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Enzymatic One-Pot Hydrolysis of Extracted Sugar Beet Press Pulp after Solid-State Fermentation with an Engineered *Aspergillus niger* Strain

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Abstract: Extracted sugar beet press pulp (SBPP) is a promising agricultural residue for saccharification and further bioconversion. Combining solid-state fermentation of SBPP with engineered *Aspergillus niger* for enzyme production followed by hydrolysis of additionally added SBPP in the same bioreactor was studied to produce a sugar solution (hydrolysate) in a one-pot process. The initial aerobic solid-state fermentations were carried out in duplicate on non-milled, wet SBPP (moisture content of 72% (*w/v*)) with an *A. niger* strain engineered for constitutive pectinase production for 96 h, and this resulted in polygalacturonase activities of up to 256 U mL^{-1} in the wet media. Afterwards, water was added to the bioreactor, and the remaining solids were suspended by stirring to dissolve the hydrolytic enzymes. Metabolic activities of *A. niger* were inactivated by a N_2-atmosphere and by increasing the temperature to 50 °C. High solid loads of milled SBPP were added to the stirred-tank reactor with a delay of 24 h to enable sugar yield calculations based on the compositional analysis of the SBPP used. The resulting final sugar concentrations of the hydrolysate after 166 h were 17 g L^{-1} D-glucose, 18.8 g L^{-1} L-arabinose, and 12.5 g L^{-1} D-galacturonic acid, corresponding to sugar yields of 98% D-glucose, 86% L-arabinose, and 50% D-galacturonic acid, respectively. Including the other sugars released during enzymatic hydrolysis in the one-pot process (D-xylose, D-mannose, D-galactose), a total sugar concentration of 54.8 g L^{-1} was achieved in the hydrolysate. The one-pot process combining hydrolytic enzyme production in solid-state fermentation with high solid loads during enzymatic hydrolysis of the milled SBPP reduces hydrolytic process costs by replacing chemical pre-treatments, enabling the in situ production of SBPP-adapted hydrolytic enzymes, as well as avoiding intermediate enzyme extraction and preparation steps.

Keywords: *Aspergillus niger* ATCC 11414; pectinases; solid-state fermentation; enzymatic hydrolysis; sugar beet press pulp; integrated process

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1. Introduction

The world's population is growing and predicted to reach 9.7 billion by 2050 [1]. Increasing consumption and food demand [2], coupled with ambitious CO_2 emission reduction targets, are some of the major challenges we are facing today [3]. The agricultural sector is responsible for about 24% of the worldwide greenhouse gas emissions [3]. The circular economy is a promising approach to reduce the emissions of greenhouse gases while meeting the increased demand for food and consumer products. The idea behind the circular economy is improving economic performance by sustainable and efficient usage of our natural resources, including a strongly reduced waste generation [4]. Additionally, innovative technologies are needed to minimize or eliminate using material from fossil or non-renewable sources [3,5]. Thereby, products can be produced sustainably, and, at the

same time, greenhouse gas emissions are reduced [3]. One possibility is the more efficient utilization of agricultural wastes, since they are available in extremely large quantities worldwide [6], are not competing with cropland for food production [7], contain valuable building blocks, e.g., for the biorefinery, and their potential is often not fully exploited, as they are often burned or landfilled [8].

Extracted sugar beet press pulp (SBPP) is one example of an agricultural residue that accrues after sucrose extraction from sugar beet. Only a small amount of this residue is used for animal feeding or concrete production, while most of it is discarded as waste [9]. The pulp consists of the sugar polymers pectin, cellulose, and hemicellulose, as well as small amounts of lignin and proteins [10–12]. The most abundant monomeric components of the sugar polymers in SBPP are D-galacturonic acid (D-GalA), arabinose, and glucose [10]. For further valorization of SBPP and to make it accessible for further biotransformations, it is necessary to release its sugar monomers [11]. Suitable methods for the degradation of SBPP are acid hydrolysis, hydrothermal techniques, and enzymatic hydrolysis [13–15]. However, chemical and thermal methods may not be sustainable due to high energy consumption [16] and the formation of non-fermentable and toxic byproducts [17]. Though less efficient compared to the chemical breakdown, enzymatic hydrolysis promises to be an energy-saving approach due to low reaction temperatures and mild pH conditions, as well as no formation of toxic byproducts [16–18].

In industrial hydrolysis processes, such as cellulosic ethanol production, the costs for enzymes are the major expenses [19]. Filamentous fungi are the major producers of hydrolytic enzymes, such as cellulases and hemicellulases [20]. A commonly used fungus for hydrolytic enzyme production is *Aspergillus niger* [21], which also shows superior pectinolytic activities, demonstrated to be further boosted by deletion of the negative pectinase regulator GaaX [22]. Previously, we evaluated different non-engineered *A. niger* strains for their natural polygalacturonase activities using controlled batch cultivations with 2% pectin minimal medium in stirred-tank bioreactors, and the *A. niger* strain ATCC 11414 was identified as the best producer [23]. The hydrolysis of 90 g L^{-1} milled SBPP was studied in subsequent submerged batch processes with sterile filtered enzyme supernatants, resulting in the release of 8.8 g L^{-1} of D-GalA, which corresponded to a D-GalA yield of ~36.4% [23]. Using engineered *Saccharomyces cerevisiae*, the resulting D-GalA solution may then be reduced within a consecutive biotransformation process with a product selectivity of 97% to the higher value product L-galactonate (L-GalOA) [24,25], which was shown to have promise, for example, as an alternative leavening agent in baking applications [26].

Bioprocess integration, combining hydrolytic enzyme production and subsequent enzymatic hydrolysis of the raw material in a one-pot process, can lower the total process costs due to the eliminated separation of the hydrolytic enzymes [27,28]. However, this is only possible if the enzyme producer is no longer able to further metabolize the released sugar monomers—either by genetic modifications or changes in the process conditions between enzyme production and subsequent enzymatic hydrolysis. Different one-pot strategies for enzyme production and enzymatic saccharification are described in the literature. Some focus on combining pre-treatment of waste material and hydrolysis in one bioreactor, such as pre-treatment and saccharification of industrial hemp residue with 2% (*w/v*) oxalic acid and enzymes generated by SSF of mixed substrates [29]. Solid-state fermentation (SSF) of sugarcane bagasse and wheat bran with a co-culture of *Aspergillus oryzae* and *Trichoderma reesei* was studied in 250 mL Erlenmeyer flasks to produce a hydrolytic enzyme mixture followed by hydrolysis of freshly added raw material and a sodium citrate buffer. The hydrolysate was supplemented with nutrients required for growth and then used for ethanol production by *Saccharomyces cerevisiae* fermentation for 9 h at 34 °C and 250 rpm with 85.3% of the theoretical yield [28].

In submerged processes usually used for hydrolytic enzyme production and subsequent enzymatic hydrolysis, the concentrations of milled solids, such as SBPP, are limited in the aqueous phase. In contrast, SSF processes enable higher solid concentrations and have the advantage of more similar culture conditions compared to natural habitats, over-

all leading to higher enzyme activities compared to submerged fermentations [30–32]. Furthermore, the risk of contamination is lower with solid media because fungi have lower moisture requirements compared to bacteria [33]. Additionally, SSF produces less wastewater and usually has lower process costs if agricultural waste is used as raw material [30,31,33,34]. Finally, the released sugar concentrations after enzymatic hydrolysis may be increased, thus contributing to the solution of one of the major challenges in the valorization of agriculture residues, resulting low sugar, and, therefore, final product concentrations [35,36].

SSF processes with *A. niger* described in the literature were mostly performed on a small-scale in shake flasks or disposable Petri dishes under static conditions [32,37–39]. For larger-scale SFF, special SFF bioreactors were used, such as stainless steel trays, column-trays, or rotating drum bioreactors [40–42]. A pilot scale SSF was applied with an *Aspergillus* species on citrus pulp and sugarcane bagasse in a packed bed bioreactor, resulting in pectinase yields between 33–41 U g^{-1} dry mass. The enzymes were separated from fermented citrus pulp and sugarcane bagasse solids by lyophilization for 24 h at −45 °C and 0.1 mbar, followed by the extraction of 5 g lyophilizated solids with 100 mL acetate buffer (pH 4.5) for the following hydrolysis of a pectin suspension [43]. To the best of our knowledge, there are no reports on the one-pot combination of SSF for producing hydrolytic enzymes with *A. niger* in combination with the enzymatic hydrolysis of an agricultural residue to yield the sugar monomers in solution.

In the present study, we combined the utilization of a polygalacturonase-deregulated *A. niger* ATCC 11414 strain (by deletion of the repressor GaaX) with a SSF setup for increased polygalacturonase secretion, and we integrated this in a one-pot approach with a subsequent enzymatic hydrolysis of milled SBPP by applying a scalable stirred-tank bioreactor for the suspension of high solid concentrations.

2. Materials and Methods

2.1. Fungal Strain

The recombinant *Aspergillus niger* strain ATCC 11414 Δ*gaaX* was generated by a CRISPR/Cas9 deletion approach, as described below. The knockout of the repressor GaaX leads to a constitutive activation of the transcription factor GaaR [22]. Thereby, the polygalacturonase production of the fungus is constitutively activated.

2.1.1. Cloning of the *A. niger gaaX* Deletion Construct

A deletion construct for the *gaaX* gene in *A. niger* was constructed via Gibson assembly [44]. The 5′-flank upstream of *gaaX*, consisting of 983 base pairs (bp), as well as the 3′-flank downstream of the coding sequence (1003 bp), amplified from *A. niger* NRRL3 gDNA, together with the AmpR-ColE1 backbone, amplified from the plasmid pYTK095, including flanking NotI recognition sites (Addgene Headquarters, Watertown, MA, USA; constructed by the John Dueber lab, UC Berkeley), were used. NotI restriction sites were used to generate a linear repair template prior to *A. niger* transformation. A CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR associated protein 9) plasmid was constructed via Golden Gate cloning, containing (i) the AMA1_2.8 fragment for stable plasmid maintenance in *A. niger* [45], (ii) the *Aspergillus nidulans pyrG* marker under its endogenous promoter and terminator as used on pFC902 to avoid recombination with the genomic *pyrG* locus in *A. niger* [46], (iii) the *A. niger* codon-optimized Cas9-NLS coding sequence under control of *A. nidulans* endogenous TEF1 promoter and *trpC* terminator as used on plasmid pFC334 [47], (iv) a single guide RNA expressed under the *A. niger* RNA Polymerase III-driven 5S rRNA promoter, as described by Zheng et al. [48], targeting the *gaaX* locus in *A. niger*, and (v) the AmpR-ColE1 backbone amplified from the plasmid pYTK095 (Addgene Headquarters, Watertown, MA, USA; constructed by the John Dueber lab, UC Berkeley) was co-transformed (pFC334, Addgene Headquarters, Watertown, MA, USA; deposited by the Uffe Mortensen lab, DTU), promoting a double-strand break in the *gaaX* genomic region to enhance homologous recombination [49].

2.1.2. Genetic Manipulation of *A. niger*

A. niger was transformed and selected via polyethylene glycol-mediated protoplast transformation according to established protocols [50]. First, a strain version of the *A. niger* ATCC 11414 lineage optimized for targeted genetic engineering was generated by disruption of the *ku70* locus by an *A. nidulans amdS* expression cassette using the same design as implemented in the *A. niger* strain MA70.15 [51,52]. Selection after transformation of the linearized *kusA* disruption cassette was carried out on minimal media, containing acetamide as the sole nitrogen source, and correct integration was verified via Southern blot. Next, the entire coding sequence of the *pyrG* locus was eliminated via transformation of a homologous repair template, containing 1 kb of genomic sequence immediately upstream and 1 kb of genomic sequence immediately downstream of the *pyrG* coding sequence. *GaaX* was knocked out via co-transformation of the above-described *gaaX* deletion construct, and the *gaaX*-targeting CRISPR/Cas9 plasmid and selection was carried out on minimal media without uracil. To validate positive transformants and to check for deletion, colony PCRs were executed using a primer binding at the end of the 5′-flank of *gaaX* and a primer binding in the genomic region downstream of the 3′-flank (Table S1). Positive strains were also tested with a second colony PCR using a primer binding in the coding sequence of *gaaX* and the primer binding downstream of the 3′-flank. Colonies containing the coding sequence were discarded.

Extraction of genomic DNA for colony PCRs was accomplished by grinding 0.5 g of freeze-dried hyphal biomass grown in liquid culture and incubating the powder in 500 µL of extraction buffer (0.2 M Tris-HCl pH 8, 0.025 M EDTA, 0.5% SDS) at 65 °C for 15 min. After the addition of 100 µL of 8 M potassium acetate solution and 15 min of centrifugation at 16,000× *g*, 300 µL of isopropanol was used to precipitate the DNA, and the pellet was washed twice with 70% ethanol and dissolved in water. After colony PCR validation, the strain was counter-selected on minimal media, supplemented with uracil and 5-fluoroorotic acid to eliminate the CRISPR/Cas9 plasmid after successful deletion of *gaaX*.

2.2. Sugar Beet Press Pulp

Südzucker AG (Mannheim, Deutschland) provided the dried extracted sugar beet press pulp (SBPP). The label on the delivered buckets was "26.03.2018, 201720217". Part of the SBPP was delivered with smaller particle sizes (milled). The dried SBPP was stored at 4 °C and once more dried at 50 °C for at least 48 h before use. The granulometric analysis of the particle size distributions was carried out in triplicates. 15 g of dried SBPP was sieved for 30 s. The results are listed in Table 1.

Table 1. Granulometric analysis of dried SBPP fractions (milled and non-milled).

SBPP Fraction	Particle Size x, mm	Fractional Share, %	Standard Deviation, %
Non-milled	4.0 < x < 6.3	10.48	3.71
	2.0 < x < 4.0	62.76	2.43
	0.5 < x < 2.0	26.76	1.74
Milled	1.0 < x < 2.0	8.28	9.61
	0.5 < x < 1.0	57.22	5.52
	x < 0.5	34.55	11.22

2.3. Sugar Beet Press Pulp Composition Analysis

The dried SBPP was crushed with glass or stainless-steel beads in a mixer mill (MM200, RETSCH GmbH, Haan, Germany) at 25 Hz for 10 min. Then, 100 mg of the crushed SBPP was incubated with 20 mL of 2 M trifluoroacetic acid at 100 °C for 6 h. Afterwards, the samples were transferred to a measuring cylinder with 2.5 mL 1 M of sodium acetate buffer and 5 mL of water and adjusted to pH 5 with 10 M NaOH and 9 M H_2SO_4. Distilled water was added to achieve a final volume of 40 mL. Before usage, the enzyme mixtures Viscozym® L (V2010) und Pectinase (P2611) from *Aspergillus aculeatus* (both Sigma-Aldrich®, Merck

KGaA, Darmstadt, Germany) were concentrated with centrifugal concentrators with a molecular weight cut-off of 10,000 Da (Vivaspin® sample concentrators, VWR International GmbH, Darmstadt, Germany) and washed three times with 50 mM of sodium acetate buffer (pH 5) and re-suspended in the initial volume (5 mL). The enzyme solutions were pooled (1:1) and diluted 1:250 in 50 mM of sodium acetate buffer (pH 5), and kanamycin (50 mg L^{-1}) was added. After acid hydrolysis, 10 mL of enzyme solution was added to 40 mL of pre-treated SBPP samples (final enzyme dilution 1:1250) in 100 mL bottles.

The enzymatic hydrolysis was carried out at pH 5, 50 °C, and shaken with a frequency of 120 min^{-1} (WiseCube, witeg Labortechnik GmbH, Wertheim, Germany) for 60 h. The concentrations of the sugars were determined via HPAEC-PAD (ICS 3000, Thermo-Fisher). The instrument setup was a Dionex AS Autosampler, a Dionex gradient mixer GM-3 (Dionex Corp., Sunnyvale, CA, USA), a CarboPac PA1 standard bore guard column (4 × 50 mm), and a CarboPac PA1 preparative IC column (4 × 250 mm, both Thermo Fisher Scientific Inc., Waltham, MA, USA). A linear gradient of 100–250 mM sodium acetate in 100 mM of sodium hydroxide solution (prepared in deionized water) was applied for 12.5 min at 1 mL min^{-1} and 30 °C. A correlation of standards with known sugar concentrations to peak area for the determination of the respective concentrations was generated (D-xylose, D-mannose, L-fuctose, L-arabinose, D-glucose, L-rhamnose, D-GalA, D-galactose, D-fructose, and D-gluconic acid) to calculate the sugar concentrations of the samples. To estimate the loss of monosaccharides during pre-treatment of the SBPP samples, 1 mL of a standard containing 1 g L^{-1} of each monosaccharide and cellulose (Avicel® PH-101, Merck KGaA, Darmstadt, Germany) was treated in the same way in parallel. The measured sugar concentrations of the SBPP samples were corrected with the resulting sugar loss compensation factors (LCF), and all composition analysis measurements were carried out in triplicates. The non-hydrolyzed biomass of the SBPP samples was determined gravimetrically in triplicates after drying at 80 °C for at least 24 h.

2.4. Preculture, Inoculum Preparation, and Cultivation Media

Forty microliter glycerol stocks of *A. niger* ATCC11414 Δ*gaaX* spores were each distributed on 39 g L^{-1} potato extract glucose agar plates (Carl Roth GmbH+Co.KG, Karlsruhe, Germany), which were supplemented with 10 g L^{-1} yeast extract, a final concentration of 1 mM uridine, and 1 mL L^{-1} of trace elements solution. After 5 days of incubation at 30 °C, the spores were harvested using a sterile 0.89% (*w/v*) NaCl solution with 0.05% (*v/v*) Tween 80. The spore suspension was filtered with sterile cotton wool and diluted to the desired spore concentration. The trace element solution (L^{-1}) consisted of the following: 10 g EDTA, 4.4 g $ZnSO_4 \cdot 7\,H_2O$, 1.01 g $MnCl_2 \cdot 4\,H_2O$, 0.32 g $CoCl_2 \cdot 6\,H_2O$, 0.32 g $CuSO_4 \cdot 5\,H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, 1.47 g $CaCl_2 \cdot 2\,H_2O$, and 1 g $FeSO_4 \cdot 7\,H_2O$ [53].

The SSF fermentation was performed with 80 g of SBPP, 200 mL of 100 mM sodium citrate citric acid buffer (pH 4.5), and 85 mL of liquid medium concentrate, resulting in final concentrations in the liquid phase of 307 mM $(NH_4)_2SO_4$, 12.3 mM KCl, 19.3 mM KH_2PO_4, 3.5 mM $MgSO_4$, 1.8 mM uridine, and 1.8 mL L^{-1} trace element solution. The initial moisture content of the SBPP was 72% (*w/v*). SBPP was autoclaved with the sodium citrate buffer, and the additives were added (sterile) afterwards.

2.5. Solid-State Fermentation

A 3.6 L stirred-tank bioreactor designed for solid substrates and enzymatic bioprocesses equipped with an anchor stirrer, a one-fold helical stirrer, and a pitched-bladed impeller (45°) (Labfors, Infors-HT, Bottmingen, Switzerland) was used for the solid-state fermentation of SBPP with *A. niger*. The stirrers shown in Figure 1 were mounted at the following positions on the axes: the anchor stirrer at the lower end of the stirrer shaft, the one-fold helical stirrer without a gap directly above the anchor stirrer, followed by the pitched-blade impeller with a gap of 3 cm. There was no stirring during SSF because the electrical drive of the bioreactor was not able to move the stirrers. The SSF processes were inoculated with a spore density of 6.25 × 10^6 spores g^{-1} SBPP by distributing them

manually in the SBPP media in a beaker glass and filling the inoculated SBPP into the bioreactor via a port in the lid using a sterile spatula until a filling height of 5 cm was achieved. Inoculation and filling of the bioreactor were performed in a clean bench (SWB Klasse 1 FAZ 3, WALDNER Holding SE & Co. KG, Wangen, Germany). The temperature was controlled at 30 °C, and the gas flow rate was fixed to 1 L of humidified sterile air h^{-1} via headspace aeration. For humidification, the sterile air was passed through a bottle filled with 500 mL of sterile deionized water at room temperature. To prevent initial spore loss, aeration was initiated 4 h after inoculation. The exhaust gas composition (O_2, CO_2) was monitored online (BlueVary, BlueSens Gas Sensor GmbH, Herten, Germany).

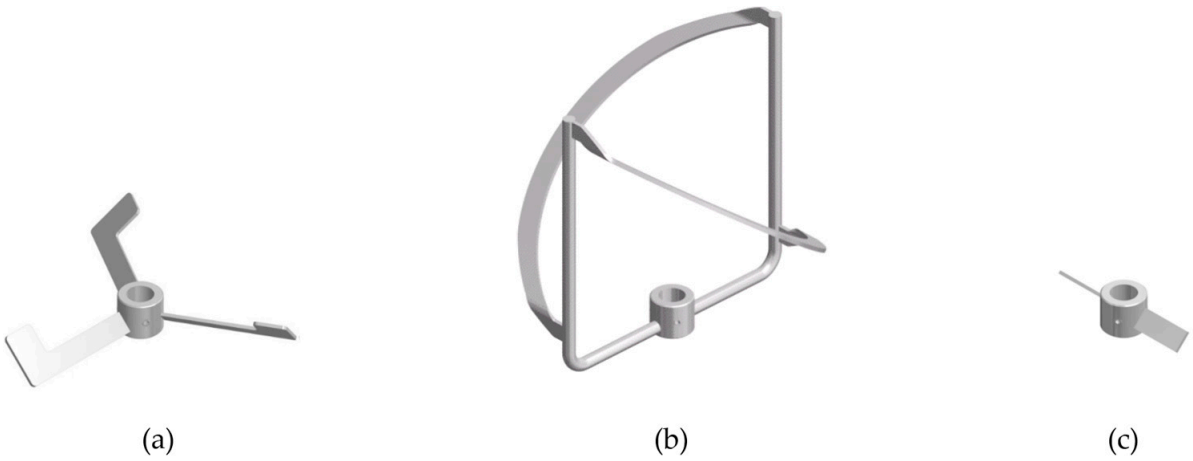

Figure 1. Stirrers made of steel used for SSF and subsequent hydrolysis of SBPP: (**a**) anchor stirrer with a maximum diameter of 14 cm and a maximum height of 2.2 cm, (**b**) one-fold helical stirrer with a maximum diameter of 14 cm and a maximum height of 13.5 cm, and (**c**) pitched-bladed impeller (45°) with a diameter of 7 cm. Stirrer figures adapted from Labfors 5 operating manual (Labfors, Infors-HT, Bottmingen, Switzerland).

2.6. Subsequent Hydrolysis of Sugar Beet Press Pulp

After the production of the hydrolytic enzymes by SSF, 1.715 L of sterile water was added to the stirred-tank bioreactor via a sterile port in the bioreactor lid. Gassing was switched to sterile nitrogen gas until the exhaust gas analyzer detected no more oxygen. The temperature was raised to 50 °C, and the agitation rate was set to 220 rpm. pH 4.5 was controlled by the addition of 1 M KOH or 0.5 M H_2SO_4. After each sampling via the sampling tube, the bioreactor was rinsed with sterile nitrogen gas until the oxygen coming into the reactor during sampling was removed. An amount of 220 g of milled SBPP previously autoclaved with 200 mL of water was added after 24 h of hydrolysis in the bioreactor.

2.7. Quantification of Sugars and Organic Acids in Hydrolysis Supernatants

Concentrations of sugars and organic acids in the hydrolysis samples were measured by HPLC (1100 Series, Agilent Technologies, Santa Clara, CA, USA) using a Rezex ROA-Organic Acid H+ (8%) LC (300 × 7.8 mm) cation exchange column with a SecurityGuard Cartridge Carbo-H (4 × 3.0 mm) pre-column (both Phenomenex Ltd., Aschaffenburg, Germany). The samples were inactivated at 90 °C for 10 min and filtered with a 0.2 µm cellulose filter (Chromafil RC20/15 MS, Macherey-Nagel GmbH & Co., KG, Dürgen, Germany). If necessary, the samples were diluted with deionized water. Separation of 20 µL samples was carried out at a constant flow rate of 0.5 mL min^{-1} of 5 mM H_2SO_4 as eluent at a column temperature of 65 °C. Sugars and organic acids were detected with a refractive index (RI) detector (1200 Series G1362A, Agilent Technologies, Santa Clara, CA, USA) and quantified using respective standards.

2.8. Gravimetric Biomass Analysis of Hydrolysis Samples

In triplicates, 2 mL of each sample were pipetted into pre-dried and weighted tubes (Safe-Lock Tube 2 mL, Eppendorf SE, Hamburg, Germany). The samples were spun down at 20,000 RCF for 10 min at 4 °C (Centrifuge 5424 R, Eppendorf SE, Hamburg, Germany). After discarding the supernatant, the remaining biomass was dried at 80 °C for at least 48 h. After cooling down, the tubes were weighed again to calculate the biomass dry weight concentration in the samples.

2.9. Total Polygalacturonase Activity Assay

Total polygalacturonase activities were determined following an adapted version of the Fructan Assay Kit protocol (Megazyme, Bray, Ireland). An amount of 100 µL of the hydrolysis supernatant (previously diluted 1:200 with 100 mM of sodium acetated buffer, pH 4.5) and 100 µL of a 10 g L^{-1} polygalacturonic acid solution (PGA, buffered in 100 mM of citrate, pH 4.5) were mixed and incubated for 60 min at 50 °C. Samples of 20 µL were taken regularly and pipetted on a PCR tray (Rotilabo®-PCR- tray, Carl Roth GmbH+Co. KG, Karlsruhe, Germany) on ice. The released reducing sugar ends were determined using a 4-hydroxybenzhydrazide solution, as described in the Megazyme protocol and measured at 405 nm with a microplate reader (Multiscan FC, Thermo Fisher Scientific Inc., Waltham, MA, USA). The first sample value measured before incubation was subtracted from each of the following sample values. One unit of total polygalacturonase activity was defined as the amount of enzyme that catalyzes the formation of one µmol D-GalA per minute at the assay conditions. All sample measurements were performed in triplicate, and the means with respective standard deviations were determined.

3. Results and Discussion

3.1. Composition of SBPP

The monomer composition of the used SBPP is shown in Table 2. The three main sugar components of the extracted SBPP under study were 21.8% (*w/w*) D-galacturonic acid (D-GalA), 21.6% (*w/w*) L-arabinose (Ara), and 13.6% (*w/w*) D-glucose (Glu).

Table 2. Dry matter composition of hydrolyzed SBPP under study in % (*w/w*), including the sugar loss compensation factors (LCF). The LCFs were multiplied by the determined values to obtain the correct share. The determined sugars were D-glucose (Glu), D-galacturonic acid (D-GalA), L-arabinose (Ara), D-galactose (Gal), D-xylose (Xyl), D-mannose (Man), L-rhamnose (Rha), L-fucose (Fuc) and D-glucuronic acid (GluA), and residual mass (RM). The results are compared to the literature and their mean values of the respective sugars [13,54,55].

	Glu	D-GalA	Ara	Gal	Xyl	Man	Rha	Fuc	GluA	RM	Total
LCF	1.56	2.78	1.80	1.80	2.13	3.41	1.80	1.66	2.16	-	
Share	13.6	21.8	21.6	6.9	1.2	0.5	6.8	0.2	0.3	28.5	101.4
[54]	23.3	19.8	23.5	5.8	1.7	1.3	2.1	0.2	n. a.	21.4	99.1
[13]	21.1	21.1	20.9	5.1	1.7	1.1	2.4	0.2	n. a.	21.4	95.0
[55]	21.7	18.9	17.3	4.3	1.5	1.5	1.1	0	n. a.	25.4	91.7
Mean	22.0	19.9	20.6	5.1	1.6	1.3	1.9	0.1	n. a.	22.7	95.2

The differences in reported data from other studies are likely due to a large extent to the biological variance of the raw material. The composition of SBPP is known to be dependent on cultivation conditions [56]. Moreover, the necessary hydrolysis protocols for the compositional analysis also have a significant influence and have not been standardized yet. D-glucuronic acid (GluA) was not analyzed in previous studies. Clear differences from previously reported SBPP compositions were observed in the L-rhamnose and D-glucose fractions. The SBPP under study showed a higher L-rhamnose fraction (6.8%) compared to the literature data (1.9%), whereas the D-glucose fraction of the SBPP under study (13.6%)

was clearly lower compared to published data (22.0%). This may be attributed to partially not hydrolyzed cellulose in the SBPP under study because the remaining dry biomass after hydrolysis is accordingly higher (28.5% compared to 22.7%) and because the cellulose control did not completely dissolve, as well. In the compositional analysis of SBPP, the mass balance was closed within the estimation error to 101.4%. We based the calculation of yields below on the measured composition of the SBPP material under study.

3.2. Integrated Process for Enzyme Production and Subsequent Hydrolysis

The SSF with the engineered *A. niger* strain was carried out with non-milled SBPP to provide cavities between the SBPP particles for sufficient aeration (Figure 2b). The filling volume of the stirred-tank bioreactor was kept as low as possible for enzyme production, and the stirrers were not operated during SSF. After 96 h of SSF, the temperature was raised to 50 °C, and the stirred-tank bioreactor was rinsed with nitrogen gas to inactivate the metabolism of *A. niger* to prevent any further sugar consumption [57,58]. Additionally, sterile water was added, and stirring was started at 220 rpm to detach and solve the hydrolytic enzymes in the water phase (Figure 2c). After 24 h, fresh sterile and milled SBPP was added to the bioreactor (Figure 2d) to start the hydrolysis. The delayed addition of milled SBPP was chosen to enable the estimation of the sugar yields during hydrolysis. The assumption was that the hydrolysis of the SBPP used for SSF would be finished within 24 h. The delayed addition of milled SBPP for hydrolysis will not be necessary for a real one-pot process combining enzyme production with SSF and hydrolysis.

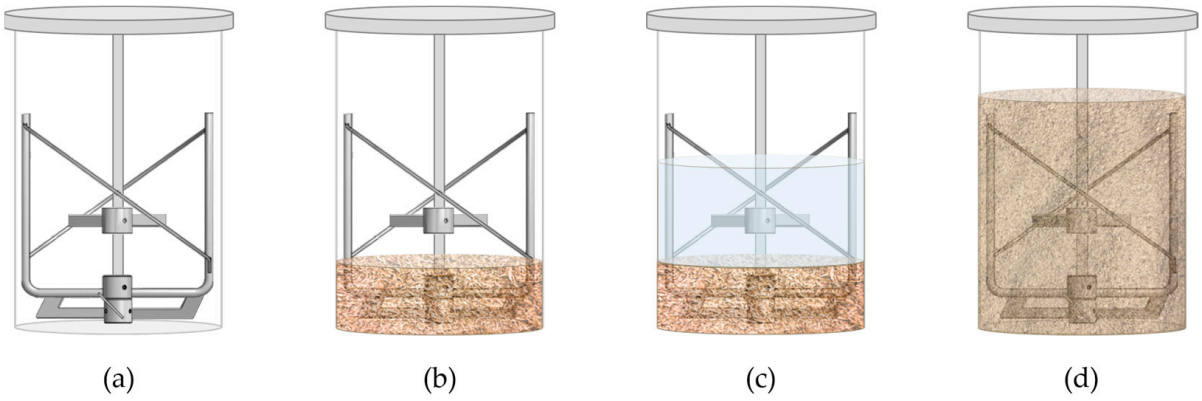

Figure 2. Process scheme for the one-pot SSF with *A. niger* for enzyme production and subsequent hydrolysis of extracted SBPP in a lab-scale stirred-tank bioreactor. (**a**) Stirrer configuration with an anchor stirrer, a one-fold helical stirrer, and a pitched-bladed impeller (from bottom to top). (**b**) Non-milled SBPP is filled in, moisturized with medium, and inoculated with *A. niger* spores. The solid level during SSF is 5 cm. (**c**) Before the initiation of the hydrolysis, 1.715 L of sterile water is added to the bioreactor with a final filling level of 12 cm, stirring is started at 220 rpm, the temperature is raised to 50 °C, and the bioreactor is rinsed with nitrogen gas to inactivate the metabolic activities of *A. niger*. (**d**) After 24 h, subsequent hydrolysis is started by adding sterile milled SBPP, resulting in a final filling level of 17 cm.

3.3. Solid-State Fermentation with Engineered A. niger

Two SSF processes were carried out with engineered *A. niger* on non-milled SBPP with an initial moisture content of 72% (*w/v*). The wet heap was inoculated with 6.25×10^6 spores per g SBPP. Sterile air was used for gassing and the CO_2- and O_2-concentrations were measured in the exhaust gas to monitor the metabolic activities of the *A. niger* cells (Figure 3). The CO_2 concentration starts to increase after ~24 h, whereas the O_2 concentration starts to decrease. Maximum metabolic activities were observed between 57–62 h after inoculation. After 96 h, metabolic activities in both SSF processes were reduced to 50% of the maximum.

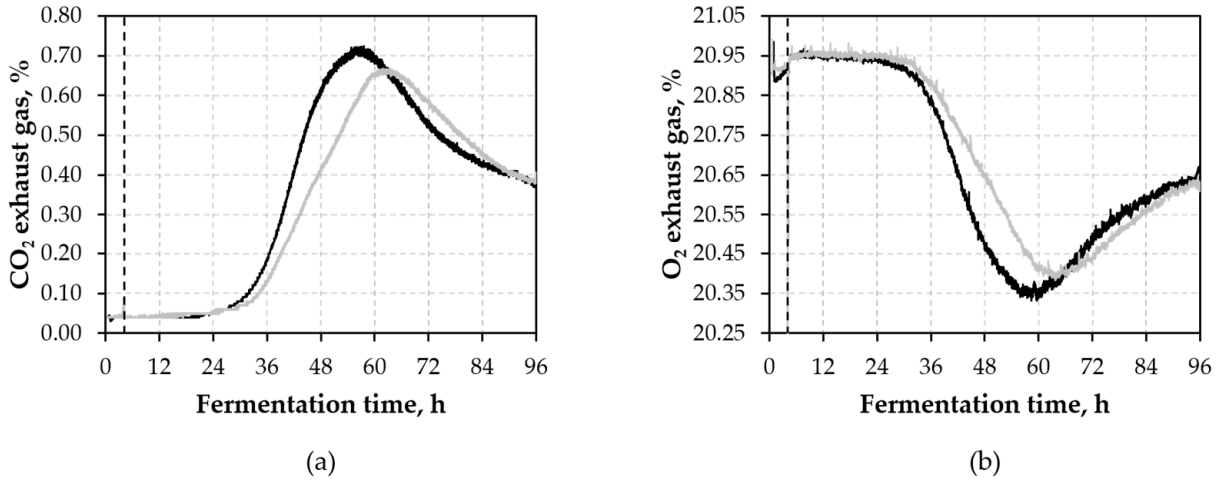

Figure 3. Carbon dioxide (CO_2) (**a**) and oxygen (O_2) (**b**) concentrations in the exhaust gases of two SSF with *A. niger* ATCC 11414 Δ*gaaX* on non-milled SBPP. The pH was buffered at pH 4.5, and the wet heap of SBPP was inoculated with 6.25×10^6 spores per L. The aeration was started with 1 L h^{-1} of sterile air after 4 h to prevent spore loss (indicated by the dashed line).

The resulting and diluted total polygalacturonase activities after 96 h SSF were 36.5 ± 5.4 U mL^{-1} (faster SSF process indicated by the black line in Figure 3) and 29.9 ± 0.6 U mL^{-1} (both measured after the addition of 1.715 L of sterile water). The estimation of the undiluted enzyme activities in the wet SSF media before hydrolysis were 256.1 ± 37.9 U mL^{-1} and 209.8 ± 4.2 U mL^{-1}, respectively. The polygalacturonase activities measured after SSF are significantly higher compared to those from a submerged fermentation experiment with this modified strain, which reached 141.12 ± 6.64 U mL^{-1} after 81 h fermentation [59]. Comparing these results to a submerged fermentation experiment with the non-modified *A. niger* ATCC 11414 strain with a polygalacturonase activity of 1.5 U mL^{-1} after 82 h fermentation, the engineered strain shows a significantly higher enzyme secretion [23]. Reported pectinase activities produced by SSF with *A. niger* on 10 g soy and wheat bran at 40% (*w/w*) moisture content were about 25 U mL^{-1} after 70 h fermentation time. The enzymes were extracted by adding 50 mL of buffer, leading to a dilution factor of 4 and resulting in an initial enzyme activity of about 100 U mL^{-1} [31].

3.4. Subsequent Hydrolysis of SBPP

After the addition of sterile water, initiating of stirring at 220 rpm, increasing the temperature to 50 °C, and rinsing of the stirred-tank bioreactor with nitrogen gas to inactivate the *A. niger* cells, autoclaved milled SBPP was added with a delay of 24 h for the hydrolysis. The biomass dry weight concentrations, including the fungal biomass, are shown in Figure 4 as a function of the hydrolysis process time. Within the first 24 h, the remaining dry mass concentration from the SSF process was reduced from ~20 g L^{-1} to ~15 g L^{-1}. Adding milled SBPP after 24 h resulted in about 119 g L^{-1} of biomass dry weight. Due to the enzymatic hydrolysis, dry mass concentrations were reduced continuously, but at a decreasing rate, reaching a final concentration of 54 g L^{-1} of dry biomass after a hydrolysis process time of 166 h. This corresponds to the enzymatic hydrolysis of about 48% of the milled SBPP added in the subsequent hydrolysis process part within 142 h.

The sugar concentrations in the liquid phase are shown in Figure 5. The sugars xylose (Xyl), mannose (Man), and galactose (Gal) cannot be separated with the applied HPLC method. The release of all sugars was finished within the first 24 h before the addition of the autoclaved milled SBPP, with the exception of glucose. Immediately after the addition of the milled SBPP, the glucose concentrations, as well as the sum of xylose, mannose, and galactose concentrations, increased instantaneously by a factor of 1.68 and 5.06, respectively. Autoclaving of the milled SBPP most probably caused this effect. Final

concentrations of 17 g L^{-1} Glu, 18.8 g L^{-1} Ara, 12.5 g L^{-1} D-GalA, and 6.5 g L^{-1} Xyl, Man, Gal, respectively, were achieved after a hydrolysis process time of 166 h, resulting in a total sugar concentration of 54.8 g L^{-1}. As none of the release rates of the sugar monomers is approaching zero after 166 h, higher sugar concentrations may be possible by increasing the hydrolysis process times.

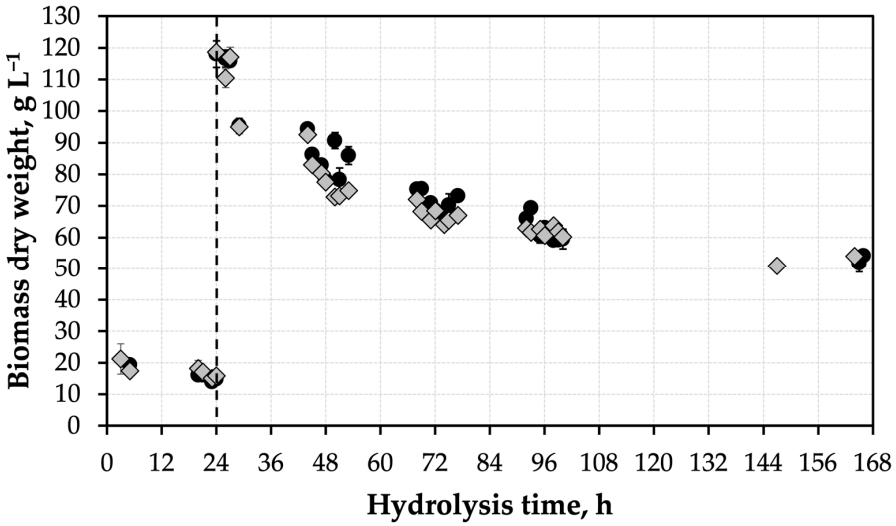

Figure 4. Biomass dry weight (BDW) during hydrolysis after SSF of SBPP with engineered *A. niger* (samples in triplicates of two one-pot processes indicated by ●, ◇). Addition of 220 g fresh milled and with 200 mL water autoclaved SBPP after 24 h hydrolysis time (indicated by a dashed line).

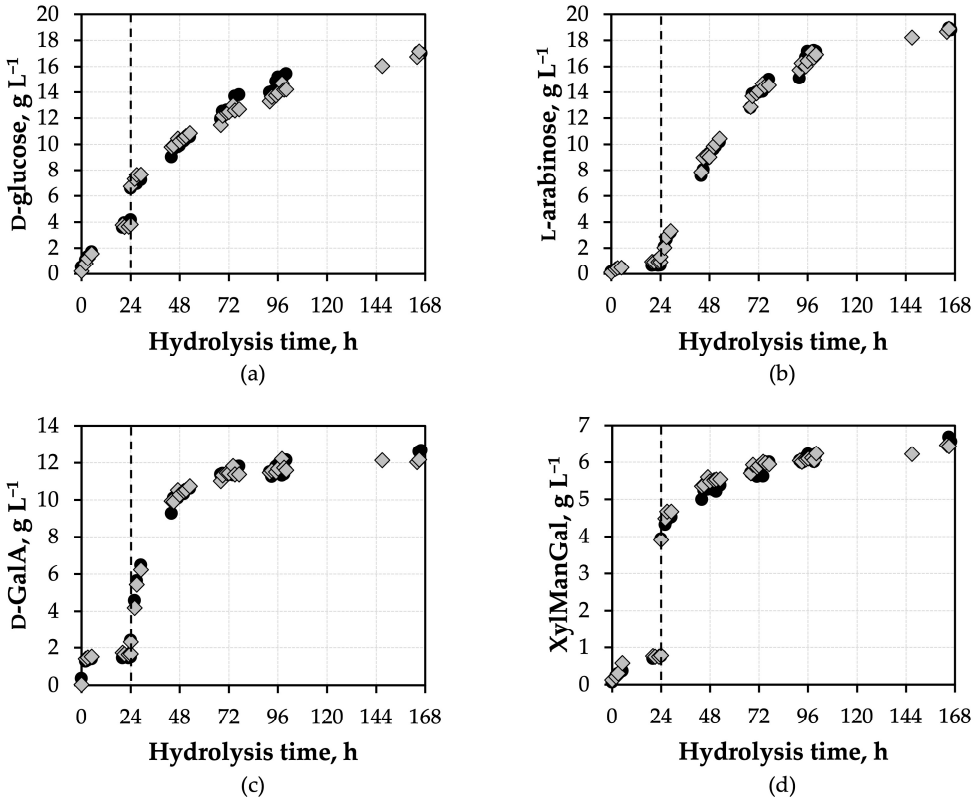

Figure 5. Sugar concentrations during hydrolysis of SBPP after SSF with engineered *A. niger* (samples of two one-pot processes indicated by ●, ◇). Addition of fresh-milled SBPP after 24 h hydrolysis time (indicated by a dashed line). (**a**) D-glucose concentrations; (**b**) L-arabinose concentrations; (**c**) D-galacturonic acid concentrations; (**d**) xylose, mannose, galactose concentrations.

The sugar yields of the hydrolysis process are shown in Figure 6 as a function of process time. Yields were calculated based on the SBPP composition analysis (Table 2) only considering the milled SBPP added after 24 h and the released sugar monomers after the addition. Glucose yield and the summed-up yield of xylose, mannose, and galactose were already ~20% and ~40%, respectively, directly after adding the autoclaved milled SBPP.

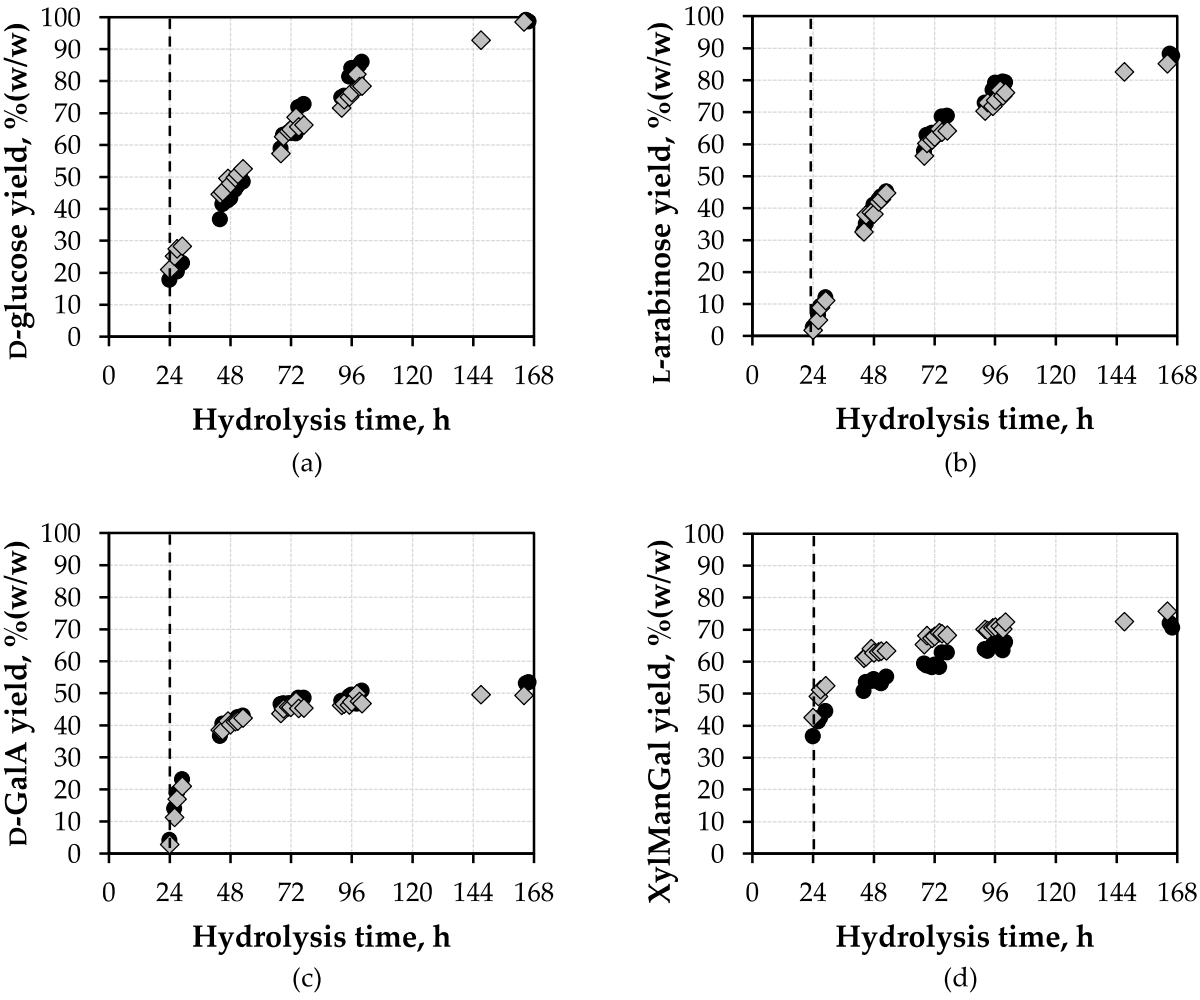

Figure 6. Sugar yields during hydrolysis of SBPP after SSF with engineered *A. niger* (samples of two one-pot processes indicated by ●, ◇). Addition of fresh-milled SBPP after 24 h hydrolysis time (indicated by a dashed line). The released sugars before 24 h were not considered for the calculation of the yields, since these were released from the remaining SBPP from the SSF. (**a**) D-glucose yield; (**b**) L-arabinose yield; (**c**) D-galacturonic acid yield; (**d**) xylose, mannose, galactose yield.

The final glucose yield after 166 h achieved 98%, but the release rate of glucose did not approach zero at the end of the hydrolysis. This may be caused by an additional release of glucose from the SBPP, which was used before in the SSF, because glucose was the only sugar that was released until the end of the 24 h delay period after SFF (Figure 5). Another explanation may be an underestimated glucose content of the SBPP under study compared to the literature data (Table 2). Considering the mean glucose content of SBPP reported in the literature (22%), the hydrolysis would result in a final glucose yield of 61% after 166 h. The final yields of the other sugar monomers after 166 h are 86% Ara and 71–75% Xyl, Man, and Gal.

The lowest yield was observed for the monomer of pectin because only 50% D-GalA was measured after 166 h of hydrolysis, although a genetically improved polygalacturonase-producing *A. niger* strain was previously applied in the SSF. After 24 h of hydrolysis,

and prior to the addition of fresh SBPP, the polygalacturonase activities decreased to 21.86 ± 1.2 U mL^{-1} (the faster SSF process is indicated by the black symbols in Figures 5 and 6) and 15.52 ± 1.9 U mL^{-1}, respectively, which correspond to a reduction to 60% and 52.4% of the initial activity, respectively. In contrast, D-GalA yields of 67% were achieved after 162 h of hydrolysis without the 24 h delay before the addition of SBPP [59]. It seems that this decrease in enzyme activity within 24 h at 50 °C before the addition of the milled SBPP might be the reason for the lower D-GalA yield, despite the fact that a temperature optimum of 50 °C was reported for the polygalacturonases from *A. niger,* and a half-life of 276 h was measured at 55 °C [60].

The achieved sugar yields of this one-pot enzyme production and hydrolysis process are comparable to the literature data of SBPP-hydrolysis processes with combinations of commercially available, concentrated enzymes for SBPP degradation. For example, an Ara yield of 94% and a glucose yield of 63–79% were reported with a multienzyme combination of fungal cellulases, hemicellulases, and pectinases with initial SBPP concentrations of 100–250 g L^{-1} within 48 h [61].

One of the major challenges of using plant biomass after hydrolysis as a substrate for fermentation is the resulting low sugar concentrations in the hydrolysate. Water evaporation for the concentration of the sugars in the hydrolysate will not be economically feasible for low-value products, such as ethanol. Complete conversion of single sugar monomers to higher-value products is thus inevitable for hydrolysates' economic utilization. As an example, the increase in D-GalA concentrations to 12 g L^{-1} shown here will improve the final concentration of the higher-value product L-galactonate, which can be produced by engineered *Saccharomyces cerevisiae* with high yields within a consecutive biotransformation process [24,25].

4. Conclusions

The one-pot process combining (i) substrate-specific hydrolytic enzyme production in SSF with engineered *A. niger* with (ii) high solid loads during enzymatic hydrolysis of the milled SBPP led to total sugar concentrations of above 50 g L^{-1} and is thus convenient for further biotransformation. Applying a one-pot process combining hydrolytic enzyme production and hydrolysis without an intermediate enzyme extraction and preparation step will help to reduce process costs. SSF of SBPP with the pectinase de-repressed *A. niger* strain showed a distinct improvement in polygalacturonase secretion compared to submerged fermentation. Further studies are necessary to improve sugar yields and concentrations up to the theoretical maxima before scale-up studies of this one-pot process make sense. In addition, studies on further integration of appropriate biotransformation steps after hydrolysis of SBPP, e.g., by the addition of an engineered whole-cell biocatalyst or by additional engineering of the *A. niger* strain already used for enzyme production, will be promising to reduce production costs further, as no intermediate purification steps will be necessary. Ideally, one of the sugar monomers released from SBPP will be used as an educt to be transformed with the highest selectivity to a value-added product, and the other sugars will serve as co-substrates. For example, the released D-galacturonic acid in the hydrolysate may be used for further biotransformation to L-galactonate by recombinant yeast, as shown before [24,25]. In addition, L-arabinose may be converted to low calorie sweetener arabitol by *Candida parapsilosis* [62]. In both biotransformations, the glucose in the hydrolysate may serve as an energy source for the yeasts and/or for cofactor regeneration.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9070582/s1, Table S1: Primers for *gaaX* gene knockout.

Author Contributions: Conceptualization, M.K. and D.W.-B. Methodology and investigation, M.K. (SSF, hydrolysis); D.S. and K.S. (SBPP analysis); K.S., M.R. and J.P.B. (microbiology). Data discussion and analysis, M.K., D.S., K.S., M.R., J.P.B. and D.W.-B. Writing—original draft preparation, M.K., K.S., M.R. and J.P.B. (engineering of *A. niger*). Writing—review and editing, J.P.B. and D.W.-B. Visualization,

M.K. Supervision, project administration, and funding acquisition, J.P.B. and D.W.-B. All authors have read and agreed to the published version of the manuscript.

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