

Effect of Dipeptides on the Growth of *Oenococcus oeni* in Synthetic Medium Deprived of Amino Acids

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Abstract. *Oenococcus oeni* has numerous amino acid requirements for growth and dipeptides could be important for its nutrition. In this paper the individual or combined effect of dipeptides on growth of *O. oeni* X₂L in synthetic media deficient in one or more amino acids with L-malic acid was investigated. Utilization of dipeptides, glucose, and L-malic acid was also analyzed. Dipeptides were constituted by at least one essential amino acid for growth. Dipeptides containing two essential amino acids, except leucine, had a more favorable effect than free amino acids on the growth rate. Gly-Gly was consumed to a greater extent than Leu-Leu and a rapid exodus of glycine to the extracellular medium accompanied it. The microorganism could use glycine in exchange for other essential amino acids outside the cell, favoring growth. In the presence of Leu-Leu, the increase in glucose consumption rate could be related to the additional energy required for dipeptide uptake.

Oenococcus oeni, the species of lactic acid bacteria (LAB) more frequently associated with malolactic fermentation (MLF), in wine has numerous nutritional requirements for growth [8, 9, 20]. Amoroso et al. [2] reported that four strains of *O. oeni* had an absolute requirement for a minimum of four amino acids for growth and in one of them (X₂L strain) the number of essential amino acids significantly increased when L-malic acid was present in the medium. Certainly L-malic and citric acids are the substrates that LAB utilizes most frequently in wine. Saguir and Manca de Nadra [18] demonstrated that citric acid metabolism was involved in the biosynthesis of aspartate-derived amino acids by *O. oeni*. Considering the amino acid requirements of *O. oeni* strains, peptide utilization may have great nutritional value for growth. On the other hand, wine has a limited amino acid content [1, 4]. Feuillat et al. [6] found that the peptide fraction extracted from wine, especially peptides of molecular weight lower than 1000, was more favorable for the growth of *O. oeni* than

the amino acid or protein fractions. Manca de Nadra et al. [12, 13] reported the proteolytic activity of *O. oeni* X₂L on the nitrogenous macromolecular fraction of white and red wines, which favored peptide release.

The aim of this work was to describe the individual or joint effect of different dipeptides as sources of amino acids on the growth of *O. oeni* X₂L in a synthetic medium supplemented with L-malic acid. At the same time the utilization of dipeptides, glucose, and L-malic acid was analyzed. The use of a chemically defined medium with L-malic acid and low amino acid concentration was necessary to evaluate the influence of dipeptides on the growth of *O. oeni* in conditions of nutritional stress, such as wine.

Materials and Methods

Microorganism. *O. oeni* X₂L was isolated from an Argentinean red wine [14, 19]. The strain was stored at -20°C in MRS medium [5] supplemented with tomato juice (15%) and glycerol (30%, v/v).

Culture media, growth conditions, and culture procedures. A chemically defined medium [11] supplemented with L-malic acid (2.5 g/L) was used as basal medium (BM) and consisted of the following composition in distilled water (per liter): D-glucose, 10 g; potassium

Table 1. Amino acid concentrations in basal medium (BM)

Amino acid	Concentration (mmol/L)
D,L-Alanine	2.24
L-Aspartic acid	1.50
L-Arginine	0.03
L-Asparagine	1.51
L-Cysteine-HCl	1.65
L-Glutamic acid	1.02
L-Glycine	4.45
L-Histidine-HCl	0.32
L-Isoleucine	0.38
L-Leucine	0.45
L-Lysine	0.34
L-Methionine	0.33
L-Phenylalanine	0.24
L-Proline	0.34
L-Serine	0.95
L-Threonine	0.42
L-Tryptophan	0.24
L-Tyrosine	0.02
L-Valine	0.26

acetate, 10 g; potassium dihydrogen orthophosphate, 2 g; sodium thioglycollate, 0.5 g; magnesium sulphate.7H₂O, 0.15 g; manganese sulphate.4H₂O, 0.02 g; ferrous sulphate.7H₂O, 0.01 g; Tween-80, 1 mg; and (in mg/L): adenine, 50; cytidylic acid, 50; deoxyguanosine, 50; guanine-HCl, 50; thymidine, 50; uracil, 50; *p*-aminobenzoic acid, 0.01; vitamin B₁₂, 0.01; calcium pantothenate, 1; D-biotin, 0.01; folic acid, 0.1; niacin, 1; pyridoxal ethyl acetal HCl, 0.5; riboflavin, 0.5; thiamine HCl, 1. Amino acid concentrations are given in Table 1. The modified BM with dipeptides (Sigma, St. Louis, MO) contained (in mmol/L): glycyl-glycine, 2.2 as a replacement for glycine; leucyl-leucine, 0.22 as a replacement for leucine; leucyl-proline, 0.45 or 0.34 as a replacement for leucine or proline respectively; prolyl-aspartic acid, 0.34 or 1.5 as a replacement for proline or aspartic acid respectively; and methionyl-proline, 0.34 or 0.33 as a replacement for proline or methionine respectively.

A semisynthetic medium where the amino acid source, except cysteine-HCl, was substituted by tryptone (4 g/l) was used for adaptation of the cells before their inoculation into the synthetic media. All media were adjusted to pH 4.8 with 1 N HCl before sterilization. The different synthetic media were sterilized in an autoclave, with heating stopped immediately on reaching 121°C. Cysteine-HCl and dipeptides sterilized by filtration through a nylon membrane (0.22 µm pore size, Millipore) were added to sterilized media.

Cultures were prepared by first growing them in MRS broth with 15% tomato juice, pH 4.8, incubated, without agitation, at 30°C (optimal conditions for *O. oeni* growth). The cells were harvested by centrifugation at the end of the exponential growth phase (34 h) and precultured three times under the same conditions in the semisynthetic adaptation medium before inoculation in synthetic media. After 72 h of incubation, the cells of the last preculture (third) were harvested by centrifugation, washed twice with sterile distilled water to avoid carry-over nutrients, and resuspended in sterile distilled water (OD₆₂₀ = 0.90). Different synthetic media were inoculated at a concentration of 6.5 × 10⁵ cfu/mL. All cultures were incubated at 30°C for 5 days and the samples were taken at various times during growth and stored frozen (-18°C) for subsequent chemical analysis.

Table 2. Effect of dipeptides as sources of amino acids on growth of *O. oeni* X₂L

Omitted amino acid	Incorporated dipeptide	µ _{max} (h ⁻¹)	A ^a
None	None	0.061 bf	1.25 a
Leucine	None	0.006 c	0.08 e
Leucine	Leu-Pro	0.056 af	1.24 a
Leucine	Leu-Leu	0.061 bf	1.32 a
Methionine	None	0.000 e	0.03 e
Methionine	Met-Pro	0.078 d	1.31 a
Proline	None	0.006 ce	0.09 e
Proline	Leu-Pro	0.062 bf	1.37 a
Proline	Met-Pro	0.077 d	1.36 a
Proline	Pro-Asp	0.077 d	1.39 a
Glycine	None	0.007 c	0.09 e
Glycine	Gly-Gly	0.078 d	1.35 a
Aspartic acid	None	0.019 c	0.39 c
Aspartic acid	Pro-Asp	0.069 bdf	1.33 a
Leu, Met, Pro, Gly	Mixture ^b	0.082 d	1.46 a

^a A: difference in cell concentration (log cfu/mL) between stationary phase and inocula.

^b Dipeptide mixture: Leu-Pro, Leu-Leu, Met-Pro, and Gly-Gly. Values with the same letter in the same column are not significantly different (*p* < 0.05).

Growth measurement. Bacterial growth was monitored by periodic spectrophotometric measurements at 620 nm using a Bausch and Lomb Spectronic-20 spectrophotometer during *O. oeni* X₂L growth. At the same times the colony-forming units (cfu/mL) were determined by plating 0.1 mL of inoculated medium on MRS medium supplemented with 20.0 g/L of agar. From these data it was possible to calculate the average growth rates.

The amino acid requirements were estimated in the medium by omitting these amino acids one at a time. They were classified into three groups according to the extent of growth (A, Table 2) in each deficient medium. From 0 to 10% of growth the amino acid was considered as essential from 10% to 50% as stimulatory, and over 50% as non-essential [2].

Kinetics of cell growth. Growth experiments were repeated at least three times. Growth data of *O. oeni* X₂L in synthetic media were modeled according to the Gompertz equation as modified by Zwietering et al. [23]:

$$Y = k + A \exp \{ \exp [(\mu_{\max}^{\Delta}) (\lambda - t) + 1] \}$$

where *y* is the log cfu/mL at the time *t*; *k* is the initial cell concentration as log cfu/mL; *A* represents the difference in cell concentration between inoculum and stationary phase; µ_{max} is the maximum growth rate as Δ log cfu/mL/h; and λ is the length of the lag phase expressed in hours.

Analytical methods. Dipeptides and amino acids were analyzed by reverse-phase HPLC (RP-HPLC) using an ISCO liquid chromatograph (ISCO, Lincoln, NE). Samples were submitted to a pre-column derivatization with *o*-phthalaldehyde (OPA). The reagent solution for derivatization consisted of 200 mg OPA in 9 mL methanol, 1 mL 0.4 M sodium borate pH 10, and 160 µL 2-mecaptoethanol (MCE). Solvents used for separation were: solvent A: methanol, 10 mM sodium phosphate buffer, pH 7.3 and tetrahydrofuran (19:80:1) and solvent

B: methanol and 10 mM sodium phosphate buffer, pH 7.3 (80:20). Solvent gradient conditions were as follows: 6 min (0 B); 10 min (15% B); 4 min (30% B); 12 min (40% B); 16 min (80% B) and 5 min (0 B). All separations were performed on a Waters Nova-Pack C18 column (150 × 3.9 mm i.d., 60 Å, 4 μm) with a flow of 1.0 mL/min. The detection was by fluorescence using a model 121 fluorimeter (340 nm excitation filter and 425 nm emission filter). Samples were injected in triplicate onto the column, after being filtered through a 0.22 μm filter. Prior to RP-HPLC analysis, all samples were diluted with 0.4 borate buffer, pH 10. Standards of amino acids and dipeptides were used to determine the concentration of free amino acids and glycyl-glycine or leucyl-leucine respectively. The standard solutions were prepared by dissolving each amino acid or dipeptide in a 0.1 N HCl solution to reach a concentration of 2.5 μmol/mL. These solutions were stored at -18°C. Aliquots of 50, 100, 200, and 500 μL of these solutions were adjusted to 25 mL with borate buffer 0.4 M pH 10.

The pre-column derivatization and the column apparatus were at room temperature.

D-Glucose was measured by the glucose oxidase method (Kit from Wiener Laboratory, Rosario, Argentina). L-Malic acid was determined by an enzymatic method (Boehringer Kit, Mannheim, Germany).

Statistical analysis. The experimental data were analyzed by one-way analysis of variance test. Variable means showing statistical significance were compared using Tukey's test (Minitab student R12). All statements of significance are based on the 0.05 level of probability [16].

Results

Effect of dipeptides on *O. oeni* X₂L growth in synthetic medium. Figure 1 shows that in BM without L-malic acid *O. oeni* grew to a low cell density (5.53×10^6 cfu/mL). The L-malic acid incorporation markedly increased the growth rate and the population level about 3.4-fold. No growth was observed in the absence of glucose.

The *O. oeni* X₂L growth parameters in BM following individual addition of Met-Pro, Leu-Pro, Pro-Asp, Gly-Gly, Leu-Leu or a dipeptide mixture containing Met-Pro, Leu-Pro, Gly-Gly and Leu-Leu in place of one or four amino acids respectively, are reported in Table 2. The strain did not grow or the growth was lower than 10% when leucine, methionine, proline or glycine were individually removed from BM, confirming that they are essential amino acids for growth. When leucine or proline were replaced by Leu-Leu or Leu-Pro respectively, the growth parameters were similar to those observed in BM. When Met-Pro or Pro-Asp were incorporated as the source of methionine or proline respectively, and Gly-Gly as a donor of glycine, the growth rate increased 26% but not the extent of growth. The removal of aspartic acid from BM reduced by 70% the growth rate and the extent of growth, confirming its stimulatory effect. When Pro-Asp was incorporated as the source of aspartic acid, the growth parameters were similar to those obtained in BM.

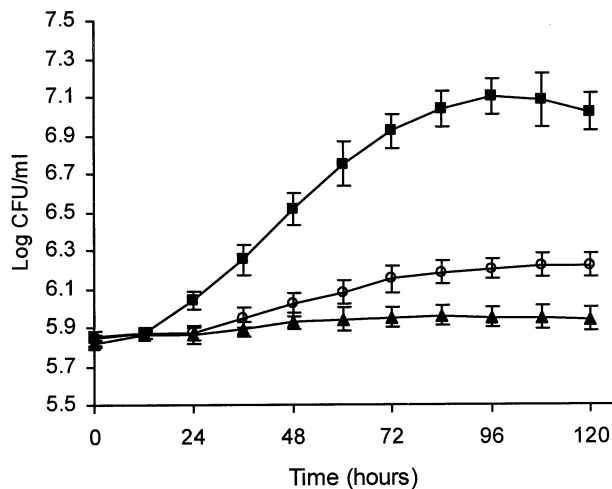


Fig. 1. *Oenococcus oeni* X₂L growth in synthetic medium. Without L-malic acid (○) with L-malic acid (■) and with L-malic acid without glucose (▲).

The addition of the dipeptide mixture in place of the respective free amino acids increased the growth rate 34%.

Dipeptide utilization by *O. oeni* X₂L. Figure 2 shows the dipeptide utilization by *O. oeni* X₂L in BM measured during bacterial growth at 24, 48, and 96 h of incubation. These experiments were carried out for Gly-Gly, which increased the growth rate, and for Leu-Leu, which did not modify growth parameters with respect to BM (Table 2). The utilization of Gly-Gly or Leu-Leu began immediately when growth began in BM deficient in glycine or leucine, respectively. Residual Gly-Gly or Leu-Leu concentrations decreased significantly at 48 h of incubation and represented 53.0% and 31.0% of the initial levels of Gly-Gly and Leu-Leu respectively. At the end of growth, Gly-Gly decreased by 1.16 mmol/L and Leu-Leu by 0.17 mmol/L, while the concentration of the hydrolysis dipeptide products increased by 0.48 mmol/L for glycine and 0.21 mmol/L for leucine. The fraction of glycine or leucine accumulated internally from dipeptides was 1.87 and 0.14 mmol/L, respectively.

The final consumption of free amino acids, shown in Table 3, was not significantly different to glycine or leucine accumulated internally from the respective dipeptide hydrolysis.

An interesting finding was the result shown in Table 4. Alanine utilization was 74% higher when Gly-Gly replaced glycine in the medium whilst no increase was observed when Leu-Leu replaced leucine in the medium.

Effect of dipeptides on glucose and L-malic acid utilization. *O. oeni* X₂L consumed 12.8%, 23.5%, and 30.5% of the initial glucose concentration at 24, 48, and

Table 3. Amino acid consumption by *O. oeni* X₂L in basal medium

Time (h)	Glycine (mmol/L)	Leucine (mmol/L)
24	0.225 ± 0.015	0.073 ± 0.002
48	1.383 ± 0.082	0.094 ± 0.005
96	1.632 ± 0.089	0.123 ± 0.005

Values are the means of three independent experiments ± SD. Initial concentrations in mmol/L: glycine 4.45, leucine 0.45.

Table 4. Alanine utilization by *O. oeni* X₂L in BM and in BM supplemented with Leu-Leu or Gly-Gly as sources of leucine or glycine respectively

Time (h)	Alanine consumption (mmol/L)		
	BM	Leu-Leu ^a	Gly-Gly ^b
24	0.420 ± 0.025	0.370 ± 0.035	0.550 ± 0.054
48	0.536 ± 0.023	0.516 ± 0.032	1.206 ± 0.043
96	0.777 ± 0.024	0.779 ± 0.036	1.339 ± 0.078

Values are the means of three independent experiments ± SD. Initial alanine concentration: 2.25 mmol/L.

^a Leu-Leu replaced leucine in BM.

^b Gly-Gly replaced glycine in BM.

96 h of incubation, respectively. L-Malic acid was co-metabolized with glucose during its growth and was completely utilized at the end of growth. Regarding glucose consumption at 24 h incubation, when Leu-Leu was added in place of leucine to BM, the amount of glucose utilization was 29% higher, even when no change was observed in the growth parameters. On the other hand when Gly-Gly was added in place of glycine, glucose utilization increased 63%, coinciding with the higher growth rate obtained in the presence of this dipeptide (data not shown).

No changes were observed in L-malic acid consumption when the dipeptides replaced free amino acids in BM.

Discussion

Saguir and Manca de Nadra [17] reported in *O. oeni* m strain, that the favorable effect of L-malic acid on bacterial growth results from an additional energy gain associated with L-malic acid decarboxylation.

When methionine, proline or glycine were replaced by Met-Pro, Pro-Asp, Gly-Gly or the dipeptide mixture containing Met-Pro and Gly-Gly in BM, *O. oeni* X₂L grew at a higher growth rate. When aspartic acid was replaced by Pro-Asp no modification of the microorganism's growth was observed. Taking account that

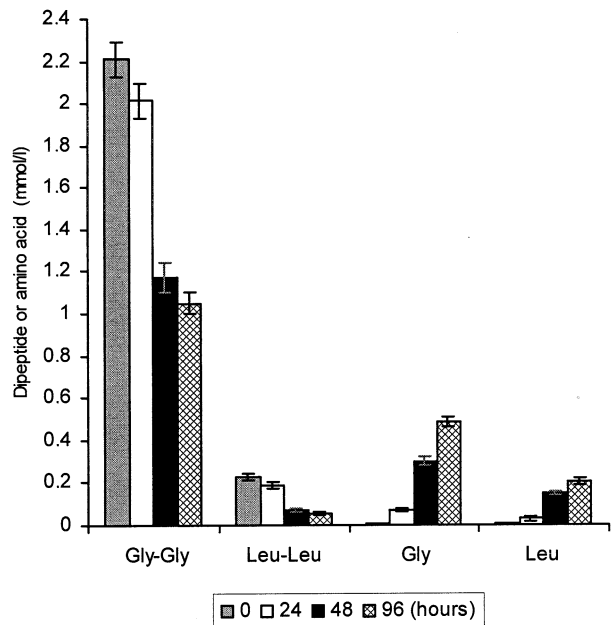


Fig. 2. Changes in concentrations of residual dipeptides and the parallel liberation of amino acids (indicated at the bottom) in cultures of *Oenococcus oeni* X₂L containing Gly-Gly or Leu-Leu as the source of glycine or leucine respectively at different incubation times.

methionine, proline, and glycine are essential amino acids for growth, the observation that dipeptides supplying two essential amino acids were more favorable than the free amino acids for growth is an important one. This could be related to the fact that dipeptides constituted by essential amino acids as the source of one of them, increase the concentration of the other present in the medium. For Leu-containing dipeptides (Leu-Leu or Leu-Pro) as the source of leucine, it was observed that the growth rate was not increased compared with that found in BM. This fact could be linked to a low utilization of leucine or Leu-containing dipeptides (Fig. 2). Foucaud et al. [7] reported that the growth response of *L. lactis* and *Leuconostoc mesenteroides* when dipeptides replaced the corresponding individual amino acid is dependent on the nature of the dipeptide.

Juillard et al. [10] emphasized the minor role of free amino acids relative to peptides during growth of Prt⁻ *L. lactis* subsp. *lactis* in milk. By contrast Van Boven and Konings [22] reported for *Lactococcus lactis* subsp. *cremoris* E8 that μ_{\max} values obtained in chemically defined media with dipeptides were the same as in amino-acid-containing media. Aredes Fernández et al. [3] reported that in *Pediococcus pentosaceus* c1 isolated from Argentinean wine a similar dipeptide mixture in place of the respective amino acids decreased the growth parameters with respect to those observed with free

amino acids, possibly due a limited amino acid uptake caused by a high concentration of peptides.

O. oeni X₂L consumed mainly glycine or glycine-containing dipeptide rather than leucine or leucine-containing dipeptide in BM, and only a small fraction of leucine was accumulated internally from Leu-Leu. So this could be linked to a better incorporation of glycine in cell material. The high Gly-Gly utilization was accompanied by an increment in the glycine efflux to the extracellular environment. Such 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. Thus, alanine utilization was 74% higher when Gly-Gly replaced glycine in the medium whilst no increase was observed when Leu-Leu replaced leucine in the medium. Rice et al. [15] reported that in *L. lactis*, alanine, threonine, and glycine were capable of exchange with “pool” glycine.

In BM supplemented with Leu-Leu in place of leucine during the first hours of incubation the higher glucose consumption could be related to the additional energy required for dipeptide hydrolysis. According to Van Boven and Konings [21], the Leu-Leu hydrolysis by whole cells of *L. lactis* ssp. *cremoris* Wg2 was dependent on the presence of the energy source lactose.

In conclusion: (i) in general, essential amino acids containing dipeptides such as methionine, glycine, and proline, had a more favorable effect than the free amino acids on the *O. oeni* X₂L growth rate but not on the extent of growth; (ii) Gly-Gly uptake was accompanied by a rapid exodus of amino acids to the extracellular medium; (iii) The higher Gly-Gly utilization significantly increased the glycine released outside the cell and the microorganism could employ it as an exchange mechanism for the incorporation of other amino acids such as alanine; (iv) glucose catabolism supplies energy for Leu-Leu uptake; and (v) L-malic acid catabolism that supplies additional energy for growth was not modified by incorporation of the individual or combined dipeptides.

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