LIQUID EFFLUENTS OF BIOETHANOL PRODUCTION FROM VARIOUS RAW MATERIALS AS MEDIA FOR XANTHAN BIOSYNTHESIS

TEČNI EFLUENTI PROIZVODNJE BIOETANOLA IZ RAZLIČITIH SIROVINA KAO MEDIJUMI ZA BIOSINTEZU KSANTANA

Bojana BAJIĆ, Siniša DODIĆ, Vladimir PUŠKAŠ, Zorana RONČEVIĆ, Jelena DODIĆ

University of Novi Sad, Faculty of Technology Novi Sad, Department of Biotechnology and Pharmaceutical Engineering,
Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
e-mail: ron@uns.ac.rs

ABSTRACT

The purpose of this paper is to examine the viability of using liquid effluents of bioethanol production from molasses, corn and a mixture of waste starch raw materials as a basis of the cultivation medium for the biotechnological production of xanthan using *Xanthomonas campestris* ATCC 13951. The dynamics of basic macronutrient consumption was used to monitor the course of xanthan biosynthesis, whereas the possibility of biosynthesis was confirmed on the basis of the rheological parameters of the cultivation broth. The success of biosynthesis was assessed according to the values of xanthan content and conversion to xanthan, which ranged from 8.78 g/L to 11.16 g/L and from 58.13 % to 73.24 % respectively when using the cultivation media based on liquid effluents of bioethanol production from different raw materials.

Key words: bioethanol, bioprocess, liquid effluents, xanthan.

REZIME

Potencijal primene tečnih efluenata industrije bioetanola u biotehnoškoj proizvodnji mnogobrojnih tržišno vrednih proizvoda, kakav je biopolimer ksantan, ogleda se u velikom sadržaju različitih organskih materija koje predstavljaju neophodne izvore nutrijenata za odgovarajući biokatalizator. Istraživanja iz okvira ovog rada obuhvatila su ispitivanje mogućnosti primene tečnih efluenata proizvodnje bioetanola iz različitih sirovina kao osnove kultivacionog medijuma u biotehnoškoj proizvodnji ksantana primenom soja *Xanthomonas campestris* ATCC 13951. Odabrani su tečni efluenti iz tri fabrike bioetanola koje kao sirovinu za proizvodnju ovog biogoriva koriste melasu, kukuruz i smešu otpadnih skrobnih sirovina. Efluenti su analizirani u pogledu parametara od značaja za biotehnošku proizvodnju i upotrebljeni za pripremu kultivacionih medijuma. Biosinteza je izvedena u Vulfovim bocama zapremine 2 l, u trajanju od 96 h. Tok biosinteze ksantana ispitan je praćenjem dinamike potrošnje osnovnih makronutrijenata, a mogućnost biosinteze je potvrđena na osnovu vrednosti reoloških parametara dobijenih kultivacionih tečnosti. Tokom kultivacije došlo je do odgovarajuće promene reologije medijuma što potvrđuju vrednosti prividnog viskoziteta, faktora konzistencije i indeksa toka. Uspješnost bioprocasa procenjena je na osnovu količine biosintetisanog ksantana i stepena konverzije supstrata u proizvod. Dobijene količine ksantana na kultivacionim medijumima koji su kao osnovu sadržali tečne efluente proizvodnje bioetanola iz različitih sirovina, su se nalazili u opsegu između 8,78 g/l i 11,16 g/l, dok su vrednosti stepena konverzije iznosile od 58,13% do 73,24%. Rezultati ovih istraživanja predstavljaju osnovu za optimizaciju proizvodnje ksantana na tečnim efluentima proizvodnje bioetanola iz različitih sirovina, sa ciljem povećanja kvaliteta i kvantiteta željenog proizvoda, uz razvoj održivog bioprocasa.

Ključne reči: bioetanol, bioprocas, tečni efluenti, ksantan.

INTRODUCTION

Bioethanol is predominantly produced in Serbia from sugar beet molasses and starch raw materials such as grains and potatoes (admittedly to a certain extent and in plants of smaller capacity) (Dodić et al., 2018). Based on the data of the Statistical Office of the Republic of Serbia for the period 2009–2016, the production of bioethanol in Serbia amounted to 60,000–65,000 hL per year. With regard to the increasing demand for bioethanol and the necessity for larger production capacities, the adequate utilization of generated effluents is of paramount importance to the competitiveness of this biofuel in the market, exerting a positive effect on the environment (Mladenović et al., 2016). Distillery wastewater, or stillage, is the aqueous by-product from the distillation of bioethanol, following the fermentation of different carbohydrate substrates such as sugar crops, starch crops, dairy products (whey) or cellulosic materials. Liquid effluents of bioethanol production pose a considerable pollution load, featuring high values of COD and BOD, nitrogen, phosphorus, potassium and heavy metals, as well as certain nutritional values. However, the characteristics of these effluents

are highly variable and greatly depend on the substrates used and several other aspects of the bioethanol production process (Wilkie et al., 2000; Banković Ilić et al., 2007). The potential of utilizing liquid effluents of the bioethanol industry for the biotechnological production of multiple profitable products, such as lactic acid and probiotics (Mojović et al., 2014), biodegradable plastic, poly β -hydroxybutyrate (Khardenav et al., 2007), and biogas (Moestedt et al., 2013), is reflected in the large amount of different organic substances, i.e. necessary sources of nutrients for a suitable biocatalyst.

Xanthan gum is an important industrial biopolymer of great commercial significance. Its widespread application, particularly in the food, pharmaceutical and petroleum industry, cosmetics, paper milling and textiles, is due to its unique rheological characteristics (Garcia-Ochoa et al., 2000). One of the biggest limiting factors for the utilization of xanthan in large-scale fermentation processes is its production cost compared to similar biopolymers. Therefore, it is important to find a more cost-effective and abundant substrate in order to increase the efficiency of this bioprocess (Lopez and Ramos-Cormenzana, 1996). Based on a number of previous studies, it can be concluded that xanthan production is possible using effluents

from the food industry and agricultural waste as a basis of the cultivation media (Bajić et al., 2017).

In order to assess the possibility of using liquid effluents of the bioethanol industry as raw materials to produce xanthan, they are required not only to be characterized but also experimentally confirmed as viable for biosynthesis, i.e. the effluents used cannot contain substances exerting an inhibitory effect on the production microorganism. Therefore, the objective of this study was to perform the xanthan biosynthesis by means of a cultivation media based on liquid effluents of bioethanol production from molasses, corn and a mixture of waste starch raw materials using *Xanthomonas campestris* ATCC 13951.

MATERIAL AND METHOD

Production microorganism

Xanthomonas campestris ATCC 13951 was used as the production microorganism in the experiments conducted. The production microorganism was stored at 4 °C on yeast-maltose (YM) agar slants (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.

Cultivation media

Liquid effluents from three bioethanol plants in Vojvodina, using molasses (W1), corn (W2) and a mixture of waste starch raw materials (W3) for biofuel production, were collected and analysed relative to the parameters of crucial importance to the biotechnological production of xanthan. The following amounts of carbon, nitrogen and phosphorus were recorded in the effluents collected: 8.42 g/L, 4.75 g/L and 0.12 g/L in W1, 5.30 g/L, 2.80 g/L and 0.12 g/L in W2, and 6.12 g/L, 2.61 g/L and 0.14 g/L in W3. On the basis of the results obtained, all the effluents analysed were diluted in order to decrease the initial nitrogen content, after which they were enriched with carbon (glucose) and phosphorus (K_2HPO_4) sources. Each media was prepared to contain a carbon source content of 15.00 g/L, a nitrogen content of 0.2 g/L and a phosphorus content of 0.045 g/L. The pH value was adjusted to 7.0 prior to sterilization by autoclaving at 121 °C and a pressure of 2.1 bars for 20 min.

Xanthan biosynthesis

The cultivation media was inoculated by adding 10 % (v/v) of inoculum prepared, under aerobic conditions, by two passages 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 °C in a laboratory shaker at 150 rpm for 48 h. The production of xanthan was carried out in 2 L Wouff bottles under aerobic conditions (an air flow rate of 1 vvm in the first 48 h, and 2 vvm thereafter) for 96 h. In the first 48 h, the biosynthesis temperature and agitation rate recorded were 26 °C and 200 rpm respectively, subsequently increasing to 32 °C and 300 rpm. The regulation of process parameters was done in accordance with the literature data (Garcia-Ochoa et al., 2000).

Product separation

After biosynthesis, the cultivation broths were pasteurized, centrifuged and used for the precipitation of the xanthan obtained in accordance with the previously described procedure (Bajić et al., 2015).

Analytical methods

Rheological properties of the cultivation broth samples were determined using a rotational viscometer (REOTEST 2 VEB MLV Prüfgeräte-Verk, Mendingen, SitzFreitel) with a double gap coaxial cylinder sensor system, spindle N. On the basis of

the measuring instrument deflection (α , Skt), shear stress (τ , Pa) was calculated relative to the defined values of shear rates (D , 1/s) using the following equation:

$$\tau = 0.1 \cdot z \cdot \alpha \quad (1)$$

where z is the constant with a value of $3.08 \text{ dyn/cm}^2 \cdot \text{Skt}$. The pseudoplastic behavior of the cultivation broth was confirmed by fitting the Ostwald-de-Waele model (Garcia-Ochoa et al., 2000) to the experimental data evaluated by a regression of the power law. The values of the consistency factor (K , $\text{Pa} \cdot \text{s}^n$), flow behavior index (n) and regression coefficient (R^2) were determined by the Excel software 2010 and used for apparent viscosity calculation (η_a , $\text{mPa} \cdot \text{s}$) using the following equation:

$$\eta_a = K \cdot D^{n-1} \quad (2)$$

In order to analyze the amounts of carbon source, nitrogen and phosphorus, the separation of solid and liquid phases in the cultivation broth samples was carried out by centrifuge at 10,000 rpm for 10 min. To determine the content of sugars, the supernatants obtained were filtered through a $0.45 \mu\text{m}$ nylon membrane (Agilent Technologies, Germany) and then analyzed by HPLC (Thermo Scientific DionexUltiMate 3000 series). The HPLC was equipped with a HPG-3200SD/RS pump, WPS-3000(T)SL autosampler ($10 \mu\text{L}$ injection loop), ZORBAX NH_2 column ($250 \text{ mm} \times 4.6 \text{ mm}$, $5 \mu\text{m}$) and RefractoMax520 detector. Acetonitrile (75 % (v/v)) was used as an eluent with a flow rate of 1.2 mL/min and an elution time of 20 min at a column temperature of 25 °C. Standard methods were used to determine residual contents of the total nitrogen and phosphorus, i.e. the Kjeldahl method (Herlich, 1990) for nitrogen and the spectrophotometric method (Gales et al., 1966) for phosphorus. A conversion rate of each nutrient analyzed (%) was calculated using the following equation:

$$\text{Nutrient conversion} = (S_0 - S) / S_0 \cdot 100 \quad (3)$$

S_0 (g/L) represents the initial nutrient content, whereas S (g/L) is the content of nutrient at the end of the bioprocess.

A conversion of carbon source into xanthan (%) was calculated using the following equation:

$$\text{Conversion} = P / C_0 \cdot 100 \quad (4)$$

C_0 (g/L) represents initial content of carbon source, whereas P (g/L) is the obtained xanthan content at the end of the bioprocess.

RESULTS AND DISCUSSION

Liquid effluents from three different plants producing bioethanol from molasses, corn and a mixture of waste starch raw materials were used as a basis of the cultivation media in order to examine the possibility of xanthan biosynthesis. The effluents obtained were analysed and prepared to contain the same initial content of basic nutrients, inoculated with *Xanthomonas campestris* ATCC 13951, followed by a biosynthesis performed under identical conditions.

The possibility of biosynthesis was confirmed based on the rheological parameters and cultivation broth apparent viscosity determined during 96 h of xanthan biosynthesis from liquid effluents of bioethanol production from different raw materials (Table 1). The viscosity of xanthan solutions is affected by the structure, level of acetyl and pyruvate groups, molecular mass and biopolymer concentration (Carignatto et al., 2011). The results obtained show an increase in the values of apparent viscosity (from 19.85 $\text{mPa} \cdot \text{s}$ to 66.22 $\text{mPa} \cdot \text{s}$ for W1, from 14.00

mPa·s to 41.47 mPa·s for W2, and from 16.83 mPa·s to 47.82 mPa·s for W3) and consistency factors (from 0.2109 Pa·sⁿ to 1.5435 Pa·sⁿ for W1, from 0.1385 Pa·sⁿ to 1.0135 Pa·sⁿ for W2, and from 0.1378 Pa·sⁿ to 1.2845 Pa·sⁿ for W3) of the cultivation broths obtained, which indicates an increase in the quantity of the produced biopolymer that affects the rheology of the system. Provided the W1, W2 and W3 values of cultivation broth apparent viscosity at the end of the bioprocess are compared, it can be seen that the highest value (66.22 mPa·s) is obtained using the liquid effluent of bioethanol production from molasses. Flow behaviour index (n) values that are lower than 1 and decrease during the bioprocess (from 0.5257 to 0.3680 for W1, from 0.5400 to 0.3585 for W2 and from 0.5780 to 0.3395 for W3) confirmed the pseudoplastic properties of the cultivation broths obtained, which is characteristic of xanthan solutions (Garcia-Ochoa et al., 2000).

Table 1. Rheological parameters and apparent viscosity of cultivation broths based on bioethanol production liquid effluents during xanthan biosynthesis

Media	Cultivation time [h]	Consistency factor [Pa·s ⁿ]	Flow behaviour index [1]	Apparent viscosity [mPa·s]
W1	48	0.2109	0.5257	19.85
	72	1.0327	0.3775	46.46
	96	1.5435	0.3680	66.22
W2	48	0.1385	0.5400	14.00
	72	0.5255	0.4239	29.79
	96	1.0135	0.3585	41.47
W3	48	0.1378	0.5780	16.83
	72	0.6640	0.3994	33.31
	96	1.2845	0.3395	47.82

In order to examine the course of biosynthesis, samples of the cultivation broths were analysed relative to carbon source, nitrogen and phosphorus contents (Figure 1). Furthermore, the success of the biosynthesis was assessed on the basis of the values of xanthan content, conversion to product and basic macronutrient conversion values (Table 2). The results obtained show that the carbon source content decreased during biosynthesis regardless of the media containing bioethanol production liquid effluents. When using W1, i.e. the cultivation media based on the effluent of bioethanol production from molasses, the content of carbon source decreased from its initial value of 15.24 ± 0.34 g/L to 3.77 ± 0.14 g/L, whereby the conversion of the most important nutrient was 75.23 %. The content of carbon source during xanthan biosynthesis using W2 decreased from 15.11 ± 0.24 g/L to 5.38 ± 0.23 g/L, whereas in the instance of W3 it decreased from 15.33 ± 0.22 g/L to 4.72 ± 0.21 g/L. However, the W2 and W3 conversion values were 64.37 % and 69.24 %, respectively. Nitrogen and phosphorus are nutrients essential for the growth of the production microorganism (Lopes et al., 2015), which is why their concentrations are expected to decrease more intensively in the first phase of biosynthesis, i.e. during the first 48h. Additionally, the value of nitrogen conversion using media based on bioethanol production effluents ranged between 44.01–54.15 %, whereas the value of phosphorus conversion ranged between 65.47–74.58%. The obtained conversion values of carbon source, nitrogen and phosphorus are particularly significant when considering that nutrients that are not utilized during biosynthesis remain in the wastewater and pose both economic and environmental losses (Bajić et al., 2017).

Fig. 1. Course of xanthan biosynthesis using liquid effluents of bioethanol production from a) molasses, b) corn, and c) starch waste

The main indicator of the xanthan biosynthesis success was its concentration in the cultivation broth. Based on the results shown in Table 2, the highest concentration of xanthan, 11.16 g/L, was obtained using the W1 medium, followed by the W3 medium with 10.08 g/L and the W2 medium with 8.78 g/L of xanthan. The conversion to xanthan values obtained for all the media applied ranged between 58.13 % and 73.24 % and is in accordance with the literature data (Rosalam and England, 2006).

Table 2. Xanthan content, conversion to xanthan and basic macronutrient conversion values for cultivation broths based on bioethanol production liquid effluents after xanthan biosynthesis

Media	Xanthan content [g/L]	Conversion to xanthan [%]	Carbon source conversion [%]	Nitrogen conversion [%]	Phosphorus conversion [%]
W1	11.16 ± 0.34	73.24 ± 0.58	75.23 ± 1.06	54.15 ± 1.30	74.58 ± 1.11
W2	8.78 ± 0.15	58.13 ± 0.91	64.37 ± 2.12	44.01 ± 0.90	65.47 ± 1.94
W3	10.08 ± 0.18	65.75 ± 0.37	69.24 ± 0.94	45.16 ± 1.85	71.36 ± 2.16

CONCLUSION

The research performed in this paper confirmed that the biopolymer xanthan gum can be produced using the cultivation media based on liquid effluents of bioethanol production from different raw materials. The largest amount of xanthan (11.16 g/L) was obtained using liquid effluents of bioethanol production from molasses as a basis of the cultivation media for xanthan production. Furthermore, the use of this effluent resulted in the highest values of all the parameters important to xanthan biosynthesis such as the conversion to xanthan (73.24 %), conversion of carbon source (75.23 %), nitrogen (54.15 %) and phosphorus (74.58 %). The results obtained in this study represent a basis for the optimization of xanthan production using liquid effluents of bioethanol production from various raw materials with the aim of increasing the quality and quantity of desired products, while developing a sustainable bioprocess.

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