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Biotechnological Valorization of Crude Glycerol from Biodiesel Industry: Xanthan Production in Lab-scale Bioreactor by *Xanthomonas euvesicatoria*

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Abstract

In this study, xanthan production by local wild-type *Xanthomonas euvesicatoria* PL4 strain in a 3 L stirred tank bioreactor from a medium containing crude glycerol generated in biodiesel industry was examined, and properties of separated biopolymer were determined. Xanthan was biosynthesized by submerged batch cultivation of producing microorganism in a medium with glycerol content of 20 g/L. The bioprocess was monitored by analysis of cultivation medium at predetermined time intervals, and its success was estimated based on the 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, and degree of total phosphorus conversion. Xanthan in concentration of 13.31 g/L was biosynthesized under the applied experimental conditions, and total glycerol, nitrogen, and phosphorous conversions of 76.23%, 64.70%, and 40.63% were achieved, respectively. The results obtained in this study suggest that the applied strain has the ability to produce xanthan with excellent properties on a crude glycerol-based medium. The findings of this study can contribute to future investigations which will aim to improve the production of xanthan on crude glycerol-based medium and find new possibilities for its application.

Keywords

biofuel industry effluent, biotechnological waste utilization, bacterial biopolymer, bioprocess monitoring, biopolymer properties

1 Introduction

Biofuels emerged as a low-carbon alternative to fossil fuels as they can lead to the reduction of greenhouse gas emissions. Biodiesel is one of the most commonly used biofuels worldwide. Expansion of biodiesel production results in the accumulation of significant amount of effluents and by-products [1]. Crude glycerol is major by-product of the biodiesel industry and is generated in the amount of 10% to 20% in relation to the volume of produced biodiesel. In general, the purity of crude glycerol obtained from biodiesel industry varies from 38% (w/w) to 96% (w/w), but its composition depends on several factors, which is why it is difficult to define its composition precisely [2, 3]. Considering that crude glycerol is impure, it requires treatment prior to discharging into the environment. Purification methods of this biodiesel industry effluent are expensive, demand high energy and water consumption and are usually for small-scale industrial applications, thus indicating the need for their utilization in crude form [2]. Several research studies have confirmed that crude glycerol can be successfully valorized in biotechnological production of xanthan and that certain strains of bacteria of the genus *Xanthomonas* possess the ability to biosynthesize this value-added biopolymer on a media containing crude glycerol as the only carbon source [4, 5].

Xanthan is microbial heteropolysaccharide of great commercial significance and, in comparison with others, this biopolymer has competitive price, and is widely applied in various industries regarding economic aspect and excellent performance [6]. Its molecular formula is $(C_{35}H_{49}O_{29})_n$ [7], while its molecular weight is usually from around $2 \cdot 10^5$ g/mol to $2 \cdot 10^7$ g/mol, and is greatly affected by the association between structural chains, as well as xanthan cultivation conditions [5, 8, 9]. Industrial production of xanthan considers the submerged cultivation of the pure bacterial culture of genus *Xanthomonas* under

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aerobic conditions on appropriately formulated media and under optimal conditions [7]. *Xanthomonas campestris* is primarily used in biotechnological production of xanthan, but other *Xanthomonas* spp., including *Xanthomonas malvacearum*, *Xanthomonas phaseoli*, *Xanthomonas axonopodis*, *Xanthomonas citri*, and *Xanthomonas euvesicatoria* can be applied for successful xanthan biosynthesis [5, 6, 10]. The cost of cultivation media is a crucial factor for commercial xanthan production and an actual rise in prices of glucose and sucrose, most commonly used carbon sources in cultivation media for xanthan production, is present. In order to reduce overall production costs, various agro-industrial by-products and effluents, including crude glycerol from biodiesel industry, were examined as alternative carbon sources [5, 11, 12]. According to the findings from previous research studies, apart from reference strain, different wild-type *Xanthomonas* strains isolated from infected plants have the ability to biosynthesize xanthan on a medium containing glycerol [3–5]. Another research proved that some *Xanthomonas* isolates are able to metabolize glycerol in higher degree than glucose [10]. Investigation into xanthan biosynthesis on crude glycerol-based media is still in its early stages, and further in-depth examination is required regarding isolation of new *Xanthomonas* strains that are able to metabolize glycerol and produce high amount of high-quality xanthan, analysis of time course profile of bioprocess, optimization of cultivation medium composition and bioprocess parameters, as well as determination of xanthan properties and potential application.

Results obtained in previous research suggest that *X. euvesicatoria* strains have a great potential for xanthan production on media containing crude glycerol as a sole carbon source [5]. However, time course profile of bioprocess, and properties of biosynthesized xanthan are poorly examined and their potential for application still remains undetermined. Therefore, in order to provide insights into time course profile of bioprocess, this study offers the first ever examination of xanthan production on crude glycerol-based medium by cultivation of local wild-type *X. euvesicatoria* PL4 strain in lab-scale stirred tank bioreactor, determination of its properties and estimation of potential application.

2 Experimental

2.1 Inoculum preparation

The strain *X. euvesicatoria* PL4, isolated from diseased pepper leave in Serbia, was used as the producing microorganism in this study. The strain was stored at 4 °C on an agar slant (YM Agar, HiMedia, India) and subcultured every four weeks within the Microbial Culture Collection of the Faculty of Agriculture in Novi Sad, Serbia.

An agar slant (YM Agar, HiMedia, India) and a liquid medium (YM Broth, HiMedia, India) was used for inoculum preparation. Inoculum was prepared in two steps, as described previously [11].

2.2 Xanthan production

Xanthan biosynthesis was performed on a medium containing crude glycerol from biodiesel production in a factory located in Serbia. Glycerol content in crude glycerol was 476.00 g/L and for cultivation medium preparation it was diluted with tap water to achieve the glycerol content of 20.00 g/L. This concentration was selected based on the results from the previous study [13]. The cultivation medium also contained a yeast extract (3.0 g/L), $(NH_4)_2SO_4$ (1.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L) and K_2HPO_4 (2.5 g/L), as suggested in earlier research [14]. 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).

The xanthan production was carried out in a 3 L stirred tank bioreactor (Biostat® A plus, Sartorius AG, Germany) with 2 L of cultivation medium. Inoculation was performed by adding 10% (v/v) of inoculum. The xanthan biosynthesis was carried out under aerobic conditions for 168 h. The optimal temperature for intensive growth and multiplication of *Xanthomonas* cells ranges from 24 °C to 27 °C, while the optimal value of this parameter for xanthan biosynthesis is in the range of 30 °C to 33 °C. The critical value of oxygen content in the stage of xanthan biosynthesis is between 6% (v/v) and 10% (v/v), however, below 6% (v/v) in addition to the reduced ability of xanthan biosynthesis, there is also a significant drop in the specific rate of oxygen uptake by the producing microorganism. The content of dissolved oxygen is closely related to the mixing speed of the cultivation medium. By mixing, the homogenization of the total volume of the medium is achieved and optimal conditions are ensured for the desired metabolic activity of the producing microorganism. It is very important that the aeration intensity and the mixing speed are adequately established in order to achieve the best possible exchange of oxygen between the medium and the dispersed air [9, 15]. All things considered, in the first 48 h, the biosynthesis was performed at a temperature of 25 °C, air flow rate of 1 volume of gas per volume of liquid per minute (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 bioprocess (300–450 rpm). 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 [15]. Since 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 [16], this parameter was regulated during the bioprocess, i.e., it was maintained above 6 by adding 1 M NaOH.

Xanthan was separated from the supernatant of cultivation medium by precipitation with cold 96% (v/v) ethanol, as described in previous study [9].

2.3 Analysis of cultivation media

The biomass concentration was determined as described in previous study by counting colony forming units (CFU) and expressed as viable cell count per milliliter of cultivation medium [9]. Agar plate (YM Agar, HiMedia, India) was used for microorganism growth and colonies counting.

Glycerol content was determined by high performance liquid chromatography by procedure described in previous study [17]. The content of total nitrogen and total phosphorus were determined using the volumetric method proposed by Kjeldahl [18] and spectrophotometric method [19], respectively. The assimilable nitrogen content, expressed as amino and ammonia nitrogen, was determined by the formol titration method [20].

The rheological behavior of cultivation medium was determined at room temperature using a rotational viscometer (REOTEST 2 VEB MLV Prufgerate-Verk, Mendingen, SitzFreitel) with double gap coaxial cylinder sensor system, spindle N. Based on deflection of measuring instrument (α, cm^2/s), shear stress (τ, Pa) was calculated under defined values of shear rates (D, 1/s; 3-1312 1/s) using Eq. (1):

$$\tau = 0.1 \cdot z \cdot \alpha \tag{1}$$

where z is the constant with the value $3.08 \cdot 10^{-1}$ Pa·cm^2/s.

The pseudoplastic behavior of the cultivation medium was confirmed by fitting the experimental data to the Ostwald-de-Waele model using the power regression. The values of the consistency factor (K, Pa·s^n), flow behavior index (n) and determination coefficient (R^2) were determined by Excel software 2013 [21] and used for calculation of medium apparent viscosity (η_a, mPa·s) from Eq. (2):

$$\eta_a = K \cdot D^{n-1} \tag{2}$$

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

2.4 Analysis of produced xanthan

The dry matter of xanthan was determined by the standard drying method at 105 °C to a constant mass [22]. The amount of ash in xanthan was determined by the standard drying method at 550 °C [22]. The Kjeldahl method was used for analysis of xanthan regarding total nitrogen content [18]. 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 [23]. The solubility of xanthan was examined by dissolving samples in water up to 1% at a medium-low temperature range (4 °C to 60 °C). The rheological properties of 1% solution of xanthan were determined at room temperature using a rotational viscometer (Reotest 2 VEB MLV Prufgerate-Verk, Mendingen, Sitzfreitel) with double gap coaxial cylinder sensor system, spindle N, as mentioned in previous study [11]. The pH value, total dissolved salts (TDS) content, and electrical conductivity of 1% aqueous solution of xanthan samples were measured at room temperature by the laboratory multiparameter analyzer Consort C863 (Consort, Belgium) with the glass (pH) and conductivity electrode, respectively.

The emulsification activity of xanthan was tested by measuring the emulsification index (EI) using a method of Cooper and Goldenberg [24] in hydrocarbons (*n*-hexane, toluene, liquid paraffin, and chloroform) and oils (sunflower, soybean, and olive oil) after 24 h of rest.

2.5 Data analysis

All experiments were carried out in triplicate, the results were averaged and standard deviations were calculated. All experimental data were processed by analysis of variance (one-way ANOVA) followed by post-hoc testing (Duncan's multiple range test). The statistical analysis was done at the significance level of $\alpha = 0.05$ using TIBCO Statistica™ 14.0.0 software [25].

3 Results and discussion

3.1 Time course of xanthan biosynthesis

In this study, xanthan biosynthesis by *X. euvesicatoria* PL4 strain in a lab-scale stirred tank bioreactor on crude glycerol-based medium was conducted in triplicate. The obtained results were processed by one-way ANOVA. Statistical analysis of the experimental data revealed a statistically significant difference between the values of each group of results, i.e., *X. euvesicatoria* PL4 cell concentration ($F = 2866.970$; $p < 0.01$), glycerol content ($F = 3175.097$; $p < 0.01$), total nitrogen content ($F = 1312.808$; $p < 0.01$),

assimilable nitrogen content (F = 712.551; p < 0.01), total phosphorus content (F = 1363.806; p < 0.01), and apparent viscosity (F = 52965.081; p < 0.01). The average values of obtained results with standard deviations are graphically presented in Figs. 1–3.

The change of biomass concentration (X) during xanthan biosynthesis in applied experimental conditions is shown in Fig. 1. According to the graphically presented results, during the first 48 h of cultivation of producing microorganism multiplied intensively and the cell concentration in the medium increased from 5.17 · 10^8 CFU/mL to 105.67 · 10^8 CFU/mL. These values suggest that exponential growth phase could be noticed since the beginning of bioprocess and lasted until 48 h of cultivation, with sharp increase in biomass content, indicating the ability of the producing microorganism to successfully metabolize crude glycerol as single carbon source, as reported previously [5]. Between 48 h and 72 h, a slower increase of biomass concentration was observed, reaching a maximum value of 111.33 · 10^8 CFU/mL in 72 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 has occurred. After 168 h of cultivation, the biomass concentration was 106.67 · 10^8 CFU/mL, indicating that biomass concentration has greatly increased during the cultivation in applied experimental conditions.

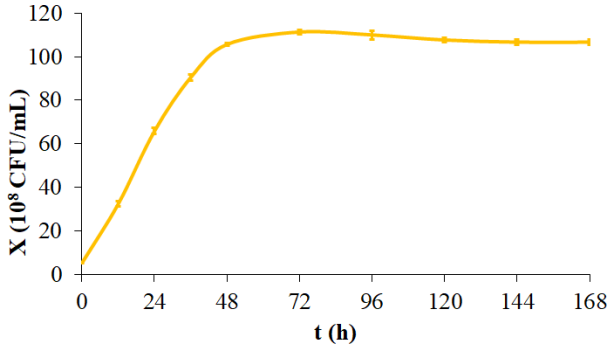

Fig. 1 Time course of *X. euvesicatoria* PL 4 cell concentration during cultivation on crude glycerol-based medium in 3 L stirred tank bioreactor

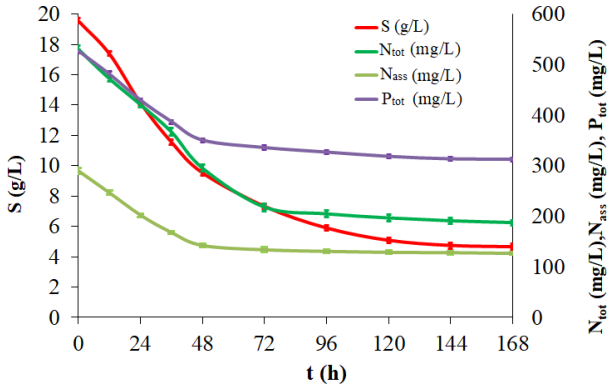

Fig. 2 Time course of glycerol content (S), total (N_{tot}) and assimilable (N_{ass}) nitrogen content, and total phosphorus content (P_{tot}) during cultivation of *X. euvesicatoria* PL4 on crude glycerol-based medium in 3 L stirred tank bioreactor

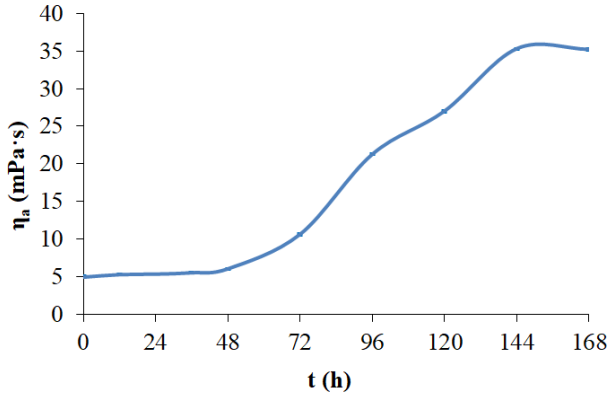

Fig. 3 Time course of apparent viscosity of crude glycerol-based medium during cultivation of *X. euvesicatoria* PL4 in 3 L stirred tank bioreactor

The change of glycerol content (S), total (N_{tot}) and assimilable (N_{ass}) nitrogen content, and total phosphorus content (P_{tot}) during the xanthan biosynthesis by *X. euvesicatoria* PL4 strain on crude glycerol-based medium in applied experimental condition is presented in Fig. 2 and as it can be noticed, consumption of glycerol, nitrogen and phosphorus was in a strong correlation with *X. euvesicatoria* PL4 growth represented in Fig. 1. The highest consumption of nutrients was observed during the exponential growth phase, due to utilization of these nutrients for cell growth, while in the stationary growth phase, i.e. after 48 h of cultivation minor consumption of nutrients could be observed, except for glycerol.

From the results represented in Fig. 2, it can be seen that during the first 48 h of cultivation there was an intense decrease in the glycerol content from 19.56 g/L to a value of 9.53 g/L. After 48 h of cultivation, the glycerol content continued to decrease, but with a lower intensity until 144 h, when the content of this nutrient was 4.73 g/L. There was no significant change in the glycerol content after 144 h of cultivation, and its value after 168 h that was 4.65 g/L.

When it comes to nitrogen, it can be noticed that similar trend for total and assimilable nitrogen was observed (Fig. 2). Within first 48 h of the cultivation in applied experimental conditions the total nitrogen content was reduced from the initial 530.67 mg/L to 295.33 mg/L, while the content of assimilable nitrogen was reduced from 289.40 mg/L to 142.33 mg/L. Observing the obtained results it can be

seen that after 48 h of cultivation there was no significant change in the total and assimilable nitrogen content, which is a consequence of the onset of the stationary growth phase of the microorganism. At the end of bioprocess, the residual concentration of total and assimilable nitrogen components was 187.33 mg/L and 126.67 mg/L, respectively. The higher consumption of total nitrogen compared to assimilable nitrogen suggests that in applied experimental conditions, besides amino and ammonia nitrogen, the cells of *X. euvesicatoria* PL4 used some other nitrogen-containing compounds such as proteins arising from yeast extract for growth and reproduction [26]. On the other hand, experimental errors in the determination of total and assimilable nitrogen should not be excluded.

Consumption of phosphorus is also examined within this study. The presented results (Fig. 2) indicate that the change in the total phosphorus content is similar to the change in the content of total and assimilable nitrogen with an intense consumption in the first 48 h of cultivation. The initial phosphorus content was reduced from 526.37 mg/L to 350.28 mg/L. Like for previously discussed nutrients, the change in the total phosphorus content in cultivation medium was insignificant after 48 h of cultivation reaching final value of 312.47 mg/L.

The change in the biomass concentration and nutrients content during the bioprocess suggest that the metabolic activity of the used producing microorganism in applied experimental conditions was efficiently realized. This indicates that all conditions for the successful xanthan biosynthesis were provided. Moreover, in the interest of establishing whether the production of xanthan occurred, rheological measurements of cultivation medium were performed. The change in the apparent viscosity of crude glycerol-based medium during the cultivation of the *X. euvesicatoria* PL4 strain is represented in Fig. 3. Although the viscosity value of the medium does not provide an exact measure of the amount of synthesized biopolymer, the simplicity of the procedure and the rapid response time are the main reasons why xanthan production, both at the laboratory and industrial level, is commonly monitored through changes in this parameter [17, 27].

The obtained results given in Fig. 3 demonstrate that the values of apparent viscosity of the crude glycerol-based medium slightly change from the initial 4.93 mPa·s up to 6.04 mPa·s in 48 h of cultivation. Further cultivation resulted with a drastic increase in the apparent viscosity of the medium up to 144 h, when its value was 35.30 mPa·s. The value of the apparent viscosity of the cultivation medium increased with lower intensity after 144 h of cultivation, and at the end of biosynthesis, the value of this parameter was 35.20 mPa·s. The change in apparent viscosity of cultivation medium is probably due the fact that viscosity is very contingent on the length of xanthan molecule chains, which is results of selected *Xanthomonas* strain, medium formulation, applied bioprocess conditions and molecular crosslinking [15, 17].

The rheological measurements conducted within this study imply that cultivation media at the end of bioprocess has pseudoplastic properties, a known characteristic of xanthan solutions [15]. The values of flow behavior index in this study decreased from 0.5665 to 0.4772, indicating that during the cultivation pseudoplastic behavior of medium became more pronounced. Lastly, the Ostwald-de-Waele model showed a good agreement with the experimental data achieved, considering that the regression coefficients were higher than 0.97 in all tests.

3.2 Success of xanthan biosynthesis

In contemplation of determining performance of xanthan biosynthesis in lab-scale stirred tank bioreactor on crude glycerol-based medium by *X. euvesicatoria* PL4 strain, xanthan concentration in medium, degree of initial glycerol conversion into xanthan, degree of metabolized glycerol conversion into xanthan, degree of glycerol conversion, degree of total nitrogen conversion, and degree of total phosphorus conversion were determined. After 168 h of the cultivation, xanthan concentration in the medium was 13.31 g/L, which is for approximately 60% higher comparing to the results obtained when the same strain was cultivated on the crude glycerol-based medium (20 g/L) in laboratory vessel with ten times smaller total volume at which around 8 g/L xanthan was produced [5]. The achieved value is also higher comparing to the values obtained in previous research, where *Xanthomonas* PL3 strain was cultivated on crude glycerol-based medium in identical bioprocess conditions, and where xanthan concentration in medium was 11.10 g/L [25]. Moreover, when observing the findings obtained within research conducted in Brazil, where cultivation of *X. campestris mangiferaeindicae* 2103 on crude glycerol-based medium (20 g/L) in a 4.5 L bioreactor resulted in production of xanthan in concentration of 5.59 g/L [4], it can be noticed that xanthan biosynthesis in the present study was highly successful if the concentration of produced biopolymer is considered as an indicator.

When it comes to conversion of nutrients, it can be concluded that degree of total, initial and metabolized glycerol conversion into xanthan was 76.23%, 68.06% and 89.30%, respectively, while the values of degree of total nitrogen and phosphorous conversion were 64.70% and 40.63%, respectively. The degree of glycerol conversion of 62.82%, total nitrogen conversion of 41.54% and total phosphorus conversion of 24.80% was obtained in previous study when *Xanthomonas* PL3 strain isolated from pepper leaves in Serbia was cultivated on crude glycerol-based medium in identical bioprocess conditions [26], indicating greater success of xanthan biosynthesis conducted within present study. The values of initial glycerol conversion of 55.26% and metabolized glycerol conversion into xanthan of 87.98% achieved in aforementioned research were lower comparing to the values achieved in the present study. Considering that in industrial conditions, the degree of carbon sources conversion into xanthan ranges from 50–85% [28], it can be noticed that in this research, very high efficiency of bioprocess has been achieved.

The results obtained in this study indicate that *X. euvesicatoria* PL4 strain demonstrates great potential for xanthan production on crude glycerol-based medium; however, productivity of this strain can be improved through further bioprocess optimization or application of genetic engineering techniques, as demonstrated by Wang et al. [29], who used a modified strain *Xanthomonas campestris* WXLB-006 and achieved a high xanthan yield.

3.3 Properties of biosynthesized xanthan

Xanthan is known as white to cream colored free-flowing powder of neutral smell and taste [15]. According to the results of characterization given in Table 1, it can be noticed that xanthan produced on crude glycerol-based medium by *X. euvesicatoria* PL4 strain occurs in its regular form as free flowing powder of cream color. As it can be seen from the data given in Table 1, the values of dry matter and ash content in produced xanthan are 92.58% and 7.08%, respectively. These values are in agreement with results from previous study when xanthan produced by reference strain *X. campestris* ATCC 13951 on crude glycerol-based medium was examined and dry matter and ash content were 93.47% and 7.83%, respectively [30]. The molecular weight of the produced xanthan was $3.17 \cdot 10^5$ g/mol and it is accordance with the values of molecular weight of xanthan obtained when the strain *X. campestris* ATCC 13951 was cultivated in the production media containing different initial glycerol concentration which were in the range from $2.64 \cdot 10^5$ g/mol to $3.17 \cdot 10^5$ g/mol [13].

Table 1 Physico-chemical properties of xanthan biosynthesized by *X. euvesicatoria* PL4 on crude glycerol-based medium in 3 L stirred tank bioreactor

Parameters	Values
Appearance	free flowing powder
Color	cream
Dry matter (%)	92.58 ± 0.60
Ash (%)	7.08 ± 0.08
Molecular mass (10^5 g/mol)	3.17 ± 0.17
Apparent viscosity of 1% aqueous solution (mPa·s)	31.73 ± 0.28
Solubility in water (4 °C to 60 °C)	complete
pH of 1% aqueous solution	5.77 ± 0.12
Electrical conductivity (mS/cm)	4.64 ± 0.27
TDS (g/L)	2.51 ± 0.07

Apparent viscosity of 1% aqueous solution of xanthan produced in present study was 31.73 mPa·s and it is higher comparing to the value of the same parameter for xanthan produced by reference strain *X. campestris* ATCC 13951 on the same medium. However, this value is much lower comparing to the value of 295.66 mPa·s obtained when commercially available xanthan (Shandong Fufeng Fermentation Co., Ltd., China) was analyzed [30]. Viscosity variations among the different xanthan samples can be attributed to the effects of salt presence in the medium or strain-specific biochemical interactions that influence the molecular structure of the produced xanthan, and the degree of polymer purity [31]. This underscores the importance of continued optimization efforts to improve the quality of xanthan produced on alternative substrates. Xanthan produced in applied experimental conditions is soluble in cold and hot water, indicating a great potential for wider application possibilities. The value of electrical conductivity is 4.64 mS/cm for the 1% aqueous solution of the analyzed xanthan sample. Relatively high electrical conductivity indicates a higher ability of xanthan to interact with other materials [32]. Nevertheless, xanthan, acting as a charge carrier, is immobile, and this conductivity is likely due to the ions emanating from the ash constituents. The pH value of 1% aqueous solution of xanthan was 5.77, while TDS was 2.51 g/L, which is important if applied in the field of environmental protection with the aim of preserving its quality. Considering all aforementioned parameters is important regarding the application of biopolymer without negative effects on the environment.

Emulsification properties of xanthan produced on medium containing waste streams and by-products from different industries are insufficiently examined and there

are only few studies which suggest that emulsification properties of xanthan mainly depend on producing strain, cultivation medium composition, process conditions and hydrocarbons/oils used [12, 30, 33]. In this study, examination of emulsification properties of xanthan, produced by cultivation of *X. euvesicatoria* PL4 strain in lab-scale bioreactor on crude glycerol-based medium, was conducted. Emulsifying activity of 0.1% (w/v) xanthan solution was determined in the presence of *n*-hexane, toluene, chloroform, liquid paraffin, sunflower oil, soybean oil and olive oil after 24 h of rest. All the experiments were conducted in triplicate. The obtained results were processed by one-way ANOVA followed by Duncan's multiple range test. Statistical analysis of the experimental data revealed a statistically significant difference between the values of each group of results ($F = 576.111$; $p < 0.01$). The average values of obtained results with standard deviations are presented graphically in Fig. 4.

Values of the emulsification index of xanthan determined when using natural oils are all above 45%, while values obtained with hydrocarbons were lower and not higher than approximately 40%. As it can be noticed from the results given in Fig. 4, the highest value of the emulsification index of 58.70% was obtained when the emulsifying activity of xanthan was examined with soybean oil. Somewhat lower values of 54.25% and 47.20% were obtained when emulsification test was conducted with olive and sunflower oil, respectively. Emulsifying activity of xanthan in natural oils is in agreement with findings from previous study where it is reported that emulsification index of xanthan produced by reference strain *X. campestris* ATCC 13951 on crude glycerol-based medium after 24 h of rest was far higher when using the natural oils against hydrocarbons [30]. Xanthan produced

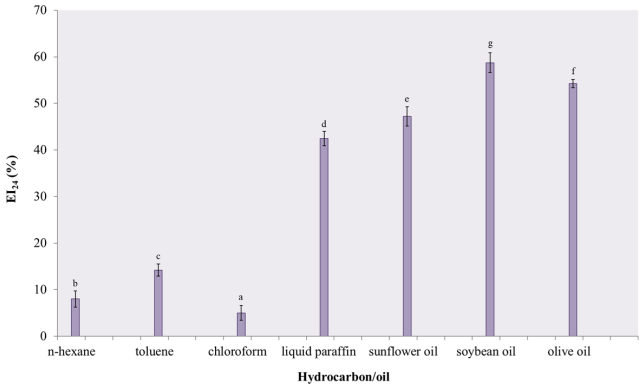

Fig. 4 Emulsification index of xanthan in different hydrocarbons/oils after 24 h of rest (bars marked with the different letters are statistically significant different according to Duncan's multiple range test)

within present study exhibited greater emulsifying activity in natural oils comparing to the xanthan produced by *X. campestris* pv. *mangiferaeindicae* IBSBF 1230 on crude glycerol-based medium (50 g/L) whose emulsifying activity after 24 h in sunflower oil was 46.5%, in olive oil 28.1%, and in soybean oil 8.3% [3]. Taking into account all previously discussed results it can be noticed that xanthan produced in applied experimental conditions exhibit excellent emulsifying activity after 24 h of rest.

The highest value of emulsification index in hydrocarbons of 42.47% was obtained with liquid paraffin. The values achieved with other hydrocarbons were much lower, with the lowest value of 5.01% obtained with chloroform. Despite the fact that emulsifying activity of produced xanthan was lower in hydrocarbons, this activity is far greater comparing to the maximal emulsifying activity of xanthan produced by reference strain *X. campestris* ATCC 13951 on crude glycerol-based medium in hydrocarbons, which was 24.29% [30], indicating that producing strain and optimization of xanthan production can affect xanthan emulsification properties.

The previously discussed results suggest that xanthan produced in the applied experimental conditions has the ability to form and stabilize emulsions with all hydrocarbons/oils tested and that the emulsifying activity of the examined biopolymer differs with the used hydrocarbons/oils. The main reason for this lies in the fact that emulsifying activity depends on the hydrophobic compound employed and interactions [34, 35]. Although emulsifying activity of produced xanthan showed better results in oils, there is still a need to examine how some properties of the system, such aqueous phase composition (salinity and pH) and temperature affect this parameter.

Xanthan is widely utilized as an ingredient of food, animal feed, and personal care products; however, its most significant application lies in the oil industry. In this sector, xanthan plays a key role in drilling muds, and serves as an essential agent for mobility control during polymer flooding processes aimed at enhancing oil recovery (EOR) [36]. Findings from previous study where *Xanthomonas campestris* strain IBSBF 2103 was cultivated on crude glycerol-based medium indicate that xanthan produced from crude glycerol has a great potential for enhance oil recovery along with other potential applications [37]. Comprehensive evaluation of xanthan properties is crucial to optimize its application in enhanced oil recovery. The insights gained from this study make a valuable contribution to the existing knowledge base,

supporting future advancements in EOR technology and indicating a sustainable path to support the circular economy as it deals with the beneficial approaches of economically justified utilization of biofuel effluent for production of biopolymer with great perspective for environmental remediation application.

4 Conclusions

The results obtained within this study have confirmed that crude glycerol generated in biodiesel industry can be used as a sole carbon source in cultivation media for successful xanthan production by local wild-type *X. euvesicatoria* PL4 strain. Apart from great properties and improved quantity of produced xanthan, a more acceptable conversion of essential nutrients was also achieved in present study. Formation of emulsion of xanthan with natural oils and liquid paraffin is reflected by the emulsification index with values higher than 40%.

Results obtained in this research are very important from an ecological aspect as they indicate promising potential for application of xanthan produced by local wild-type *Xanthomonas* strain on medium containing crude glycerol from biodiesel production in oil recovery processes. The findings from this study represent valuable source of information for further investigations to harness the unique properties of xanthan for efficient and sustainable environmental remediation and related applications.

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