NAD(P)H regeneration is the key for heterolactic fermentation of hexoses in *Oenococcus oeni*

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***Oenococcus oeni* (formerly *Leuconostoc oenos*) can perform malolactic fermentation, converting L-malate to L-lactate and carbon dioxide, in wines. The energy and redox potential required to support the growth of the micro-organism are supplied mainly by the consumption of carbohydrates via the heterolactic pathway. In the first steps of hexose metabolism two molecules of NAD(P)⁺ are consumed, which must be regenerated in later reactions. The aim of this work was to test if aerobic growth of *O. oeni* promotes higher cell yields than anaerobic conditions, as has been shown for other lactic acid bacteria. *O. oeni* M42 was found to grow poorly under aerobic conditions with glucose as the only carbohydrate in the medium. It was demonstrated that O₂ inactivates the enzymes of the ethanol-forming pathway, one of the two pathways which reoxidizes NAD(P)⁺ cofactors in the heterolactic catabolism of glucose. These results suggest that the regeneration of cofactors is the limiting factor for the aerobic consumption of glucose. When external electron acceptors, such as fructose or pyruvate, were added to glucose-containing culture medium the growth of *O. oeni* was stimulated slightly; fructose was converted to mannitol, oxidizing two molecules of NAD(P)H, and pyruvate was transformed to lactate, enabling the regeneration of NAD⁺. The addition of cysteine seemed to suppress the inactivation of the ethanol-forming pathway enzymes by O₂, enabling glucose consumption in aerobic conditions to reach similar rates to those found in anaerobic conditions.**

Keywords: aerobic glucose catabolism, NADH, ethanol-forming pathway, *Leuconostoc oenos*

INTRODUCTION

Oenococcus oeni is a lactic acid bacterium able to carry out malolactic fermentation (MLF), which is the bio-conversion of malic acid, present in wine, to lactic acid and CO₂. The general biochemistry of aerobic carbohydrate metabolism in *O. oeni* has been successfully elucidated (Salou *et al.*, 1994). *O. oeni* obtains energy to grow from the catabolism of sugar via heterolactic fermentation. The metabolic end-products of this catabolic pathway are lactic acid, acetic acid, ethanol and

CO₂ (Fig. 1) (Wibowo *et al.*, 1985; Cogan & Jordan, 1994). The relative yields of acetic acid and ethanol are related to the availability of NAD(P)⁺ in the cell. NAD(P)H produced during the first steps of the heterolactic catabolism of sugars is reoxidized by the conversion of pyruvic acid to lactic acid, as well as by the conversion of acetyl phosphate to ethanol. Although lactic acid bacteria (LAB) have a fermentative metabolism, some *Leuconostoc* strains exhibit higher biomass production when cultured in the presence of O₂ (Plihon *et al.*, 1995). Under aerobic conditions, O₂ acts as an electron acceptor oxidizing NADP(H) by NAD(P)H oxidases. Other compounds can also be used as electron acceptors, e.g. pyruvic acid or fructose (Ito *et al.*, 1983). Fructose is catabolized by the heterolactic pathway but can also be transformed into mannitol by mannitol dehydrogenase, a collateral reaction where NAD(P)H is regenerated (Veiga-da-Cunha *et al.*, 1993). The channel-

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Abbreviations: ADH, alcohol dehydrogenase; EFP, ethanol-forming pathway; LAB, lactic acid bacteria; MBB, MLO basal broth; MBBG, MLO basal broth plus glucose (55 mM); X_{max}, maximum biomass.

Fig. 1. Schematic representation of carbon and energy flows through the central metabolic pathways of *O. oeni* M42. Numbers represent: 1, mannitol dehydrogenase; 2, phosphate acetyltransferase; 3, acetaldehyde dehydrogenase; 4, ADH; 5, lactate dehydrogenase; 6, pyruvate oxidase; 7, erythritol-4-phosphate dehydrogenase.

ling of sugar through these two alternative pathways depends on the NAD(P)H/NAD(P)⁺ balance within the cell.

The aim of this work was to test whether O₂ or other electron acceptors could be used to improve the sugar consumption and growth of *O. oeni*. Moreover, NAD(P)H oxidases and the ethanol-forming-pathway activities of *O. oeni* have been studied under aerobic and anaerobic conditions. Elucidating such physiological aspects should allow problems associated with the production of malolactic starter cultures to be solved.

METHODS

Strain cultivation and maintenance. *O. oeni* CECT M42 was isolated from a Macabeo white wine by Pardo & Zúñiga (1992) and maintained in 20% (v/v) glycerol frozen at -20 °C, or freeze-dried. The strain was grown routinely on medium for *Leuconostoc oenos* (MLO; Caspritz & Radler, 1983) and maintained on MLO plates at 4 °C until needed.

Media and cultivation conditions

Effect of anaerobic and aerobic conditions on catabolism of sugar and growth. *O. oeni* M42 was grown in 200 ml MLO basal broth (MBB), i.e. MLO without sugars, citrate, tomato juice or cysteine, in a 350 ml Biostat-Q multiple bioreactor (B. Braun). Sugars (glucose or fructose) were supplied to the medium before sterilization at a final concentration of 55 mM.

MBB plus 55 mM glucose (MBBG) was used to test the effect of adding external electron acceptors. The compounds added were pyruvate (20 mM), fructose (20 mM), or L-cysteine.HCl (1.5, 7.5, 15 or 30 mmol l⁻¹; Sigma). In each case the pH was adjusted to 4.8 with 1 M KOH. After autoclaving, media were supplemented with 0.01 g l⁻¹ filter-sterilized D-pantothenic acid. The bioreactors were inoculated with an initial biomass concentration of 2 mg l⁻¹ from a pre-culture grown in flasks at 28 °C for 48 h, without stirring. The cultures in the bioreactors were grown at 28 °C with constant stirring (200 r.p.m.). The aerobic or anaerobic conditions were obtained by a continuous flush (0.50 l min⁻¹) of sterile air or N₂, respectively. O₂ partial pressure was continuously controlled and registered in bioreactors to ensure aeration conditions (anaerobic, pO₂ < 5%, or aerobic, pO₂ > 95%, as required). Microbial growth was recorded by periodic sampling of the culture broth. Optical densities of samples were measured at 600 nm. A calibration curve was constructed to correlate OD₆₀₀ and dry weight (g l⁻¹). Specific growth rates were calculated from the cell mass profiles as described by Salou *et al.* (1994). The changes in pH of the media were recorded. The concentrations of residual sugars and the final products of fermentation were quantified in the broth at the end of the experiments, by HPLC (see 'Analysis of chemical compounds').

Effect of anaerobic and aerobic conditions on the internal pools of NADH and NADPH. A separate experiment was designed to determine the effect of aeration conditions on the pool concentrations of the cofactors and on the activity of cell enzymes related to their reoxidation. Cells were grown in 1 l MBBG under N₂ flushing, in a 2 l Biostat-B bioreactor (B. Braun). When the culture reached a level of 0.08 g cell dry matter per litre, 200 ml was aseptically transferred to another bioreactor and then continuously aerated, whereas the rest of the culture continued growing anaerobically. The experiments lasted 5 d in both reactors, sparged with either N₂ or air. Samples were collected at the end of this period to measure the ethanol forming pathway (EFP) enzyme activities. Duplicate experiments were performed for each culture condition.

Analysis of chemical compounds. Samples were withdrawn directly from the bioreactors through the sampling ports and subsequently stored at -20 °C. For chemical analysis, supernatants from centrifuged samples (16000 r.p.m., 30 min) were filtered through a C-18 cartridge (Sep-Pak, Waters) and then through a 0.22 µm membrane filter. The concentrations of sugars, organic acids and ethanol in the fermentation broth were quantified by HPLC using an HPX-87H Aminex ion-exclusion column as previously described (Maicas *et al.*, 1999). ATP yields were determined from experimental lactate and acetate concentrations as described by Firme *et al.* (1994).

Preparation of cell-free extracts. Culture broth was withdrawn from the bioreactor into an ice-cooled bottle, centrifuged and washed twice with 10 mM potassium phosphate buffer (pH 7.0, 4 °C) and once with 3 mM Tris/HCl (pH 7.0, 4 °C). Cells were then resuspended in 2 ml 6.4 mM Tris/HCl (pH 8.4, 4 °C) and disrupted by breaking them in a vortex with 1 vol. of 0.1 mm glass beads for 10 min. Cells and cell extracts from the anaerobic cultures were maintained under N₂ flushing to prevent exogenous oxidation. The suspension was centrifuged (16000 r.p.m., 30 min, 2 °C) and the cell-free supernatant was pooled in a pre-cooled Eppendorf tube and stored at -20 °C for further analyses.

Protein quantification in the cell-free extracts. The Micro BCA Protein Assay Reagent (Pierce) was used to measure the protein concentration in the cell-free extracts, according to the manufacturer's instructions, using BSA as a standard.

Analysis of *in vitro* enzyme activities. Enzyme assays were performed at 30 °C in quartz cuvettes with a 1 cm light path using a Beckman model DU-7 spectrophotometer. The activity of alcohol dehydrogenase (ADH; EC 1.1.1.1) was measured in 3 ml of a reaction mixture containing 0.2 M glycine/NaOH buffer (pH 9.0), 3.3% (v/v) ethanol, 7.5 mM β -NAD⁺ and 150 μ g protein extract (Klingenberg, 1985). Enzyme activity was calculated using the increase in A_{340} resulting from the coenzyme reduction. The reaction for ADH (NADP⁺) (EC 1.1.1.2) activity determination was identical to that described above, except that β -NAD⁺ was replaced by β -NADP⁺. For acetaldehyde dehydrogenase (acetylating) (EC 1.2.1.10) determination, the coenzyme reduction was measured in a final volume of 3 ml containing: 0.2 M K₂HPO₄/KH₂PO₄ buffer pH 7.4; 10 mM 2-mercaptoethanol; 5 mM acetaldehyde; 50 mM β -NAD⁺ (or β -NADP⁺); and, 150 μ g protein extract. Phosphate acetyltransferase (EC 2.3.1.8) activity was determined by measuring acetyl phosphate decomposition in the presence of coenzyme A and arsenate according to the method of Kelly & Patchett (1996). NAD(P)H oxidase activities were assayed in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 25 μ M β -NADH (or β -NADPH) (Anders *et al.*, 1970). The reactions were started by adding the extract; the decreases in A_{340} were monitored. One unit of enzyme activity was defined as the amount of enzyme reducing 1 μ mol coenzyme min⁻¹; the specific activity was expressed as mmol (g protein)⁻¹ min⁻¹. The functionality of the assays was verified with enzymes purchased from Sigma.

Enzyme inactivation *in vitro*. Protein extracts were obtained from cells grown on MBBG under anaerobic conditions. Cells were recovered from culture medium at late-exponential phase and extracts were made as described above. Extracts were air-flushed in 0.2 M glycine/NaOH buffer for 6 h (with or without 3 mM cysteine). Samples were periodically withdrawn and ADH activity was measured as described. The enzyme activity of unflushed extracts was recorded and used as a control.

Quantification of cofactors. Assays were performed in cuvettes equilibrated at 30 °C using a Perkin-Elmer model LS-50 Luminescence spectrometer with excitation at 340 nm and emission peak at 460 nm. Buffers and stock solutions were prepared with ultrapure water and were filtered through 0.1 μ m filters. Each enzyme was prepared at the highest possible concentration, and fresh dilutions were made each day. Standard solutions of pyrimidine nucleotides were prepared each day and stored on ice. Standard substrate solutions were prepared daily and were assayed spectrophotometrically on the day of use.

β -NAD⁺ quantification was performed in 1.5 ml 0.1 M Tris/HCl containing 0.4 M hydrazine hydrate (pH 8.5). The reaction was started by adding 0.5% (v/v) ethanol. When temperature equilibration was completed (1–2 min), 7.5 μ l ADH (EC 1.1.1.1) (566 U ml⁻¹) was added. After 2–3 min, 7.5 μ l standard solution (0.1 mM β -NAD⁺) or cell-free extracts were added to the reaction solution. β -NADH production was measured by reading the increase of fluorescence. β -NADP⁺ quantification was performed in 1.5 ml 0.05 M triethanolamine/HCl, 10 mM MgCl₂, 5 mM EDTA (pH 7.4) as described for β -NAD⁺ quantification, but using 0.1 M glucose 6-phosphate as substrate and 50 μ M β -NADP⁺ as cofactor. The reaction was catalysed by glucose-6-phosphate dehydrogenase:NADP⁺ oxidoreductase (EC 1.1.1.49) (27.8 U ml⁻¹). NAD(P)H quantification was carried out by luminescence as described by Klingenberg (1985). Anaerobic conditions were maintained by N₂-flushing to prevent indirect effects, when required.

RESULTS AND DISCUSSION

Effect of anaerobic or aerobic conditions on growth and metabolic end-products during heterolactic catabolism of sugars

Glucose was completely metabolized by *O. oeni* M42 through the heterolactic pathway (Fig. 1) under anaerobic conditions, allowing a high biomass-to-glucose conversion yield, $Y = 5.23 \text{ g (mol sugar)}^{-1}$ (Fig. 2). The maximum specific growth rate, 0.08 h⁻¹, corresponded to the first 24 h of growth. The maximum biomass (X_{max}) was 0.30 g l⁻¹. The analysis of metabolic end-products showed equimolar amounts of lactate and ethanol in addition to significant levels of acetate (Table 1).

The X_{max} reached by *O. oeni* grown aerobically on glucose was very poor, $X_{\text{max}} = 0.05 \text{ g l}^{-1}$ (Fig. 2). Less than 5% (w/w) of the available glucose was metabolized, and the relative proportions of metabolic end-products were different to those found under anaerobic conditions (Table 1). These results contrast with those reported by Lucey & Condon (1986) and Sakamoto & Komagata (1996), in which *Leuconostoc mesenteroides* cells cultured aerobically grew better than those cultured anaerobically. LAB do not usually grow well under vigorous aeration and it could be said that the metabolism of LAB in the presence of O₂ is unusual. However, Lucey & Condon (1986) and Sakamoto & Komagata (1996) found that *Leuconostoc mesenteroides* cultured aerobically grew better than when cultured anaerobically because of the presence of inducible NAD(P)H oxidases, leading the cells to gain an ATP molecule from the transformation of acetyl phosphate to acetate. Plihon *et al.* (1995) also reported an improvement in *Leuconostoc mesenteroides* growth under aerobic conditions when the culture was supplied with sparged air or pure O₂. In some cases, LAB do not benefit from O₂, but they are not inhibited by its presence, e.g. *Lactobacillus plantarum* (Murphy & Condon, 1984) and *Lactococcus lactis* (Cogan *et al.*, 1989). However, *O. oeni* M42

Fig. 2. Growth of *O. oeni* M42 in MBB plus 0.5 g cysteine l⁻¹. ○, glucose + air-flow; □, glucose + N₂-flow; ●, fructose + air-flow; ■, fructose + N₂-flow. Samples were incubated at 28 °C and an initial pH of 4.8. Cultures were stirred continuously at 200 r.p.m.

Table 1. Quantification of substrates and end-products in the metabolism of hexoses by *O. oeni* M42

O. oeni M42 was grown in MBBG or MBB plus fructose (55 mM) under different aeration conditions. Samples were incubated at 28 °C and an initial pH of 4.8. All determinations were made in triplicate with at least two different extracts. ND, Not detected.

Aeration conditions	Substrate	Consumed sugar (mmol l ⁻¹)	Products (mmol l ⁻¹)					Carbon recovery (%) [*]
			Lactic acid	Acetic acid	Glycerol	Ethanol	Mannitol	
Anaerobic	Glucose	55.0	44.4	21.7	0.2	45.8	ND	95.3
	Fructose	55.0	20.2	31.1	0.0	0.4	36.9	99.1
Aerobic	Glucose	4.5	5.3	3.6	0.3	1.1	ND	97.0
	Fructose	38.4	12.7	24.4	0.3	0.9	20.4	92.0

^{*}The carbon imbalance is attributed to some incorporation of hexoses into biomass and to the production of minor unidentified compounds.

Table 2. Activities of some enzymes involved in the regeneration of NAD(P)H by *O. oeni* M42

Measurements were made with crude extracts. All determinations were made in triplicate with at least two different extracts. ND, Not detected.

Enzyme	Aeration condition	Cofactor	Specific activity [mmol (g protein) ⁻¹ min ⁻¹]
NADH oxidase	Aerobic	NADH	ND
	Anaerobic	NADH	ND
	Aerobic	NADH (+ FAD)	0.32 × 10 ⁻³
	Anaerobic	NADH (+ FAD)	ND
NADPH oxidase	Aerobic	NADPH	0.61 × 10 ⁻³
	Anaerobic	NADPH	ND
	Aerobic	NADPH (+ FAD)	0.82 × 10 ⁻³
	Anaerobic	NADPH (+ FAD)	ND
Alcohol dehydrogenase	Aerobic	NADH	ND
	Anaerobic	NADH	718
	Aerobic	NADPH	ND
	Anaerobic	NADPH	89
Acetaldehyde dehydrogenase	Aerobic	NADH	ND
	Anaerobic	NADH	0.34
	Aerobic	NADPH	ND
	Anaerobic	NADPH	0.02

growth was almost completely inhibited under aerated conditions. This inhibition of growth could be due either to the toxicity of O₂ to cells, to the lack of NAD(P)H oxidases, or to the inactivation of the EFP enzymes. In anaerobic bacteria, if a peroxidase system for detoxification is not present, the H₂O₂ formed kills the cells. This effect was discarded in *O. oeni* M42 when it was found that cells cultured aerobically on fructose produced 20-fold more biomass than when cultured on glucose (Fig. 2), and 70 % of the fructose was consumed.

NADH- and NADPH-oxidase activities were recorded under aerobic conditions for *O. oeni* (Table 2), although the reported levels were probably not sufficient to

efficiently reoxidize the cofactors given that growth was negligible under aerobic conditions. On the other hand, cells grown under anaerobic conditions produced up to 0.30 g cell dry matter per litre of medium. The lack of significant activity for NAD(P)H oxidases under aerobic conditions has been previously reported in other LAB (Nuraida *et al.*, 1992; Warriner & Morris, 1995). Although NADH oxidases appear to be widely distributed among LAB, some strains lack them, whereas NADPH oxidases have only rarely been reported in LAB (Warriner & Morris, 1995). Warriner & Morris (1995) described the inability of *Lactobacillus hilgardii* to benefit from the presence of O₂, which was explained by the lack of a NAD(P)H oxidase system. However, the

Table 3. Concentration of cofactors and NAD(P)⁺:NAD(P)H ratios under different atmospheric conditions and growth phases

Glucose was used as the carbon source. Samples were incubated at 28 °C and an initial pH of 4.8. The values shown are means of triplicate assays.

Aeration condition	Cofactor [mmol (g protein) ⁻¹]			Ratio [NAD(P) ⁺ :NAD(P)H]
	NAD ⁺	NADP ⁺	NAD(P)H	
Anaerobic	12.54	12.99	1.44	17.67
Aerobic	12.88	6.61	5.01	3.89

absence of NAD(P) oxidases is not enough to explain low cell growth under aerobic conditions, because Lucey & Condon (1986) reported that the aerobic growth of a mutant of *Leuconostoc mesenteroides* X2 lacking NADH oxidase was similar to its growth under anaerobic conditions, with the relative proportions of lactate and ethanol being the same under aerobic and anaerobic conditions. The low glucose consumption under aerobic conditions, and the low quantity of ethanol produced, suggested that the EFP was very inefficient. Ito *et al.* (1974, 1983) suggested that the effect of aeration on glycolysis in *Leuconostoc mesenteroides* was the consequence of the inability of cellular activities to reoxidize NAD(P)H via the EFP. The results of our enzyme assays to check EFP activity in *O. oeni* showed that both alcohol and acetaldehyde dehydrogenase (NAD⁺ and NADP⁺ dependent) activities were high enough to support cell growth in glucose-containing anaerobic cultures (Table 2); however, these activities could not be detected in cell extracts transferred to aerobic conditions. This suggests that the EFP enzymes were inhibited by O₂, thus stopping the normal way in which cofactors are reoxidized during the heterofermentative catabolism of glucose (Fig. 1, Table 2). Although it could be possible that O₂ represses the synthesis of EFP enzymes instead of inhibiting the enzymes, it is reasonable to assume that the enzymes are constitutive, as has been reported for *Leuconostoc mesenteroides* (Sakamoto & Komagata, 1996). This route is essential for reoxidation of the cofactors produced in the first steps of heterolactic sugar catabolism, and inhibiting this pathway would seriously compromise cellular development. There is evidence that O₂ inhibits enzyme activity of *O. oeni* *in vivo*: transfer of an aliquot of a bioreactor culture grown on glucose from anaerobic to aerobic conditions resulted in loss of activity of EFP enzymes after 1 h. In addition, we found that normal activity in anaerobic extracts dramatically decreased when they were flushed with air.

Oxidized and reduced cofactors were quantified in extracts of cells from cultures grown on glucose aerobically and anaerobically. No differences were found in the NAD⁺ concentration, but the NADP⁺ concentration was half as low in the aerobic extracts as compared to the anaerobic extracts (Table 3). The ratio between

oxidized and reduced cofactors was 4.5-fold higher in extracts cultured under anaerobic conditions than those cultured under aerobic conditions (Table 3). These results suggest that *O. oeni* cells grown aerobically on glucose did not reoxidize the NAD(P)H properly because the EFP enzymes were inhibited by O₂, and as a consequence growth was seriously compromised.

Results obtained from the analysis of the end-products of fructose catabolism showed that this sugar was mainly oxidized to acetate [$Y_{\text{acetate}} = 0.63$ mol acetate (mol sugar)⁻¹], mannitol [$Y_{\text{mannitol}} = 0.53$ mol mannitol (mol sugar)⁻¹] and lactate [$Y_{\text{lactate}} = 0.33$ mol lactate (mol sugar)⁻¹], whereas ethanol was hardly produced (Table 1). In this case, cells attained a final biomass of 0.12 g l⁻¹, 2.4-fold higher than on glucose, and although EFP enzymes were inhibited by O₂, the reoxidation of NAD(P)H was achieved due to the reduction of fructose to mannitol, via mannitol dehydrogenase (Veiga-da-Cunha *et al.*, 1992, 1993). This was confirmed by the relative quantities of mannitol and ethanol derived from fructose (Table 1). It was also observed that twofold more acetate was produced with respect to lactate, implying that some pyruvate was channelled to acetyl phosphate by pyruvate oxidase, resulting in extra ATP production, which improves growth (Divies *et al.*, 1994). Cells grown on fructose, either anaerobically or aerobically, exhibited lower X_{max} values than cells grown on glucose under anaerobic conditions (Fig. 2). This was a consequence of the fact that a large proportion of the fructose was converted to mannitol without gaining ATP, whereas glucose was fully transformed to lactate, ethanol, acetate and CO₂, with a gain of at least 1 mol ATP per mol glucose.

Aerobic growth in a glucose medium supplied with reducing substrates

The previous results suggest that adding reducing agents to MBBG under aerobic conditions could improve the regeneration of cofactors to some extent. Given that ADH and acetaldehyde dehydrogenase were inhibited by O₂ in *O. oeni* M42, pyruvic acid, fructose or cysteine were added to MBBG in order to test the ability of these products to reoxidize NAD(P)H and, hence, to improve

Fig. 3. Aerobic growth of *O. oeni* M42 in MBB containing: glucose, ●; glucose + pyruvic acid, △; glucose + fructose, □. Samples were incubated at 28 °C and an initial pH of 4.8. Cultures were stirred continuously at 200 r.p.m.

growth. Growth on glucose alone was negligible (Fig. 3). When pyruvic acid was supplied it was completely metabolized to lactic acid and acetic acid, reoxidizing NADH and leading to higher biomass production (Fig. 3). The stoichiometrical balance showed a two-threefold greater yield of acetic acid than when *O. oeni* was grown on glucose alone (Table 4), whilst higher levels of ATP [$Y_{ATP} = 0.11 \text{ mol ATP (mol sugar)}^{-1}$] were produced. The addition of pyruvic acid resulted in 20-fold higher levels of biomass production ($X_{max} = 0.09 \text{ g l}^{-1}$) than those seen in its absence (Fig. 3). Nevertheless, as lactate dehydrogenase can reoxidize NADH, but not NADPH, addition of pyruvic acid to MBBG does not allow complete reoxidation of NADPH generated in the first reactions of the carbohydrate pathway and, therefore, glucose could not have been exhausted. Ito *et al.* (1983) reported a beneficial effect of pyruvic acid on *Leuconostoc mesenteroides* growth when it was used as an external electron acceptor.

The addition of the same quantity of fructose (20 mM) to MBBG allowed its reduction to mannitol, with NAD(P)H molecules being oxidized via mannitol dehydrogenase (Fig. 1). Hence, 80% (w/w) fructose was reduced to mannitol to generate reducing equivalents,

while the remainder, together with 7.1 mM glucose, was equimolarly converted to lactate and acetate (Table 4). This permitted $X_{max} = 0.10 \text{ g l}^{-1}$, a little higher than that obtained with pyruvic acid (Fig. 3). The simultaneous consumption of both glucose and fructose led to higher yields of acetate, compared to those seen in glucose cultures. Glucose is known to be metabolized in the heterolactic pathway, whereas fructose is mainly devoted to NAD(P)H reoxidation in the mannitol pathway (Maicas *et al.*, 1999).

The data presented in Table 5 indicate that the addition of quantities of cysteine, ranging from 0–30 mM, have a beneficial effect on glucose consumption and bacterial growth. The higher the quantity of supplied cysteine, the higher the degradation of glucose and the greater final biomass reached. When 30 mM cysteine was added, biomass production ($X_{max} = 0.28 \text{ g l}^{-1}$) and the consumption of glucose mirrored that in an O_2 -free atmosphere. Lactic acid and ethanol production from cultures grown on MBBG plus cysteine were shown to be equimolar, by analysis of the end-products of metabolism. The other end-product found in the culture broth was acetic acid, the yield of which varied from 0.2–0.6 (where yield is mmol acetic acid produced l^{-1} versus mmol glucose consumed l^{-1}), depending on the levels of cysteine supplied (Table 5). Part of this acetate would come from cysteine that was metabolized via pyruvate to yield extra ATP, as has been described in other LAB (Bruinenberg *et al.*, 1997). Additionally, two extra NADH molecules were reoxidized.

The ethanol:lactic acid ratio was 1.0:1.1 when cysteine was added, showing that the EFP works and leads to the heterolactic catabolism of glucose under aerobic conditions. The combination of the thiol groups of cysteine with O_2 would avoid the direct O_2 inhibition of EFP enzymes. ADH and acetaldehyde dehydrogenase activities were not detected in extracts (Table 2); however, when these extracts were added with 3 mM cysteine some activity was recovered. The effect of cysteine on the enzyme activity was more accurately assayed in anaerobic extracts, as the protein concentration in aerobic extracts was very low. Aliquots of the anaerobic extract, with or without 3 mM cysteine, were air-

Table 4. Quantification of substrates and end-products in the aerobic metabolism of hexoses by *O. oeni* M42

O. oeni M42 was grown in MBBG plus reducing substrates, where indicated. Samples were incubated at 28 °C and an initial pH of 4.8. All determinations were made in triplicate with at least two different extracts. ND, Not detected.

Consumed substrates (mmol l^{-1})			Products (mmol l^{-1})					Carbon recovery (%)*
Glucose	Fructose	Pyruvic acid	Lactic acid	Acetic acid	Glycerol	Ethanol	Mannitol	
4.5	0	0	5.3	3.6	0.3	1.1	ND	97.0
7.1	20.0	0	9.5	9.0	0.7	0.4	16.0	89.4
3.6	0	20.0	19.1	10.4	0.2	1.1	ND	99.1

* The carbon imbalance is attributed to some incorporation of hexoses into biomass and to the production of minor unidentified compounds.

Table 5. Quantification of substrates and end-products in the metabolism of glucose by *O. oeni* M42

O. oeni M42 was cultured aerobically in MBBG plus different quantities of cysteine. Samples were incubated at 28 °C and an initial pH of 4.8. All determinations were made in triplicate with at least two different extracts.

Substrates (mmol l ⁻¹)		Products (mmol l ⁻¹)		
Cysteine added	Glucose consumed	Lactic acid	Acetic acid	Ethanol
0.0	1.0	1.0	0.0	1.0
1.5	21.5	19.8	4.6	20.0
7.5	26.4	23.4	16.7	26.4
15.0	33.7	29.9	18.5	33.7
30.0	52.5	46.5	21.7	52.5

Fig. 4. Activity of ADH. ●, Unflushed anaerobic extract; □, air-flushed extract without cysteine; ■, air-flushed extract with 3 mM cysteine; △, air-flushed extract incubated without cysteine for 6 h, followed by incubation with 3 mM cysteine for 30 min. All determinations were made in triplicate.

flushed. The specific activity of ADH, the most active enzyme in this pathway, before contact with air was 605 mmol (g protein)⁻¹ min⁻¹. After air-flushing (2, 4 and 6 h) the ADH activity decreased progressively (Fig. 4). An aliquot of the 6 h aerated extract was subsequently incubated with 3 mM cysteine for 30 min prior to enzyme determination, and 90 % of the initial activity was recovered. The addition of 3 mM cysteine to the extracts during aeration also permitted a high maintenance of activity, supporting O₂-quenching by cysteine (Fig. 4).

Cysteine is usually included in the composition of media used to cultivate LAB because it is an antioxidant. In this work we have demonstrated that the provision of cysteine to MBBG under aerobic conditions enhances the catabolism of glucose and, as a consequence, the growth of *O. oeni*.

In conclusion, we have proved that *O. oeni* M42 does not benefit from the presence of O₂ in the atmosphere, as has already been observed for other LAB (Lucy & Condon, 1986). The absence of active NAD(P)H oxi-

dases and the inhibition of EFP enzymes by O₂ resulted in a low NAD(P)⁺:NADP(H) ratio and, as a consequence, growth of *O. oeni* M42 on MBBG was negligible (Fig. 3). Growth can be improved by adding other substrates that act as electron acceptors, such as fructose (Nuraida *et al.*, 1992) or cysteine, to glucose cultures. This is of great interest when the objective is to obtain the highest biomass possible, as in the manufacture of commercial starters for malolactic fermentation.

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