

# Effects of Inhibitory Environmental Factors on Growth of *Oenococcus oeni* CCSYU2068 for Malolactic Fermentation of Cider Production

Y. Xu<sup>1,2</sup>, G. Zhao<sup>1</sup>, H. Pan<sup>1</sup> and J. Li<sup>1</sup>

## ABSTRACT

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The effects of several inhibitory factors (sulfur dioxide, pH and ethanol) on the growth of lactic acid bacteria and the subsequent malolactic fermentation (MLF) were studied by inoculation of different culture strains of *Oenococcus oeni*, the major lactic acid bacteria (LAB) in cider production. After comparing their organoleptic properties, three strains of *Oenococcus oeni* were selected from indigenous and commercial sources and their inhibitory effects on cell growth and MLF examined. The malolactic bacteria expressed variations in tolerance to the environmental conditions of pH, sulfur and ethanol concentration. Isolated from an indigenous cider production facility, *O. oeni* L4 had a better capacity with constant growth even when the concentration of SO<sub>2</sub> was 50 ppm, ethanol 10% (v/v) and pH 3.0. *O. oeni* L4 showed better properties for metabolizing the major acids: malic, lactic and acetic acid. The decomposition mean rate of malic acid was as high as 228.52 mg/L per day with a low acetic acid concentration of 101.78 mg/L under the stress conditions of cider production.

**Key words:** Cider production, inhibitory factors, malolactic fermentation, *Oenococcus oeni*.

## INTRODUCTION

Cider production involves two main fermentation processes, alcoholic fermentation and malolactic fermentation. Malolactic fermentation (MLF), the secondary fermentation by lactic acid bacteria (LAB), is considered not only to be a desirable process for deacidification, but also contributes to the complexity of fruit wine flavor and confers a degree of microbiological stability<sup>11,21,24</sup>. *Oenococcus oeni* (formerly known as *Leuconostoc oenos*) is a gram-positive heterofermentative coccus usually present in pairs or chains. This is the major lactic acid bacteria known to carry out MLF after alcoholic fermentation in the modern fruit wine industry<sup>7,9,11,13</sup>. Although there are numerous papers describing MFL in fruit wine making,

most of these deal with winemaking or yeast characteristics and malolactic fermentation technology in cider production<sup>2,5,8,20,23,28</sup>. Few papers have been published describing *O. oeni* growth in cider must after alcohol fermentation or malolactic fermentation in this inhibitory environment. The use of malolactic bacteria (MLB) for cider production is of particular interest in China where there is an increasing market demand for ciders. China has an annual apple production of 21 million tons.

It is well known that the application of a starter culture to induce MLF has not been employed in cider production due to lack of adaptation of cultures and hence MLF failure. The selection of an appropriate LAB and understanding its tolerance to the environment is essential for MLF<sup>9,16,17</sup>. Malolactic fermentation encouraged by an appropriate inoculum of pure culture *O. oeni* has become popular over the last few years in fruit wine production<sup>10,12,25</sup>. The MLF process is unpredictable and can permit growth of undesirable microorganisms that cause spoilage of the product. Cider is not a favorable media for the growth of *O. oeni*. There are a number of environmental factors in cider production, such as low pH and temperature, presence of ethanol and sulfur dioxide, which are inhibitory to the growth of *O. oeni* strains. For example, a temperature ~15°C is not optimal for the growth and fermentation of *O. oeni* bacteria due to changes in the enzymatic functions relevant to growth and related metabolism<sup>9,22</sup>. Sulfur dioxide is added to control oxidation and prevent growth of spoilage microorganisms, resulting in inhibition of oxidizing enzymes<sup>6</sup>. In addition, other inhibitory compounds from yeast metabolism, such as ethanol can affect growth and even survival of *O. oeni*. The growth of a LAB strain in the cider making process is rather complex because of these inhibitory compounds in a hostile environment and the bacterial growth requirements<sup>1,6,15</sup>. These environmental and nutritional complications, as well as the diversity of the *O. oeni* strains, suggests that the fermentative characterization of malolactic bacteria and inhibitory factors are very relevant to the growth of *Oenococcus* strains and the MLF process can affect cider product quality<sup>14</sup>. An understanding of *O. oeni* adaptation and resistance to environmental stress in terms of biodiversity would be valuable to basic research and industrial applications of MLF in cider making.

The objective of this study was to evaluate the effects of major environmental parameters on growth of lactic acid bacteria used in cider production and malolactic fer-

<sup>1</sup>Laboratory of Brewing Microbiology and Applied Enzymology, School of Biotechnology, Key Laboratory of Industrial Biotechnology, Ministry of Education, Southern Yangtze University, 170 Huihe Rd, Wuxi, Jiangsu, P.R. China 214036.

<sup>2</sup>Corresponding author. E-mail: yxu@sytu.edu.cn

**Table I.** Softness index of ciders fermented by different *O. oeni* strains.

Strain	Alcohol % (v/v)	Tannin <sup>a</sup> (g/L)	Total acids <sup>b</sup> (g/L)	Softness index <sup>c</sup>
<i>O. oeni</i> L1	6.3 ± 0.3	0.326 ± 0.009	2.78 ± 0.08	3.19 ± 0.16
<i>O. oeni</i> L2	6.7 ± 0.2	0.317 ± 0.003	2.70 ± 0.12	3.68 ± 0.22
<i>O. oeni</i> L3	6.5 ± 0.4	0.331 ± 0.011	2.28 ± 0.05	3.89 ± 0.21
<i>O. oeni</i> L4	6.8 ± 0.2	0.318 ± 0.007	2.65 ± 0.09	3.83 ± 0.17
<i>O. oeni</i> L5	6.3 ± 0.2	0.339 ± 0.011	2.43 ± 0.11	3.53 ± 0.20
<i>O. oeni</i> L6	6.5 ± 0.3	0.304 ± 0.008	2.60 ± 0.11	3.60 ± 0.11
<i>O. oeni</i> L7	5.6 ± 0.2	0.316 ± 0.004	2.70 ± 0.14	2.58 ± 0.20
Control <sup>d</sup>	6.1 ± 0.3	0.339 ± 0.007	4.56 ± 0.09	1.20 ± 0.07

<sup>a</sup> grams of gallic acid per liter of cider.

<sup>b</sup> grams of malic acid per liter of cider.

<sup>c</sup> Softness index = ethanol concentration (% v/v) minus [total acid (g/L) and tannin concentration (g/L)].

<sup>d</sup> cider fermented without subsequent malolactic fermentation.

mentation. By comparing the differences in fermentation characteristics between *O. oeni* strains from different sources and subjecting three of them to the main inhibitory environmental factors (sulfur dioxide, pH, and ethanol) the effect on the growth of *O. oeni* strains and malolactic fermentation was investigated.

## MATERIALS AND METHODS

### Microorganisms

*O. oeni* strain L4 was isolated and screened from the cellar of a cider winery in Shangdong, China. It was labeled as CCSYU 2068 and taxonomically identified, physiologically characterized and stored in the Culture Collection of Southern Yangtze University (CCSYU). Other strains were *O. oeni* L1 (CECT4029, originally isolated from German wine), L2 (CECT4727, originally isolated from wine must), L3 (CECT4100, originally isolated from wine), L5 (CECT218, originally isolated from red wine of California), L6 (CECT4730, originally isolated from Australian wine) from Coleccion Espanola de Cultivos Tipo (CECT, Valencia Span), *O. oeni* L7 from a commercial source (Lallemand SA, Toulouse, France) and a typical apple wine yeast strain *Saccharomyces cerevisiae* CCTCC M 201022 (China Center Type Culture Collection, Wuhan, China)<sup>26,27</sup>.

### Fermentation conditions

Concentrated apple juice (70.8°Brix, bright and enzymatically treated) was supplied by Yantai North Andrew Juice Co. Ltd (Shangdong, China) and diluted with distilled water (1:6) to 13–14°Brix, and adjusted to a titration acid concentration of 5.0 g/L by addition of malic acid. Fermentations were carried out in pre-sterilized 5-L flasks with diluted concentrated apple juice by inoculating the *Saccharomyces cerevisiae* strain at a final concentration of 10<sup>6</sup> CFU/mL. Alcoholic fermentation was carried out at 15°C for ~15 days until specific gravity reached approximately 1.005 for MLF with a final concentration of sulfite of 20 mg L<sup>-1</sup>. Other fermentation conditions are as reported previously<sup>26,27</sup>.

For the lactic bacterium starter culture, MRS synthetic medium was used as a basal medium for plate culture with the following composition (g L<sup>-1</sup>): glucose, 10; fructose, 5; tryptone (Difco), 10; yeast extract (Oxoid), 5;

MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.05; cysteine/HCl, 0.5; diammonium citrate, 3.5; Tween-80 (Sigma), 0.2 mL per liter; tomato juice 100 mL<sup>28</sup>. The pH of the medium was adjusted to 4.8. The inoculum was transferred into an apple juice broth for the starter culture inducing MLF and supplemented with yeast extract 0.5%<sup>7</sup>. The apple juice broth was sterilized by autoclaving (Tomy SS-325, Tokyo, Japan) under 0.08 Mpa for 25 min. *O. oeni* strains were cultivated statically at 30°C for 6 days until stationary phase was reached and cells collected for sequential malolactic fermentation.

In this sequential MLF inoculation fermentation, a high cell inoculum (10<sup>6</sup> CFU mL<sup>-1</sup>) was used to start the malolactic fermentation at the end of the alcoholic fermentation. The malolactic fermentation in 1-L flasks was conducted at 20°C for 7–14 days. To investigate the influence of sulfite and ethanol, LAB were grown in the same medium but with the addition of sulfite and ethanol.

### Viable counts

The growth of lactic acid bacteria in pure culture was monitored by periodic viable counts and expressed as colony-forming units (CFU) per mL of solution. MSR medium supplemented with 100 ppm cycloheximide to inhibit yeast growth was used<sup>7</sup>.

### Analytical methods

Samples were collected aseptically through the fermentor sampling port and alcohol was determined by densitometry at 20°C after distillation as described by Ough and Amerine<sup>18</sup>. The pH value was measured using a 320 pH meter (Mettler Toledo, Switzerland). Titratable acidity was determined by titration against 0.1 N NaOH to pH 8.2 with malic acid and calculated in grams of malic acid per liter of cider in total acids. Tannin concentration was measured according to Singleton and Rossi (Folin-Ciocalteu method) and measured in grams of gallic acid per liter of cider<sup>4</sup>. The organic acids malic, acetic and lactic acid were analyzed by HPLC (Agilent 1100, Palo Alto, CA, USA). A Zorbox SB-C18 analytical column (250 × 4.6 mm, 5 μm, MAC-MOD Analytical, Inc., Chadds Ford, PA) was used under the following conditions: column temperature, 30°C; mobile phase, 0.1M K<sub>2</sub>HPO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub> pH 2.5; flow rate, 0.5 mL/min, and 5 uL volume injection. Column effluents were monitored at 215 nm.

Unless stated otherwise, the chemicals and solvents used in this study were analytical grade purchased from Sigma, Fluka (Germany). All experiments were conducted in triplicate.

## RESULTS AND DISCUSSION

### Effect of *O. oeni* strains from different sources on sensory quality and chemical composition of cider

To observe the practical implications of the environmental factors effect on MLB and major acid metabolism, seven different sources *O. oeni* strains, most originally isolated from winemaking, were selected to undergo malolactic fermentation for cider production. A sensory evaluation by a professional panel of 12 on the final sensory quality of each cider found that only three strains, *O. oeni* L2, L3 and L4 expressed better organoleptic characteristics and better mouthfeel. This variation in adaptation of strains of *O. oeni* to the fermentative environment suggested that the inhibitory response to environmental changes varied depending on physiological properties such as pH, the concentration of ethanol and total SO<sub>2</sub>.

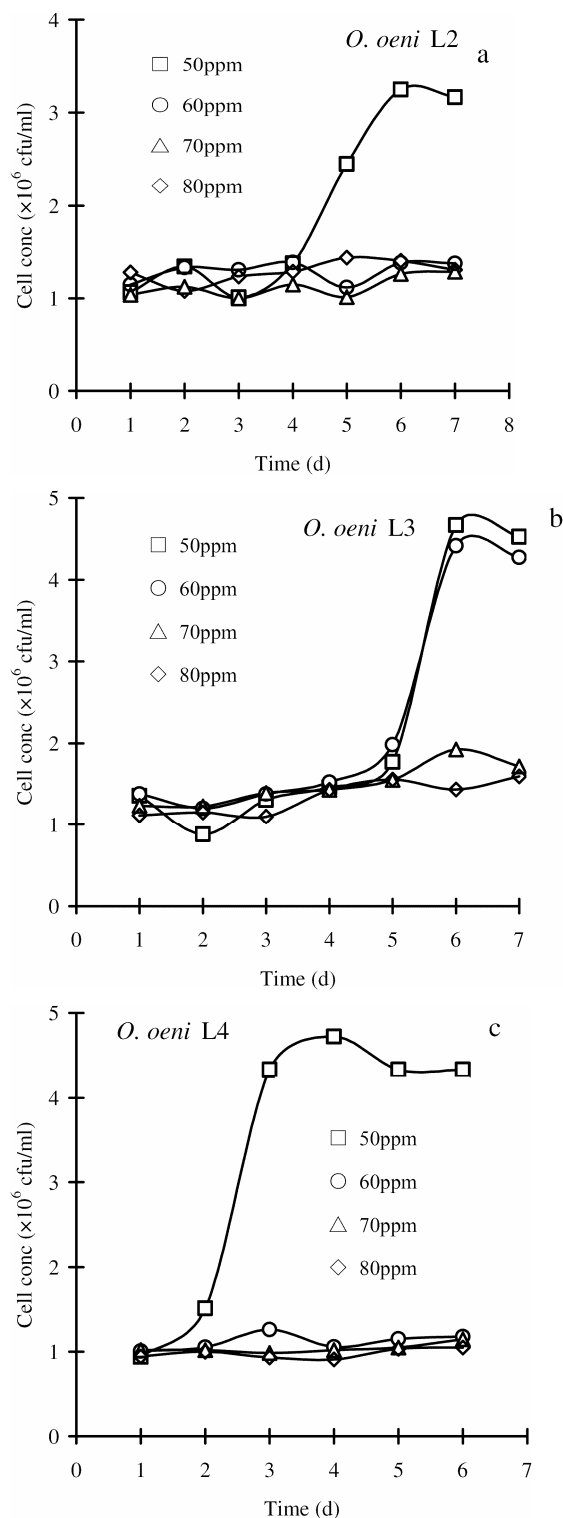
The contribution to cider flavor quality of malolactic fermentation required evaluation including the use of a softness index to judge the flavour harmony and complexity of the cider. Softness index has been used in fruit winemaking<sup>21</sup> and is calculated by the equation: ethanol concentration minus total acids and tannin concentration. Table I shows that cider samples fermented by *O. oeni* L2, L3 and L4 showed a higher softness index of 3.68–3.89. Compared to the cider subjected only to an alcoholic fermentation without MLF (control in Table I), the three cider samples after MLF conducted by L2, L3 and L4 showed more body, mouthfeel and a longer after-taste. This sensory impact of MLF on cider reflected the strains' difference in stress response and environmental adaptation.

Considering LAB diversity in nature<sup>3,19</sup> and that malolactic fermentation must be able to improve the cider flavor quality without adverse conditions, the *O. oeni* strains L2, L3 and L4 were further studied to obtain an understanding of the effects of environmental factors on lactic acid bacterial strains as result of their better enological characteristics.

### Effect of sulfur dioxide in cider must on cell growth of *O. oeni*

Fig. 1 shows that three strains (L2, L3 and L4) exhibited growth inhibition and their tolerance to SO<sub>2</sub> differed depending on the levels of sulfur dioxide (added as potassium metabisulphite). All three strains showed little proliferation when the SO<sub>2</sub> concentration was above 70 ppm in cider must. As an inhibitor of oxidizing enzymes and an antioxidant, sulfur dioxide has been used in cider production for many years and prevents proliferation of desirable and undesirable microorganisms. Although the authorized limit is 200 ppm total SO<sub>2</sub> and International Organizations recommend its total elimination because of health concerns, a higher tolerance to SO<sub>2</sub> of the LAB strain is a practical control to prevent proliferation of undesirable bacteria.

There was a difference in growth between the three strains. *O. oeni* L2 and L3 grew slowly from the second day to sixth day, while *O. oeni* L4 growth entered into stationary phase within four days (Fig. 1a and Fig. 1b). *O. oeni* L3, however, still exhibited growth under a SO<sub>2</sub> con-



**Fig. 1.** Effect of added concentration of sulphur dioxide on cell growth of *O. oeni* L2 (a), *O. oeni* L3 (b), and *O. oeni* L4 (c) under condition of pH 3.5 and 6% (v/v) ethanol. Sulphur dioxide (□) 50 ppm, (○) 60 ppm, (△) 70 ppm, (◇) 80 ppm.

centration of 60 ppm. The amount of SO<sub>2</sub> required also depends on the pH value present. The SO<sub>2</sub> concentration was 50 ppm with an initial pH of 3.5 for this MLF, whereas the usual concentration in cider making is 50 ppm–150 ppm SO<sub>2</sub> at pH 3.0–3.8.

### Effect of initial pH on cell growth of *O. oeni*

As indicated in Fig. 2 when the concentration was 50 ppm SO<sub>2</sub> and ethanol 6% (v/v) growth was very slow at pH 3.0–3.5. The pH value had a greater impact on the growth of all three strains of *O. oeni* in cider than the SO<sub>2</sub> concentration. For lactic acid bacteria growth the optimal pH is 5.5.

It was observed that *O. oeni* L2 growth occurred when cider must pH was at 3.2 and 3.5. No growth was observed at pH 3.0 (Fig. 2a). *O. oeni* L3 grew quickly at pH 3.0 and 3.2 whereas no growth was observed at pH 3.5 (Fig. 2b). Only strain *O. oeni* L4 exhibited rapid growth at a pH ranging from 3.0 to 3.5 (Fig. 2c). All however, required a time of more than 10 days at the lower pH value. In general, a lower pH presents more resistance to the lactic acid bacteria growth to some extent and malolactic bacteria vary in tolerance to a lower pH environment.

### Effect of concentration of ethanol on cell growth of *O. oeni*

Three chosen strains *O. oeni* L2, L3 and L4 were tested for ethanol tolerance (Fig. 3). When ethanol concentrations ranged from 6% (v/v) to 12% (v/v) in a cider matrix at pH 3.5 and sulfur dioxide of 50 ppm, the three strains showed growth delay with increasing ethanol percentage and the strains exhibited obvious inhibition beyond 8% (v/v) with the exception of *O. oeni* L4.

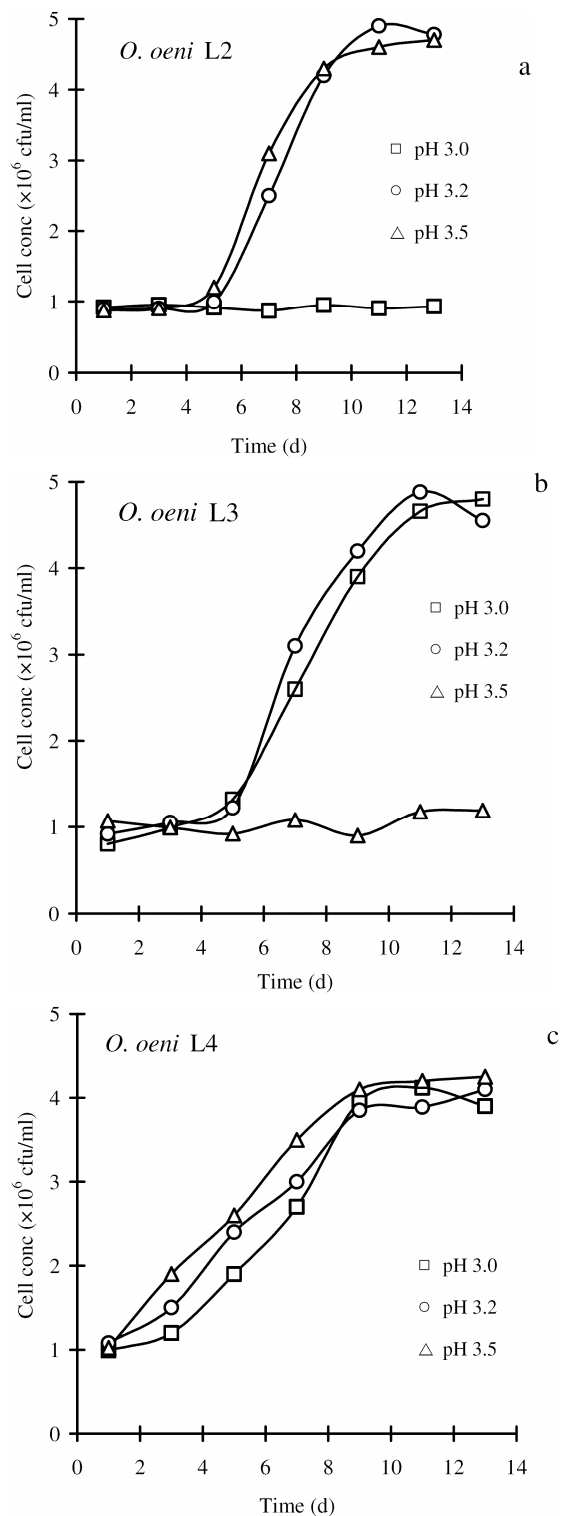
Strain *O. oeni* L4 grew into stationary phase in four days, two days earlier compared to *O. oeni* L2 and L3 with a high cell density of  $4.8 \times 10^6$  CFU/ml (Fig. 3c), and grew when ethanol concentration exceeded 10% (v/v). However, with a four to five day delay of growth, *O. oeni* L3 and *O. oeni* L2 grew to a cell density of about  $4.5 \times 10^6$  CFU/ml even in an ethanol concentration of 8% (v/v) (Fig. 3a and 3b). More than 10% (v/v) ethanol concentration could give great resistance to lactic acid bacteria and inhibit their growth. Although ethanol is the main metabolic product from ethanol fermentation by *Saccharomyces cerevisiae*, the malolactic fermentation strains' tolerance to ethanol responded differently to cell growth, and only the ethanol resistant strain could conduct a successful malolactic fermentation in sequential fermentations.

### Effect on malolactic fermentation

Fig. 4 shows the time course of malic acid degradation and lactic acid formation by *O. oeni* strains at initial pH 3.5, ethanol 6% (v/v) and free SO<sub>2</sub> 12.4 mg/L for 15 days. Among the three LAB stains, *O. oeni* L4 exhibited the highest decomposition mean rate of 228.52 mg/L per day for the conversion of malic acid to lactic acid (Table II), indicating its higher tolerance to environmental stress. In contrast, *O. oeni* L2 depleted malic acid at a rate of 29.75 mg/L per day. Under the same environmental conditions, the effect of environmental stress on lactic acid bacteria growth caused an impact on the malolactic fermentation

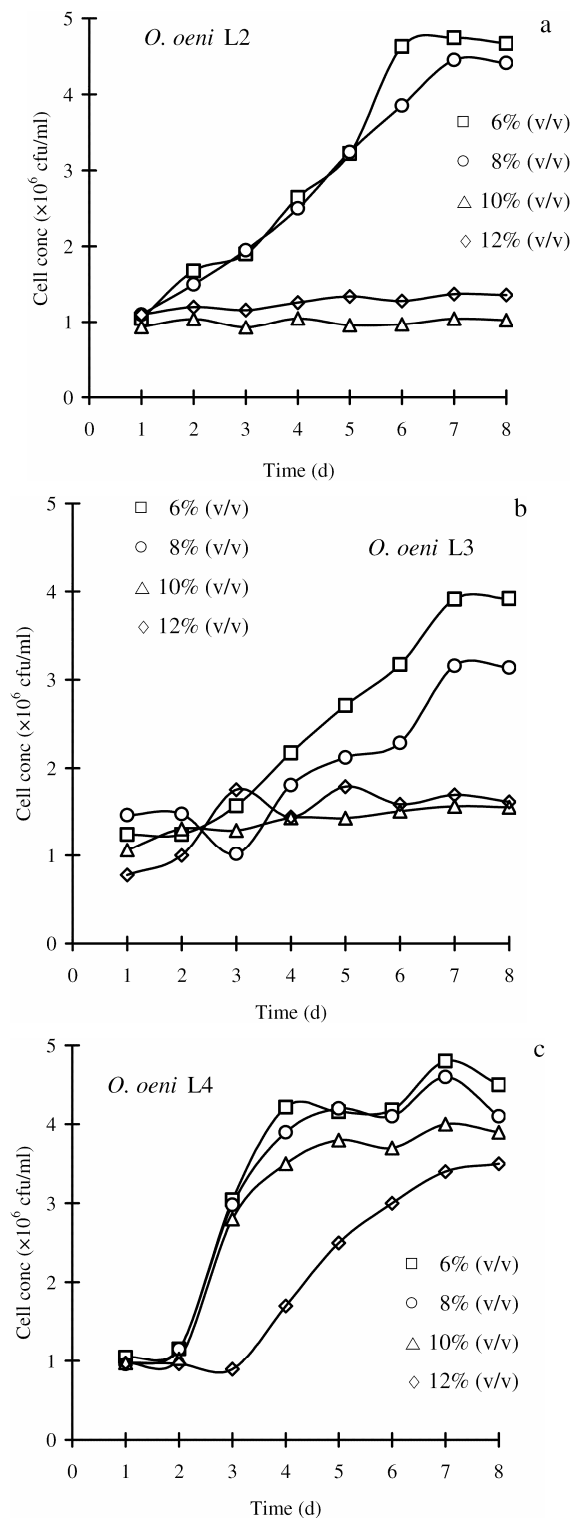
by changing the major acid composition and concentration (Table II).

As shown in Table II, there are differences between the three strains' ability to produce acetic acid. In regard to their ability to decompose malic acid, *O. oeni* L4 and L3



**Fig. 2.** Effect of initial pH on the cell growth of *O. oeni* L2 (a), *O. oeni* L3 (b), and *O. oeni* L4 (c) under condition of 50 ppm sulphur dioxide and 6% (v/v) ethanol. (□) pH 3.0, (○) pH 3.2, (△) pH 3.5.

decreased almost the same degree of total acid (~50% with the reduction of 2.44 g and 2.66 g of acid respectively), but there was an increase of 105.06 mg of acetic acid per liter for *O. oeni* L3 and 20.78 mg of acetic acid



**Fig. 3.** Effect of the concentration of ethanol on the cell growth of *O. oeni* L2 (a), *O. oeni* L3 (b), and *O. oeni* L4 (c) under condition of 50 ppm sulphur dioxide and pH 3.5. Ethanol (□) 6% v/v, (○) 8% v/v, (△) 10% v/v, (◇) 12% v/v.

per liter for *O. oeni* L4. When observing the inhibitory effect on MLB and selecting a good malolactic strain, it should be noted that the acetic acid concentration increase during malolactic fermentation can bring unpleasant tastes to cider. Figure 4 shows that environmental factors affect the major acids metabolic flux and impact flavor compounds. The MLB of *O. oeni* L4 is the most suitable strain for MLF, with a high tolerance and ability to adapt to MLF stress conditions for cider production.

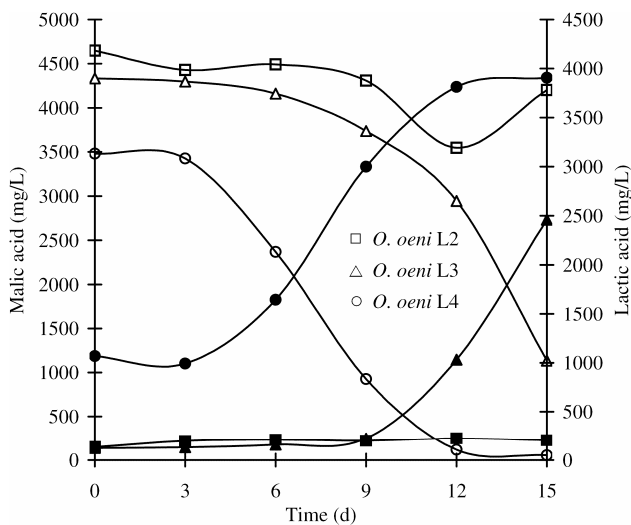
## CONCLUSIONS

The results presented in this work describe the environmental inhibitory effects and factors in cider production on growth and malolactic fermentation (MLF) for three selected *O. oeni* lactic acid bacteria. By selecting appropriate LAB to drive malolactic fermentation on the basis of their organoleptic characteristics, the three strains of *O. oeni* were used to observe the different influences of three major variable parameters (sulfur dioxide, pH, and ethanol) in cider production on LAB growth in subsequent malolactic fermentation. It was observed that malolactic bacterial expression varied in tolerance to environmental conditions such as pH, sulfur and ethanol at different levels. The local strain LAB *O. oeni* L4 showed a better adaptation to environmental parameters with constant growth,

**Table II.** The effect of different *O. oeni* strains on the major acids during malolactic cider fermentation.

After MLF	<i>O. oeni</i> L2	<i>O. oeni</i> L3	<i>O. oeni</i> L4
Decomposition mean rate of malic acid (mg/L-d)	29.75	213.21	228.52
Increment value of acetic acid (mg/L)	25.92	105.06	20.78
Acetic acid (mg/L)	106.92	186.06	101.78
Total acid <sup>a</sup> (g/L)	3.48	2.57	2.35
Reduction of total acid (g/L)	1.57	2.44	2.66
Degree of reduced acids (%)	31.33	48.70	53.09
Increment value of pH	0.07	0.26	0.26

<sup>a</sup>grams of malic acid/L of cider.



**Fig. 4.** Malic acid (open symbol) and lactic acid (solid symbol) evolution during malolactic fermentation. (□) *O. oeni* L2, (△) *O. oeni* L3, (○) *O. oeni* L4.

even when the concentration of SO<sub>2</sub> and ethanol were 50 ppm and 10% (v/v) respectively at pH 3.0. The decomposition mean rate of malic acid by *O. oeni* L4 was as high as 228.52 mg/L per day with the least acetic acid formed during malolactic fermentation. An understanding of the starter culture features adapted to the environment would allow better performance of malolactic fermentations. *O. oeni* L4 had better metabolic properties for major acids in that its decomposition mean rate of malic acid was as high as 228.52 mg/L per day, with a low acetic acid concentration of 101.78 mg/L under the stress conditions encountered in cider production.

## ABBREVIATIONS

MLF malolactic fermentation  
LAB lactic acid bacteria  
MLB malolactic bacteria

## ACKNOWLEDGEMENTS

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