THE BIOTECHNOLOGICAL PRODUCTION OF XANTHAN ON VEGETABLE OIL INDUSTRY WASTEWATERS. PART I: MODELLING AND OPTIMIZATION

Xanthan is a complex exopolysaccharide produced by the plant-pathogenic bacterium Xanthomonas campestris pv. campestris [1]. It has a wide range of applications, especially in the food industry due to its various beneficial properties, including emulsion stabilization, temperature stability, compatibility with food ingredients, and its pseudoplasticity. Additionally, it is widely used in the pharmaceutical, cosmetic, agricultural, and petroleum industry [2]. Due to its increasing use in the aforementioned industries, the world market of xanthan is expected to rise significantly in the next decade and reach the value of 987.7 million US dollars by the year 2020, according to the report of the Grand View Research Inc.

Keywords: biotechnological production, cultivation media, wastewaters, RSM, xanthan biosynthesis.
Sucrose, which are common carbon sources, can reduce the price of the final product [3,4]. Based on various scientific papers published in the current literature it can be observed that xanthan production is possible using food industry and agricultural waste effluents as a basis for cultivation media, and several bacterial strains that belong to the genus Xanthomonas, in most cases the species Xanthomonas campestris. Several waste effluents, such as molasses [4-6], date fruit waste [7,8], cheese whey [9,10], tapioca pulp [11], potato residues [12,13], sugar beet pulp [3], citrus waste [14], acid-hydrolyzed wastes from melon, watermelon, cucumber and tomato [15], chestnut flour [16], ram horns [17], sugar cane broth [18], cassava starch [19,20] were successfully used for its production. Additionally, wastewaters of the food industry are used instead of expensive synthetic cultivation media [21]. Potential raw material sources for xanthan production come from all food industries, which produce large amounts of wastewaters rich in compounds necessary for microorganism growth and multiplication. Furthermore, our previous research showed that, among others, vegetable oil industry wastewaters can be used as a substrate for biotechnological xanthan production [22,23].

Most of these studies confirmed the possibility of using various wastewaters and emphasized the need for optimizing the composition of the media based on them. This is particularly important considering the fact that wastewaters obtained from different food processing industries will necessarily have different characteristics and compositions. Defining the appropriate composition of cultivation media is one of the crucial factors for the growth of the microorganism and the production of xanthan. The optimized cultivation media, which is based on food processing industry wastewater, will ensure an uninterrupted metabolism of the production microorganism and consequently the highest yield and quality of the obtained biopolymer. Therefore, choosing an appropriate experimental design in order to optimize cultivation media composition would be a step towards obtaining a higher product yield. In a large number of experimental design methods, response surface methodology (RSM) has been comprehensively applied for its minimal amount of experiments required and effective process optimization. Additionally, this statistical method utilizes quantitative data in order to determine and simultaneously resolve multivariate equations. RSM is a statistical technique based on the essential principles of statistics, which aim to execute experimental planning, to build empirical models, and to evaluate the effect of independent variables on the desired variable response [24-26]. This method has been applied in the optimization of medium composition and other critical variables which have an influence on the production of biopolymers such as curdlan gum, xanthan gum, gellan gum, welan gum, pullulan, dextran, levan etc. [27].

Therefore, the aim of this study is the numerical and graphical optimization of the macronutrients in the cultivation media based on vegetable oil industry for the xanthan production, by using the RSM approach. Mathematical relationships were used to describe the obtained responses and the developed models were applied to find an optimal cultivation media composition. The obtained optimization results will represent a basis for increasing the scale and efficiency of this biotechnological process.

**EXPERIMENTAL**

**Production microorganism**

As a production microorganism, the referent culture Xanthomonas campestris ATCC 13951, was used for experiments. The inoculum was prepared in two steps - first, by refreshing the culture by incubation for 24 h, at 26 °C, on yeast maltose (YM®, Difco) agar slants, and second, by double passaging of the microorganism on the synthetic YM® (Difco) media for 36 h, at 26 °C. Samples were spontaneously aerated and externally stirred (laboratory shaker, 150 rpm).

**Cultivation media**

In accordance with the defined aim of the research and applied experimental plan, 15 cultivation media based on vegetable oil industry wastewater were prepared. Wastewater obtained from vegetable oil industry located in Vojvodina were first analysed to determine its composition significant for the biotechnological production. Initial total nitrogen content was 0.018 g/L, total phosphorus was 0.0037 g/L, COD value was 7240 mg/L, BOD value was 3200 mg/L and the obtained wastewater did not contain any digestible sugars. Based on the obtained results, all cultivation media were enriched with glucose as the carbon source, yeast extract and (NH₄)₂SO₄ as the nitrogen source (in 2:1 ratio), while K₂HPO₄ was added as the phosphorus source. The amounts of aforementioned nutrients are defined by the experimental plan. All cultivation media were additionally enriched with 0.05% of MgSO₄·7H₂O. The pH value of the cultivation media was set to 7.0 and sterilized in an autoclave at 121 °C and overpressure of 1.1 bar during 20 min.

**Cultivation**

The biotechnological process of xanthan production was carried out under the same experimental con-
ditions in 15 erlenmeyer flasks, containing one-third of the volume, of the wastewater based media. The inoculation was performed with 10 vol.% of the inoculum. A rotational laboratory shaker at 150 rpm was used for aeration and stirring of cultivation media. Cultivation was performed at a temperature of 26 °C for the first 48 h, after which it was increased to 30 °C and kept at this value until the end of the process, which lasted for 96 h [32].

Analytical methods

At the end of the process, samples of the cultivation broths were analysed. Rheological properties of the cultivation broth samples were determined using a rotational viscometer (REOTEST 2 VEB MLV Prüfgeräte-Verk, Mendingen, SitzFreielt), with a double gap coaxial cylinder sensor system, spindle N. Rheological parameters were calculated according to the Ostwald de Vaele equation.

The samples of the cultivation broth were centrifuged at 10,000 rpm for 30 min (Hettich Rotina 380R, Germany) and the obtained supernatants were used to determine residual carbon content. The supernatants were filtered through a 0.45 μm nylon membrane (Agilent Technologies, Germany) and then analyzed by HPLC (Thermo Scientific Dionex UltiMate 3000series). The HPLC instrument was equipped with a HPG-3200SD/RS pump, WPS-3000(T)SL autosampler (10 μL injection loop), ZORBAX NH2 column (250 mm×4.6 mm, 5 μm) and a RefractoMax520 detector. 75 vol.% acetonitrile was used as the eluent at a flow rate of 1.2 mL/min and an elution time of 20 min at a column temperature of 25 °C.

In order to determine residual nitrogen and phosphorus content, prior to centrifugation (10,000 rpm, 30 min) the obtained cultivation broths were diluted with 4 volumes of the distilled water. From the obtained supernatants the residual nitrogen content was determined by the Kjeldahl method [28], and a standard method was used to determine the residual phosphorus content [29].

Product separation

Xanthan was recovered by precipitation with 96 vol.% ethanol in the presence of KCl as the electrolyte. Ethanol was gradually added to the supernatant at 15 °C until the alcohol content in the mixture was 60%, at constant stirring. A saturated solution of KCl was added when half of the necessary ethanol amount was poured into the supernatant in a quantity to obtain a final content of 1 vol.%. After precipitation, the mixture of xanthan was kept at 4 °C, for 24 h and then centrifuged (4000 rpm, 15 min). The precipitate was dried to a constant mass at 60 °C and this data was used to calculate the xanthan yield.

Statistics and data analysis

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize responses (output variables: Y1, Y2,...,Y3) which are influenced by several experimental factors or independent variables (input variables: X1,X2,...,X3). Optimization by Box-Behnken design under RSM vividly underscores interactions between variables and their effects. Box-Behnken design is rotatable second-order designs based on three-level incomplete factorial designs, and has been used for the optimization of several processes by a significant number of researchers. A total of 15 experiments is needed for the three-level three-factorial Box-Behnken experimental design [30,31]. The experimental factors and their values (g/L) are: X1 - carbon source content (10.00-30.00), X2 - nitrogen content (0.02-0.20); and X3 - phosphorus content (0.0045-0.045). For the description of the responses a second-degree polynomial model was fitted to data:

\[ Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \]

where \( b_0 \) represents the intercept, \( b_i \) represents the linear, \( b_{ii} \) the quadratic and \( b_{ij} \) represents the interaction effect of the examined factors. Selected responses are xanthan yield (Y1), cultivation media apparent viscosity (Y2), residual carbon content (Y3), residual nitrogen content (Y4) and residual phosphorus content (Y5).

Experimental data were subjected to statistical analysis using software Statistica 9.0 (StatSoft, USA). Response surface curves were plotted with a constant value of one of the parameters, while remaining two parameters were varied. The obtained data was analyzed using the software package Design Expert 8.1 (StatEase, Inc., USA) and the method of desirability function was applied for numerical and graphical optimization and determination of optimal values of examined parameters.

RESULTS AND DISCUSSION

In accordance with the defined aim and experimental plan of this research, the media for xanthan biosynthesis were defined with vegetable oil industry wastewater as the basis. The values of varied experimental factors and their responses as well as the calculated value of the xanthan conversion are shown in Table 1. Carbon source, nitrogen, and phosphorus...
contents were chosen as experimental factors because they represent macronutrients that are essential for the biosynthesis of xanthan as well as for the metabolism of production microorganism. The success of the performed biosynthesis was determined by the xanthan yield and the apparent viscosity of the cultivation media, while the residual carbon, nitrogen, and phosphorus contents indicate the economic and ecological efficiency of the process.

The obtained values of xanthan yield ranges from 8.19 to 16.17 g/L and the highest values are achieved with 30.00 g/L of initial glucose content. Moreover, when using the highest examined initial amount of glucose, the xanthan conversion is the lowest and ranges between 49.80-53.90%, but according to the available literature data [32] the xanthan conversion is between 50-85%, and the obtained values are in this range. When using 10.00 g/L of carbon source, conversion is between 81.88-95.10%, while when using 20.00 g/L of glucose, conversion values are lower (54.38-68.85%). Additionally, based on the obtained results it can be seen that the conversion value decreases with increasing of the initial carbon content. It is evident that no matter how much carbon source is added to the cultivation media, xanthan yield approximately reaches the maximal value of 15 g/L. It is evident that when using this experimental conditions and cultivation media composition, the aforementioned amount of produced xanthan changes the rheology of the system and decreases the rate of oxygen mass transfer that is necessary for xanthan biosynthesis as well as for metabolism of the production strain. The absence of sufficient amount of dissolved oxygen inhibits further biosynthesis as well as consumption of carbon source in the media, which affects high residual carbon content in media and consequently decrease of the conversion value [40].

The obtained values indicate that vegetable oil industry wastewaters can be used as a basis for cultivation media for xanthan production, due to the absence of inhibitory substances that would endanger Xanthomonas campestris metabolism.

All the obtained cultivation broths showed a pseudoplastic type of flow, which is characteristic of the xanthan solutions [2] and the rheological parameters (data not shown), consistency factor (obtained values are in the range between 0.4878 and 0.5725) and flow behaviour index (obtained values are in the range between 0.4878 and 0.5725) were used for calculating the apparent viscosity of cultivation media.

In industrial systems, when the amount of residual carbon content is lower than 5 g/L xanthan biosynthesis is considered as finished. Based on these results, it can be concluded that when using the highest examined glucose content (30.00 g/L), the process is economically and ecologically inefficient. Based on the obtained results it can be concluded that, compared to initial media nitrogen contents, nitrogen content was significantly reduced in all experiments (50.00-94.50%), while the amount of residual nitrogen is slightly higher than the nitrogen content of the util-

### Table 1. Combinations of experimental factors and obtained response values after completed biosynthesis of xanthan

<table>
<thead>
<tr>
<th>Experimental factors</th>
<th>Responses</th>
<th>Conversion&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C source content, g/L</td>
<td>N content, g/L</td>
<td>P content, g/L</td>
</tr>
<tr>
<td>10.00</td>
<td>0.02</td>
<td>0.025</td>
</tr>
<tr>
<td>30.00</td>
<td>0.02</td>
<td>0.025</td>
</tr>
<tr>
<td>10.00</td>
<td>0.20</td>
<td>0.025</td>
</tr>
<tr>
<td>30.00</td>
<td>0.20</td>
<td>0.025</td>
</tr>
<tr>
<td>10.00</td>
<td>0.11</td>
<td>0.0045</td>
</tr>
<tr>
<td>30.00</td>
<td>0.11</td>
<td>0.0045</td>
</tr>
<tr>
<td>10.00</td>
<td>0.11</td>
<td>0.045</td>
</tr>
<tr>
<td>30.00</td>
<td>0.11</td>
<td>0.045</td>
</tr>
<tr>
<td>20.00</td>
<td>0.02</td>
<td>0.045</td>
</tr>
<tr>
<td>20.00</td>
<td>0.20</td>
<td>0.0045</td>
</tr>
<tr>
<td>20.00</td>
<td>0.20</td>
<td>0.045</td>
</tr>
<tr>
<td>20.00</td>
<td>0.20</td>
<td>0.045</td>
</tr>
<tr>
<td>20.00</td>
<td>0.11</td>
<td>0.025</td>
</tr>
<tr>
<td>20.00</td>
<td>0.11</td>
<td>0.025</td>
</tr>
<tr>
<td>20.00</td>
<td>0.11</td>
<td>0.025</td>
</tr>
</tbody>
</table>

<sup>a</sup>Conversion [%] = 100(P/S0); P – xanthan yield (g/L); S0 – initial C source content (g/L)
ized wastewater only in the experiments in which the amount of added nitrogen was highest. Phosphorus content is also significantly reduced compared to the initial content in the cultivation media (46.68-68.10%). In addition to producing a high-value product, significant environmental benefits can be achieved throughout the minimization of the residual nutrients, especially nitrogen and phosphorus, which increase the organic load of the process effluents and must be treated before being discharged into the environment. Additionally, the unutilized nutrients that remain in the cultivation broth represent an economical wastage and a problem during product separation and purification.

Statistical analysis of experimental results

By applying RSM, it is possible to design experiments, build models, search for optimal conditions for desirable responses and evaluate the interaction of factors that may influence process efficiency with a reduced number of experiments [33]. The response surface method was selected for the optimization and fitting of results of xanthan production using vegetable oil industry wastewater as a basis of the cultivation media. For responses obtained after experiments, a second degree polynomial model was established to evaluate and quantify the influence of the variables. Table 2 shows the results of the statistical analyses, while Table 3 shows the ANOVA results for selected responses.

The results were statistically processed by analysing the variance at the significance level of \( \alpha = 0.05 \) (confidence interval 95%). The coefficient of determination \( (R^2) \) and the model \( p \)-value were used to estimate the adequacy of the model. The significance of each coefficient in the obtained second-degree polynomial model is determined by the \( p \)-value and statistically significant coefficients \( (p < 0.05) \) are marked in Table 2.

For the xanthan yield response, the coefficient of determination was 0.972 which points to a high correlation between observed and predicted values. The most significant effects are the linear and squared effect for the initial carbon source content as well as the interaction effect of nitrogen and phosphorus

Table 2. Regression equation coefficients for selected responses; C - coefficient; * - effect significant at \( p < 0.05 \) confidence level

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>( Y_1 )</th>
<th>( Y_2 )</th>
<th>( Y_3 )</th>
<th>( Y_4 )</th>
<th>( Y_5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-0.076</td>
<td>0.976</td>
<td>25.999*</td>
<td>0.0001</td>
<td>-6.726*</td>
</tr>
<tr>
<td>Linear</td>
<td>( b_1 )</td>
<td>1.011*</td>
<td>0.003</td>
<td>0.891*</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>( b_2 )</td>
<td>1.670</td>
<td>0.920</td>
<td>-137.6*</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>( b_3 )</td>
<td>8.322</td>
<td>0.289</td>
<td>-1.185</td>
<td>0.8524</td>
</tr>
<tr>
<td>Quadratic</td>
<td>( b_{11} )</td>
<td>-0.016*</td>
<td>0.014</td>
<td>0.013*</td>
<td>0.0016</td>
</tr>
<tr>
<td></td>
<td>( b_{12} )</td>
<td>-96.724</td>
<td>0.121</td>
<td>415.87*</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>( b_{13} )</td>
<td>-20.475</td>
<td>0.102</td>
<td>5.903</td>
<td>0.5329</td>
</tr>
<tr>
<td>Interaction</td>
<td>( b_{12} )</td>
<td>0.101</td>
<td>0.830</td>
<td>3.072*</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>( b_{13} )</td>
<td>-0.247</td>
<td>0.269</td>
<td>0.120</td>
<td>0.5150</td>
</tr>
<tr>
<td></td>
<td>( b_{13} )</td>
<td>62.482*</td>
<td>0.037</td>
<td>-127.5*</td>
<td>0.0011</td>
</tr>
</tbody>
</table>

Table 3. Analysis of variance (ANOVA) of the modelled responses; \( DF \) - degrees of freedom; \( SS \) - sum of squares; \( MS \) - mean square

<table>
<thead>
<tr>
<th>Response</th>
<th>( Y_1 )</th>
<th>( Y_2 )</th>
<th>( Y_3 )</th>
<th>( Y_4 )</th>
<th>( Y_5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( DF )</td>
<td>( SS )</td>
<td>( MS )</td>
<td>( DF )</td>
<td>( SS )</td>
</tr>
<tr>
<td>( Y_1 )</td>
<td>5</td>
<td>3.253</td>
<td>0.6506</td>
<td>10</td>
<td>2555.42</td>
</tr>
<tr>
<td>( Y_2 )</td>
<td>5</td>
<td>2.41</td>
<td>0.483</td>
<td>10</td>
<td>3350.32</td>
</tr>
<tr>
<td>( Y_3 )</td>
<td>5</td>
<td>1.013</td>
<td>0.2026</td>
<td>10</td>
<td>1338.54</td>
</tr>
<tr>
<td>( Y_4 )</td>
<td>5</td>
<td>0.000023</td>
<td>0.000005</td>
<td>10</td>
<td>0.002792</td>
</tr>
<tr>
<td>( Y_5 )</td>
<td>5</td>
<td>0.000172</td>
<td>0.000034</td>
<td>10</td>
<td>0.169177</td>
</tr>
</tbody>
</table>
Viscosity, including apparent viscosity, depends on the amount of xanthan in the cultivation broth, molecular weight and structures formed between the biopolymer molecules, which all are influenced by the basic nutrient contents, among other parameters [34]. The most significant effects on apparent viscosity of cultivation media are the linear and squared effect for the initial carbon source and nitrogen content, as well as the effects of interaction between the carbon source and nitrogen and between nitrogen and phosphorus content.

During xanthan biosynthesis, the carbon source is used both for the cell growth and for the production of xanthan, for which the presence of suitable phosphorus and nitrogen sources are necessary and is of great importance [35]. Therefore, it is expected that residual carbon content response is affected by initial contents of these nutrients (linear effects of carbon source, nitrogen and phosphorus and squared effect of initial nitrogen and phosphorus). This very clearly indicates that carbon source consumption can be managed by limiting nitrogen and phosphorus contents, or the ratio of these nutrients.

The significant effects on the response for residual nitrogen content is the linear and squared effect of initial phosphorus and the squared effect of initial nitrogen content, while for residual phosphorus response the most significant is the linear and squared effect of initial phosphorus content, which indicates that these nutrients are important for the production microorganism metabolism in the initial phases of cultivation.

Values of the coefficient of determination, for all the obtained responses, are high and have a value close to 1.00 and indicate a good fit of experimental data to the second degree polynomial equation. Only for the value of the response for residual nitrogen content is somewhat lower (0.939). The model F-values for xanthan yield (\( Y_1 \)), cultivation media apparent viscosity (\( Y_2 \)), residual carbon content (\( Y_3 \)), residual nitrogen content (\( Y_4 \)) and residual phosphorus content (\( Y_5 \)) imply that models for selected responses are significant. Additionally, comparisons between experimental and predicted values for the obtained responses are graphically showed in Figure 1.

**Mathematical model for xanthan yield**

Statistical analysis and graphical analysis of the data were performed using Statistica 9.0 software (StatSoft, USA). Plotting responses for xanthan yield as a function of two factors drew response surface plots, while the third factor had the mean value from the

![Figure 1. Comparison of experimentally obtained data for selected responses (xanthan yield, apparent viscosity of cultivation broths, residual carbon, residual nitrogen and residual phosphorus) and the values predicted by the models.](image-url)
selected range. Figure 2 presents the effect of initial carbon source, nitrogen and phosphorus content on xanthan yield.

Figure 2. Xanthan yield value as a function of two variables at constant value of third: a - phosphorus content (0.025 g/L); b - nitrogen content (0.11 g/L); c - carbon source content (20.00 g/L).

The response surface shown in Figure 2a undoubtedly shows that at the phosphorus content of 0.025 g/L, the initial nitrogen content in examined range has very low effect on xanthan yield, while the effect of the carbon source content (10.00-30.00 g/L) is clear, and increasing the carbon source content in the xanthan production media increases the yield of the desired product. Additionally, the most significant effects are the linear ($p = 0.003$) and squared ($p = 0.014$) effect for the initial carbon source content, confirming that the concentration of carbon source affects the xanthan yield [36]. These results are in agreement with previously reported findings in the literature [37] showing that the carbon source had a more positive effect on xanthan yield than the nitrogen source. The response surface shows that the maximum yield of xanthan (15.00-16.00 g/L) is obtained at a carbon source concentration above 26.00 g/L, for all applied initial nitrogen content. Therefore, a high concentration ratio of carbon source to the limiting nutrient, which can be nitrogen, phosphorus, or sulphur positively influence xanthan biosynthesis and when the nitrogen is used as the limiting nutrient, a high C/N ratio is required to stimulate xanthan production [38].

Figure 2b shows that the effect on xanthan yield of initial carbon source and phosphorus contents at a constant value of nitrogen content (0.11 g/L) is similar to the effect of initial carbon source and nitrogen content at a constant value of phosphorus content (Figure 2a). From the response surface, it can be seen that maximum xanthan yield (15.00-16.00 g/L) is obtained at carbon concentrations higher than 26.00 g/L, in the entire range of applied phosphorus concentration, but somewhat higher yield values (about 16.00 g/L) can be observed when using lower amounts of initial phosphorus (0.01-0.03 g/L). The graph shows that at constant nitrogen content, changing phosphorus content does not significantly affect xanthan yield. Moreover, at constant phosphorus content, changing carbon source content significantly affects xanthan yield in the entire examined range.

The effect of initial nitrogen and phosphorus contents on the obtained xanthan yield during biosynthesis at carbon source content of 20.00 g/L is shown on Figure 2c, as predicted by the model. The interaction effect of nitrogen and phosphorus contents has a statistically significant effect ($p = 0.036$) on xanthan yield and their positive interaction (Table 2) indicates their synergetic effect. The lower values of xanthan yield (about 10.00 g/L) were obtained at the highest used phosphorus content (about 0.040-0.045 g/L) and the lowest used nitrogen content (< 0.04 g/L), as well as at the lowest used phosphorus content (< 0.015 g/L) and the highest used nitrogen content (about 0.18-0.20 g/L). Figure 2c also shows that the maximum xanthan
yield (about 13 g/L) was obtained when the cultivation media contained the uniform ratio of nitrogen and phosphorus in the entire examined ranges. Based on the obtained results and in the applied experimental conditions it can be concluded that ratio of the nitrogen and phosphorus content in the cultivation media containing wastewater from vegetable oil industry, in order to achieve highest possible xanthan yield, needs to be approximately 4 to 1 (N:P = 4:1).

Optimization of cultivation medium for xanthan production

This research used the desirability function method in order to obtain the optimum composition of cultivation media that is based on vegetable oil industry wastewater for the production of the biopolymer xanthan. The final goal of the application of the response surface method is process optimization, so the developed models are used for simulation and optimization. This method consists of converting individual responses into individual desirability functions whose values range from 0 to 1. The overall desirability function is equal to the geometric average of individual desirability functions, and its validity is assessed using the aforementioned method. The desirability method is recommended due to its simplicity, availability in the software and provides flexibility in weighting and giving importance for individual response [39].

In order to determine the optimal content of carbon, nitrogen and phosphorus in media based on vegetable oil industry wastewater that is necessary for unimpeded xanthan production, the specific intervals for requested response values were set. The defined interval for xanthan yield ranged between 12.00 and 16.17 g/L, considering that all the values that are lower than these are not justified for downstream processing. The defined interval for cultivation media apparent viscosity is 23.87-65.87 mPa·s, which represents the entire obtained range. The residual carbon interval values were set to 0.78-5.00 g/L, because 5.00 g/L is the amount that represents the critical amount for xanthan biosynthesis in industrial systems. Also, the defined ranges for residual nitrogen and residual phosphorus ranged between 0.0070-0.01 g/L and 0.0015-0.01 g/L, respectively, in order to decrease the initial amounts of nitrogen and phosphorus used for the experiments.

The usual numerical optimization method produced a large number of combinations of varied parameters for which the total desirability function was 1.000, and Table 4 shows 10 selected solutions.

For the requested optimization conditions, using the applied method, the software assesses correct values for all factors/responses relevant to optimization (Table 4). Based on the obtained results it can be seen that the optimized amount of carbon source content is between 14.31 and 14.99 g/L, except for one value of 16.27 g/L, the optimized amount of initial phosphorus content is equal in all optimization combinations, and is 0.02 g/L, and the optimized amounts of initial nitrogen contents are very different and ranges between 0.02-0.14 g/L. In production conditions on the industrial scale it is impossible to achieve experimental factor values (contents of carbon source, nitrogen and phosphorus) exactly as assessed by the optimization process which would, together with the maximum total desirability function value, enable an ideal prediction of response values (xanthan yield, apparent viscosity of cultivation media, residual carbon, residual nitrogen and residual phosphorus). Thereby it is necessary to assess intervals of experimental factor values for which the assessed response values will be acceptable. These intervals of experimental factor values, which represent the optimal condition, can be visualized graphically by superimposing the contours for the various response surfaces in an overlay plot [33]. Therefore, Figure 3 shows overlay plots of the selected responses.

Table 4. Selected optimization results: estimated values; desirability function, \( D = 1 \); \( P \) content= 0.02 g/L

<table>
<thead>
<tr>
<th>Solution</th>
<th>C source content, g/L</th>
<th>N content, g/L</th>
<th>Xanthan yield, g/L</th>
<th>Apparent viscosity, mPa·s</th>
<th>Residual C, g/L</th>
<th>Residual N, g/L</th>
<th>Residual P, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.73</td>
<td>0.04</td>
<td>12.05</td>
<td>37.83</td>
<td>4.40</td>
<td>0.0077</td>
<td>0.0089</td>
</tr>
<tr>
<td>2</td>
<td>14.78</td>
<td>0.03</td>
<td>12.03</td>
<td>38.72</td>
<td>4.17</td>
<td>0.0086</td>
<td>0.0078</td>
</tr>
<tr>
<td>3</td>
<td>16.27</td>
<td>0.02</td>
<td>12.52</td>
<td>42.11</td>
<td>4.90</td>
<td>0.0082</td>
<td>0.0091</td>
</tr>
<tr>
<td>4</td>
<td>14.80</td>
<td>0.14</td>
<td>12.11</td>
<td>33.38</td>
<td>5.00</td>
<td>0.0100</td>
<td>0.0099</td>
</tr>
<tr>
<td>5</td>
<td>14.99</td>
<td>0.03</td>
<td>12.06</td>
<td>39.32</td>
<td>4.30</td>
<td>0.0079</td>
<td>0.0088</td>
</tr>
<tr>
<td>6</td>
<td>14.48</td>
<td>0.12</td>
<td>12.00</td>
<td>33.34</td>
<td>4.87</td>
<td>0.0091</td>
<td>0.0094</td>
</tr>
<tr>
<td>7</td>
<td>14.31</td>
<td>0.10</td>
<td>12.00</td>
<td>33.67</td>
<td>4.76</td>
<td>0.0081</td>
<td>0.0095</td>
</tr>
<tr>
<td>8</td>
<td>14.68</td>
<td>0.13</td>
<td>12.03</td>
<td>33.46</td>
<td>4.95</td>
<td>0.0098</td>
<td>0.0094</td>
</tr>
<tr>
<td>9</td>
<td>14.74</td>
<td>0.13</td>
<td>12.06</td>
<td>33.52</td>
<td>4.99</td>
<td>0.0099</td>
<td>0.0094</td>
</tr>
<tr>
<td>10</td>
<td>14.39</td>
<td>0.11</td>
<td>12.01</td>
<td>33.52</td>
<td>4.82</td>
<td>0.0086</td>
<td>0.0093</td>
</tr>
</tbody>
</table>
(xanthan yield, apparent viscosity of cultivation media, residual carbon, residual nitrogen and residual phosphorus content) as a function of carbon source and nitrogen content in the examined ranges and with constant initial phosphorus content value of 0.02 g/L (a), as well as nitrogen and phosphorus contents in the examined ranges, and with constant initial carbon source content value of 15.00 g/L (b).

The light grey fields represent the ranges of varied parameters (experimental factors) in which all the requested conditions were satisfied. Dark grey fields on the other hand, represent the ranges of parameters where one or more of the requested conditions weren’t satisfied.

Overlay plot presented in Figure 3a shows that at initial phosphorus content of 0.02 g/L, xanthan production is possible when 15.00-16.00 g/L of carbon and 0.02-0.11 g/L of nitrogen were added to the cultivation media based on vegetable oil industry wastewater. Then it is possible to obtain yields of around 12.00 g/L of xanthan with residual carbon and nitrogen of 5.00 and 0.01 g/L, respectively. The obtained results are in accordance with previously published data [7,17] which showed that low nitrogen content has a positive effect on xanthan production. If the cultivation media based on vegetable oil industry wastewater is initially enriched with 15.00 g/L, according to the overlay plot shown on Figure 3b it can be seen that nitrogen and phosphorus which are necessary to be added range from 0.02-0.09 g/L and 0.01-0.02 g/L, respectively. In this case, maximum xanthan yield is obtained (12.00 g/L) with residual carbon, nitrogen and phosphorus values of around 5.00, 0.01 and 0.01 g/L, respectively.

Validation of the model

In order to validate the developed models, three confirmation experiments were carried out using the average experimental factor values (carbon source 15.5 g/L, nitrogen content 0.065 g/L and phosphorus content 0.0145 g/L) of the obtained optimal cultivation media composition range. For the actual responses, the calculated average results of three measurements, for xanthan yield, apparent viscosity of cultivation media, residual carbon, residual nitrogen and residual phosphorus were 12.06±0.19 g/L, 38.32±2.08 mPa·s, 3.87±0.27 g/L, 0.015±0.002 g/L and 0.009±0.001 g/L, respectively. The values predicted with developed models for xanthan yield, apparent viscosity of cultivation media, residual carbon, residual nitrogen and residual phosphorus were 11.79g/L, 38.67 mPa·s, 3.953 g/L, 0.016 g/L and 0.008 g/L, respectively. Based on the obtained results, it can be seen that experimental values are in excellent agreement with the predicted values for all modelled responses.

CONCLUSION

The obtained results proved that the Box-Behnken experimental design and response surface methodology represent an efficient approach for optimization of cultivation media based on vegetable oil industry wastewater, in terms of carbon source, nitrogen and phosphorus content, for the production of xanthan. The
values predicted by the developed models for xanthan yield, apparent viscosity of cultivation media, residual carbon, residual nitrogen and residual phosphorus were 11.79 g/L, 38.67 mPa·s, 3.953 g/L, 0.016 g/L and 0.008 g/L, respectively, when using the initial values of the carbon source (15.5 g/L), nitrogen (0.065 g/L) and phosphorus (0.0145 g/L) contents. Using the same initial values of experimental factors, experimentally obtained values for xanthan yield, apparent viscosity of cultivation media, residual carbon, residual nitrogen and residual phosphorus were 12.06±0.19 g/L, 38.32±2.08 mPa·s, 3.87±0.27 g/L, 0.015±0.002 /L and 0.009±0.001 g/L, respectively, which validates the developed models. Additionally, the obtained results indicate that vegetable oil industry wastewaters have a lot of potential for the efficient production of cost-effective xanthan. Further research should consist of optimizing process parameters in order to increase yield and quality of xanthan, as well as defining the kinetics and kinetic parameters as an important step towards industrializing this biotechnological process.

REFERENCES
B-Ž. BAJIĆ et al.: THE BIOTECHNOLOGICAL PRODUCTION OF XANTHAN...


BOJANA Ž. BAJIĆ
DAMJAN G. VUČUROVIĆ
SINIŠA N. DODIĆ
ZORANA Z. RONČEVIĆ
JOVANA A. GRAHOVAC
JELENA M. DODIĆ
Univerzitet u Novom Sadu,
Tehnološki fakultet Novi Sad,
Katedra za biotehnologiju i
farmaceutsko inženjerstvo, Bulevar
cara Lazara 1, 21000 Novi Sad,
Srbija

NAUČNI RAD

BIOTEHNOLOŠKA PROIZVODNJA KSANTANA NA OTPADNOJ VODI IZ PROIZVODNJE JESTIVOG ULJA. DEO I: MODELOVANJE I OPTIMIZACIJA

Cilj ovog rada je utvrđivanje mogućnosti primene otpadne vode iz proizvodnje jestivog ulja kao osnove kultivacionog medijuma za proizvodnju ksantana, primenom Xanthomonas campestris ATCC 13951, kako bi se otpadna voda iz jedne grane industrije upotrebila kao sirovina u drugoj. Takođe, cilj je i optimizacija sastava medijuma čija je osnava otpadna voda u pogledu sadržaja ugljenika, azota i fosfora. Eksperimenti su izvedeni u skladu sa Box-Behnken eksperimentalnim planom sa tri faktora na tri nivoa (glukoza: 10,00-30,00 g/L, azot: 0,02-0,20 g/L, fosfor: 0,0045-0,045 g/L) i tri ponavljanja u centralnoj tački. Uspešnost biosinteze je procenjena na osnovu analize dobijenih kultivacionih tečnosti i određivanja količine ksantana, prividnog viskoziteta kultivacione tečnosti, kao i sadržaja rezidualnog ugljenika, azota i fosfora. Postupak odzivne površine je primenjen za određivanje vrednosti optimalnog sadržaja izvora ugljenika, azota i fosfora. Na osnovu rezultata grafičke optimizacije, za intervale zahtevanih vrednosti odziva, definisani model predviđa da je proizvodnja ksantana moguća kada se u kultivacioni medijum čija je osnova otpadna voda iz proizvodnje jestivog ulja dodat 15,00-16,00 g/L izvora ugljenika, 0,02-0,09 g/L azota i 0,01-0,02 g/L fosfora.

Ključne reči: biosintezas kantana, biotehnološka proizvodnja, kultivacioni medijum, otpadne vode, RSM.