EFFECT OF THE INITIAL GLYCEROL CONCENTRATION IN THE MEDIUM ON THE XANTHAN BIOSYNTHESIS

Zorana Z. Rončević, Bojana Ž. Bajić, Jovana A. Grahovac, Siniša N. Dodić, Jelena M. Dodić

University of Novi Sad, Faculty of Technology Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

This study is concerned with the effect of different initial glycerol concentrations in the medium on xanthan production by Xanthomonas campestris ATCC 13951. Xanthan biosynthesis was carried out in batch mode under aerobic conditions at a temperature of 30°C and agitation rate of 150 rpm for 7 days. The process efficiency was estimated based on the values of raw xanthan yield, average molecular weight of the polymer and residual content of glycerol, total nitrogen and phosphorus. Based on these results, the initial concentration of glycerol as a carbon source in the production medium was suggested. In the applied experimental conditions, high raw xanthan yield (12.15 g/l) of good quality (M_w = 2.86·10^5 g/mol) and the lowest amount of residual nutrients (glycerol 2.75 g/l, nitrogen 0.46 g/l and phosphorus 0.67 g/l) was achieved in the medium with the initial glycerol content of 20 g/l. The obtained results are the basis for optimization of xanthan production on glycerol containing media in order to increase the product yield and quality.

KEY WORDS: xanthan, Xanthomonas campestris, medium composition, glycerol

INTRODUCTION

Xanthan, or xanthan gum, is one of the most important microbial polysaccharides produced by Xanthomonas campestris and by other Xanthomonas species. This natural polysaccharide is an industrial biopolymer of great commercial significance. Due to rheological properties of xanthan solutions, like high viscosity at low concentrations, pseudoplasticity and stability over a wide range of temperatures, pH values and electrolyte concentrations, this polymer is used in food, cosmetics, pharmaceuticals, paper, paint, textiles and adhesives, as well as in the oil and gas industry (1).

The xanthan functionality is a direct consequence of its unique chemical structure. Xanthan is a heteropolysaccharide with a very high molecular weight, consisting of D-glucosyl, D-mannosyl and D-glucuronyl acid residues in a molar ratio of approximately 2:2:1 and variable proportions of O-acetyl and pyruvyl residues. The variations in the

* Corresponding author: Zorana Z. Rončević, University of Novi Sad, Faculty of Technology Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: ron@uns.ac.rs
cultivation media compositions and environmental conditions, as well as in the Xanthomonas strains used for the production are the factors that can influence the structure and molecular weight of this biopolymer (2).

In order to produce xanthan, Xanthomonas campestris needs several nutrients, including micronutrients (e.g. potassium, iron, and calcium) and macronutrients such as carbon and nitrogen. Changes in the medium composition by using different carbon sources affect the xanthan yield and quality (3). Carbon sources commonly used in the xanthan biosynthesis are glucose and sucrose. However, industrial xanthan production on glucose or sucrose containing media is not cost effective (4). A significant cost reduction can be achieved by using less expensive substrates, such as different wastewaters (5). Considering that glycerol is the main by-product of biodiesel manufacturing process, growing biodiesel production will lead to large surpluses of waste glycerol, which makes it an interesting substrate (6). One of the possible solutions to reduce the problems caused by waste glycerol disposal into the environment is its use as a carbon and energy source for microbial growth in industrial microbiology. Glycerol bioconversion into valuable products, such as 1,3-propanediol, dihydroxyacetone, ethanol, succinic acid, propionic acid, citric acid, pigments, polyhydroxyalcanoate, biosurfactants etc. has been discussed previously (7). Also, glycerol has been proposed as a cheap and available substrate for xanthan production (8-10).

The aim of this study was to investigate the effect of different initial glycerol concentrations in the medium on the xanthan production by Xanthomonas campestris ATCC 13951. Based on the process efficiency estimated from raw xanthan yield, average molecular weight of polymer and residual nutrients content, an initial glycerol concentration in the production medium was suggested.

**EXPERIMENTAL**

**Producing microorganism**

In the experiments the strain Xanthomonas campestris ATCC 13951 was used as the producing microorganism. It was stored at 4°C on yeast maltose (YM) agar slant containing: 15.0 g/l glucose, 3.0 g/l yeast extract, 3.0 g/l malt extract, 5.0 g/l peptone and 20.0 g/l agar. The culture was subcultured at four-week intervals.

**Cultivation media**

The medium used for the preparation of inoculum was YM broth containing: 15.0 g/l glucose, 3.0 g/l yeast extract, 3.0 g/l malt extract and 5.0 g/l peptone. The xanthan production was performed on the glycerol containing media. In order to determine the effect of different initial glycerol content on the xanthan biosynthesis, glycerol (Lach-Ner, Czech Republic) in concentrations of 10.0 g/l, 20.0 g/l, 30.0 g/l, 40.0 g/l, 50.0 g/l and 60.0 g/l was added to the production media (media I-VI, respectively). These media also contained 3.0 g/l yeast extract (HiMedia, India), 1.5 g/l (NH₄)₂SO₄, 0.3 g/l MgSO₄·7H₂O and 3.0 g/l K₂HPO₄. The pH value of the media was adjusted to 7.0±0.2 and then sterilized by autoclaving at 121°C and pressure of 2.1 bars for 20 min.
Biosynthesis conditions

The xanthan production was carried out simultaneously in 300 mL Erlenmeyer flasks with 100 mL of the cultivation medium with the corresponding composition (media I-VI). Inoculation was performed by adding 10% (v/v) of inoculum prepared in aerobic conditions, on YM broth, at 28°C in a laboratory shaker at 150 rpm for 48 h. The biosynthesis was carried out in batch mode under aerobic conditions for 7 days at a temperature of 30°C and agitation rate of 150 rpm.

Product separation

After the biosynthesis, the product separation from the cultivation media was carried out in order to evaluate xanthan production. In this study, xanthan was recovered from the supernatant of cultivation broth, obtained using an ultracentrifuge (Hettich Rotina 380 R, Germany) at 10,000 rpm for 10 min, by precipitation with 96% (v/v) ethanol in the presence of KCl as the electrolyte. Ethanol was gradually added with constant stirring to the supernatant cooled at 15°C until the alcohol content in the mixture was 60% (v/v). A saturated solution of KCl was added when one half of the needed ethanol amount was poured into the supernatant in a quantity in order to reach 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 3500 rpm for 15 min (Tehtnica LC-321, Slovenia). The precipitated polymer was then dried to constant weight at 60°C to determine raw xanthan yield.

Determination of average molecular weight

The average molecular weight of the raw xanthan was obtained by measuring flow time of xanthan solutions in 0.1 M NaCl using Ubbelhode capillary viscosimeter. The temperature was kept at 25°C using a circulatory water bath. The value of the intrinsic viscosity ([η]) was determined and applied to calculate the polymer average molecular weight (M_W) using the Mark-Houwink type equation proposed by Milas et al. (11):

\[ [\eta] = 1.7 \times 10^{-7} M_W^{1.14} \]  \[1\]

Analysis of the cultivation broth

At the end of the process, samples of the cultivation broths were analyzed. Rheological properties of the cultivation broth samples were determined using a rotational viscosimeter (REOTEST 2 VEB MLV Prüfgeräte-Verk, Mendingen, SitzFreitael) with a double-gap coaxial cylinder sensor system, spindle N. The volume of the samples was 10 mL. Based on rheological data and using the Ostwald-de Waele model, apparent viscosity (\(\mu\)) was calculated.

The separation of the solid from the liquid phase in the samples of cultivation broth were carried out by centrifuging at 10,000 rpm for 10 min (Hettich Rotina 380 R, Germany). In the further research, only the liquid phase of cultivation media was used to determine residual nutrient content.
The obtained supernatants were filtered through a 0.45 μm nylon membrane (Agilent Technologies, Germany) and then analyzed by HPLC (Thermo Scientific Dionex UltiMate 3000 series) to determine residual glycerol content. The HPLC system was equipped with a HPG-3200SD/RS pump, WPS-3000(T)SL autosampler (10 μl injection loop), ZORBAX NH2 column (250 mm x 4.6 mm, 5 μm) and RefractoMax520 detector. The eluent was 70 % (v/v) acetonitrile, with a flow rate of 1.0 mL/min and an elution time of 20 min at column temperature of 30°C. The residual content of total nitrogen was determined by the Kjeldahl method (12), and residual total phosphorus content was determined by spectrophotometric method (13).

RESULTS AND DISCUSSION

Since the product yield and quality depend on the concentration of carbon source in the media (3), the strain _Xanthomonas campestris_ ATCC 13951 was cultivated in the production media containing different initial glycerol concentration. The raw xanthan yield, apparent viscosity of cultivation broth and the average molecular weight of polymer were determined as indicators of the xanthan production efficacy. The results of these experiments are presented in Table 1.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Raw xanthan [g/l]</th>
<th>μ [mPa·s]</th>
<th>M_w [g/mol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>9.82</td>
<td>0.0192</td>
<td>2.64·10^5</td>
</tr>
<tr>
<td>II</td>
<td>12.15</td>
<td>0.0328</td>
<td>2.86·10^5</td>
</tr>
<tr>
<td>III</td>
<td>13.59</td>
<td>0.0355</td>
<td>3.15·10^5</td>
</tr>
<tr>
<td>IV</td>
<td>14.65</td>
<td>0.0451</td>
<td>3.12·10^5</td>
</tr>
<tr>
<td>V</td>
<td>15.13</td>
<td>0.0476</td>
<td>2.98·10^5</td>
</tr>
<tr>
<td>VI</td>
<td>15.81</td>
<td>0.0499</td>
<td>3.17·10^5</td>
</tr>
</tbody>
</table>

In the applied experimental conditions, the xanthan production yield increased with the increase in the initial glycerol concentration in the medium (Table 1). The highest raw xanthan yields of 15.81 g/l and 15.13 g/l were obtained in the media with a glycerol content of 50 g/l (medium V) and 60 g/l (medium VI). In the media with the glycerol concentrations of 20-40 g/l (media II-IV), which are the most preferred concentrations of carbon sources in the media for xanthan production (3), high product yields (12.15-14.65 g/l) were also achieved. According to the literature data (14), if the content of the carbon source in the production medium is 20 g/l, maximum xanthan yields of 14.74 g/l, 13.23 g/l, 12.32 g/l and 12.10 g/l was obtained with contents of glucose, sucrose, maltose and soluble starch, respectively. The raw xanthan yield (12.15 g/l) obtained for medium II indicates that the medium with 20 g/l glycerol content is suitable for the xanthan biosynthesis.

The type and concentration of carbon source in the cultivation media greatly affect the xanthan quality (3). In this study, the xanthan quality was estimated based on the values of the apparent viscosity and average molecular weight. The results shown in Table 1 in-
dicate that the viscosity values follow the same trend as the xanthan yield values. The apparent viscosity of cultivation broths increased with rising initial glycerol content. The values of this parameter ranged from 0.0192 to 0.0499 mPa·s. Since the viscosity of the cultivation broth also depends on the quantity of xanthan, the average molecular weight is a better indicator of the quality of the synthesized biopolymer. The values reported in the literature for the xanthan molecular weight are usually between $10^5$ and $10^6$ g/mol (15). The average molecular weights of the raw xanthan obtained in this research ranged from 2.64 to $3.17 \cdot 10^5$ g/mol (Table 1). These results suggest that there is no significant difference in the quality of the synthesized polymers. Therefore, different initial glycerol concentrations in the cultivation medium do not significantly affect the quality of the xanthan produced under the applied experimental conditions.

Since xanthan is a secondary metabolite of \textit{Xanthomonas} strains it is essential to study the effect of the key medium components on the producing microorganisms’ growth, as well as on the biosynthesis, and find their optimum concentration in order to increase the product yield and quality. Also, when defining the composition of the production media it must be taken into account that the higher process efficiency can be achieved when the residual nutrient content is minimal. In addition, the unused nutrients represent losses from an economic viewpoint. Wastewaters with a significant organic load must undergo a demanding treatment process before being released into the environment, which reduces the profitability of the process. In this study, the residual content of glycerol, total nitrogen and phosphorus were determined, since they are the most important nutrients. The glycerol conversion was also calculated. The obtained results are presented in Table 2.

\textbf{Table 2. Residual content of glycerol, total nitrogen and phosphorus and glycerol conversion}

<table>
<thead>
<tr>
<th>Medium</th>
<th>Residual glycerol [g/l]</th>
<th>Glycerol conversion [%]*</th>
<th>Residual nitrogen [g/l]</th>
<th>Residual phosphorus [g/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.01</td>
<td>99.00</td>
<td>0.49</td>
<td>0.53</td>
</tr>
<tr>
<td>II</td>
<td>2.75</td>
<td>86.25</td>
<td>0.46</td>
<td>0.47</td>
</tr>
<tr>
<td>III</td>
<td>11.46</td>
<td>61.81</td>
<td>0.50</td>
<td>0.53</td>
</tr>
<tr>
<td>IV</td>
<td>20.12</td>
<td>49.71</td>
<td>0.46</td>
<td>0.50</td>
</tr>
<tr>
<td>V</td>
<td>28.84</td>
<td>42.31</td>
<td>0.49</td>
<td>0.52</td>
</tr>
<tr>
<td>VI</td>
<td>36.55</td>
<td>39.09</td>
<td>0.49</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*Conversion [%] = ($S - S_0$)/$S$ \cdot 100
$S_0$ – initial nutrient content [g/l]
$S$ – residual nutrient content [g/l]

The results shown in Table 2 indicate that the residual glycerol content increased with the increase in the initial glycerol concentration in the medium. At the same time, the values of glycerol conversion showed a decrease. The lowest content of this nutrient was achieved in medium I (0.01 g/l) and medium II (2.75 g/l). Also, in these media, the glycerol conversion was the highest and amounted to 99.00 % and 86.25 %, respectively. For a large-scale xanthan production a medium with the initial glycerol concentration of 20 g/l is more suitable because the achieved yield is by 23.73 % higher than for the medium with 10 g/l of glycerol.
As it can be seen from Table 2, the residual content of total nitrogen is in the range of 0.46-0.50 g/l, and of phosphorus in the range of 0.67-0.73 g/l. Although the residual contents of these nutrients are not dependent on the initial glycerol concentration in the cultivation medium, it is evident that the lowest content of nitrogen and phosphorus was found in the medium containing 20 g/l glycerol (medium II). This medium also achieved highest nitrogen and phosphorus conversion rates. However, all calculated conversion values for both nutrients were very low, being in the range of 13.99-21.16 % for nitrogen and 13.14-22.33 % for phosphorus. These results suggest that the media contained high amounts of the initial nitrogen and phosphorus. Therefore, further research should be focused on the medium composition optimization in order to increase efficiency, and hence the profitability of the process.

Based on the obtained results, the xanthan production efficiency in the applied experimental conditions was estimated. Since the cultivation of the selected producing strain on medium II achieved high raw xanthan yield of good quality and lowest content of glycerol, total nitrogen and phosphorus, the medium with the initial glycerol concentration of 20 g/l was suggested for xanthan production, which is in agreement with the finding reported in the literature (10).

CONCLUSIONS

In this study, the possibility of xanthan production on media with glycerol as carbon source was confirmed. Based on the high raw xanthan yield (12.15 g/l) of good quality ($M_w = 2.86 \cdot 10^5$ g/mol) and the lowest content of residual nutrients (glycerol 2.75 g/l, nitrogen 0.46 g/l and phosphorus 0.67 g/l), the medium with the initial glycerol concentration of 20 g/l was suggested for production of this valuable biopolymer. The results obtained in this study present the basis for optimization of xanthan production on glycerol containing media, in terms of other medium components, as well as environmental conditions in order to increase the product yield and quality.

REFERENCES


за овим шећерима указују на потребу примене алтернативних сировина мање тржишне вредности. Глицерол, главни нуспроизвод производње биодизела, је веома јефтин и доступан супстрат за производњу овог високовредног биополимера. Употреба глицерола као извора угљеника у подлогама за биосинтезу ксантанна смањила би трошкове производње, али и еколошке проблеме изазване његовим накупљањем у животној средини. Циљ овог рада је испитивање утицаја различитих почетних концентрација глицерола у подлози за производњу ксантанна применом Xanthomonas campestris ATCC 13951. Ефикасност процеса процењена је на основу вредности приноса сировог ксантанна, средње молекулске масе полимера и резидалних садржаја нутријената након чега је предложен почетни садржај овог извора угљеника у подлози за биосинтезу. У примењеним експерименталним условима, висок принос ксантанна (12,15 г/л) добар квалитета \(M_w = 2,86 \cdot 10^5\) г/моль и минимальан резидалан садржај нутријената (глицерол 2,75 г/л, азот 0,46 г/л и фосфор 0,67 г/л) постиже се у подлози са почетном концентрацијом глицерола од 20 г/л. Добијени резултати представљају основу за оптимизацију производње ксантанна на подлози са глицеролом са циљем повећања приноса и квалитета производа.

**Кључне речи:** ксантан, Xanthomonas campestris, састав подлоге, глицерол

Received: 24 September 2014.
Accepted: 17 October 2014.