Influence of Carbon Dioxide and Nitrogen Source on Sustainable Production of Succinic Acid from *Miscanthus* Hydrolysates

Mariusz Kuglarz and Monika Rom

Abstract—**This study presents sustainable succinic acid production from lignocellulosic hydrolysates, using A. succinogenes, which consumes CO2 during succinic fermentation. Optimal combination of carbon source (gaseous and/or solid MgCO$_3$, 15-30 g/dm^3) and nitrogen source (mixtures of yeast extract and hydrolyzed rapeseed cakes: 100:0, 80:20, 60:40, 50:50 and 40:60 % w/w) were experimentally determined. Obtained results stated that simultaneous addition of gaseous CO$_2$ and MgCO$_3$ (20-30 g/dm^3) resulted in the highest sugar conversion (89-95%) and succinic yields (66-74%). Additionally, CH$_4$ content in biogas, used as CO$_2$ source, was increased by 21-22% and reached 91-92% after succinic fermentation with addition of MgCO$_3$ (20 g/dm^3). The course of succinic fermentation confirmed that 50% of nitrogen dosage can be added as rich in amino acids rapeseed cakes, originated from biodiesel production. Succinic fermentation with gaseous CO$_2$ as the only CO$_2$ or after replacing more than 50% of nitrogen dosage as rapeseed cakes, resulted in increased acetic and formic acids production (by-products of succinic fermentation). Optimal conditions of succinic fermentation (CO$_2$ source and dosage, waste nitrogen source) identified in this study can pave the way towards sustainable production of succinic acid from lignocellulosic biomass.**

Index Terms—**Succinic acid, biomass, hydrolysates after pretreatment, nitrogen source, carbon dioxide, biogas.**

I. INTRODUCTION

Green technology is becoming a driving force in chemical industry because of the current need to decrease environmental impacts, caused by petrochemical industry. Succinic acid in particular, can be used as precursor for the production of biodegradable polymers, food, fine chemicals, green solvents and pharmaceuticals [1]. Succinic acid, also known as amber acid or butanedioic acid, has been recognized as one of the top twelve building-blocks chemicals [2]. Currently, this compound is mainly produced petro-chemically through hydrogenation of maleic anhydride, a process which is considered as a relatively expensive and contributes to environmental problems. Alternatively, bio-based production of succinic acid through microbial fermentation is considered as a very attractive, due to the fact that it can contribute to the abatement of CO$_2$ emissions as this gas is consumed during succinic fermentation [3], [4].

Among number of bacterial strains able to generate succinic acid through anaerobic fermentation, *Actinobacillus succinogenes* is considered as one of the most promising ones for industrial applications [5], [6], mainly due to its ability to ferment wide range of carbon sources, i.e. glucose, xylose, arabinose, galactose etc. [5]. However, effective production of succinic acid (building block chemical) from lignocellulosic hydrolysates, is strictly connected with the amount of sugars released during biomass hydrolysis (pre-treatment). According to the current state of knowledge, pretreatments with organic solvents are very effective in lignocellulosic biomass hydrolysis and lead to smaller sugar losses compared to most commonly used acid-based, alkali or oxidative methods [7]-[10]. Hydrolyzed biomass is converted into succinic acid through microbial fermentation (including *Actinobacillus succinogenes*) and carbon dioxide availability and nitrogen source constitute the most important parameters influencing effectiveness of succinic fermentation (sugar conversion rates, succinic yields). In particular, CO$_2$ supply is the crucial factor determining succinic yield, the ratio of succinic acid to other by-products as well as the degree of sugar utilization. Besides carbon source, nitrogen source and its dosage have a direct impact on the economy of succinic fermentation. In most cases, yeast extract, being rich in amino acids, vitamins and trace elements is considered as favorable nitrogen source in fermentation processes. However, yeast extract is treated as one of the most expensive medium component and sustainable succinic acid production should eliminate or decrease its usage, by application of waste products, rich in amino acids.

This study presents sustainable succinic acid production from hydrolysates after glycerol-based pretreatment, using *A. succinogenes* 130Z (ATCC 55618). The aim of this study was to analyze the influence of carbon dioxide and nitrogen source on the effectiveness of succinic fermentation. Optimal combination of carbon source (gaseous and solid MgCO$_3$) and nitrogen source (mixtures of yeast extract and rapeseed cake) were experimentally determined. According to best of our knowledge, this is the first study evaluating the usage of biogas after co-digestion processes (CO$_2$ source) and rapeseed cakes (nitrogen source) for the production of succinic acid from real hydrolysates, obtained from *lignocellulosic* biomass.

II. MATERIALS AND METHOD

A. Feedstock

Hydrolysates (35.6 $\pm$ 0.7 g-glucose/dm^3 and 8.25 $\pm$ 0.3

g-xylose/dm^3) obtained after biomass pretreatment with glycerol-based method at optimized conditions (80% w/w of waste glycerol after biodiesel production + 1.25% w/v H_2SO_4 as a catalyst) and subsequent hydrolysis with the most effective enzymatic mixture (10% w/w Cellic® CTec2, 5% w/w *β-Glucanase* and 1% w/w Cellic® HTec2) were used as feedstock for succinic acid production. Pretreatment conditions and dosage of enzymatic cocktails were established during our previous studies [11].

B. Procedure of Succinic Fermentation

Before fermentation, hydrolysates were autoclaved at 121 ℃ for 20 minutes as well as mixed with experimental medium (nutrients) at 75:25 (v/v), which was selected as the most optimal ratio, based on our previous studies [12]. Succinic fermentation was conducted in triplicates, using identical 3 dm^3 fermenters (Sartorius BIOSTAT Aplus, Germany) with an initial working volume of 1.0 L, operated at 37 ℃ and 150 rpm for 48 h. In each case, about 5% (v/v) of exponentially growing inoculum was added. The strain of *A. succinogenes* 130Z (DSM 22257) was obtained from DSMZ and was stored in glycerol at - 80 ℃ until used. Seed culture medium was composed of (g/dm^3): glucose (10.0), yeast extract (5.0 or 10.0), $NaHCO_3$ (10.0), $NaH_2PO_4·2 H_2O$ (9.6), $K_2HPO_4·3 H_2O$ (20.3). During start-up of the process, N_2 gas was used to create anaerobic conditions in fermenters. Prior to start of batch fermentation, pH was adjusted to 6.8 with 50% H_3PO_4 and 0.05 mL of sterile Antifoam 204 was added. In case of pH decrease below 6.8 during fermentation due to acid production and insufficient carbonate–bicarbonate buffering capacity, NaOH solution (8 M) was added.

Two sets of succinic fermentation were conducted. Firstly, the influence of carbon dioxide source and dosage were taken into account. Solid $MgCO_3$ (15-30 g/dm^3) and biogas (containing 75% of CH_4 and 25% of CO_2) were used as carbon dioxide. The biogas originated from our previous sewage sludge and kitchen biowaste co-digestion experiments [13]. This part of experiment was conducted using 5:1 gas-liquid ratio and atmospheric pressure (101.3 kPa). In order to increased CO_2 solubility, the process was conducted at increased pressure, i.e. (140 kPa). For monitoring the pressure inside fermenters, a pressure gauge was mounted with a relief valve. The biogas was recirculated during fermentation and changes in CH_4 and CO_2 concentrations were recorded. Considering the fact that gaseous CO_2 exhibits a relatively poor solubility, biogas (containing CO_2) was supplemented with solid $MgCO_3$ (15-30 g/dm^3).

During the second set of succinic acid production, the impact of nitrogen source (yeast extract and/or rapeseed cakes after biodiesel production) was taken into account. The rapeseed cake dry matter (d.m.) content amounted to 950±20 g/kg and the main components of this substrate included: total protein 340±25 g/kg, cellulose 148±10 g/kg, hemicellulose 54.0±5.0 g/kg, insoluble lignin 150±8.5 g/kg, ash 48.2±2.5 g/kg. Total amino acid content in cakes before hydrothermal processing amounted to 310±10 g/kg. The hydrothermolysis of rapeseed cake was performed in a batch reactor (Minotavr-1), at 210-220 ℃, which is in consistent with previous studies on effective amino acids conversion from rapeseed cakes [14]. The fermentation was conducted using the following ratios of yeast extract and rapeseed cake (% w/w): 100:0, 80:20, 60:40, 50:50 and 40:60. Nitrogen dosage (yeast extract and/or rapeseed cakes) amounted to 5 and 10 g/dm^3, which is in the range commonly used for succinic fermentation, using *A. succinogenes* [3], [11]-[13].

One milliliter samples were taken periodically (after 0, 3, 6, 12, 15, 18, 24, 30, 36 and 48 h) and used for analysis of sugars (glucose, xylose), and acids (succinic-, acetic- and formic). Succinic acid yield (Y_{SA}) was calculated as the amount of succinic acid (g/dm^3) obtained per 1 g/dm^3 of sugars (glucose + xylose) consumed. Sugar utilization was calculated as the difference between initial sugar content (g/dm^3) and sugar content after succinic acid production (g/dm^3).

C. Analytical Methods

Concentrations of sugars and organic acids (succinic, acetic, formic) were measured by using high performance liquid chromatography HPLC (Agilent 1260 Infinity, Germany) equipped with a Bio-Rad Aminex HPX-87H column at 63 ℃ and ultraviolet (UV) and refractive index (RI) detector (67162A, Germany), using 4mM H_2SO_4 as eluent at 0.6 mL/min flow rate. The pH was measured using a standard pH meter. All chemicals used in this study were of analytical grade. Dissolved carbon dioxide content in fermentation broths was determined using an InPro 5000i sensor coupled with Mettler Toledo (M400).

All results are presented as average values were compared statistically. One-way ANOVA test followed by HSD tests were used for multiple comparisons between samples, with the level of significant set at 0.05. The same letters represent data equivalent statistically ($p > 0.05$).

III. Results and Discussion

A. Succinic Fermentation Using Different Carbon Sources

Carbon dioxide constitutes one of the major substrates used for biosynthesis of succinic acid, while, CO_2 source and dosage influence the metabolic flux as well as effectiveness of succinic acid production [15]. Firstly, the succinic acid fermentation was conducted, using gaseous CO_2 (biogas containing 75% CH_4 and 25% CH_4). The process conducted at atmospheric pressure (101.3 kPa, CO_2 partial pressure 40 kPa) allowed to utilize about 55% of available sugars (glucose and xylose) and resulted in a relatively low succinic yield - 54% (0.54 g-succinic acid/g-sugar consumed (Table I, Figure 1). Process conducted at increased pressure, i.e. 140 kPa (CO_2 partial pressure of 56 kPa) and thus at higher CO_2 solubility [6], had no significant impact on sugar utilization, whereas, succinic yield was increased by 7% (Table I). These succinic yields are slightly lower than yields previously reported, using model biogas mixture (60% CH_4 and 40% CO_2). However, the current study was based on biogas containing significantly lower CO_2 content (25% vol.) compared to previous studies [6].

In many previous studies on bio-succinic acid production, salts ($MgCO_3$, $NaHCO_3$ or $CaCO_3$) are used as carbon source. In particular, $MgCO_3$ was identified as the most effective CO_2 supplier and pH control agent [4], [16].

In these cases, the highest succinic titers (22-23 g/dm^3) and yields (74-76%) were obtained after addition of 25-30 g/dm^3

$MgCO_3$ as carbon dioxide source (data equivalent statistically, data statistically equivalent, $p > 0.05$) (Table I). It should also be highlighted that the degree of sugar conversion and succinic yields were improved by 67-70% and 37-40%, respectively compared to the process with biogas as the CO_2 source (Fig. 1). Significantly lower succinic titers and yields were obtained for $MgCO_3$ dosage of 15-20 g/dm^3, which proved that availability of CO_2 plays a crucial role during succinic acid biosynthesis and promotes the carbon flow towards SA production branch of TCA (tricarboxylic acid) cycle [3], [4].

TABLE I: Influence of Carbon Dioxide Source on Succinic Acid Production

CO_2 source	Sugar util. (%)	Succinic yield (%)	*Byproducts (%)*
Biogas[a] (101.3 kPa)	54.7±3.5c	54.4±2.5de	34.5±1.4a
Biogas (140 kPa)	55.7±2.0c	58.1±2.1cd	36.6±2.1a
$MgCO_3$ 15 g/dm^3	74.2±2.6b	48.6±4.7e	27.5±1.4b
$MgCO_3$ 20 g/dm^3	80.2±4.6b	63.1±2.2cd	28.5±1.5bc
$MgCO_3$ 25 g/dm^3	91.4±0.9a	74.3±2.4ab	24.7±1.2d
$MgCO_3$ 30 g/dm^3	93.2±0.8a	76.1±2.5a	24.2±1.2d
CO_2[a]+15 g/dm^3 $MgCO_3$	79.3±3.8b	55.1±3.6de	28.9±1.3b
CO_2[a]+20 g/dm^3 $MgCO_3$	89.3±1.5a	65.7±3.0bc	25.8±1.4cd
CO_2[a]+25 g/dm^3 $MgCO_3$	94.6±1.3a	72.8±4.3ab	25.9±1.3cd
CO_2[a]+30 g/dm^3 $MgCO_3$	94.2±1.9a	73.7±3.9ab	27.0±1.1cd

(a - fermentation with biogas as carbon dioxide source, conducted at pressure of 101.3 kPa, average values $n = 3$, ±standard deviation, the same letters represent data equivalent statistically $p > 0.05$)
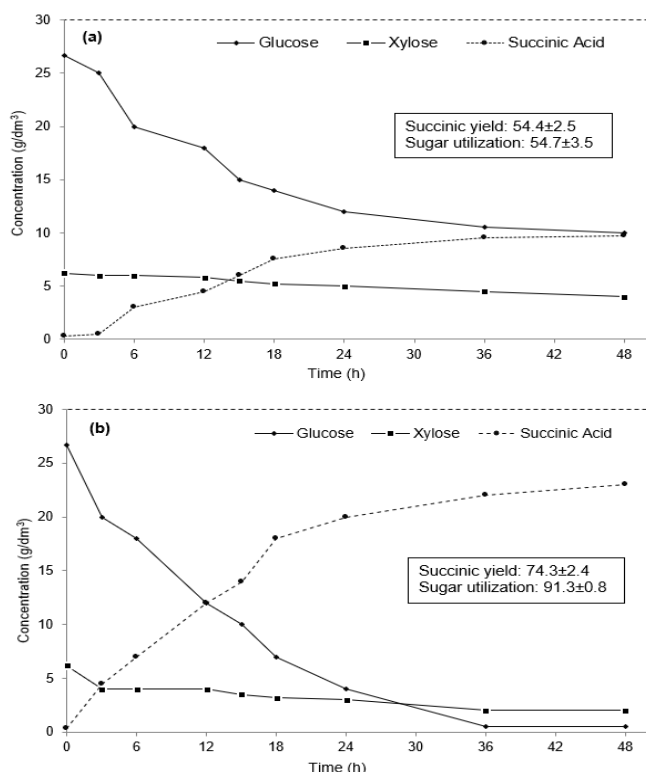

Fig. 1. Course of succinic acid fermentation (a- biogas used as carbon source, 101.3 kPa, yeast extract used as nitrogen source: 10 g/dm^3; b - simultaneous usage of biogas, 101.3 kPa and solid $MgCO_3$: 25g/dm^3, yeast extract used as nitrogen source at total dosage10 g/dm^3).

During the second part of the experiment, simultaneous effect of gaseous CO_2 and solid $MgCO_3$ on the performance of succinic fermentation, was taken into account. When $MgCO_3$ was added with the supply of gaseous CO_2; the following compounds: CO_2, HCO_3^- and CO_3^{2-} would become in the equilibrium in the fermentation broth [15]. The highest succinic titer and yield amounted to 22-23 g/dm^3 and 73-74%, respectively and these values were obtained for $MgCO_3$ dosage of 25-30 g/dm^3 (300-400 mM CO_2) (data equivalent statistically, data statistically equivalent, $p > 0.05$) (Table I). Slightly lower succinic yields were recorded for $MgCO_3$ dosage of 20 g/dm^3 (Table I). In these conditions, the dosage of carbon sources significantly exceeded the maximum CO_2 solubility (139 mM) [15]. This proves that high succinic yields are obtained in conditions of excess CO_2 content. This is in accordance with previous reports stating that the highest succinic yields (69-71%, 0.69-0.71 g/g) were obtained for carbonate dosage of 300-500 mM [17]. Obtained succinic yields are within the values most commonly reported for lignocellulosic hydrolysates [2], [12]. It should be highlighted that maximal theoretical molar yield of succinic acid per mole of glucose corresponds to 1.71 (12/7) (Eq. (1)) and this equation equals to 1.12 g/g when expressed as a mass unit. This theoretical maximal yield would be achieved anaerobically and represents a theoretical situation where the microorganisms do not use substrate for growth, maintenance or an external electron acceptor.

$$C_6H_{12}O_6 + \frac{6}{7}CO_2 \rightarrow \frac{12}{7}C_4H_6O_4 + \frac{6}{7}H_2O \qquad (1)$$
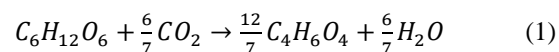
Based on our results, it can be stated that the availability of CO_2 is crucial for the bio-succinic acid production, and *A. succinogenes* consumes CO_2 during fermentation. The stoichiometric reaction cited above suggests that 1 kg of succinic acid will utilize 0.37 kg of CO_2. Therefore, it is evident that bio-succinic acid production could contribute to the abatement of CO_2 emissions. An additional purpose of using CO_2 for biosynthesis of succinic acid was to purify the biogas used as CO_2 source. CH_4 content in biogas after succinic fermentation with gaseous CO_2 source increased by 10-11% and reached the level of 82-84% ±1.5%. There was no a significant impact of increased CO_2 pressure on the final CH_4 content in biogas (88% ±1.5%) (Table II). An increase of CH_4 content from 85% to 95% was reported in previous studies [6]. However, as previously mentioned, these authors used biogas containing significantly higher CO_2 content (40% vol.) compared to present study (25% vol.). As regards the simultaneous usage of gaseous CO_2 and $MgCO_3$ (dosage of 25-30 g/dm^3), it was observed that CH_4 content after succinic fermentation, was not significantly increased (76-77% vol.). Such conditions reflected high CO_2 content, originated from carbonate. Whilst, the CH_4 content in biogas increased by 21-23% with a lower dosage of $MgCO_3$ (15-20 g/dm^3) (Table II). In these cases, the final CH_4 content in biogas treated is in consistence with values possible to obtain via commercially available purification methods, e.g. biogas treatment with water scrubbing or using chemical scrubbers with amine solutions [18].

All in all, these results proves the positive impact of simultaneous addition of gaseous CO_2 and solid $MgCO_3$ on the course of succinic fermentation, both in regards of succinic yield and biogas purification (Table II). What is more, fermentation with simultaneous CO_2 source (biogas/MaCO3) resulted in almost 90% sugar utilization, with complete glucose consumption and about 50%

bioconversion of xylose into carboxylic acids. Whereas, the process with biogas as the only CO_2 source resulted in significant residual glucose and almost 70% of initial xylose present in the solution (Fig. 1). This proves that *Actinobacillus succinogenes* prefers glucose as substrate rather than C_5 sugars (e.g. xylose) [11], [12].

TABLE II: INFLUENCE OF SUCCINIC FERMENTATION ON BIOGAS COMPOSITION

CO_2 source	CH_4 (% vol.)	CO_2 (% vol.)
Biogas (101.3 kPa)	76.3±2.0	21.8±1.8
Biogas (140 kPa)	82.1±2.0	15.4±1.8
Biogas (140 kPa) + 15 g/dm³ $MgCO_3$	91.8±2.2	5.17±1.2
Biogas (140 kPa) + 20 g/dm³ $MgCO_3$	91.3±2.0	5.97±1.3
Biogas (140 kPa) + 25 g/dm³ $MgCO_3$	78.8±2.5	17.1±2.0
Biogas (140 kPa) + 30 g/dm³ $MgCO_3$	77.5±2.1	17.3±2.0

Depending on succinic fermentation conditions, including type of CO_2 source and its dosage, other metabolites, such as acetic, formic, lactic acid or ethanol, can be produced in different amounts [19]. In the present study, acetic- and formic acid were produced as the main fermentation by-products. In the initial growth phase, succinic, acetic and formic acids were simultaneously produced. It was noticed that during succinic fermentation with biogas as the only CO_2 source; by-products (acetic and formic acids) content significantly exceeded 30% of total fermentation products (succinic, acetic and formic acid) (Table I).

As compared to the succinic acid, which is formed via C_4 pathway, i.e. carboxylation of phosphoenolpyruvate and NADH (nicotinamide adenine dinucleotide) consumption within the reductive branch of TCA cycle), - acetic and formic acids are formed via C_3 pathway [20]. By-products concentration (acetic and formic acids) during fermentation with $MgCO_3$ or $MgCO_3$+biogas was stable and did not exceed 30% of total fermentation products (succinic, acetic and formic acid, Table I). This showed that applied CO_2 concentration was enough to increase C_4 flux towards higher succinic acid production.

B. Succinic Fermentation Using Different Nitrogen Sources

Fermentation with yeast extract as a nitrogen source started without any lag phase and higher values of sugar utilization and succinic titers were obtained after application of higher yeast extract dosage (i.e. 10 g/dm³) compared to dosage of 5 g/dm³ (Table III). This shows that yeast extract is a favorable component of medium, used for bio-succinic synthesis. However, yeast extract is considered as one of the most expensive medium component (200 USD/kg of extract suitable for microbial growth) [21]. Based on the results obtained, it was concluded that fermentation assays containing mixtures of yeast extract/rapeseed cakes, in which at least 50 % of yeast extract was present, also started without any lag phase. In such cases, sugar utilization and succinic yields amounted to 84-87% and 67-68% (Table III) (data statistically equivalent, $p > 0.05$). This is in accordance with previous studies stating that rapeseed meal (e.g. pretreated by dilute acid method and hydrolysed with pectinase) can be used as carbon and nitrogen source, for production of succinic acid by *A. succinogenes* [22]. Significantly higher

residual sugar contents and lower succinic yields were obtained after application of more than 50% of rapeseed cakes as nitrogen dosage (Table III), which proves that it is difficult to completely replace yeast extract in the original fermentation medium, by rapeseed cakes as nitrogen sources. Yeast extract, being rich in amino acids, vitamins and trace elements is considered as favorable nitrogen source in fermentation processes.

TABLE III: INFLUENCE OF NITROGEN SOURCE ON SUCCINIC ACID PRODUCTION

YE:RK (% w/w)	Sugar util. (%)	Succinic acid (g/dm³)	Succinic yield (%)	Byproducts (%)
100:0[a]	81.7±3.0ab	14.6±1.0b	54.5±1.8b	32.6±1.8b
100:0[b]	89.3±1.5a	19.2±1.4a	65.2±3.2a	27.1±2.0b
80:20[b]	86.8±1.7a	19.3±0.6a	67.7±1.7a	28.8±1.7b
60:40[b]	86.8±1.8a	19.2±1.2a	67.1±3.4a	29.0±1.8b
50:50[b]	83.8±1.7a	18.8±1.0a	68.3±2.9a	30.4±1.6b
40:60[b]	74.6±3.5b	15.4±1.0b	61.9±3.2ab	39.1±1.7a

(a - total nitrogen source of 5 g/dm³, b- total nitrogen source of 10 g/dm³, YE – yeast extract, RK - rapeseed cakes, average values n=3, ±standard deviation, the same letters represent data equivalent statistically p > 0.05)
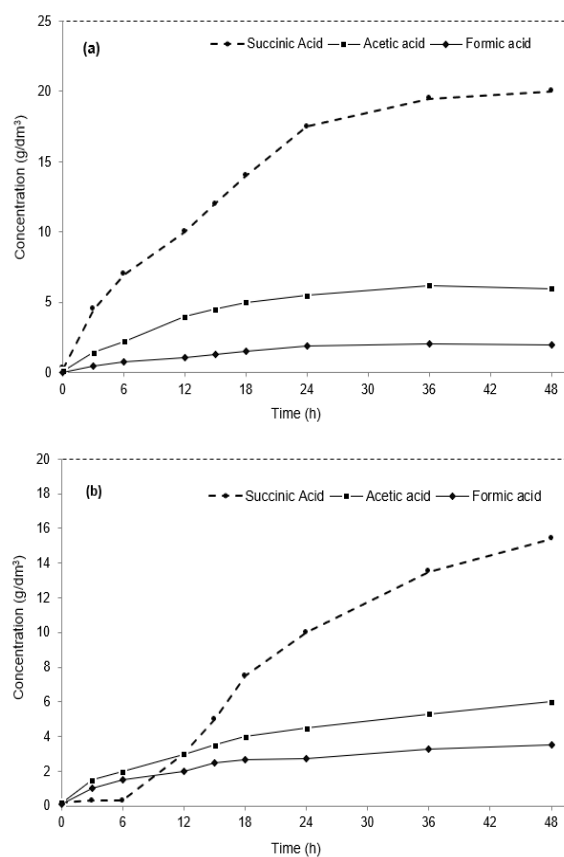

Fig. 2. Influence of nitrogen source on the course of succinic fermentation (a - fermentation with ratio of yeast extract to rapeseed cakes 50:50, b - fermentation with ratio of yeast extract to rapeseed cakes 40:60; nitrogen dosage in both analyzed processes: 10 g/dm³).

Succinic fermentation with addition of more than 50% of rapeseed cakes resulted in increased production of acetic and formic acids (by-products of succinic fermentation) (Fig. 2). What is more, succinic production started with a 6 h lag phase after addition of 60% nitrogen dosage as rapeseed cakes (Fig. 2). During the first hours of fermentation, by-products (acetic and formic acids) significantly exceeded succinic production. Such disturbances in succinic fermentation were not

observed after addition of $\leq 50\%$ nitrogen dosage as rapeseed cakes. After 18-24 h of the process, the production of acetic and formic acids ceased, which is connected with low cell-biomass generation during stationary phase and consequently lower energy requirements [3]. In this case, carbon flux shifts towards succinic acid production (Fig. 2). It should be mentioned that increased by-products concentration in fermentation broths influences negatively the final product (succinic acid) separation. It is estimated that downstream processing of succinic acid can make more than 50-60% of total costs and is attributed to recovery and refining [6]. Acetic and formic acids can be effectively removed via vacuum distillation as their boiling points are significantly lower than boiling point of succinic acids [23].

Based on the results presented, it can be stated that rapeseed cakes can be used as component of fermentation medium and partially ($\leq 50\%$) replace yeast extract. It is in accordance with other reports stating that alternative nitrogen sources are not able to completely eliminate yeast extract during succinic fermentation, e.g. by addition of corn steep liquor (CLS). In this study, it was found that this substrate could not completely replace enriched yeast extract without adding additional ingredients in order to meet physiological demands of *A. succinogenes* [24].

IV. Conclusion

The results obtained in this study clearly confirmed that hydrolisates obtained from *Miscanthus×giganteus* after glycerol pretreatment can be considered as a promising feedstock for succinic acid production. The study focused on two the most important process parameters, which influence succinic yields and sugar conversion rates: carbon dioxide availability and nitrogen source.

1) These results proved the positive impact of simultaneous addition of gaseous CO_2 (biogas) and solid $MgCO_3$ on the course of succinic fermentation and succinic yield.
2) Costly yeast extract used during succinic fermentation can be successfully replaced ($\leq 50\%$ of its dosage) by rapeseed cakes, i.e. a residual material from pressing of oil from rapeseed.

However, the complex composition of oil cakes requires further research to confirm which component (of organic nitrogen) has the most significant impact on the effectiveness of succinic acid and other metabolites production. Further research should also include an effective concept of succinic broths purification and downstream processes, which would allow to obtain succinic acid ($> 98\%$) suitable for further chemical transformations.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M. Kuglarz conducted the research on succinic fermentation using different carbon and nitrogen sources as well as analyzed hydrolysates and final products in succinic broths, using HPLC method. M. Kuglarz also prepared the manuscript for publication; M. Rom prepared hydrolysates for succinic fermentation, participated in hydrolysates analyses (carbohydrate, final products) and developed statistical evaluation of obtained results. All authors had approved the final version.

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M. Kuglarz is Ph.D. in technical sciences, discipline: environmental engineering, Silesian University of Technology, Faculty of Energy and Environmental Engineering, Poland, degree given by the Resolution of the Faculty Council, 24 February 2012. He worked as quest post-doc during didactic and scientific internship at Technical University of Denmark, DTU Environmental Engineering, April to October 2012. He participated in an internship and training program "TOP 500 Innovators Science-Management-Commercialization Program", Haas School of Business, University of California, Berkeley, USA, 9 weeks (May-July 2013). Later, he took part in research exchange at Technical University of Denmark, DTU Environmental Engineering, 5 visits (2015-2018), at the invitation of a foreign institution. He is now employed at University of Bielsko-Biala, Faculty of Materials, Civil and Environmental Engineering, Institute of Environmental Protection and Engineering, Poland, current position, assistant professor and senior researcher. His scientific and research activity includes more than 60 scientific publications, including 15 publications in journals included in the database of Journal Citation Reports (JCR); total impact factor (IF): 40,825 (all obtained after Ph.D degree). He participated in 24 scientific conferences (international and national), delivered 7 oral speeches and presented 17 posters. In 2018, he was awarded for the best presentation during a conference, organized by Elsevier (BIORESTEC 2018 Best Poster Awards). His involvement in professional activities was honored with 4 Rector's Awards (2010-2014, 2016, 2017, 2018), including the category "for obtaining the highest value of IF index (impact factor)", in the University scale.

Monika Rom is researcher and laboratory specialist. She has more than 10 years of experience. She is also a trainer in prestigious program Top Minds, an initiative of Association Top 500 Innovators and the Polish-American Fulbright Commission. She has been working on different aspects of environmental engineering, including feedstock compositional analyses. She is the member of TOP 500 Association of Innovators, participated in several post-doctoral programs, including: 9 week Science-Management-Innovation program at University of California, Berkeley (edition 2012).