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Single-Cell Oil Production by Engineered *Ashbya gossypii* from Non-Detoxified Lignocellulosic Biomass Hydrolysate

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Abstract: In this work, microbial lipid production from non-detoxified Eucalyptus bark hydrolysate (EBH) with oleaginous xylose-utilizing *Ashbya gossypii* strains was explored. The best producing strain from a set of engineered strains was identified in synthetic media mimicking the composition of the non-detoxified EBH (SM), the lipid profile was characterized, and yeast extract and corn steep liquor (CSL) were pinpointed as supplements enabling a good balance between lipid accumulation, biomass production, and autolysis by *A. gossypii*. The potential of the engineered *A. gossypii* A877 strain to produce lipids was further validated and optimized with minimally processed inhibitor-containing hydrolysate and high sugar concentration, and scaled up in a 2 L bioreactor. Lipid production from non-detoxified EBH supplemented with CSL reached a lipid titer of 1.42 g/L, paving the way for sustainable single-cell oil production within the concept of circular economy and placing lipids as an alternative by-product within microbial biorefineries.

Keywords: *Ashbya gossypii*; optimization; lipid production; bio-jet fuel; by-product valorization; biorefineries

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1. Introduction

Lipids can be used for a variety of applications, ranging from the food industry to biodiesel production. The two major sources of lipids are vegetable oils and animal fats, which require extensive land usage and have a considerable carbon footprint, thus raising environmental and economic concerns [1]. Microbial oils (single-cell oils, SCOs) represent a more sustainable option for producing lipids [2,3], since these biotechnological approaches do not depend directly on environmental/climatic factors, like weather conditions or seasonal availability, nor compete with animal feed or food stocks. Furthermore, microbial systems allow the tailoring of fatty acid (FA) composition, which can be achieved by genetically manipulating the FA metabolism of microbial cells [4]. Another advantage is the potential of microorganisms to utilize various and cheap carbon sources for their growth.

One example of these substrates is lignocellulosic hydrolysates, which are obtained from lignocellulosic materials, such as wood waste or agricultural residues. These materials are abundant, renewable, and contain fermentable sugars that can be valorized to ethanol [5] and/or high-value chemicals [6], but a pretreatment is mandatory (enabling the selective separation of the main components, including cellulose, intact or solubilized hemicellulose, and lignin) in the scope of biomass biorefinery [7]. The pretreated biomass is then hydrolyzed by enzymes to release the C6 and C5 (if required) sugars [8], which is a crucial step in the biorefinery process [9]. Pretreatments often resort to chemicals like acids and alkalis to facilitate enzymatic hydrolysis and often lead to the production of toxic components in the hydrolysate [10]. An additional step for the removal of these toxics can be used to enable the growth of microorganisms in the hydrolysate, at the cost of a portion of available sugars being lost [11]. Also, this additional step increases the

production costs, which may negatively affect the economic competitiveness of the process. To bypass this, research aims to develop cost-effective SCO production processes from non-detoxified lignocellulosic hydrolysates [12]. Other inexpensive residue by-products can be used as supplements to enhance fermentation conditions, such as corn steep liquor (CSL). This residue has been included in different media used for microbial growth [13,14] and very-high-gravity fermentations [15] as a source of amino acids, vitamins, and minerals.

A. gossypii is a filamentous hemiascomycete industrially used to produce riboflavin, a process in which downstream product recovery is facilitated due to the ability of this fungus to undergo autolysis during the late stationary phase of growth or at low temperatures [16]. In addition to riboflavin, engineered *A. gossypii* strains are capable of producing other compounds of interest for the food and feed industry, such as the nucleosides inosine and guanosine [17], folic acid [18], polyunsaturated fatty acids (PUFAs) (up to 35% of total SCOs) [19] and other microbial oils [20], γ-lactones [21], orotic acid [22], and limonene [23].

Extensive engineering of the *A. gossypii* FA metabolism has generated strains with high levels of lipid accumulation (up to 40% (*w*/*w*) in sugar-based media or 70% (*w*/*w*) in oil-supplemented media) [20,24] as well as tunable lipid profiles [4,19]. Beyond that, with the recent discovery of the importance of the profile and amount of FAs accumulated for the *A. gossypii* ability for de novo production of γ-lactones [21], further applications can be envisioned for lipid-accumulating *A. gossypii* strains.

To enable efficient and tailored lipid production, several process conditions must be optimized, especially when residues like non-detoxified lignocellulosic hydrolysates are targeted as substrates. Several efforts at the metabolic engineering level were developed to pave the way for efficient substrate usage. The potential of oleaginous xylose-utilizing *A. gossypii* strains was described for the production of SCOs from media containing mixed formulations of detoxified corn cob hydrolysates, sugarcane molasses, and crude glycerol [20]. In this work, we took one step further towards real process applications by demonstrating the potential of these oleaginous strains for SCO production from non-detoxified Eucalyptus bark hydrolysate (EBH), an abundant residue from the pulp and paper industry, with large amounts being generated annually. Eucalyptus pulp and paper wastes are conventionally left in the field to enrich the soil or burn for electricity or heat production, but due to their composition, other valorization routes, like ethanol production, have recently been explored [7,25]. Strain performance was first studied in synthetic media mimicking the composition of the non-detoxified hydrolysate, containing the major inhibitors that could hinder growth. SCO production by the best-performing strain was then characterized using different nitrogen and micronutrient sources in the synthetic and real non-detoxified hydrolysate. The impact of cell lysis onset on the physiology of the strains and on the productivity of the process was studied with the aim of enhancing SCO production in non-detoxified EBH.

2. Materials and Methods

2.1. A. gossypii Strains and Culture Conditions

Three engineered *A. gossypii* strains were used in this study: the xylose-utilizing A729 strain [26], and two oleaginous strains derived from A729: A842 and A877 [20] (Table 1). Stock cultures were maintained at −80 °C as spores suspended in spore buffer (20% (*v*/*v*) glycerol, 0.8% (*w*/*v*) NaCl with 0.025% (*v*/*v*) Tween 20). Shake-flask cultures were performed in 250 mL Erlenmeyer flasks containing 50 mL of medium, at 30 °C and 200 rpm orbital shaking (25 mm orbit) [22]. Bioreactor cultures were performed in a 2 L bioreactor (RALF Advanced, Bioengineering AG, (Wald, Switzerland) containing 1 L of production medium, at 28 °C or 30 °C and 500 rpm, with 2.0 vvm aeration. The culture pH was kept at 7.0 ± 0.1 via the addition of 6.5 g/L $CaCO_3$ [22]. Temperature, O_2, and pH probes were used to measure these parameters. Foaming was reduced with the automatic addition of a silicon anti-foaming agent (Sigma-Aldrich) controlled by a foam probe.

Table 1. *A. gossypii* strains utilized in this study.

Strain	Parental Strain	Genotype	Relevant Phenotype	Reference
A729	ATCC 10895	*ACL107Cp::loxP-AgGPDp, AGR324Cp::loxP-AgGPDp, ABR229Cp::loxP-AgGPDp, ADR304W::loxP-AgGPDp-Bspta-AgPGK1t, AGR034W::loxP-AgGPDp-AnxpkA-AgENO2t*	Ino^-, Xyl^+	[26]
A842	A729	A729, *ACR165W*$^{850-1139}$*::loxP, AAR071W::loxP-AgGPDp-aar071w*$^{T1975G,\ T3463G}$	Ino^-, Xyl^+, oleaginous	[20]
A877	A842	A842, *AFR171W::loxP-KanMX-loxP-AgGPDp-AgACR140C-AgPGK1t*	Ino^-, Xyl^+, oleaginous, $G418^R$	[20]

2.2. Media Composition and Preparation

Agar-solidified Ashbya Full Medium (AFM; 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 1 g/L myo-inositol, 15 g/L agar) [22] was used to prepare fragmented mycelia for shake-flask inoculation. Several production media were tested during this study (Table 2). Eucalyptus bark was collected in the pulp mill of Cacia (Aveiro, Portugal) from The Navigator Company and used as feedstock. When received, the Eucalyptus bark was homogenized in a defined lot and stored in plastic containers at room temperature. Each lot was chemically characterized to determine the exact polysaccharides' content for further use. The dry matter content was 59% (*m*/*m*), with total polysaccharides of 45.5 g/100 $g_{\text{oven-dried solids}}$, of which there was 33.3 g/100 $g_{\text{oven-dried solids}}$ and 12.2 g/100 $g_{\text{oven-dried solids}}$ of glucan and xylan, respectively. A commercial cellulolytic enzyme (also containing highly active xylanases) cocktail from Novozymes (Cellic® CTec3 HS) was used for enzymatic hydrolysis. The preparation of EBH was as follows: prior to the enzymatic hydrolysis step, a pretreatment stage was carried out to release fermentable monosaccharides from the feedstock. Pretreatment was based on a proprietary non-catalyzed steam explosion technology without the addition of acids and using only high-pressure steam, initially developed by the company STEX®, and since 2019, the technology developments have been made in partnership with LNEG. This steam explosion step was carried out in a 320 L reactor coupled to a 4000 L blow tank where pretreated biomass is discharged. After pretreatment, the solid fraction was washed with water at room temperature, chemically characterized and directly used for the enzymatic hydrolysis stage. Eucalyptus bark hydrolysate was obtained after 48 h enzymatic saccharification of the pretreated solid at an initial 175 g/L solid concentration (oven-dried basis), by applying Cellic® CTec3 at a dosage of 3% (*m*/*m* oven-dried solids). This suspension was incubated with the enzyme preparation at 50 °C in a 600 L stirred tank reactor, and the resulting hydrolysate was centrifuged (12,000× *g*, 15 min, 4 °C) to remove the unreacted solids and then appropriately stored (frozen) until use. The hydrolysate was analyzed using HPLC. After defrosting, the hydrolysate was centrifuged again to remove the remaining solids and sterilized using 0.22 µm mixed cellulose ester sterile membrane filters. The final composition of this EBH was as follows: 55.52 g/L glucose, 7.2 g/L xylose, 5.29 g/L acetic acid, 0.66 g/L formic acid, and 0.01 g/L HMF. A second batch of EBH with concentrated sugars (EBHcs), 84.05 g/L glucose and 8.65 g/L xylose, was used for bioreactor fermentation. Synthetic hydrolysate-mimicking media (SM) containing these major components of EBH and 1 g/L myo-inositol (to prevent auxotrophic starvation) was supplemented with yeast extract (YE; Liofilchem), corn steep liquor (CSL, Sigma-Aldrich), or yeast nitrogen base without amino acids and ammonium sulfate (YNB; BD Difco) containing ammonium sulfate (AS) or asparagine (Asp). EBH containing 1 g/L myo-inositol was supplemented with YE and CSL, as indicated in Table 2. The C/N ratios for each formulation are indicated

in Table 2. The pH of each media was adjusted to 6.8–7.0 with KOH (5 mol.L^{-1}), and 6.5 g/L $CaCO_3$ was added to maintain the culture pH during fermentation [27,28].

Table 2. Media formulations used for SCO production. As nitrogen and micronutrient sources, yeast extract (YE), corn steep liquor (CSL), or yeast nitrogen base without amino acids and ammonium sulfate (YNB) containing ammonium sulfate (AS) or asparagine (Asp) were used as indicated. For each formulation, the approximate C/N ratio is indicated, which for YE and CSL was calculated based on the information provided by the supplier on the product's technical sheet.

Media	Concentration (g/L)				C/N Ratio
	YE	1x YNB + AS	1x YNB + Asp	CSL	
SM-2 to 10 YE	2 to 10	-	-	-	~150 to ~30
$SM_{1/2}$(5YE)	5	-	-	-	~30
$SM_{1/5}$(2YE)	2	-	-	-	~30
SM-YNBAS	-	5	-	-	29
SM-YNBAsp	-	-	2	-	74
SM-5 to 15 CSL	-	-	-	5 to 15	~65 to ~22
EBH-2 to 10 YE	2 to 10	-	-	-	~150 to ~30
EBH-15/17CSL	-	-	-	15 or 17	~22 or ~19

2.3. Lipid Production

Fragmented mycelia for shake-flask inoculation were prepared by collecting mycelia grown for 2 days at 30 °C from agar-solidified AFM, digesting with 7.5 mg/mL of lysing enzymes from Trichoderma harzianum (Sigma-Aldrich) until homogeneously dispersed mycelia (≈1 h) is obtained, washed with spore buffer and resuspended in liquid AFM (1.5 mL/plate) [20]. This mixture was used to inoculate 100 mL of AFM in 500 mL flasks to achieve an initial optical density at 600 nm (OD) of 0.5. After 20 h of growth, OD was measured, and the required biomass was filtered to inoculate 50 mL of production medium to an initial OD of 0.5, in 250 mL flasks [21]. Samples were taken at several time points to establish growth curve tendencies and evaluate sugar consumption besides lipid production and accumulation [20,26]. To overcome the interference of $CaCO_3$ in OD and cell dry weight (CDW) measurements, a solution of 0.9% NaCl and 3 mol.L^{-1} HCl was added to the samples (at a 1:4 proportion) to dissolve the $CaCO_3$. CDW was determined via filtration of culture samples, collection of the washed mycelia into pre-weighed dried tubes, and drying at 105 °C until constant weight (~24 h) [21].

2.4. HPLC Analyses

For EBH, monosaccharides (glucose and xylose) were measured with HPLC using Agilent 1260 series (Waldbronn, Germany) equipped with refractive index (RI) and diode array (DAD) detectors, the latter being set at a fixed wavelength of 280 nm for furans (hydroxymethylfurfural and furfural). An Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) was used, operating at 50 °C with 5 mmol.L^{-1} sulfuric acid as a mobile phase at a flow rate of 0.4 mL/min.

To determine glucose, xylose, acetic acid, formic acid, and ethanol concentrations throughout the fermentation, samples from production cultures were analyzed with HPLC using the BioRad Aminex HPX-87H (300 × 7.8 mm) column operating at 60 °C and 5 mmol.L^{-1} sulfuric acid as a mobile phase at a flow rate of 0.6 mL/min [22]. Formic and acetic acids were quantified using a DAD detector (Shimadzu, Columbia, MA, USA) at a wavelength of 210 nm, while glucose, xylose, and ethanol were quantified using an RI detector (Shimadzu, Columbia, MA, USA). Concentrations were calculated from calibration curves prepared from pure standards.

2.5. Lipid Extraction and GC-MS Quantification

Mycelia collected via filtration from 2.5 mL culture samples was dried at 105 °C as indicated above. Dried biomass (15–20 mg) was macerated in screw-capped glass tubes using a glass rod and resuspended in 1 mL of 2.5% (*v*/*v*) sulfuric acid in methanol solution, with 50 µg of heptadecanoic acid as an internal standard. Tubes were closed with Teflon caps to avoid solvent evaporation and incubated at 80 °C for 100 min. The reaction was stopped with 1 mL of 1 mol.L^{-1} NaCl, to which 0.5 mL of n-hexane was added to extract FAMES through vigorous shaking [20]. Tubes were left untouched for 10 min for the phases to separate. Approximately 0.3 mL of the upper organic phase was recovered, dehydrated with excess anhydrous sodium sulfate, and transferred to 2 mL glass vials closed with Teflon caps [20]. Samples were kept at −20 °C until analysis.

GC-MS analysis was carried out in a QP2020 NX 2 GC-MS (Shimadzu), using a Stabilwax-MS column (30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness; Restek) [26]. Helium was used as a carrier gas at a constant flow rate of 1 mL/min at a constant split ratio of 1:20, and the injected sample volume was 1 µL. The injector was kept at 250 °C and the interface at 200 °C. The oven program was as follows: initial temperature of 90 °C for 5 min, then ramped up to 190 °C at 12 °C/min, and finally to 230 °C at 4 °C/min. MS detection was in the range of 35 to 500 m/z. FAMEs retention indexes and mass spectra were identified by comparison with the commercial standard FAME37 (Supelco) and also confirmed via similarity analysis with the NIST17 library. Total FAMEs quantification was calculated using the standard internal pattern method, in reference to 50 µg/mL of heptadecanoic acid C17:0 (Sigma) [26].

2.6. Nile Red Lipid Staining

To observe lipid bodies accumulation inside the microbial cells, Nile red (Sigma, St. Louis, MO, USA) staining agent was used. Mycelia were collected via centrifugation, and pellets were resuspended in PBS pH 7.5 to a final OD of 1.25, to which 0.02 mL of 50 µg/mL Nile red solution in acetone was added. Samples were incubated for 8 min at room temperature and then observed in an Olympus BX51 microscope (Tokyo, Japan) equipped with a fluorescence illuminator and a fluorescence cube with an excitation filter of 530–550 nm and a barrier filter of 590 nm.

3. Results and Discussion

3.1. Performance of Xylose-Utilizing Oleaginous A. gossypii Strains in Non-Detoxified EBH-Mimicking Media

Xylose-utilizing oleaginous *A. gossypii* strains were previously shown to be able to accumulate ~40% of CDW in lipid content using detoxified industrial wastes as feedstock [20]. In this study, we aimed to explore the potential of these strains for lipid production using non-detoxified Eucalyptus bark hydrolysate (EBH) as a substrate. To assess their growth and lipid performance, we first characterized their behavior in a synthetic medium mimicking the composition of non-detoxified EBH (SM) supplemented with 10 g/L yeast extract (YE).

In SM-10YE, all strains exhibited similar growth curves up to 48 h (Figure 1A). However, beyond this point, their growth rates decelerated, likely due to the onset of autolysis and spore formation (Figure S1). Autolysis, being a part of *A. gossypii* physiology, may have potential benefits in downstream intracellular metabolite recovery if well controlled [29]. Despite autolysis, all strains continued to consume the sugars in the culture, with >90% of glucose uptake by all strains when the cultures were stopped at ~120 h (Figure 1A). However, xylose was not as efficiently consumed as glucose (Figure S2), likely due to the requirement for NADPH for the activity of xylose reductase in the engineered fatty acid metabolism [20]. This could lead to the carbon flux being diverted towards lipid synthesis instead of biomass production, especially when high biomasses are achieved [30].

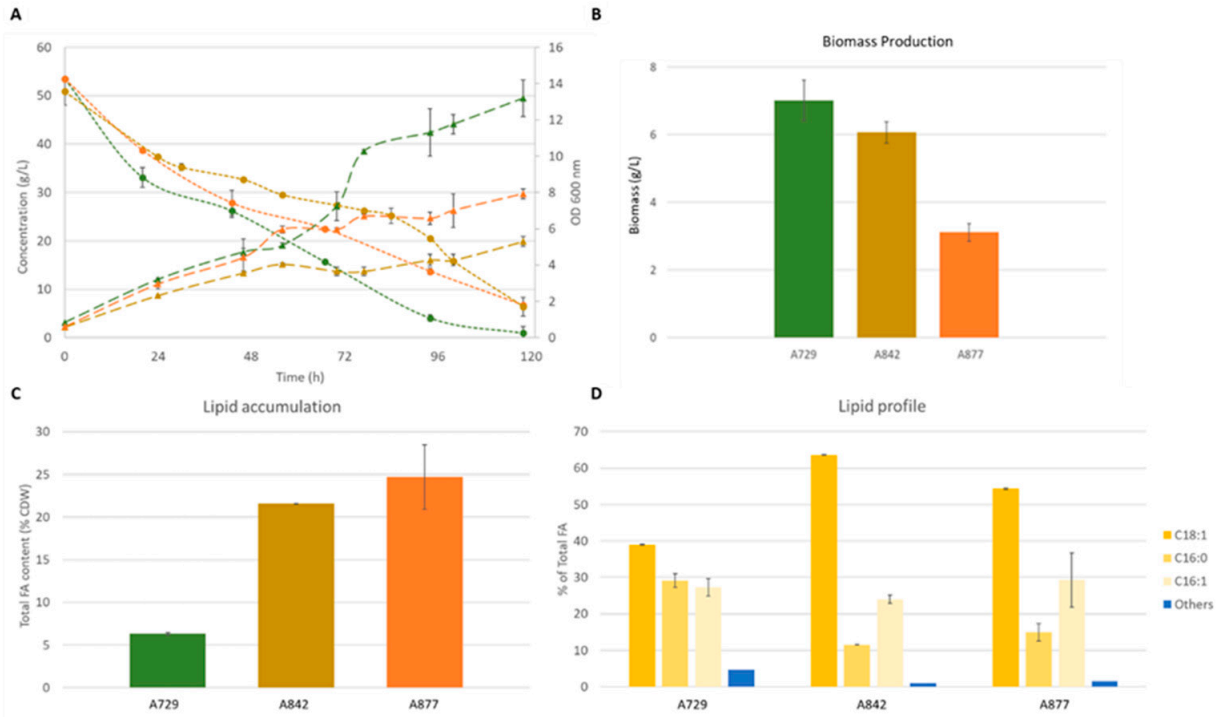

Figure 1. Performance of engineered *A. gossypii* strains in SM-10YE. (**A**) Growth and glucose consumption. OD is represented by triangles, in the secondary Y axis. Glucose is represented by circles. Strain A729 is in a dark green color, strain A842 in dark gold, and strain A877 in orange. (**B**) Biomass concentration at 120 h. (**C**) Total lipid content at 120 h. (**D**) Lipid profiles of strains at the same time point. The data represent average values and standard deviations of biological duplicates. Where not seen, error bars were smaller than the symbols.

Despite strain A842 and A877 showing lower final CDW compared to A729 (Figure 1B), their intracellular lipid content, analyzed by GC-MS, was higher (Figure 1C). Strain A877 exhibited the highest lipid accumulation, particularly as oleic acid (C18:1), along with lower relative amounts of other fatty acids, such as C16:0 and C16:1 (Figure 1D). Nile red staining further confirmed the presence of larger lipid bodies in the mycelia of strain A877 compared to A729 (Figure S1).

Considering the higher lipid accumulation and slightly better growth profile than strain A842, strain A877 was selected for further investigation of lipid production in non-detoxified EBH.

3.2. *A. gossypii* A877 Performance Using Different Media Supplementations

A. gossypii A877 did not grow on EBH without nitrogen and micronutrient supplementation. To address this, three different sources of nitrogen and micronutrients were used to supplement SM: yeast extract (YE), YNB containing ammonium sulfate (SM-YNBAS), and YNB containing asparagine (SM-YNBAsp) (Table 2). To find a balance between lipid accumulation, biomass production, and autolysis, the concentration of YE in SM was adjusted to 10, 5, and 2 g/L. The SM media carbon content was also adjusted by dilution of the SM-10YE ($SM_{1/2}$(5YE) and $SM_{1/5}$(2YE)).

By comparing the performance of *A. gossypii* A877 in these media (Figures 2 and S3), it was observed that biomass and mycelia autolysis decreased with decreasing YE concentration. Autolysis and spore formation were also observed in YNBAsp (around 48 h) and YNBAS (around 72 h). Surprisingly, the presence of high nitrogen concentrations in the media caused autolysis onset, likely due to oxygen depletion. In yeasts, anoxia was reported to induce transient oxidative stress, changes in mitochondrial respiration related proteins, and ROS production [31]. Bioreactor fermentation in SM-10YE showed that oxygen levels decreased as biomass production increased, coinciding with autolysis onset, at

20 h (Figure S4). Anoxia triggered by high nitrogen concentration may have contributed to this effect. Ensuring sufficient oxygenation of the culture is vital to prevent collapse and maximize biomass and lipid production. Notably, the highest C/N ratio of SM-YNBAsp and SM-2YE (Table 2) supported the best lipid accumulation (Figure 2C), leading to lipid contents of up to ~33% at the end of the fermentation. The highest lipid titer was reached in SM-YNBAsp, due to the combination of high lipid accumulation and high biomass production in this medium (Figure 2). These results indicate that balancing autolysis, biomass production, and lipid accumulation is critical for optimized lipid production by *A. gossypii* A877.

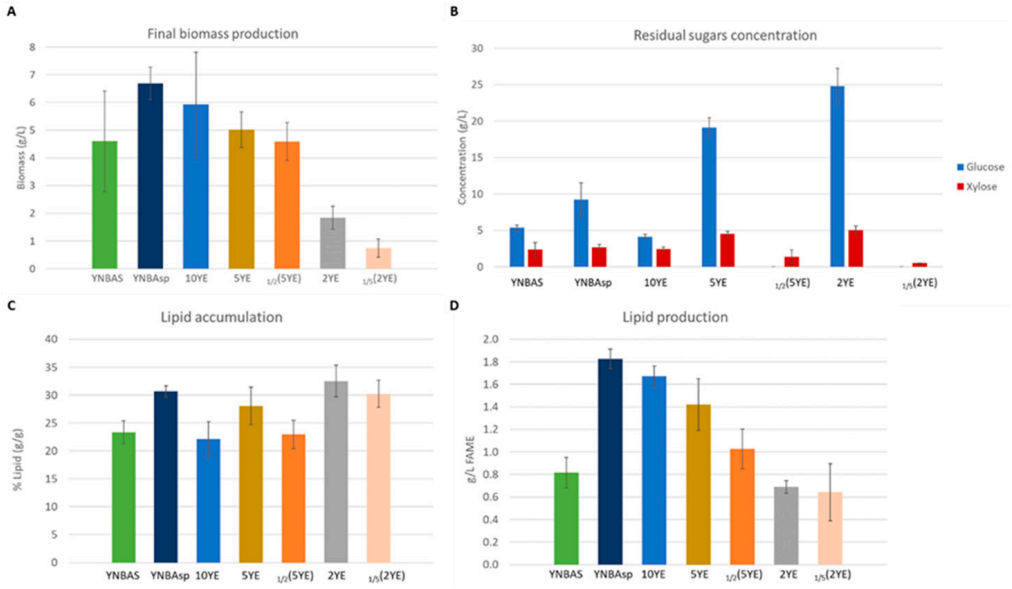

Figure 2. Performance of *A. gossypii* A877 in SM-YNBAS, SM-YNBAsp, SM-10YE, SM-5YE, SM-2YE, SM1/2(5YE), and SM1/5(2YE). Final biomass production (**A**) and residual sugars concentration (**B**) at 168 h for YNBAS, YNBAsp, and 10YE, and at 144 h for 5YE, 2YE, 1/2(5YE), and 1/5(2YE). Lipid accumulation (**C**) and production (**D**) at the same time points. The data represent average values and standard deviations of biological duplicates.

Envisioning a cost-efficient bioprocess, CSL at different C/N ratios was used to supplement SM (Table 2). A range of 5 to 15 g/L CSL was tested to find the amount of CSL that would lead to the best biomass/lipid production balance in SM. Increasing CSL concentrations led to increasingly higher optical densities and biomass production by *A. gossypii* A877 (Figure 3A,B). Interestingly, autolysis onset was verified only at 42 h or later. The highest CSL concentrations tested (lowest C/N ratios) boosted biomass production, which promoted lipid production (Figure 3C,D). Once again, these results highlighted the importance of maintaining the balance between biomass and lipid accumulation in order to improve the total lipid titer.

Taken together, these results pinpointed 5 g/L YE as the SM supplementation enabling the best balance between lipid accumulation, biomass production, and autolysis by *A. gossypii* A877. Moreover, 15 g/L CSL also proved to be a viable option to avoid autolysis while supporting reasonable lipid titer.

3.3. Lipid Accumulation and Production by Strain A877 in Non-Detoxified EBH

Based on the results obtained in SM, *A. gossypii* A877 was further evaluated for lipid production in real non-detoxified EBH supplemented with 5 or 10 g/L YE, or 15 g/L CSL (Figure 4). In non-detoxified EBH, *A. gossypii* A877 showed faster growth and sugar consumption profiles than in YE. By reducing YE to 5 g/L, the growth profile improved, with less pronounced autolysis. Nile red staining showed big intracellular lipid droplets and deformed mycelia due to the excess lipid accumulation (Figure S5). Lipid droplets and

spores were also visible outside of lysed cells (Figure S5G,H). Notably, 5 g/L YE supported a final biomass of 14 ± 0.8 g_{CDW}/L after 110 h (Figure 4A), with almost complete sugar consumption (Figure 4B). These conditions exhibited great potential for lipid production using non-detoxified lignocellulosic biomass hydrolysate. Although *A. gossypii* A877 did not grow in EBH without supplementation, biomass production was higher in supplemented EBH than in SM (Figures 2–4), which may be ascribed to the presence of small amounts of micronutrients important for biomass production in EBH.

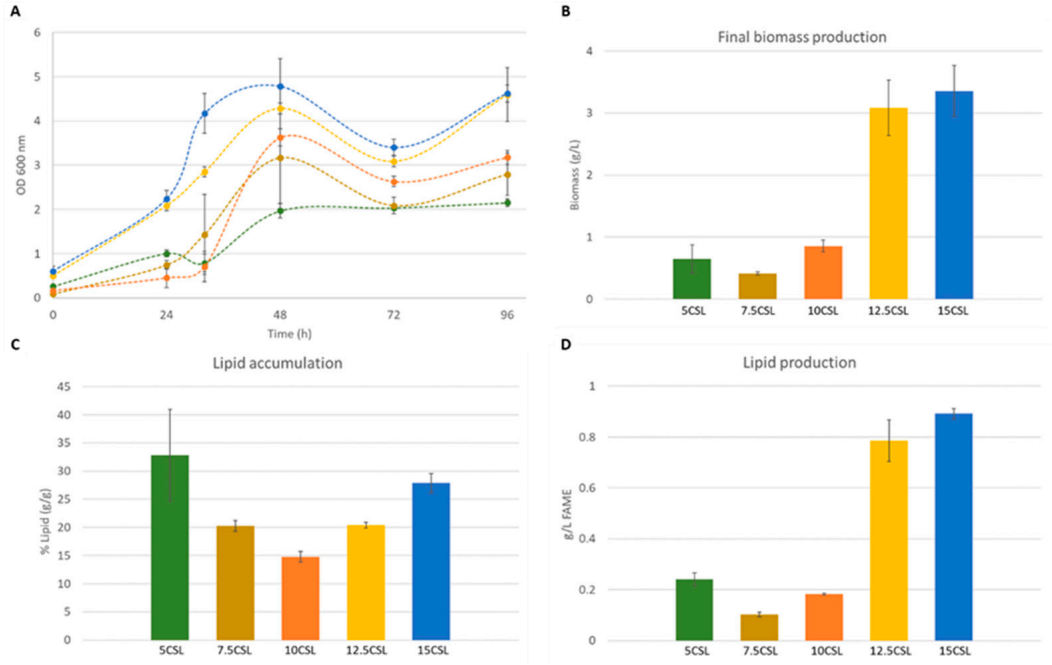

Figure 3. Performance of *A. gossypii* A877 in SM supplemented with different CSL concentrations. Growth curves (**A**) and biomass concentration (**B**) at the point of total sugar consumption (120 h for supplementation with 5 (green), 7.5 (brown), and 10 g/L (orange), and 96 h for 12.5 (yellow) and 15 (blue) g/L). Lipid accumulation (**C**) and production (**D**) at the same time points. The data represent average values and standard deviations of biological duplicates.

Based on these results in flask, bioreactor fermentations in EBH-5YE (~60 g/L total sugars) (at 30 °C) or EBHcs-17CSL (~90 g/L total sugars) (at 28 °C) were subsequently performed, with the latter providing better conditions for lipid production (Figure 5). Despite lower lipid accumulation and longer fermentation time, higher biomass production in EBHcs-17CSL supported a lipid titer of 1.42 g/L at 78 h, which corresponds to a productivity two times higher than that reached in EBH-5YE. This culture only reached anoxia later in the fermentation course (Figure 5B), which could be a potential reason for later autolysis onset and, thus, enhanced biomass production. Autolysis, which typically occurs during the later stages of the mycelia life cycle, was triggered early in all non-detoxified EBH cultures in flask and bioreactor, with the exception of EBHcs-17CSL in bioreactor. The stress responsible for autolysis was hypothesized to be anoxia, caused by insufficient oxygen supplementation to meet the demands of the rapidly growing biomass. The presence of excess nitrogen could be a cause for this, resulting in fewer cells utilizing the nutrients during the productive phase. In yeast, the availability of a high carbon content at a time of nitrogen depletion (high C/N after complete nitrogen fixation by biomass during the trophic phase) creates conditions that divert the carbon flux toward lipid accumulation instead of biomass production [32–34]. After onset, autolysis remained during fermentation and was cumulative in all tested media. The high biomass production obtained in EBHcs-17CSL compared to EBH-5YE in bioreactor suggests that slow initial growth leads to less oxygen deprivation and consequently to less autolysis (Figure 5). Thus, finding a balance

between growth, available oxygen, autolysis, and carbon flux is critical for optimized lipid production by *A. gossypii* A877.

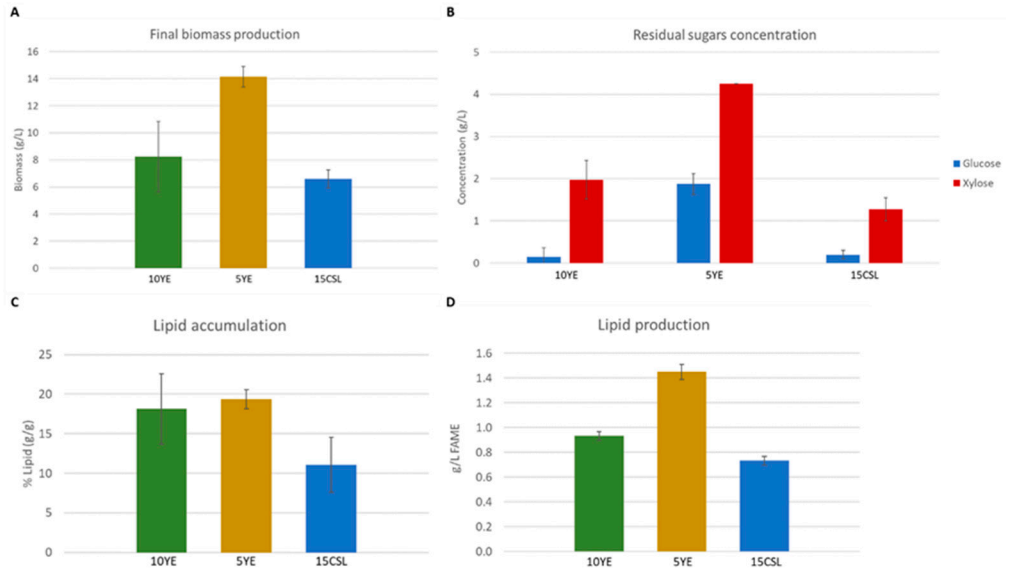

Figure 4. Performance of *A. gossypii* A877 in EBH media supplemented with 10 g/L YE (10YE), 5 g/L YE (5YE), and 15 g/L CSL (15CSL). Final biomass production (**A**) and residual sugars concentration (**B**) at 84 h (10YE) and at 110 h (5YE and 15CSL). Lipid accumulation (**C**) and production (**D**) at the same time points. The data represent average values and standard deviations of biological duplicates.

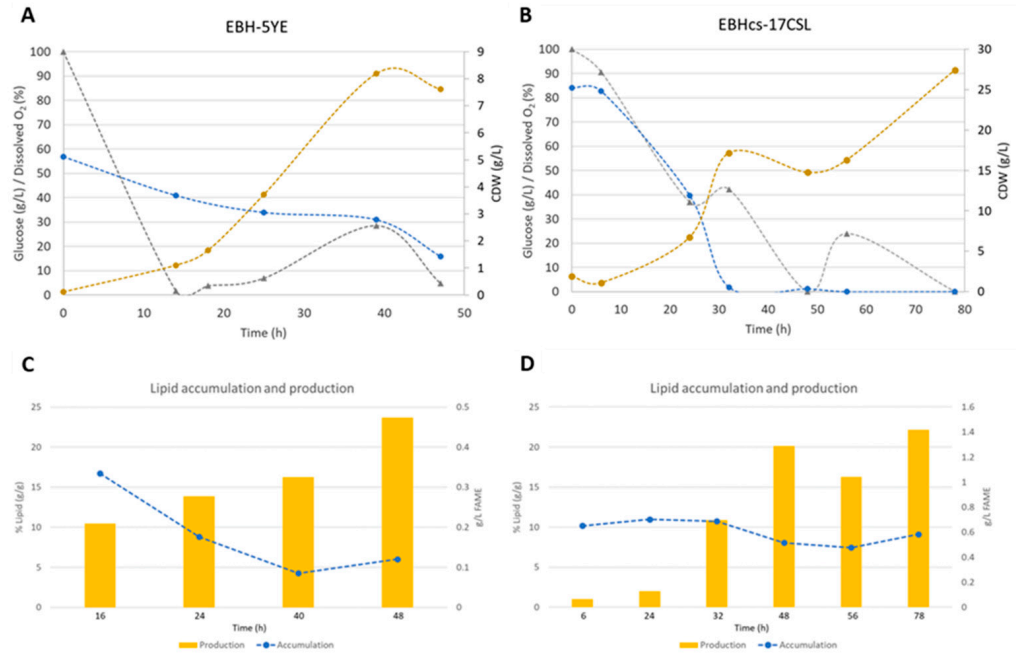

Figure 5. Performance of *A. gossypii* A877 in bioreactor cultivations with EBH-5YE (**A**) and EBHcs-17CSL (**B**). (**A**,**B**) Glucose concentration is represented by blue lines and dissolved O_2 by grey lines, both read on the primary Y axis. Biomass concentration is represented by gold lines and is read on the secondary Y axis. Lipid accumulation (blue line, primary Y axis) and production (yellow bars, secondary Y axis) are shown at different time points for EBH-5YE (**C**) and EBHcs-17CSL (**D**). For the condition EBH-5YE, two experiments were performed, with a variance of 28%.

A. gossypii A877 demonstrated significant potential for lipid production using media composed exclusively of low-cost and renewable substrates, namely non-detoxified Eu-

calyptus bark hydrolysate supplemented with corn steep liquor (Figures 4 and 5). While lipid accumulation and titer attained in other microorganisms may be higher (Table 3), the usage of renewable and inexpensive substrates, namely a mixture composed exclusively of non-detoxified Eucalyptus bark hydrolysate and corn steep liquor instead of detoxified hydrolysate media and/or supplemented with expensive nutrients, together with the possibility of fine-tuning the aimed lipid profile in *A. gossypii*, opens new perspectives for designed SCO sustainable production.

Table 3. Lipid titer (g/L) and content (% g/g) of other *A. gossypii* A877 study and other oleaginous yeasts in different hydrolysate media compositions. G: glucose, X: xylose, S: sucrose.

Microorganism	Medium Composition (g/L)	Lipid Titer (g/L)	Lipid Content (%)	Reference
A. gossypii A877	Non-detoxified EBH G: 84.0 X: 8.65	1.42	11.0	This study
A. gossypii A877	50% detoxified corn cob hydrolysate + 4% molasses supplemented with YE and tryptone G: 2.60 X: 15.1 S: 20.3	3.90	38.1	[20]
Y. lipolytica YSXID	Miscanthus hydrolysates (Fed-batch) supplemented with YNB containing amino acids and ammonium sulfate G: 35.2 + 32.7 X: 32.8 + 22.8	12.01	42.4	[34]
C. curvatus	Corn stover enzymatic hydrolysates supplemented with YE, ammonium sulfate, and trace elements G: 18.8 X: 14.5	4.6	39.4	[33]

4. Conclusions

In this study, we have demonstrated the remarkable capability of engineered *A. gossypii* strains to grow and produce lipids, achieving a lipid titer of up to 1.42 g/L in non-detoxified EBH with minimal processing supplemented with CSL. This finding opens up new avenues for the development of a low-cost, bio-integrated process for lipid production, leveraging non-detoxified lignocellulosic biomass as a sustainable feedstock.

The present study has also highlighted the critical importance of considering environmental factors that can induce stress and trigger early autolysis in *A. gossypii*. In particular, the availability of oxygen emerged as a crucial parameter influencing both biomass and target metabolites production. Autolysis onset can compromise biomass and metabolite yields, necessitating further investigation to optimize biomass production and establish a well-balanced oxygen supplementation strategy. Addressing these challenges will be instrumental in achieving even higher lipid and metabolite yields in future studies.

The results showcased in this study mark a notable stride towards the progress of bio-based lipid production as an alternative by-product within biorefineries, exemplifying their commitment to holistic biomass utilization. By understanding and optimizing the factors influencing strain performance with minimal hydrolysate processing, we can lay the foundation for cost-effective and sustainable lipid production processes. Future research should focus on refining oxygenation strategies and further exploring *A. gossypii* to produce tunable lipid profiles. The knowledge gained from this work contributes to the growing field of industrial biotechnology and has significant implications for a more sustainable and economically viable bio-based economy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9090791/s1, Figure S1: Nile red staining of lipid bodies inside the mycelia; Figure S2: Sugar consumption and metabolite production during the fermentation course; Figure S3: Metabolite production during the fermentation course; Figure S4: Performance of *A. gossypii* A877 in bioreactor cultivations; Figure S5: Nile red staining of lipid bodies.

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