International Journal of Environmental Engineering– IJEE
Volume 2: Issue 2 [ISSN: 2374-1724]
Publication Date: 30 October, 2015

Wastewaters from rose wine production as substrate for xanthan production

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Abstract* — Wineries and other grape processing industries annually generate large amounts of solid and liquid waste streams. Winery wastewaters are the waste product of many independent processing and cleaning operations in wineries. As a consequence of the working period and the winemaking technologies, as well as the cleaning practices, volumes and composition of winery wastewaters greatly vary over the year. Due to high organic load, the disposal of these effluents in the environment causes several issues. The bioconversions of winery wastewaters in valuable products are an important alternative to overcome these ecological problems. The aim of this study was to examine the possibility of xanthan production from media based on four wastewaters obtained from different stages of the rose wine production process, i.e. wastewaters collected during the washing of the crusher, press and tanks after clarification of must and fermentation. Process efficacy was estimated based on raw xanthan yield and rheological characteristics of media. In applied experimental conditions, raw xanthan yields were in the range between 8.03 g/L and 17.52 g/L. Also, all cultivation media showed pseudoplastic behavior. Obtained results suggest that wastewaters from rose wine production have a great potential to be used as a substrate for production of this high value biopolymer.

***Keywords* — environmental engineering, winery wastewaters, biotechnological xanthan production**

I. *Introduction*

Wine is an alcoholic beverage produced by the fermentation of grape juice. Winemaking requires the implementation of several unit operations: grape reception, must production, fermentation, decanting, maturation-stabilization, filtration and transportation-disposal. In the unit operations performed in wineries, distilleries and other grape processing industries, large volumes of waste streams are generated annually. These include organic waste (solids, skins, etc.), inorganic wastes (diatomaceous earth, bentonite clay and perlite), greenhouse gases (CO_2, volatile organic compounds, etc.) and wastewaters. It is estimated that a winery produces between 1.3 and 1.5 kg of residues per liter of wine produced, 75% of which is wastewaters (1).

Wastewaters from the wine production, called winery wastewaters, mainly originate from various washing operations after the crushing and pressing of grapes, as well as the rinsing of fermentation tanks, barrels and other equipment or surfaces, from the washing of bottles and from cooling. During the course of each year, volumes and composition of wastewaters greatly vary in relation to the working period (vintage, racking or bottling), the size of the winery and the used winemaking technologies (production of white, rose, red or special wines) (2, 3).

Winery wastewaters are characterized by high concentrations of organic matter and a variable content of suspended solids. Chemical oxygen demand (COD) concentrations vary from 500 to 45000 mg/L, total suspended solids (TSS) from 12 to 7300 mg/L, and pH value from 3.5 to 7.0 (2). In addition, winery wastewaters contain all the typical substances of grapes and wine, such as sugars, nitrogen, phosphorus, alcohols, organic acid and high-molecular weight compounds, e.g. polyphenols, tannins and lignins. Also, the residues from winemaking (yeasts, clarifying and fining agents, etc.) and sterilising agents used for the treatment of tanks and facilities can be found in the winery effluents (1, 4).

Due to its specific composition and high organic load, winery wastewaters represent a serious ecological problem in all wine producing countries. The disposal of winery effluents in creeks, rivers and on soils represents unacceptable environmental risks. Therefore, an increasing number of researches are aiming to develop efficient technologies for the degradation of the organic matter present in winery wastewaters. A wide range of biological processes, such as aerobic and anaerobic digestion and advanced oxidation processes, as well as various combinations among them have been proposed (5, 6). However, the most suitable procedures seem to be treatments involving recycling rather than detoxification. The production of useful microbial products has been shown to be one of the most promising alternatives (7).

In previous studies, the use of different wastewaters from food industry as substrates for biotechnological xanthan production was described (8, 9). Xanthan is the most important microbial polysaccharide industrially produced by *Xanthomonas campestris*. Due to its unique rheological properties, this biopolymer is widely used in the food, cosmetic, pharmaceutical, petrochemical and other industries as a thickening agent, stabilizer, or emulsifier, and combined with other polymers as a gelling agent (10, 11).

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Considering that xanthan biosynthesis is possible on various waste liquid effluents, winery wastewaters are an interesting potential substrate for biotechnological production. The use of winery wastewaters to produce xanthan would eliminate the environmental problems caused by waste disposal and reduce production costs of this high value biopolymer.

The aim of this study was to examine the possibility of xanthan production using *Xanthomonas campestris* on wastewaters obtained from different stages of the rose wine production process. The rheological properties of the cultivation media, raw xanthan yield and degree of sugar conversion into product were determined as indicators of successful xanthan biosynthesis.

II. Materials and methods

A. Producing microorganism

In these experiments the strain *Xanthomonas campestris* ATCC 13951 was used as the producing microorganism. The strain of producing microorganism was stored at 4^oC on yeast maltose (YM) agar slant (containing: 15.0 g/L glucose, 3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone and 20.0 g/L agar) and subcultured every four weeks.

B. Substrates

Wastewaters from rose wine production collected during the washing of the crusher (WW1), press (WW2) and tanks after clarification of must (WW3) and fermentation (WW4) were obtained from a domestic winery located in vineyards of Fruska Gora, Vojvodina, Serbia. These waste streams were analyzed in terms of sugar, total nitrogen and phosphorus content. Based on obtained results winery wastewaters were diluted or enriched by addition of glucose to an initial sugar content of 25 g/L, which was followed by adding $CaCO_3$ in concentration of 2 g/L. The substrates were adjusted to pH value of 7.0 and used as cultivation media for xanthan production with no addition of nitrogen and phosphorus.

C. Biosynthesis conditions

The xanthan production was carried out in 3 L laboratory bioreactor (Biostat® A plus, Sartorius AG, Germany) with 2 L of cultivation medium. The laboratory bioreactor with the appropriate substrate was sterilized by autoclaving at 121^oC and pressure of 2.1 bars for 20 min. The sterile medium was inoculated by adding 10% (v/v) of inoculum prepared by double passaging in aerobic conditions, on YM broth (containing 15.0 g/L glucose, 3.0 g/L yeast extract, 3.0 g/L malt extract and 5.0 g/L peptone), at 26^oC in a laboratory shaker at 150 rpm for 48 h. The biosynthesis was carried out in batch mode under aerobic conditions (air flow rate of 1 vvm in the first 48 h, and 2 vvm afterwards) for 120 h. In the first 48 h, the biosynthesis temperature and agitation rate were 26°C and 200 rpm, after which they were increased to 32^oC and 300 rpm, respectively. Regulation of process parameters was done in accordance with the literature data (11).

D. Product separation

After biosynthesis, the product separation from cultivation medium was carried out to evaluate the xanthan production. In this study, xanthan was recovered from supernatant of cultivation broth, obtained using ultracentrifuge (Hettich Rotina 380 R, Germany) at 10000 rpm for 10 min, by precipitation with 96% (v/v) ethanol in the presence of KCl as the electrolyte. Ethanol was gradually added to the supernatant cooled at 15^oC until the alcohol content in mixture of 60% (v/v) with constant stirring. A saturated solution of KCl was added when half of the needed ethanol amount was poured into the supernatant in a quantity to reach a final content of 1% (v/v). Obtained mixture was kept at 4°C for 24 h in order to dehydrate the precipitated xanthan, and then centrifuged at 3500 rpm for 15 min (Tehtnica LC-321, Slovenia). The precipitated polymer was then dried to constant weight at 60^oC in order to determine raw xanthan yield. Ethanol used for precipitation of xanthan was recycled by distillation.

E. Analytical methods

At the end of the process, samples of cultivation broths were analyzed. Rheological properties of cultivation broth samples were determined using rotational viscometer (REOTEST 2 VEB MLV Prüfgeräte-Verk, Mendingen, SitzFreitel) with double gap coaxial cylinder sensor system, spindle N. Volume of samples was 10 mL. Based on deflection of measuring instrument (α, Skt) shear stress (τ, Pa) was calculated, under defined values of shear rates, using the following equation:

$$\tau = 0.1 \cdot Z \cdot \alpha \qquad (1)$$

where Z is the constant with the value 3.08 ($dyn/cm^2 \cdot Skt$). According to Ostwald-de Waele model, which describes viscosity of pseudoplastic fluids, and calculated values of shear stress, rheological parameters were determined.

In the analyzed samples, contents of sugar, total nitrogen and phosphorus were determined. Samples were filtered through 0.45 μm nylon membrane (Agilent Technologies, Germany) and then analyzed by HPLC (Thermo Scientific Dionex UltiMate 3000 series) to determine residual sugar content. HPLC instrument was equipped with pump HPG-3200SD/RS, autosampler WPS-3000(T)SL (10 μL injection loop), column ZORBAX NH_2 (250 mm x 4.6 mm, 5 μm) and detector RefractoMax520. 75% (v/v) acetonitrile was used as eluent with flow rate of 1.2 mL/min and elution time of 20 min at column temperature of 25^oC. Content of nitrogen was determined by the Kjeldahl method (12), and phosphorus content was determined using spectrophotometric method (13).

III. Results and discussion

Winery wastewaters, obtained from different stages of production process, are effluents with very complex composition. Therefore, it is difficult to fully qualitatively and quantitatively characterize these waste streams. For the purposes of this research, wastewaters from rose wine production (WW1-WW4) were analyzed only in terms of the content of nutrients which are important for biotechnological xanthan production, i.e. concentration of sugar, total nitrogen and phosphorus were determined. Analyses of raw materials show that the compositions of the most important nutrient for xanthan biosynthesis in these effluents were typical for winery wastewaters (data not shown). Based on obtained results cultivation media were prepared as previously described, and all experiments were performed under identical conditions.

The success of the xanthan production on wastewaters obtained from different stages of rose wine production process in applied experimental conditions was determined based on raw xanthan yield and degree of sugar conversion into product. Obtained results are presented in Table I. Although all media contained the same initial sugar content and nitrogen and phosphorus contents were in the range optimal for the xanthan biosynthesis, in applied experimental conditions raw xanthan yields were in the range between 8.03 g/L and 17.52 g/L. The lowest product yields were obtained from medium WW1 (8.03 g/L) and medium WW2 (9.34 g/L), while the highest product yield came from medium WW4 (17.52 g/L). High yields were also achieved from the medium WW3 (14.68 g/L). Wastewaters collected during the washing of the tanks after clarification of must (WW3) and fermentation (WW4) contained higher contents of various organic acids, vitamins and other growth promoting compounds in comparison with wastewaters generated during the washing of the crusher (WW1) and press (WW2). Taking this into account, obtained results for raw xanthan yield are confirmed with the literature data that organic acids have a stimulatory effect on xanthan production (14). In addition, as opposed to other media (WW1-WW3), medium WW4 contained both inorganic and organic nitrogen which is a consequence of yeast cells autolysis. Since the use of organic nitrogen in cultivation medium improves xanthan biosynthesis (15), the highest raw xanthan yield obtained from medium WW4 was expected. Values of sugar conversion into product in medium WW3 and WW4 were 58.70% and 70.08%, respectively, which is in accordance with literature data (15), while these values are significantly lower in medium WW1 (32.14%) and WW2 (37.36%).

TABLE I. RAW XANTHAN YIELD AND SUGAR CONVERSION INTO PRODUCT

Winery Wastewaters	*Raw xanthan yield, P [g/L]*	*Conversion[a] [%]*
WW1	8.03	32.14
WW2	9.34	37.36
WW3	14.68	58.70
WW4	17.52	70.08

a. *Conversion [%] =* $P/S_0 \cdot 100$

Quality of xanthan produced in applied experimental conditions was evaluated based on the rheological behavior of cultivation media after biosynthesis. The rheological properties were determined from relationship between shear rate and shear stress shown in Figure 1. Flow curves represent pseudoplastic type of flow which is characteristic of xanthan solutions (11).

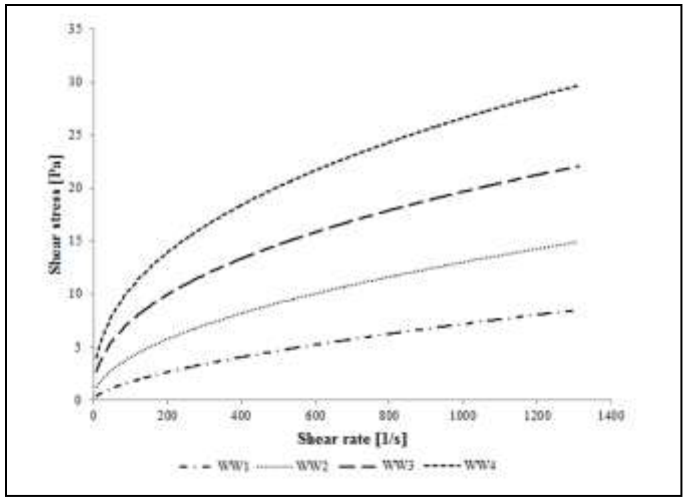

Figure 1. *Effect of the shear rate on shear stress for media based on winery wastewater after biosynthesis*

Pseudoplastic characteristics of media based on winery wastewaters are also confirmed by values of flow behavior index (n) and correlation coefficient (R^2) given in Table II. Flow behavior index represents a level of deviation from Newtonian flow behavior. For pseudoplastic fluids, such as xanthan solutions, this parameter should be $0<n<1$. Values of flow behavior index for media used in this research (WW1-WW4) were in the range of 0.4027-0.6237.

TABLE II. RHEOLOGICAL PARAMETERS AND CORRELATION COEFFICIENT FOR MEDIA BASED ON WINERY WASTEWATERS AFTER BIOSYNTHESIS

Winery Wastewaters	*Consistency factor, K*	*Flow behavior index, n*	*Correlation coefficient,* R^2
WW1	0.0966	0.6237	0.980
WW2	0.3908	0.5074	0.997
WW3	1.0575	0.4231	0.997
WW4	1.6478	0.4027	0.996

Viscosity of xanthan solutions strongly depends on its structure and concentration (11). Given that the viscosity and consistency factor (K) are proportional, values of consistency factor (Table II) indicate different quality and quantity of synthesized polymer. Values of flow behavior index, high values of consistency factor and results of product yield suggest that medium WW4 contained the highest amount of xanthan with good quality, while medium WW1 contained the lowest amount of xanthan with the lowest quality. Also, based on values of rheological parameters as well as the raw xanthan yield obtained for all media indicate the possibility of xanthan production on media based on wastewaters from rose wine production. This creates an opportunity for further research in order to optimize the xanthan production on winery wastewaters in terms of medium composition and environmental conditions.

In addition to producing a high value product, this research aimed to examine the possibility of application of biotechnological processes for the reduction of organic load in winery wastewaters, which was estimated by calculating the degree of sugar, nitrogen and phosphorus conversions. Conversions were calculated based on nutrients content in cultivation media before biosynthesis (S_0, N_0, P_0) and stillage obtained after ethanol distillation (S, N, P). Values of sugar, nitrogen and phosphorus conversions were in the ranges 63.72-74.27, 61.54-71.11 and 86.92-92.99, respectively (Table III). The high values of nutrient conversions indicate that xanthan production can significantly reduce organic load of waste streams, where the sugar is mostly metabolized in biosynthesis phase, and nitrogen and phosphorus in the phase of intensive growth of the producing microorganism cells.

TABLE III. VALUES OF SUGAR, NITROGEN AND PHOSPHORUS CONVERSION

Winery Wastewaters	*Sugar conversion[a] [%]*	*Nitrogen conversion[b] [%]*	*Phosphorus conversion[c] [%]*
WW1	63.72	71.11	92.99
WW2	72.41	66.67	91.01
WW3	72.81	62.34	88.95
WW4	74.27	61.54	86.92

a. *Conversion [%]* $= (S_0\text{-}S)/S_0 \cdot 100$

b. *Conversion [%]* $= (N_0\text{-}N)/N_0 \cdot 100$

c. *Conversion [%]* $= (P_0\text{-}P)/P_0 \cdot 100$

IV. Conclusions

In this study, the possibility of xanthan production on wastewaters obtained from four different stages of the rose wine production process was examined. Based on the pseudoplastic behavior of cultivation media and high values of raw xanthan yield (8.03-17.52 g/L) it can be concluded that the winery wastewaters have a great potential to be used as a substrate for production of this high value biopolymer. Also, in this research application of biotechnological processes was proposed as possible solution for the reduction of organic load in winery wastewaters. The results obtained in this paper could be the basis for optimization of xanthan production on media based on winery wastewaters in order to achieve higher product yields and reduce the negative impact on environment caused by disposal of these wastes.

Acknowledgment

The study is result of the investigations conducted within the Project number 114-451-1484/2014-02 funded by Provincial Secretariat for Science and Technological Development of Autonomous Province of Vojvodina.

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