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scientific note

# Effect of Physico-Chemical Factors on Growth of Starter Culture for Wine Malolactic Fermentation

# Djelovanje fizikalno-kemijskih parametara na rast starter kulture za jabučno-mliječnu fermentaciju vina

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#### Summary

The induction of the malolactic fermentation (MLF) by inoculating a wine with selected malolactic bacteria has, recently, received immense enologists' attention because of its positive influence on quality and sensory characteristics of wine. The strains of Leuconostoc oenos adapted to grow in adverse conditions of fermentation (i.e., low pH values and temperature, high concentrations of SO<sub>2</sub> and ethanol) were the most frequently used.

Since MLF of wines is performed in adverse conditions for bacterial growth in this paper the effects of temperature and pH lower than optimal, and different ethanol and SO2 concentrations on the growth of strain UR were investigated with optimal value of at least one factor of growth (T and/or pH). The simultaneous effect of these factors combined by Graeco-Latin Square method was also investigated. The optimal conditions determined for the growth of the investigated strain are: 37 °C and pH = 6.5. The growth of Leuconostoc oenos UR was good at 25 °C, while lower pH (3.0 and 4.0) and higher ethanol volume fractions ( $\varphi = 10$  and 12%) inhibited bacterial growth significantly. The investigation of SO2 concentration (30, 50 and 100 mg/L) showed that only the highest SO2 concentration in medium slightly suppressed bacterial growth after 24 hours of cultivation. The growth of L.oenos UR in the conditions combined by Graeco-Latin Square method was significantly suppressed. Namely, after 24 hours of growth under such conditions, the maximal cell concentration ratio (CCR) was only 15 % of CCR value obtained at the same unfavourable pH, but in otherwise optimal conditions. The ratio between sums of squares of separate variables and total sums of squares (expressed as a percent), estimates the inhibitory influence of each variable in Greaco-Latin Square Design on the growth of L.oenos UR. It was estimated that the share of medium pH value is the most significant: 73.0 % and 88.4 % after 24 hours and 120 hours of growth, respectively.

#### Introduction

Lactic acid bacteria make a significant contribution to the quality and stability of wines by conducting malolactic fermentation (MLF). Wine composition and, conse-

#### Sažetak

Poticanje jabučno-mliječne fermentacije (JMF) u vinima inokulacijom odabranim bakterijama mliječne kiseline zaokuplja sve veću pažnju enologa radi povoljnih utjecaja na kakvoću i senzorske karakteristike vina. U tu svrhu najčešće se koriste sojevi vrste Leuconostoc oenos prilagođeni rastu u nepovoljnim uvjetima pod kojima se provodi fermentacija (tj. niske vrijednosti pH i temperature, visoke koncentracije SO<sub>2</sub> i etanola).

U radu su istraženi optimalni uvjeti rasta (pH podloge i temperatura) bakterije L.oenos soj UR u MRS podlozi u svrhu proizvodnje starter kulture za JMF. Budući da se JMF u vinima provodi u uvjetima nepovoljnim za bakterijski rast, u radu je istražen i utjecaj temperatura i vrijednosti pH nižih od optimalnih, različitih koncentracija etanola i SO2 na rast soja UR, uz uvijet da je bar jedan od parametara rasta (T i/ili pH) bio optimalan. Isto je tako istražen i utjecaj tih parametara kombiniranih metodom grčko-latinskog kvadrata. Optimalni uvjeti za rast istraživanog soja su 37 °C i pH podloge 6,5. Pri 25 °C L.oenos UR raste dobro, dok niže vrijednosti pH podloge (3,0 i 4,0 ) i veći volumni udjeli etanola ( $\varphi=10$  i 12 %) značajno inhibiraju bakterijski rast. Od istraživanih koncentracija SO2 (30, 50 i 100 mg/L) samo je u podlozi s najvećom koncentracijom SO2 uočen nešto slabiji rast nakon 24 sata uzgoja. Rast L.oenos UR značajno je potismut u uvjetima kombiniranim prema grčko-latinskom kvadratu. Naime, nakon 24 sata rasta u ovim uvjetima, maksimalni postignuti faktor preživljavanja (CCR) iznosio je samo 15 % od vrijednosti CCR dobivene pri istom nepovoljnom pH, ali uz inače ostale optimalne uvjete rasta. Odnosom suma kvadrata pojedinih varijabli i ukupne sume kvadrata (izražene kao postotak) određen je inhibitorski utjecaj pojedine varijable u grčko-latinskom kvadratu na rast L.oenos UR. Udjel vrijednosti pH podloge je najznačajniji: 73,0 % nakon 24 sata, odnosno 88,4 % nakon 120 sati rasta istraživane bakterije.

quently, wine quality are altered by these bacteria, because some wine components are utilized as substrates for growth, and metabolic end products are excreted into the wine. Many qualitative studies, and more recent quantitative data have shown that *Leuctonostoc oenos* is the species most frequently occurring in wine (1-3) and is mainly responsible for MLF. MLF is considered desirable in high acid wines produced from grapes grown in relatively cool climates because such wines become more palatable after deacidification (4). Although it is desirable, initiation of MLF is often difficult (5) since many factors inhibit the growth of malolactic bacteria in wine. The most important ones are the low pH, high concentration of ethanol and sulfur dioxide.

In this study the effect of selected physico-chemical factors on growth kinetics of *Leustonostoc oenos* strain UR, potentially usable starter culture for wine MLF, was investigated.

### Experimental

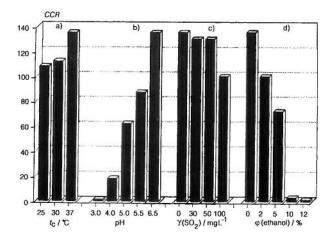
The Leustonostoc oenos strain UR used in this study was kindly provided by Professor A. Maujen, Faculte de Sciences, Reims. Routinely, the strain was subcultured twice a month on MRS (Biolife, Milano) slants (pH = 5.0) at 30 °C (6). Inoculum was prepared by cultivation of bacterium in MRS broth (pH = 5.0) in thermostat at 30 °C for 16 hours. All experiments were performed stationary in 1 L capacity E. flasks and 700 mL of MRS broth. Inoculation size was 10 mL/L. The separate effect of temperature (25, 30 and 37 °C), pH (3.0, 4.0, 5.0, 5.5 and 6.5), ethanol ( $\varphi = 2$ , 5, 10 and 12%) and SO<sub>2</sub> ( $\gamma = 30$ , 50 and 100 mg/L), and the effect of the same factors combined by Graeco-Latin Square Design (Table 1, 7) on bacterial growth were investigated. Graeco-Latin Square Design allows simultaneous investigation of four factors (i.e., T, pH, SO<sub>2</sub> and ethanol) each at p levels (p = 3 was chosen) in only p<sup>2</sup> runs (Table 1). To investigate the effect of factors separately, factor values were the following: the pH of the medium 6.5 (except in experiments with different pH values), and growth temperature 37 °C (except in experiments with different temperatures). In each case, the pH value was adjusted with ortho-phosphoric acid before sterilization and confirmed afterwards. The SO2 in the form of K2S2O5 and abs. ethanol were added to the media after sterilization. Bacterial growth was determined by plate count method placing the 10 mL of sample decimal dilutions (min. two) in triplicate, on MRS agar and incubating at 37 °C for 48 hours. The cell concentration of Loenos UR after 24 hours (separate factors), and 24 and 120 hours (combined factors) of growth was determined. The results were expressed as the concentration of cells, i. e., the number of cells, corresponding to the number of colony forming units (CFU) per milliliter of culture (N). The cell concentrations ratio (CCR) i. e., the ratio between concentration of bacterial cells after 24  $(N_{24})$ and 120 (N<sub>120</sub>) hours of growth and starting cells concentration  $(N_0)$  were taken for comparing the influence of investigated factors on bacterial growth.

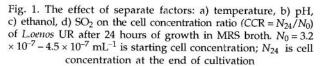
#### Results

The influence of separate parameters (i.e., T, pH,  $SO_2$  and ethanol) on *CCR* of *L.oenos* UR is shown in Fig.1. The optimal growth conditions for *L.oenos* in MRS broth were  $t_c = 37$  °C and pH = 6.5 (Fig. 1.a and b). The growth ratio for cells grown at 25 °C was about 3/4th of that obtained in optimal conditions. Decrease of pH value from 6.5 to 3.0 (Fig. 1.b) and increase of ethanol volume factors from 5 to 12 % (Fig. 1.c) significantly influenced the *L.oenos* UR growth. The CCR of *L.oenos* UR slightly de-

Table 1. Design of experiment by Graeco-Latin Square method (according to 7), with four factors each at p levels in  $p^2$  runs Tablica 1. Plan pokusa prema grčko-latinskom kvadratu (prema 7), sa četri varijable, svaka na p razina u  $p^2$  kombinacija

Factors Čimbenici	Levels Razine P	<i>Media</i> (p² runs) <i>Podloge</i> (p² kombinacija)								
		1	2	3	4	5	6	7	8	9
SO <sub>2</sub> γ/mgL <sup>-1</sup> Columns Stupci	SO <sub>2</sub> -1 SO <sub>2</sub> -2 SO <sub>2</sub> -3	10	30	50	10	30	50	10	30	50
pH Rows Redovi	pH-1 pH-2 pH-3	3.0	3.0	3.0	3.5	3.5	3.5	4.0	4.0	4.0
Ethanol Etanol φ/% Latin letters Latinska slova	A B C	4	7	10	7	10	4	10	4	7
Temperature Temperatura $t_c/^{\circ}C$ Greek letters Grčka slova	$egin{array}{c} lpha \ \gamma \ eta \end{array}$	15	25	20	20	15	25	25	20	15





Slika 1. Djelovanje pojedinih parametara: a) temperatura; b) pH; c) etanol; d) SO<sub>2</sub> na faktor preživljavanja ( $CCR = N_{24}/N_0$ ) L.oenos UR nakon 24 sata uzgoja u MRS podlozi.  $N_0 = 3.2 \times 10^{-7} - 4.5 \times 10^{-7}$  mL<sup>-1</sup> je početna koncentracija stanica;  $N_{24}$  je koncentracija stanica na kraju uzgoja

creased by addition of SO<sub>2</sub> in the medium (Fig. 1.d). The cell concentration ratio after 24 and 120 hours of bacterial growth in media combined by the Graeco-Latin Square method (Table 1) are shown in Fig. 2.

#### Discussion

The extent of physico-chemical factors known to affect the growth of malolactic bacteria (i.e., pH, T, SO<sub>2</sub> and ethanol) greatly depends on the species (3) or even the bacterial strain (8,9). Among leustonostocs the Leuconostoc ocnos is the species reported to be almost exclusively present in wines, wineries and vineyards (1) and is considered as the malolactic bacterium »par excellence«. Although it was found that the optimal temperature for growth of Leuconostoc oenos is between 20 and 25 °C (10,11) and pH between 4.4 and 4.6, the best growth of L.oenos UR was obtained at  $t_c = 37$  °C, and pH = 6.5. Examining the influence of separate factors on Locnos UR growth at least one factor (pH and/or T) of growth was optimal. The highest negative effect on bacterial growth produced the low pH value of growth medium (below 5.0, Fig. 1.b) , and the high alcohol content (above  $\varphi = 5$  %, Fig. 1.c). However, although the bacterial growth was significantly suppressed, even at the lowest pH and highest alcohol concentration some growth occurred (Fig. 1). The cumulative negative effect of investigated factors is evident. In media with combined factors (Fig. 2) much lower CCR, either after 24 or 120 hours of growth were obtained, compared to the bacterial growth resulted from investigations of separate factors (Fig. 1). For example, the maximal CCR after 24 hours of growth, achieved in medium 8 (Fig. 2), was about 85 % smaller than that obtained in medium with the same pH (pH = 4.0) but without the influence of the other growth inhibiting factors (Fig. 1). This is in accordance with the published data (3,8,12).

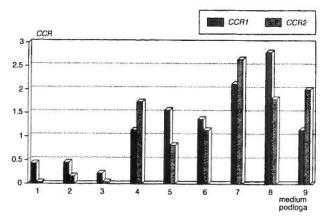


Fig. 2. The influences of SO<sub>2</sub>, ethanol, starting pH and temperature interactions (media 1–9) on the cell concentration ratios (CCR) of *L.oenos* UR in MRS broth after 24 ( $CCR_1 = N_{24}/N_0$ ) and 120 ( $CCR_2 = N_{120}/N_0$ ) hours of cultivation

Slika 2. Utjecaj djelovanja različitih koncentracija  $SO_2$ , etanola, početnih vrijednosti pH podloge i temperatura (podloge 1–9) na faktore preživljavanja (CCR) bakterije *L.oenos* soj UR u MRS podlozi nakon 24 ( $CCR_1 = N_{24}/N_0$ ) i 120 ( $CCR_2 = N_{120}/N_0$ ) sati uzgoja

Namely, the negative effect of pH lowering is increasing when the medium contains SO<sub>2</sub>, because at lower pH values (below 5.0) the free SO<sub>2</sub> concentration (known antimicrobial agent in wine), increases (13). According to the literature (8,14) alcohol tolerance of malolactic bacteria is greatly affected by temperature and pH, and it decreases as the temperature rises and pH decreases. Ethanol is known as the main inhibitor of bacterial growth, but L.oenos UR can grow in medium with  $\varphi = 10\%$  of ethanol (Fig. 1.d), even at pH below optimal value and in the presence of the other inhibiting factors (Fig. 2, medium 7). In combined factors conditions the best cell concentration ratios were achieved in medium 8 and 7 after 24 and 120 hours of growth, respectively. Results presented in Fig. 2 indicate that Locnos UR is able to adapt and even grow in unfavorable conditions. Namely, the CCR values obtained after 120 hours of growth in media 4, 7 and 9 (Fig. 2) were by 35, 20 and 43 % higher than after 24 hours of growth in the same media, respectively. The ratio between sums of squares of individual variables (SS<sub>Rows</sub>, SS<sub>Columns</sub>, SS<sub>Latin letter</sub> and SS<sub>Greek letter</sub>) and total sums of squares (SS<sub>Total</sub> = 5.67 for  $N_{24}/N_0$  and SS<sub>Total</sub> = = 7.32 for  $N_{120}/N_0$ ), expressed as a percent, was used to estimate the negative influence of each variable in Graeco--Latin Square Design on the growth of Locnos UR. The obtained values for pH, SO2, ethanol and temperature are 73.0, 12.7, 10.6 and 3.4 % (for  $N_{24}/N_0$ ) and 88.4, 6.7, 1.9 and 2.9 % (for  $N_{120}/N_0$ ), respectively.

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