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# XANTHAN PRODUCTION ON CRUDE GLYCEROL BY LAB-SCALE BIOREACTOR CULTIVATION OF LOCAL Xanthomonas ISOLATE

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**Abstract**

Intensive development of the global biodiesel industry has led to the generation of a large excess of crude glycerol, which is impure, and its disposal into the environment is unacceptable without previous purification. Since purification costs are high, the application of crude glycerol in biotechnological production of value-added products represents a promising solution for a sustainable utilization of this effluent. The aim of this study was to examine xanthan biosynthesis by the *Xanthomonas* PL 3 strain on a medium containing crude glycerol from biodiesel production in a laboratory-scale bioreactor in order to further scale up this bioprocess. Xanthan was produced by submerged cultivation in a crude glycerol-based medium (glycerol content of 20 g/L) in a 3 L lab-scale bioreactor (working volume of 2 L), under aerobic conditions for 168 h (0-48 h: 25°C, 1 vvm and 200 rpm; 48-168 h: 30 °C, 2 vvm, and agitation rate adjusted according to the dissolved oxygen concentration which was maintained at values higher than 30%). The bioprocess was monitored by the analysis of cultivation medium samples in predetermined time intervals, and its success was estimated based on the xanthan concentration in the medium, separated biopolymer average molecular weight and degree of nutrient conversion. In the applied experimental conditions, 11.10 g/L of xanthan with the average molecular weight of $2.85 \cdot 10^5$ g/moL was biosynthesized. At the end of this bioprocess, the degree of total glycerol, nitrogen and phosphorous conversion was 62.82%, 41.54% and 24.80%, respectively. Results obtained in this study suggest that the *Xanthomonas* PL 3 strain has the ability to produce xanthan of good quality on cultivation media containing crude glycerol from the biodiesel industry.

**Keywords:** Biotechnological production, biopolymer, xanthan, *Xanthomonas* isolate, crude glycerol, biodiesel industry effluent.

## 1. INTRODUCTION

Xanthan is a natural polysaccharide which is produced by metabolic activity of Gram-negative bacteria from the genus *Xanthomonas* (Rottava et al. 2009). Outstanding rheological properties, biodegradability, non-toxic nature and biocompatibility make this anionic polysaccharide widely used in the food, biomedical, pharmaceutical, petrochemical, chemical and textile industries (Demirci, Palabiyik, Apaydın, Mirik, & Gumus 2019; Kalia & Choudhury 2019; Riaz, Iqbal, Jiang, & Chen 2021). Xanthan has been classified as a food additive number E 415 by the European List of Permitted Food Additives, and the United States Food and Drug Administration has given the GRAS status (Generally Recognized as Safe) to an ethanol precipitate of xanthan(Dzionek, Wojcieszyńska, & Guzik 2022). This polysaccharide is composed of glucose, mannose and glucuronic acid units, and its molecular weight distribution depends on the association between structural chains. Therefore, the molecular weight of xanthan usually varies from around $2 \cdot 10^5$ g/moL to $2 \cdot 10^7$ g/moL (Badwaik, Giri, Nakhate, Kashyap, & Tripathi 2013; Garcí-Ochoa, Santos, Casas, & Gómez 2000)

Xanthan occurs as a white or cream-colored free-flowing powder of neutral smell and taste (Khan, Park, & Kwon 2007). Different *Xanthomonas* species, such as *X. malvacearum*, *X. phaseoli*, *X. axonopodis* and *X. eu-*

*vesicatoria*, have a great potential for successful xanthan biosynthesis, but *X. campestris* is the species most commonly used for the industrial production of xanthan (Petri 2015; Zahovic, Dodic, Grahovac, Grahovac, & Trivunovic 2021). Commercially, production of xanthan is mainly performed as aerobic submerged batch cultivation of the reference strain *X. campestris* ATCC 13951 on a medium of appropriate composition and under optimal conditions. The success of xanthan production is largely influenced by the cultivation medium composition, producing strains and bioprocess parameters (Zahović et al. 2022). Sucrose and glucose are mostly used as carbon sources in the media for xanthan production (Brandão et al. 2013). Considering that the cost of substrate is an important factor for commercial xanthan production and that the prices of the aforementioned sugars are rising, it can be noticed that there is a need for the exploitation of more economical carbon sources in order to reduce the overall production costs (Wang, Wu, Zhu, & Zhan 2016). Despite the fact that there are various alternative substrates of lower market value which may be used as a carbon source in the cultivation medium for xanthan production, crude glycerol from the biodiesel industry proved to be one of the most promising (de Jesus Assis et al. 2014; Wang et al. 2016). Results from several studies have confirmed that besides the reference strain, different wild-type *Xanthomonas* strains isolated from infected plants possess the ability to biosynthesize xanthan on a medium with glycerol as a sole carbon source (Brandão et al. 2013; Trindade, Munhoz, & Burkert 2015; Zahović et al. 2022). Moreover, some *Xanthomonas* isolates are able to metabolize glycerol to a higher degree than glucose (Zahović et al. 2021). Research related to the xanthan biosynthesis on glycerol-based media is still in initial stages, and there is a need for a more detailed examination regarding the isolation of new *Xanthomonas* strains that are able to metabolize glycerol and produce a large amount of high-quality xanthan, as well as regarding the optimization of the cultivation medium composition and process parameters. The aim of this study is to examine xanthan biosynthesis by the cultivation of the *Xanthomonas* PL 3 strain, isolated from pepper leaves, on a medium containing crude glycerol from biodiesel production in a domestic factory, in a laboratory-scale bioreactor, with an additional aim to further scale up this bioprocess. Estimation of the bioprocess success was based on the quantity and quality of isolated xanthan and the conversion rate of the most important nutrients at the end of biosynthesis.

## 2. MATERIALS AND METHODS

### 2.1. Producing microorganism

The strain *Xanthomonas* PL 3, isolated from pepper leaves in Serbia, was used as the producing microorganism in this experiment. The strain was stored at 4°C on an agar slant (Yeast Maltose Agar, HiMedia, India) and subcultured every four weeks within the Microbial Culture Collection of the Faculty of Agriculture in Novi Sad, Serbia.

### 2.2. Cultivation media

An agar slant (Yeast Maltose Agar, HiMedia, India) was used for strain storage, and a commercial liquid medium (Yeast Maltose Broth, HiMedia, India) was used for its incubation during inoculum preparation. Xanthan production was performed on a medium containing crude glycerol from biodiesel production in a factory located in the Republic of Serbia. Glycerol content in crude glycerol was around 50% (w/v) and its content in the cultivation medium was adjusted to 20.00 g/L. This concentration was selected based on the results from the previous study (Roncevic, Bajic, Grahovac, Dodic, & Dodic 2014). The cultivation medium also contained a yeast extract (3.0 g/L), $(NH_4)_2SO_4$ (1.5 g/L), $K_2HPO_4$ (3.0 g/L) and $MgSO_4 \cdot 7H_2O$ (0.3 g/L). The pH value of all used media was adjusted to 7.0±0.2 and then sterilized by autoclaving (121°C, 2.1 bar, 20 min).

### 2.3. Inoculum preparation

Inoculum was prepared in two steps. Producing microorganism was subcultured on an agar slant and incubated at 25°C for 48 h. Inoculum I preparation involved suspending the producing microorganism cells in the commercial liquid medium. The suspension was then incubated in aerobic conditions at 25°C and 150 rpm (laboratory shaker KS 4000i control, Ika® Werke, Germany) for 48h. Inoculum II preparation was performed by adding 10% (v/v) of Inoculum I in the commercial liquid medium and incubation in conditions identical to those for Inoculum I preparation.

### 2.4. Xanthan production

The xanthan production was carried out in a 3 L laboratory bioreactor (Biostat® A plus, Sartorius AG, Germany) with 2 L of cultivation medium. Inoculation was performed by adding 10% (v/v) of inoculum prepared as previously described. Cell concentration in the medium at the beginning of cultivation was $4.7 \cdot 10^8$ CFU/mL. The xanthan biosynthesis was carried out under aerobic conditions for 168 h. In the first 48 h, the biosynthesis was

performed at a temperature of 25°C, air flow rate of 1 vvm and agitation rate of 200 rpm. Afterwards, temperature and air flow rate were increased to 30°C and 2 vvm, respectively, while agitation rate was corrected as needed and according to the dissolved oxygen concentration which was maintained at values higher than 30% during the biosynthesis. During xanthan production, the pH of the cultivation medium decreased from neutral to values close to 5 due to the production of organic acids and xanthan, which contains acid groups (Garcí-Ochoa et al. 2000). Considering the fact that the optimum pH for the bacterial growth range is between 6 and 7.5 and the optimum pH range for the xanthan production is between 7 and 8 (Sherley & Priyadharshini 2015), this parameter was regulated during the bioprocess, i.e., it was maintained above 6.0 by adding 5.0 M KOH. The dissolved oxygen concentration was maintained at values greater than 30% of saturation during the entire bioprocess by regulating the aeration intensity and mixing speed as recommended in the literature (Casas, Santos, & Garcí–Ochoa 2000).

### 2.5. Xanthan separation

At the end of biosynthesis, xanthan was separated from the supernatant of the cultivation medium by precipitation with cold 96% (v/v) ethanol in the presence of potassium chloride. Ethanol was gradually added to the supernatant at constant stirring until the alcohol content in the mixture was 60% (v/v). A saturated solution of potassium chloride was added when half of the necessary ethanol amount was poured into the supernatant in such a quantity as to obtain a final content of 1% (v/v). After precipitation, the mixture of xanthan was kept at 4 °C for 24 h and then centrifuged (4,000 rpm, 15 min). The precipitate was dried to a constant mass at 60 °C and this data was used to calculate the xanthan concentration in the medium.

### 2.6. Determination of xanthan molecular weight

The average molecular weight of the separated xanthan was estimated based on the intrinsic viscosity of its 1% (w/v) solution in 0.1 M sodium chloride using the Mark–Houwink type equation (Milas, Rinaudo, & Tinland 1985).

### 2.7. Determination of biomass concentration

The biomass concentration was expressed as viable cell count per milliliter of cultivation medium and is determined by counting colony forming units (CFU). The samples of the cultivation medium, taken under aseptic conditions, were serially diluted in a sterile saline solution and plated on agar plates (Yeast Maltose Agar, HiMedia, India), which were incubated at 25°C for 48 h. The living bacterial cell on the plate was grown into a colony, and the viable cell count in the cultivation medium was then calculated by multiplying the final number of colonies by the dilution factor.

### 2.8. Determination of nutrient content

The samples of cell-free cultivation media taken in previously defined time intervals and obtained by centrifugation at 10,000 rpm for 15 min (Rotina 380 R, Hettich Lab Technology, Germany) were analyzed for glycerol, total and assimilable nitrogen and total phosphorus contents. Glycerol content was determined by high performance liquid chromatography (HPLC). The samples were filtered through a 0.45 □m nylon membrane (Agilent Technologies Inc, Germany) and then analyzed. The HPLC instrument (Thermo Scientific Dionex UltiMate 3000 series) was equipped with a HPG-3200SD/RS pump, WPS-3000(T)SL autosampler (10 □L injection loop), Zorbax NH2 column (250 mm × 4.6 mm, 5 □m) and RefractoMax520 detector. Acetonitrile 70% (v/v) was used as eluent with a flow rate of 1 mL/min and elution time of 10 min at a column temperature of 30°C. The contents of total nitrogen and phosphorus were determined using the volumetric method proposed by Kjeldahl (Helrich 1990) and spectrophotometric method (Gales, Julian, & Kroner 1966), respectively. The assimilable nitrogen content, expressed as amino and ammonia nitrogen, was determined by the formol titration method (Zoecklein, Fugelsang, Gump, & Nury 1999). The nutrient content results were used to calculate the degree of crude glycerol, total nitrogen and total phosphorus conversion (K, %) using Equation 1:

$$\mathrm{K} = \frac{(Y_0 - Y)}{Y_0} \cdot 100 \qquad (1)$$

where $Y_0$ is initial nutrient content (g/L), while Y is residual nutrient content (g/L). The results for glycerol content after inoculation and xanthan concentration in the medium were used to calculate the degree of initial glycerol conversion into xanthan ($K_{P/S}$, %) using Equation 2:

$$\mathrm{K_{P/S}} = \frac{P}{S_0} \cdot 100 \qquad (2)$$

where $S_0$ is the initial glycerol content (g/L) and P is the xanthan concentration in the medium at the end of the bioprocess (g/L). The initial and residual glycerol content results as well as xanthan concentration in the medium

were used to calculate the degree of metabolized glycerol conversion into xanthan ($K_{P/\Delta S}$, %) using Equation 3:

$$K_{P/\Delta S} = \frac{P}{S_0} \cdot 100 \quad (3)$$

where $S_0$ is the initial glycerol content (g/L), S is the residual glycerol content (g/L), and P is the xanthan concentration in a medium at the end of bioprocess (g/L).

### 2.9. Determination of cultivation medium rheological behavior

The rheological behavior of cultivation medium samples taken in previously defined time intervals was evaluated using a rotational viscometer (REOTEST 2 VEB MLV Prufgerate-Verk, Mendingen, SitzFreitel) with double gap coaxial cylinder sensor system, spindle N. Based on the deflection of the measuring instrument ($\alpha$, Skt), shear stress (□, Pa) was calculated under defined values of shear rates (D, 1/s) using Equation 4:

$$\tau = 0.1 \cdot z \cdot \alpha \quad (4)$$

where z is the constant with the value 3.08 dyn/cm$^2$·Skt.

The pseudoplastic behavior of the cultivation medium was confirmed by fitting the experimental data to the Ostwald-de-Waele model using power regression. The values of the consistency factor (K, Pa·s$\eta$), flow behavior index (n) and determination coefficient ($R^2$) were determined by Excel software 2013 and used for the calculation of medium apparent viscosity ($\eta_a$ , mPa·s) from Equation 5:

$$\eta_a = K \cdot D^{n-1} \quad (5)$$

where D is a shear rate with the value of 100s$^{-1}$.

## 3. RESULTS AND DISSCUSION

The xanthan biosynthesis by the cultivation of the *Xanthomonas* PL3 strain, isolated from pepper leaves, in a 3 L lab-scale bioreactor on a crude glycerol-based medium was performed to investigate the possibility of biotechnological utilization of this effluent from the biodiesel industry. The bioprocess flow in the applied experimental conditions was monitored by the analysis of cultivation medium samples, taken in previously defined time intervals, in terms of biomass content, the content of essential nutrients for biotechnological production of this biopolymer, and rheological behavior. The obtained values of the analyzed parameters are graphically represented in Figure 1. The bioprocess success was assessed based on the xanthan concentration in the medium, the average molecular weight of the separated biopolymer, and degree of conversion of the most important nutrients. The results of these analyses are summarized in Table 1. Xanthan biosynthesis was performed under controlled conditions, which included measurement and regulation of pH and temperature, as well as dissolved oxygen concentration, by the adjustment of mixing speed and aeration intensity. The change of biomass concentration (X), glycerol content (S), total ($N_{tot}$) and assimilable ($N_{ass}$) nitrogen content, total phosphorus content ($P_{tot}$) and medium apparent viscosity (□$_a$) during the xanthan biosynthesis by *Xanthomonas* PL 3 on a crude glycerol-based medium in the applied experimental conditions is presented in Figure 1.

The results for biomass concentration shown in Figure 1 indicate that the viable cell count of *Xanthomonas* PL 3 increased from the beginning of the cultivation. As it can be seen from the graphically presented results, during the first 48 h of cultivation the producing microorganism multiplied intensively and the cell concentration in the medium increased from $4.7 \cdot 10^8$ CFU/mL to $100.8 \cdot 10^8$ CFU/mL. This indicates that the exponential growth phase of the producing microorganism occurred at the beginning of the bioprocess and lasted for 48 h. Between 48 h and 72 h, a slower increase in biomass concentration was observed, reaching a maximum value of $1.16 \cdot 10^{10}$ CFU/mL at the 72nd h. After 72 h of cultivation, there was no significant change in the biomass concentration and it can be concluded that the stationary phase of producing microorganism growth occurred. After 168 h of biosynthesis, the biomass concentration was $115 \cdot 10^8$ CFU/mL. According to the available literature, in order to achieve a successful production of xanthan, the value of the initial biomass concentration should be in the range from $10^6$ CFU/mL to $10^8$ CFU/mL (Pejin 2003). The results shown in Figure 1 indicate that the glycerol content in the cultivation medium generally decreased during the entire bioprocess. The initial glycerol content in the medium was 20.09 g/L and, according to the results presented in Figure 1, it can be noticed that, during the first 48 h of cultivation, there was an intense decrease in the glycerol content to a value of 11.74 g/L. After 48 h, the glycerol content continued to decrease, but with a slightly lower intensity until the 144th h, when the content of this nutrient was 7.51 g/L. After 144 h of cultivation, there was no significant change in the glycerol content in the medium, and its value at the 168th h was 7.47 g/L. This suggests that if there is no carbon source consumption, xanthan biosynthesis does not occur in the stationary phase of producing microorganism growth (Palaniraj & Jayaraman 2011) and bioprocess duration could be shortened by 24 h. Considering the ob-

tained results which show that intensive metabolic activity of the *Xanthomonas* PL 3 strain happened in the first 48 h of cultivation in the 3 L lab-scale bioreactor, it can be concluded that the used producing strain has a great ability to metabolize crude glycerol in the applied experimental conditions. This is also supported by the findings from previous studies when performing the bioprocess by the same producing strain in smaller volumes and less intensive bioprocess conditions (Zahović et al. 2022; Zahović & Trivunović 2021).

Nitrogen sources in xanthan production media present the growth-limiting factor for *Xanthomonas* cells (Garcí-Ochoa et al. 2000). Graphical representation of the obtained results given in Figure 1 shows that the initial content of total nitrogen in the medium decreased intensively from the very beginning of the bioprocess. Consequently, the total nitrogen content was reduced from the initial 910 mg/L to 560 mg/L within the first 48 h. It can be noted that the period of intensive consumption of nitrogen components corresponded to the period in which the exponential growth phase of the producing microorganism took place. Observing the results from Figure 1 it can be seen that, after 48 h of cultivation, there was no significant change in the total nitrogen content, which is a consequence of the onset of the stationary growth phase of the microorganism. At the end of the bioprocess, the residual concentration of overall nitrogen components was 532 mg/L. The change in the content of assimilable nitrogen during the cultivation of the *Xanthomonas* PL 3 strain on the crude glycerol-based medium is also shown in Figure 1.

A trend of change of assimilable nitrogen identical to that of the total nitrogen content can be observed. In this case, the content of assimilable nitrogen decreased from 593.60 mg/L to 316.40 mg/L within the first 48 h. After this period, there was no significant change in the content of assimilable nitrogen, and, after 168 h of cultivation, its value was 263.20 mg/L. The obtained results confirm that the intensive reduction of assimilable nitrogen content is in accordance with the consumption of total nitrogen. The higher consumption of total nitrogen compared to assimilable nitrogen indicates that in applied experimental conditions, besides amino and ammonia nitrogen, the cells of the *Xanthomonas* PL 3 strain used some other nitrogen-containing compounds, such as proteins originating from a yeast extract, for growth and reproduction (Ozdal & Kurbanoglu 2019). However, experimental errors in the determination of total and assimilable nitrogen should not be excluded. The obtained results shown in Figure 1 suggest that an intensive consumption of phosphorus from the very beginning of the cultivation of the *Xanthomonas* PL 3 strain on the glycerol-based medium is evident. Given that phosphorus is the limiting nutrient for *Xanthomonas* cell growth and therefore for xanthan biosynthesis, it is expected that the change in phosphorus content follows the change in nitrogen content (Garcí-Ochoa, Santos, & Fritsch 1992). Analyzing the presented results, it can be noted that the change in the total phosphorus content is similar to the change in the content of total and assimilable nitrogen during the bioprocess. The initial phosphorus content of 646.43 mg/L decreased intensively in the first 48 h when it reached 504.24 mg/L. The change in the total phosphorus content in the cultivation medium was insignificant after 48 h of cultivation, and, at the end of cultivation, phosphorus content was 486.08 mg/L. Observing the results for the change in the content of the most important nutrients in the crude glycerol-based medium during the cultivation of the *Xanthomonas* PL 3 strain, it can be concluded that the metabolic activity of the used producing strain in the applied experimental conditions was easily carried out, i.e., that the biomass concentration has increased, and that all conditions for the successful xanthan biosynthesis were provided. In order to determine whether the production of xanthan occurred, rheological measurements of the cultivation medium were performed. The measured values were fitted with a power law equation of the Ostwald-de Waele model in order to define the values of the flow behavior index and the consistency factor, on the basis of which the apparent viscosity of the cultivation medium was calculated. The change in the apparent viscosity of the crude glycerol-based medium during the cultivation of the *Xanthomonas* PL 3 strain is also represented in Figure 1. The obtained results show that the value of the apparent viscosity of the medium during biosynthesis did not change significantly in the first 48 h. In that period, this value increased from the initial 4.29 mPa·s to 4.83 mPa·s. After 48 h of cultivation, a drastic increase in the apparent viscosity of the cultivation medium was noticed up to 144 h, when its value was 34.82 mPa·s. Observing the results shown in Figure 1 , it can be seen that the value of the apparent viscosity of the cultivation medium slightly increased after 144 h of cultivation, and at the end of biosynthesis, the value of this parameter was 35.22 mPa·s. The rheological measurement showed that all samples have pseudoplastic properties, a known characteristic of xanthan solutions (Garcí-Ochoa et al. 2000), considering that the values of flow behavior index in this study were $0 < n < 1$. The values of this parameter decreased from 0.6719 to 0.4846 indicating that during the cultivation pseudoplastic behavior of the medium became more pronounced. Further, the Ostwald-de-Waele model showed a good agreement with the experimental data, since the regression coefficients were higher than 0.91 in all tests. As the consistency factor is proportional to the viscosity, the increase in values of this parameter during

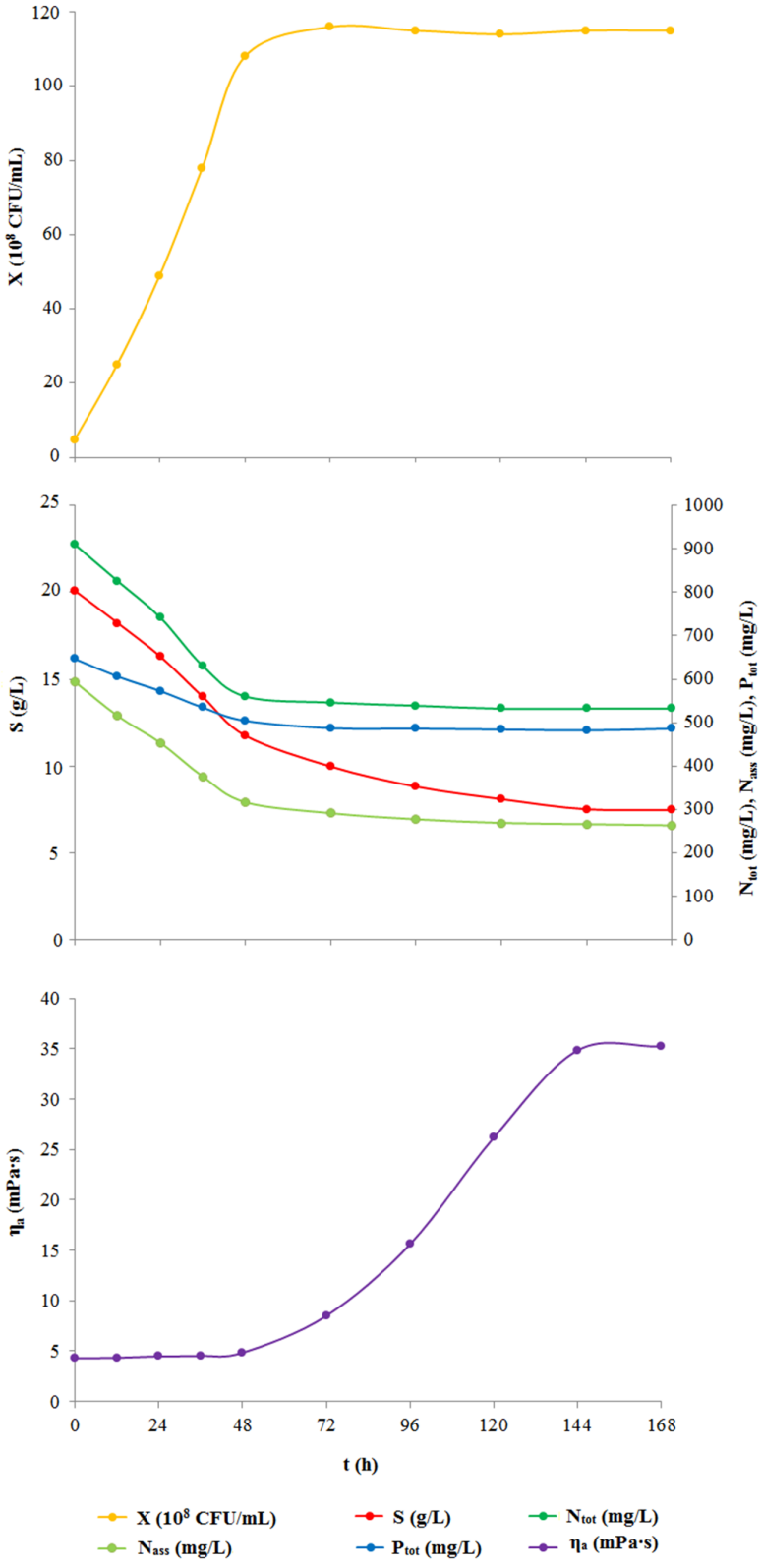


**Figure 1.** The flow of xanthan biosynthesis by *Xanthomonas* PL 3 in 3 L lab-scale bioreactor on crude glycerol-based medium in terms of (X), glycerol content (S), total ($N_{tot}$) and assimilable ($N_{ass}$) nitrogen content, total phosphorus content ($P_{tot}$) and medium apparent viscosity ($\eta_a$).

**Table 1.** Indicators of the success of xanthan biosynthesis by *Xanthomonas* PL 3 in 3 L lab-scale bioreactor on crude glycerol-based medium.

| Indicator | P (g/L) | KP/S (%) | KP/□S (%) | KS (%) | KNtot (%) | KPtot (%) | Mw (105 g/moL) |
|---|---|---|---|---|---|---|---|
| Value | 11.10 | 55.26 | 87.98 | 62.82 | 41.54 | 24.80 | 2.85 |

*P - xanthan concentration in medium; $K_{P/S}$ - degree of initial glycerol conversion into xanthan; ($K_{P/\Delta S}$ - degree of metabolized glycerol conversion into xanthan; $K_S$ - degree of glycerol conversion; $K_{Ntot}$ - degree of total nitrogen conversion: $K_{Ptot}$ - degree of total phosphorus conversion; $M_w$ - average molecular weight of xanthan.*

the bioprocess from 0.0194 Pa·s$^n$ to 0.3781 Pa·s$^n$ indicates that the viscous nature of the cultivation medium at the end of biosynthesis can be observed (Soto-Caballero et al. 2016). In addition, the discussed changes in the value of apparent viscosity of the medium during the cultivation of the used producing strain are reliable confirmation that xanthan was produced in the applied experimental conditions. In order to investigate the performance of xanthan production on a crude glycerol-based medium, selected indicators of bioprocess success (xanthan concentration in the medium, degree of initial glycerol conversion into xanthan, degree of metabolized glycerol conversion into xanthan, degree of glycerol conversion, degree of total nitrogen conversion, degree of total phosphorus conversion and average molecular weight of xanthan) were determined, and the obtained results (Table 1) were further discussed. After the cultivation, xanthan concentration in the medium was 11.10 g/L. This value is higher compared to the values obtained in previous researches, where xanthan was produced by *Xanthomonas* strains, isolated from pepper leaves and crucifers on a crude glycerol-based medium, but in smaller volumes (300 mL Erlenmeyer flasks and 2.0 L Woulff bottles), and where xanthan concentration in media varied from around 5.00 g/L to 8.00 g/L (Rončević, Zahović, et al. 2020; Zahović et al. 2022; Zahović & Trivunović 2021). Moreover, xanthan concentration obtained in this study is also higher in comparison with the results obtained when reference strain *Xanthomonas campestris* ATCC 13951 was cultivated on a medium containing crude glycerol (15.00 g/L). In that research, xanthan concentration in media varied from 6.77 g/L to 7.22 g/L (Rončević, Bajić, et al. 2020). Comparing the aforementioned results to those obtained in a research conducted by scientists from Brazil, in which a xanthan content of 5.59 g/L was achieved during the *Xanthomonas campestris mangiferaeindicae* 2103 cultivation on a crude glycerol-based medium (20 g/L) in a 4.5 L bioreactor (de Jesus Assis et al. 2014), it can be concluded that xanthan biosynthesis in the present study was highly successful if the concentration of the produced biopolymer is considered as an indicator. This indicates that the producing strain, medium composition, process parameters, as well as bioprocess volume have a great effect on xanthan biosynthesis.

Besides xanthan production, the conversion of important nutrients presents a very significant indicator of bioprocess success. The obtained results (Figure 1 ) indicate that the content of glycerol, total nitrogen and total phosphorus content in the medium decreased during xanthan biosynthesis in the applied experimental conditions. At the end of the bioprocess, the degree of glycerol conversion was 62.82% while the initial and metabolized glycerol conversions into xanthan were 55.26% and 87.98%, respectively (Table 1). The conversion of glycerol achieved in this study is in agreement with the degree of glycerol conversion of 63.14% obtained in an earlier study when the same strain was cultivated on a crude glycerol-based medium but in a smaller volume and less intensive bioprocess conditions, especially in terms of aeration and agitation (Zahović & Trivunović 2021). However, metabolized glycerol conversion into xanthan of 36.11% in the aforementioned research conducted by the authors was far lower compared to the value of 87.98% (Table 1) achieved in the present study. This indicates that an increase in bioprocess volume leads to better conditions in terms of homogenization and aeration, which, together with the increase in inoculum incubation time, have a positive effect on glycerol conversion into xanthan and contribute to better dissolution of oxygen, necessary for successful xanthan production. Taking into account that in industrial conditions, the degree of carbon sources conversion into xanthan ranges from 50 to 85% (Rosalam & England 2006), it can be concluded that in this research, a very high efficiency of the bioprocess has been achieved. In addition to carbon sources, nitrogen and phosphorus sources are also essential nutrients in cultivation media for xanthan production. According to the data presented in Table 1, the total nitrogen conversion achieved within this study was 41.54%, and this value is greater compared to the value of 33.07% achieved in an earlier study when the cultivation of the same producing strain on a crude glycerol-based medium was performed in a 2.0 L Woulff bottle (Zahović & Trivunović 2021) for 168 h with the inoculum prepared for 72 h. The value of total phosphorus conversion of 24.80% obtained in the present research is slightly higher compared to the result obtained in the aforementioned research when the degree of total phosphorus conversion during the cultivation of the

same producing strain on a crude glycerol-based medium was 21.77% (Zahović & Trivunović 2021). This confirms that an increase in bioprocess volume and inoculum incubation time has a positive effect on nutrient consumption by the applied *Xanthomonas* strain. Additionally, the results obtained in this research using the *Xanthomonas* strain isolated from pepper leaves are higher compared to the results obtained in an earlier study, where *Xanthomonas* strains, isolated from crucifers, were cultivated on a crude glycerol-based medium in a 2.0 L Woulff bottle for 168 h (with inoculum prepared for 72 h) and where total nitrogen and total phosphorus conversion rates were in the range from about 23-30% and from about 18–22%, respectively (Rončević, Zahović, et al. 2020). The quality of xanthan represents another important indicator of the success of this bioprocess and can be estimated based on several parameters, such as the viscosity of its solutions, composition, molecular weight, etc. (Lopes, Lessa, Silva, & Cerda 2015). In this study, the average molecular weight of separated xanthan was used as the biopolymer quality indicator. Observing the results presented in Table 1 it can be noticed that the average molecular weight of xanthan produced on a medium containing crude glycerol from biodiesel production is 2.85 $\cdot 10^5$ g/moL. This result is in agreement with the results obtained in an earlier study, where xanthan biosynthesis was performed on a crude glycerol-based medium in a smaller volume by different *Xanthomonas* strains, isolated from crucifers and pepper leaves. That study reported the average molecular weight of separated biopolymers in the range from 5 $\cdot 10^4$ g/moL to 3.0 $\cdot 10^5$ g/moL (Zahović et al. 2022). By analyzing all the results given in Figure 1 and Table 1, it was determined that xanthan biosynthesis by the cultivation of the *Xanthomonas* PL 3 strain in a 3 L lab-scale bioreactor on a medium containing crude glycerol from biodiesel production in a domestic factory was successful. Despite the fact that some of the nutrient conversion values obtained in the applied experimental conditions were relatively low, or not as high as expected, the performed bioprocess resulted in reduced glycerol, nitrogen and phosphorus contents, and it is important for the purpose of minimizing the negative impact of crude glycerol from biodiesel production on the environment.

## 4. CONCLUSIONS

In accordance with the defined aim, this study examined the flow of xanthan biosynthesis by the cultivation of the *Xanthomonas* PL 3 strain, isolated from pepper leaves, in a lab-scale bioreactor on a medium containing crude glycerol from biodiesel production in a factory located in the Republic of Serbia. The results of this study have confirmed that crude glycerol generated by the domestic biodiesel industry can be used as a sole carbon source in cultivation media for successful xanthan production by the local *Xanthomonas* isolate. Besides the good quality and quantity of the produced biopolymer, acceptable conversion of essential nutrients was also achieved within this research. The results of this study have great importance from an ecological point of view, considering the fact that the biotechnological production of xanthan on a cultivation medium containing crude glycerol from the biodiesel industry represents a promising solution for the sustainable valorization of this effluent. Moreover, the results obtained in this study represent valuable information that can be used in future investigations related to the optimization of the bioprocess in terms of increasing the xanthan yield and quality, the process scale-up, as well as the estimation of possible applications of this biopolymer.

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