* T_{73} was studied by growing the yeast under agitation (highly aerobic) or no agitation (minimally aerobic) of cultures. As Table 1 shows, highly aerobic conditions encourage notably the production of esters by non-*Saccharomyces* yeast, whereas, on the contrary, this stimulation effect on the ester production by *S. cerevisiae* T_{73} was observed for the defined minimally aerobic conditions.

H. uvarum and *S. cerevisiae* T_{73} produced low levels of ethyl acetate (ranging from 20 to 30 mg/l) in comparison with those yielded by *H. guilliermondii* (250 mg/l) or *P. anomala* (more than 4000 mg/l) in highly aerobic culture conditions. The highest isoamyl acetate concentration was found in *P. anomala* 48-h cultures grown in highly aerobic conditions, being approximately 7- and 19-fold greater than that produced by *S. cerevisiae* T_{73} cultures in minimally and highly aerobic conditions, respectively. With respect to 2-phenylethyl acetate, *H. guilliermondii* yielded much higher concentrations than the other yeast strains, particularly under highly aerobic culture conditions. *H. guilliermondii* and *S. cerevisiae* T_{73} produced similar levels of isobutyl acetate, whereas at 48 h *P. anomala* yielded a 15-fold greater quantity of this ester. With respect to non-acetate esters, *P. anomala* yielded higher concentration of diethyl succinate than the other strains either in highly or minimally aerobic conditions. In *S. cerevisiae* T_{73} strain cultures this ester was only detected under minimally aerobic growing conditions. However, the *S. cerevisiae* strains were the greatest producer of both ethyl caproate and ethyl

Table 1
Production of esters by *H. guilliermondii* 11104, *H. uvarum* 1444, *P. anomala* 10590 and *S. cerevisiae* T_{73} when grown under highly aerobic or minimally aerobic culture conditions

Component (mg/l)[a]		Yeast strains											
		H. guilliermondii 11104			*H. uvarum* 1444			*P. anomala* 10590			*S. cerevisiae* T_{73}		
		8 h	24 h	48 h	8 h	24 h	48 h	8 h	24 h	48 h	8 h	24 h	48 h
Isoamyl acetate	A	3.06	3.37	0.80	0.15	0.06	n.d.	0.44	3.76	10.21	0.02	0.75	0.55
	B	0.01	0.23	0.48	n.d.	0.01	n.d.	0.05	0.21	0.48	0.07	1.31	1.53
2-Phenylethyl acetate	A	33.83	163.80	109.60	0.11	0.19	0.12	0.36	1.18	0.12	0.08	0.13	0.10
	B	3.55	21.92	28.88	0.03	0.04	0.22	0.04	0.12	0.27	0.04	0.17	0.25
Ethyl acetate	A	408.70	204.90	250.50	10.61	28.84	18.14	257.10	1502.00	4143.00	n.d.	5.67	21.93
	B	2.99	14.67	46.12	n.d.	8.38	33.56	36.91	145.10	668.60	0.16	6.20	12.48
Ethyl caproate	A	n.d.	0.01	0.01	n.d.	n.d.	n.d.	n.d.	0.03	0.04	0.01	0.10	n.d.
	B	n.d.	0.01	0.01	n.d.	n.d.	n.d.	n.d.	0.01	0.03	0.01	0.22	0.15
Ethyl caprylate	A	0.01	0.02	0.01	n.d.	n.d.	n.d.	0.01	0.01	0.02	0.05	0.16	0.01
	B	0.01	0.01	0.01	n.d.	0.01	n.d.	0.01	0.06	0.05	0.04	0.72	1.03
Diethyl succinate	A	n.d.	n.d.	n.d.	n.d.	n.d.	0.35	0.43	2.71	2.62	n.d.	n.d.	n.d.
	B	n.d.	n.d.	n.d.	n.d.	0.20	n.d.	0.64	1.11	1.17	0.14	0.37	0.47
Isobutyl acetate	A	0.18	0.26	0.16	n.d.	n.d.	0.08	n.d.	0.32	1.93	n.d.	n.d.	n.d.
	B	n.d.	n.d.	n.d.	n.d.	n.d.	0.05	n.d.	n.d.	n.d.	0.02	0.14	0.12

n.d.: not detected.
A: highly aerobic conditions; B: minimally aerobic conditions.
[a]Mean values for three replicas. Standard errors were always lower than 20% of mean values.

caprylate in cultures grown under minimally aerobic conditions.

4. Discussion

It is generally believed that esters produced during wine fermentation contribute significantly to the desirable aspects of the fermentation bouquet of wine (Rapp and Mandery, 1986). The characteristic fruity flavours of wine and other grape-derived alcoholic beverages are primarily due to a mixture of hexyl acetate, ethyl caproate and ethyl caprylate (apple-like aroma), isoamyl acetate (banana-like aroma), and 2-phenylethyl acetate (fruity and flowery flavor). In yeast strains, AATase is the key enzyme for the production of acetate esters during fermentation (Fujii et al., 1994), and the *ATF1*-encoded AATase from *S. cerevisiae* is the best-studied acetyl-transferase activity (Yoshioka and Hashimoto, 1981; Malcorps et al., 1991; Fujii et al., 1994, 1997). Although it is well known that non-*Saccharomyces* wine yeasts are good producers of esters and despite the fact that several authors have suggested their use as mixed starters together with *S. cerevisiae* to improve the sensory properties of wine (Mateo et al., 1991; Moreno et al., 1991; Zironi et al., 1993; Gil et al., 1996; Romano et al., 1997), there is a lack of information about acetate ester-forming activity in yeasts of oenological origin. In our work, two approaches have been followed to study the production of acetate esters by non-*Saccharomyces* wine yeasts. The first approach, based on the double coupling system activity (Oda et al., 1996), allowed us to screen for the presence of acetate ester forming activities with different substrate specificities by adding the alcohol to be esterified to a decane solution that acts as an extractive system for the esters formed. In a second approach to obtain information about the production of esters in physiological growth conditions, yeasts were grown in liquid medium under highly aerobic or minimally aerobic conditions without adding the alcohol to be esterified, in order to determine the profile of esters formed.

Using the double coupling system, we have selected yeast strains belonging to the genera *Hanseniaspora* and *Pichia* that are able to promote the esterification of various alcohols such as ethanol, geraniol, isoamyl alcohol and 2-phenyl ethanol. Neither of the yeast strains esterified linalool, maybe

because it is a tertiary alcohol. *P. anomala* 10590 showed a wide range of substrate specificity, being able to esterify four of the five alcohols tested. Within the genus *Hanseniaspora*, three of the five species tested showed the double coupling system activity, although with different substrate specificity. The *H. guilliermondii* 11104 strain esterified ethanol, geraniol and isoamyl alcohol, whereas two *H. uvarum* strains were able to produce ethyl acetate and isoamyl acetate (*H. uvarum* 1444) or only ethyl acetate (*H. uvarum* 11105). Species belonging to the genus *Pichia* such as *P. kluyveri*, *P. quercuum* and *P. heedii*, have been described previously as having a strong double coupling activity of acetyl-CoA formation and alcohol esterification might be due to AATase activity (Oda et al., 1996, 1999; Oda and Ohta, 1997), but there are no data concerning the genus *Hanseniaspora*. The two wine *S. cerevisiae* species did not produce any of the esters tested, in agreement with the results obtained by Oda and Ohta (1997). The highly aerobic conditions in the plate assays could explain the lack of acetate ester forming activity in *S. cerevisiae* strains (Fujii et al., 1997).

Non-*Saccharomyces* wine yeasts that can grow during the early stages of wine fermentation when respiration may be important, are capable of anaerobic as well as aerobic growth. However, there is hardly any data about the effect of aeration culture conditions on ester synthesis by non-*Saccharomyces* wine yeast. Inoue et al. (1997) reported that *Han. mrakii* could produce a large amount of isoamyl acetate when the cells were cultured under aerobic conditions in contrast with *S. cerevisiae*, which could not produce the ester. We have studied the effect of aeration conditions on the formation of acetate esters by the yeast strains selected from the plate assay and by *S. cerevisiae* T_{73}, a widely used commercial wine yeast strain. Low levels of acetate esters were found when non-*Saccharomyces* yeast strains were grown under minimally aerobic conditions or when *S. cerevisiae* T_{73} was grown under highly aerobic conditions. Our results on liquid media indicate that when grown under highly aerobic conditions, *H. guilliermondii* 11104 and *P. anomala* 10590 produce greater concentrations of acetate esters than *S. cerevisiae* T_{73} growing under the same aeration conditions or even under minimally aerobic conditions. On the contrary, *S. cerevisiae* T_{73} strain was the best producer of those non-acetate esters, such as ethyl caproate and ethyl caprylate, which play an important role on wine aroma. This suggests that both kinds of esters could become from different metabolic pathways involving different enzymatic activities. Ethyl acetate was the major component produced by non-*Saccharomyces* wine yeasts. Similar results were described for the overexpression of *ATF1* in wine and brewing yeast strains (Lee et al., 1995; Lilly et al., 2000). Excessively high concentrations of ethyl acetate do not improve the aroma of young wines, but it has been reported that the negative effects of high levels of ethyl acetate might be reduced during bottle aging (Lilly et al., 2000). Also non-*Saccharomyces* wine yeasts produced increased concentrations of esters with a fruity aroma. *H. guilliermondii* 11104, in particular, was found to be a potent 2-phenylethyl acetate producer and *P. anomala* 10590 produced significant amounts of isoamyl acetate. Although *H. uvarum* has traditionally been described as a good ester producer (Mateo et al., 1991; Sponholz, 1993; Romano et al., 1997), the strains that we have tested were not outstanding with respect to the synthesis of any of the esters studied.

This study has revealed the potential of non-*Saccharomyces* wine yeasts to produce acetate ester forming activities with different substrate specificities, which could be employed to improve the sensory properties of wine. *H. guilliermondii* 11104 and *P. anomala* 10590 produce significant amounts of 2-phenylethyl acetate and isoamyl acetate, respectively, suggesting their possible use in mixed starters for wine production although further detailed studies are required in this respect. Our research is now focused on the purification of the *H. guilliermondii* 11104 and *P. anomala* 10590 acetate ester forming enzymes.

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