International Journal of Food Microbiology 134 (2009) 201–210

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: Potential applications in the wine industry

Philippe Renault [a,b], Cecile Miot-Sertier [a], Philippe Marullo [a,b], Purificacion Hernández-Orte [c], Laure Lagarrigue [a], Aline Lonvaud-Funel [a], Marina Bely [a,*]

[a] *Université Bordeaux, UMR 1219, INRA, ISVV, Villenave d'Ornon, 33140, France*
[b] *SARCO, LAFFORT Bordeaux, 33072, France*
[c] *University Zaragoza, Faculty of Sciences, Zaragoza, 50009, Spain*

ARTICLE INFO

Article history:
Received 14 April 2009
Received in revised form 16 June 2009
Accepted 20 June 2009

Keywords:
Non-*Saccharomyces*
Torulaspora delbrueckii
Wine fermentation
Selection
Volatile acidity
Genetic characterization

ABSTRACT

In this study, several strains of *Torulaspora delbrueckii* yeast species were evaluated in the laboratory for their enological properties. In a preliminary step, the ability of different molecular methods to discriminate among *T. delbrueckii* strains was compared. A combination of 7 PCR methods was able to separate 21 strains into 18 groups, while an REA-PFGE method allowed, in one experiment, the separation into 19 groups. The *T. delbrueckii* strains used presented a wide phenotypic variability in fermentation behaviour, e.g. Lag Phase (LP) duration, T50 parameter (time necessary to ferment half the sugar), and ethanol production. These 3 parameters have to be considered for industrial selection, particularly the LP duration. The majority of *T. delbrueckii* strains produced 8 to 11% and 7 to 10% ethanol vol. at 17 °C and 24 °C, respectively, with a maximum ethanol concentration of 12.35 at 17 °C and 10.90% vol. at 24 °C. The phenotypic variability of this species was also reflected in volatile acidity, glycerol, and aroma production. These experiments confirmed the low volatile acidity and glycerol production of this species and revealed a difference in osmotic stress response, compared to *Saccharomyces cerevisiae*. *T. delbrueckii* presented high fermentation purity and produced low levels of undesirable volatile compounds, such as hydrogen sulphide and volatile phenols.

1. Introduction

Spontaneous alcoholic fermentation of grape must is a complex process, involving the sequential action of several yeast genera and species, found on grapes and in must. The early stages in fermentation are dominated by the growth of non-*Saccharomyces* yeasts, characterized by low fermentative capacity. After the first few days' fermentation, they die off, due to the increasing concentration of ethanol (Heard and Fleet, 1985, 1986). Subsequently, *Saccharomyces cerevisiae*, an ethanol-tolerant species, takes over the fermentation. Quantitative studies of wine-making ecology have shown that significant levels of certain non-*Saccharomyces* species survive for longer periods than previously thought (Cabrera et al., 1988; Egli et al., 1998; Fleet, 2003; Heard and Fleet, 1985; Herraiz et al., 1990; Lema et al., 1996; Moreno et al., 1991; Pardo et al., 1989; Pina et al., 2004; Zott et al., 2008). Indeed, several authors have reported the influence of non-*Saccharomyces* yeast species on wine quality (Cabrera et al., 1988; Ciani and Ferraro, 1998; Ciani and Picciotti, 1995; Fleet, 2003, 2008; Herraiz et al., 1990; Lema et al., 1996; Romano et al., 2003) and evaluated the biotechnological interest of their enzymatic activities (e.g. esterases, β-glucosidase, and proteases), assumed to enhance fruit aromas in wine (Fernández-González et al., 2003; Fleet, 2008; Rosi et al., 1994; Strauss et al., 2001).

Recent metabolic and analytical profiles of non-*Saccharomyces* yeasts showed that *Torulaspora delbrueckii* species (formerly *Saccharomyces rosei*) had a positive impact on the flavour of alcoholic beverages (Ciani and Maccarelli, 1998; Ciani and Picciotti, 1995; Herraiz et al., 1990; Moreno et al., 1991). Indeed this species exhibits low production of undesirable compounds, such as acetaldehyde, acetoin, acetic acid, and ethyl acetate (Cabrera et al., 1988; Ciani et al., 2006; Ciani and Maccarelli, 1998; Ciani and Picciotti, 1995; Herraiz et al., 1990; Martinez et al., 1990; Peynaud, 1956; Plata et al., 2003; Viana et al., 2008). In view of its high fermentation purity, *T. delbrueckii*, in mixed or sequential culture with *S. cerevisiae*, was proposed as a way of minimising acetic acid production in wine under standard or high-sugar conditions (Bely et al., 2008; Ciani et al., 2006; Lafon-Lafourcade et al., 1981). The aromatic potential of this species has also been investigated: Viana et al. (2008), Plata et al. (2003), and Ciani and Maccarelli (1998) showed the low capacity of *T. delbrueckii* to produce esters, while Hernández-Orte et al. (2008) suggested that this species significantly modulated the levels of various varietal aroma compounds (nor-isoprenoids, terpenols, benzenoids, volatile phenols, vanillin, and lactones), by hydrolysing their respective precursors.

Nevertheless, despite increasing interest in *T. delbrueckii* species in wine applications, most studies to date only focused on a few strains, so no satisfactory overview of the wine-making properties of this

* Corresponding author. Tel.: +33 5 5757 5866; fax: +33 5 5757 5813.
E-mail address: marina.bely@univ-pau.fr (M. Bely).

0168-1605/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2009.06.008

Table 1
Strains of *T. delbrueckii* used.

Strain	Code	Collection	Origin
CLIB 230	A	CIRM-Levures	Unknown
CLIB 503	B		Sugar refinery
CLIB 504	C		Fermented rice
CLIB 505	D		Fermented milk
CLIB 506	E		Bot
CLIB 507	F		Grape berry (Burgundy)
CLIB 1134	G		Grape berry (Burgundy)
CLIB 1135	H		Grape berry (Burgundy)
CLIB 1136	I		Grape berry (Burgundy)
CLIB 1137	J		Grape berry (Burgundy)
CLIB 1138	K		Grape berry (Burgundy)
31703	L	MUCL	Grape must (Bordeaux)
27828	M		Unknown
L0470	N	IOEB	Grape must (Bordeaux)
L0544	O		Grape must (Bordeaux)
L0543	P		Grape must (Bordeaux)
L0631	Q		Grape must (Bordeaux)
L0630	R		Grape must (Bordeaux)
IFR 5	S	DGAM	Grape must (Tokaj)
IFR 7	T		Grape must (Tokaj)
AN07 Y01	U	ADRIA	Yogurt

CIRM-Levures: Centre international de ressources microbiennes, Thivernal-Grignon, France.
MUCL: Mycothèque de l'Université catholique de Louvain, Belgique.
IOEB: Collection de la faculté d'oenologie de l'Université de Bordeaux, France.
DGAM: Department of genetics and applied microbiology, University of Debrecen, Hungary.
ADRIA: Adria Normandie, secteur recherche et développement, Villers-Bocage, France.

species was available. Only Ciani and Maccarelli (1998) studied the fermentation properties of a wide sample of yeasts (90 strains), but measured only a few oenological parameters in a grape must modified with yeast extract. In this study, we considered a collection of 21 *T. delbrueckii* strains, isolated from various foods. The first step was to compare their genetic profile, using different molecular methods: RAPD-PCR (Random Amplification of Polymorphic DNA), microsatellite fingerprinting, and REA-PFGE (Restriction Endonuclease Analysis Pulse-Field Gel Electrophoresis). This was necessary to discriminate *T. delbrueckii* at the strain level, paving the way for clone selection programs. Secondly, the phenotypes of this species were characterized by analyzing fermentation traits and aromatic profiles, in accordance with oenological practice. The final aim of this work was to investigate potential biotechnological applications of this species in mixed starters with *S. cerevisiae*.

2. Materials and methods

2.1. Microorganisms and media

Twenty one *T. delbrueckii* strains of various origins were used (Table 1). In a few tests, three *S. cerevisiae* strains were also assayed: Zymaflore ST (Laffort Oenologie, France, CLIB 2026 (CIRM collection)), Zymaflore VL1 (Laffort Oenologie, CLIB 2015 (CIRM collection)), and SB from the IOEB collection.

Yeasts were grown at 24 °C on complete agar YEPD medium (1% yeast extract, 1% peptone, 2% dextrose) solidified with 2% agar.

2.2. Genetic characterization of T. delbrueckii strains

2.2.1. Extraction of nucleic acid from cultures

Strains were grown for 3 days in 10 mL YEPD broth at 24 °C, then the cultures were centrifuged at 5000 g for 10 min. The pellets were washed twice with TE (10 mmol/L Tris–HCl, 1 mmol/L EDTA pH8). The nucleic acids were extracted using the protocol described by Cocolin et al. (2000) and quantified using a BioRad SmartSpec™ spectrophotometer (Marnes la Coquette, France).

2.2.2. PCR conditions

DNA extracted from *T. delbrueckii* strains was subjected to RAPD-PCR using different primers: M13, M14, Coc, OPA02, and OPA09 (Yu et al., 1997; Zapparoli et al., 2000) and PCR fingerprinting, using microsatellite primers (GTG)5 and (GAC)5 (Baleiras Couto et al., 1996) (Table 2).

Reactions took place in a final volume of 25 µL for RAPD-PCR and 50 µL for PCR fingerprinting, containing 10 mmol/L Tris–HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1% Triton X100, 0.2 mg/mL BSA, 3.12% Glycerol, 0.2 mmol/L of each dNTP, 2.5U Taq-polymerase (Q.BIOgene, Montreal, Canada) and primers. A BioRad thermocycler was used for amplification. Following amplification, 10 µL samples of the PCR products were analysed on 3% agarose gels (Eurobio, Les Ulis, France) in 1X Tris–acetate EDTA (40 mmol/L tris–acetate, 1 mmol/L EDTA pH 8) at 110 V for 1.5 h.

Table 2
PCR conditions.

PCR Type	Primers			PCR cycles			
	Name	Sequence	Primer concentration in reaction mix	First cycle: denaturation	Cycle	Cycle numbers	Last cycle: extension
RAPD	M13 (Zapparoli et al., 2000)	5′-GAGGGTGGCGGTTCT-3′	2 µmol/L	94 °C, 5 min	D: 94 °C, 1 min A: 45 °C, 1 min E: 72 °C, 2 min	40	72 °C, 5 min
RAPD	M14 (Zapparoli et al., 2000)	5′-GAGGGTGGGGCCGTT-3′	2 µmol/L	94 °C, 5 min	D: 94 °C, 1 min A: 45 °C, 1 min E: 72 °C, 2 min	40	72 °C, 5 min
RAPD	Coc (Zapparoli et al., 2000)	5′-AGCAGCGTGG-3′	0.8 µmol/L	94 °C, 5 min	D: 94 °C, 1 min A: 40 °C, 1 min E: 72 °C, 2 min	32	72 °C, 10 min
RAPD	OPA02 (Yu et al., 1997)	5′-TGCCGAGCTG-3′	4 µmol/L	94 °C, 5 min	D: 94 °C, 1 min A: 36 °C, 1 min E: 72 °C, 2 min	39	72 °C, 10 min
RAPD	OPA09 (Yu et al., 1997)	5′-GGGTAACGCC-3′	4 µmol/L	94 °C, 5 min	D: 94 °C, 1 min A: 36 °C, 1 min E: 72 °C, 2 min	39	72 °C, 10 min
Fingerprinting with microsatellite primers	(GTG)5 (Baleiras Couto et al., 1996)	(GTG)5	0.2 µmol/L	95 °C, 5 min	D: 95 °C, 15 sec A: 55 °C, 45 sec E: 72 °C, 1.5 min	35	72 °C, 4 min
Fingerprinting with microsatellite primers	(GAC)5 (Baleiras Couto et al., 1996)	(GAC)5	0.2 µmol/L	95 °C, 5 min	D: 95 °C, 15 sec A: 45 °C, 45 sec E: 72 °C, 1.5 min	35	72 °C, 4 min

D: denaturation; A: annealing; E: extension.