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The essential amino acids requirements for *Oenococcus Oeni* growth and organic acids metabolism

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Lactic acid bacteria are characterized by numerous nutritional requirements. The influence of amino acids on the growth of *Oenococcus oeni* SD-2a and metabolism of organic acids were determined. The microorganism in basal medium grew to 2.47×10^8 CFU·ml^{-1} biomass with maximum growth rate of 0.0072 h^{-1}. When glutamic acid and cysteine were independently omitted from basal medium, no growth was observed, indicating that these amino acids are essentials for bacterial growth. When L-malate and citric acids were added individually or together to basal media deficient in glutamic acid or cysteine, they played a beneficial role in bacterial growth and L-malic acid utilization; and both acid combined had a stimulatory effect on the rate of growth and L-malic acid utilization. When glutamic acid or cysteine concentration increased in the same media, bacterial maximum growth rate (μmax) and final biomass production increased; the rate of L-malate acid utilization, L-lactic acid production or citric acid consumption also increased.

Key words: *Oenococcus oeni*, amino acids, organic acids.

INTRODUCTION

Oenococcus oeni is the main lactic acid bacteria (LAB) species involved in malolactic fermentation (MLF) in wine, which is a very important stage of sparkling in wine production (Lonvaud-Funel, 1999; Versari et al., 1999). Fermentation of L-malate by *O. oeni* is of physiological significance in wine, which contains high amounts of C4-dicarboxylic acid. Malate is metabolized to L-lactic acid and CO_2 (L-malate→L-lactate+CO_2), and it also makes wines microbiologically more stable (Versari et al., 1999; Maicas, 2001). LAB has been characterized by their numerous nutritional requirements, especially of nitrogen sources (Fourcassie et al., 1992; Saguir and Manca de Nadra, 2002; Terrade and Orduňa, 2009). Fourcassie et al. (1992), studying the effect of 18 amino acids on growth, D-glucose fermentation and malolactic activity of six strains of *O. oeni*, found that all strains had an absolute requirement for four amino acids and needed six others for optimum growth. Vasserot et al. (2001) have reported for *O. oeni* grown in synthetic medium with low L-aspartic acid concentration, that with increasing L-aspartic acid concentration, maximum bacterial growth rate and maximum bacterial biomass production also increased. Saguir and Manca de Nadra (2002) reported that for *O. oeni* growing in the absence of amino acids of the aspartate family, no growth was observed when the essential amino acid, L-asparagine or L- isoleucine was remo-

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Abbreviations: **LAB**, Lactic acid bacteria; **MLF**, malolactic fermentation; **CFU**, colony-forming units; **HPLC**, high performance liquid chromatography; **BM**, basal medium.

ved from the basal medium. Terrade and Orduňa (2006, 2009) reported that arginine was essential for the growth of *O. oeni* EQ54 and *O. oeni* VFO, but did not stimulate growth of two *O. oeni* strains. L-malic and citric acids are certainly the substrates that wine lactic acid bacteria degrade most frequently in their natural environment. On the other hand, the free amino acid concentration in wine is limited. Amino acid catabolism could play an important role in the ability to obtain energy in nutrient-limited environments. However, the catabolic pathway of many amino acids remains partially undefined in LAB.

Oenococcus oeni SD-2a is an indigenous LAB strain isolated from indigenous wine of China. It is proven that *O. oeni* SD-2a has a better adaptability to stress environment in wine, such as pH, ethanol, cold and sulfur dioxide (Li et al., 2006). With the excellent characters, great deals of concerns have given to *O.oeni* SD-2a recently (Li et al., 2009; Zhang et al., 2012). However, there are few reports on the nutrition demand of *O.oeni* SD-2a, especially on nitrogen source.

This paper described the amino acid requirements by *Oenococcus oeni* SD-2a and the effect of L-malic and/or citric acids on the growth of *Oenococcus oeni* SD-2a, determined during the absence of each essential amino acid from culture medium. The possible roles of L-malic and citric acids as precursors of anabolic compounds for the synthesis of essential amino acids were also reported.

MATERIALS AND METHODS

Bacterial strain, media and culture conditions

O. oeni SD-2a isolated from Chinese wines was used (Li et al., 2006). The culture media ATB contained ($g \cdot l^{-1}$): glucose, 10; yeast extract, 5; peptone, 10; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$ 0.05; cysteine/HCl 0.5; and tomato juice 250 ml. The medium pH was adjusted to 4.8 with HCl/KOH, sterilized in an autoclave heating for 20 min on reaching 121°C. The basal medium (BM) had the following composition in deionized water ($g \cdot l^{-1}$): glucose 10; potassium acetate, 10; KH_2PO_4, 2; sodium thioglycollate, 0.5; $MgSO_4 \cdot 7H_2O$, 0.15; $MnSO_4 \cdot 4H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; Tween 80, 1 mg; and yeast nitrogen base without amino acids (YNB), 0.67. Amino acid concentrations are given in Table 1. The semi-BM medium, where the amino acids source, except cysteine-HCl, was substituted by tryptone (4 $g \cdot l^{-1}$) was used for the cells adaptation before inoculating into the synthetic media. L-malate and citric acids were incorporated individually or together to the semi-BM and BM at 18.25 and 3.7 $mmol \cdot l^{-1}$, respectively. All media except ATB were adjusted to pH 4.8 with HCl/KOH, and then sterilized by 0.22 µm filtration membrane.

The stock strain was reactivated by two successive transfers in ATB medium, then inoculated, without agitation; at 25°C was harvested at the end of exponential growth phase (84 h) and precultured under the same conditions in the semi-BM medium. After 84 h of incubation, the cells from the last transfer were harvested by centrifugation, washed twice with sterile 0.9% NaCl solution to avoid the carry-over of essential nutrients and re-suspended in sterile NaCl. BM media were inoculated at a concentration of 5×10^6 $CFU \cdot ml^{-1}$. All cultures were incubated statically at 25°C for about four days.

Bacterial growth was monitored by periodic spectrophotometric measurements at 600 nm using UNICO UV-3802 spectrophotometer and determined as colony forming units per milliliter ($CFU \cdot ml^{-1}$). Decimal dilutions were prepared from the suspension before amino acid was omitted and plated on ATB agar. From these data it was possible to calculate the average of growth rates.

Analytical methods

The concentrations of L-malate and citric acids were analyzed by high performance liquid chromatography (HPLC). The concentrations of organic acid standard varied from 0.5 to 3.0 $g \cdot l^{-1}$ for L-malate acid; 0.2 to 1.0 $g \cdot l^{-1}$ for citric acid. The prepared standard solutions of organic acids were stored at 4°C. A HPLC system (Waters 600E Series, American) equipped with a pump system, a UV/V detector (Waters-2487) is monitored at 210 nm. A symmetry ShieldTM RP18 column (5 µm, 25 cm × 4.6 mm) was kept at 35°C, with an injection volume of 20 µl. The mobile phase consisted of two solvents: 97% solvent A, pure methanol (chromatogram class) and 3% solvent B, aqueous solutions of KH_2PO_4 in 0.01 $mol \cdot l^{-1}$, adjusted to pH 2.8 with H_3PO_4. Flow rate of 0.6 $ml \cdot min^{-1}$ was employed. L-lactic acid was measured by enzymatic methods (Boheringer Kits, Mannheim, Germany).

Statistical analysis

All results presented in this paper are the average of three independent replicate assays. The variations were <5%.

RESULTS

Growth of *Oenococcus oeni* SD-2a in 19 media deficient in one amino acid

Strains were cultivated in 19 different synthetic media lacking one amino acid, prepared by modifying the composition of amino acids mixture of the basal media (Table 1), and the BM is used as reference (Saguir et al., 2002). Table 2 shows the bacterial maximum growth rate and cell density of *O.oeni* SD-2a in media without one amino acid. The microorganism in BM grew to 2.47×10^8 $CFU \cdot ml^{-1}$ biomass with maximum growth rate 0.0072 h^{-1}.

The individual lack of the amino acids of the glutamate family, glutamic acid, proline and arginine from BM shows that no growth was observed in the medium without glutamate, and a diminution of 29.2 and 22.2% was observed on the growth rate due to lack of proline and argnine, respectively.

The results obtained in the absence of each member of the aspartate family, aspartate, asparingine, lysine, methionine, threonine and isoleucine shows that all these amino acids except isoleucine had a stimulatory effect on growth with the following growth rate diminution: aspartate, 27.8%; threonine, 38.9%. However, the absence of isoleucine is not the essential amino acid for *O.oeni* SD-2a growth in BM as previously reported (Saguir and Manca de Nadra, 2002).

The absence of each member of pyruvate family, alanine, valine or leucine shows that there is no apparently decrease on growth rate, but the cell density is reduced by 21.5, 42.5 and 34.4%, respectively.

Table 1. Amino acid in basal medium.

Compound	Concentration ($g \cdot l^{-1}$)
L-Glutamic acid	0.15
L-Proline	0.04
L-Arginine	0.005
L-Aspartic acid	0.2
L-Asparagine	0.2
L-Lysine	0.05
L-Methionine	0.05
L-Threonine	0.05
L-Isoleucine	0.05
L-Alanine	0.2
L-Leucine	0.06
L-Valine	0.03
L-Serine	0.1
L-Cysteine-HCl	0.2
L-Glycine	0.3
L-Phenylalanine	0.04
L-Tyrosine	0.004
L-Tryptophan	0.05
L-Histidine	0.05

The individual removal of members of the serine family, serine, cysteine or glycine, from BM shows that no growth was observed in the medium without cysteine and a diminution of 15.3% was observed in the growth rate due to omission of glycine.

The results obtained in the absence of members of the aromatic amino acids family, phenylalanine, tyrosine or tryptophane confirmed that there is no apparently decrease on growth. While histidine omitted in BM showed that the cell density is decreased by 44.1%. In all, the essential amino acids of *O. oeni* SD-2a are glutamic acid and cysteine.

Effect of organic acids on *Oenococcus oeni* SD-2a growth and metabolism of L-malate, L-lactic and citric acids

L-malate and citric acids were added individually or together to BM deficient in glutamic acid or cysteine (Table 3). Results indicate that the addition of organic acids led to the omission of gutamic acid and cysteine exigencies, suggesting that malate and citric acids played a beneficial role in bacterial growth; and both organic acids combined had a stimulatory effect on the rate of growth. The addition of L-malate acid almost restored the bacterial growth to the same cell density as that obtained in reference media (Table 2). In the absence of glutamic acid and presence of both malate and citric acids, the cell density increased almost threefold compared with that in malate acid supplement. The result is similar to media deficient of cysteine due to the presence of both organic acids.

The effect of organic acid on L-malic acid consumption and L-lactic acid production are illustrated in Figure 1. There was no possible growth when glutamic acid or cysteine was omitted from BM (Table 3). When citric acid was added to the BM deficient in glutamic acid and cysteine, *O.oeni* SD-2a could not utilize it to grow, but with the supplement of L-malic acid, *O.oeni* SD-2a could utilize it to synthesis substance which is necessary for bacterial development. 9.25 and 7.15 $mmol \cdot l^{-1}$ L-malic acid was consumed and almost transferred into L-lactic acid when L-malic acid was added to BM deficient in glutamic acid and cysteine, respectively. With addition of both organic acids, the amounts of L-malic acid consumed are more than that observed in one organic acid supplement: 10.25 and 10.05 $mmol \cdot l^{-1}$ for deficiency of glutamic acid and cysteine, respectively. The results suggest that *O. oeni* SD-2a is able to use L-malic acid as biosynthetic precursor for synthesis of the essential amino acids and the citric acid played a positive role in L-malic acid utilization, especially in the absence of cysteine (Figure 1). The utilization of citric acid by bacteria is shown in Figure 2. *O. oeni* SD-2a began to metabolize this organic acid at 24 h cultivation and finished as the bacterial growth period end; but the metabolism is not complete. When glutamic acid is absent in media, 25 and 29.6% citric acid was utilized for the presence of citric acid and both organic acids, respectively. When cysteine was omitted in media, 30.9% and 33.4% citric acid was consumed for supplement of citric acid and both organic acids. The final consumption amounts in BM added with organic acid in the absence of cysteine are more than that observed in the same media in the absence of glutamic acid, suggesting that citric acid may remedy the deficiency of cysteine (Figures 1 and 2).

Effect of glutamic acid or cysteine concentration on *Oenococcus oeni* SD-2a growth and matebolism of L-malate, L-lactic and citric acids

The effects of glutamic acid and cysteine concentration on the growth of *O.oeni* SD-2a in basal media together with L-malic and citric acids are shown in Table 4. When glutamic acid concentration increased from 0 to 1 $mmol \cdot l^{-1}$, bacterial maximum growth rate (μmax) increased from 0.0093 to 0.0267 h^{-1}, and final biomass production increased almost twice to 1.35×10^9 $CFU \cdot ml^{-1}$; but when the concentration of glutamic acid increased over 1 $mmol \cdot l^{-1}$, there was inhibitory effect on bacterial growth, the cell density reduced to 7.27×10^8 and 1.96×10^8 $CFU \cdot ml^{-1}$, for 6 and 10 $mmol \cdot l^{-1}$, respectively. However, the highest growth rate is 0.0389 in 6 $mmol \cdot l^{-1}$ cysteine concentration, and cell density is increased to 1.49×10^9 $CFU \cdot ml^{-1}$. Bacteria were inhabited when cysteine concentration increased over 6 $mmol \cdot l^{-1}$.

The effects of glutamic acid and cysteine concentration on L-malate acid consumption and L-lactic acid production are illustrated in Figure 3. Like the trends in bacterial growth, most L-malate acid was consumed and it was mostly metabolized to L-lactic acid and CO_2 in glutamic

Table 2. Growth of *O.oeni* SD-2a in basal media deficient in one amino acid.

Omitted amino acid	μmax	Final biomass (CFU·ml^{-1})
None	0.0072	2.47×10^8
Glu	0.0000	NG
Pro	0.0051	1.44×10^8
Arg	0.0056	1.63×10^8
Asp	0.0052	2.10×10^8
Asn	0.0058	1.45×10^8
Lys	0.0068	2.23×10^8
Met	0.0062	1.30×10^8
Thr	0.0044	1.21×10^8
Ile	0.0071	1.98×10^8
Ala	0.0069	1.94×10^8
Leu	0.0068	1.42×10^8
Val	0.0070	1.62×10^8
Ser	0.0069	1.85×10^8
Cys	0.0000	NG
Gly	0.0061	2.17×10^8
Phe	0.0066	1.83×10^8
Tyr	0.0070	2.24×10^8
Trp	0.0068	1.88×10^8
His	0.0069	1.38×10^8

NG, No growth.

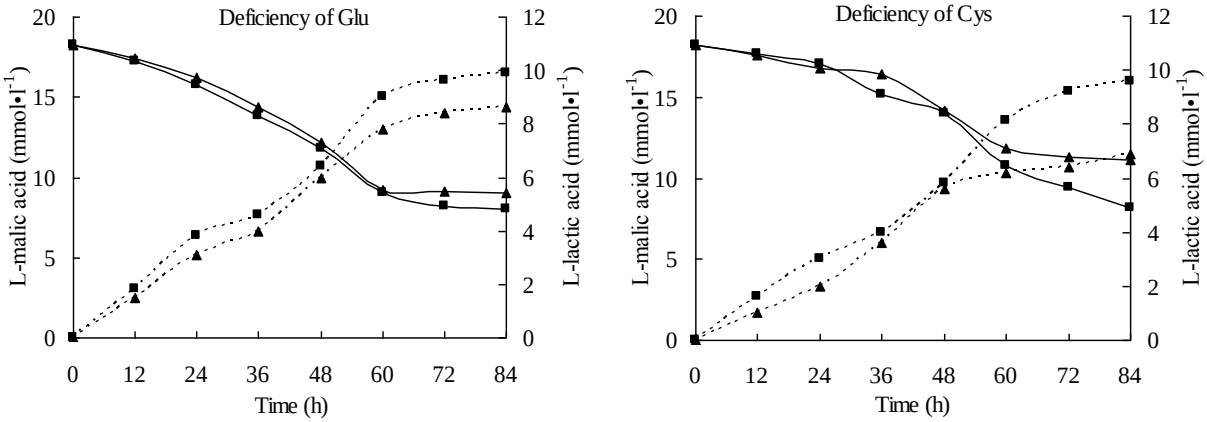

Figure 1. L-malic acid consumption (solid line) and L-lactic acid production (dashed line) by *O.oeni* SD-2a cultivate in BM added with organic acid and deficient in glutamic acid or cysteine. ▲, Addition of L-malic acid; ■, addition of both acid.

acid concentration of 1 mmol·l^{-1} and cysteine concentration of 6 mmol·l^{-1}. The rate of L-malate acid utilization increased when the concentration of glutamic acid increased from 0 to 1 mmol·l^{-1}, and it was reduced when the concentration increased over 1 mmol·l^{-1}. Similarly, the rate of L-lactic acid production was stimulated when cysteine concentration increased from 0 to 6 mmol·l^{-1}, but was inhibited when it increased over 6 mmol·l^{-1}.

The metabolism on citric acid of *O.oeni* SD-2a is not completed in various essential amino acid concentrations (Figure 4). However, the result of citric acid consumption is similar to the result in malate acid. The rate of citric acid utilization climbed to 38 and 42% in glutamic acid concentration of 1 mmol·l^{-1} and cysteine, 6 mmol·l^{-1}, respectively.

DISCUSSION

In a previous investigation, Amoroso et al. (1993) suggested

Table 3. Effect of organic acids on the growth of *O.oeni* SD-2a growing in different media deficient in essential amino acid.

Omited amino acid	Media type	μmax	Final biomass (CFU·ml^{-1})
Glu	BM	0.0000	NG
	BM+L-malic acid	0.0077	2.51×10^{8}
	BM+citric acid	0.0000	NG
	BM+both	0.0116	7.05×10^{8}
Cys	BM	0.0000	NG
	BM+L-malic acid	0.0061	2.17×10^{8}
	BM+citric acid	0.0000	NG
	BM+both	0.0135	7.26×10^{8}

NG, No growth.

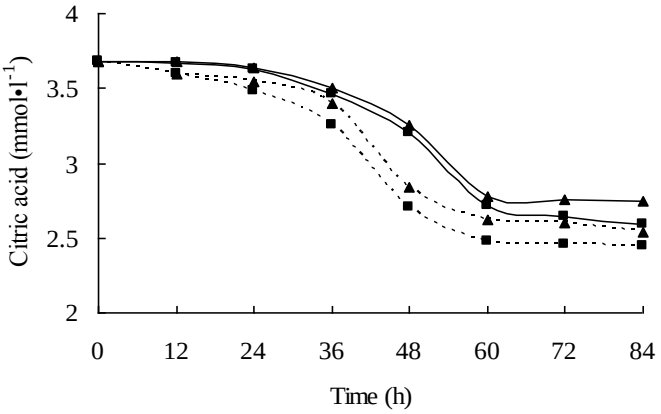

Figure 2. Citric acid consumption by *O.oeni* SD-2a cultivate in BM added with organic acid and deficient in glutamic acid (solid line) or cysteine (dashed line). ▲, Addition of L-malic acid; ■, addition of both acid.

that four amino acids are the essential amino acids for four strains of *Oenococcus oeni*. The amino acid requirements of *O.oeni* SD-2a were examined in this study, and it was found that glutamic acid and cysteine are the essential amino acids of *O.oeni* SD-2a. Meanwhile, both acids are also identified as the crucial amino acids in many other stains of *O. oeni* (Fourcassie et al., 1992; Pedro et al., 2004). There is a hypothesis that *O. oeni* cannot synthesize cysteine and glutamic acids put forward by Vasserot et al. (2001), and linked to a fully inoperative citric acid cycle.

Besides, some other amino acids were proved to be essential acids for *O. oeni* when omitted from media, such as asparagine and isoleucine (Saguir and Manca de Nadra 1997; Terrade and Orduña, 2006). However, in our study, *O. oeni* SD-2a is not sensitive to isoleucine but indeed has 28% growth rate reduction in media without asparagines (Table 2). Also in previous researches, it is reported that many amino acids have stimulating effects on growth of *O. oeni* (Hughes et al. 1997; Saguir and Manca de Nadra, 2002). Tonon and Lonvaud-Funel (2000) showed that arginine produced more maintenance energy, so that *O. oeni* strains are able to metabolize arginine to take advantage of the additional energy source for growth. But this argument was disproved few years later; Terrade and Mira de Orduña (2009) indicated that arginine did not stimulate growth of the two *O. oeni* strains studied, and arginine may play a role in wine microbiological stability. It is demonstrated that in a medium absent of L-aspartic acid, bacterial growth was reduced from 33 to 48%, depending on the strain used (Fourcassie et al. 1992), and the present results obtained are similar to it. Aredes Fernandez et al. (2003) reported that glycine efflux could be used by *O. oeni* X2L to exchange for other essential amino acids outside the cell, favoring its growth rate under poor nutritional conditions.

Vasserot et al (2001) studied the effect of different L-aspartic acid concentration on the metabolism of *O.oeni*, and found out that the applicable concentration for strains growth is 0.3 mmol·l^{-1}. While we examined the influence of concentration of glutamic acid and cysteine on bacterial growth, metabolism of L-malic acid and citric acid, result obtained is similar with that in the study of Vasserot (2001): low concentration could not satisfy the bacterial nutrition demands but high concentration brought inhibitory effect on strains.

In lactic acid bacteria, the physiological function of the malolactic and citrate fermentation pathways is the generation of proton motive force that was used as energy for cellular processes. The results of organic acids addition individually or together to the media deficient in one amino acid suggest that essential amino acids can be synthesized from intermediaries metabolically derived from components of the medium such as L-malic and citric acids. As L-malic acid was mostly recovered as L-lactic acid in all media, it is possible to conclude that *O. oeni* SD-2a is unable to use L-malic acid as biosynthetic precursor for essential amino acids synthesis. But some reports hypothesize that part of citric acid play an important role in synthesis of some amino acids (Saguir and

Table 4. Effects of the essential amino acid concentration on the growth of *Oenococcus oeni* SD-2a growing in basal media in the presence of both L-malic and citric acids.

Essential amino acid concentration (mmol·l^{-1})		μmax	Final biomass(CFU·ml^{-1})
Glu	0	0.0093	6.94×10^{8}
	0.15	0.0104	7.01×10^{8}
	0.3	0.0138	8.16×10^{8}
	1	0.0267	1.35×10^{9}
	6	0.0133	7.27×10^{8}
	10	0.0026	1.96×10^{8}
Cys	0	0.0141	7.31×10^{8}
	0.15	0.0145	7.73×10^{8}
	0.3	0.0188	8.82×10^{8}
	1	0.0232	9.77×10^{8}
	6	0.0389	1.49×10^{9}
	10	0.0203	8.68×10^{8}

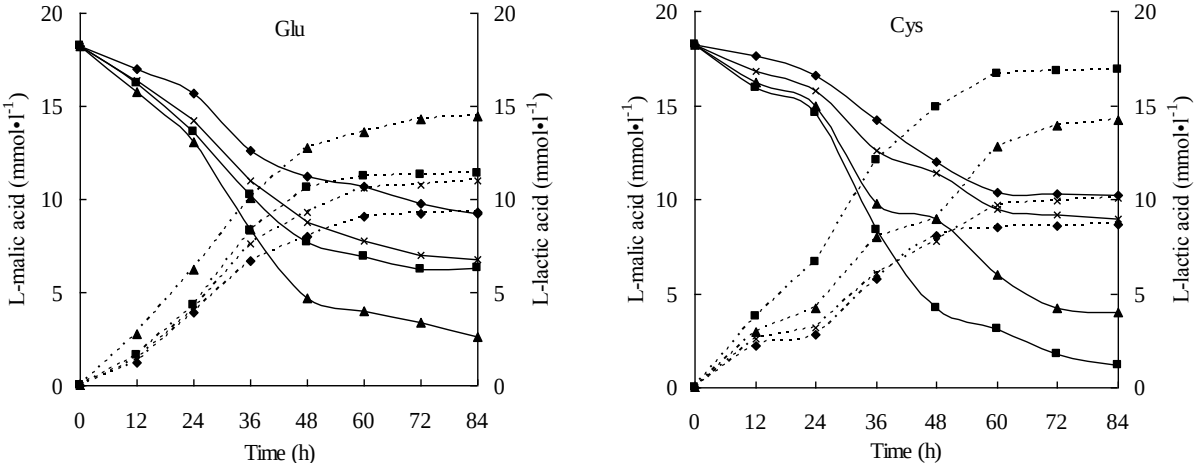

Figure 3. L-malic acid consupmtion (solid line) and L-lactic acid production (dashed line) by *O.oeni* SD-2a metabolism in each BM added with both L-malic and citric acids varing glutamic acid and cysteine concentration. ◆, 0 mmol·l^{-1}; ▲, 1 mmol·l^{-1}; ■, 6 mmol·l^{-1}; ×, 10 mmol·l^{-1}.

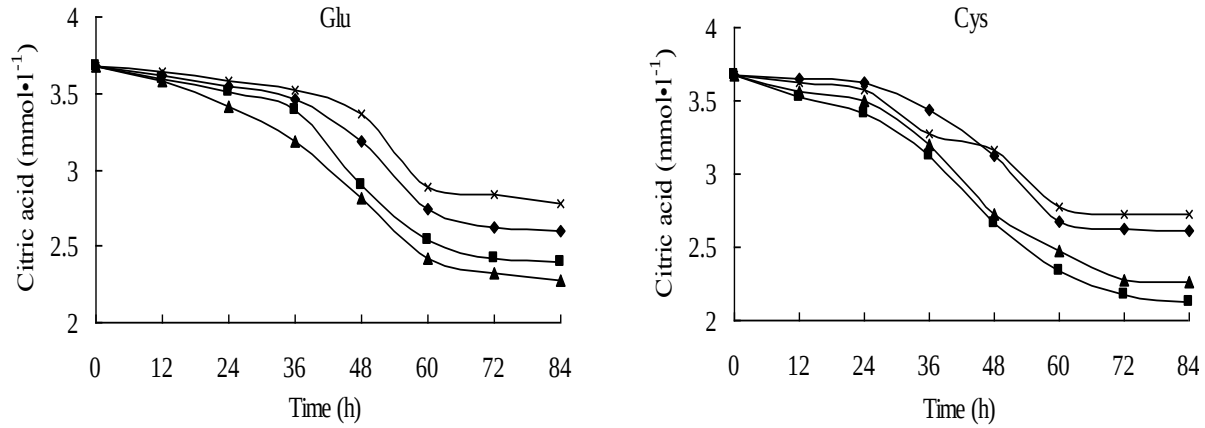

Figure 4. Citric acid consumption by *O.oeni* SD-2a metabolism in each BM medium added with both L-malic and citric acids varing glutamic acid and cysteine concerntration. ◆, 0 mmol·l^{-1}; ▲, 1 mmol·l^{-1}; ■, 6 mmol·l^{-1}; ×, 10 mmol·l^{-1}.

Manca de Nadra, 2002); also, citric acid is used as an electron acceptor, allowing acetate formation and ATP production (Cook and Russell, 1994).

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