Journal of Applied Microbiology 2002, **93**, 295–301

Effect of L-malic and citric acids metabolism on the essential amino acid requirements for *Oenococcus oeni* growth

F.M. Saguir[1] and M.C. Manca de Nadra[1,2]

[1]Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán and [2]Centro de Referencia para Lactobacillus (CERELA), Tucumán, Argentina

388/12/01: received 14 December 2001, revised 15 April 2002 and accepted 24 April 2002

F.M. SAGUIR AND M.C. MANCA DE NADRA. 2002.

Aims: The purpose of this work was to study the effect of L-malic and/or citric acids on *Oenococcus oeni* m growth in deficient nutritional conditions, and their roles as possible biosynthetic precursors of the essential amino acids.

Methods and Results: Bacterial cultures were performed in synthetic media. Bacterial growth rate was reduced or annulled when one amino acid was omitted from basal medium, especially for members of aspartate family, except lysine. The organic acids increased or restored the growth rates to the respective reference values. In each medium deficient in one essential amino acid, the L-malic acid utilization was accompanied by an increase of L-lactic acid concentration and accounted for approximately 100% L-malic acid consumed. D-Lactic acid formation from glucose decreased in the medium without cysteine. Except for tyrosine, the recovery of glucose–citrate as D-lactic acid was lower than in the complete medium when asparagine, isoleucine or cysteine were excluded. The ethanol and acetate production was not modified.

Conclusions: L-Malic and citric acids favoured *Oenococcus oeni* m growth in nutritional stress conditions. Specifically citric acid was involved in the biosynthesis of the aspartate-derived essential amino acids and glucose in the cysteine biosynthesis.

Significance and Impact of the Study: Such beneficial effect of L-malic and citric acids on amino acids requirements of *Oenococcus oeni* m have great significance considering the low amino acids concentration in wine.

INTRODUCTION

Oenococcus oeni is the major species found in wine during malolactic fermentation. It is the best adapted to the low pH and high ethanol concentration that characterize wine after alcoholic fermentation (Wiwobo *et al*. 1985; Kunkee 1991). Due to nutritional complexity of lactic acid bacteria, they are frequently cultivated in complex or natural media for different applications. However, the complexity of these media is such that metabolic pathways are difficult to characterize precisely. *Oenococcus oeni* has numerous growth requirements, especially of nitrogen sources for growth (Garvie 1967; Tracey and Britz 1989; Fourcassie *et al*. 1992). In a previous investigation, Amoroso *et al*. (1993) reported that four strains of *Oenococcus oeni* had an absolute requirement for a minimum of four amino acids, that only L-asparagine was essential for growth of all strains and that vitamins and bases were generally stimulatory. The addition of L-malic and citric acids avoided some amino acid exigencies, suggesting that in terms of nutritional requirements these acids played a beneficial role. Moreover both organic acids allowed the growth of the *Oenococcus oeni* m strain when different combinations of essential amino acids such as asparagine and isoleucine, asparagine and cysteine, or isoleucine and cysteine were successively omitted from synthetic medium (Saguir and Manca de Nadra 1997). The substrate mixtures could avoid both carbon flux and energy limitations. 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 acids concentration in wine is limited. Colagrande *et al*. (1984) reported the presence of only small amounts (less than 10 mg l^{-1}) of methionine, leucine or isoleucine in Champagne-based wine. Arginine and proline are usually considered as major components of grape juices

Correspondence to: Dra. María Cristina Manca de Nadra, CERELA, Chacabuco 145, 4000, Tucumán, Argentina (e-mail: mcmanca@unt.edu.ar).

and wines (Amerine and Oug 1980). The general assumption is that biomass synthesis in lactic acid bacteria is predominantly from building blocks present in the culture medium (Schmit *et al.* 1992). However, this dissociation between catabolism of the energetic substrates (glucose, organic acids) and carbon assimilation from organic nitrogen sources (amino acids) may be less complete in deficient nutritional conditions, with some exchange of carbon flux between the different types of carbon substrates.

This paper describes the effect of L-malic and/or citric acids on the specific growth rate of *Oenococcus oeni* m, determined during the absence of each essential amino acid from culture medium. A synthetic medium was used to characterize precisely the possible roles of L-malic and citric acids as precursors of anabolic compounds for the essential amino acids synthesis.

MATERIALS AND METHODS

Bacterial strain

The strain *Oenococcus oeni* m was isolated from an Argentinean red wine (Manca de Nadra and Strasser de Saad 1987; Strasser de Saad and Manca de Nadra 1987). The strain was stored at −20°C in MRS medium (De Man *et al.* 1960) supplemented with tomato juice (15%) and glycerol (30%, v/v).

Media, growth conditions and culture procedures

A chemically defined medium (Ledesma *et al.* 1977) was used as basal medium (BM) with the following composition in distilled water (in l^{-1}): Glucose, 10 g; potassium acetate, 10 g; potassium dihydrogen orthophosphate, 2 g; sodium thioglycollate, 0·5 g; magnesium sulphate.$7H_2O$, 0·15 g; manganese sulphate.$4H_2O$, 0·02 g; ferrous sulphate.$7H_2O$, 0·01 g; Tween 80, 1 mg; and (in mg l^{-1}): adenine, 50; cytidylic acid, 50; deoxyguanosine, 50; guanine HCl, 50; thymidine, 50; uracil, 50; *p*-aminobenzoic acid, 0·01; vitamin B12, 0·001; calcium pantothenate, 1; D-biotin, 0,01; folic acid, 0·1, niacin, 1; piridoxal ethyl acetal HCl, 0·5; riboflavin, 0·5; thiamine HCl, 1. Amino acid concentrations are given in Table 1. Different modified synthetic media were prepared by modifying the composition of amino acids mixture of the basal medium as stated in the text. A semisynthetic medium, where the amino acids source, except cysteine-HCl, was substituted by tryptone (4 g l^{-1}) was used for the cells adaptation before inoculation into the synthetic media. L-Malic and citric acids were incorporated individually or together to the semisynthetic, basal and modified media at 2·5 and 0·7 g l^{-1}, respectively. All media were adjusted to pH 4·8 with 10 mol l^{-1} KOH or HCl before sterilization. The different synthetic media were sterilized in an autoclave, with heating stopped immediately on reaching 121°C. Cysteine-HCl sterilized by filtration was added to sterilized media.

Table 1 Amino acids in the basal medium

Compound	Concentration (g l^{-1})
L-Glutamic acid	0·15
DL-Alanine	0·20
L-Arginine	0·005
L-Asparagine	0·20
L-Cysteine HCl	0·20
L-Phenylalanine	0·04
L-Histidine HCl	0·05
L-Isoleucine	0·05
L-Leucine	0·06
L-Lysine HCl	0·05
L-Methionine	0·05
L-Proline	0·04
L-Serine	0·10
L-Tyrosine	0·004
L-Threonine	0·05
L-Tryptophan	0·05
L-Valine	0·03
L-Glycine	0·3

For the final culture in the experimental synthetic media the cells grown in MRS with tomato juice (15%), pH 4·8, incubated, without agitation, at 30°C were harvested at the end of exponential growth phase (26 h) and precultured under the same conditions in the semisynthetic medium. After 72 h of incubation the cells from the last transfer were harvested by centrifugation, washed twice with sterile distilled water to avoid the carry-over of essential nutrients and resuspended in sterile distilled water to $O.D._{620\ nm} = 0{\cdot}90$. Synthetic media were inoculated at a concentration of 6×10^6 cells ml^{-1}. All cultures were incubated statically at 30°C for 10 d.

Growth measurement

Bacterial growth was monitored by periodic spectrophotometric measurements at 620 nm using Bausch and Lomb Spectronic-20 spectrophotometer and by direct counting of cells. From this data it was possible to calculate the average of growth rates.

Analytical methods

D-Glucose was analysed by the glucose oxidase method (Kit from Wiener Laboratory, Rosario, Argentina). L-Malic, citric and acetic acids were measured by enzymatic methods (Boheringer Kits, Mannheim, Germany). In order to measure the production of acetate from glucose or citrate metabolism, cells were cultivated under the same conditions

as described in the text, in the basal medium with glucose without potassium acetate.

L- and D-lactic acids were determined using specific L-lactate dehydrogenase from rabbit muscle (Sigma) and D-lactate dehydrogenase from *Lactobacillus leichmanii* (Sigma). Diacetyl, acetoin and 2,3-butanediol formation was analysed as a combined value according to the colorimetric method of Hill *et al*. (1954) modified by Branen and Keenan (1970).

Statistical analysis

To validate the methods, the Student's test was used. Three replicate determinations were carried out.

RESULTS

Effect of organic acids on *Oenococcus oeni* m growth in deficient synthetic media

Table 2 shows the maximum growth rate (μ_{max}) and cell density of *Oenococcus oeni* m and the effect of organic acids, L-malic, citric or both acids in synthetic media lacking one amino acid. The microorganism grew a small extent ($2{\cdot}51 \times 10^8$ cfu ml^{-1}) in basal medium with a growth rate of 0·041 h^{-1}. Organic acids strongly increased (2- to 9-fold) the extent of growth of *Oenococcus oeni* strain in the synthetic medium with all amino acids required and in the presence of both L-malic and citric acids the growth parameters were maximal ($1{\cdot}99 \times 10^9$ cfu ml^{-1} and 0·094 h^{-1}).

The individual suppression of the amino acids of the glutamic acid family led to reduced growth rates, 48·8% for glutamate, 41·5% for proline and 51·2% for arginine, associated with a reduction of final biomass of about 50%.

The results obtained in the absence of each member of the aspartate family, lysine, methionine, threonine, isoleucine and asparagine, show that no growth was possible when asparagine or isoleucine were removed from basal medium, confirming that they are essential amino acids for growth. It should be noted that there was a greatly diminished growth rate in the media lacking methionine (82·9%) or threonine (68·3%). Both amino acids also had a stimulatory effect on bacterial growth. The absence of lysine, a nonessential amino acid for *Oenococcus oeni* m growth (Amoroso *et al*. 1993), in the basal medium did not modify the growth parameters. The absence of each member of the pyruvate family, alanine, valine or leucine, shows that all these amino acids had a stimulatory effect on growth with the following growth rate diminution: for alanine 26·83%, leucine 68·3% and valine 75·6%.

The individual removal of members of the serine family, serine, cysteine or glycine, from basal medium shows that no growth was observed in the medium without cysteine and a diminution of 29·3 and 58·6% was observed on the growth

Table 2 Effect of L-malic and/or citric acids on the growth of *Oenococcus oeni* m growing in basal media deficient in one amino acid

Omitted Amino acid	BM		BM + L-malic acid		BM + citric acid		BM + both organic acids	
	μ_{max}	Final biomass (cfu ml^{-1})	μ_{max}	Final biomass (cfu ml^{-1})	μ_{max}	Final biomass (cfu ml^{-1})	μ_{max}	Final biomass (cfu ml^{-1})
None	0·041	$2{\cdot}5 \times 10^8$	0·069	$5{\cdot}72 \times 10^8$	0·092	$1{\cdot}26 \times 10^9$	0·094	$1{\cdot}99 \times 10^9$
Glu	0·021	$1{\cdot}19 \times 10^8$	0·048	$2{\cdot}80 \times 10^8$	0·091	$6{\cdot}30 \times 10^8$	0·062	$1{\cdot}00 \times 10^9$
Pro	0·024	$1{\cdot}20 \times 10^8$	0·048	$2{\cdot}80 \times 10^8$	0·091	$6{\cdot}20 \times 10^8$	0·092	$2{\cdot}06 \times 10^9$
Arg	0·020	$1{\cdot}20 \times 10^8$	0·069	$5{\cdot}70 \times 10^8$	0·091	$1{\cdot}26 \times 10^9$	0·093	$9{\cdot}91 \times 10^8$
Asp	0·019	$1{\cdot}18 \times 10^8$	0·060	$2{\cdot}70 \times 10^8$	0·080	$5{\cdot}90 \times 10^8$	0·092	$2{\cdot}00 \times 10^9$
Asn	0·000	NG	0·051	$5{\cdot}70 \times 10^8$	0·048	$5{\cdot}90 \times 10^8$	0·079	$2{\cdot}10 \times 10^9$
Lys	0·039	$2{\cdot}58 \times 10^8$	0·069	$5{\cdot}80 \times 10^8$	0·088	$6{\cdot}20 \times 10^8$	0·090	$1{\cdot}13 \times 10^9$
Met	0·007	$1{\cdot}21 \times 10^8$	0·056	$5{\cdot}61 \times 10^8$	0·091	$6{\cdot}30 \times 10^8$	0·094	$2{\cdot}10 \times 10^9$
Thr	0·013	$1{\cdot}2 \times 10^8$	0·052	$2{\cdot}70 \times 10^8$	0·063	$6{\cdot}50 \times 10^8$	0·065	$1{\cdot}00 \times 10^9$
Ile	0·000	NG	0·067	$2{\cdot}82 \times 10^8$	0·092	$6{\cdot}30 \times 10^8$	0·094	$2{\cdot}10 \times 10^9$
Ala	0·030	$1{\cdot}30 \times 10^8$	0·064	$5{\cdot}70 \times 10^8$	0·091	$6{\cdot}20 \times 10^8$	0·093	$1{\cdot}90 \times 10^9$
Leu	0·013	$1{\cdot}30 \times 10^8$	0·069	$5{\cdot}70 \times 10^8$	0·093	$1{\cdot}26 \times 10^9$	0·093	$1{\cdot}99 \times 10^9$
Val	0·010	$1{\cdot}20 \times 10^8$	0·060	$2{\cdot}80 \times 10^8$	0·051	$6{\cdot}24 \times 10^8$	0·052	$1{\cdot}05 \times 10^9$
Ser	0·029	$1{\cdot}24 \times 10^8$	0·048	$2{\cdot}78 \times 10^8$	0·090	$1{\cdot}20 \times 10^8$	0·096	$1{\cdot}95 \times 10^9$
Cys	0·000	NG	0·037	$2{\cdot}33 \times 10^8$	0·085	$5{\cdot}54 \times 10^8$	0·094	$1{\cdot}96 \times 10^9$
Gly	0·017	$1{\cdot}00 \times 10^8$	0·052	$2{\cdot}35 \times 10^8$	0·055	$6{\cdot}10 \times 10^8$	0·058	$8{\cdot}60 \times 10^8$
Phe	0·019	$1{\cdot}24 \times 10^8$	0·069	$5{\cdot}81 \times 10^8$	0·034	$5{\cdot}91 \times 10^8$	0·095	$1{\cdot}97 \times 10^9$
Tyr	0·000	NG	0·069	$5{\cdot}7 \times 10^8$	0·060	$6{\cdot}00 \times 10^8$	0·068	$1{\cdot}19 \times 10^9$
Trp	0·016	$1{\cdot}12 \times 10^8$	0·069	$5{\cdot}7 \times 10^8$	0·084	$1{\cdot}26 \times 10^8$	0·087	$9{\cdot}57 \times 10^8$

NG: no growth.

rate by omission of serine and glycine, respectively. The results obtained in the absence of members of the aromatic amino acids family, phenylalanine, tyrosine or tryptophane, confirmed that tyrosine is an essential amino acid (Amoroso *et al*. 1993) and in its absence the microorganism did not grow. The growth rate diminished 53·7 and 61% in the absence of phenylalanine and tryptophane, respectively, and the bacterial growth was reduced of about 50%. The addition of the organic acids individually or in combination to the media deficient in one amino acid increased or, moreover, restored the growth rate and the bacterial growth to the respective reference values. Thus the growth rate obtained in synthetic medium without methionine increased eightfold by L-malic acid supplementation and it was similar to control values when the media were supplemented with citric or citric plus L-malic acids. In the basal media without *Oenococcus oeni* m growth, organic acids supplementation almost restored the growth to the same cell density as that obtained in control media, and the growth rate was raised to the same value as that observed in control medium by (a) addition of L-malic and/or citric acid to the medium deprived of isoleucine, (b) addition of L-malic plus citric acids to the medium deprived of cysteine and (c) addition of L-malic acid to the medium without tyrosine. In absence of asparagine and presence of L-malic, citric or both organic acids the growth rates were 26·6%, 48% and 15% lower, respectively, than in control media. This was also observed in the media without cysteine plus L-malic acid, and without tyrosine plus citric acid or both. The results suggest that the essential amino acids can be synthesized from intermediaries metabolically derived from components of the medium such as L-malic and citric acids.

In order to elucidate if the carbon flux associated with the metabolism of organic acids can be related with the synthesis of amino acids, the utilization of L-malic and citric acids and their metabolic products was measured after 120 h incubation at 30°C.

Time course of L-malic acid metabolism

In complete basal medium L-malic acid was totally utilized and it was almost completely recovered as L-lactic acid and CO_2 (malolactic fermentation) as previously described (Saguir and Manca de Nadra 1996). The time course of L-malic acid utilization and L-lactic acid formation was studied in basal media lacking an essential amino acid for the *Oenococcus oeni* m growth, in presence of 18·65 mmol l^{-1} L-malic acid (Fig. 1). The utilization of L-malic acid began immediately growth began in the basal medium without asparagine, isoleucine or tyrosine and it was consumed more rapidly after 40 h incubation. In these conditions after 80 h incubation *Oenococcus oeni* m degraded nearly all of the initial L-malic acid, especially in absence of tyrosine

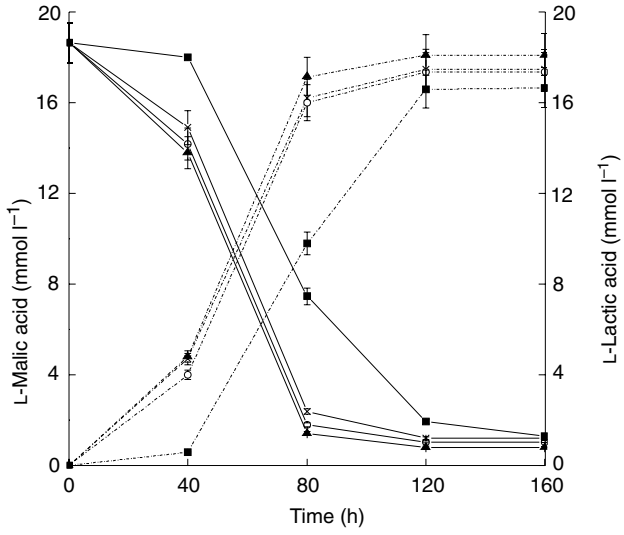

Fig. 1 L-Malic acid consumption (solid line) and L-lactic acid production (dashed line) by *Oenococcus oeni* m metabolism in each basal medium deficient in one essential amino acid. Without: Aspn (○); Ile (×); Cys (■); Tyr (▲)

(17·98 mmol l^{-1}) and it was accompanied by a corresponding increase in concentration of L-lactic acid. When cysteine was omitted from basal medium, *Oenococcus oeni* m began to use the dicarboxylic acid at 40 h incubation at 30°C coinciding with the end of lag growth phase. In this condition, L-malic acid was consumed at a lower rate than those observed when asparagine, cysteine or tyrosine were individually eliminated from basal medium and this could be related with the lower growth rate reached by this strain in absence of cysteine (Table 2). L-Lactic acid continued to accumulate into the medium throughout the period of L-malic acid degradation and accounted for approximately 100% L-malic acid consumed (Fig. 1).

The production of L-lactic acid from L-malic acid by *Oenococcus oeni* m in the cited media could explain why the final pH values were higher (about 0·15 unit) than initial pH value (data not shown). In addition to L-lactic acid, independently of essential amino acid omitted from culture medium, D-lactic acid and ethanol were found (Fig. 2). In absence of asparagine, isoleucine or tyrosine approximately equimolecular amounts of these products were formed according to the theoretical stoichiometry of glucose metabolism. No acetoin, diacetyl and 2,3-butilenglicol were detected. Only the absence of cysteine was able to reduce the recovery of glucose as D-lactic acid, from 96% to 72%.

Time course of citric acid metabolism

Saguir and Manca de Nadra (1996) reported that in complete basal medium with citric acid, D-lactic acid production was

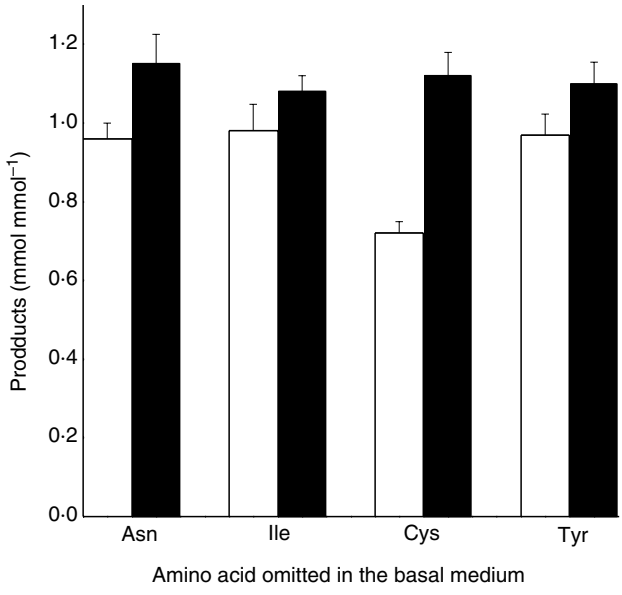

Fig. 2 D-Lactic acid (□) and ethanol (■) production from glucose by *Oenococcus oeni* m. Results are the ratio of amount of D-glucose consumed and D-lactic acid or ethanol produced in each basal medium deficient in one essential amino acid (indicated at the bottom)

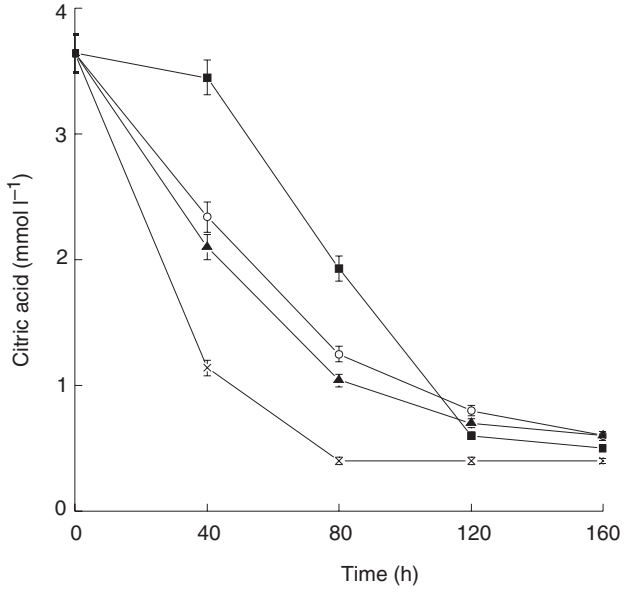

Fig. 3 Citric acid consumption by *Oenococccus oeni* m metabolism in each basal medium deficient in one essential amino acid. Without: Aspn (○), Ile (×); Cys (■); Tyr (▲)

directly proportional to the glucose–citrate cometabolism. The addition of citrate showed a change in the analytical balance of the end products of glucose metabolism: ethanol from glucose diminished from 1 : 1 to 1 : 0·6 and acetic acid was produced with an enhancement of ATP production via acetate kinase. No diacetyl, acetoin and 2,3-butanediol were produced from citrate and acetic acid was formed in molar ratio 1 : 1 (mmol mmol^{-1}). Figure 3 and Table 3 show the time course of citric acid utilization and metabolic products formation in basal media lacking an essential amino acid for *Oenococcus oeni* m growth, in presence of 3·64 mmol l^{-1} citric acid, respectively. The result of citric acid utilization in the medium without cysteine was similar to that observed in presence of L-malic acid (Fig. 1). The citric acid utilization was faster when isoleucine was omitted from culture medium than in absence of tyrosine, asparagine or cysteine. This result coincided with the higher growth rate reached by *Oenococcus oeni* m in the basal medium without isoleucine in presence of citric acid (Table 2). At 80 h incubation *Oenococcus oeni* m consumed nearly all of the initial citric acid in the medium lacking isoleucine (87·1%). When cysteine, tyrosine or asparagine were individually eliminated from basal medium the final consumption of citric acid was 85·7, 78·6 and 77·1%, respectively, at 120 h incubation at 30°C. At this time (Table 3), a 100% recuperation of glucose–citrate utilization as D-lactic acid was observed only when tyrosine was omitted from the complete medium, in the same way as for basal medium. The recovery of glucose–citrate as D-lactic acid was 27, 21 and 18% lower than in the complete basal medium with citric acid, when asparagine, isoleucine or cysteine were removed from culture medium, respectively. Ethanol and acetate production from glucose–citrate metabolism by *Oenococcus oeni* m was not affected by the omission of asparagine, isoleucine, cysteine or tyrosine from culture medium and no diacetyl, acetoin or 2,3-butanediol were produced.

Table 3 Product stoichiometries of *Oenococcus oeni* m growing on glucose + citric acid in each basal medium deficient in one essential amino acid

Amino acid omitted in the basal medium*	D-Lactic acid (mmol mmol^{-1} glu + cit)	Ethanol (mmol mmol^{-1} glu)	Acetate (mmol mmol^{-1} glu + cit)
L-Asparagine	0·73 ± 0·03	0·76 ± 0·03	0·43 ± 0·02
L-Isoleucine	0·79 ± 0·04	0·71 ± 0·02	0·46 ± 0·02
L-Cysteine	0·82 ± 0·03	0·61 ± 0·03	0·53 ± 0·03
L-Tyrosine	1·03 ± 0·04	0·65 ± 0·03	0·49 ± 0·02

* BM contained: 55·5 mmol l^{-1} glucose (glu), 3·64 mmol l^{-1} citrate (cit).

When L-malic and citric acids metabolism was simultaneously studied, similar results were found.

DISCUSSION

The data obtained demonstrated that the nutrient limitation by amino acids had a significant inhibitory effect on the maximum growth rate and the maximum biomass production of *Oenococcus oeni* m in poor nutritional conditions. A similar result was reported by Vasserot *et al.* (2001) for *Oenococcus oeni* growing in synthetic medium with low concentration of L-aspartic acid, since when L-aspartic concentration increased from 0 to 0·15 mmol l^{-1}, bacterial maximum growth rate increased from 0·19 to 0·24 h^{-1} and the bacterial maximum biomass production increased from 0·09 to 0·15 mg l^{-1}. The results of organic acids addition individually or in combination to the media deficient in one amino acid suggest that the 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 completely recovered as L-lactic acid on a yield close to 1 : 1 (mmol $mmol^{-1}$) in all media, it is possible to conclude that *Oenococcus oeni* m is unable to use the dicarboxylic acid as biosynthetic precursor for the essential amino acids synthesis. However, biochemical energy gain associated with malolactic fermentation must be taken into account as additional advantage to carry out these anabolic reactions. Except for cysteine, the fact that D-lactic acid and ethanol formed from glucose fermentation were similar to those observed in the basal medium containing all 18 amino acids in presence of glucose and L-malic acid, supports the idea that the lack of one of these essential amino acids did not also produce changes in the glucose metabolism. By contrast in absence of cysteine the lower recovery of D-lactic acid from glucose metabolism indicates that the glucose metabolism could be involved in its biosynthesis.

The studies on citric acid metabolism in the media deprived of an essential amino acid show interesting findings. The lower recovery of glucose–citrate as D-lactic acid in the media lacking asparagine, isoleucine or cysteine, implies that under these conditions of limited amino acid supply, *Oenococcus oeni* m could be able to obtain carbon source for the synthesis of these essential amino acids through the catabolism of glucose or citrate. As the cysteine elimination from basal medium supplemented with L-malic or citric acid was always accompanied with a carbon imbalance from glucose or glucose–citrate to D-lactic acid, respectively, it seems reasonable that glucose could be involved in the cysteine formation under the studied conditions. In bacteria, cysteine is synthesized from serine by incorporation of sulphide or thiosulphate (Kitabatake *et al.* 2000) and serine is synthesized from 3-phosphoglycerate. This late compound is an intermediate in the glucose metabolism. In bacteria asparagine is formed from aspartate by an amidation reaction (Reitzer and Magasanik 1982; Hughes *et al.* 1997). Isoleucine biosynthesis starts with the deamination of threonine, also coming from aspartate, catalysed by threonine deaminase. As in the absence of asparagine or isoleucine the lower production of D-lactic acid was only observed when citrate was present in the culture medium, we can concluded that part of citrate metabolism was diverted by *Oenococcus oeni* m for these amino acids synthesis, via oxalacetate. Moreover, the fact that the amount of D-lactic acid recovered from glucose–citrate metabolism was 5% higher in the absence of isoleucine than in the medium without asparagine (Table 3) supports the idea that citric acid utilization would be involved primarily in the asparagine biosynthesis. The conversion of oxalacetate to aspartate proceeds in bacteria in a single step reaction catalysed by a transaminase. During studies aiming at the elucidation of citrate transport by *Leuconostoc oenos* (Ramos *et al.* 1994), it was observed that approximately 20% of the radioactive labelled provided in citrate remained inside the cells. Ramos *et al.* (1995) demonstrated in *Leuconostoc oenos* isolated from wine that at least 10% of the citric acid supplied was converted to aspartate that was not excreted to the extracellular medium.

As in presence of citrate *Oenococcus oeni* m grew at a growth rate 48% higher in the medium lacking isoleucine than asparagine (Table 2), it can be hypothesized that the branched-chain amino acids leucine and valine present in the medium could be stimulating isoleucine biosynthesis. Van der Sluis *et al.* (2000) reported that in *Zygosaccharomyces rouxii* like in *Saccharomyces cerevisiae* the activity of threonine deaminase is stimulated by valine and leucine and inhibited by isoleucine. As acetic acid produced from citrate catabolism was not affected by the omission of one essential amino acid from basal medium, we can conclude that acetic acid was not involved in the essential amino acids biosynthesis. Collins and Bruhn (1970) reported that acetate from citrate metabolism was incorporated into cell lipids of *Lactococcus lactis* subsp. *lactis* var. *diacetylous*.

In conclusion, we demonstrate that malic and citric acids affected *Oenococcus oeni* m growth in nutritional stress conditions. This behaviour was related to their role as biosynthetic precursors of amino acids. Specifically citric acid was involved in the biosynthesis of aspartate-derived amino acids: asparagine and isoleucine.

ACKNOWLEDGEMENTS

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT).

REFERENCES

Amerine, M.A. and Oug, C.S. (1980) *Methods for Analysis of Must and Wines*. p. 341. New York: John Wiley and Sons.

Amoroso, M.J., Saguir, F.M. and Manca de Nadra, M.C. (1993) Variation of nutritional requirements of *Leuconostoc oenos* by organic acids. *Journal of International Des Science, Vigne et Du Vin* **27**, 135–144.

Branen, A.L. and Keenan, T.W. (1970) Diacetyl, acetoin and 2,3-butanediol production by *Lactobacillus casei*. *Journal of Dairy Science* **53**, 632–635.

Colagrande, O., Silva, A. and Casoli, A. (1984) Acides aminés dans les vins mousseux. *Connaissance de la Vigne et Du Vin* **18**, 27–47.

Collins, E.B. and Bruhn, J.C. (1970) Pole of acetate and pyruvate of *Streptococcus diacetilactis*. *Journal of Bacteriology* **103**, 597–604.

De Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of Lactobacilli. *Journal of Applied Bacteriology* **23**, 130–135.

Fourcassie, P., Makaga-Kabinda-Massard, E., Belardi, A. and Maujean, A. (1992) Growth, glucose utilization and malolactic fermentation by *Leuconostoc oenos* strains in 18 media deficient in one amino acid. *Journal of Applied Bacteriology* **73**, 489–496.

Garvie, E. (1967) The growth factor and amino acid requirement of the genus *Leuconostoc*, including *Leuconostoc paramesentero* (sp. Nov.) and *Leuconostoc oenos*. *Journal of General Microbiology* **48**, 439–447.

Hill, E.C., Wenzel, F.W. and Barreto, A. (1954) Colorimetric method for the microbiological spoilage in citrus juices. *Food and Technology* **8**, 168–171.

Hughes, C.A., Beard, H.S. and Matthews, B.F. (1997) Molecular cloning and expression of two cDNAs enconding asparagine synthetase in soybean. *Plant Molecular Biology* **33**, 301–311.

Kitabatake, M., So, M.W., Tumbula, D.L. and Soll, D. (2000) Cysteine biosynthesis pathway in the archaeon *Methanosarcina barkeri* encoded by acquired bacterial genes? *Journal of Bacteriology* **182**, 143–145.

Kunkee, R.E. (1991) Some roles of L-malic acid in the malolactic fermentation in wine making. *FEMS Microbiology Reviews* **88**, 55–72.

Ledesma, O.V., Ruiz Holgado, A. and Oliver, G. (1977) A synthetic medium for comparative nutritional studies of Lactobacilli. *Journal of Applied Bacteriology* **42**, 123–133.

Manca de Nadra, M.C. and Strasser de Saad, A.M. (1987) Evolution of lactic acid bacteria during the different stages of vinification of Cafayate (Argentina) wine. *Microbiology-Aliments-Nutrition* **5**, 235–240.

Ramos, A., Lolkema, J.S., Santos, H. and Konings, W.N. (1994) Uniport of anionic citrate and proton consumption in citrate metabolism generates a proton motive force in *Leuconostoc oenos*. *Journal of Bacteriology* **176**, 4899–4905.

Ramos, A., Lolkema, J.S., Konings, W.N. and Santos, H. (1995) Enzymes basis for pH regulation of citrate and pyruvate metabolism by *Leuconosotc oenos*. *Applied and Environmental Microbiology* **61**, 1303–1310.

Reitzer, L.J. and Magasanik, B. (1982) Asparagine synthetase of *Klebsiella aerogenes*: properties and regulation of synthesis. *Journal of Bacteriology* **151**, 1299–1313.

Saguir, F.M. and Manca de Nadra, M.C. (1996) Organic acids metabolism under different glucose concentrations of *Leuconostoc oenos* from wine. *Journal of Applied Bacteriology* **81**, 393–397.

Saguir, F.M. and Manca de Nadra, M.C. (1997) Growth and metabolism of *Leuconostoc oenos* in synthetic media. *Microbiologie-Aliments-Nutrition* **15**, 131–138.

Schmitt, P., Diviès, C. and Cardona, R. (1992) Origin of end-products from the co-metabolism of glucose and citrate by *Leuconstoc mesenteroides* subsp. *cremoris*. *Applied Microbiology and Biotechnology* **36**, 679–683.

Strasser de Saad, A.M. and Manca de Nadra, M.C. (1987) Isolation and identification of the lactic acid bacteria from Cafayate (Argentina) wines. *Microbiology-Aliments-Nutrition* **5**, 45–49.

Tracey, R.P. and Britz, J.P. (1989) The effect of amino acids on malolactic fermentation by *Leuconostoc oenos*. *Journal of Applied Bacteriology* **171**, 280–284.

Van der Sluis, C., Smit, B.A., Hartmans, S., ter Schure, E.G., Tramper, J. and Wijffels, R.H. (2000) Regulation of aspartate-derived amino-acid metabolism in *Zygsaccharomyces rouxii* compared to *Saccharomyces cerevisae*. *Enzyme and Technology* **27**, 151–156.

Vasserot, Y., Dion, C., Bonnet, E., Maujean, A. and Jeandet, P. (2001) A study into the role of L-aspartic acid on the metabolism of L-malic acid and D-glucose by *Oenococcus oeni*. *Journal of Applied Microbiology* **90**, 380–387.

Wiwobo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. and Lee, T.H. (1985) Occurrence and growth of lactic acid bacteria in wine: a review. *American Journal of Enology and Viticulture* **36**, 302–313.