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EFFECT OF SUCROSE CONCENTRATION ON OXALIC ACID BIOSYNTHESIS BY *ASPERGILLUS NIGER*

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Summary. The aim of the work was to determine the influence of initial sucrose concentration in fermentation medium on oxalic acid synthesis by *Aspergillus niger* W78C in submerged batch culture. Cultivations were conducted in synthetic medium containing sucrose in the concentration of 100, 125, 150, 175 or 200 $g \cdot dm^{-3}$. As the result of the present investigation, the optimal sucrose concentration was found at the level of 125 $g \cdot dm^{-3}$. In medium containing this amount of carbon source, 70.2 $g \cdot dm^{-3}$ of oxalic acid was obtained. When substrate concentration was the highest, 200 g dm^{-3}, the amount of product was only 5.2 $g \cdot dm^{-3}$ higher than in medium with 125 $g \cdot dm^{-3}$ of sucrose. Moreover, an increase of the initial sucrose concentration resulted in process time extension, an increase of citric and gluconic acids concentration and a decrease of productivity and oxalic acid yield.

Key words: oxalic acid, *Aspergillus niger*, biosynthesis, sucrose, carbon source

INTRODUCTION

Oxalic acid (OA) is the simplest aliphatic dicarboxylic acid naturally present in every leafy vegetable and hence is present in every vegetable food product [Massey 2007, Cefola and Pace 2015]. It has many uses: beside metal treatment, chemical and pharmaceutical applications, oxalic acid may be used in biohydrometallurgy [Biswas et al. 2013, Kursunoglu and Maya 2015], bleaching [Aghaie et al. 2009, Musiał et al. 2011], waste water treatment [Wei et al. 2013, Abraham et al. 2014] and wastes utilisation [Sun and Qiu 2012, Asghari et al. 2013]. In recent years there is a growing interest in oxalic acid application in food industry as a preservative food additive [Jin et al. 2014, Riuz-Jimenez et al. 2014, Cefola and Pace 2015, Li et al. 2016, Wang et al. 2016].

At present oxalic acid is produced mostly by chemical processes, such as oxidation of carbohydrates, olefins and glycols. These methods have a negative impact on the environ-

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ment, therefore biological methods of its production are necessary [Emeko 2015, Betiku et al. 2016, Mai et al. 2016]. Among the microorganisms that produce oxalic acid, the fungus *Aspergillus niger* is favoured as the most efficient [Musiał et al. 2011, Walaszczyk et al. 2014, Emeko et al. 2015, Betiku et al. 2016].

Synthesis of organic acids by *A. niger* is an adaptive reaction to environmental changes. Oxalic acid is secreted when the pH of the medium is close to neutrality. Its role is to lower the pH quickly and thus limit the growth of competitive microorganisms, mainly bacteria. Citric acid is synthesised when the pH of the environment is low, in order to buffer the medium and prevent the pH increase. Production of gluconic acid is not aimed at acidifying the medium. Biotransformation of glucose to gluconate is a mechanism to bound glucose into compound unavailable to competing organisms. This last process is the most efficient at pH 5.0–5.5, in which many bacteria have their optimum of growth. The formed gluconic acid is later used by *A. niger* as a carbon source [Andersen et al. 2009, Poulsen et al. 2012].

Carbohydrates have been widely tested as substrates for oxalic acid biosynthesis, among them: glucose [Mandal and Banerjee 2005, Walaszczyk et al. 2014], sucrose [Strasser et al. 1994, Cameselle et al. 1998, Musiał et al. 2005, Walaszczyk et al. 2015], milk whey [Casamelle et al. 1998], sugar beet molasses [Podgórski and Leśniak 2003], cashew apple juice [Emeko et al. 2015, Betiku et al. 2016] or corncobs [Mai et al. 2016]. Nevertheless, sucrose was used in the first defined medium for organic acids synthesis by *A. niger* [Currie 1917] and it was recognized by Strasser et al. [1994] as a suitable carbon source for oxalic acid production. In citric acid bioproduction by *A. niger* sucrose was a better carbon source than glucose and its optimal concentration was set between 13 and 18% [Walaszczyk et al. 2014]. In oxalic acid biosynthesis, the initial sucrose concentration was mostly either 100 or 150 $g \cdot dm^{-3}$ [Strasser et al. 1994, Cameselle et al. 1998, Foryś and Podgórski 2004, Mandal and Banerjee 2005, Musiał et al. 2005, Walaszczyk et al. 2015].

Investigating the influence of substrate concentration on oxalic acid secretion is a very interesting issue considering that there is little research in this field. Podgórski and Leśniak [2003] tested beet molasses and stated that optimal initial sucrose concentration should be 120 $g \cdot dm^{-3}$, while Santoro et al. [1999] obtained the best results using milk whey in the amount of 100 $g \cdot dm^{-3}$. When glucose was the substrate, the highest oxalic acid concentration was reached when sugar amount was 150 $g \cdot dm^{-3}$ [Walaszczyk et al. 2014].

The aim of this work was to examine the influence of initial sucrose concentration on oxalic acid synthesis by *A. niger* in submerged batch cultures in defined media.

MATERIAL AND METHODS

Microorganism

Aspergillus niger W78C strain from the collection of Biotechnology and Food Analysis Department, Wroclaw University of Economics (Poland) was used in this study.

Medium preparation and culture conditions

The only carbon and energy source for microorganism was sucrose in the form of white sugar. The synthetic medium consisted (per 1 dm^{-3}) of: sucrose 100, 125, 150, 175 or 200 g, NH_4NO_3 1.89 g, KH_2PO_4 0.32 g, $MgSO_4 \cdot 7H_2O$ 0.64 g, $ZnSO_4 \cdot 7H_2O$ 0.97 mg, $CuSO_4 \cdot 5H_2O$ 0.86 mg, $FeSO_4 \cdot 7H_2O$ 1.64 mg, $MnSO_4 \cdot H_2O$ 1.02 mg, distilled water. After sterilization, the medium was inoculated with spores of *A. niger* in the amount of about $3 \cdot 10^4$ per flask. The experiments were carried out 16 to 40 days in 30°C in 750 cm^3 flasks filled with 125 cm^3 of the medium, put on rotary shaker working with the frequency of 150 min^{-1}. The pH was set at 6.0 before sterilisation and regulated manually at this level by the addition of 8 M KOH every second day beginning from the sixth day. The end of the process was set by the lack of increase of total acidity of the medium measured titrimetrically with 0.1 M NaOH, taking into account the amount of KOH added for pH regulation.

Analytical methods

The dry weight of the biomass was determined gravimetrically by drying harvested and filtered biomass at 105°C to a constant weight. The concentrations of organic acids and sugars were determined using HPLC on Rezex ROA Organic Acid column coupled to a UV detector at 210 nm and an RI detector. The column was eluted with 0.0025 M H_2SO_4 at room temperature and a flow rate of 0.5 $ml \cdot min^{-1}$.

Mathematical and statistical methods

Volumetric oxalic acid production rate (productivity) (Q_P) was calculated as:

$$Q_P = \frac{P}{t} \ [g \cdot dm^{-3} \cdot day^{-1}]$$

where: P – oxalic acid concentration [$g \cdot dm^{-3}$],
t – time [day].

Oxalic acid yield ($Y_{P/S}$) was calculated as:

$$Y_{P/S} = \frac{P}{S} \cdot 100 \ [\%]$$

where: P – oxalic acid concentration [$g \cdot dm^{-3}$],
S – initial sucrose concentration [$g \cdot dm^{-3}$].

Chemical selectivity (*HF*) of oxalic acid biosynthesis was calculated as:

$$HF = \frac{P}{P + CA + GA} \cdot 100 \ [\%]$$

where: P – oxalic acid concentration [$g \cdot dm^{-3}$],
CA – citric acid concentration [$g \cdot dm^{-3}$],
GA – gluconic acid concentration [$g \cdot dm^{-3}$].

The statistical analysis of the results was done using MS Excel 2013 and Statistica 12.0 (StatSoft, Inc. 2014). The one-factor analysis of variance (ANOVA) with a confidence level $p \leq 0.05$ was conducted.

Evaporation was less than 15% and was included in calculations.

RESULTS AND DISCUSSION

The first defined medium for organic acids synthesis by *A. niger* elaborated by Currie [1917] contained sucrose in the concentration of 125–150 $g \cdot dm^{-3}$. Later research with this substrate was conducted in media containing sucrose in the amount of 100 [Strasser et al. 1994, Mandal and Banerjee 2005] or 150 $g \cdot dm^{-3}$ [Cameselle et al. 1998, Foryś and Podgórski 2004, Musiał et al. 2005, Walaszczyk et al. 2015]. In order to examine the influence of initial sucrose concentration on oxalic acid biosynthesis, five levels of its concentration were tested throughout this work: 100, 125, 150, 175 and 200 $g \cdot dm^{-3}$. The amounts of product, accompanying acids and dry mass obtained in media with different initial sucrose concentration are presented in Figure 1.

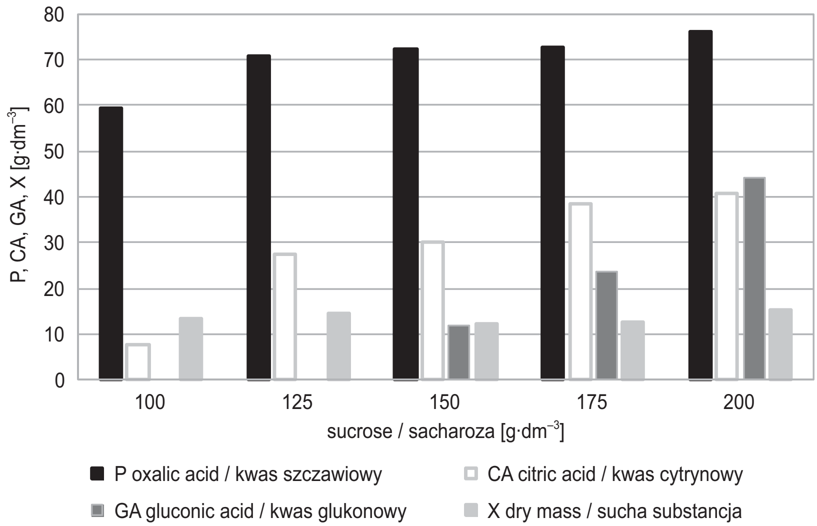

Fig. 1. The maximum concentration of oxalic acid in media with different initial sucrose concentration. The concentration of citric acid, gluconic acid and dry mass are given from the day the maximum oxalic acid concentration was reached

Rys. 1. Najwyższe stężenie kwasu szczawiowego w podłożach z różnym początkowym stężeniem sacharozy. Stężenie kwasu cytrynowego, kwasu glukonowego i zawartość suchej substancji podano z dnia, w którym osiągnięto najwyższe stężenie kwasu szczawiowego

The one-factor analysis of variance of organic acids concentration showed statistical significance of obtained results. In the medium with the lowest initial sucrose concentration (100 $g \cdot dm^{-3}$), the lowest oxalic acid concentration was obtained (59.3 $g \cdot dm^{-3}$) – Figure 1. The increase of substrate amount to 125 $g \cdot dm^{-3}$ caused the increase of product concentration to 70.8 g·dm^{-3}. Further increase of initial sugar concentration in the medium was not so effective: when the sucrose concentration was 200 g·dm^{-3}, the amount of oxalic acid reached 76.0 g·dm^{-3}, which was only 5.2 g·dm^{-3} higher than in the medium with the substrate in the concentration of 125 g·dm^{-3}. Such high amounts of oxalic acid were not reported in the available literature. Strasser et al. [1994] obtained 38.4 g·dm^{-3} and Mandal and Banerjee [2005] only 7.63 g·dm^{-3} of oxalic acid from 100 g·dm^{-3} of sucrose. Cameselle et al. [1998], Foryś and Podgórski [2004], Musiał et al. [2005] and Walaszczyk et al. [2015] used medium with sucrose in concentration of 150 g·dm^{-3} and obtained respectively: 33.8, 61.2, 46.0 and 44.4 g·dm^{-3}.

Figure 2 shows the profile of oxalic acid concentration depending on initial sucrose concentration in the medium. The higher the initial concentration of the substrate was, the slower the product amount raised and the longer the time of reaching the maximum of product concentration was: from 16 days in the medium with sucrose in the concentration of 100 g·dm^{-3} to 40 days in the medium with 175 and 200 g·dm^{-3} of the substrate.

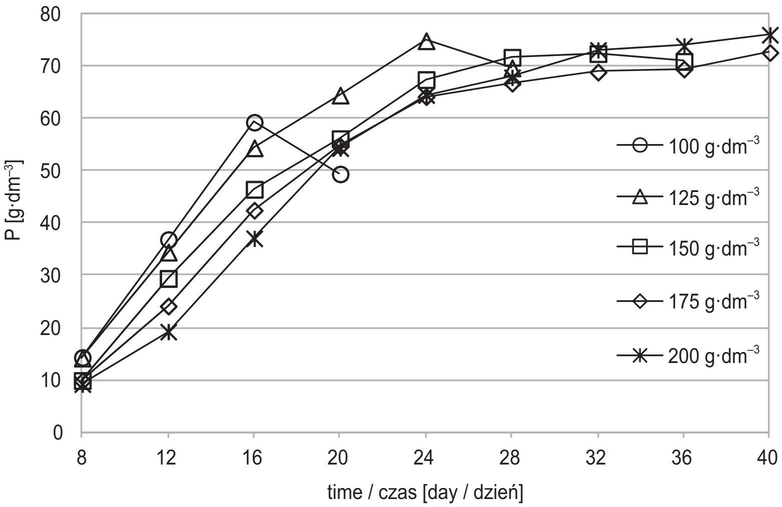

Fig. 2. Profile of oxalic acid concentration depending on initial sucrose concentration in medium

Rys. 2. Kształtowanie się stężenia kwasu szczawiowego w zależności od początkowego stężenia sacharozy w podłożu

In all the experiments, apart from oxalic acid, *A. niger* secreted also citric and gluconic acids, but at the end of the process citric acid was present in all media and gluconic only when the initial sucrose concentration was 150 g·dm^{-3} or higher (Fig. 1). The final citric acid amount grew from 7.9 to 40.7 g·dm^{-3} and gluconic acid from 0.0 to 44.3 g·dm^{-3} with the increase of the initial sucrose concentration from 100 to 200 g·dm^{-3}.

Figure 3 presents changes in citric and gluconic acids concentration depending on the initial sucrose concentration in the medium. Citric acid was synthesised in all medium variants. The lower the initial sugar concentration in the medium was, the quicker citric acid concentration reached the maximum. It is worth noting that the maximum of citric acid concentration in medium with 100, 125 and 150 g·dm^{-3} of sucrose was always earlier than the maximum of oxalic acid concentration (compare Fig. 2 and 3a). Only in the cultures with higher initial substrate amount, 175 and 200 g·dm^{-3}, citric acid concentration grew constantly to the end of the process.

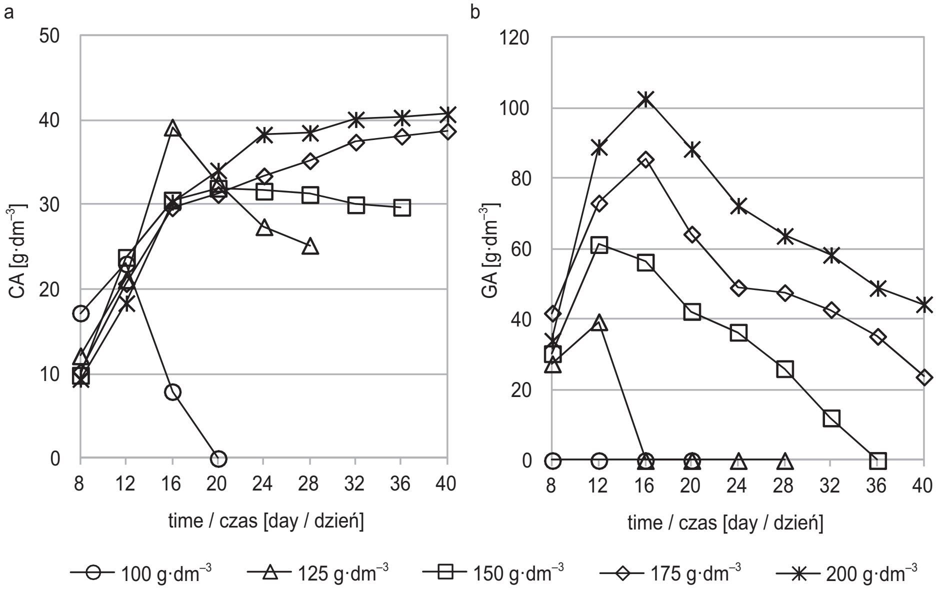

Fig. 3. Profiles of citric (a) and gluconic (b) acids concentration depending on initial sucrose concentration in medium

Rys. 3. Kształtowanie się stężenia kwasu cytrynowego (a) i glukonowego (b) w zależności od początkowego stężenia sacharozy w podłożu

Production of gluconic acid by *A. niger* is a process of oxidation of available glucose into unavailable to other organisms compound that could be used later as a carbon source for oxalic acid synthesis in the case of lack of easier assailable sources [Andersen et al. 2009, Poulsen et al. 2012]. Formation of gluconic acid is indicated as the main disadvantage of usage of sucrose and glucose as carbon sources in bioprocesses with *A. niger* [Strasser et al. 1994, Cameselle et al. 1998, Walaszczyk et al. 2014]. In the experiments presented in this paper gluconic acid was detected in all medium variants beside the one with the lowest initial sugar concentration (Fig. 3b). It was probably because the substrate was completely used to produce oxalic and citric acids to acidify the medium and there was no glucose left to store it as gluconic acid. As it can be seen in Figure 3b, the higher initial sucrose amount in the medium was, the higher maximum concentration of gluconic acid was observed. In all the experiments with gluconic acid present, after reaching its maximum, the concentration of the acid decreased because it was probably reused as a carbon source for oxalic acid production. The same process course and conclusion were noted by other authors [Strasser et al. 1994, Cameselle et al. 1998, Musiał et al. 2005, Poulsen et al. 2012, Walaszczyk et al. 2014]. In most cases described in the literature, the final gluconic acid concentration was higher than oxalic [Strasser et al. 1994, Cameselle et al. 1998, Musiał et al. 2005]. In all experiments reported in this paper the final amount of gluconic acid was always lower than oxalic (Fig. 1), despite the maximum gluconic acid concentration was even 102.5 $g \cdot dm^{-3}$ (Fig. 3b).

Because *A. niger* cultivated in defined media with sucrose as carbon source secretes three organic acids: oxalic, citric and gluconic (Fig. 1), the chemical selectivity of the process is one of the most important parameters of results evaluation. Oxalic acid biosynthesis processes described in literature had mostly a rather low selectivity, in the range between 29–41% [Strasser et al. 1994, Cameselle et al. 1998, Musiał et al. 2005], mostly because of gluconic acid produced in the amount greater than oxalic. Only Walaszczyk et al. [2015] reported chemical selectivity of the used strain at the level of almost 70%. Figure 4 presents the levels of this parameter in the experiments described in this paper. The chemical selectivity of processes was high, between 47.2–88.2%, but it decreased with the increase of the initial sugar concentration. Also productivity and oxalic acid yield lowered from 3,7 $g \cdot dm^{-3} \cdot day^{-1}$ and 59% to 1.9 $g \cdot dm^{-3} \cdot day^{-1}$ and 38% respectively when the substrate amount grew from 100 to 200 $g \cdot dm^{-3}$ (Fig. 4). Values of these two parameters were on a quite good level, considering that Musiał et al. [2005] reported that in their experiments productivity of oxalic acid on sucrose media was high,

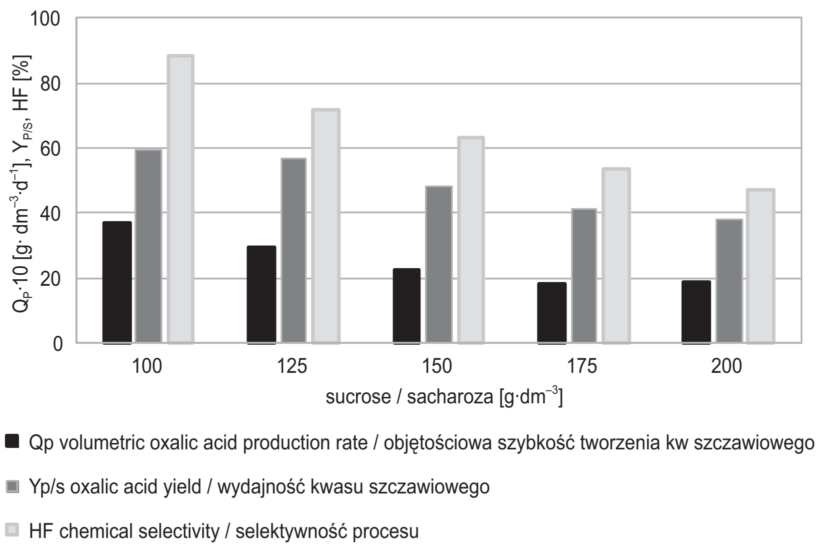

Fig. 4. Volumetric oxalic acid production rate, oxalic acid yield and chemical selectivity of oxalic acid biosynthesis depending on initial sucrose concentration in the medium. The values of the parameters were calculated with data from the day the maximum oxalic acid concentration was reached

Rys. 4. Kształtowanie się objętościowej szybkości produkcji kwasu szczawiowego, wydajności kwasu szczawiowego oraz współczynnika selektywności procesu biosyntezy kwasu szczawiowego w zależności od początkowego stężenia sacharozy w podłożu. Wartości parametrów obliczono na podstawie danych z dnia, w którym osiągnięto najwyższe stężenie kwasu szczawiowego

6.7 $g \cdot dm^{-3} \cdot day^{-1}$, but the oxalic acid yield they obtained was low, only 31%; Cameselle et al. [1998] reached productivity of 4.2 $g \cdot dm^{-3} \cdot day^{-1}$ and yield of 23%; Strasser et al. [1994] noted 3.8 $g \cdot dm^{-3} \cdot day^{-1}$ and 38% and Walaszczyk et al. [2015] 2.7 $g \cdot dm^{-3} \cdot day^{-1}$ and 29% of productivity and yield respectively.

CONCLUSIONS

Sucrose is a suitable carbon source for oxalic acid synthesis by *A. niger*. Investigating the optimal substrate concentration in culture medium it was found that the increase of initial sucrose concentration caused the increase of product concentration, but the biggest rise was between 100 and 125 $g \cdot dm^{-3}$ of the substrate. The amounts of accompanying citric and gluconic acids increased with the grow of the initial sugar concentration in the medium, also the time of the process extended, and volumetric production rate and oxalic acid yield decreased. After analysis of obtained data, the optimal initial sucrose concentration was recognized as 125 $g \cdot dm^{-3}$.

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WPŁYW STĘŻENIA SACHAROZY JAKO ŹRÓDŁA WĘGLA W PROCESIE BIOSYNTEZY KWASU SZCZAWIOWEGO PRZEZ *ASPERGILLUS NIGER*

Streszczenie. Celem badań było określenie wpływu początkowego stężenia sacharozy, będącej jedynym źródłem węgla, na proces biosyntezy kwasu szczawiowego przez *Aspergillus niger* metodą wgłębnej hodowli okresowej. W badaniach stosowano szczep *A. niger* W78C. Hodowle prowadzono w podłożach syntetycznych zawierających sacharozę w stężeniu 100, 125, 150, 175 lub 200 $g \cdot dm^{-3}$. W podłożu o najniższym początkowym stężeniu sacharozy, tj. 100 $g \cdot dm^{-3}$, uzyskano najniższe stężenie kwasu szczawiowego, tj. 59,3 $g \cdot dm^{-3}$. Podwyższenie stężenia substratu do 125 $g \cdot dm^{-3}$ spowodowało zwiększenie ilości produktu do 70,8 $g \cdot dm^{-3}$, czyli o 19,1%. Dalszy wzrost stężenia cukru w podłożu nie był już tak skuteczny, gdyż w wariancie z najwyższym początkowym stężeniem sacharozy, tj. 200 $g \cdot dm^{-3}$, uzyskano tylko o 5,2 $g \cdot dm^{-3}$ (czyli o 7,4%) więcej kwasu szczawiowego niż w podłożu, w którym ilość substratu była równa 125 $g \cdot dm^{-3}$. Wzrost początkowego stężenia substratu ze 100 do 200 $g \cdot dm^{-3}$ powodował wydłużenie czasu procesu, wzrost stężenia kwasów towarzyszących: cytrynowego i glukonowego, oraz spadek wydajności kwasu szczawiowego i szybkości jego tworzenia. Na podstawie uzyskanych stężeń produktu w podłożach z różnym początkowym stężeniem substratu oraz po analizie parametrów procesu (wydajności kwasu szczawiowego i objętościowej szybkości jego produkcji) uznano, że najkorzystniejsze stężenie sacharozy w tym procesie wynosi 125 $g \cdot dm^{-3}$.

Słowa kluczowe: kwas szczawiowy, *Aspergillus niger*, biosynteza, sacharoza, źródło węgla