Desalination and Water Treatment
www.deswater.com

doi:10.5004/dwt.2017.20497

73 (2017) 155–163
April

Utilization of membrane processes for separation of succinic acid after fermentation of *Miscanthus* biomass

Mariusz Kuglarz[a,*], Klaudiusz Grübel[a], Jolanta Bohdziewicz[b]

[a]*Faculty of Materials, Civil and Environmental Engineering, Institute of Environmental Protection and Engineering, University of Bielsko-Biala, Willowa 2, 43-309 Bielsko-Biala, Poland, Tel. +48 338279181, Fax +48 338279101, email: mkuglarz@ath.bielsko.pl (M. Kuglarz)*
[2]*Faculty of Energy and Environmental Engineering, Institute of Water and Wastewater Engineering, Silesian University of Technology, Konarskiego 18, 44-100 Gliwice, Poland*

Received 30 June 2016; Accepted 9 January 2017

ABSTRACT

The study presents succinic acid production from biomass of *Miscanthus*, integrated with downstream processing of fermentation broth. The pre-treatment procedure, used to loosen the lignocellulosic structure, was based on temperature (140 and 180°C) and sulfuric acid addition (0.0, 0.5, 0.75, 1.0, 1.5 and 2.0% w/v). The pretreatment after addition of 1.5% H_2SO_4 at 140°C ensured insignificant cellulose losses, the highest hemicellulose solubilization (60%) as well as effectively facilitated enzymatic saccharification (glucose yield: 80%). Succinic acid yield amounted to 73%, resulting in following mixture of carboxylic acids (% vol. of total acids): 64% succinic, 21% acetic and 15% formic acid. In the present study, an effective method of succinic acid (the target product) separation, using membrane processes (100–300 Da), was presented. Filtration using DK-NF membrane conducted at pH 6.8, followed by diafiltration, was selected as the most appropriate for the treatment of analysed fermentation broth. During diafiltration of retentate after NF treatment, the succinic acid content reached 95% of total acids present in the treated mixture. Whilst, the recovered permeate, containing mostly water, can be recycled and used again for biomass dilution in the pre-treatment step.

Keywords: Membrane separation; Carboxylic acids; Succinic acid; Commercial membranes (100-300 Da); Fermentation broth; Biomass pretreatment; Diafiltration; Water reuse

---

1. Introduction

Production of succinic acid, one of the top 12 building-block chemicals, through microbial fermentation has recently received worldwide attention. However, the key problems connected with biological succinic acid production are: cheap raw material selection and effective separation of target product from fermentation broth. As regards the cheap feedstock, lignocellulosic materials are the most abundant land-based resources on the planet. *Miscanthus*, perennial C4 crop, and specifically the species of *Miscanthus giganteus*, is of particular interest, because it combines high biomass production and sugar content with a low environmental impact. Similarly to other lignocellulosic biomasses, *Miscanthus* contains a tight structure (lignocellulosic complex), which makes it difficult to be used directly as feedstock for biofuels/biochemicals production. Therefore, the biomass pretreatment is necessary and the most commonly method used for lignocellulosic biomass is steam treatment, facilitated by addition of acids (most commonly H_2SO_4). During the acid-based pretreatment, the hemicellulose is hydrolyzed into its monomers, mainly xylose as well as arabinose, galactose. However, this method may produce some inhibitors (e.g. furfural, HMF - hydroxymethylofurfural)

---

*Corresponding author.

Presented at the EDS conference on Desalination for the Environment: Clean Water and Energy, Rome, Italy, 22–26 May 2016.

1944-3994 / 1944-3986 © 2017 Desalination Publications. All rights reserved.

and is not effective in biomass delignification. Such compounds present in the hydrolysate can negatively impact on fermentation processes as well as broth purification. The biomass pretreatment should avoid or at least minimize generation of undesirable products, connected with carbohydrates and lignin degradation [1]. *Miscanthus giganteus* is a limitedly examined feedstock for biofuels/biochemical production compared to other lignocellulosic biomass (e.g. wheat straw, rapeseed straw, willow). Previous reports confirm the positive influence of one-step dilute acid pretreatment on *Miscanthus giganteus*, i.e. after 0.73% of sulfuric acid at 150°C for 6.1 min [2], or 1–4% v/v of sulfuric acid at 121°C for 20 min [3]. The available literature also mentions an effective two-steps pretreatment, including dilute acid presoaking (0.75% sulfuric acid at 100°C for 14 h), followed by wet explosion at 170°C for 5 min [4], or 2% NaOH at 50°C for 2 h, followed by 1% H_2SO_4 treatment [5]. However, two-steps processes are usually connected with higher operating costs. The above mentioned studies describe only a limited range of dilute-acid pretreatment conditions (sulfuric concentration/temperature applied) and according to our knowledge, steam/acid based pretreatment before biological succinic acid production, from *Miscanthus giganteus*, has never been analyzed.

The second important issue strictly connected with developing bio-succinic acid production is an effective separation of succinic acid from fermentation broth, as downstream processing of succinic acid can make more than 50–60% of total costs and is attributed to recovery and refining [6]. Succinogenes produce not only succinic acid, but also other by-products such as: formate, acetate and lactate etc., which is connected with low succinate purity in the fermentation broth. The initial step of broth separation is the removal of: cells, colour, as well as protein residues, which is mostly achieved through centrifugation. The main separation step is the recovery of carboxylic acids from fermentative broth. The traditional separation methods include the precipitation of the insoluble calcium salts of carboxylic acids. $Ca(OH)_2$ is usually added to neutralize the fermentation broth and precipitate succinate. Calcium succinate is recovered by filtration, and free acid is released from the precipitates by adding sulfuric acid. The disadvantages of this process are connected with generation of large amounts of solid and slurry wastes [7]. Besides this conventional method, several other separation processes have been proposed to separate carboxylic acids, including succinic acid. For example, high molecular weight amines are known to be effective extracting agents for organic acids. However, total recyclability of the organic phase and downstream processing costs should also be taken into account. Adsorption process is connected with short lifetime of adsorbents, low capacity, and additional filtration. Whilst, direct distillation is an energy-intensive process [8]. The last step is the final purification of succinic acid, which can be realized through crystallization, facilitated by previous vacuum evaporation (if necessary; depending on previous steps of separation), i.e. the technology which is well-developed, effective and currently applied in the industry [9]. For further development of separation techniques, new technologies or more effective combinations of existing methods need to be developed. During the last few years, ultrafiltration (UF) was successfully applied for clarifying fermentation broths after succinic fermentation. This process allowed to remove almost all microorganisms cells (>99%), majority of the proteins (>80%) as well as resulted in a clearer solution compared to centrifugation [10]. Recent findings on fouling during ultrafiltration should help to apply this technology at an industrial scale in the near future [11]. Currently, pressure-driven membrane processes such as nanofiltration (NF), and reverse osmosis (RO) have gained much attention. RO membranes have a dense, ultrathin skin layer on the polymeric supports. NF membranes have looser structures than RO membranes and usually have more charged groups on the membrane surface. NF and RO have a wide range of applications as regards fermentation broth purification and separation [12]. Nanofiltration, operated in a diafiltration mode, has recently been successfully applied in an integrated membrane-assisted process—for the treatment carob pod-based model and simulated broth [13]. However, further research is necessary in order to prove the application of membrane filtration as the key step in succinic acid separation systems. Especially, in case of real fermentation broths, originated from wide range of biomass types. According to our knowledge, the usage of membrane processes (NF/RO) for the separation of succinic acid produced from *Miscanthus* hydrolysates after steam/dilute sulfuric acid pretreatment, has never been analysed.

The study presents succinic acid production from biomass of *Miscanthus*, integrated with succinic acid recovery from fermentation broth. Effective method to separate the target product from fermentation broth, using membrane filtration (NF and RO), was presented. Organic acids can be effectively retained or pass through NF or RO membranes by adjusting the pH of feed stream. Therefore, the purpose of this study was to analyze the retention of organic acids and their salts (succinate, acetate, formate) by NF or RO membranes. Additionally, the influence of biomass processing, prior to downstream processing, on the membrane fouling was analyzed. Retentate after the filtration in conditions considered as the most appropriate was further washed with water, in a diafiltration mode. This step of treatment was expected to enrich the solution in succinic acid and effectively remove residual by-products (acetic- and formic acid).

2. Materials and methods

2.1. Feedstock

Biomass of *Miscanthus* (*Miscanthus giganteus*), used in this study, came from a farm located in the north of Poland (Pomeranian province). The biomass was planted on plowed soil. Seedlings from domestic plantations were put to a depth of 3–5 cm. Dry biomass was chopped using a shredder (4–5 cm length) and ground to particles of 4–5 mm; using a cutting mill. The dry matter (DM) content was 93–94% (w/w). The biomass contained: cellulose (44.1 ± 2.2%), hemicellulose (24.9 ± 1.5%), including 20.8 ± 1.05% of xylan as well as insoluble lignin (24.8 ± 1.5%) and 2.65 ± 0.1% of ash.

2.2. Biomass hydrolysis

The pre-treatment procedure was based on temperature (140 and 180°C) and sulfuric acid addition (0.0, 0.5, 0.75, 1.0, 1.5 and 2.0% w/v); conducted at solid content of 10% (w/w) feedstock/water. After H_2SO_4 addition, mixtures were steam treated in a batch reactor at 140°C for 20 min or at 180°C for 10 min. The slurry (after pretreatment) was separated into solid fraction (water insoluble fraction, WIS) and liquid fraction. Solids separated were dried at 55°C for 24 h and used as feedstock for enzymatic hydrolysis. Enzymatic process was conducted at a solid loading of 5% (w/v) in a 50 mM sodium citrate buffer, for 48 h, at 50°C; pH 4.8. The following enzymes loadings were applied: Celluclast 1.5 L (Celluclast, 30 FPU/g glucan) derived from *Trichoderma ressei* and Novozyme 188 (Novozyme, 20 IU/g glucan) from *Aspergillus niger*.

2.3. Succinic fermentation

Succinic acid was produced in a batch mode, using 200 mL sealed anaerobic bottles; 100 mL working volume. The ratio of hydrolysate:medium amounted to 75:25, as initially established during our previous studies [14,15]. *A. succinogenes* 130Z (DSM 22257) was used for fermentation. All tests were repeated four times. Bottles were flushed with N_2 inoculated with 5% (v/v) of exponentially growing inoculum (CDW ≈ 3 g/L). Bottles were incubated at 37°C for 48 h. Seed culture medium composition, synthetic media preparation and composition as well as details about succinic acid procedure have been previously described [14–15].

2.4. Fermentation broth purification

2.4.1. Initial purification

Residue after succinic acid production was immediately centrifuged at 10.000 g for 10 min. This clarification allowed to remove large particles, cells, impurities from yeast and nutrients used for fermentation. Activated carbon (10% w/v) was mixed with the filtrate for 1 h to remove the organic impurities that contributed to the brown/yellow colour of the broth and then the supernatants were filtered (0.2–0.45 µm pore size filters).

2.4.2. Membrane materials and filtration processes

2.4.2.1. Separation of carboxylic acids from fermentation broth (step 1) The SW30XLE membrane produced by Dow (100 Da, classified as reverse osmosis) and DK membrane produced by GE company (150–300 Da, classified as nanofiltration) were used for filtrations. SW30XLE membrane was characterized by the following parameters: retention coefficient: NaCl 99.5%; $MgSO_4$ 99.5%; contact angle of 45°. In case of DK membrane, these coefficients amounted to 40% (NaCl) and 98% ($MgSO_4$); contact angle amounted to 37°. Filtration processes were performed in a device (type GH-100–400, Osmonics), working in a dead-end mode, using membrane active surface of 36.3 cm^2 (flat sheet membranes). The processes were conducted under trans-membrane pressure of 2 MPa; pH (feedstock): 3.0, 6.8, 8.5; rotary velocity of the stirrer was maintained at the level of 200 rpm/min.

2.4.2.2. By-products washing from permeate after NF (step 2) Membrane filtration was conducted in order to effectively separate succinic acid from remaining amounts of acetic and formic acid present in the retentate after NF filtration (Fig. 1). The process was conducted in the cross direction (called cross - flow), using Osmonics Inc. (type SEPA CF – HP) module. The membrane (SW30XLE, 100 Da), with the active surface of 144 cm^2, was used. Linear flow velocity over the membrane surface was 2 m/s. Initial volume of the feed solution amounted to 3 L. Retentate was recycled and the same amount of ultra-pure water was added as the volume of permeate withdrawn; in 1 h intervals.

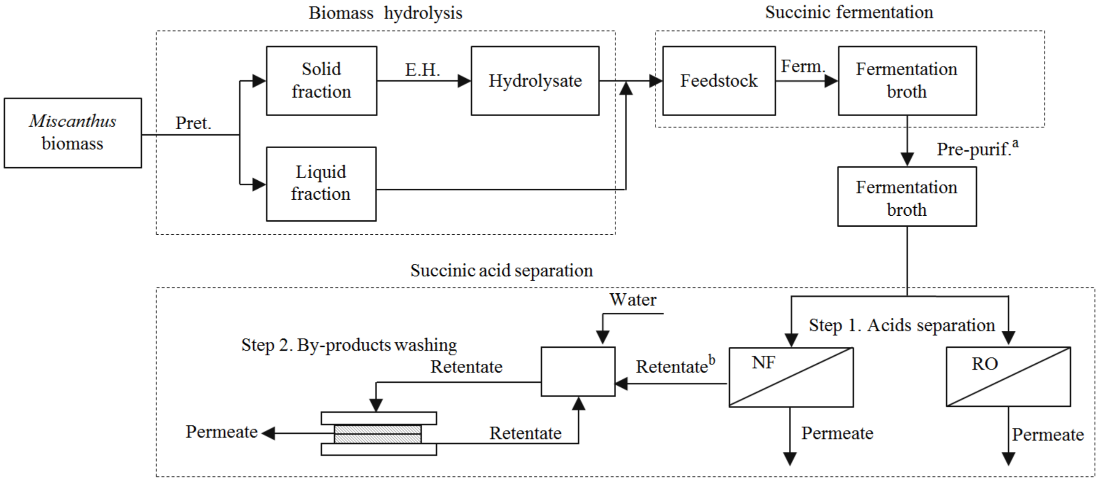

Fig. 1. Concept of bio-succinic production from *Miscanthus* after pretreatment, integrated with succinic acid separation from fermentation broth (Pret. - steam/acid pretreatment, E.H. - enzymatic hydrolysis, Ferm. - succinic fermentation, NF - nanofiltration, RO - reverse osmosis, [a] - pre-purification of fermentation broth by centrifugation and activated carbon treatment, [b] - retentate after NF filtration considered as the most appropriate option after 1 step of separation and used as feedstock for 2 step).

2.5. Calculations

2.5.1. Effectiveness of hydrolysis and succinic acid fermentation

Sugar yields after hydrolysis (pretreatment/enzymatic hydrolysis) were based on the amount of glucose, xylose, or their sum released into liquid fraction and related to initial cellulose, hemicellulose content or total amount of sugar in the biomass before hydrolysis. Obtained values were expressed as percentage. Details information as well as description of formulas used can be found in our previous reports [14,15]. Succinic acid yield was expressed as the amount of succinic acid obtained (g) per 1 g of sugars (glucose + xylose) consumed and presented as percentage.

2.5.2. Fermentation broth separation

Effectiveness of membrane processes used for carboxylic acids separation from broth was based on the value of carboxylic acids (succinic-, acetic, formic- and lactic) retention and volumetric permeate flux values.

2.5.2.1. Retention

In filtration experiments, the retention of the component (R_i) was defined as follows (1):

$$R_i\% = \left(1 - \frac{C_p \cdot V_p}{C_f \cdot V_f}\right) \cdot 100\% \tag{1}$$

where C_p is the concentration of the particular component in the permeate, g/L; C_f is the concentration of the particular component in the feed, g/L; V_p is the volume of the permeate after filtration, L; V_f is the volume of the feedstock used for filtration, L.

2.5.2.2. Volumetric permeate flux Efficiency of the membrane processes was determined according to the following formula (2):

$$J_V = \frac{V}{F \cdot t} L / m^2 \cdot h \tag{2}$$

where J_v is the volumetric permeate flux, L/m^2·h; V is the permeate collected after the particular period of time (t), L; F is the active membrane surface (m^2); t is the filtration time (h).

2.6. Methods

The scope of analyses included: total solids (TS), volatile solids (VS), ash, pH [16] as well as sugar monomers, organic acids (succinic-, lactic-, formic- and acetic acid) and inhibitors (furfural and 5-hydroxymethyl-2-furaldehyde HMF) by means of high performance liquid chromatography HPLC (Agilent 1260); equipped with a BioRad Aminex HPX-87H column equipped with refractive index (RI) detector (RID 1362A) and ultraviolet (UV). Sugars in solid samples as well as Klason lignin content were determined by two-steps hydrolysis in acidic conditions. All chemicals used in this study were of analytical grade (Sigma Aldrich).

2.7. Statistical evaluation

In the present study, results are presented as average values (n = 4) with standard deviations (±). The characteristics of the pretreated biomass as well as data obtained during succinic acid downstream processes were compared statistically. One-way ANOVA test followed by Tukey's HSD tests were used for multiple comparisons between samples. The level of significance was set at 0.05. In order to confirm the normal distribution and homogeneity of variances, Shapiro–Wilk and Levene test were used, respectively. Finally, data significantly equivalent were indicated by the same letters.

3. Results and discussion

3.1. Biomass hydrolysis

All the conditions of the pretreatment had a significant effect on the biomass composition, i.e. cellulose, hemicellulose and lignin content (Table 1). Recovery of WIS (water insoluble solids) ranged from 59% to 84%; depending on the conditions of pretreatment applied. In both range of temperature, the highest glucan increase in pretreated biomass was recorded for the acid concentration of 1.5–2%, i.e. 22–23% (140°C) and 30–34% (180°C) (Table 1). For all treatments conditions, except when temperature of 180°C and 2% acid was used, more than 97% of the glucan was retained in the solid fraction. Negative influence of too high acid concentration used for pretreatment, has previously been reported and caused cellulose losses due to formation of glucose degradation products [15,17,18]. High glucan loss (>9% of glucan) during above mentioned pretreatment conditions (180°C/2% H_2SO_4) did not allow to take this assay for further consideration (Table 1).

An increase in glucan content was associated with solubilization of hemicellulose fraction through pretreatment, which is the main advantage of the dilute-acid pretreatment methods [19]. The hemicellulose content in solid fraction gradually decreased with increasing severity of the pre-treatment, which proves that high temperature facilitated by sulfuric acid is powerful catalyst for removing hemicellulose from *Miscanthus* biomass (Table 1). The hemicellulose released into liquid fraction consisted mostly of xylose, while, low arabinose concentration (less than 0.5 g/L, data not shown) was also found in liquid after pretreatment. The highest amounts of xylose released (58–60% of initial hemicellulose) was received after pretreated at 140°C and 1.5–2.0% acid addition. In case of pretreatment at 180°C, significantly lower values of xylose (Table 1) were released into liquid fraction; due to significant sugar losses. For all pretreatment trials at 180°C, the hemicellulose losses amounted to 20.3–45.8% of initial hemicellulose in biomass. Whilst, such losses after treatment at 140°C were lower than 10% (calculated based on results of solid and liquid fraction, Table 1). Sugar losses, especially from hemicellulose fraction, are commonly observed in methods involving high temperature and acid addition [17]. Such losses can be connected with biomass degradation products, e.g. furfural, hydroxymethylfurfural (HMF), organic acids, etc. In our case, relatively low concentrations of furfural (<0.1 g/L) and HMF (<0.23 g/L) (data not shown) were recorded during the experiment. This can be supported by

Table 1
Characteristics of solid and liquid fractions after pretreatment at 140°C and 180°C (average values $n = 4$, ± standard deviations, the same letters represent data equivalent statistically $p > 0.05$)

Pretreatment	Solid fraction				Liquid fraction	
	Glucan, % DM	Xylan, % DM	Lignin, % DM	WIS, %	Glucan, %*	Xylan, %*
Pretreatment temperature: 140°C						
0.0% H_2SO_4	47.9 ± 1.9e	20.7 ± 0.9a	25.3 ± 0.9b	84 ± 3a	n.d.	21.3 ± 2.3h
0.5% H_2SO_4	50.0 ± 1.2de	17.9 ± 0.5b	25.6 ± 1.4b	81 ± 1ab	n.d.	32.1 ± 3.5fg
0.75% H_2SO_4	50.6 ± 1.2de	15.5 ± 1.3bc	26.3 ± 1.5b	80 ± 1bc	n.d.	41.2 ± 3.0de
1.0% H_2SO_4	51.5 ± 1.7de	14.5 ± 0.8cd	26.8 ± 1.3ab	79 ± 2bcd	n.d.	44.5 ± 0.8d
1.5% H_2SO_4	53.8 ± 1.3cd	12.4 ± 0.7def	27.4 ± 1.6ab	77 ± 2bcd	n.d.	57.8 ± 1.4ab
2.0% H_2SO_4	54.1 ± 1.4cd	11.7 ± 0.9ef	27.8 ± 1.3ab	76 ± 2cd	n.d.	60.2 ± 2.0a
Pretreatment temperature: 180°C						
0.0% H_2SO_4	52.0 ± 1.8de	17.9 ± 1.3b	25.5 ± 1.3b	76 ± 1cd	1.2 ± 0.3b	25.2 ± 3.0gh
0.5% H_2SO_4	53.1 ± 1.3cd	13.4 ± 0.7cd	25.9 ± 1.3b	75 ± 2cd	1.3 ± 0.3b	34.1 ± 3.0de
0.75% H_2SO_4	53.4 ± 1.1cd	10.1 ± 0.6fg	28.4 ± 1.4ab	74 ± 2de	1.7 ± 0.7ab	42.0 ± 2.0d
1.0% H_2SO_4	55.7 ± 2.7bc	9.05 ± 0.4g	29.2 ± 1.8ab	72 ± 3ef	1.4 ± 0.3b	42.7 ± 2.6d
1.5% H_2SO_4	57.4 ± 2.6ab	6.05 ± 0.4h	28.8 ± 1.9ab	67 ± 2f	1.9 ± 0.8ab	47.7 ± 1.7cd
2.0% H_2SO_4	59.2 ± 1.0a	0.85 ± 0.8i	30.8 ± 2.1a	59 ± 2g	3.5 ± 0.4a	52.2 ± 2.9bc

*glucose or xylose released into liquid fraction during pretreatment and expressed as percentage of initial glucan, xylan content in biomass before pretreatment, n.d. – not detected or below detection limit.

the statement that relatively low concentrations of furfural and HMF are generated during pretreatment at temperatures below 190°C [18]. Whilst, organic acids (1.0–3.0 g CH_3COOH/L and 0.5–2.5 g HCOOH/L) were observed as the main degradation products and its occurrence is mostly associated with hydrolysis of acetylated hemicelluloses.

Pretreatment at 140°C after addition 1.5–2.0% of acid (most of the parameters statistically equivalent, $p > 0.05$, Table 1) was selected as the most appropriate for the analyzed species of *Miscanthus* biomass. Finally, taking into consideration the fact that pretreatment at higher acid concentration is strictly connected with corrosion risk, formation of inhibitors as well as can cause inhibition during further processing (enzymatic hydrolysis, succinic fermentation) [19], – biomass pretreated at 140°C after addition of 1.5% acid was selected as the most appropriate for *Miscsnthus* biomass and enzymatically hydrolyzed; using the commercial enzymes mixtures.

Glucose yield of untreated biomass amounted to 25% (data not shown), which indicates that biomass of Miscanthus is relatively difficult to hydrolyze. Similar values are achieved in case of many untreated lignocellulosic biomasses [20]. Applied pre-treatment influenced the effectiveness of enzymatic hydrolysis in a positive way, reaching maximum values of about 70% (glucose yield) and 50% (xylose yield) after 24 h of the process. Further extending of enzymatic hydrolysis did not impact on its effectiveness (Fig. 2, data equivalent statistically). Lower values of xylose yield during enzymatic hydrolysis can be explained by the fact that most of the xylose was released during pretreatment (Table 1), and thus the proportion of more resistant and less accessible hemicellulose increased [15,17]. Considering effectiveness of pretreatment and enzymatic hydrolysis in total, the mixture of liquid fraction (after pretreatment) and hydrolysate (after enzymatic hydrolysis) contained 23.2 g/L of sugars, including 13.6 g/L glucose and 9.6 g/L xylose (calculated based on results presented in Table 1 and Fig. 2). This mixture was used as a feedstock for succinic acid production in a further step of the experiment (Table 2).

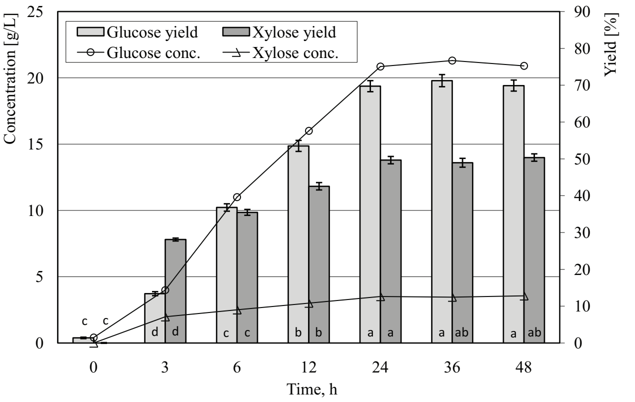

Fig. 2. Enzymatic hydrolysis course of biomass after dilute-acid pretreatment (1.5% of sulfuric acid at 140°C) (average values $n = 4$, ± standard deviations, the same letters represent data equivalent statistically $p > 0.05$).

3.2. Succinic acid production

Hydrolysates generated after 1.5% H_2SO_4 pretreatment, followed by enzymatic hydrolysis, were selected for succinic acid fermentation in anaerobic bottles (Table 2). Glucose was completely consumed, while, about 10% of initial xylose was still present after the process. Glucose is, in most

Table 2
Characteristics of feedstock for succinic production, fermentation broth after succinic acid production as well as fractions recovered during broth membrane filtration

Compound	Succinic fermentation		Succinic acid separation			
			I step		II step	
	Feedstock[a]	Broth	Retentate RO (pH 3.0)[b]	Retentate NF (pH 6.8)[c]	Retentate[d]	Permeate[d]
Glucose, g/L	10.2 ± 0.8	n.d.	n.d.	n.d.	n.d.	n.d.
Xylose, g/L	7.3 ± 0.5	0.72 ± 0.1	0.68 ± 0.1	0.25 ± 0.1	n.d.	n.d.
Succinic acid, g/L	n.d.	12.2 ± 0.5	10.7 ± 0.4	9.85 ± 0.2	9.47 ± 0.05	0.25 ± 0.02
Acetic acid, g/L	1.5 ± 0.2	4.02 ± 0.3	0.32 ± 0.1	1.28 ± 0.1	0.28 ± 0.02	0.66 ± 0.03
Formic acid, g/L	1.1 ± 0.2	2.92 ± 0.2	0.29 ± 0.1	0.93 ± 0.1	0.20 ± 0.02	0.48 ± 0.02

[a]mixture of hydrolysate (65% vol.) and liquid fraction after pretreatment 1.5% acid at 140°C (35% vol.), [b]retentate collected after the most appropriate condition of RO filtration, [c]retentate collected after the most appropriate condition of NF filtration, [d]retentate or permeate after 10 h of membrane washing, in a diafiltration mode, n.d. – not detected/below detection limit.

cases, utilized first by *A. Succinogenes*, and some xylose left was also previously observed. Enhanced sugar conversion can be achieved after ensuring stable pH and mixing condition during the whole experiment, which can be realized in completely controlled reactors [15]. In the current study, the succinic acid yield of 73% was achieved (Fig. 3). However, it should be taken into account that a significant content of feedstock constituted xylose (40% of total sugars), which is usually connected with lower succinic yields compared to glucose [15,21]. The production of succinic acid is usually accompanied by other metabolites, such as acetic, formic, lactic acid or ethanol; depending on fermentation conditions and pretreatment method used [15]. In the present study, acetic – (21% of total acids in fermentation broth) and formic acid (15% of total acids in fermentation broth) were produced as the main by-products (Fig. 3). However, it should be highlighted that some acetic and formic acids were present in the feedstock before succinic fermentation as a result of biomass pretreatment. Additionally, small amounts of lactic acid (<1 g/L, data not shown) was produced, which indicated some imbalances of the process connected with cell growth decrease [22]. However, above mentioned disadvantages of the *Miscanthus* biomass fermentation in anaerobic bottles can be overcome by tests in controlled conditions.

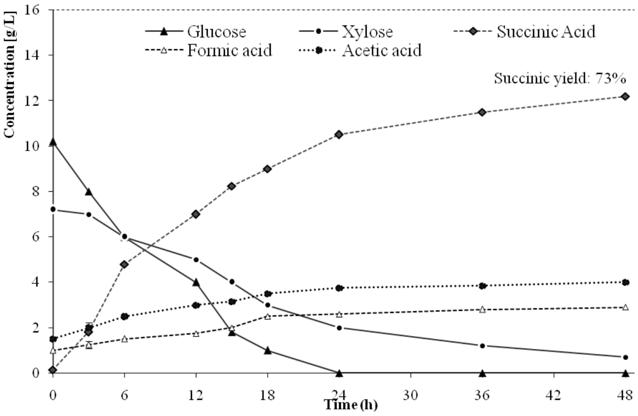

Fig. 3. Time course of succinic acid fermentation (hydrolysate after 1.5% H_2SO_4 pretreatment, followed by enzymatic hydrolysis; fermentation medium to hydrolysate ratio: 25:75 vol.).

3.3. Separation of carboxylic acids from fermentation broth

The effectiveness of NF/RO membrane filtration is influenced by many factors, including pH of the feed solution, molecular weight of compounds as well as membrane surface charge [23]. Carboxylic acids are classified as weak acids and their retentions are highly pH-dependent. Thus, the influence of pH feed solution on the effectiveness (rejection/permeation) and efficiency of membrane processes (permeate flux) was analysed.

3.3.1. Effectiveness of acids separation

Using reverse osmosis or nanofiltration membrane, succinate, the major product of the fermentation, was to be retained in the retentate, while the monovalent by-product salts were to be removed by permeation. For SW30XLE (reverse osmosis) membrane, the rejection of succinic acid (MW: 118.09 Da), which have an MW larger than MWCO of the membrane (100 Da) was very high (>88%) irrespective of pH value (Fig. 4). Whilst, the acetic – (MW: 60.05 Da) and formic acid (MW: 46.03 Da) were only retained at 8–10% for the pH of 3.0 (un-dissociated forms). Taking into account the fact that there is no significant difference in diffusivity between succinic, acetic and formic acids [24], the difference in molecular weights between separated compounds was considered as the main separation factor of un-dissociated acids. Increasing the pH value of feed solution to the level of 6.8 and 8.5 caused an increase in acids rejection. The rejection reached the value of 98–99%, 95–96% and 91–92% for ionic form of succinic, acetic and formic acid, respectively (Fig. 4). In this case, the increase of pH value from neutral (natural value of fermentation broth) to the level of about 8.5 did not have a significant influence on retention values (data equivalent statistically, Fig. 4). Since analysed organic acids were in dissociated forms at pH 6.8–8.5, an increase of the rejection could be explained by electric repulsion [25].

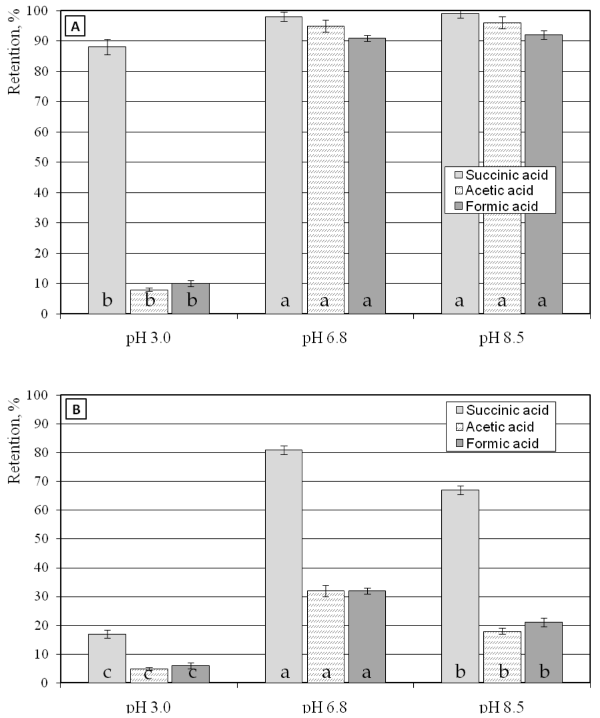

Fig. 4. Influence of pH feed solution on the retention of carboxylic acids ((A) – filtration with SW30XLE membrane, (B) – filtration with DK membrane, the same letters represent data equivalent statistically $p > 0.05$).

As it could have been expected, NF (DK 150–300 Da membrane) turned out to be ineffective for the pH 3.0 of feed solution (all analysed acids in un-dissociated froms; pK_a = 3.76–5.6). In this case, only about 17% of succinc acid was retained (Fig. 4). In general, the rejection of carboxylic acids in NF membranes is not very high at low pH, – due to the low hindrance effect of NF membranes. This is in agreement with study on butyric acid separation [25]. At higher pH values, the negatively charged membrane highly rejects negatively charged acids, due to the electrostatic repulsion effect. What is more, NF membranes have more negatively charged groups on their surface than RO membranes, which helps to reject divalent ions. In our case, the succinic acid rejection increased to 81% as a result of pH increased to 6.8 (Fig. 4). Dissociated forms of acetic and formic acid (pK_a = 3.75–4.76) were also more effectively rejected (31–32%), most probably due to repulsion effect. However, a decrease in carboxylic acid rejection was observed in case of pH 8.5 (data different statistically, Fig. 4). In our case, ionic strength of the solution increased from 69.0 mM/L L to 76.5 mM/L as a result of pH adjustment to 8.5, using NaOH. It can be partially explained by the fact that high ion concentration, used for pH adjustment, suppresses the electrical double layer on the charged membrane, thus weakening electrostatic repulsion and minimizing the rejection of negatively charged acids at higher pH [26]. What is more, NF membranes carry more charged groups in the membrane matrix at high pH and those groups have been postulated to push adjacent polymers apart, resulting in membrane swelling and an increased molecular weight cut off [26–27]. This could have a negative influence on the rejection values.

3.3.2. Permeate flux values

Similarly to retention of target products, the values of permeate flux are also very important from the technological and economical point of view. Significant decrease in permeate flux is considered as the main disadvantage of membrane application, as it leads to shorter lifetime of the filtration material. Moreover, fouling requires membrane cleaning, which is connected with additional costs, as water/chemicals are used. The water fluxes amounted 44 L/m^2 h (SW30XLE, classified as RO membrane) and 58 L/m^2 h (DK, classified as nanofiltration membrane), which is strictly connected with membrane characteristics and filtration conditions (Fig. 5). Filtration of analysed fermentation broths was connected with evident decreases in permeate fluxes. In case of nanofiltration, the values stabilized at 45–46 L/m^2·h (pH = 3.0), 39–40 L/m^2·h (pH = 6.8, natural value of fermentation broth after succinic production) and 29–30 L/m^2·h (pH = 8.5). Whilst, values after reverse osmosis reached significantly lower values compared to nanofiltration processes. Flux values reached 13–14 L/m^2·h (pH = 3.0), 8.5–9.0 L/m^2·h (pH = 6.8) and 4.8–5.0 L/m^2·h (pH 8.5) (Fig. 5). In principle, residues after anaerobic processes are difficult to treat, as they contain various components added for anaerobic processes (salts

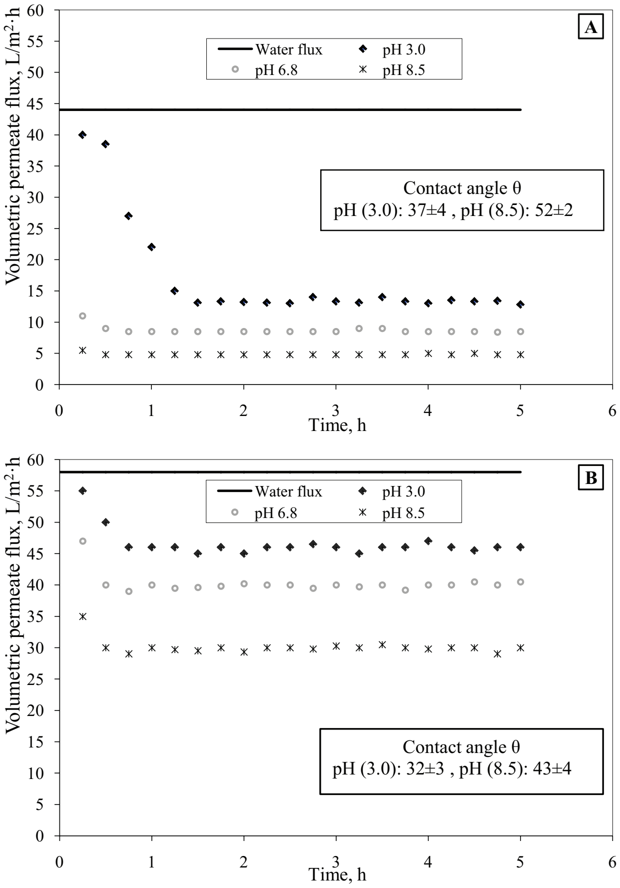

Fig. 5. Influence of feed solution pH value on the volumetric flux values ((A) – filtration with reverse osmosis membrane SW30XLE; (B) – filtration with DK-NF membrane).

added for microbial growth), organic compounds originated from nitrogen source used (e.g. amino acids, peptides etc.) as well as VFA [12,28,29]. Additionally, negative influence of increased pH of feed solution – on the permeate flux was observed (Fig. 5). This trend can be accounted for the fact that salts addition for pH adjustment increases the osmotic pressure of feed solutions, which can have a negative influence on permeate flux values. What is more, analysed membranes changed their properties towards hydrophilic properties at acidic pH (3.0), which was proved by the higher membrane contact angle. Whilst, the membranes used exhibited more hydrophobic properties at alkaline conditions, which could have been the reason of lower permeate flux (Fig. 5).

Taking into consideration the presented retentions of succinic acid/succinate as the target product and permeate flux values, filtration using DK-NF membrane conducted at pH of 6.8 was selected as the most appropriate for the treatment of analysed fermentation broth. In this case, only about 7% (on average) lower retention of succinic acid was observed compared to the best results obtained during RO filtration at pH of 3.0 (Fig. 4). What is more, filtration at 6.8 (natural value of broth after succinic fermentation) does not require pH adjustment, which can be connected with lower operating costs.

3.3.3. By-products washing from retentate

The retentate after nanofiltration conducted at pH 6.8 contained 82% of succinic acid (calculated as percentage of all acids, Table 2), which is significantly higher than succinic content in the fermentation broth (64% of total acids) (Fig. 3). The retentate was treated with the application of SW30XLE membrane, operated in a diafiltration mode (Fig. 6). Succinic acid, the major product of fermentation, was retained and its losses did not exceed 4% during the washing step. Whilst, acetic and formic acids, by-products of fermentation, were effectively removed by permeation. After 10 h of membrane filtration, the succinic acid amounted to 95% of total acids content (Table 2). Moreover, high water usage diluted the acids recovered in the permeate. The recovered permeate after 10 h of filtration contained less than 1.5 g/L of acids (Table 2) and can be recycled and used again for biomass dilution during pre-treatment step (biomass solvent) or other technological purposes. Nowadays, separation and purification of succinate from the fermentation broth is still an economical obstacle for its biological production; water recovery and recyclability from stillage is a challenge and important aspect, which can improve the performance of bio-succinic production from lignocellulosic biomass [30,31]. However, detailed research is necessary in order to test the usage of such water as a solvent for biomass processing.

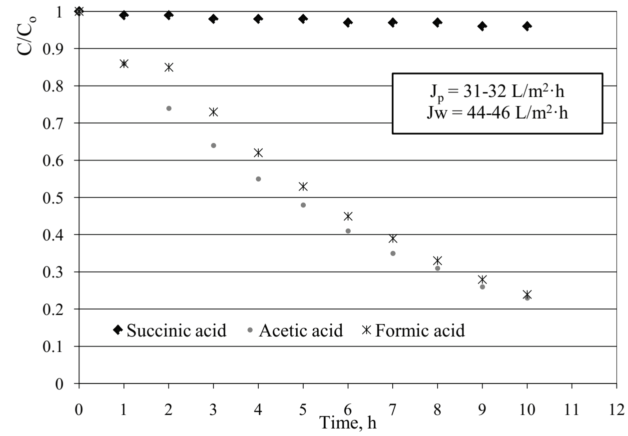

Fig. 6. Removal of residual by products from retentate using membrane filtration, in a diafiltration mode (C_o – acids concentration in retentate before filtration, C – acids concentration in retentate during diafiltration, J_p – permeate flux of the analysed sample, J_w – water permeate flux).

A stable value of permeate flux was observed during the process (31–32 L/m^2·h), which constitutes about 70% of permeate flux of distilled water (Fig. 6). After 10 h of filtration, flush with distilled water applied for 10 h effectively recovered almost 95% of membrane permeability (data not shown). This indicates that the fouling was reversible during cross-flow filtration (diafiltration) of the sample treated with NF during the first step of carboxylic acid separation. The usefulness of membrane diafiltration for the treatment of residues after anaerobic digestion has recently gained a lot of attention. For example, effective combined UF-NF systems for fermentation broth treatment or ethanol recovery have been presented [12,32,33]. The washing step presented above using membranes is only one of the possible option for the final removal of residual by-products (acetic and formic acid) from fermentation broth. Another possible option is water evaporation, which can be integrated with distillation of volatile carboxylic acids, since distillation of these compounds in petrochemical industry is commonly practiced. Evaporation is a very simple and effective method to remove volatile acids, but this process is commonly considered as energy demanding. The presented concept of succinic acid separation from fermentation broth after *Miscanthus* biomass conversion has to be verified in subsequent studies, for an extended period of operation. An economic evaluation would be needed to verify the optimum operating conditions established in the present study.

4. Conclusions

The study presents the concept of succinic acid production, based on biomass of *Miscanthus* after steam/acid pretreatment. Hydrolysates generated after the most appropriate pretreatment conditions (140°C/1.5% H_2SO_4, followed by enzymatic hydrolysis) were successfully used as feedstock for succinic acid production (succinic yield of 73%). Succinic acid, considered as one of the top building-block chemicals of the future, was successfully separated from fermentation broth by means of membrane processes. Filtration using DK-NF membrane conducted at pH 6.8 (I step), followed by diafiltration (II step) allowed to reach 95% content of succinic acid in the mixture of carboxylic acids. The recovered permeate, containing mostly water and insignificant amounts of acids, – can be recycled to the pre-treatment step (biomass solvent) or used for other technological purposes. Membrane techniques used for separation of target products can significantly enhance the performance of bio-succinic production from lignocellulosic biomass in the near future. However, further research as well as economic evaluation would be needed to verify the effectiveness of applied membrane filtrations.

Acknowledgments

This work was supported by the University of Bielsko-Biala (Poland) within internal funds for development of young scientists as well as EU project POKL04.01.02-00-196/09-00 "Construction – a bridge between tradition and modernity in the region". The research was also conducted during 3 months internship (M. Kuglarz, University of Bielsko-Biala, Poland) in the Silesian University of Technology (Faculty of Energy and Environmental Engineering, Institute of Water and Wastewater Engineering, Environmental Chemistry and Membrane Processes Research Group).

References

[1] A.T.W.M. Hendriks, G.G. Zeeman, Pretreatments to enhance the digestibility of lignocellulosic biomass. Biores. Technol., 100 (2009) 8–10.

[2] W.C. Lee, W.C. Kuan, Miscanthus as cellulosic biomass for bioethanol production. Biotechnol. J., 10(6) (2015) 840–854.

[3] N. Xu, W. Zhang, S.F. Ren, F. Liu, C.Q. Zhao, H.F. Liao, Z.D. Xu, J.F. Huang, Q. Li, Y.Y. Tu, B. Yu, Y.T. Wang, J.X. Jiang, J.P. Qin, L.C. Peng, Hemicelluloses negatively affect lignocellulose crystallinity for high biomass digestibility under NaOH and H_2SO_4 pretreatments in Miscanthus, Biotechnol. Biofuels, 5 (2012) 58.

[4] A. Sørensen, P.J. Teller, T. Hilstrøm, B.K. Ahring, Hydrolysis of Miscanthus for bioethanol production using dilute acid presoaking combined with wet explosion pre-treatment and enzymatic treatment. Biores. Technol., 99 (2008) 6602–6607.

[5] S.L. Si, Y. Chen, C.F. Fan, H.Z. Hu, Y. Li, J.F. Huang, H.F. Liao, B. Hao, Q. Li, L.C. Peng, Y.Y. Tu, Lignin extraction distinctively enhances biomass enzymatic saccharification in hemicelluloses-rich Miscanthus species under various alkali and acid pretreatments, Biores. Technol., 183 (2015) 248–254.

[6] Q.Z. Li, X.L. Jiang, X.J. Feng, J.M. Wang, C. Sun, H.B. Zhang, M. Xian, H.Z. Liu, Recovery processes of organic acids from fermentation broths in the biomass-based industry, J. Microbiol. Biotechn., 26 (2016) 1–8.

[7] J.B. McKinlay, C. Vieille, J.G. Zeikus, Prospects for a biobased succinate industry, Appl. Microbiol. Biot., 76 (2007) 727–740.

[8] T. Kurzrock, D. Weuster-Botz, Recovery of succinic acid from fermentation broth. Biotechnol. Lett., 32 (2010) 331–339.

[9] Y.Q. Sun, L. Yan, H.X. Fu, Z.L. Xiu, Salting-out extraction and crystallization of succinic acid from fermentation broths. Process Biochem., 49 (2014) 506–511.

[10] C.X. Wang, Q. Li, H. Tang, W. Zhou, D.J. Yan, J.M. Xing, Y.H. Wan, Clarification of succinic acid fermentation broth by ultrafiltration in succinic acid bio-refinery. J. Chem. Technol. Biotechnol., 88 (2013) 444–448.

[11] C.X. Wang, Q. Li, H. Tang, D.J. Yan, W. Zhou, J.M. Xing, Y.H. Wan, Membrane fouling mechanism in ultrafiltration of succinic acid fermentation broth. Biores. Technol., 116 (2012) 366–371.

[12] J.M. Prodanović, V.M. Vasic, Application of membrane processes for distillery wastewater purification - a review. Desal. Water Treat., 51 (2013) 3325–3334.

[13] P.A. Sosa, C. Roca, S. Velizarov, Membrane assisted recovery and purification of bio-based succinic acid for improved process sustainability. J. Membr. Sci., 501 (2016) 236–247.

[14] I.B. Gunnarsson, M. Kuglarz, D. Karakashev, I. Angelidaki, Thermochemical pretreatments for enhancing succinic acid production from industrial hemp (Cannabis sativa L.), Biores. Technol., 182 (2015) 58–66.

[15] M. Kuglarz, M. Alvarado-Morales, D. Karakashev, I. Angelidaki, Integrated production of cellulosic bioethanol and succinic acid from industrial hemp in a biorefinery concept, Biores. Technol., 200 (2016) 639–647.

[16] APHA, Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association Fed., Washington, DC, 2005.

[17] M. Kuglarz, I.B. Gunnarsson, S.E. Svensson, T. Prade, E. Johansson, I. Angelidaki, Ethanol production from industrial hemp: Effect of combined dilute acid/steam pretreatment and economic aspects, Biores. Technol., 163 (2014) 236–243.

[18] S.J. Horn, M.M. Estevez, H.K. Nielsen, R. Linjordet, V.G.H. Eijsink, Biogas production and saccharification of Salix pretreated at different steam explosion conditions. Biores. Technol., 102 (2011) 7932–7936.

[19] N. Brosse, A. Dufour, X.Z. Meng, Q.N. Sun, A. Ragauskas, Miscanthus: a fast- growing crop for biofuels and chemicals production. Biofuels, Bioprod. Biorefin., 6 (2012) 580–598.

[20] A. Pakarinen, P. Maijala, F.L. Stoddard, A. Santanen, P. Tuomainen, M. Kymäläinen, L. Viikari, Evaluation of annual bioenergy crops in the boreal zone for biogas and ethanol production. Biomass Bioenerg., 35 (2011) 3071–3078.

[21] K.-Q. Chen, J.A. Li, J.F. Ma, M. Jiang, P. Wei, Z.-M. Liu, H.J. Ying, Succinic acid production by actinobacillus succinogenes using hydrolysates of spent yeast cells and corn fiber, Biores. Technol., 102 (2011) 1704–1708.

[22] M.I. Kim, N.J. Kim, L. Shang, Y.K. Chang, S.Y. Lee, H.N. Chang, Continuous production of succinic acid using an external membrane cell recycle system, J. Microbiol. Biotechnol., 19 (2009) 1369–1374.

[23] C. Bellona, J.E. Drewes, P. Xu, G. Amy, Factors affecting the rejection of organic solutes during NF/RO treatment – a literature review. Water Res., 38 (2004) 2795–2809.

[24] H.G. Nam, C. Park, S.H. Jo, Y.W. Suh, S. Mun, Continuous separation of succinic acid and lactic acid by using a three-zone simulated moving bed process packed with Amberchrom-CG300C. Process Biochem., 47 (2012) 2418–2426.

[25] Y.H. Cho, H.D. Lee, H.B. Park, Integrated membrane processes for separation and purification of organic acid from a biomass fermentation process, Ind. Eng. Chem. Res., 51 (2012) 10207–10219.

[26] B. Xiong, T.L. Richard, M. Kumar, Integrated acidogenic digestion and carboxylic acid separation by nanofiltration membranes for the lignocellulosic carboxylate platform. J. Membr. Sci., 489 (2015) 275–283.

[27] A. Braghetta, F. DiGiano, W. Ball, Nanofiltration of natural organic matter: pH and ionic strength effects, J. Environ. Eng., 123(7) (1997) 628–641.

[28] J. Bohdziewicz, M. Kuglarz, Treatment of post-digestion liquors with the application of struvite precipitation and reverse osmosis, Desal. Water Treat., 51(1) (2013) 366–373.

[29] M. Kuglarz, K. Grübel, J. Bohdziewicz, Chemical precipitation and ammonia air stripping as effective pre-treatment methods before membrane filtration of co-digestion effluents, Desal. Water Treat., 55(6) (2015) 1672–1682.

[30] A. Luiz, T. Handelsman, G. Barton, H. Coster, J. Kavanagh, Membrane treatment options for wastewater from cellulosic ethanol biorefineries, Desal. Water Treat., 53 (2015) 1547–1558.

[31] C.S. López-Garzón, A.J.J. Straathof, Recovery of carboxylic acids produced by fermentation. Biotechnol. Adv., 32 (2014) 873–904.

[32] T. Brás, M.C. Fernandes, J.L.C. Santos, L.A. Neves, Recovering bioethanol from olive bagasse fermentation by nanofiltration, Desalin. Water Treat., 51 (2013) 4333–4342.

[33] Y. He, G. Chen, Z. Ji, Li. Shunxing, Combined UF–NF membrane system for filtering erythromycin fermentation broth and concentrating the filtrate to improve the downstream efficiency, Sep. Purif. Technol., 66 (2009) 390–396.