DEVELOPMENT OF A RELIABLE AND EASY METHOD FOR SCREENING *OENOCOCCUS* CARBOHYDRATE CONSUMPTION PROFILE

B. HOCINE, Zohra JAMAL, L. RIQUIER,
Aline LONVAUD-FUNEL and Marguerite DOLS-LAFARGUE*

UMR 1219 Œnologie, INRA - Université de Bordeaux, ISVV, 210, chemin de Leysotte,
CS 50008, 33882 Villenave d'Ornon, France

Abstract

Aims: The aim of this study was to develop a colorimetric test to determine *Oenococcus* carbohydrate consumption profile.

Methods and results: A semi-defined growth medium which enabled efficient bacterial growth and medium acidification in the presence of glucose was developed. The inoculum size and the presence of citrate in the medium were optimized. Acidification of the medium was revealed by adding bromocresol green and was shown to be perfectly correlated with D-glucose, D-fructose, L-arabinose, D-xylose, L-rhamnose, D-cellobiose or D-trehalose consumption by 23 distinct *O. oeni* strains and one *O. kitaharae* strain (confirmed with HPLC analysis).

Significance and impact of the study: Efficient bacterial growth is essential before malic acid degradation occurs in wine. The method developed here will facilitate the comparison of numerous *O. oeni* strains and their ability to grow by using the various carbohydrates present in wine prior to malolactic fermentation.

Keywords: bacterial phenotype, *Oenococcus oeni*, *Oenococcus kitaharae*, colorimetric test, wine

Résumé

Objectif : Cette étude présente la mise au point d'un test colorimétrique adapté à la caractérisation des profils de consommation de glucides par les bactéries du genre *Oenococcus*.

Méthodes et résultats : Un milieu de croissance semi-défini, assurant à la fois une croissance bactérienne efficace et une acidification significative du milieu en présence de glucose, a été élaboré. Une attention particulière a été portée au volume de l'inoculum et à la présence de citrate dans le milieu. L'acidification du milieu, évaluée visuellement après ajout de vert de bromocrésol, s'est révélée en parfaite corrélation avec la consommation le D-glucose, D-fructose, L-arabinose, D-xylose, L-rhamnose, D-cellobiose ou D-tréhalose par 23 souches distinctes d'*O. oeni* et une souche d'*O. kitaharae* (mesurée par analyse HPLC).

Impact de l'étude : une phase préliminaire de croissance est indispensable avant la dégradation de l'acide malique par *O. oeni* dans le vin. Or, pour croître et réaliser la fermentation malolactique, les bactéries de l'espèce *O. oeni* doivent utiliser les rares sources de carbone présentes dans le vin en fin de fermentation alcoolique. La méthode développée ici facilitera la comparaison de nombreuses souches bactériennes du point de vue de leur capacité à utiliser les divers glucides présents dans le vin.

Mots clés : phénotype bactérien, *Oenococcus oeni*, *Oenococcus kitaharae,* test colorimétrique, vin

manuscript received the 20th October 2009 - revised manuscript received the 7th January 2010

*Corresponding author: dols@enscbp.fr

J. Int. Sci. Vigne Vin, 2010, **44**, n°1, 31 - 37

INTRODUCTION

In wine, malolactic fermentation (MLF) results in the bacterial conversion of L-malic acid into L-lactic acid and carbon dioxide with a subsequent reduction of wine acidity (Kunkee, 1967, Lonvaud-Funel, 1999). Several bacterial species have the capability to carry out malolactic fermentation, but *Oenococcus oeni* is the most reliable to induce malolactic fermentation, even in relatively acidic wines (Pilone and Kunkee, 1972, Dicks *et al.*, 1995, Lonvaud Funel, 1999, Mills *et al.*, 2005). Malate is not a growth substrate for bacteria, even though it generates a proton motive force which stimulates growth (Pilone and Kunkee, 1976, Cox and Henick-Kling, 1989; Loubière *et al.*, 1992; Salema *et al.*, 1996a and b). Other substrates are thus needed to support efficient bacterial growth before rapid malic acid transformation occurs (Tracey and Van Rooyen, 1988, Salou *et al.*, 1991; Saguir and Manca de Nadra, 1996, Miranda *et al.*, 1997). As a result, the wine carbohydrates serving as source of energy for *O. oeni* are of particular interest to the oenologists. Indeed, a strain that can use a large number of carbohydrates might better succeed inoculation in different wines as compare to a strain that can only use a limited number of sugars (Unden and Zaunmüller, 2009).

Because of its low growth rate, low growth pH, and specific growth factor requirements, API galleries (Analytical Profile Index) are not suitable for *O. oeni* characterization. Moreover, the need for a complex growth medium makes it difficult to test for substrate fermentation (Pilone and Kunkee, 1972). Indeed, in the past, most carbohydrate utilization profiles were determined by comparing the final OD obtained in the presence of carbohydrate with the one obtained in the same complex medium without carbohydrate (Peynaud and Domercq, 1968; Pilone and Kunkee, 1972; Beelman *et al.*, 1977). However, this method was sometimes misleading. The most reliable method still consists of measuring each carbohydrate concentration before and after bacterial growth. However, this requires a specific quantitative method for each carbohydrate, which can be time consuming particularly when a large number of strains have to be studied.

O. oeni is a heterofermentative lactic acid bacterium: hexoses are converted to equal amounts of lactate, CO_2 and ethanol or acetate, while pentoses are converted to equal amounts of lactate and acetate (Kandler, 1983; Unden and Zaunmüller, 2009). Therefore, carbohydrate catabolism decreases the pH of the growth medium. The aim of our study is thus to develop an easy and reliable test based on colour change upon growth medium acidification, specifically suited to determine *O. oeni* carbohydrate consumption profile.

MATERIALS AND METHODS

1. Bacterial strains

Twenty three strains of *Oenococcus oeni* were used in this study. Five of them were commercial malolactic starters (VO, PSU-1, B1, IOEB SARCO 277, IOEB SARCO 450) while the other eighteen were strains from the IOEB collection (ATCC BAA-1163, IOEB SARCO 396, IOEB SARCO 396bis, IOEB SARCO 729, IOEB SARCO 730, IOEB SARCO 733, IOEB SARCO 1491, IOEB 0205, IOEB 8403, IOEB 8406, IOEB 8413, IOEB 8417, IOEB 8419, IOEB 8802, IOEB 8905, IOEB 8908, IOEB 9220, IOEB 9624). One strain of *O. kitaharae* DSM17330 was also used.

2. Medium composition

Bacteria were grown in a medium (MRSa) adapted from MRS (de Man *et al.*, 1960) with the following composition ($g.l^{-1}$): yeast extract, 2; beef extract, 4; bactopeptone, 5 ; sodium acetate, 2.5; trisodium citrate, 1; K_2HPO_4, 1 ; $MgSO_4$, 7 H_2O, 0.1 ; $MnSO_4$, H_2O, 0.05; and Tween 80, 0.5 ml. The carbon source (D-glucose, D-fructose, L-rhamnose, L-arabinose, D-cellobiose, D-trehalose or D-xylose) was added to a final concentration of 10 $g.l^{-1}$. The pH was adjusted to 5.0 or 5.5 and the medium was sterilized for 20 min at 121 °C.

Bacteria were also grown in a semi-defined medium (SMD) containing : casamino-acids 10 $g.l^{-1}$; sodium acetate 3,4 $g.l^{-1}$; KH_2PO_4 1 $g.l^{-1}$; $MgSO_4$, $7H_2O$ 0.1 $g.l^{-1}$; $MnSO_4$, $4H_2O$ 0.1 $g.l^{-1}$; ammonium citrate 2.0 $g.l^{-1}$; bactotryptone 5 $g.l^{-1}$; yeast nitrogen base 6.7 $g.l^{-1}$; and adenine, uracyl, thymine, guanine 5 $mg.l^{-1}$ each. Carbohydrate (D-glucose, D-fructose, L-rhamnose, L-arabinose, D-cellobiose, D-trehalose, or D-xylose) was added to a final concentration of 10 $g.l^{-1}$ (unless otherwise stated in the text). The carbohydrate solutions were prepared as 50 $g.l^{-1}$ solutions and sterilized 20 min at 121°C, while the base solution was prepared as a 2X solution and sterilized by filtration (0.2 µm cut off). Before sterilization, the pH was adjusted to 5.0 or 5.5.

3. Growth conditions

Stock cultures of *O. oeni* strains were kept at -80 °C. They were first grown in MRSa-glucose medium (pH 5.0) at 26°C without agitation.

For the carbohydrate consumption profile analysis, MRSa-glucose grown cells (one-week old) were prepared as follows: cells were centrifuged (10 000 RPM, 4°C, 5 min), washed with NaCl 9‰ and re-suspended in SMD base 2X in order to obtain an OD_{600} equal to 1. This cellular suspension (10% of the final culture volume, unless otherwise stated in the text) was then used to

inoculate the medium containing the carbohydrate (2 ml-well microplates were used). Non-inoculated medium with the carbohydrate and inoculated medium without the carbohydrate were prepared as negative controls.

4. Analytical methods

After a two-week incubation, the absorbance (600 nm, OD_{600}) of the cell suspension was measured. The culture supernatant was collected after centrifugation (10 000 x g, 4 °C, 5 min) and kept at -20 °C for HPLC analysis.

The concentration of carbohydrates, lactic acid, acetic acid, glycerol, erythritol, acetoin butanediol, and ethanol in the supernatant were measured by anion exchange chromatography (Aminex HPX87H column, Bio-Rad) using a Waters system (Milford, USA) consisting of a pump (Waters 600), an injector (Waters 717) and a refractometer (Waters 2414). The eluant (H_2SO_4 5mM) had a constant flow rate of 0.5 $ml.min^{-1}$ at room temperature.

5. Colorimetric assay

Two hundred microliters of the cell suspension were mixed with 40 µl of Bromocresol green (0.25 $g.l^{-1}$ in aqueous solution) in a 250 µl microplate. The reactions (change of colour) were read visually.

6. Pulsed-field gel electrophoresis.

Bacteria were harvested during the exponential growth phase, embedded in agarose slices for DNA extraction, digested with NotI, and analyzed through pulsed-field gel electrophoresis (PFGE) as previously described (Gindreau *et al.*, 1997).

RESULTS AND DISCUSSION

1. Growth medium optimization

Carbohydrate degradation by *O. oeni* results in lactic acid and ethanol or acetic acid accumulation, with a subsequent pH decrease. In order to develop a colorimetric test, we first searched for a growth medium that would enable significant bacterial growth and pH decrease. For that purpose, two randomly selected *O. oeni* strains were grown in MRSa-glucose (5 $g.l^{-1}$) or SMD-glucose (5 $g.l^{-1}$) medium with initial pH equal to 5.0 and 5.5 (Table 1). All the media assayed enabled significant growth and pH decrease. However, SMD supported less OD increase in the absence of glucose than MRSa (mean final OD in SMD_{water} = 0.179 and mean final OD in $MRSa_{water}$ = 0.266), regardless of the initial pH of the medium. This « residual » growth could rely on the protein and polysaccharide content of MRS and SMD bases. Thus the difference between the final OD of inoculated culture in the presence of glucose and the final OD of inoculated culture in the presence of water (ΔOD) was higher in SMD (Table 1). In addition, the pH decrease associated with glucose consumption was more important in SMD than in MRSa medium, especially with an initial pH of 5.5. No significant pH decrease was observed in the absence of glucose in both growth media. The omission of acetate and/or citrate, which could contribute to the buffer strength of the MRSa or SMD base, did not modify the pH decrease associated with growth (data not shown). Based on these results, the subsequent experiments were all performed in SMD medium containing citrate and acetate, and an initial pH of 5.5.

2. Colour indicator

Bromocresol green appeared to be the most appropriate pH indicator for the pH range covered by SMD carbohydrate inoculated medium. In the presence of bromocresol green (0.08 $g.l^{-1}$), the non-acidified medium was blue, whereas below pH 5.1, the medium was clearly green (Figure 1).

3. Test reliability

The twenty four bacterial strains were then grown in SMD pH 5.5 in the presence of water, D-glucose, L-arabinose, D-cellobiose, D-fructose, L-rhamnose, D-trehalose or D-xylose (10 $g.l^{-1}$). Table 2 indicates, for each test, the number of positive strains deduced from

Table 1. Determination of the appropriate medium for colorimetric test development. Strains B1 and PSU-1 were grown in the presence of either water or glucose (5 $g.l^{-1}$). n = 2 assays.

		Strain B1		Strain PSU-1	
Base	Initial pH	ΔpH (a)	ΔOD (b)	ΔpH (a)	ΔOD (b)
MRSa	5.0	-0.20 ± 0.05	0.30 ± 0.01	-0.15 ± 0.02	0.25 ± 0.05
	5.5	-0,35 ± 0.04	0.29 ± 0.06	-0.25 ± 0.03	0.20 ± 0.03
SMD	5.0	-0.29 ± 0.04	0.45 ± 0.10	-0.16 ± 0.07	0.50 ± 0.02
	5.5	-0.38 ± 0.05	0.40 ± 0.03	-0.40 ± 0.05	0.48 ± 0.05

(a) difference between initial and final pH of inoculated culture in the presence of glucose.
(b) difference between final OD of inoculated culture in the presence of glucose and final OD of inoculated culture in the presence of water.

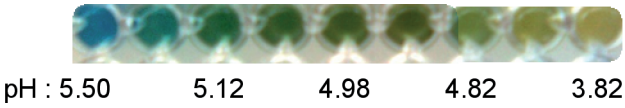

Figure 1. SMD-glucose + bromocresol green (30 mg.l^{-1}) colour depending on pH.

the OD, colorimetry and HPLC analysis after two weeks of incubation. The HPLC test constituted the reference. It was considered positive when at least 30% of the carbohydrate had been catabolized, which corresponded to a production of at least 20 mM lactic acid. The frontier between a positive and a negative result was clearly identified in all cases since the lactic acid final concentration was much higher than 20 mM in positive wells and lower than 10 mM in negative wells. Small quantities of secondary products (erythritol, glycerol, acetoin, butanediol) were produced in a proportion varying from 0% to 5% of the carbohydrate consumed (mol per mol), depending on the strain and on the carbohydrate considered, but these did not interfere with the pH decrease induced by organic acid formation.

For the colorimetric test, a well containing non-inoculated SMD medium pH 5.5 with bromocresol green was used as a negative reference (blue colour). No colour change was observed in wells containing inoculated SMD_{water} and in several wells containing inoculated SMD plus carbohydrate. These accounted for the negative colour tests. By contrast, the wells having a green coloration (similar to that obtained at pH 5.12 on figure 1 or even more yellow) were considered positive tests. We did not obtain any intermediate coloration. Each assay where a colour change was observed also presented a significant decrease of the carbohydrate concentration, with a concomitant production of lactic acid. Indeed, the list of positive strains (i.e. strains able to use each single carbohydrate) obtained from the colorimetric test perfectly reflected the one obtained from the HPLC test (data not shown).The colour test gave no false positive or false negative results (Table 2).

For the OD test, a difference between final OD of inoculated culture in the presence of glucose and in the presence of water (ΔOD) higher than 0.2 constituted a positive result. The number of strains able to use either L-arabinose, D-cellobiose, D-fructose, L-rhamnose, D-trehalose or D-xylose based on ΔOD was always higher than that obtained from the HPLC analysis. The rate of false positive results varied from 0% to 21% depending on the carbohydrate (Table 2). No false negatives (i.e. ΔOD<0.2 but positive HPLC test) were observed with the OD test. In order to decrease the rate of false positives, the ΔOD was then considered significant at ΔOD>0.3 (Table 2, column d). This did not significantly change the rate of false positives but it introduced false negatives. Cellobiose was the carbohydrate that induced the highest number of misleading OD changes. There was no relationship between the strain and the number of false positive results, even though, three strains presented a significant OD increase in SMD_{water} medium (Table 2). The OD criterion alone (commonly used to determine *O. oeni* carbohydrate phenotype) was not completely reliable.

The final lactic and acetic acid concentration after arabinose exhaustion is indicated in table 3. There was no significant variation among the positive strains, suggesting that the main metabolic pathways active in

Table 2. Comparison of the number of positive strains obtained with HPLC, OD and colorimetric analysis (over 24 *Oenococcus* strains assayed in SMD pH 5.5, initial substrate concentration 10 g.l^{-1}) with the OD, colour and HPLC test.

Total number of strains	Number of positive based upon HPLC analysis (a)	Number of positive based upon colour change (b)	Number of positive based upon OD change (c)	Number of positive based upon OD change (d)
24	0	0	3	2
	24	24	24	23 (1 fn)
	22	22	24 (2 fp)	21 (2fn, 2fp)
	15	15	20 (5 fp)	20 (5 fp)
	20	20	22 (2 fp)	21 (1 fp)
	0	0	1 (1 fp)	1 (1fp)
	23	23	24 (1 fp)	22 (2fn, 1fp)
	0	0	3 (3 fp)	3

(a) Substrate consumption between inoculated and un-inoculated medium > 30% or increase of lactic acid concentration >20 mM.
(b) Change in final colour between SMD-carbohydrate and SMD-water medium.
(c) for SMD-water assays: final OD >0.3, and for SMD-carbohydrate assays: difference between final OD of SMD-carbohydrate and SMD-water ΔOD> 0.2 (number of false positive, fp, and false negative, fn)
(d) for SMD-water assays: final OD >0.4, and for SMD-carbohydrate assays: difference between final OD of SMD-carbohydrate and SMD-water ΔOD> 0.3 (number of false positive, fp, and false negative, fn)

Table 3. Lactic and acetic acid formation and final OD observed on arabinose exhaustion (SMD pH 5.5 + arabinose 5 g.l^{-1}). The positive assays where arabinose was not completely consumed do not appear in the table.

Strain	Lactic acid (a) mM	Acetic acid (a) mM	Final OD
450	35	32	0.527
730	33	33	0.562
396	33	32	0.576
1491	34	35	0.598
PSU-1	35	37	0.600
8802	32	32	0.600
8905	31	32	0.618
8908	34	36	0.673
9224	33	36	0.701
8406	33	33	0.858
B1	35	38	0.898
8403	33	36	0.918
9220	34	34	0.952
8419	34	31	0.956
8417	34	33	1.054

(a) final acid concentration – initial acid concentration ; According to Unden and Zaumüller (2009), the expected main metabolic pathways are : 1 arabinose (33 potential) → 1 lactic acid + 1 acetic acid
1 citric acid (8 potential) → 1 lactic acid + 1 acetic acid
A carbon recovery of 85-90% is generally expected for *Leuconostoc* fermentation balance (Salou *et al*., 1994; Dols lafargue *et al.*, 1997).

the various strains were identical. On the contrary, the final OD measured after arabinose assimilation significantly varied from one strain to the other, indicating varying growth efficiency on this carbohydrate. The same phenomenon was observed with other carbohydrates (data not shown). This could be due to the distinct strain abilities to use medium components other than carbohydrates as auxiliary growth substrates. Different levels of cell maintenance or energy sinks could also explain the differences in OD yields between the strains. This certainly contributes to the lower reliability of the OD criterion compared with the other two (colorimetric and HPLC analysis).

4. Phenotypes comparisons

The phenotypes obtained for the 24 strains were compared with those described in the literature (Table 4). All the strains studied metabolized glucose. The second substrate most frequently used by the 23 *O. oeni* strains was trehalose. Trehalose is not a grape carbohydrate, but it is released by yeasts at the end of alcoholic fermentation. As a result, it can be very interesting for a malolactic starter to be able to metabolize trehalose. Arabinose, fructose and cellobiose were also frequently used by the *O. oeni* strains in our study. None of the strain assayed metabolized rhamnose. Indeed, our results were in accordance with those in the literature (Table 4). Particular attention was paid on pentose phenotype which was proposed by Peynaud and Domercq (1968) as a basis for recognizing « *Leuconostoc oinos* » (later named *L. oenos* and now known as *Oenococcus oeni*) among wine cocci. Indeed, 92% of the strains in our study were able to grow on arabinose as the sole carbohydrate while 97% of the strains studied by Peynaud and Domercq (1968) did (OD test). On the contrary, none of the 24 strains we studied grew on xylose. In the study by Peynaud and Domercq (1968), the number of strains having such a phenotype was also low (13%). From their data, three positive strains on xylose (24 x 0.13) were expected in our study. This is exactly the number of positive strains we obtained from the biased OD test. Does *O. oeni* really have the ability to metabolize xylose or are these 13% in the Peynaud and Domercq studies false positives? Peynaud and Domercq also specified that when a strain grew on xylose, this was its favourite substrate and growth was very fast. The analysis of a greater number of strains with a more accurate test might be necessary to isolate xylose positive strains.

Twelve strains out of the 23 *O. oeni* studied (52%) had the same phenotype: they used glucose, trehalose, arabinose, fructose and cellobiose but did not grow in the presence of xylose or rhamnose. This phenotype was shared by 19/45 strains (42%) in the study by Peynaud and Domercq (1968). Among the 24 strains studied, *O. kitaharae* DSM 17330 and *O. oeni* PSU-1 were the only ones for which a phenotype was available (Beelman *et al.*, 1977, Endo and Okada, 2006). As shown in table 4, the phenotype of *O. kitaharae* DSM 17330 was identical to the one found in the literature. On the contrary, there was a discrepancy between our results and those by Beelman *et al*. (1977) concerning PSU-1 growth on arabinose. PFGE controls first confirmed that we really

Table 4. *O. oeni* and *O. kitaharae* strains phenotype comparisons.

Carbohydrate assayed	Present study (a)	Peynaud and Domercq, 1968 (a)		Endo and Okada 2006 (b)	Present study (b)	Beelman *et al.*, 1977 (b)	Present study (b)
Numbers of strains	23 *O. oeni*	235 wine cocci	45 *L. «oinos»*	6 *O. kitaharae*	*1 O. kitaharae DSM17330)*	1 *O. oeni* PSU-1	1 *O. oeni* PSU-1
D-glucose	100%		100%	+	+	+	+
L-arabinose	95%	97%	100%	-	-	-	+
D-cellobiose	61%		77%	Depends	+	+	+
D-fructose	83%		100%	+	+	+	+
L-rhamnose	0%		0%	-	-	-	-
D-trehalose	96%		91%	+	+	+	+
D-xylose	0%	13%	NI (c)	-	-	-	-
D-xylose and D-arabinose		5%	0%				

(b) +/- indicate the ability of the candidate strain to use the indicated carbohydrate. (c) NI: not indicated.

Table 5 - Influence of inoculum size on arabinose metabolism by PSU-1 (SMD pH 5.5 + arabinose 5 g.l^{-1}).

Inoculum size	1%	2%	5%	10%	20%
Colour change	+	+	+	+	+
Final OD	0.590	0.610	0.606	0.720	0.686
Arabinose consumed (HPLC test)	90%	100%	100%	100%	100%

Table 6. Influence citrate on arabinose metabolism by *O. oeni* PSU-1 and two others *O. oeni* strains (SMD pH 5.5 + arabinose 5 g.l^{-1}).

Growth medium	SMD arabinose with citrate			SMD arabinose without citrate		
strain	PSU-1	277	450	PSU-1	277	450
Colour change	+	+	+	-	-	-
Final OD	0.639	0.702	0.551	0.178	0.201	0.234
Arabinose consumed (HPLC test)	100%	100%	100%	15%	12%	21%

used PSU-1 (data not shown). The origin of the differences in PSU-1 arabinose phenotype was first thought to be linked to differences in the inoculum size (10% in our study and 1% in the study by Beelman *et al.* 1977). However, as shown in table 5, no phenotype differences concerning arabinose metabolism were observed when the inoculum size was changed.

Another difference was the growth medium composition. SMD medium contains citrate while the BB medium used by Beelman *et al.* (1977) did not. As shown in table 6, the omission of citrate in the SMD base strongly reduced growth and lactic acid production on arabinose. As reported by Salou *et al.* (1994), citrate is a very poor growth substrate for *O. oeni*. However, the electrogenic transport of citrate across the membrane results in an electrochemical gradient which can favour the proton motive force (pmf) dependent transport of other compounds such as arabinose (Ramos *et al.,* 1994; Zaumüller and Unden, 2009). Indeed, *O. oeni* PSU-1 genome displays several pentose/H+ symporters belonging to the pmf-dependant major facilitator superfamily (Mills *et al.*, 2005; Lorca *et al.*, 2007). The presence of citrate in the growth medium is thus essential for unambiguous pentose consumption tests.

CONCLUSION

For neutral substrate consumption profile determination, we recommend the use of SMD medium (see composition above) complemented with 10 g.l^{-1} of the tested, substrate, inoculated (10%) with a washed cell suspension (OD_{600} equal to 1), and after a two-week incubation, a control of acidification by means of bromocresol green (30 mg/l) addition. A negative control (inoculated without substrate) is needed to confirm colour change in positive strains.

Further studies involving more strains and more neutral substrates found in grape juice or wine (glucose, fructose, trehalose, mannose, ribose, raffinose, sucrose, galactose, arabinose, xylose, melibiose) will be of great interest for

O. oeni diversity characterization and it will be easy and faster with the test developed in this study.

REFERENCES

Beelman R. B., Gavin III A. and Keen R. M., 1977. A new strain of *Leuconostoc oenos* for induced malolactic fermentation in eastern wines. *Am. J. Enol. Vitic.*, **28**, 159-165.

Cox D. J. and Henick Kling T., 1989. Chemiosmotic energy from malolactic fermentation. *J. Bacteriol.*, **171**, 5750-5752.

De Man J. C., Rogosa M. and Sharpe M. E., 1960. A medium for the cultivation of *Lactobacilli. J. Appl. Bacteriol*., **23**, 130-135.

Dicks L. M. T., Dellagio F. and Collins M. D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni*. *Int. J. Syst. Bacteriol*., **45**, 395-397.

Dols M., Chraibi W., Remaud-Simeon M., Lindley N. D. and Monsan P., 1997. Growth and energetics of *Leuconostoc mesenteroides* NRRL B-1299 during metabolism of various sugars and their consequences for dextransucrase production. *Appl. Environ. Microbiol.*, **63**, 2159-2165.

Endo A. and Okada S., 2006. *Oenococcus kitaharae* sp. nov., a non-acidophilic and non-malolactic-fermenting *Oenococcus* isolated from a composting distilled shochu residue. *Int. J. Syst. Evol. Microbiol.*, **56**, 2345-2358.

Gindreau E., Joyeux A., De Revel G., Claisse O. and Lonvaud-Funel A., 1997. Evaluation of the settling of malolactic starters within the indigeneous microflora of wines. *J. Int. Sci. Vigne Vin*, **31**, 197-202.

Henick-Kling T., Sandine W. E. and Heatherbell D.A., 1989. Evaluation of malolactic bacteria from Oregon wines. *Appl. Environ. Microbiol*., **55**, 2010-2016.

Kandler O., 1983. Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek*, **49**, 209-224.

Kunkee R. E., 1967. Malolactic fermentation. *Adv. Appl. Microbiol*., **9**, 235-249.

Lonvaud Funel A., 1999. Lactic acid bacteria and the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek*, **76**, 317-331.

Lorca G. L., Barabote R. D., Zlotopolski V., Tran C., Winnen B., Hvorup R. N., Stonestrom A. J., Nguyen E., Huang L. W., Kim D. S. and Saier M. H. Jr., 2007. Transport capabilities of eleven gram-positive bacteria: comparative genomic analyses. *Biochim. Biophys. Acta*, **1768**, 1342-1366.

Loubière P., Salou P., Leroy M. J., Lindley N. D. and Pareilleux A., 1992. Electrogenic malate uptake and improved growth energetics of the malolactic bacterium *Leuconostoc oenos* grown on glucose malate mixtures. *J. Bacteriol.*, **174**, 5302-5308.

Mills D. A., Rawsthaorne H., Parker C., Tamir D. and Makarova K., 2005. Genomic analysis of *Oenococcus oeni* PSU-1 and its relevance to winemaking. *FEMS Microbiol. Rev*., **29,** 465-475.

Miranda M., Ramos A., Veiga-da-Cunha M., Loureiro-Dias M. C. and Santos H., 1997. Biochemical basis for glucose induced inhibition of malolactic fermentation in *Leuconostoc oenos*. J. *Bacteriol.*, **179**, 5347-5354.

Peynaud E. and Domercq S., 1968. Étude de quatre cents souches de coques hétérolactiques isolées de vins. *Ann. Inst. Pasteur*, **19**, 159-170.

Pilone G. J. and Kunkee R. E., 1972. Characterization and energetics of *Leuconostoc oenos* ML34. *Am J. Enol. Vitic.*, 23, 61-70.

Pilone G. J., and Kunkee R. E., 1976. Stimulatory effect of malolactic fermentation on the growth rate of *Leuconostoc oenos* ML34. *Appl. Environ. Microbiol*., **32**, 405-408.

Ramos A., Poolman B., Santos H., Lolkema J. S. and Konings W. N., 1994. Uniport of anionic citrate and proton consumption in citrate metabolism generates a proton motive force in *Leuconostoc oenos*. *J. Bacteriol*., **176**, 4899-4905.

Saguir F. M. and Manca de Nadra M. C., 1996. Organic acid metabolism under different glucose concentrations of *Leuconostoc oenos* from wine. *J. Appl. Bacteriol*., **81**, 393-397.

Salema M., Capucho I., Poolman B., San Romao M. V. and Loureiro Dias M. C., 1996a. *In vitro* reassembly of the malolactic fermentation pathway of *Leuconostoc oenos*. *J. Bacteriol*., **178**, 5537-5539.

Salema M., Lolkema J. S., San Romao M. V. and Loureiro Dias M. C., 1996b. The proton motive force generated in *Leuconostoc oenos* by L-malate fermentation. *J. Bacteriol.*, **178**, 3127-3132.

Salou P., Leroy M. J., Goma G. and Pareilleux A., 1991. Influence of pH and malate-glucose ratio on the growth kinetics of *Leuconostoc oenos*. *Appl. Microbiol. Biotechnol.* **36**, 87-91.

Tracey R. P. and Van Rooyen T. J., 1988. Utilization of glucose, fructose and malic acid by malolactic bacteria: effect of ethanol and formation of mannitol and volatile acids. *J. Appl. Bacteriol*., **65**, 113-118.

Unden G. and Zaunmüller T., 2009. Metabolism of sugars and organic acids by lactic acid bacteria from wine and must. 135-147. *In: Biology of microorganisms on grapes in musts and in wines*. ed. Konig *et al.*, Springer Verlag, Berlin Heidelberg.

Zaunmüller T. and Unden G., 2009. Transport of sugars and sugar-alcohols by lactic acid bacteria. 149-163. *In: Biology of microorganisms on grapes in musts and in wines*. ed. Konig *et al.*, Springer Verlag, Berlin Heidelberg.