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Utilization of Different Types of Glucose Oxidase for Reduction of Glucose Concentration in Synthetic Grape Juice

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Abstract

One of the most promising techniques for oxidation of glucose into a gluconic acid is the utilization of the enzyme glucose oxidase. In order to optimize the process, two types of enzymes were used as catalysts for glucose oxidation in several model synthetic grape juices. The first one is a food grade enzyme Alphamalt Gloxy 5080 from *Aspergillus niger*. The other one is pure enzyme from *Aspergillus niger*, used as a sole or in a combination with catalase isolated from beef liver. Both the pure glucose solution and the synthetic grape juice were used as substrates for enzymatic pretreatment. The Alphamalt Gloxy 5080 enzyme, used in a concentration of 1 g/L, showed 77.60% substrate conversion of the glucose used in a concentration of 10 g/L. the pure glucose oxidase having concentration of 25 mg/L converted only 1.32% of glucose, while when combined with 15 μL catalase, the conversion was even 49.25%.

Key words: glucose, glucose oxidase, oxidation of glucose, grape juice, enzymatic treatment

Introduction

A demand for wines with lower alcohol concentration has increased in the last two decades. Even the International Organization of Vine and Wine (OIV) in cooperation with Food Agricultural Organization (FAO) and World Health Organization (WHO) are focused nowadays to examine all the positive effects of the consumption of alcohol free and low alcohol wines on the consumer's health (Di Lorenzo et al., 2008).

There are different techniques for reducing the amount of alcohol in wines. They can be classified according to the stage of wine production. They are typically used: pre, during or post fermentation (Schmidtke et al., 2012).

Post-fermentation techniques are characterized by eliminating a portion of ethanol from the finalized wine. Some of these are reverse osmosis (Bui et al., 1986), osmotic distillation and pervaporation (Takacs et al., 2007), use of spinning cone column (Belisario-Sanchez et al., 2012). These processes are expensive, they demand specific equipment and can provoke changes in aromatic composition (Heux et al., 2006).

Fermentation technologies with novel yeast strains (Novoigt et al., 2002) and genetically modified *Sacharomyces cerevisae* that divert grape carbon compounds from ethanol to other metabolites or for biomass production, are also some of the novel modes to produce low alcohol wines (Varela et al., 2012). Malherbe et al., (2003) managed to express the *Aspergillus niger* gene encoding a glucose oxidase in *Saccharomyces cerevisiae* and thus to decrease the alcohol production during fermentation.

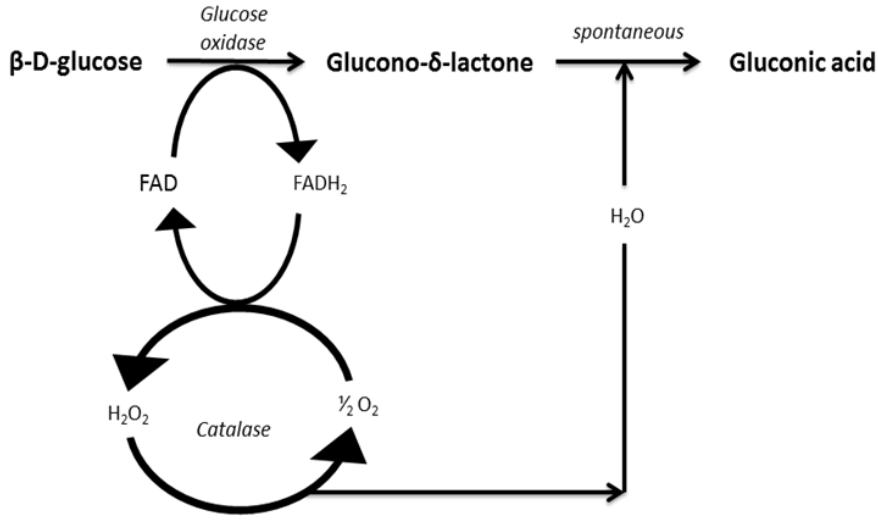
Prefermentation techniques assume lowering of glucose concentration in grape juice instead of elimination of ethanol from wine after the alcoholic fermentation. These techniques are: early grape harvest (Kontoudakis et al., 2011), adjusting vine leaf area to crop ratio (Stoll et al., 2009) and enzymatic methods such as utilization of glucose oxidase (Pickering et al., 1999). Early grape harvest significantly alters the qualitative parameters of the must (Kontoudakis et al., 2011). Early grape harvest gives unripe grapes, which means that in the must there is insufficient amount of color and aromatic compounds needed for obtaining typicity of wine (Kontoudakis et al., 2011). Adjusting the vine leaf area to crop ratio is a very interesting and promising viticultural method for moderation of the concentration of fermentable sugars in harvested grapes, however there is need for more research to determine the optimal conditions for obtaining grapes with oenologically acceptable organoleptic characteristics (Schmidtke et al., 2012).

Enzymatic methods are faster, highly specific methods that minimize loss or alteration of desirable organoleptic qualities and off-flavor development (Schmidtke et al., 2012).

The enzyme glucose oxidase (EC 1.1.3.4) catalyzes only the oxidation of the beta form of glucose. Grape must contains approximately equal amounts of glucose and fructose. Therefore, the theoretical maximal reduction on alcohol production can be 50%. In practice, alcohol reductions range from less than 4% to 40%. The efficiency of glucose oxidation depends on the enzyme concentration, pH, oxygen concentration and temperature (Pickering et al., 1998).

Treatment of grape juice with glucose oxidase could convert glucose into gluconic acid which can't be metabolized by wine yeasts (Rensburg & Pretorius, 2000).

The pH value of the medium plays an important role in maintaining the proper conformation of the enzyme (Bankar et al., 2009). The mechanism of the enzymatic oxidation of glucose into gluconic acid proceeds via two steps as presented in the Scheme 1.



Scheme 1. Oxidation of glucose by glucose oxidase
Оксидација глукозе уз помоћ глукоза-оксидазе

There are only few literature data of utilization of glucose oxidase for lowering the alcohol level in wines. Thus, Biyela et al., (2009), have used the commercial bakery preparation Gluzyme Mono 10.000 BG (Gluzyme) to reduce the glucose content of synthetic grape juice before fermentation.

Results showed up to 0.5% v/v less alcohol at an enzyme concentration of 20 kU compared to the control samples. Pickering et al., (1999a) analyzed the effect of utilization of glucose oxidase into the process of wine production.

This enzyme has converted glucose into a gluconic acid, but was deactivated in the presence of alcohol in concentrations greater than 1% by volume (Pickering et al., 1998). The low pH of wine was found to be a dominant limiting factor in the rate and extent of glucose conversion by glucose oxidase (Pickering et al., 1999b). Reduced-alcohol white wine from glucose treatment had a significantly modified taste (showing increased acidity) and appearance, although aroma, and mouth feel characteristics appeared relatively unaffected (Pickering et al., 1999c).

The final product of glucose oxidase enzymatic reaction is hydrogen peroxide, a known antimicrobial agent, which explains the antimicrobial activity against lactic acid bacteria and acetic acid bacteria (Malherbe et al., 2003).

Since there is scarce literature data about utilization of glucose oxidase in pretreatment of the red grape must, the aim of this work is to establish the reaction system for reduction of alcohol level in red wines. Thus, evaluation of two different enzymatic preparation was performed on both the pure glucose solution as a substrate and on the minimal model must. The time course of the enzymatic reactions was studied by using different concentrations of enzymatic preparations. The effect of catalase coupling with the pure glucose oxidase on the glucose conversion was also examined.

Materials and Methods

Alphamalt Gloxy 5080 (Muhlenchemie, Germany) is commercial preparation of glucose oxidase obtained from non pathogenic, not genetically modified *Aspergillus niger* strain. This food grade enzyme has activity of 10500 units per gram (U/g) and low catalase activity. Alphamalt Gloxy 5080 is used in baking industry with dosage of 0.5-2 g/100kg flour. Glucose oxidase (EC 1.1.3.4) used was lyophilized glucose oxidase from *Aspergillus niger*. This pure enzyme produced by Merck (Germany) had the activity of 8 U/mg. The liquid catalase (Merck, Germany) used was obtained from beef liver and had an activity of 1300000 U/mL.

Two types of media were used to evaluate the efficiency of glucose oxidase. In the first experiment two glucose solutions were prepared with concentrations of 10 g/L and 20 g/L glucose. The glucose was dissolved in buffer (0.1 M citrate buffer) with pH 5.5. In subsequent trials, Minimal Model Must medium (MMM) was used with concentration of glucose of 10 g/L.

This MMM medium contained organic acid concentration similar to those found in grape must obtained from ripe grapes.

The acid concentrations were adjusted as follows: 4.0 g/L tartaric acid, 1.5 g/L malic acid and 0.5 g/L citric acid. The pH of this medium was adjusted to pH 3.50 using sodium hydroxide (10 M NaOH).

All trials were carried out in 100 mL Erlenmeyer flasks with each containing 50 mL of medium. The Erlenmeyer flasks were sealed with a porous lead. Assays of 1 mL total were taken every 6 hours for 5 days.

The reaction was carried out on orbital shaker (Certomat R, B.Brown Biotech International) with 150 rot/min (facilitating the dissolution of oxygen) and with a constant temperature of 30˚C. Alphamalt was used in two different concentrations, 1 and 2 g/L. The pure glucose oxidase was used in concentration of 25 mg/L. The catalase was added in the same moment as the glucose oxidase, at the beginning of the reaction. The concentration of the catalase used was 500 µl/L.

D-glucose was measured using DNS method for determination of reduced sugars (Miller, 1959). Every measurement was performed in triplet. The absorbance was measured on spectrophotometer Cary 50 Scan (Varian) at 540nm wavelength.

The pH value of all flasks was measured at the beginning and at the end of the trial period. The pH meter used was Sartorius Basic pH Meter PB-11.

Results and Discussion

Pure glucose solution as a substrate for glucose conversion

When the activity of the enzyme Alphamalt Gloxy 5080 from the fungus *Aspergillus niger* was evaluated by using the medium with 10 g/L glucose as a substrate, the equilibrium was reached very quickly, at the 48th hour of the reaction (Figure 1).

By this time, in the reaction system with the enzyme used in a concentration of 1g/L, the conversion of the glucose was 77. 60% and when the enzyme was used in a concentration of 2 g/L the substrate conversion was 73.39%.

The analysis of the reaction systems with also 10 g/L glucose, but now with pure glucose oxidase as a catalyst, showed large differences in effectiveness depending on whether the enzyme was used separately or in a combination with catalase. While the glucose oxidase used as a sole enzyme converted only the 1.32% glucose, its utilization in combination with catalase from beef liver resulted in even 49.25% conversion of the glucose (Figure 2).

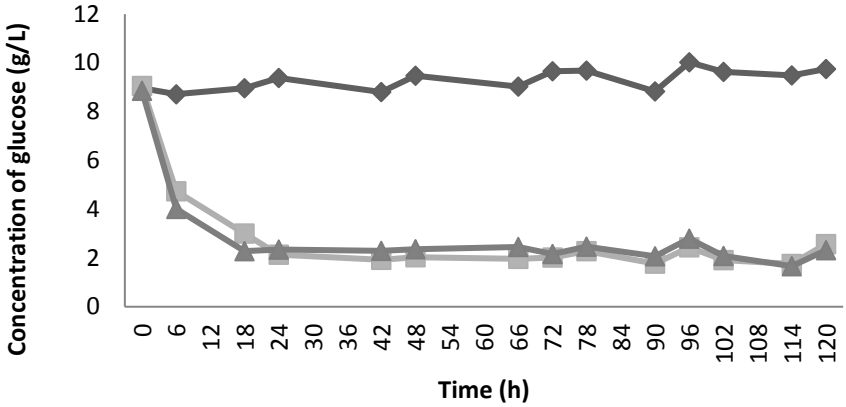


Fig. 1. Conversion of glucose into gluconic acid by the commercial Alphamalt Gloxy 5080 glucose oxidase using the medium with 10 g/L glucose. The initial concentration of glucose, control (♦) was lowered by utilizing 1 g/L (■) and 2 g/L (▲) enzymatic buffered solution.

Конверзија глукозе у глуконску киселину уз помоћ препарата Alphamalt Gloxy *5080 глукоза оксидаза у медијуму са 10 g/L глукозе. Почетна концентрација глукозе – контрола (♦) је смањена коришћењем 1 g/L (■) и 2 g/L (▲) ензиматског пуферованог раствора*

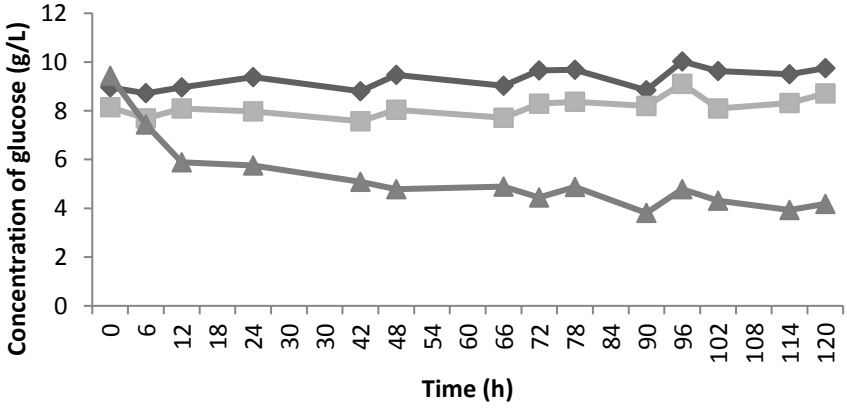


Fig. 2. The conversion of the glucose with initial concentration of 10 g/L (control: ♦) with pure glucose oxidase (GOX 25 mg/L ■) and in a combination with catalase (GOX 25 mg/L+CAT 25 μL ▲)

Конверзија глукозе са почетном концентрацијом од 10 g/L (контрола: ♦) са чистом глукоза оксаидазом (GOX 25 mg/L ■) и у комбинацији са каталазом (GOX 25 mg/L+CAT 25 μL ▲)

These results are easily explainable if we consider that the commercial Alphamalt Gloxy 5080 glucooxidase preparation has catalase activity as well, which is not the case with the pure glucooxidase from *Aspergillus niger*. It is rather obvious that these two enzymes should be coupled in order to obtain a powerful enzymatic system for glucose oxidation, as is already reported in the literature (Pickering, 1997).

In the reaction system with pure glucose oxidase, similarly as in the reaction system with Alphamalt Gloxy 5080, the reaction equilibrium was reached at the 48th hour of reaction.

When the glucose concentration of the medium was increased from 10 to 20 g/L, the enzymatic solution of Alphamalt Gloxy 5080 used in a concentration of 1 g/L has converted 52.99% of the glucose at the 48th hour of the reaction. At the same duration of the reaction, the utilization of the enzymatic preparation used in a concentration of 2 g/L resulted in the conversion yield of even 74.66% (Figure 3).

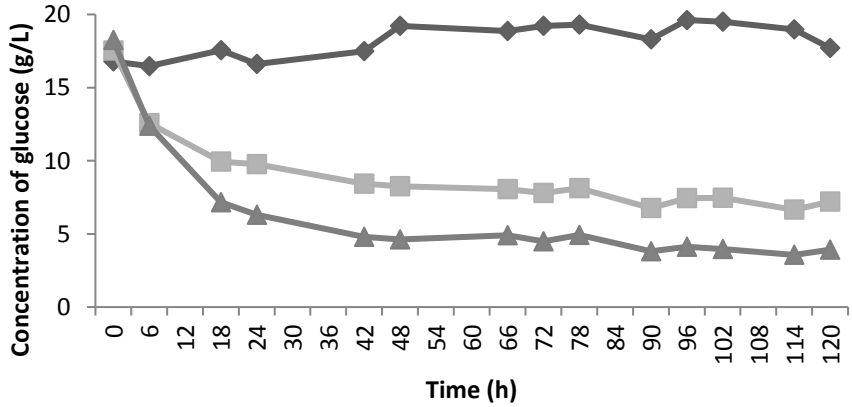


Fig. 3. Conversion of glucose into gluconic acid by the commercial Alphamalt Gloxy 5080 glucose oxidase using the medium with 20 g/L glucose. The initial concentration of glucose, control (♦) was lowered by utilizing 1 g/L (■) and 2 g/L (▲) enzymatic buffered solution.
Конверзија глукозе у глуконску киселину уз помоћ препарата Alphamalt Gloxy 5080 глукоза оксидаза у медијуму са 20 g/L глукозе. Почетна концентрација глукозе – контрола (♦) је смањена коришћењем 1 g/L (■) и 2 g/L (▲) ензиматског пуферованог раствора

The interesting parameter for the reaction progress and effectiveness in the drop of the pH values of the reaction media (Table 1 and Table 2).

As can be seen from Table 1, the lowest pH value corresponds well to the preparation that was most active and effective in glucose conversion, Alphamalt Gloxy 5080 in a concentration of 2 g/L.

Tab. 1. pH values of the reaction systems with 10 g/L glucose at the beginning of the reaction (initial pH) and at the end of the reaction (final pH value)
Вриједности pH реакционих система са 10 g/L глукозе на почеку реакције (почетна pH) и на крају реакције (завршна pH)

pH value in solution with 10 g/L glucose *pH у раствору са 10 g/L глукозе*					
	control *контрола*	1 g/L Alphamalt	2 g/L Alphamalt	25 mg/L GOX	25 mg/L GOX + 25µL CAT
initial pH value *почетна pH*	5.60	5.60	5.60	5.60	5.60
final pH value *завршна pH*	5.60	5.04	4.92	5.51	5.16

The same opinion holds for the enzymatic reaction conducted in the medium with 20 g/L (Table 2). The lowest final value of pH of 4.53 was measured for Alphamalt Gloxy 5080 in a concentration of 2 g/L.

Tab. 2. pH values of the reaction systems with 20 g/L glucose at the beginning of the reaction (initial pH) and at the end of the reaction (final pH value)
Вриједности pH реакционих система са 20 g/L глукозе на почеку реакције (почетна pH) и на крају реакције (завршна pH)

pH value in solution with 20 g/L glucose *pH у раствору са 20 g/L глукозе*			
	control *контрола*	1 g/L Alphamalt	2 g/L Alphamalt
initial pH value *почетна pH*	5.60	5.60	5.60
final pH value *завршна pH*	5.60	4.73	4.53

Minimal model must as a substrate for glucose conversion

Since the initial pH of the minimal model must is low and it has a value of 3.55, it greatly affects the enzymatic activity. Thus, the enzymatic preparation of pure glucose oxidase showed a very low, negligible activity in the model must medium. However, the Alphamalt Gloxy 5080 showed relatively high activity even at such a low pH value. Thus, used in the concentration of 1 g/L it converted 59.85% of glucose and used in the concentration of 2 g/L it converted even 67.05% of glucose (Figure 4).

It seems that this commercial enzymatic preparation, produced for bakery application and immobilized in a whey flower, is protected from the negative effect of the low pH value (Pickering, 1997; Biyela *et al*., 2009).

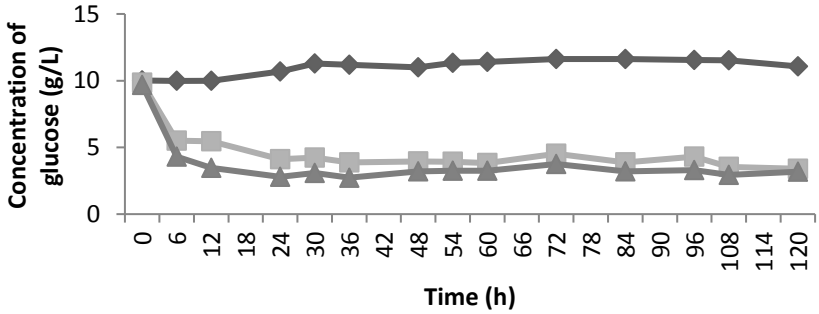


Fig. 4. Conversion of glucose from the minimal model into gluconic acid by the commercial Alphamalt Gloxy 5080 glucose oxidase using the medium with 10 g/L glucose. The initial concentration of glucose, control (♦) was lowered by utilizing 1 g/L (■) and 2 g/L (▲) enzymatic buffered solution.
Конверзија глукозе у минималном моделу у глуконску киселину препаратом Alphamalt Gloxy *5080 глукоза оксидаза у медијуму са 10 g/L глукозе. Почетна концентрација глукозе – контрола (♦) је смањена коришћењем 1 g/L (■) и 2 g/L (▲) ензиматског пуферованог раствора*

The same conclusion for the effectiveness of the Alphamalt Gloxy 5080 that showed best performances even at the low pH values could be seen from the results presented in the Table 3. The effectiveness of the glucose conversion by Alphamalt can be observed by the decrease of the pH value, from the initial 3.55 to 3.27. However, the pH value remains the same, or had a very small decrease in the trial with pure glucose oxidase.

Tab. 3. pH values of the reaction systems with1 g/L glucose in the minimal model must at the beginning (initial pH) and at the end of the reaction (final pH)
Вриједности pH у минималном моделу са 1 g/L глукозе на почеку реакције (почетна pH) и на крају реакције (завршна pH)

pH value in MMM with 10 g/L glucose *pH у моделу MMM са 20 g/L глукозе*					
	control *контрола*	1 g/L Alphamalt	2 g/L Alphamalt	25 mg/L GOX	25 mg/L GOX + 25μL CAT
initial pH value *почетна pH*	3.55	3.55	3.55	3.55	3.55
final pH value *завршна pH*	3.55	3.27	3.27	3.56	3.53

Conclusion

The commercial enzyme Alphamalt and the pure glucose oxidase showed very different results in converting glucose in used media. The effectiveness of glucose oxidase largely depends on whether it is used as a sole or in combination with catalase (conversion of 1.32% of glucose, and 49.25% respectively). Alphamalt showed greater conversation rate because of the presence of low catalase activity in the preparation. The value of pH affects the efficiency of the enzyme, thus pure glucose oxidase showed negligible activity in MMM with pH 3.5. However, this pH value was not inhibitory for Alphamalt. This commercial enzyme is immobilized in white flour which means that it is protected from environmental influences. Thus the immobilization as a technique often used in enzyme technology can be considered as a method for protection of the enzyme from negative influence from the environment, such as low pH value and high concentration of substrate.

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Употреба различитих врста глукоза-оксидазе за смањење концентрације глукозе у синтетичком соку од грожђа

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Сажетак

Једна он најперспективнијих техника за оксидацију глукозе у глуконску киселину је коришћење ензима глукоза-оксидаза. У циљу оптимизације процеса, двије врсте ензима су коришћене као катализатори за оксидацију глукозе, у неколико модела синтетичког сока од грожђа. Први ензим је хранидбеног типа Alphamalt Gloxy 5080 изолован из *Aspergillus niger*. Други ензим је пречишћени тип ензима из *Aspergillus niger*, коришћен појединачно или у комбинацији са каталазом изолованом из говеђе јетре. Као супстрат за ензиматски предтретман коришћен је раствор чисте глукозе и синтетичког сока грожђа. Alphamalt Gloxy 5080 ензим, који је коришћен у концентрацији од 1 g/L имао је учинак од 77,60% конверзије подлоге глукозе у концентрацији од 10 g/L. Раствор чисте глукозе у концентрацији од 25 mg/L имао је конверзију глукозе од само 1.32% а у комбинацији са 15 μL каталазе конверзију од чак 49.25%.

Кључне ријечи: глукоза, лукоза-оксидаза, оксидација глукозе, сок од грожђа, ензиматски третман

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