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Increase Butanol Production from Corn Straw by Mineral Compounds Supplementation

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Abstract: In this study, two types of fermentation methods: SSF and consolidation SHF/SSF were used for production of acetone-butanol-ethanol (ABE) from corn straw as a feedstock. *Clostridium acetobutylicum* DSM1731 was used as the fermenting organism. Corn straw was thermochemically pretreated and then hydrolyzed using three types of enzymes. The impact has been investigated on the effect of mineral compounds supplementation ($(NH_4)_2SO_4$, $MgSO_4$, $(NH_4)_3PO_4$) on ABE productivity and butanol content. From the SSF, where mineral salts were supplemented into the fermentation medium, it was found that the maximum ABE and butanol concentrations were 28.35 g/L and 24.03 g/L, respectively, corresponding to a productivities of 0.295 g/L/h (ABE) and 0.250 g/L/h (butanol). In the consolidation SHF/SSF method with mineral compounds supplementation, the maximum ABE and butanol concentrations were 31.35 g/L and 28.64 g/L, respectively, corresponding to productivities of 0.327 g/L/h (ABE) and 0.298 g/L/h (butanol). Compared to control samples, mineral salts supplementation had a positive effect on cellular metabolic activities, leading to an earlier initiation of the solventogenesis stage. In supplemented samples, an increase in the rate of ABE fermentation by *Clostridium* was observed.

Keywords: lignocellulosic biomass; biofuels; enzymatic hydrolysis; ABE fermentation

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1. Introduction

Due to its renewable nature, plant biomass is the cheapest raw material with the greatest potential as an energy carrier, which can serve as an excellent solution to meet current and future fuel needs. It is also a carbon neutral resource over its life cycle [1]. One of the methods of biomass processing is biochemical conversion, which is based on the fermentation processes, i.e., alcoholic, acetone-butanol-ethanol (ABE) and methane. They lead to the production of liquid and gaseous biofuels, which are the result of the metabolic activity of microorganisms. Many kinds of plant biomass can be used for the production of biobutanol. These can be energy crops, plant biomass from agricultural crops or agricultural waste such as corn cobs, oat ears, wood chips, corn and grain stalks, and all types of straw, e.g., wheat, rye, oats, and corn straw [2–4]. More attention should be given to these raw materials that are not used for food purposes and do not compete with food supplies, e.g., lignocellulosic biomass, which is one of the most abundant renewable feedstocks on the planet [1,5,6]. The feedstock price plays a major role in the economic viability of fermentative butanol production, accounting for up to 60% of the total cost of ABE fermentation [1].

Biobutanol can be produced through fermenting sugars by anaerobic bacteria, usually *Clostridium* sp. The process is known as ABE fermentation, since microorganisms produce three solvents, namely acetone, n-butanol, and ethanol, in their metabolic pathway. The most popular strains used in ABE fermentation are *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, *C. cadaveris*, *C. pasteurianum*, *C. sporogenes*, *C. tetanomorphum and C. saccharoperbutylacetonicum*. *Clostridium* sp. is a Gram positive bacteria. These bacilli are

mesophilic with optimal temperatures of 10–45 °C and require anaerobic conditions in order to grow [7–9].

Clostridia strains are non–cellulolytic microbes and hence cannot use cellulose as a carbon source. Therefore, it is essential to convert polysaccharides into monosaccharides by pretreatment (such as mechanical treatment, acidic treatment, alkali treatment, thermal treatment, and biological treatment) or enzymatic hydrolysis [10]. Solventogenic clostridia are one of the few organisms able to ferment not only hexoses from hydrolyzed cellulose (glucose, galactose, and mannose), but also pentoses from hemicelluloses (xylose and arabinose) [11]. Therefore, to produce butanol, agricultural waste containing cellulose and hemicellulose can also be used [12]. Xin et al. [13] reported that the butanol concentration increases when xylose is present in the fermentation medium, as it promotes acetic acid reassimilation in the solventogenesis stage of ABE fermentation. Solventogenic clostridia, such as *C. acetobutylicum* and *C. beijerinckii,* are the microorganisms most commonly used for butanol production from lignocellulosic and starchy biomass [14–16].

ABE fermentation consists of two distinct characteristic phases, namely acidogenesis and solventogenesis. The first phase (acidogenesis stage) occurs at the initial growth phase of the *Clostridia* (usually within the first 24 h), during which metabolites like acetate, butyrate, hydrogen, and carbon dioxide are the major products produced [17–19]. During the first phase, the pH of the growth medium reduces. The second one (solventogenesis stage) occurs at the stationary phase of the microbial growth (usually after 48 h). In this phase, acetic and butyric acids formed during the acidogenic phase are re-assimilated and used in the production of acetone, butanol and ethanol [20,21].

The amount of simple sugars in the hydrolysate are one of the major factors influencing the butanol production, which can be regulated through the hydrolysis process and pretreatment. In the literature, many researchers indicate the threshold value of sugar concentration in the medium, at which the change of the acid-forming phase into the solvatogenic phase occurs [22]. Ezeji et al. [23] reported that the butanol production in continuous culture fermentation of corn starch by *C. beijerinckii* decreases when the simple sugars concentration drops to <0.05 g/L.

Conversion technology for liquid biofuels from lignocellulosic biomass can be classified into two of the most frequently applied processes: (1) separate hydrolysis and fermentation (SHF), and (2) simultaneous saccharification and fermentation (SSF) [4,24,25]. In the SHF method, all processes (enzymatic hydrolysis and fermentation) are run in separate steps under its optimal conditions. SSF fermentation is carried out in same reaction vessel and is based on the fact that the monosaccharides obtained after the hydrolysis of cellulose are immediately fermented by the microorganisms. The main limitation of this method is the optimal temperature for cellulase activity (45–60 °C) compared to the conditions of ABE fermentation (30–37 °C) [26–28]. However, some researchers have shown that in the saccharification of cellulose between 37 °C and 60 °C there was no overall significant difference [29]. Krishna et al. [30] reported that when using cellulase on the saccharification, the difference in sugar yields in the range of 35 °C–50 °C was a maximum 8% for 35 °C. Raw materials get higher concentrations of ABE mixture by different SHF and SSF methods. It is difficult to predict which method the feedstock will be characterized by better fermentation productivity. Sasaki et al. [25], using 6% wood chips as substrate, obtained ABE concentrations equal to 15.29 g/L using the SHF method. Compared to the SSF method, it was 12% higher: 13.41 g/L. However, Ibrahim et al. [31], using the SSF method (4.45 g/L), obtained a 44% higher concentration of the ABE mixture compared to the SHF method (2.51 g/L) using the oil palm empty fruit bunch (OPEFB) as a substrate.

In order to make better use of the lignocellulosic substrate in the fermentation process, it is necessary to optimize the fermentation conditions that affect the cell growth of bacteria, product concentration, yield, and productivity. Our previous studies reported that the application of mineral mix (ammonium sulfate, dipotassium phosphate, magnesium sulfate) and soybean oil into fermentation medium contributes to an increase in the productivity of ethanol from starch [32]. It was reported that mineral compounds are

involved in the metabolic pathways of microorganisms as the cofactors of enzymes or had a protective effect on cells. The addition of zinc to the fermentation process enhanced the stress tolerance of yeast cells induced by thermal conditions and activates enzymes in glycolytic pathways [33,34]. According to the literature, several researchers investigated ABE fermentation from lignocellulosic substrates with nutrient additions such as P2 solution (as a semisynthetic medium containing buffer, minerals, and vitamins) or yeast extract [35,36]. Minerals are supplemented in the lignocellulosic hydrolysates, which can be characterized by too poor chemical composition and nutritional deficiency for microorganisms. Wu et al. [37] studied the effect of zinc supplementation on ABE fermentation by *C. acetobutylicum*. They reported that zinc addition facilitated the ABE fermentation process. The butanol and ABE productivities increased, correspondingly, to 0.32 and 0.53 g/L/h from 0.18 and 0.30 g/L/h compared to the control sample without zinc supplementation.

Biobutanol is receiving renewed interest due to its potential in terms of its physicochemical properties, such as low water miscibility, energy content and having an octane number a similar level to that of gasoline, and its blending ability with gasoline at any proportion [38–40]. The biobutanol can be mixed up to 30% (*v/v*) with gasoline without the need to alter current vehicle or engine technologies. This is because biobutanol's energy value is similar to gasoline, and the density of the mixture of biobutanol and gasoline is slightly higher compared to pure gasoline [41]. Relative to bioethanol, butanol has higher energy content, lower volatility and is also less hygroscopic, and is corrosive to the existing infrastructure [17]. Finally, biobutanol demonstrates an overall low order of toxicity and is more biodegradable under aerobic conditions [1,37].

In the present study, alkali-pretreated corn straw was enzymatically hydrolyzed with the use three types of enzymes: cellulase, hemicellulase, and xylanase. To investigate efficient ABE production from the resulting hydrolysates, two types of fermentation methods: SSF and consolidation SHF/SSF, were applied using bacteria, namely *C. acetobutylicum* DSM 1731. In this article, the effect of mineral compounds supplementation (such as ammonium sulfate, magnesium sulfate, and ammonium phosphate) on acetone-butanol-ethanol fermentation was studied with the aim of improving butanol concentration and productivity.

2. Materials and Methods

2.1. Substrate

The research material included corn straw. Before pretreatment, the raw material was dried at 50 °C over 48 h (until constant weight), and ground in a cutting mill (ZBPP, Bydgoszcz, Poland). This allowed a particle size reduction to 0.5–1.0 mm. The moisture and dry organic matter content of corn straw were 6.8% and 94.3%, respectively.

2.2. Bacterial Strain and Culture Medium

C. acetobutylicum DSM 1731 (purchased from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) was used to produce ABE. The cells were inoculated in an anaerostat with the use of GasPacks-Kit to 411 DSMZ medium consisting of fresh potatoes (washed, peeled and sliced) 200.0 g, $CaCO_3$ 2.0 g, Na-resazurin solution (0.1% *w/v*), 0.5 mL, D-Glucose 6.0 g, L-Cysteine-HCl × H_2O 0.5 g, distilled water 1000.0 mL and cultured at 37 °C for 48–72 h without any agitation.

2.3. Alkaline Pretreatment and Enzymatic Hydrolysis

The pretreatment stage includes the essential steps to reduce feedstock crystallinity and particle size (mechanical pretreatment) and the decomposition of its structure in order to the increase of surface area contact between cellulosic fibers with enzymes (thermochemical pretreatment).

In order to delignify lignocellulosic feedstock and make it more accessible for enzymatic hydrolysis, the substrate (10 g of corn straw) was subjected to a preliminary pretreatment with a calcium hydroxide solution (prepared by dissolving 0.50 g/g $Ca(OH)_2$ in 130 mL of distilled water) at 135 °C for 30 min. To remove toxic compounds produced

during the pretreatment of lignocellulosic biomass, a detoxification process was carried out. The detoxification process was conducted using activated carbon (in 1:5 ratio) in 80 ± 2 °C for 2 h, with continuous agitation at 150 rpm. In order to complete the pretreatment step, the sample was cooled to 50 °C in a water bath. The pretreated cellulosic material was used for subsequent saccharification and ABE fermentation.

The biomass obtained after the thermochemical pretreatment was subjected to an enzymatic hydrolysis process in order to release the sugar monomers. The feedstock was hydrolyzed for 4 h using a complex of cellulase, hemicellulase and xylanase produced from *Trichoderma reesei*, *Aspergillus* sp. and *Aspergillus oryzae*, respectively. As the effect of the enzymes, the viscosity of the liquid was decreased. Enzymatic hydrolysis was performed at 50 °C on a rotary shaker at 140 rpm, with pH 5.0. A complex of enzymes were used: Cellic Ctec2 (150 FPU/mL, Novozymes A/S, Bagsværd, Denmark), Viscozyme L (100 FBGU/g, Novozymes A/S, Bagsværd, Denmark), and Pentopan Mono BG (≥2500 units/g, Novozymes A/S, Bagsværd, Denmark).

The sugar conversion was calculated as follows [42]:

$$\alpha_{cvs} = \left(1 - \frac{W_{res}}{W_{all}}\right) \times 100\%, \quad (1)$$

where: α_{cvs}—sugar conversion (%), W_{res}—residue cellulose content (%), W_{all}—cellulose content of treated corn straw (%).

All enzymatic hydrolysis experiments were performed in triplicate, and the means were calculated.

2.4. Fermentation Strategies

A simultaneous saccharification and fermentation (SSF) process and consolidation SHF/SSF process was conducted after the corn straw was pretreated. During this processes the monosaccharides available in the medium (C6 and C5 sugars) are converted to a mixture of solvents (ABE). The SSF method (simultaneous saccharification and fermentation) assumes the combining of hydrolysis and acetone-butanol-ethanol (ABE) fermentation into a single operation, which is performed in a single reactor, at 37 °C, for 96 h, without pH control. To increase the initial monosaccharides concentration in the SHF/SSF method, the SSF process was modified. In the first stage, which was characteristic for the separate hydrolysis and fermentation (SHF) method, the lignocellulosic substrate was subjected to enzymatic hydrolysis at the optimum temperature for enzymes (50 °C) for 4 h. The substrate was then cooled to 37 °C and inoculated with *C. acetobutylicum* DSM 1731. The method became SSF, since the saccharification (due to the presence of cellulose enzymes in the substrate) and the fermentation (due to *C. acetobutylicum* inoculation) were functioning simultaneously. The initial pH of the medium for both strategies was pH 5.0. The pH was controlled by using 3 M NaOH and 0.5 M H_2SO_4.

To study the effect of mineral compounds supplementation on ABE fermentation, the medium was supplemented with three mineral salts: ammonium sulfate $(NH_4)_2SO_4$ (in the amount of 1.5 g/L), magnesium sulfate $MgSO_4$ (0.75 g/L), and ammonium phosphate $(NH_4)_3PO_4$ (0,75 g/L).

Batch fermentations were performed in a 2-L fermentor with a working volume of 1.5 L, under anaerobic conditions. A constant temperature was kept inside the fermentation chamber by a circulation thermostat H200-H22 (PolyScience, Niles, IL, USA) connected to its water jacket. An anaerobic environment was produced by using purified N_2 gas into the above the surface of the biomass and to the fermentation medium. Fermentation experiments were performed in triplicate.

The sugar fermentation rate was calculated as follows:

$$r_{csp} = \frac{(W_{all} - W_{res}) \times 1.1 - c_{res}v}{vt_{fp}}, \quad (2)$$

where: r_{csp}—sugar consumption rate (g/L/h), W_{res}—residue cellulose content (%), W_{all}—cellulose content of treated corn straw (%), c_{res}—residue sugars concentration (g/L), v—total volume of the fermentation medium (L), t_{fp}—fermentation period (h).

2.5. Analytical Methods

Moisture and dry organic matter were measured according to Polish standard methods PN-92/P-50092. To determine moisture, the samples were dried at 105 °C over 2 h, to constant weight. After this, to quantitate dry organic matter, the materials were mineralized in an oven at 550 °C for 3 h. The total sugar concentration was determined by the Lane-Eynon method. Ethanol concentration (g/L) was determined using a Carl–Zeiss refractometer and alcohol tables, with an earlier prepared 100 mL sample, using the distillation method. Samples were analyzed in triplicate.

An HP 6890 gas chromatograph with a flame ionization detector (FID) was used to determine the content of butanol and acetone of the ABE mixture. A CP-WAX 57-CB capillary column with dimensions of 50 m × 0.25 mm × 0.30 μm (Agilent Technologies, Santa Clara, CA, USA) was used to separate the compounds. Gas chromatograph (GC) operating parameters were as follows: dispenser temperature—210 °C, detector temperature—240 °C sample volume—1 μL, GC oven temperature program-from 40 °C to 160 °C with a rate of 10 °C/min, flow rate of the carrier gas (helium) through the column—30 mL/min. A computer analytical station with Hewlett Packard Chem-Station software was used for integrating the signal and for reporting.

Cellulose, hemicellulose, and lignin contents were determined by the method such as van Soest using the FOSS Fibertec®8000 device (FOSS Analytical A/S, Hillerød, Denmark) equipped with a hot and cold extraction unit. The analysis involved the extraction of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL). This was in accordance with the manufacturer's methodology, ISO 13906:2008 and ISO 16472:2006. The cellulose concentration was calculated by subtracting the ADL value from the ADF content. The hemicellulose concentration was calculated by subtracting the ADF value from the NDF content.

3. Results and Discussion

3.1. Chemical Composition of the Lignocellulosic Feedstock

Clostridium sp. are not able to directly ferment polysaccharides (cellulose and hemicellulose) as a carbon source, therefore an alkali pretreatment followed by enzymatic hydrolysis for corn straw to the ABE fermentation was used. The studies conducted by Kotarska et al. [43] showed that the treatments with alkali $Ca(OH)_2$ is an effective method to remove lignin and a part of hemicelluloses. It was found that the thermochemical pretreatment led to the delignification process, which was an essential step to prepare the raw material prior to the enzymatic release of fermentation sugars. The removal of lignin bonds resulted in a better degradation of the cellulose and hemicellulose fractions during enzymatic hydrolysis because the availability of the biomass surface for cellulolytic enzymes was increased.

One of the steps of preparation of the raw material for ABE fermentation was to carry out detoxification in order to remove compounds that inhibit the growth of anaerobic bacteria, and thus the process of acetone-butanol-ethanol fermentation (ABE). The main toxic products generated during the pretreatment of lignocellulosic biomass were furfural, 5—(hydroxymethyl)furfural (HMF), and phenolic compounds. Activated charcoal adsorption was used to remove inhibitors from hydrolysates in these studies because it has high capacity to absorb compounds and is neutral for sugar and acetic acid [44]. The enzymatic hydrolysis was carried out with the use of a combination of three enzymes: cellulase, hemicellulase, and xylanase. The whole process takes place in three successive stages: cellulase adsorption on the cellulose surface, cellulose hydrolysis to glucose, and cellulase desorption from the material surface. In order to evaluate the effectiveness of the enzymatic hydrolysis, the changes in composition of corn straw were compared before

and after the pretreatment. Furthermore, the amounts of fermentable sugars generated by enzymatic saccharification were determined, Table 1. The contents of cellulose, hemicellulose, and lignin were 19.54 ± 0.45%, 16.41 ± 1.59%, and 4.28 ± 0.20%, (*v/v*) on a dry weight (DW), respectively, and the remaining ingredients were ash and other components (pectins, proteins, and fats), Table 1.

Table 1. Chemical composition of corn straw before and after thermochemical pretreatment and enzymatic hydrolysis.

Chemical Composition	Raw Corn Straw	Treated Corn Straw
Cellulose (% DW)	19.54 ± 0.45	6.55 ± 0.09
Hemicellulose (% DW)	16.41 ± 1.59	5.21 ± 0.17
Total lignin (% DW)	4.28 ± 0.20	3.18 ± 0.15
Other [1] (% DW)	59.77 ± 2.16	85.06 ± 3.21
Total sugars (g/L)	NA [2]	39.78 ± 0.13
Sugar conversion (%)	NA	72.70 ± 1.68

Notes: The table shows mean values and standard deviations. [1] Other—ash + extractives, [2] NA—not available.

The data presented in Table 1 show that the alkaline and enzymatic hydrolysis decreased the content of the cellulose, hemicellulose, and lignin by about 66.5%, 68.3%, and 25.7%, respectively, as compared to the control samples. This result shows that thermochemical pretreatment is a very important step in improving enzymatic saccharification. Table 1 summarizes the amounts of total sugars and sugar conversion from corn straw obtained by chemical pretreatment using $Ca(OH)_2$ and enzymatic hydrolysis. It has been found that two-step pretreatment generated about 39.78 g/L of monosaccharides (with sugar conversion degree of above 70%), which was suitable for the initiation of the biobutanol production in ABE fermentation. With a low amount of sugar, *Clostridium* converts the remaining sugar into more acids than solvents, since the passage from acidogenesis to solventogenesis is disturbed [45,46].

3.2. Application of SSF and Consolidation SHF/SSF Methods

The SSF method is an effective process for biofuel production from biomass [47]. To improve the performance of butanol production, a consolidation SHF/SSF process (instead of conventional SSF) was conducted in this work. The modification consisted in carrying out the enzymatic hydrolysis in optimal conditions for the action of cellulolytic enzymes (i.e., at 50 °C) for a period of 4 h. The biomass was then inoculated with *C. acetobutylicum* and ABE fermentation was carried out under optimal conditions for bacteria, at 37 °C. A consolidation of the SSF and SHF process was aimed to take advantage of the benefits of both of these methods. The initial stage of the hydrolysis SHF/SSF method was performed under conditions that are optimal for cellulases, which ensured a higher concentration of simple sugars at the early stage of fermentation, before moving the operation mode to SSF. The ABE fermentation was initiated before the maximum hydrolysis conversion level was reached. The aim of the study was to determine the effective fermentation mode for the cellulosic biobutanol production, using *C. acetobutylicum* DSM 1731.

The ABE fermentation performance was compared with the results of research by other authors, and shown in Table 2. In most studies, the SSF method carried out by anaerobic bacteria remain great challenges owing to the fermentation rate (low initial sugar concentration) and the mismatched temperature of hydrolysis and fermentation.

Table 2. Comparative performance of ABE fermentation via SSF process.

Feedstock	Microorganism	Fermentation Method	ABE			Reference
			Conc. (g/L)	Productivity (g/L/h)	Yield (g/g)	
Wood chip	*C. acetobutylicum*	SSF	13.4	0.09	0.27	[25]
OPEFB [1]	*C. acetobutylicum*	SSF	7.4	0.06	0.16	[48]
Switchgrass	*C. acetobutylicum*	SSF	12.3	0.10	0.26	[26]
Wheat straw	*C. beijerinckii*	SSF	11.9	0.27	0.42	[35]
Wheat bran	*C. beijerinckii*	SSF	11.8	0.16	0.32	[16]
Corn straw	*C. acetobutylicum*	SSF	17.1	0.20	0.22	[42]
Corn straw	*C. acetobutylicum*	SSF	18.2	0.30	0.31	[49]
Corn straw	*C. acetobutylicum*	SSF	21.3	0.22	0.54	This study
Corn straw	*C. acetobutylicum*	SHF/SSF	23.2	0.24	0.58	This study

Notes: [1] OPEFB—oil palm empty fruit bunch.

It was found that the ABE concentration from various lignocellulosic feedstock on the results by other authors in general ranged from 7.4 to 18.2 g/L (Table 2). In our studies was obtained a relatively high ABE concentration when using the SSF and SHF/SSF processes. In the SSF method, the concentration of ABE was 21.3 g/L, corresponding to an ABE productivity of 0.22 g/L/h. The ABE concentration was higher by 3.1 g/L and 4.2 g/L compared to the results from other authors, who also used corn straw as a substrate for ABE fermentation [42,49]. Wu et al. [49] and Li et al. [42] reported on the ABE production of 18.2 g/L and 17.1 g/L using *C. acetobutylicum*, corresponding to the yield of 0.31 g/g and 0.22 g/g. In contrast, the consolidation SHF/SSF process resulted in the highest ABE production, cause introducing pre-saccharification had increased the initial monosaccharides concentration obtained from degradation of the polysaccharides (cellulose and hemicellulose), before the SSF procedure. It was found that the ABE concentration increased from 21.3 g/L (SSF method) to 23.2 g/L (SHF/SSF method), which is equivalent to a 9% increment. The ABE yield in the SHF/SSF method (0.58 g/g) was 7% higher when compared to the simultaneous process in SSF (Table 2). Qureshi et al. [35] reported on the production of 11.9 g/L of total ABE from wheat straw through an SSF method using *C. beijerinckii*. In this study, an ABE yield of 0.42 g/g was achieved, which was 28% lower when compared to the SHF/SSF method used in our studies. Whereas Sasaki et al. [25], using wood chip as substrate, obtained 13.4 g/L of ABE, corresponding to an ABE yield of 0.27 g/g. The ABE yield was 53% lower than that of the SHF/SSF method used in our studies.

3.3. Effect of Supplementation of Mineral Compounds on ABE Fermentation

The studies also examined the efficiency of ABE fermentation the addition to the use of mineral compounds. The key fermentation metrics, including ABE productivity, sugar consumption rate, and ABE mixture composition were tested. Different processes have been used for the production of butanol from pretreated biomass, including simultaneous saccharification, fermentation (SSF), and consolidation SHF/SSF. The effect of mineral compounds supplementation as a source of nitrogen and nutrients on acetone–butanol–ethanol (ABE) fermentation was investigated. In this study three mineral compounds were used, including ammonium phosphate (dose 0.75 g/L), ammonium sulfate (dose 1.5 g/L), and magnesium sulfate (dose 0.75 g/L). The control sample consisted of a treated cellulosic biomass with no added mineral salts. Figures 1 and 2 show the time course of butanol production and ABE productivity depending on the fermentation method and the mineral compounds supplementation into the medium.

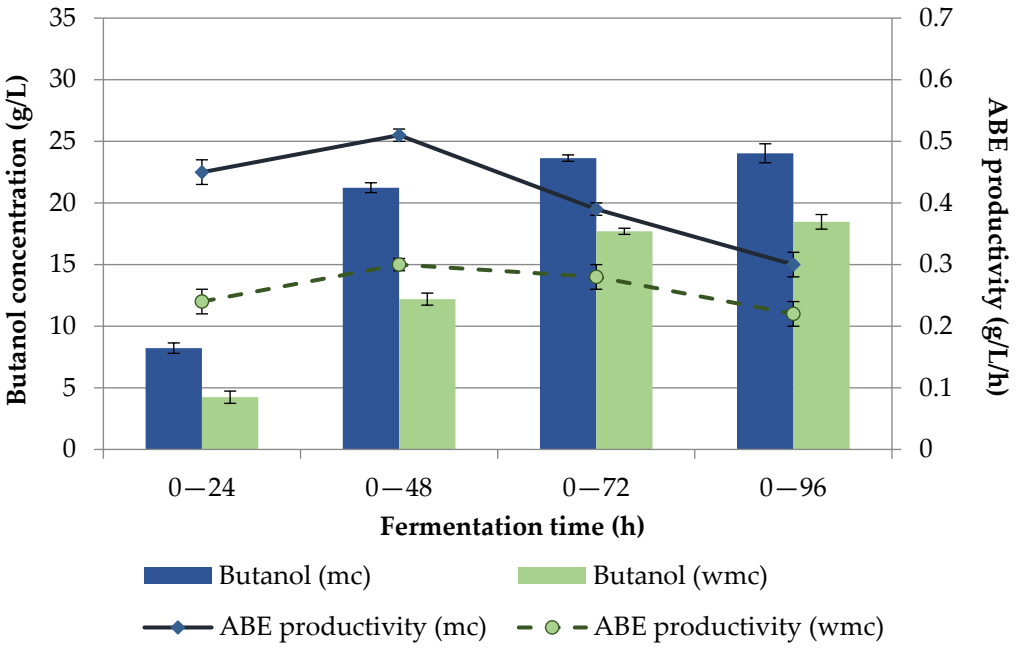

Figure 1. Butanol production and ABE productivity with simultaneous hydrolysis and fermentation (SSF) processes (SSF mc—with mineral compounds; SSF wmc—without mineral compounds).

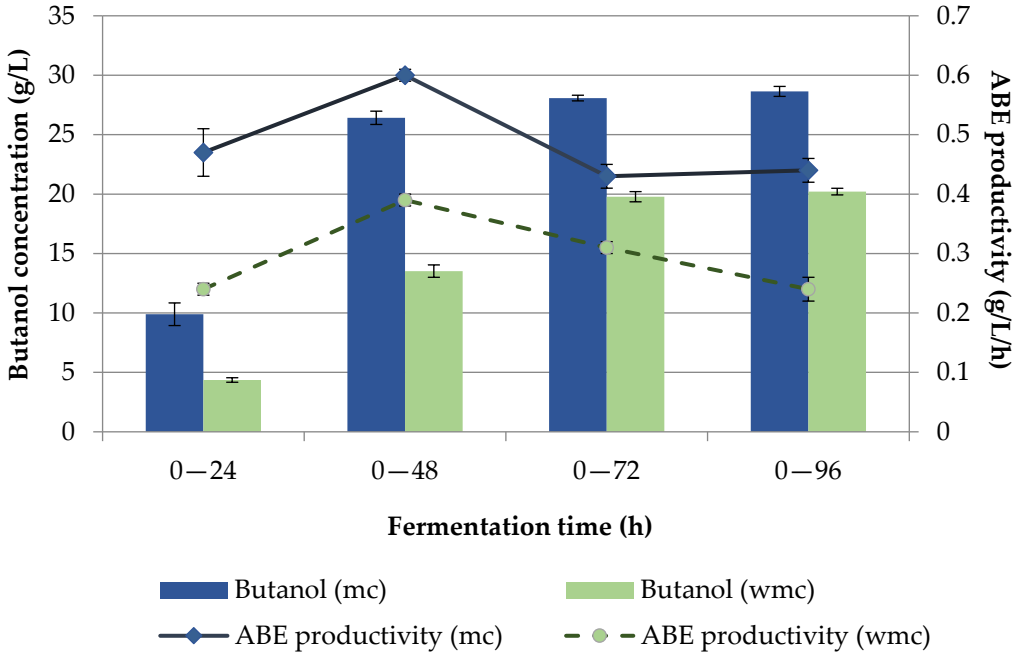

Figure 2. Butanol production and ABE productivity through consolidation SHF and SSF (simultaneous saccharification and fermentation) processes (SHF/SSF mc—with mineral compounds; SHF/SSF wmc—without mineral compounds).

The ABE productivity was calculated as the total ABE concentration in g/L divided by the fermentation time (h) and expressed as g/L/h. The fermentation time is defined as the difference in the period between inoculation and the end of fermentation expressed in h. Based on the results in Figures 1 and 2, it was found that the drastic increase of butanol production was observed following a fermentation time of 24 h for both the SSF and consolidation SHF/SSF methods. At that time (in 48 h), the concentration of butanol was 12.20 g/L–for the SSF process without mineral compounds (SSF wmc), corresponding

to a ABE productivity of 0.299 g/L/h and 13.52 g/L–for consolidation SHF/SSF process without mineral compounds (SHF/SSF wmc), corresponding to a ABE productivity of 0.331 g/L/h. This indicated that about 66–67% of the overall butanol concentration was obtained at 48 h. However, when mineral compounds were supplemented into the medium, about 88–92% of the total butanol concentration was obtained in 48 h. In the SSF with mineral compounds, the butanol amount at 48 h was about 21.24 g/L, with 0.511 g/L/h of ABE productivity. In the SHF/SSF consolidation with mineral compounds, the butanol concentration at 48 h was about 26.42 g/L, with 0.596 g/L/h of ABE productivity.

The data presented in Figures 1 and 2 and Table 3 show that the mineral compounds supplementation has a stimulating effect on the *Clostridium* and contributes to an increase in the productivity of fermentation. In supplemented samples with ammonium and magnesium salts, an increase in the content of butanol and total ABE productivity was observed compared to the controls.

Table 3. Characteristics of acetone–butanol–ethanol fermentation of the corn straw under SSF and SHF-SSF processes with and without mineral compounds.

Products and Fermentation Parameters	SSF		SHF/SSF	
	With Mineral Compounds	Without Mineral Compounds	With Mineral Compounds	Without Mineral Compounds
Acetone (g/L)	$1.85^{c} \pm 0.06$	$1.23^{a} \pm 0.04$	$1.83^{c} \pm 0.03$	$1.64^{b} \pm 0.04$
Butanol (g/L)	$24.03^{c} \pm 0.77$	$18.47^{a} \pm 0.59$	$28.64^{d} \pm 0.42$	$20.21^{b} \pm 0.28$
Ethanol (g/L)	$2.48^{d} \pm 0.10$	$1.64^{c} \pm 0.02$	$0.86^{a} \pm 0.02$	$1.31^{b} \pm 0.02$
Total ABE (g/L)	$28.35^{c} \pm 0.61$	$21.33^{a} \pm 0.52$	$31.33^{d} \pm 0.37$	$23.15^{b} \pm 0.30$
Incubation time (h)	96	96	96	96
ABE yield (g/g)	$0.71^{c} \pm 0.02$	$0.54^{a} \pm 0.01$	$0.79^{d} \pm 0.02$	$0.58^{b} \pm 0.02$
Butanol yield (g/g)	$0.60^{c} \pm 0.02$	$0.46^{a} \pm 0.02$	$0.72^{d} \pm 0.02$	$0.51^{b} \pm 0.01$
ABE productivity (g/L/h)	$0.295^{c} \pm 0.005$	$0.222^{a} \pm 0.006$	$0.327^{d} \pm 0.004$	$0.241^{b} \pm 0.003$
Butanol productivity (g/L/h)	$0.250^{b} \pm 0.006$	$0.192^{a} \pm 0.008$	$0.298^{c} \pm 0.006$	$0.211^{a} \pm 0.003$
Fermentable sugars (g/L)	$26.48^{b} \pm 0.25$	$24.26^{a} \pm 0.08$	$27.20^{c} \pm 0.13$	$24.81^{a} \pm 0.18$
Sugar fermentation rate (g/L/h)	$0.148^{b} \pm 0.003$	$0.128^{a} \pm 0.001$	$0.155^{c} \pm 0.002$	$0.130^{a} \pm 0.002$

Notes: The table shows mean values and standard deviations. Mean values designated by different letters and placed in the same row differ statistically significantly at $p < 0.05$; $n = 3$.

In the SSF, without mineral compounds supplementation, the total ABE (acetone, butanol, ethanol) concentration was 21.33 g/L, and butanol was 18.47 g/L in a fermentation time of 96 h, with productivities representing 0.222 and 0.192 g/L/h, respectively. The butanol productivity (g/L/h) was calculated as the ratio of butanol concentration (g/L) to the fermentation time (h). It was found that when mineral salts were supplemented into the fermentation medium, the maximum butanol and total ABE amount increased by 30% and 33%, respectively, which consequently increased concentration to 24.03 g/L (butanol) and 28.35 g/L (ABE) in a fermentation time of 96 h, with productivities representing 0.250 and 0.295 g/L/h, respectively. In the SSF with mineral compounds (SSF mc), an ABE yield of 0.71 g/g and butanol yield of 0.60 g/g were calculated. These values were higher than in the control sample without mineral addition (SSF wmc) by 24% (0.54 g/g) and 23% (0.46 g/g), respectively. In the SHF/SSF with mineral compounds (SHF/SSF mc), the ABE and butanol yield were 0.79 g/g and 0.72 g/g, respectively. This is equivalent to 13% (ABE yield) and 16% (butanol yield) increments when compared to the SHF/SSF without mineral addition (SHF/SSF wmc).

Based on the results in Figure 1, a higher concentration of butanol was found in each day of the process: about 3.9 g/L over the period of 0–24 h, 9.0 g/L over 0–48 h, 5.9 g/L over 0–72, and 5.6 g/L over 0–96 h compared to those achieved with the control without mineral compounds supplementation.

In the SHF/SSF method the addition of ammonium phosphate $(NH_4)_3PO_4$, ammonium sulfate $(NH_4)_2SO_4$, and magnesium sulfate $MgSO_4$ into the medium increased the

rate of ABE fermentation by *Clostridium* and the concentration of butanol and total ABE (acetone, butanol, ethanol). The mineral salts supplementation had a positive effect on the microorganisms activities, leading to an earlier initiation of the solventogenesis stage. This was confirmed by the fact that a DSM 1731 strain can produce 9.89 g/L of butanol during the first 24 h as compared to the control sample, which can produce 5.36 g/L. Over the period of 0–48 h, 0–72 h, and 0–96 h, the amount of butanol was higher: about 12.9 g/L, 8.3 g/L, and 8.4 g/L, respectively. Compared to the control sample without mineral compounds supplementation, the final ABE and butanol concentrations were increased to 31.33 and 28.64 g/L from 23.15 and 20.21 g/L, which consequently increased productivities by 36% (ABE) and 41% (butanol). Birch and Walker [50] proved that during fermentation, magnesium ions protect the yeast cells against ethanol, osmotic and temperature. Rees and Stewart [51] reported that over 300 enzymes require the presence of Mg^{2+} as a cofactor, including that necessary for glycolytic, alcohol, and fatty acid biosynthesis. The interference in the fermentation process through applying mineral compounds does not radically change the biobutanol production technology, but it improves the fermentative abilities of bacteria by stimulation of biological processes occurring in cells and biochemical processes proceeding in the fermentation media.

The synthesis of butanol, acetone and ethanol ceased after 96 h for both methods. The monosaccharides (polysaccharide breakdown products) obtained in the process of chemical pretreatment and enzymatic hydrolysis were almost completely utilized for ABE production by the cells of the microorganisms. For the SSF method with mineral compounds supplementation, the amount of fermentable sugars was 26.48 g/L (88% of fermentable sugars were utilized), while for the consolidation SHF/SSF method with mineral compounds supplementation, the amount of fermentable sugars were 27.20 g/L (90% of fermentable sugars were utilized). Therefore, the value of the sugar fermentation rate was 0.148 g/L/h and 0.155 g/L/h, respectively (Table 3). These values were higher than from the control samples without the addition of mineral salts by 16% (for the SSF) and 19% (for the SHF/SSF).

3.4. The Mass Balance of Pretreatment and ABE Fermentation in Relation to Two Methods: SSF and SHF/SSF

Figure 3 presented the polysaccharides balance in the pretreatment process (thermochemical and enzymatic hydrolysis) and ABE fermentation. It was expressed in relation to the SSF method and the SHF/SSF consolidation method (with and without mineral compounds supplementation). The doses of minerals are presented in Section 2.4. The mass balance indicates that the content of polysaccharides (cellulose, hemicellulose) and lignin of 1 kg of raw material was 335.0 g and 39.9 g, respectively. After the process, the amount of the above-mentioned polysaccharides and lignin in hydrolysate was 12.9 g and 3.5 g, respectively, and the final solid content was about 11%.

After 72 h of fermentation, the ABE (acetone–butanol–ethanol) ratio in the fermentation medium was 0.6:8.5:0.9, with about 85% butanol obtained from the SSF method with mineral compounds supplementation. Meanwhile, the ratio of acetone–butanol–ethanol from consolidation SHF/SSF was 0.6:9.1:0.3, with about 91% butanol. For both methods, the butanol content in the overall ratio of solvents was much higher as compared to that (60%) in the typical ABE fermentation. It was found that the highest concentration of ABE and butanol were obtained in the consolidation SHF-SSF method with mineral compounds supplementation. The amount of ABE in this method was higher by 11%, compared to SSF with mineral compounds supplementation. The data presented in Figure 3 show that butanol concentration in the consolidation SHF/SSF method with supplementation was higher by 8.43 g/L compared to the SHF/SSF without supplementation, by 4.61 g/L compared to the SSF method with supplementation, and by 10.17 g/L to SSF without supplementation. The ABE fermentation in the SHF/SSF also proceeded faster than in the SSF method, since the fermentable sugars in the SHF/SSF method were available to initiate the butanol production. The SSF method has limited fermentable sugars availability

due to a different temperatures for saccharification and fermentation, which is why in the consolidation SHF/SSF method can be increased enhance the productivity as compared to SSF. This is because the *Clostridium* inoculated after 4 h of saccharification, whereas the fermentable sugars were readily available by microorganisms.

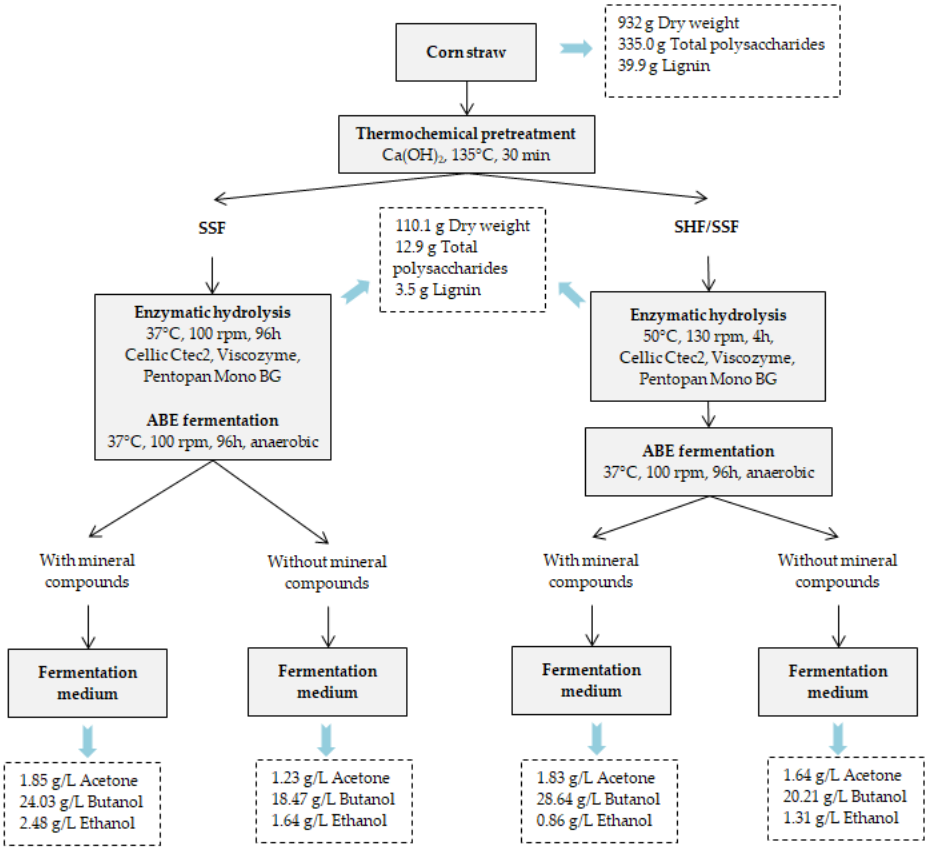

Figure 3. Mass balance of the process ABE fermentation including pretreatment and enzymatic hydrolysis.

As far as we know, there are a few studies about the cellulosic butanol production with the modified SSF and SHF methods. Cheng et al. [4] demonstrated a process to convert agricultural waste into butanol using a combination of SHF with simultaneous saccharification and fermentation (sequential SHF–SSF) processes. They reported that the maximum butanol concentration for rice straw and bagasse were 2.92 g/L and 2.29 g/L, respectively. Furthermore, Husin et al. [29] conducted the DSSF (delayed simultaneous saccharification and fermentation) process of sago hampas to improve biobutanol amount and productivity. The concentration of butanol was achieved at approximately 4.62 g/L, with a yield and productivity of 0.11 g-biobutanol/g-sugar and 0.06 g/L·h, respectively.

4. Conclusions

This study has shown that the macro-elements and micro-elements play a significant role in fermentation processes. The highest potential of butanol production from cellulosic biomass among the tested methods was obtained in the developed consolidation SHF/SSF method with mineral compounds. For the experiments with mineral salts addition into the fermentation medium, higher ABE and butanol concentrations were achieved for the SSF and SHF/SSF methods. Our experimental results validated the positive effect of ammonium sulfate, magnesium sulfate and ammonium phosphate supplementation on the rate of ABE fermentation and butanol production. Compared to the sample control without mineral compounds, earlier initiation of solventogenesis occurred, making the about 88–92% of the total butanol concentration was obtained within 48 h of ABE fermentation. In addition, it is

important to note that adding minerals into the fermentation medium does not radically change the biobutanol production technology but visibly improves the ABE production.

The ratio of acetone–butanol–ethanol from the consolidation SHF/SSF method with mineral compounds supplementation was much higher: 0.6:9.1:0.3, with about 91% butanol as compared to that in the typical ABE fermentation.

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