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# VARIATION OF NUTRITIONAL REQUIREMENTS *OF LEUCONOSTOC OENOS* BY ORGANIC ACIDS

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**Summary :** *Leuconostoc oenos strains : m, $L_2$, ST, $X_2L$, have been isolated from different Argentinian red wines. Amino acids, vitamins and base requirements were determined in a synthetic medium containing L- malic acid, citric acid or both organic acids. The organisms differed in their requirements. The growth of the four L. oenos strains studied was greater in the synthetic medium with L-malic acid plus citric acid. In this medium, L. oenos m, $L_2$, and ST eliminated their amino acid requirements, however $X_2L$ in the same medium increased these requirements. In respect of the vitamin and base requirements, L. oenos m and $L_2$ showed no significant difference by the addition of L-malic acid, citric acid or both organic acids to the synthetic medium. L. oenos ST needed L-malic acid plus citric acid to avoid these requirements, while the $X_2L$ strain increased its requirements with both organic acids.*

## INTRODUCTION

Lactic acid bacteria are important in winemaking for the following reasons : they deacidify the wine conveniently, improve the organoleptic characteristics and provide a biological stability of the final product. This results from the bacterial transformation of malic acid to lactic acid and carbon dioxide, during malolactic fermentation. This fermentation can be achieved by the genera *Lactobacillus*, *Leuconostoc* and *Pediococcus*, of which *L. oenos* species has been considered as the most appropriate organism due to its high tolerance to ethanol, sulphur dioxide and high wine acidity (DAVIS et *al.*,1986 ; DAVIS et *al.*, 1988). With respect to the nutritional requirements, FEUILLAT et *al.* (1977) show that *L. oenos* have the least demand for specific nitrogen compounds. This fact is of practical importance to wine, especially after alcoholic fermentation, due to the low nitrogen compounds concentration (FEUILLAT et *al.*, 1985). GARVIE (1967) has reported that *L. oenos* has an absolute need for arginine, cysteine, glutamic acid, isoleucine, tyrosine and valine while certain strains also require aspartic acid, glycine, histidine, leucine, methionine, phenylalanine, serine, threonine and tryptophan for optimun growth in a synthetic medium. Regarding the essential amino acids, only arginine and glutamic acid appear to be available in high enough concentration at the end of alcoholic fermentation. (FEUILLAT et *al.*,. 1985).

The aim of this work is to determine if nutritional requirements of amino acids, vitamins and bases of *L. oenos* strains isolated from Argentinian red wines are affected by the presence of L-malic acid and citric acid, present in must and wine.

## MATERIALS AND METHODS

### I — MICROORGANISM

*Leuconostoc oenos* strains : m, $L_2$, ST and $X_2L$ were isolated from different Argentinian red wines (MANCA DE NADRA and STRASSER DE SAAD, 1987a, 1987b ; STRASSER DE SAAD and MANCA DE NADRA, 1987).

### II — MEDIA FOR MAINTAINING CULTURES

The cultures were grown in a liquid medium, MRS (DE MAN et *al.*, 1960) supplemented with 15 p. cent tomato juice. Sterilization was carried out for 15 min. at 118°C.

The cells were transferred from MRS to a modified semi-synthetic medium, where the amino acid source was constituted by tryptone (4 g/l) and added to cysteine hydrocloride. Cultures of strains required several transfers through media before rapid growth was obtained.

### III — MEDIA FOR DETERMINING AMINO ACIDS, VITAMINS AND BASES

The nutritional requirements of strains were tested by comparing their growth in the synthetic medium (LEDESMA et *al.*, 1977) with growth in the medium lacking each of the growth factor constituents. The composition of the synthetic medium (g/l) was as follows :

- Potassium acetate : 10,
- d-glucose : 10,
- Potassium dihydrogen orthophosphate : 2,
- Sodium thioglycollate : 0,5,
- Magnesium sulphate, 7 $H_2O$ : 0.15,
- Manganous sulphate, 4 $H_2O$ : 0.02,
- Ferrous sulphate, 7 $H_2O$ : 0.01,
- Tween 80 : 1.1.

Amino acids, vitamins and base concentrations are given in the table I.

The following organic acids were added to the semi-synthetic and synthetic media: citric acid (0.5 g/l) and L-malic acid (2.5 g/l). The pH of the media were adjusted at 4.8.

The incubation was performed at 30°C. The synthetic and semi-synthetic media were sterilized in autoclave and heating was immediately stopped at 117°C. After cooling, the solution of L-cysteine hydrochloride previously sterilized by filtration was added, except when the cysteine requirement was being investigated.

**TABLE I**

**Amounts of amino acids, vitamins and bases included in the synthetic medium**

| Compound | Concentration (g/l) | Compound | Concentration (g/l) |
|---|---|---|---|
| L-Glutamic acid | 0.15 | Adenine | 0.05 |
| DL-Alanine | 0.20 | Cytidylic acid | 0.05l |
| L-Arginine HCL | 0.005 | Deoxyguanosine | 0.05 |
| L-Asparagine | 0.20 | Guanine HCL | 0.05 |
| L-Cysteine HCL | 0.20 | Thymidine | 0.05 |
| L-Phenylalanine | 0.04 | Uracil | 0.05 |
| L-Histidine HCL | 0.05 | | |
| L-Isoleucine | 0.05 | | |
| L-Leucine | 0.06 | p-Aminobenzoic acid | 0.00001 |
| L-Lysine HCL | 0.05 | Vitamin B12 | 0.000001 |
| L-Methionine | 0.05 | d-Biotin | 0.00001 |
| L-Proline | 0.04 | Calcium pantothenate | 0.001 |
| L-Serine | 0.10 | Folic acid | 0.0001 |
| L-Tyrosine | 0.004 | Niacin | 0.001 |
| L-Threonine | 0.05 | Pyridoxal ethyl acetal HCL | 0.0005 |
| L-Triptophan | 0.05 | Riboflavin | 0.0005 |
| L-Valine | 0.03 | Thiamin HCL | 0.001 |

IV — INOCULUM PREPARATION.

After incubation for 4 days, the cells from the last transfer in the semi-synthetic medium were harvested. The cells were washed twice with sterile distilled water by centrifugation and the sediment was resuspended in sterile distilled water to give a final absorbance of 0.7 at 620 nm (end of exponential phase of growth). 0.05 ml of cultures were inoculated into each of the amino acids, vitamins and bases test media. The tubes were incubated at 30°C for 7 days.

IV — GROWTH MEASUREMENTS

Growth was estimated for optical density measurements at 620 nm. From this data the nutritional requirements were classified into three groups according to the extent of growth in each deficient medium. From 0 p. cent to 10 p. cent of growth the nutritional requirements are considered as essential (E), 10 p. cent and 50 p. cent stimulatory (S), over 50 p. cent non-essential (NE).

## RESULTS AND DISCUSSION

### I — INFLUENCE OF ORGANIC ACIDS

The maximum growth of the four *L. oenos* strains was observed in the synthetic medium with L-malic acid and citric acid (figure 1). This effect implies that the malolactic reaction must generate some energetic advantage, probably through a stimulated utilization of carbon sources (PILONE and KUNKEE, 1976). SALOU et *al.* (1991) found that the addition of malate resulted in a marked stimulation of growth of a *L. oenos* strain. For COX and HENICKKLING (1989), the catabolism of malate might increase the proton motive force and internal pH of the cells, reducing the need to use ATP to maintain pH homeostasis. HARVEY and COLLINS (1962) and COGAN (1987) observed the favourable effect of the presence of the citric acid on the growth of *Streptococcus* and *Leuconostoc*. It has been proposed that citrate could enhance the formation of some essential cellular constituents that should be synthesized more slowly with glucose alone. Citrate might also enhance bacterial growth by providing an extra source of ATP (COGAN, 1987).

### II — AMINO ACID REQUIREMENTS

The amino acids requirements are shown on the table II.

The amino acid requirements differentiated according to the various strains. Only L-asparagine was required by all of them in the synthetic medium. In this medium *L. oenos* m required L-isoleucine, L-cysteine and L-tyrosine for growth, however they had a stimulatory effect in the presence of citric acid. With L-malic acid some of these amino acids were not required and the others had a stimulatory effect. When L-malic plus citric acids were present, they were not required. *L. oenos* $L_2$ in the synthetic medium required L-asparagine, L-phenylalanine, L-histidine, L-isoleucine, L-leucine, L-cysteine, L-threonine and L- tryptophan. In presence of citric acid *L. oenos* $L_2$ required DL-alanine, L- asparagine, L-histidine, L-isoleucine, L-methionine, L-proline and L- arginine. In the medium with L-malic acid, the $L_2$ strain only needed L-phenylalanine and L-histidine. With L-malic acid and citric acid, L-asparagine, L- histidine, L-isoleucine, L-treonine and L-tryptophan had a stimulatory effect and the other amino acids were not required. *L. oenos* ST grew similary in the synthetic medium and with the addition of L-malic acid.

The majority of the amino acids were required in the medium with citric acid. Only L-arginine was necessary with both organic acids. *L. oenos* $X_2L$ required L-asparagine, L-phenylalanine, L-histidine and methionine in the synthetic medium, these requirements increased when L-malic acid and citric acid were added independently or combined into the medium. Thus, the L-malic acid and citric acid avoided the requirements of amino acids of the m, ST and $L_2$ strains but did not succeed with the $X_2L$ strain. TRACEY and BRITZ (1989) found that certain strains of *L. oenos* yielded similar growth in a synthetic medium and in the same medium with L-malic acid where six amino acids were omitted. It is possible that there is another limiting factor than the amino acids studied. In our case L-malic acid and citric acid could supply intermediaries for the amino acids synthesis.

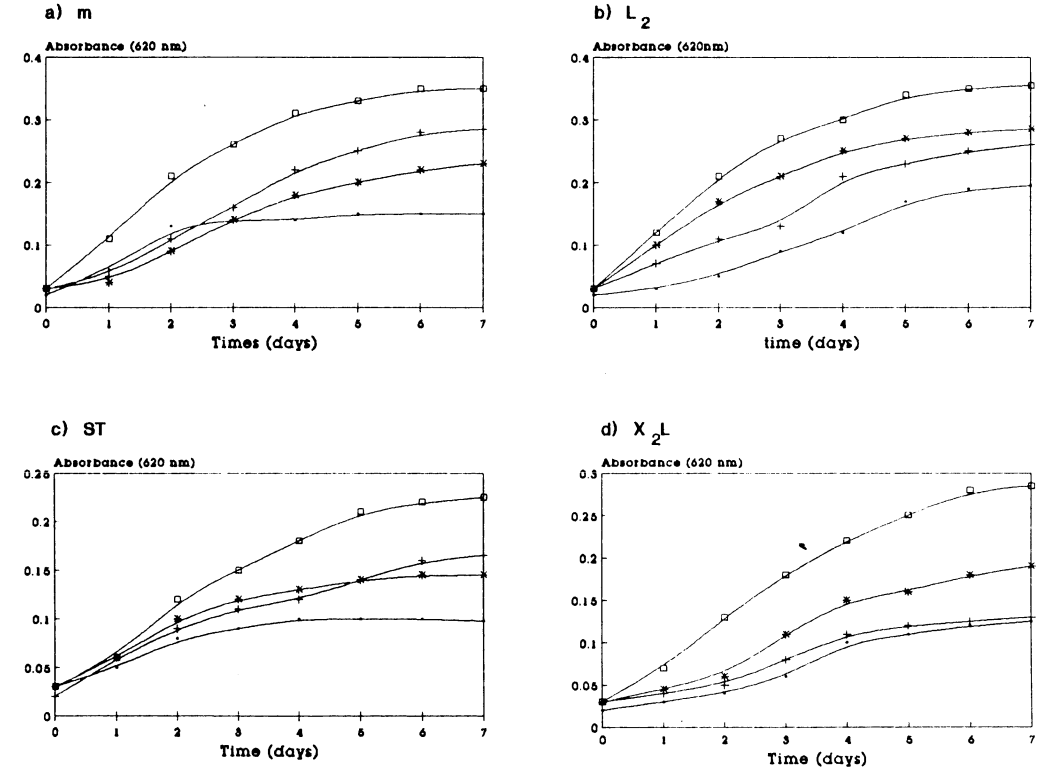


Figure 1

Fig. 1 — Increase in the absorbance (620 nm) in the synthetic medium (━), with citric acid (+), with malic acid (*), with L. malic acid plus citric acid ( )

*L. œnos* strains : a) m; b) $L_2$ ; c) ST ; d) $X_2L$

**TABLE II**

**Nutritional requirements of amino acids of *Leuconostoc oenos* m, $L_2$, ST and $X_2L$**

| Amino acid omitted | Synthetic medium | | | | + citric acid* | | | | + L-malic acid** | | | | + L-malic acid + citric acid | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strains | | | | Strains | | | | Strains | | | | Strains | | | |
| | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ |
| L-Glutamic acid | S | NE | E | NE | S | S | NE | S | NE | S | E | S | S | S | NE | S |
| DL-Alanine | S | S | E | S | S | E | E | S | NE | S | E | NE | NE | S | S | E |
| L-Aspagarine | E | E | E | E | S | E | E | E | NE | S | E | S | NE | S | NE | E |
| L-Phenylalanine | S | E | E | E | S | S | E | S | NE | E | E | S | NE | NE | S | S |
| L-Histidine HCL | S | E | E | E | S | E | S | E | NE | E | E | E | NE | S | NE | NE |
| L-Isoleucine | E | E | E | S | S | E | S | S | S | S | E | S | NE | S | S | E |
| L-Leucine | S | E | E | S | NE | S | NE | S | NE | S | E | E | NE | NE | NE | E |
| L-Cysteine HCL | E | E | E | S | S | S | E | E | S | NE | E | E | NE | NE | NE | S |
| L-Lysine | NE | S | E | S | S | S | E | E | NE | NE | E | E | S | NE | NE | E |
| L-Methionine | S | S | E | E | S | E | NE | E | NE | NE | E | E | NE | NE | S | S |
| L-Proline | S | S | E | NE | S | E | E | NE | S | S | E | E | NE | S | NE | S |
| L-Serine | S | NE | E | NE | NE | S | NE | S | S | NE | E | E | NE | S | S | NE |
| L-Tyrosine | E | S | E | S | S | S | E | E | NE | S | E | S | S | S | NE | E |
| L-Threonine | S | E | E | S | S | S | E | S | S | S | E | E | S | S | NE | E |
| L-Tryptophan | S | E | E | S | NE | S | E | S | NE | NE | E | E | S | S | S | S |
| L-Valine | S | S | E | S | S | S | NE | S | S | S | E | E | S | S | NE | E |
| Glycine | S | S | E | S | S | S | NE | S | S | S | E | E | S | NE | S | E |
| L-Aspartic acid | S | S | E | NE | S | S | E | E | S | NE | E | S | NE | S | S | S |
| L-Arginine | S | S | E | S | NE | E | E | S | NE | NE | E | S | S | NE | E | S |

E : essential ; NE: non essential ; S: stimulatory

Synthetic medium added with : * 0.5 g/l citric acid; ** 2.5 g/l L-malic acid

*L. oenos* ST needed L-aspartate in the synthetic medium for its growth but not in the same medium with both organic acids. This amino acid could be synthesized by a transaminationary reaction from glutamate and oxaloacetate. *L. oenos* m only required L- asparagine in the synthetic medium ; *L. oenos* $L_2$ needed it in the synthetic medium and with citric acid, but not in the same media with L-malic acid or both organic acids. Asparagine could be formed from aspartate and ammonium with the hydrolysis of ATP. Thus aspartate could be obtained from L-malic acid for $L_2$ strain.

III — VITAMIN REQUIREMENTS

The vitamin requirements are shown on the table III.

In the synthetic medium and in presence of citric, L-malic or both organic acids, vitamins were not essential for *L. oenos* m. *L. oenos* $L_2$ neither required vitamins in the synthetic medium, with L-malic acid or L-malic acid plus citric acid but with citric acid they needed p-$NH_2$ benzoic acid, pyridoxal and thiamine. *L. oenos* ST required all the vitamins in the synthetic medium and with L-malic acid, however when citric or both organic acids were present the requirements were eliminated. *L. oenos* $X_2L$ needed folic acid in all cases. P-aminobenzoic acid and thiamine were also required in the synthetic medium. With L-malic acid, $X_2L$ needed d-biotine and niacine. In the presence of L-malic acid plus citric acid, d-biotine, pyridoxal and vitamin B12 were necessary for its growth.

Thus, the requirements for the m and $L_2$ strains were not greatly modified by the addition of L-malic, citric or both organic acids. *L. oenos* ST required p-aminobenzoic acid and the other vitamins in the synthetic medium. With both organic acids, p-aminobenzoic acid was not required. This vitamin would be formed as an intermediary of the aromatic amino acid synthesis that could be carried out by *L. oenos* ST in the presence of L-malic acid plus citric acid (table I).

The $X_2L$ strain showed lower requirements for vitamins in the synthetic medium. In the medium without p-aminobenzoic acid a similar result to *L. oenos* ST was observed. Vjt B12 was not required in the synthetic medium, but with L-malic acid plus citric acid was essential, probably due to its greater consumption. The last reaction of methionine biosynthesis could be catalysed by Vit B12. Methionine was required in the synthetic medium but not with both organic acids (table I). Therefore, this amino acid would be synthesized by *L. oenos* $X_2L$ in presence of L-malic acid plus citric acid, increasing Vit B12 requirements.

IV — BASE REQUIREMENTS

The base requirements are shown on the table IV.

*L. oenos* m. needed no base for its growth in any assayed media. With citric acid or both organic acids, the majority of bases had a stimulatory effect. *L. oenos* $L_2$ did not require a base in the synthetic medium. In the presence of citric acid it required desoxiguanosine, guanine and uracil. With L-malic acid it needed only adenine and with both organic acids only uracil. *L. oenos* ST required of all them in the synthetic medium and with L-malic acid. With citric acid only uracil was necessary.

**TABLE III**

**Nutritional requirements of vitamins of *Leuconostoc oenos* m, $L_2$, ST and $X_2L$**

| Vitamin omitted | Synthetic medium | | | | + citric acid * | | | | + L-malic acid ** | | | | + L-malic acid + citric acid | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strains | | | | Strains | | | | Strains | | | | Strains | | | |
| | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ |
| Folic acid | S | S | E | E | S | NE | NE | E | NE | NE | E | E | S | S | NE | E |
| p-aminobenzoic acid | S | NE | E | E | S | E | E | NE | NE | S | E | S | S | NE | NE | S |
| d-biotine | S | S | E | S | S | S | NE | S | S | NE | E | E | S | NE | S | E |
| Pyridoxal | S | S | E | S | NE | E | NE | S | S | S | E | S | NE | NE | S | E |
| Niacin | S | S | E | S | S | S | NE | S | NE | E | E | E | S | S | NE | S |
| Pantothenate | S | S | E | S | NE | NE | NE | S | S | S | E | NE | S | S | S | S |
| Riboflavin | S | S | E | NE | S | S | NE | S | S | NE | E | S | NE | S | S | S |
| Thiamine | S | S | E | E | S | E | NE | S | S | S | E | S | S | NE | NE | S |
| Vit B12 | S | S | E | S | S | S | NE | S | NE | S | E | S | NE | S | S | E |



**TABLE IV**

**Nutritional requirements of bases of *Leuconostoc oenos* m, $L_2$, ST and $X_2L$**

| Base omitted | Synthetic medium | | | | + citric acid * | | | | + L-malic acid ** | | | | +L-malic + citric acid | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strains | | | | Strains | | | | Strains | | | | Strains | | | |
| | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ | m | $L_2$ | ST | $X_2L$ |
| Adenine | S | S | E | S | NE | S | NE | NE | S | E | E | S | S | S | S | NE |
| Cytidylic acid | S | S | E | S | S | E | NE | S | NE | NE | E | E | S | NE | NE | E |
| Deoxiguanosine | NE | S | E | E | S | E | NE | S | NE | S | E | NE | S | S | S | E |
| Guanine | S | S | E | NE | S | E | NE | S | S | S | E | NE | S | S | S | S |
| Thymidine | S | S | E | S | S | S | NE | NE | S | S | E | NE | S | NE | S | E |
| Uracil | NE | S | E | S | S | E | E | S | NE | S | E | S | NE | E | S | NE |

E : essential ; NE : non essential ; S : stimulatory

Synthetic medium added with : * 0.5 g/l citric acid ; ** 2.5 g/l L-malic acid

In the presence of L-malic acid plus citric acid, the bases were not required. *L. oenos* $X_2L$ needed desoxiguanosine in the synthetic medium. With citric acid any of the bases was required. In presence of L-malic acid, $X_2L$ needed cytidylic acid, and with both organic acids, $X_2L$ required cytidylic acid, adenine and thymidine.

In conclusion, the results presented in this paper suggest that a better understanding of malate-citrate metabolism related to nitrogen compounds would be useful when producting lactic acid bacteria for use in winemaking.

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