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POTENTIAL OF DIFFERENT *Xanthomonas campestris* STRAINS FOR XANTHAN BIOSYNTHESIS ON WASTE GLYCEROL FROM BIODIESEL PRODUCTION

POTENCIJAL RAZLIČITIH *Xanthomonas campestris* SOJEVA ZA BIOSINTEZU KSANTANA NA OTPADNOM GLICEROLU IZ PROIZVODNJE BIODIZELA

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ABSTRACT

A rapid expansion of the biodiesel industry has created various ecological issues relative to crude glycerol disposal. Xanthan biosynthesis is considered one of the sustainable solutions for minimizing the adverse effects of waste crude glycerol on the environment. The initial phase of xanthan production on crude glycerol entails the screening of producing microorganism. Therefore, the purpose of this study is to examine the possibility of xanthan production on a crude glycerol-based medium using different Xanthomonas campestris strains. The bioprocesses performed were assessed according to the rheology of the media considered, amounts of xanthan produced and conversion degrees of the most important nutrients present. The pseudoplastic behavior of all the media considered, the amounts of xanthan produced (5.22-7.67 g/L) and the degrees of crude glycerol, total nitrogen and phosphorus conversion (34.44-57.61 %, 23.04-30.35 % and 18.20-22.28 %, respectively) suggest that crude glycerol, after additional bioprocess optimization, can be a suitable raw material for the industrial production of xanthan.

***Key words**: waste utilization, crude glycerol, xanthan production, Xanthomonas campestris*

REZIME

Ubrzana ekspanzija industrije biodizela tokom poslednjih nekoliko decenija rezultovala je različitim ekološkim problemima usled odlaganja velikih količina netretiranih efluenata, naročito sirovog glicerola. Kako bi se smanjio negativan uticaj na životnu sredinu, predložene su brojne strategije za upravljanje sirovim glicerolom koje su u skladu sa konceptom održivog razvoja. Među njima je biosinteza ksantana jedno od perspektivnih rešenja. Prvi korak u razvoju postupka proizvodnje ksantana na sirovom glicerolu je skrining proizvodnih mikroorganizama. Stoga je cilj ovih sitraživanja bio ispitivanje mogućnosti proizvodnje ksantana na medijumu čija je osnova sirovi glicerol primenom različitih Xanthomonas campestris sojeva. U okviru eksperimentalnog dela, izvedena je kultivacija četiri soja Xanthomonas campestris izolovana sa listova različitih biljaka iz porodice kupusnjača (CB, CF, 12-2 i Xp3-1) na medijumu sa sirovim glicerolom kao izvorom ugljenika. Biosinteza ksantana izvedena je u Woulff-ovim bocama zapremine 2,0 l u aerobnim uslovima, submerznom tehnikom kultivacije, pri optimalnim vrednostima procesnih parametara u toku 168 h. Reologija kultivacionog medijuma, količina proizvedenog ksantana i stepen konverzije važnih nutrijenata su određeni kako bi se ispitala uspešnost izvedenih bioprocesa. Pseudoplastično ponašanje svih medijuma, dobijene vrednosti za količinu ksantana (5,22-7,67 g/l) i stepeni konvezije sirovog glicerola, ukupnog azota i ukupnog fosfora (34,44-57,61%, 23,04-30,35% i 18,20-22,28%, respektivno) ukazuju na to da sirovi glicerol, nakon dodatne optimizacije bioprocesa, može biti pogodna sirovina za proizvodnju ksantana u uvećanim razmerama, što ukazuje da rezultati dobijeni u ovim istraživanjima predstavljaju osnovu za potencijalnu industrijalizaciju ispitivanog biotehnološkog postupka.

***Ključne reči:** iskorišćenje otpada, sirovi glicerol, proizvodnja ksantana, Xanthomonas campestris.*

INTRODUCTION

Increased global concerns about growing energy demands, limited reserves of fossil fuels and negative impacts of the conventional fossil fuels use on the environment (particularly greenhouse gas emissions) have placed a new emphasis on alternative energy sources. Liquid biofuels produced from renewable resources are considered important alternative energy sources (*Quispe et al., 2013*). Therefore, biodiesel has recently been receiving increased attention because it is renewable, sustainable, biodegradable, efficient, environmentally friendly, and directly usable in diesel engines without major engine modifications (*da Silva et al., 2009*). Accordingly, the biodiesel industry has become one of the most rapidly growing industries in the world, with a global biodiesel production of about 25.2 million tons in 2014 (indicating a constantly growing trend) (*He et al., 2017*).

The biodiesel production involves the utilization of different categories of catalysts such as alkalis, acids, and enzymes. Alkalis and acids are divided into homogeneous and heterogeneous catalysts. Due to their high activity and low costs, sodium hydroxide and potassium hydroxide are the most commonly used catalysts for the transesterification of oils in biodiesel production (*Chatzifragkou and Papanikolaou, 2012*). Homogeneous base catalysts can be corrosive, and thus their removal from the resulting biofuel is particularly problematic (often resulting in the formation of stable emulsions and soaps). Heterogeneous catalysis is more efficient as it ensures an easier separation of the catalyst from the final product. Moreover, the possibility of reusing this catalyst renders the process of

heterogeneous catalysis economically justified (*Sulaiman et al., 2020*).

Owing to the increasing global demand for biodiesel and the necessity for larger biodiesel production capacities, the appropriate utilization of generated effluents is of paramount importance to the competitiveness of biodiesel in the market and the economic and environmental sustainability of commercial biodiesel production (*Hejna et al., 2016*). Crude glycerol is the main by-product of the biodiesel industry. On balance, crude glycerol accounts for a 10-20% share of the total biodiesel production. Increased biodiesel production results in large surpluses of crude glycerol, which poses a serious environmental hazard and thus a disposal issue (*Quispe et al., 2013*).

Glycerol from biodiesel production is an impure compound requiring significant assets for purification, which impedes its use in the food and pharmaceutical industries (*Brandao et al., 2013*). Therefore, the use of this effluent as a carbon and energy source for microbial growth in industrial biotechnology is a more suitable application. However, the presence of different impurities (namely methanol, ethanol, inorganic salts, metals, long chain fatty acids and soaps) in crude glycerol may inhibit the metabolic activity of producing strains, which can result in the reduced biomass growth and bioconversion of glycerol into desired products (*Konstantinović et al., 2016*). Accordingly, the use of this effluent as a carbon and energy source for microbial growth is possible provided producing microorganisms are tolerant to impurities in crude glycerol or additional treatments are performed. Untreated glycerol may substitute conventional carbohydrates (namely glucose, sucrose and starch) in some bioprocesses, e.g. the production of bioethanol, butanol, biosurfactants, pigments and some acids such as lactic, citric, propionic and succinic acids (*da Silva et al., 2009*). In addition, crude glycerol is an interesting substrate often exploited in xanthan production (*de Jesus Assis et al., 2014*). The bioconversion of glycerol into xanthan and other valuable bioproducts reduces the production costs of desired metabolites and mitigates the ecological impacts caused by the accumulation of this industrial stream in the environment (*Johnson and Taconi, 2007; Hejna et al., 2016*).

Xanthan is the most commercially produced microbial polysaccharide obtained by the *Xanthomonas campestris* cultivation. It is widely used as a thickening or stabilizing agent in the food, pharmaceutical, and oil-recovery industry. This biopolymer is well-known for its unique rheological properties or pseudoplastic behavior, i.e. high viscosity at low shear, stability over a broad range of temperature and pH value, and high resistance to shear degradation in aqueous solutions (*García-Ochoa et al., 2000; Petri, 2015*). Xanthan is frequently employed in the food industry to obtain a higher viscosity of food products (*Gilani et al., 2011*).

On an industrial scale, xanthan is generally produced in a batch mode under aerobic conditions by the submerged cultivation of the pure bacterial culture *Xanthomonas campestris* in bioreactors with mechanical mixing on appropriately formulated media under optimal conditions (*Ozdal and Kurbanoglu, 2018*). The only effluent that remains after xanthan biosynthesis is stillage. The stillage from xanthan production is comparable to distillery stillage and is characterized by high values of chemical and biological oxygen consumption, low pH values and a high content of organic matter. There are numerous treatments for the degradation and utilization of organic matter present in the stillage, but the production of high-value products is one of the most promising alternatives (*Đuran et al., 2018*).

Xanthan production is usually performed using the reference strain *Xanthomonas campestris* ATCC 13951 on a medium with glucose or sucrose (*Kumara et al., 2012*). However, the increasing market price of and the demand for these sugars suggest that they may no longer be economically acceptable raw materials. In order to reduce the costs of medium preparation and overall xanthan production, the use of different waste streams as less expensive carbon sources are recommended (*Reis et al., 2010*). Their underutilization can be accounted for by the inability of the reference strain to successfully metabolize other carbon sources such as glucose or sucrose (*Roseiro et al., 1992*). In addition, it is important to note that xanthan can be produced by different bacteria of the genus *Xanthomonas* such as *Xanthomonas campestris*, *Xanthomonas phaseoli* and *Xanthomonas malvacearum.* However, *Xanthomonas campestris* is the most widely used for the industrial production of xanthan (*Leela and Sharma, 2000*). All the *Xanthomonas campestris* strains are phytopathogenic and infect various plants, some of which are important for agriculture such as cabbage, tomatoes, beans and peppers (*García-Ochoa et al., 2000*).

The purpose of this study is to examine the possibility of xanthan biosynthesis on a crude glycerol-based medium using different *Xanthomonas campestris* strains. The bioprocesses performed were assessed according to the rheology of the media considered, amounts of xanthan produced and conversion degrees of the most important nutrients present.

MATERIAL AND METHOD

Producing microorganism

A total of four *Xanthomonas campestris* strains isolated from different cruciferous plants (CB, CF, 12-2 and Xp3-1) were used as the producing microorganisms in the experiments conducted. The cultures were stored at 4 °C on an agar slant (YMA®, HiMedia, India) and subcultured at four-week intervals.

Cultivation media

A commercial medium (YMB®, HiMedia, India) was used for the inoculum preparation, whereas xanthan production was performed on a crude glycerol-based medium as the sole carbon source. A total of 20.0 g/L of crude glycerol was used, which was obtained from a domestic biodiesel factory (Belgrade, Serbia) that uses waste oil as raw material for biodiesel production. The following properties of the crude glycerol considered were determined: a glycerol content of 60.88 % (w/w), a phosphorus content of 0.015 % (w/w), a sulfur content of 1.58 mg/kg, a sodium content of 199.83 mg/kg, a potassium content of 3.73 % (w/w), a calcium content of 6.76 mg/kg, a magnesium content of 2.31 mg/kg, a moisture content of 4.84 % (w/w), a methanol content of 0.45 % (w/w), an organic matter content of 95.12 % (w/w), a density of 1.12 g/cm^3 and a kinematic viscosity of 74.97 mm^2/s. The cultivation medium also contained 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 the medium was adjusted to 7.0 ± 0.2 and then sterilized by autoclaving at 121 °C and 2.1 bar for 20 min.

Xanthan production

The xanthan production was carried out in a 2.0 L Woulff bottle with 1.5 L of the cultivation medium. The sterile medium was inoculated by adding 10 % (v/v) of inoculum prepared by a double passage procedure under aerobic conditions at 25 °C and 150 rpm (using the laboratory shaker KS 4,000i control, Ika® Werke, Germany) for 48 h. The biosynthesis was carried out in a batch mode under aerobic conditions (an air flow rate of 1 vvm in the first 48 h, and 2 vvm afterwards) for 168 h. In the first 48 h, the temperature was 25 °C and the agitation rate was 200 rpm, which thereafter increased to 30 °C and 300 rpm, respectively.

Xanthan separation

At the end of biosynthesis, the xanthan obtained was separated from the cultivation medium supernatant by precipitation with cold 96 % (v/v) ethanol in the presence of potassium-chloride as electrolyte. The supernatant was obtained using an ultracentrifuge (Hettich Rotina 380 R, Germany) at 10,000 rpm for 15 min. Ethanol was gradually added to the supernatant with constant stirring until the alcohol content in mixture was 60 % (v/v). Prior to this, a saturated solution of potassium-chloride was added into the cell-free supernatant to obtain a final content of 1 % (v/v). The obtained mixture was kept at 4 °C for 24 h in order to dehydrate the precipitated xanthan, and then centrifuged at 4,000 rpm for 15 min (Tehtnica LC-321, Slovenia). The precipitated biopolymer was dried at 60 °C to a constant weight in order to determine the quantity of xanthan produced. The ethanol used for xanthan precipitation was recycled by distillation.

Determination of the cultivation medium rheological properties

The rheological behavior of the cultivation medium samples taken at the end of the bioprocesses performed were evaluated using a rotational viscometer (REOTEST 2 VEB MLV Prufgerate-Verk, Mendingen, SitzFreitel) with the double gap coaxial cylinder sensor system spindle N. Relative to the measuring instrument deflection (α, Skt), the shear stress (τ, Pa) was calculated (at the specific values of shear rates (D, 1/s)) using the following equation:

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

where z is a constant of 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 the power regression. The values of the consistency factor (K, Pa·s^n), the flow behavior index (n) and the coefficient of determination (R^2) were determined using Excel 2013, which was employed for computing the medium apparent viscosity (η_a, mPa·s) using Eq. 2:

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

where D is a shear rate of 100 1/s.

Determination of nutrients content

The samples of cell-free cultivation media taken after inoculation and 168 h of cultivation, obtained by centrifugation at 10,000 rpm for 15 min (Rotina 380 R, Hettich Lab Technology, Germany), were analyzed for crude glycerol, total nitrogen and total phosphorus contents.

The glycerol content was determined using the high pressure liquid chromatography (HPLC). Prior to analysis, the samples were filtered through a 0.45 μm nylon membrane (Agilent Technologies Inc, Germany). The HPLC instrument (Thermo Scientific DionexUltiMate 3000 series) was equipped with the HPG-3200SD/RS pump, the WPS-3000(T)SL autosampler (10 μL injection loop), the ZORBAX NH2 column (250 x 4.6 mm, 5 μm) (Agilent Technologies Inc, USA) and the Refracto Max 520 detector (ERC Inc, Japan). The acetonitrile solution (70 % v/v) was used as eluent at a flow rate of 10 mL/min and the elution time of 10 min at a column temperature of 30 °C.

The nitrogen content was determined using a method proposed by Kjeldahl (*Helrich, 1990*), whereas the phosphorus content was estimated using the spectrophotometric method (*Gales et al., 1966*).

The nutrient content results were used to calculate crude glycerol, total nitrogen and total phosphorus conversion (K, %) using Eq. 3:

$$K = \frac{(S_0 - S)}{S_0} \cdot 100 \quad (3)$$

where S_0 is the initial nutrient content (g/L), whereas S is the residual nutrient content (g/l).

Statistical analysis

All the experiments were carried out in triplicate and the results were averaged. The data obtained were processed using the analysis of variance (One-Way ANOVA). Significant differences between the means were determined by the Duncan's multiple range test at a significance level of α = 0.05 using the Statistica 13.2 software (Dell Inc., USA).

RESULTS AND DISCUSSION

The xanthan production under the experimental conditions was evaluated according to the rheological behavior of the crude glycerol-based media after the cultivation of the *Xanthomonas campestris* strains considered. The rheological properties were determined relative to the shear rate and shear stress values obtained (Figure 1). The flow curves represent a pseudoplastic flow type, which is a well-known property of xanthan solutions (*García-Ochoa et al., 2000*).

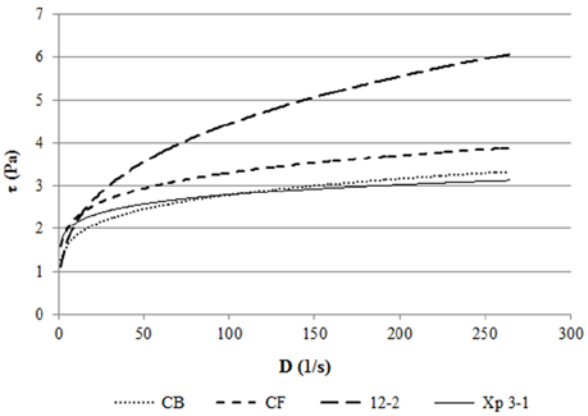

Figure 1. Effect of the shear rate on shear stress for the crude glycerol-based media considered after the cultivation of different Xanthomonas campestris strains

The pseudoplastic properties of the crude glycerol-based cultivation media were also confirmed by the flow behavior index values (n) shown in Table 1. The flow behavior index represents a level of deviation from the Newtonian flow behavior. The flow behavior index is equal to 1 for Newtonian fluids, greater than 1 for dilatants, and less than 1 for pseudoplastic fluids (*Björn et al., 2012*). The flow behavior index values obtained for the media samples analyzed in this research were in the range of 0.1178-0.3204.

As the consistency factor is proportional to the viscosity, the different values of this parameter shown in Table 1 indicate the difference in the quantity and quality of xanthan produced under the experimental conditions by the cultivation of different *Xanthomonas campestris* isolates on a crude glycerol-based medium. The Ostwald-de-Waele model results were in good agreement with the experimental data because the determination coefficients were higher than 0.90 in all the tests conducted (Table 1). Moreover, the values of the apparent viscosity in Table 1 indicate that the highest cultivation medium viscosity

(44.41 mPa·s) was obtained when using the strain 12-2, whereas the lowest apparent viscosity of the cultivation medium (27.87 mPa·s) was achieved by the CB strain cultivation.

Table 1. Rheological parameters of the crude glycerol-based media after the cultivation of different Xanthomonas campestris strains

Producing microorg.	Consistency factor, K [Pa·s^n]	Flow behavior index, n [1]	Coefficient of determination, R^2	Apparent viscosity, η_a [mPa·s]
CB	1.2005	0.1829	0.925	27.87
CF	1.3609	0.2311	0.930	39.45
12-2	1.0156	0.3204	0.924	44.41
Xp 3-1	1.6207	0.1178	0.916	27.88

The values of glycerol, total nitrogen and total phosphorus conversion in the crude glycerol-based media after the cultivation of different *Xanthomonas campestris* strains are shown in Table 2. In addition to xanthan production, the conversion of important nutrients is a very significant indicator of the bioprocess success. The results obtained indicate that the glycerol, total nitrogen and total phosphorus content of the media considered decreased during the xanthan biosynthesis. It can be seen that the glycerol conversion ranged from 34.44 % to 57.61 %, the nitrogen conversion ranged from 23.04 % to 30.35 %, and the phosphorus conversion ranged from 18.20 % to 22.28 %. The highest conversion values of crude glycerol (57.61 %), total nitrogen (30.35 %) and total phosphorus (22.28 %) were obtained when the Xp 3-1 strain was cultivated under the experimental conditions. This value of crude glycerol conversion is greater than that obtained in previous research when the reference strain *Xanthomonas campestris* ATCC 13951 was applied on a crude glycerol-based medium (*Zahović et al., 2019*). The lowest values of crude glycerol (34.44 %), total nitrogen (23.04 %) and total phosphorus (18.20 %) conversion were obtained using the CF, CB and 12-2 isolates, respectively. Although all the nutrient conversion values obtained are not high, the cultivation of the *Xanthomonas campestris* strain Xp3-1 on the crude glycerol-based medium considered results in reduced glycerol, nitrogen and phosphorus contents, which is important for minimizing the negative impact of such waste on the environment.

Table 2. Conversion degree of the most important nutrients present in the crude glycerol-based media considered after the cultivation of different Xanthomonas campestris strains

Producing microorganism	Crude glycerol conversion [%]*	Total nitrogen conversion [%]*	Total phosphorus conversion [%]*
CB	48.75 ± 0.67^b	23.04 ± 0.37^a	19.81 ± 0.54^c
CF	34.44 ± 0.43^a	24.64 ± 0.35^b	19.01 ± 0.42^b
12-2	35.31 ± 0.44^a	26.12 ± 0.62^c	18.20 ± 0.31^a
Xp 3-1	57.61 ± 0.54^c	30.35 ± 0.51^d	22.28 ± 0.41^d

**Values in the same column marked with the same letter are not significantly different at α = 0.05 according to the Duncan's multiple range test.*

According to the experimental plan, xanthan was precipitated and the conversion of sugar into product was calculated in order to determine the success of the performed bioprocesses. The results obtained are summarized in Table 3. The highest xanthan concentration in the crude glycerol-based medium (7.67 g/L) was obtained using the Xp 3-1 strain. The results values are higher than the previously published data which showed that the cultivation of the reference strain *Xanthomonas campestris* ATCC 13951 on the crude glycerol resulted in the accumulation of 6.68 g/L xanthan in the medium (*Zahović et al., 2019*).

Sugar conversion into product represents the amount of carbon sources converted into xanthan. According to the results shown in Table 3, the initial sugar conversion into the desired metabolite under the experimental conditions ranged from 26.11 % to 38.36 %. Conversely, the metabolized sugar conversion into biopolymers ranged from 64.10 % to 79.18 %, depending on the *Xanthomonas campestris* strains employed.

Table 3. Xanthan concentration in the crude glycerol-based media after the cultivation of different Xanthomonas campestris strains and the conversion of sugar into product

Producing microorganism	Xanthan concentration, P [g/L]***	Initial sugar conversion* [%]***	Metabolized sugar conversion** [%]***
CB	6.61 ± 0.39^b	33.07 ± 0.48^c	67.66 ± 0.44^b
CF	5.22 ± 0.21^a	26.11 ± 0.37^a	73.12 ± 0.59^c
12-2	5.85 ± 0.44^{ab}	29.27 ± 0.27^b	79.18 ± 0.52^d
Xp 3-1	7.67 ± 0.53^c	38.36 ± 0.42^d	64.10 ± 0.40^a

** Initial sugar conversion [%] = $P/S_0 \cdot 100$*

*** Metabolized sugar conversion [%] = $P/(S_0\text{-}S) \cdot 100$*

****Values in the same column marked with the same letter are not significantly different at α = 0.05 according to the Duncan's multiple range test.*

The results obtained indicate that all the strains considered can be used as xanthan producing microorganisms on a crude glycerol-based medium. High values of all the indicators of bioprocess success suggest that *Xanthomonas campestris* Xp 3-1 represents the most appropriate producing strain under the set experimental conditions.

CONCLUSION

In this study, the possibility of xanthan production on a crude glycerol-based medium as a sole carbon source using different *Xanthomonas campestris* strains was confirmed. The efficacy of such production was estimated according to the rheology of the media considered, amounts of xanthan produced and conversion degrees of the most important nutrients present. The results obtained for all the parameters considered suggest that the *Xanthomonas campestris* Xp 3-1 strain can be a suitable producing microorganism for the industrial production of xanthan on a crude glycerol-based medium. The findings in this study can provide a basis for the optimization of xanthan production on a crude glycerol-based medium, with an aim of increasing the xanthan yield and quality. In addition to producing a high-value product, this research particularly focused on reducing xanthan production costs and minimizing the negative impact of waste crude glycerol on the environment.

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