INFULENCE OF CULTIVATION TIME ON XANTHAN BIOSYNTHESIS ON EFFLUENTS FROM WHITE WINE PRODUCTION

UTICAJ VREMENA TRAJANJA KULTIVACIJE NA BIOSINTEZU KSANTANA NA EFLUENTIMA IZ PROIZVODNJE BELOG VINA

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ABSTRACT

*In this study, the influence of cultivation time on xanthan biosynthesis by using *Xanthomonas campestris* ATCC 13951 on effluents from different stages of white wine production was examined in order to determine the critical bioprocess parameters. The obtained results show that after 96h of cultivation there is no significant change in the concentration of sugar components in all investigated media, and consequently there is no significant production of biopolymer, which indicates that the reduction of cultivation time for 24 h is possible without negative effect on the yield of desired product. It has also been found that the consumption of total nitrogen was noticeably higher in relation to the amount of ammonia and amino compounds, indicating that the wastewaters generated during the white wine production contain other nitrogen components that the applied producing strain can metabolize.*

Key words: xanthan, *Xanthomonas campestris*, winery wastewaters, cultivation time.

REZIME

*Ksantan je komercijalno najznačajniji mikrobiološki heteropolisaharid koji nastaje metaboličkom aktivnošću bakterija roda *Xanthomonas* na medijumu sa glukozom. Međutim, nespecifični zahtevi proizvodnog mikroorganizma u pogledu izvora ugljenika, kao i sve veća potražnja i povećanje cene komercijalne glukoze ukazuju na potrebu primene alternativnih sirovina manje tržišne vrednosti. Efluenti različitih grana prehrambene industrije, među kojima su i otpadne vode vinarija, zbog svog biodegradabilnog karaktera predstavljaju interesantne supstrate za biotehnošku proizvodnju ksantana i nalaze se u žiži istraživanja u oblasti proizvodnje i unapređenja postupka dobijanja mikrobioloških polisaharida. Upotrebom ovih otpadnih tokova za biosintezu ksantana smanjuju se troškovi proizvodnje, ali i ekološki problemi izazvani njihovim nakupljanjem u životnoj sredini. U okviru ovih istraživanja ispitano je uticaj vremena trajanja kultivacije referentnog soja *Xanthomonas campestris* ATCC 13951 na biosintezu ksantana na efluentima iz različitih faza proizvodnje belog vina sa ciljem utvrđivanja kritičnih parametara bioprocasa. Dobijeni eksperimentalni podaci pokazuju da u svim ispitivanim medijumima nakon 96 h kultivacije ne dolazi do značajne promene koncentracije šećernih komponenti, a samim tim ni do značajne produkcije biopolimera, zbog čega je moguće skratiti bioprocen za 24 h bez negativnog uticaja na prinos željenog proizvoda. Pored toga, utvrđeno je da je rezidualni sadržaj šećera u svim medijumima veći od 5 g/l što je ekonomski neisplativo za biotehnošku proizvodnju. Takođe, dobijeno je da je potrošnja ukupnog azota bila veća u odnosu na količinu utrošenih amino i amonijskih jedinjenja što ukazuje na to da se u otpadnim vodama generisanim tokom proizvodnje belog vina nalaze i druge azotne komponente koje primenjeni proizvodni soj može da metaboliše.*

Ključnereči: ksantan, *Xanthomonas campestris*, otpadne vode vinarije, vremekultivacije.

INTRODUCTION

Xanthan or xanthan gum is the most important microbiological heteropolysaccharide composed of glucose, mannose, and glucuronic acid units. Due to its excellent rheological properties xanthan has widely been used as an additive in various industrial and biomedical applications such as food and food packaging, cosmetics, water-based paints, toiletries, petroleum, oil-recovery, construction and building materials, and drug delivery (Palaniraj and Jayaraman, 2011). Industrially, this biopolymer is produced by the Gram-negative bacterium *Xanthomonas campestris* on glucose and sucrose containing media in batch production mode (Rosalam and England, 2006). Factors, such as the bacterial strain, culture medium, growth conditions, type of bioreactor and operation mode can influence xanthan production (Zabot et al., 2012). The composition of the culture medium that provides the necessary nutrients for microbial growth plays an important role in the biosynthesis, molecular structure, and yield of xanthan. The culture medium represents 20–30% of the total production cost, therefore optimization of its type and the concentration of its components (mainly the carbon source) can reduce the costs and lead to accumulation of a higher quality biopolymer (Lopes et al.,

2015). Due to the non-specific requirements of the producing microorganism in terms of carbon source, many researchers have begun to assess alternative carbon sources for xanthan production, in an attempt to reduce production costs (Habibia and Khosravi-Daranib, 2017). In addition, the increasing demand and relatively high market price of commercial glucose indicate the need for the application of alternative cheaper substrates for xanthan production (Mudoi et al., 2013). The food industry effluents, including the winery wastewaters, due to their biodegradable character, represent interesting substrates for xanthan biotechnological production. Also, these alternative substrates are in the focus of research in the field of production and improvement of microbiological polysaccharides. The use of mentioned waste streams for xanthan biosynthesis reduces the overall production cost and the ecological problems caused by their accumulation in the environment (Niknezhad et al., 2015).

Wineries produce large amount of wastewaters generated by numerous activities during the wine production that mainly include cleaning of tanks and barrels, washing the floors and equipment, rinsing the transfer lines, bottling facilities, filtration units, product losses and rainwaters diverted into the wastewaters management system (Chapman et al., 2001). Waste effluent obtained in different fractions of the production will

necessarily have different characteristics and components. The differences will primarily be reflected in the most predominant compounds in these kinds of wastewaters such as soluble sugars (fructose and glucose), organic acids (tartaric, lactic and acetic), alcohols (glycerol and ethanol) and high molecular weight compounds, such as polyphenols, tannins and lignin (Conradie et al., 2014). However, overall wastewaters from wineries, due to its high organic and inorganic load, the large volumes produced and its seasonal variability, represent huge ecological problem and require adequate treatment prior to discharge into the environment (Bustamante et al., 2005). In order to minimize the negative environmental impact and recycle the wastes generated by the wine industry, different treatment methods have been proposed (Mosse et al., 2011). A promising alternative is bioconversion of these waste effluents into value-added products. In previous research, xanthan production was suggested as a possible solution for utilization of wastewaters from wine industry (Bajić et al., 2015).

The objective of this paper was to investigate the influence of cultivation time on xanthan biosynthesis by using *Xanthomonas campestris* ATCC 13951 on effluents from different stages of white wine production. Wastewaters from white wine production collected from different parts of the process, after crushing and pressing of grape, as well as clarification of must and fermentation, were used, and in order to determine the critical bioprocess parameters the change of the most important nutrient content and apparent viscosity were analyzed during the cultivation.

MATERIAL AND METHOD

Producing microorganism

The reference strain *Xanthomonas campestris* ATCC 13951 was used as the producing microorganism in these experiments. The pure culture was stored at 4°C on agar slant (YMA®, HiMedia, India) and subcultured every four weeks.

Cultivation media

Wastewaters from different stages of white wine production generated in domestic winery located in vineyards of Fruška Gora, Vojvodina, Serbia, were used as raw materials. The cultivation media based on the effluents collected during washing the crusher, press and tanks after clarification of must by flotation, and fermentation were prepared as previously described (Bajić et al., 2015).

Xanthan production

The xanthan production was carried out in 3 L laboratory bioreactor (Biostat® A plus, Sartorius AG, Germany) with 2 L of cultivation medium. The sterile medium was inoculated by adding 10 vol.% of inoculum prepared by double passaging procedure in aerobic conditions, on YM broth (YMB®, HiMedia, India), at 25°C on a laboratory shaker at 150 rpm for 36 h. The biosynthesis was carried out in batch mode under aerobic conditions (airflow rate of 1 vvm in the first 48 h, and 2 vvm afterwards) for 120 h. In the first 48 h, biosynthesis temperature and agitation rate were 26°C and 200 rpm, after which they were increased to 32°C and 300 rpm, respectively.

Analysis of cultivation broth

The samples of cultivation broth taken in defined time during the biosynthesis were analyzed in term of its chemical composition. The content of sugars, total and assimilable nitrogen, as well as total phosphorus were determined in supernatants obtained by centrifugation of cultivation broth at 10000 rpm for 10 min (Hettich Rotina 380 R, Germany). To determine sugars content supernatants were filtered through a 0.45 µm nylon membrane (Agilent Technologies, Germany) and then analyzed by HPLC instrument (Thermo Scientific

Dionex UltiMate 3000 series) under appropriate conditions (Bajić et al., 2015). The contents of total nitrogen and phosphorus were determined using volumetric method proposed by Kjeldahl (Herlich, 1990) and spectrophotometric method (Gales et al., 1966), respectively. The assimilable nitrogen content, expressed as amino and ammonia nitrogen, was determined by the Formol titration method (Zoecklein et al., 1999).

The apparent viscosity of cultivation broth samples taken in defined time intervals was calculated based on results of measurement on rotational viscometer ("Reotest 2 RV-2", Medingen GmbH, Germany) with double gap coaxial cylinder sensor system and spindle N, as described by Rončević et al. (2017).

Data analysis

All experiments were carried out in triplicate, and the obtained results are represented as mean values with standard deviations. The experimental data were processed by one-way analysis of variance (One-Way ANOVA). Significant differences between the means were determined by Duncan's multiple range test at the significance level of $\alpha=0.05$ using Statistica 13.2 software (Dell Inc., USA).

RESULTS AND DISCUSSION

Winery wastewaters are effluents with high organic and inorganic load and very complex composition. In previous studies, wastewaters from different stages of white wine production were analyzed in terms of parameters that are commonly used for characterization of winery waste streams (Puškaš et al., 2015), as well as the most important nutrients for xanthan biosynthesis (Bajić et al., 2015; Rončević et al., 2017). Considering that the concentration of biopolymer in the cultivation broth at the end of the bioprocess is about 20-30 g/L (Sherley and Priyadarshini, 2015), and that the industrial xanthan production tends to achieve the conversion rate of the carbon source into the desired product of about 50-80%, it is evident that the application of media with high initial sugar content in this biotechnological process is not justified. Therefore, in this study the cultivation media based on wastewaters generated during the various stages of the white wine production were prepared to contain 25 g/L of initial sugar. The xanthan production was performed in the laboratory bioreactor under the same conditions using reference strain of *Xanthomonas campestris* ATCC 13951. The consumptions of the most important nutrients for xanthan biosynthesis were examined during the cultivation, and the obtained results are presented in Figure 1.

The results presented in Figure 1 and data obtained by statistical analysis indicate that sugar content in all used media was changed significantly during the bioprocess. Although, at the beginning of the cultivation there was no statistically significant difference in the amount of the sugar component in all media ($p=0.517$), the dynamic of its change during the biosynthesis was not the same, probably, due to different concentrations of other essential nutrients in all investigated effluents. The sugar content in the medium based on wastewater from crusher washing (Figure 1a) was not changed significantly ($p=0.262$) during the first 12 h of biosynthesis. After mentioned time, due to the metabolic activity of used producing microorganism sugar content in this medium intensively decreased until 96 h ($p<0.001$), after which its change was not statistically significant ($p=0.171$).

The residual sugar content in medium based on wastewater generated during washing the crusher was 9.40 g/L. The sugar content in other investigated media was intensively reduced from the beginning of the cultivation.

Fig. 1. Changes in the contents of sugar (S), total nitrogen (N), assimilable nitrogen (N_a) and total phosphorus (P) during xanthan biosynthesis on media based on wastewaters from different stages of white wine production: a) crushing, b) pressing, c) flotation, d) fermentation

In the media based on wastewaters from washing the tanks after must clarification (Figure 1c) and fermentation (Figure 1d) sugar consumption was noticed up to 96 h. The decrease of sugar components in these media was not statistically significant after 96 h, which confirm the p -values of 0.193 and 0.173, respectively. At the end of biosynthesis the sugar content in obtained cultivation broths were 7.91 g/L and 8.75 g/L, respectively. Intensive decrease of sugar content in medium based on the wastewater from press washing was noticed up to 84 h ($p < 0.001$). Then, the consumption of sugar components until the end of the biosynthesis was not detected ($p = 0.384$), and the residual sugar content was 8.24 g/L. Comparing the amount of sugar that is left in the cultivation broth at the end of biosynthesis it is concluded that there is no statistically significant difference between the investigated media in term of the residual sugar content ($p = 0.104$). On the other hand, there is no significant change in the concentration of the sugar components in all investigated media after 96 h of cultivation. Since the producing microorganism cells use the carbon source to fulfill their energy demand, growth and xanthan biosynthesis, it is clear that there is no significant production of biopolymer after the mentioned period. Based on these theoretical facts, it is evident that reduction of cultivation time for 24 h is possible without negative effect on the desired product yield in all investigated media.

According to the results shown in Figure 1, a significant difference in the amount of total nitrogen in all media at the beginning of the bioprocess can be noticed ($p < 0.001$). The higher initial nitrogen content was determined in media based on wastewaters obtained after must clarification (413.67 mg/L) and fermentation (323.67 mg/L), while lower concentrations of initial nitrogen, 89.28 mg/L and 103.45 mg/L, were determined in media based on wastewaters obtained after crushing and pressing of grape, respectively. Taking into account the composition of used raw materials (Rončević et al., 2017), as well the media preparation procedure, which included only

correction of sugar content (Bajić et al., 2015), the obtained results are as expected. The statistical analysis of the obtained data showed that the total nitrogen content was significantly changed during the cultivation of producing strain on all investigated media, which clearly indicates that there was no inhibition of its metabolic activity. However, the varied dynamics of the change in mentioned nutrient content indicate differences in the intensity of cell metabolism and the moment of the beginning of a certain growth phase. A slow consumption of total nitrogen in media based on wastewaters generated during crusher washing ($p = 0.060$) is recorded during the first 12 h of cultivation, responding to the lag growth phase. Faster nitrogen consumption ($p < 0.001$) could be noticed between 12 h and 60 h of cultivation, which indicated a utilization of this nutrient for cell growth. The stationary phase occurred after 60 h of cultivation, which confirms the unchanged nitrogen content up to the end of bioprocess ($p = 0.139$). Compared to the sugar content, which intensively decreased from the beginning of the cultivation in the medium based on the wastewater from washing the tank after fermentation (Figure 1d), significant consumption of total nitrogen components was not detected during the first 12 h of cultivation ($p = 0.089$). Significant total nitrogen consumption was noticed between 12 h and 60 h ($p < 0.001$), whereas after 60 h of cultivation significant changes in this nutrient concentration were not recorded ($p = 0.179$). The total nitrogen content in the cultivation broth at the end of bioprocess was 169.67 mg/L. The concentration of total nitrogen in media based on the wastewaters obtained after washing the press (Figure 1b) and tank after must clarification (Figure 1d) was significantly changed up to 60 h ($p < 0.001$) and 48 h ($p < 0.001$) of cultivation, respectively. Further changes in the concentration of this nutrient in both cultivation broths was not observed ($p = 0.116$ and $p = 0.194$, respectively). Residual total nitrogen content in this media determined at the end of bioprocess were 103.45 mg/L and 223.00 mg/L, respectively. The assimilable nitrogen content determined during the cultivations is also

presented in Figure 1. As it can be seen from graphically presented results, the higher initial concentrations of assimilable nitrogen were determined in the media based on wastewaters generated during washing the tanks after must clarification (90.02 mg/L) and fermentation (84.33 mg/L), while lower concentrations of this nutrient, 22.47 mg/L and 13.07 mg/L, were determined in media based on wastewaters obtained after crusher and press washing, respectively. The results of statistical analysis indicate that there was not statistically significant difference in the content of assimilable nitrogen between medium based on wastewater from washing the tanks after must clarification and fermentation ($p=0.093$), although statistically significant difference in the amount of total nitrogen in these media was noticed ($p<0.001$). On the other hand, medium based on wastewater from crusher and press washing contained equal amounts of total nitrogen, while the significant difference in the concentration of the assimilable nitrogen was noticed ($p=0.012$). Analyzing the obtained curves for assimilable nitrogen consumption it can be observed that the concentration of this nutrient was significantly decreased from the beginning of the cultivation in all used media. Consumption of amino and ammonia nitrogen does not need to be reflected in the consumption of total nitrogen if the concentration of assimilable nitrogen compounds is significantly lower than total nitrogen concentration, which was detected in these experiments. However, dynamic of changes in the content of total and assimilable nitrogen matched in all used media except in the medium based on the wastewater from washing the tank after fermentation (Figure 1d) where intensive decrease in the total nitrogen content was detected up to 60 h of cultivation, while the significant consumption of assimilable nitrogen compounds was noticed up to 48 h ($p<0.001$). At the end of the bioprocess a not-metabolized amount of assimilable nitrogen in this medium was 10.23 mg/L. The producing strain cultivations on the media based on the wastewaters from the crusher and press washing were characterized by significant decrease in assimilable nitrogen content up to the 60 h ($p<0.001$) while further consumption of the amino and ammonia nitrogen in these media was not significant ($p=0.090$ and $p=0.069$, respectively). At the end of cultivation, media based on the wastewaters from crusher and press washing contained 5.26 mg/L and 1.88 mg/L of assimilable nitrogen, respectively. Significant consumption of assimilable nitrogen in the medium based on the wastewater from washing the tank after must clarification (Figure 1c) was recorded up to 48 h ($p<0.001$) after which change in the content of this nutrient was not significant ($p=0.176$). Residual concentrations of assimilable nitrogen in observed media was 9.21 g/L. Although the dynamics of assimilable nitrogen consumption in all media were different, the applied producing strain was metabolized equal amount of the assimilable nitrogen compounds in applied experimental condition. According to the obtained results, the amount of metabolized amino and ammonia nitrogen ranged from 11.19 mg/L to 88.00 mg/L. On the other hand, consumption of the total nitrogen was from 39.42 mg/L to 223.00 mg/L, which are significantly higher values compared to the values of assimilable nitrogen. These differences indicate that wastewaters from white wine production contain other nitrogen compounds that used *Xanthomonas campestris* strain can metabolize. Graphically presented results (Figure 1) and results of statistical analysis indicate that the total phosphorus consumption was significant from the beginning of cultivation in all investigated media. The time in which the phosphorus was intensively consumed coincides with a period of intense reduction in total nitrogen content. This was observed for all used media except for the medium based on the crusher and press washing wastewaters (Figure 1a and 1b). In these media, a significant decrease in phosphorus content was 12 h shorter than

the period of total nitrogen consumption, i.e. up to 48 h of cultivation ($p<0.001$), after which it was insignificant up ($p=0.085$ and $p=0.097$, respectively). This is, probably, the result of the smaller initial content of total phosphorus in comparison with initial assimilable nitrogen concentration in these media due to which the producing strain was faster metabolized fermentable phosphorus compounds. The residual phosphorus concentrations in mentioned media were 4.28 mg/L and 3.87 mg/L, respectively. Intensive decrease in total phosphorus content was recorded up to 48 h in medium based on the wastewater generated during the flotation tank washing, after which a significant change in the amount of this nutrient was not observed ($p=0.304$). The residual content of total phosphorus in this cultivation medium was 8.93 mg/L. The consumption of total phosphorus in medium based on the wastewater obtained after washing the fermentation tank was intensive up to 60 h of cultivation, while the observed change was not statistically significant until 120 h ($p=0.147$), when the analyzed nutrient was detected in a quantity of 22.53 mg/L. Statistical analysis showed that the amount of unused phosphorus compounds in the cultivation broths detected at the end of the biosynthesis was significantly different among the experiments ($p<0.001$) except in the cultivation broths obtained after biosynthesis on wastewaters from crusher and press washing ($p=0.576$). The analysis of samples taken from the mentioned cultivation media after inoculation showed the same results.

Based on the changes in the content of the most important nutrients in the white wine wastewaters containing media, the metabolic activity of the producing strain under the applied conditions was confirmed. Additionally, in order to verify the xanthan production on investigated effluents, rheological measurements were performed from the moment of bioprocess conditions regulation. The obtained results indicate that in applied condition the xanthan biosynthesis occurs (Bajić et al., 2015). The apparent viscosity was calculated using the values of rheological parameters for all cultivation broths and their changes during the cultivation are shown in Figure 2. Statistical analysis of the experimental data suggest that apparent viscosity of cultivation media based on the wastewaters generated in different stages of white wine production was significantly changed during bioprocess ($p<0.001$), while the results presented in Figure 2 indicate the different dynamics of the changes in this parameter, which is probably a consequence of variations in the quantity and quality of the xanthan in investigated media. The values obtained at the beginning of rheological measurements were significant different. Hence, in 48 h of cultivation, the most viscous media were those that contained wastewaters from washing the tanks after flotation (10.72 mPa·s) and fermentation (14.58 mPa·s), while lower viscosity was measured in media based on the wastewater after press (8.15 mPa·s) and crusher washing (8.91 mPa·s). Significant increase in apparent viscosity from the beginning to the end of the cultivation was observed in the media based on the wastewater generated after grape crushing, must clarification and fermentation ($p<0.001$). At the end of biosynthesis the apparent viscosity of mentioned media were 14.60 mPa·s, 53.13 mPa·s, 76.58 mPa·s, respectively. It is important to note that the consumption of carbon sources after 96 h of cultivation was not significant in all media, indicating the end of bioprocess in applied conditions. Since the viscosity of xanthan cultivation broths does not depend only on its concentration, but also on the biopolymer molecular weight and the presence of the pyruvate and acetate groups in the molecule (Reis et al., 2010), it is evident that the increase in the viscosity after 96 h of cultivation is the consequence of the biopolymer quality changes. Namely, the applied producing strain, depending on the medium composition and the applied process conditions, synthesizes the macromolecules with a certain length

and structure. These molecules, thanks to the pyruvate and acetate groups, are linked during the bioprocess via the bivalent ions present in the cultivation broth. This is particularly expressed in cultivation media that contained calcium carbonate as a buffering agent (Rosiro et al., 1992). Considering that the preparation of cultivation medium for these experiments included the addition of the calcium carbonate, it can be assumed that the mentioned factors are the reason for the increase in values of apparent viscosity in the period when sugar consumption was not significant.

Fig. 2. Apparent viscosity of media based on winery wastewaters

CONCLUSION

In accordance with the defined aim, in this research the influence of the cultivation time on xanthan biosynthesis by *Xanthomonas campestris* ATCC 13951 on effluents from different stages of white wine production was examined in order to determine the critical bioprocess parameters. Based on the change in the content of the most important nutrients, metabolic activity of the producing strain on all used media under applied conditions was confirmed. Also, the change in the values of apparent viscosity confirmed the successful xanthan production in all investigated media. Furthermore, the obtained results show that in all media after 96h of cultivation there is no significant consumption of sugar components, and consequently there is no significant production of biopolymer, which indicate that the reduction of cultivation time for 24 h is possible without negative effect on the yield of desired product. It has also been found that the consumption of total nitrogen was noticeably higher compared to the consumption of ammonia and amino compounds, indicating that in the wastewaters generated during the production of white wine there are other nitrogen components that the applied producing strain can metabolize. These results represent the basis for further optimization of xanthan production on the effluents from the white wine production with the aim of potential industrialization of the xanthan production process.

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