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Influence of Temperature and Carbon Dioxide on Fermentation of Cabernet Sauvignon Must

Marin Berovič[1,2], Jan Mavri[1], Mojmir Wondra[3], Tatjana Košmerl[3] and Dejan Bavčar[4]*

[1]Department of Chemical, Biochemical and Ecological Engineering, University of Ljubljana, Hajdrihova 19, SI-1115 Ljubljana, Slovenia

[2]National Institute of Chemistry, Hajdrihova 19, SI-1115 Ljubljana, Slovenia

[3]Department of Food Technology, Biotechnical Faculty, University of Ljubljana Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

[4]Department of Enology, Agricultural Institute of Slovenia, Hacquetova 17, SI-1001 Ljubljana, Slovenia

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Summary

In the process of wine fermentation temperature and the amount of carbon dioxide present represent parameters that can be easily monitored and controlled. The influence of variation of the process temperature and the fluxes of additional inlet gaseous carbon dioxide in *Saccharomyces bayanus* fermentation of Cabernet Sauvignon grape must on the accumulation of biomass and production of metabolites was studied. All experiments with temperature and redox potential control on-line were performed in a 10-litre laboratory stirred tank reactor. Metabolites of *Saccharomyces bayanus* fermentation comprising higher alcohols (1-propanol, 2-butanol, isoamyl alcohol), as well as reducing sugars, were measured off-line by gas and high pressure liquid chromatography.

Key words: fermentation, temperature, carbon dioxide, redox potential

Introduction

In living organisms oxidation-reduction systems play so intimate and such an essential part that life itself might be defined as a continuous oxidation-reduction reaction. It is not surprising, therefore, that theoretical speculations and experimental studies on oxidation and reduction processes in animals and plants have been actively pursued since the isolation of oxygen over 150 years ago *(1).*

A redox potential measurement represents the sum of the potentials of all oxido-reduction processes in a particular fermentation. In this measurement, the metabolic activity in microbial cells plays the most significant role *(2)*, and it is influenced by reversible oxido-reduction couples, irreversible reductors and by the activity of free oxygen and hydrogen *(3).* A redox potential measurement depends on the pH value, equilibrium constant, oxido-reduction potentials in the substrate and the dissolved oxygen concentration *(4).*

Redox potential was used as a relevant parameter for bioprocess monitoring in several aerobic processes

* Corresponding author; Phone: ++386 1 2419 510; Fax: ++386 1 4760 300; E-mail: marin.berovic@uni-lj.si

with various microorganisms: *Pseudomonas (5)*, *Actinomyces levoris (6)*, *Corynebacterium glutamicum (7)*, as well as in anaerobic processes where the significance of the redox potential has been defined *(8–10)*. In the production of xylitol by a recombinant *Saccharomyces cerevisiae* containing the xyl-1 gene of *Pichia stipatis*, the xylitol yield of the substrate increased with the increase of xylose activity, which was indicated by a high value of the redox potential *(11)*. The redox potential was also used as a key control parameter in the on-line estimation of viable cells in a hybridoma culture at various DO levels *(12)*.

In citric acid fermentation by *Aspergillus niger*, it was found that for a high yield production, particular redox potential levels of fermentation are of essential importance *(13,14)*. The redox potential was also very successfully used as a key parameter in the scale-up of submerged citric acid fermentation from a 10-litre laboratory scale to the 100- and 1000-litre scale in geometrically non-similar stirred tank reactors, where redox time profile regulation, over-aeration and mixing were used *(14,15)*.

Materials and Methods

Microorganism

Selected dry yeast (*Saccharomyces bayanus*, Lalvin QA23 Lallemand Inc., Montreal, Canada, H1W2N8) was used in all the experiments. Reactivation of 2.5 g of yeast was done according to the producer, in 200 mL of water diluted Cabernet Sauvignon grape must at 25 °C for 30 min. The inoculum was equivalent to 0.3 g of dry yeast per litre of must.

Fermentation substrate

In the present experiments grape must of Cabernet Sauvignon, vintage 1998, from the Vipava Agroind 1894 Winery (Reg. No. GO-810/98), with the concentration of reducing sugars of 270 g/L and total acidity of 7.8 g (tartaric acid)/L, was used. The amount of 0.25 g/L of Fermevit (100 g of Fermevit contains: thiaminchloride hydrate 220 mg, ammonium phosphate 85 g and potassium bicarbonate 15 g) (TOKIS, Slovenia) was added to the substrate. A working volume of 10 L was used in all experiments. The substrate was not sulphurized before the beginning of the fermentation.

Fermentor

All laboratory scale experiments were performed in the standard 10-litre working volume stirred tank reactor (Bioengineering AG, Switzerland). The fermentor was equipped with a sterilizable Ingold pH electrode, Ingold redox electrodes (platinum as indicator and calomel as reference electrode), Ingold pO_2 electrodes and pCO_2 electrodes, together with an automatic temperature control unit.

For on-line measurements of pH, temperature, oxygen partial pressure and redox potential in the bioreactor, SHIVA control sofware (BIA d.o.o., Slovenia) was installed. All the experiments were performed at low stirrer speed N = 50 rpm. Carbon dioxide (Messner, Austria), at a gas flow of Q_g = 40 L/h, was injected 24 h after the inoculation.

To prevent the oxidation of the substrate, in all experiments the fermentor headspace was aerated with nitrogen (Q_g = 0.1 L/min). To check for anaerobic conditions or the absence of air/oxygen leakage into the fermentation broth, pO_2 control was applied, and the value of pO_2 = 0 was maintained.

Biomass

The volume of 20 mL of the sample was centrifuged at 4000 rpm for 5 min. The sediment was washed twice with deionized water. Biomass was determined as dry weight after filtering and drying at 105 °C for 24 h.

Analysis

Saccharomyces bayanus metabolites as higher alcohols (1-propanol, 2-butanol, isoamyl alcohol) as well as reducing sugars were determined off-line by gas chromatography according to Ough and Amerine (*16*). A GC Varian Model GC 3700, Austria, was used, with *n*-butanol as internal standard. An HPLC isochratic system including an HPLC type 64 pump, an injection valve with 20-μL injection loop, a UV-VIS spectrophotometer no. A0293 with 10-μL flow cell (0.1 response time) with Eurochrom 2000 software and data processing system were applied. All instruments were obtained from Knauer, Germany.

Organic acids, reducing sugars, ethanol and higher alcohols (1-propanol, 2-butanol, isoamyl alcohol) in grape must and wine were analysed by HPLC in accordance with the validated methods proposed by BIO--RAD (*17*). Samples were filtered through 0.45-μm membranes and analysed using a 300 × 7.8 mm Aminex HPX-87H organic acid analysis cation exchange column (Bio-Rad Laboratories). Elution was performed at 65 °C. The mobile phase was 0.005 M H_2SO_4 in Milli-Q water. The pump was operated at a flow rate of 0.5 mL/min ($0.008 \cdot 10^{-3}$ L/s). The injection volume was 20 μL. The eluting compounds were monitored by a fixed wavelength ultraviolet (UV-VIS) detector at 210 nm. This detector was connected in series with a refractive index (RI) detector. Glucose, fructose and ethanol were detected by RI. The peaks were quantified using external standard calibration. The components were identified by a comparison of their retention times with those of the standards. Quantification was performed using external standards prepared from pure compounds.

Each experiment was performed in triplicate and the average result of each series is presented. A mixing speed of 50 rpm was applied in all experiments.

Results and Discussion

In wine technology the redox potential might represent a fast, accurate and reliable measurement which also gives very important information related to the oxidation and reduction ability of the wine, its alteration and its influence on quality and stability (*18*). Several process parameters influence wine yeast metabolism and therefore the final quality of the products. The process temperature and the amount of carbon dioxide

present are two parameters that significantly influence the rate of yeast metabolism and the intensity of the formation of the product. In this sense redox potential represents an important insight into the metabolic activity of a microbial culture. The influence of temperature and carbon dioxide on yeast metabolism could be well reflected in the on-line measurement of redox potential, which can also be applied as a tool for efficient regulation of the process and fermentation control *(19).*

In this research the influence of temperature and of the inlet flux of gaseous carbon dioxide on *S. bayanus* wine yeast fermentation metabolism of Cabernet Sauvignon grape must was studied.

Temperature represents one of the most significant parameters affecting the rate of the fermentation process and the production of various metabolic products that are reflected in the final wine quality. According to the Arrhenius equation, higher fermentation temperature, up to 35 °C, increases the rate of metabolic reactions. This change is also significantly reflected in the ratio between the activities of oxidants and reductants presented in the Nernst equation /1/.

$$E = E_{\mathrm{calomel}} + RT/nF \ln a_{\mathrm{ox}}/a_{\mathrm{red}} \qquad /1/$$

where E_{calomel} = 244 mV E_0

In the first part of our experiments fermentation temperatures of 18, 22 and 26 °C were applied. Ethanol and higher alcohols (1-propanol, 2-butanol, isoamyl alcohol), as the products of *Saccharomyces bayanus* metabolism, were measured off-line. The highest biomass and ethanol production were detected at 26 °C (Figs. 1 and 2). Yeast multiplication occurred much faster at higher temperature compared to that at lower temperature. The amount of ethanol production could also indicate the intensity of primary metabolism. In relation to the anaerobic conditions of the biosynthesis, available glucose and fructose were converted to ethanol, higher alcohols, CO_2, water and the reaction free energy was converted to ATP.

The increase of fermentation temperature also influences the production of 1-propanol, 2-butanol and isoamyl alcohol (Figs. 3a, b and c). In the case of 1-propanol, a maximum of 18 mg/L was detected at 26 °C after 92 h, while at 22 °C the same amount was found 52 h later, after 142 h of fermentation. At 18 °C, 16 mg/L of 1-propanol was detected after 142 h (Fig. 3a).

In the case of 2-butanol, fermentation produced 25 mg/L at 26 °C after 118 h, 17 mg/L at 22 °C after 164 h and 15 mg/L at 18 °C after 140 h of fermentation (Fig. 3b).

Similar results were found related to the production of isoamyl alcohol. A maximum of 345 mg/L was detected at 26 °C after 90 h, while at 22 °C nearly the same amount (348 mg/L) was found after a delay of 32 h, after 122 h of fermentation (Fig. 3c). At the temperature of 18 °C, 290 mg/L of isoamyl alcohol was detected after 140 h.

Similarly to the growth of biomass and ethanol, higher alcohol production is also related to the activation of the enzymes involved in ammonia anabolism that takes place during the growth of biomass. A higher

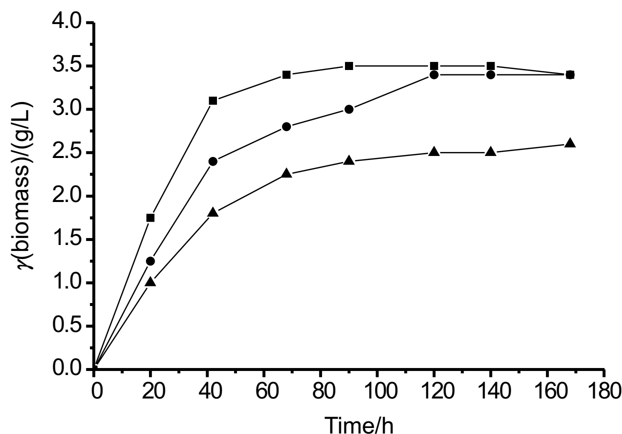

Fig. 1. Biomass accumulation (▲T = 18 °C, ● T = 22 °C, ■ T = 26 °C)

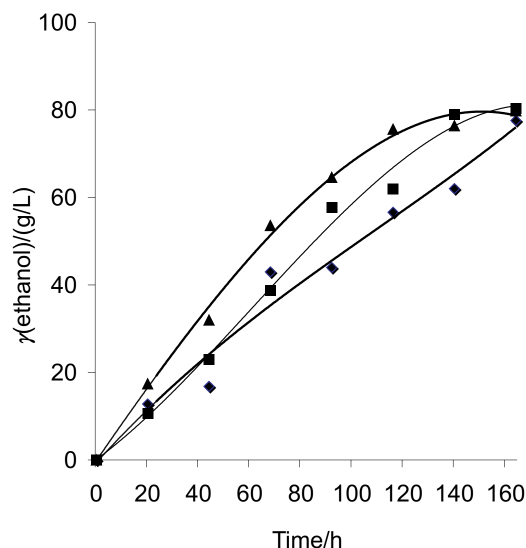

Fig. 2. Ethanol accumulation (◆ T = 18 °C, ■ T = 22 °C, ▲ T = 26 °C)

process temperature induces an increase in the enzyme activity by the reduction of the activation energy of the bioprocess. Fermentation temperature lower than 18 °C reduces the rate of *Saccharomyces bayanus* metabolism and therefore the production of ethanol and higher alcohols (Figs. 3a, b and c).

Of the available sugars in the must, consumption of glucose started first. At a temperature of 26 °C, glucose was totally consumed after 120 h, while at 22 °C this occurred after 140 h and at 18 °C after 164 h. The consumption of fructose at various temperatures paralleled that of glucose, while the rates of consumption decreased with the decrease of the fermentation temperature. In fermentation at 18 °C 20 g/L of residual fructose were detected after 168 h. The highest temperature of fermentation resulted in faster yeast metabolism, related to faster glucose and fructose uptake (Fig. 4). This was also indicated in a faster decrease in redox potential (Fig. 5).

Wine fermentation generally shows a growth pattern consisting of four phases – lag, exponential growth,

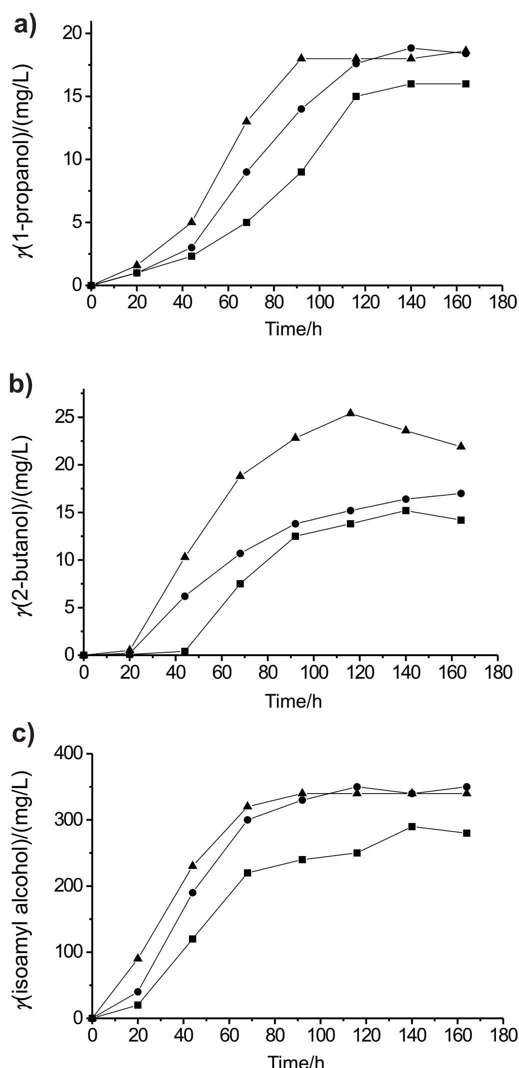

Fig. 3. Production of a) 1-propanol, b) 2-butanol and c) isoamyl alcohol at various fermentation temperatures (■ T =18 °C, ● T =22 °C, ▲ T =26 °C)

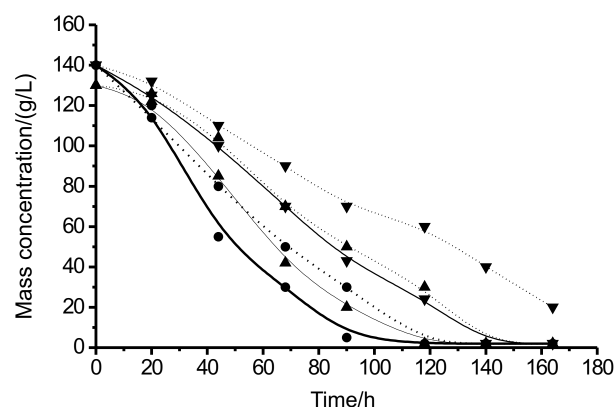

Fig. 4. Consumption of glucose (solid lines) and fructose (dotted lines) (▼ T = 18 C, ▲ T = 22 °C, ● T = 26 °C)

stationary and decline phases. During the lag phase a pseudo-aerobic fermentation process occurs, which is significantly important. In this phase a significant amount of biomass is produced and most of the acetaldehyde is synthesized *(20,21)*. High levels of acetaldehyde may cause the inhibition of the yeast growth and could significantly diminish the glucose consumption rate. In the next phase the activation of the enzyme alcohol dehydrogenase starts. This enzyme converts acetaldehyde into ethanol and the reductive conditions in the system are achieved.

During the exponential phase of yeast growth, larger amounts of ethanol production are achieved. This phase is followed by an abrupt decrease of redox potential. Soon after that the stationary phase follows, when the accumulated ethanol finally blocks microbial growth and prevents yeast metabolism. In this phase the redox potential reaches a steady value, which stays unchanged until the end of fermentation. The inhibiting effect of ethanol concentration on the yeast growth rate depends strongly on the fermentation temperature *(22)*.

Redox potential changes are well related to the production of ethanol. At the minimum measured value of redox potential, the concentration of ethanol reaches its maximal value and becomes constant until the end of fermentation. The results of this process are also reflected in the experimental changes of on-line redox potential values (Fig. 5).

At 18 °C the lag phase extended from 0 to 24 h. The exponential phase, from 24 to 35 h, followed and then from 35 to 155 h of fermentation it turned to the stationary phase. At the temperature of 22 °C the lag phase was 10 h shorter, from 0 to 14 h, and after a somewhat more extended exponential growth phase from 14 to 24 h, it turned from 24 to 155 h into the stationary phase. In the last run at 26 °C the lag phase took only 4 h. From 4 to 24 h it turned into a more extended exponential growth phase, which after 24 up to 155 h turned into the stationary phase. At the fermentation temperature of 26 °C, the minimum redox potential value of –150 mV was obtained after only 20 h. After this, the redox potential slowly decreased again and at 120 h it became constant at its minimal value of –240 mV.

The difference between the initial measured redox potential value and the minimal one (ΔE) was 430 mV at 26 °C, while at 18 °C it was only 280 mV. This means that the process at 26 °C was converted into a more stable and reductive environment (Fig. 5).

In the second group of experiments the influence of additional gaseous CO_2 was studied. The presence of higher CO_2 partial pressure reduces the cell activities, resulting in lower ethanol production as well as in the biosynthesis of higher alcohols. Introducing additional carbon dioxide to the fermentation broth, it was possible to reduce the transport of metabolites from the yeast cells towards the liquid phase. A higher concentration of CO_2 in the cell decreases cell activities and is also significantly reflected in the product synthesis (Figs. 6a, b and c).

Introduction of carbon dioxide results in additional resistance against pyruvate decarboxylation towards acetaldehyde, since a change in the balance between carboxylation and decarboxylation reactions was obtained *(23)*. The major inhibitory effects of CO_2 result in changes of yeast membrane composition and its perme-

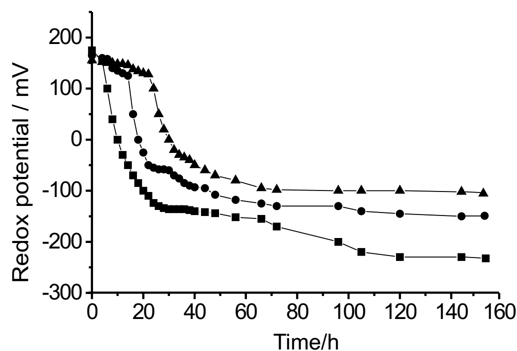

Fig. 5. Redox potential in Cabernet Sauvignon must (▲ T=18 °C, ● T =22 °C, ■ T =26 °C)

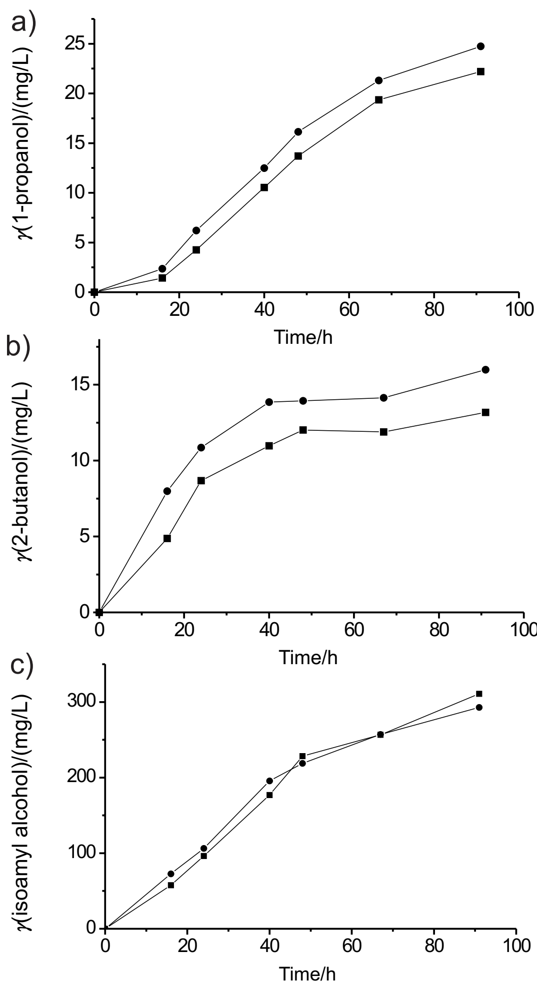

Fig. 6. Production of a) 1-propanol, b) 2-butanol and c) isoamyl alcohol (■ with additional CO_2 and ● without addition of CO_2, at T = 26 °C)

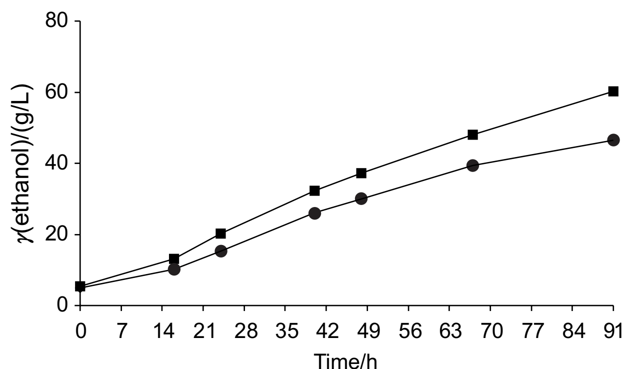

Fig. 7. Production of ethanol at T = 26 °C (■ with introduction of CO_2, ● without introduction of CO_2)

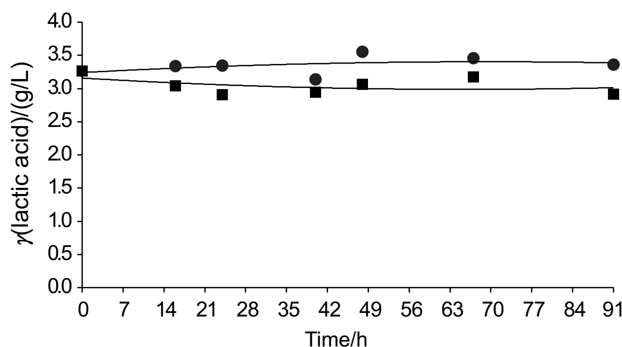

Fig. 8. Production of lactic acid at T = 26 °C (■ with addition of CO_2, ● without addition of CO_2)

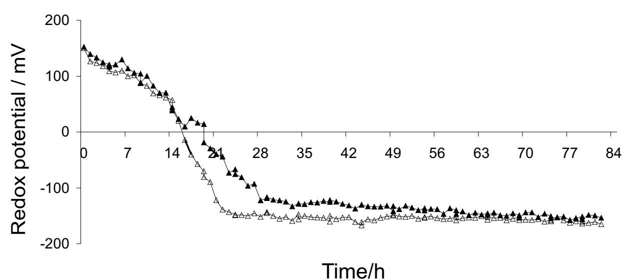

Fig. 9. Redox potential in Cabernet Sauvignon must (▲ with addition of CO_2, Δ without addition of CO_2)

ability *(24).* As a possible consequence limited reoxidation of NADH and dehydrogenase enzymes may occur. The yeast metabolism is therefore much slower and results mostly in reduced production of ethanol and other higher alcohols.

Increased production of ethanol, as a reductive substance, is also reflected in a decrease of redox potential. By introduction of additional CO_2 it was possible to reduce the rates of enzyme activities and ethanol biosynthesis (Fig. 7). In contrast, the introduction of CO_2 does not inhibit lactic acid biosynthesis (Fig. 8). The flux of carbon dioxide inhibits the metabolic activities of the yeast cell, which has the consequence of a slower decrease of redox potential compared to the case without the application of CO_2 (Fig. 9).

Conclusions

The first lag phase or pseudo-aerobic phase was found to be a period of significant importance. In this phase the essential amount of biomass needed for the

further fermentation process was produced and most of the acetaldehyde was excreted.

At a higher fermentation temperature of 26 °C, this phase was the shortest, while in the experiments at 18 and 22 °C it was much more prolonged and the transient phase where microbial growth turns from aerobic to anaerobic growth was much more pronounced. These changes were well indicated by redox potential measurements. A low oxygen concentration at the start of the fermentation was found to be of essential importance for the establishment of the quantity of yeast biomass needed for further fruitful fermentation process. In the exponential growth phase that follows, the enzyme alcohol dehydrogenase, reducing acetaldehyde into ethanol, was activated. The rate of its activity also increased at higher fermentation temperatures.

Additional input of carbon dioxide also influenced the reduction of *Saccharomyces bayanus* metabolic activity, which was reflected in lower ethanol and 2-butanol biosynthesis, while the production of 1-propanol, isoamyl alcohol as well as of lactic acid was not affected *(25).*

Variation of the process temperature, as well as additional input of carbon dioxide could be applied as useful regulatory parameters controlling the rate of the fermentation process, and the production of *Saccharomyces bayanus* metabolites.

Finally, there are also some new remarkable findings, related to the agreement of the redox potential with the measurements themselves, which have to be considered in further investigations. According to the version of the Nernst equation /2/, it is likely that the platinum electrode provides a catalytic surface upon which the oxidation of ethanol (and/or some other oxidizable components) is coupled with the reduction of oxygen (or protons).

$$E_\mathrm{h} = E_0 + RT/nF \ln a_\mathrm{oxidants} / a_\mathrm{reductants} \qquad /2/$$

So it seems that the potential established at the electrode is the one at which the anodic current matches the cathodic current for these processes. Instead it appears that the platinum redox electrode is somewhat selective in those compounds which significantly affect the measurements, so different readings could be obtained if other supposedly »inert« electrodes such as gold or glassy carbon were used *(26).*

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References

1. L. F. Hewitt: *Oxidation-Reduction Potentials in Bacteriology and Biochemistry*, Livingstone, Edinburgh (1950) pp. 8–12.

2. M. Berovic: Submerged citric acid fermentation in stirred tank and bubble column reactor, *PhD thesis*, University of Ljubljana (1986).

3. I. L. Rabotnova, *Die Bedeutung physikalisch-chemischer Faktoren für die Lebenstätigkeit der Bakterien*, Fischer-Verlag, Jena (1963) pp. 62–78.

4. A Ishizaki, H. Shibai, Y. Hirose, *Agr. Biol. Chem. 38* (1974) 2399–2405.

5. R. P. Tengerdy, *Biotechnol. Bioeng. 3* (1961) 255–272.

6. V. I. Sukharevich, E. P. Yakovljeva, V. A. Tsyganov, N. N. Shvezova, *Mikrobiologya*, *39* (1970) 981–985.

7. M. K. Radjai, R. T. Hatch, T. W. Cadman, *Biotechnol. Bioeng. Symp. 14* (1984) 657–672.

8. M. Peck, D. P. Chynoweth, *Biotechnol. Bioeng. 39* (1992) 1151–1160.

9. B. Heppner, G. Zellner, H. Diekmann, *Appl. Microbiol. Biotechnol. 36* (1992) 6.

10. S. Beck, B. Schink, *Arch. Microbiol. 163* (1995) 182–187.

11. J. Hallborn, M. F. Gorwa, N. Meinander, M. Penttila, S. Keranen, B. Hanhagerdal, *Appl. Microbiol. Biotechnol. 42* (1994) 326–333.

12. K. Eyer, E. Heinzle, *Biotechnol. Bioeng. 49* (1996) 277–283.

13. M. Berovic, A. Cimerman, *Eur. J. Appl. Microbiol. Biotechnol. 16* (1982) 185–188.

14. M. Berovic, *Biotechnol. Bioeng. 64* (1999) 552–557.

15. M. Berovic, M. Rošelj, M. Wondra, *Food Technol. Biotechnol. 38* (2000) 193–201.

16. C. S. Ough, M. A. Amerine: *Methods for Analysis of Musts and Wines*, John Wiley & Sons Inc., New York (1988) pp. 80–108.

17. BIO-RAD *Guide to Aminex HPLC Columns for Food and Beverage Analysis*, Richmond (1997) pp. 17–24.

18. Z. Dikanovic-Lucan, A. Palic, *Bulletin de L'O.I.V.* (1995) 762–779.

19. L. M. Balakireva, V. M. Kantere, I. L. Rabotnova, *Biotechnol. Bioeng. Symp. 4* (1974) 769–780.

20. J. Ribéreau-Gayon, E. Peynaud, P. Sudraud, P. Ribéreau-Gayon: *Traité d'Œnologie. Vol. 3: Vinifications, Transformations du Vin*, Dunod, Paris (1976).

21. P. Martinez, L. Perez Rodriguez, T. Benitez, *Am. J. Enol. Vitic. 48* (1997) 160–168.

22. R. B. Boulton, V. L. Singleton, L. F. Bisson, R. E. Kunkee: *Principles and Practices of Winemaking*, The Chapman & Hall Enology Library, New York (1996) pp. 36–47.

23. G. M. Herad, G. H. Fleet, *J. Appl. Bacteriol. 65* (1988) 23–28.

24. R. S. Jackson: *Wine Science*, Academic Press, San Diego (2000) pp. 120–137.

25. P. Ribéreau-Gayon, D. Duburdieu, B. Doneche, A. Lonvaud: *Handbook of Enology*, *Vol. 1*, *The Microbiology of Wine and Vinifications*, John Wiley & Sons, Ltd., New York (2000) pp. 88–127.

26. P. A. Kilmartin, H. Zou, *Electroanalysis*, *13* (2001) 1347–1350.

Utjecaj temperature i ugljičnog dioksida na fermentaciju mošta Cabernet Sauvignon

Sažetak

Temperaturu i količinu ugljičnog dioksida u procesu fermentacije lako je pratiti i kontolirati. Istraživan je utjecaj promjena temperature vrenja i protjecanja plinovitog ugljičnog dioksida pri fermentaciji mošta Cabernet Sauvignon sa *Saccharomyces bayanus* nakupljanjem biomase i proizvodnjom metabolita. Svi su pokusi provedeni u laboratorijskom reaktoru od 10 L s miješalicom uz stalno praćenje temperature i redoks-potencijala. Metaboliti dobiveni fermentacijom *S. bayanus*, tj. viši alkoholi (1-propanol, 2-butanol, izoamilni alkohol), kao i reducirajući šećeri određeni su tijekom fermentacije plinskom i tekućinskom kromatografijom.