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Microbial lipids from industrial wastes using xylose-utilizing *Ashbya gossypii* strains

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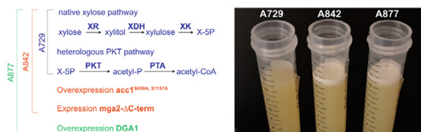
G R A P H I C A L   A B S T R A C T



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A B S T R A C T

This work presents the exploitation of waste industrial by-products as raw materials for the production of microbial lipids in engineered strains of the filamentous fungus *Ashbya gossypii*. A lipogenic xylose-utilizing strain was used to apply a metabolic engineering approach aiming at relieving regulatory mechanisms to further increase the biosynthesis of lipids. Three genomic manipulations were applied: the overexpression of a feedback resistant form of the acetyl-CoA carboxylase enzyme; the expression of a truncated form of Mga2, a regulator of the main Δ9 desaturase gene; and the overexpression of an additional copy of *DGA1* that codes for diacylglycerol acyltransferase. The performance of the engineered strain was evaluated in culture media containing mixed formulations of corn-cob hydrolysates, sugarcane molasses or crude glycerol. Our results demonstrate the efficiency of the engineered strains, which were able to accumulate about 40% of cell dry weight (CDW) in lipid content using organic industrial wastes as feedstocks.

1. Introduction

The sustainable and circular bioeconomy constitutes a new challenge for the near future that aims at using renewable biological resources to produce food, materials and energy. Hence, a sustainable bioeconomy can turn organic wastes and residues into valuable resources (European Commission, 2018). In this regard, the development of new technologies and microbial biocatalysts for the biotransformation of industrial wastes into high-value products is an important issue for the industrial biotechnology. Consequently, different approaches are being implemented for the valorization of waste low-cost substrates such as lignocellulosic hydrolysates, crude glycerol, molasses, waste cooking oil, wastewaters and animal or plant food waste (Qin et al., 2017; Ravindran and Jaiswal, 2016). Indeed, the lignocellulosic biorefinery industry has received much attention from the EU and represents an important element of the future European bioeconomy (Hassan et al., 2019). These strategies broaden horizons for the sustainable production of biofuels, nutraceuticals, enzymes, bioactive compounds, bioplastics and nanoparticles, among others (Ravindran and Jaiswal, 2016).

Microbial lipids can be used for the production of many valuable chemicals with applications as biofuels, nutraceuticals and oleochemicals (Lennen and Pfleger, 2013). Microbial lipids are produced by a wide range of microorganisms such as filamentous fungi, microalgae,

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bacteria and yeast, which can be cultivated in a controlled bioreactor environment. In addition, some microbial biocatalysts can be grown using low-cost waste substrates and, therefore, represent an ideal element for a sustainable bioeconomy (Qin et al., 2017).

Ashbya gossypii is a filamentous hemiascomycete that is currently exploited for the industrial production of riboflavin (Revuelta et al., 2017). A complete molecular toolbox for strain engineering is available for *A. gossypii*, including genomic, bioinformatic, and biotechnological tools (Aguiar et al., 2015; Dietrich et al., 2004; Jiménez et al., 2019; Ledesma-Amaro et al., 2018). In terms of bioprocessing, *A. gossypii* has the ability to grow using low-cost substrates and presents inexpensive downstream processing (Schwechheimer et al., 2016), thereby making this fungus a suitable microbial chassis for the implementation of efficient bioprocesses. Thus, *A. gossypii* has been proposed as an efficient biotechnological chassis for the production of different high-added value compounds such as folic acid (Serrano-Amatriain et al., 2016), nucleosides (Ledesma-Amaro et al., 2015a), recombinant proteins (Aguiar et al., 2017), γ-lactones (Silva et al., 2019) and microbial lipids (Ledesma-Amaro et al., 2018, 2015b).

In this regard, the production of microbial lipids in *A. gossypii* from xylose and sugarcane molasses has been reported using different metabolic engineering strategies (Díaz-Fernández et al., 2017; Lozano-Martínez et al., 2017). The endogenous xylose-utilizing pathway was overexpressed, together with an heterologous phosphoketolase pathway, channeling the xylose carbon flux towards the biosynthesis of microbial lipids (Díaz-Fernández et al., 2017). In addition, the expression of a truncated form of Mga2, a regulator of the main Δ9 desaturase gene *OLE1* (Fig. 1), was demonstrated to be effective for increasing the biosynthesis of fatty acids (FA) in culture media containing either glucose or sugarcane molasses (Lozano-Martínez et al., 2017). However, the production of lipids did not exceed 25% of the cell dry weight (CDW), thereby suggesting that regulatory mechanisms might hamper the biosynthesis of lipids in *A. gossypii*.

The existence of major bottlenecks due to feedback inhibition of lipogenic enzymes has been described (Fig. 1): the acetyl-CoA carboxylase (Acc1), which is the initial and rate-limiting enzyme of FA biosynthesis, can be inhibited both by saturated FA and by Snf1-dependent phosphorylation (Qiao et al., 2015; Woods et al., 1994). Also, the fatty acyl-CoA pool can regulate the activity of the FA synthase (FAS), the Acc1 and the Δ9 desaturase (Ole1) in yeasts (Neess et al., 2015; Qiao et al., 2015). Indeed, previous works succeeded at increasing the biosynthesis of lipids by abolishing those regulatory bottlenecks (Pfleger et al., 2015). For example, the simultaneous overexpression of Δ9 desaturase, Acc1 and diacylglycerol acyltransferase (Dga1) contributed to the reduction of saturated FAs and resulted in a significant increase of the lipid titer in *Yarrowia lipolytica* (Qiao et al., 2015). Also, the use of feedback resistant mutants of *ACC1* and the overexpression of *DGA1* have been proved to be successful metabolic engineering strategies aimed at increasing the lipid productivity (Blazeck et al., 2014; Shi et al., 2014).

This work presents a combined approach both for alleviating regulatory bottlenecks of lipid biosynthesis and utilizing different industrial wastes for the production of microbial lipids in *A. gossypii*. Hence, engineered strains of *A. gossypii* are reported to accumulate up to 40% of CDW, following a multi-wastes valorization approach, using corn-cob lignocellulosic hydrolysates (CCh), sugarcane molasses or crude glycerol. The utilization of these industrial by-products as efficient feedstocks for microbial fermentation is further discussed.

2. Materials and methods

2.1. *A. gossypii* strains and general growth conditions

The *A. gossypii* GXX-PX strain (A729) was used as the parental strain

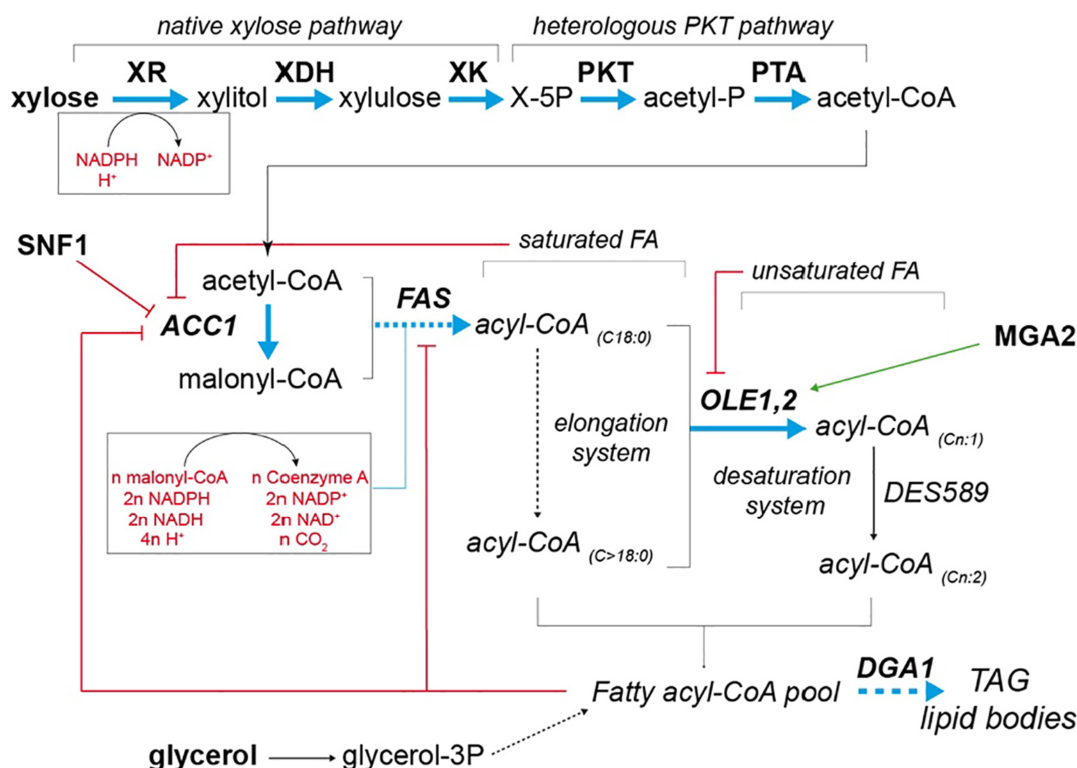

Fig. 1. Schematic representation of the biosynthesis of lipids. A simplified model of the biosynthesis of lipids from xylose is depicted. Engineered targets are indicated in bold. Negative regulation is indicated by red lines; positive regulation is indicated by green lines. Blue arrows denote metabolic steps that were activated in this work. The cofactor balances of XR and FAS reactions are shown in red. XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulose kinase; PKT, phosphoketolase; PTA, phosphotransacetylase; ACC1, acetyl-CoA carboxylase; FAS, FA synthase; OLE1, 2, Δ9 desaturase; DGA1, diacylglycerol acyltransferase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Díaz-Fernández et al., 2017). *A. gossypii* spore isolation and sporulation conditions were as described previously (Jiménez et al., 2005). Cultures were grown at 28 °C and 200 r.p.m. using either MA2-rich medium (Jiménez et al., 2005) or AFM-rich medium (Aguiar et al., 2014) with the indicated carbon sources. Concentrations of 250 mg/L for geneticin (G418) (Gibco-BRL) were used where indicated.

2.2. Gene manipulation

For the overexpression of an additional copy of *DGA1*, an overexpression integrative cassette was constructed using a Golden Gate assembly method as previously described (Ledesma-Amaro et al., 2018). The integrative module comprised recombinogenic flanks targeting the *AFR171W* locus and a *loxP-KanMX-loxP* selection marker. The *DGA1* ORF was placed under the *AgGPD1* promoter (P_{AgGPD1}) and the *AgPGK1* (T_{AgPGK1}) terminator sequences. For the overexpression of a feedback resistant form of Acc1 an overexpression integrative cassette targeting the *ACC1* gene was designed. First, an overexpression plasmid was constructed *in vitro* comprising recombinogenic flanks targeting the *ACC1* locus, a *loxP-KanMX-loxP* selection marker, the P_{AgGPD1} promoter and the *ACC1* wild-type coding sequence (3799 nt). Second, site-directed mutagenesis by inverse PCR was followed to sequentially introduce T1975G (S559A) and T3463G (S1155A) mutations using mutagenic primer pairs. The final construct was linearized and the transformation cassette was obtained by enzymatic restriction with *Not*I. The expression of a truncated form of Mga2 was carried out by deleting the C-terminal part (residues 850-1139) of AgMga2 as previously described (Lozano-Martínez et al., 2017).

Spores of *A. gossypii* were transformed with the corresponding cassettes as described previously (Jiménez et al., 2005), and positive clones were selected in G418-containing medium. Homokaryon clones were obtained by sporulation of the primary transformants. The correct genomic integration of each cassette was confirmed by analytical PCR followed by DNA sequencing. The *loxP* repeated inverted sequences present in the *loxP-KanMX-loxP* marker enabled the selection marker to be eliminated by expressing a Cre recombinase and subsequently reused (Aguiar et al., 2014; Serrano-Amatriain et al., 2016).

2.3. Preparation and detoxification of corn cob hydrolysate (CCh)

Corn cob was mixed with water at liquid solid ratio of 8 g/g and submitted to hydrothermal treatment under non-isothermal conditions (Tmax of 205 °C), following the operational conditions previously described (Garrote et al., 2008), in a 2 L stainless steel reactor (Parr Instruments Company) equipped with Parr PDI temperature controller (model 4848). After treatment, autohydrolysis liquor was recovered by filtration and submitted to dilute acid treatment (0.5% w/w H_2SO_4 for 165 min at 125 °C), as described elsewhere (Rivas et al., 2006). The CCh was neutralized with $CaCO_3$ until pH 5.

Inhibitor compounds (namely, furfural, HMF and phenolic compounds) were removed using activated charcoal and ionic exchange resins (Cunha et al., 2019). CCh was mixed with activated charcoal (10 g of hydrolysate/g of activated charcoal) for 60 min in agitation for phenolic compounds removal. After that, the hydrolysate was submitted to ion exchange resins treatment. Hydrolysate was mixed with Amberlite IR-120 resin (H + form) at ratio of 10 g of resin/g of hydrolysate for 60 min in agitation. Then, the cationic resin was removed by filtration and the hydrolysate was mixed with Mto-Dowex anionic resin (OH– form) at ratio 20 g of resin/g of acetic acid.

Finally, the pH of the detoxified CCh was adjusted to 6.8 with KOH (5 M) before sterilization by filtration and then CCh was added to the media at a final concentration of 50% v/v, in order to keep the concentration of xylose within the range of 10–15 g/L.

2.4. Preparation and detoxification of crude glycerol

Crude glycerol was kindly provided by CVR-Centre for Waste Valorisation (Guimarães, Portugal) and, before use, it was prepared as previously described (Ruhal et al., 2011) with slight modifications. The raw material was first diluted in distilled water (1:4 v/v) to reduce its viscosity. Then, the pH of the mixture was adjusted to 3 with HCl (6 M) to convert soap into free FA. The precipitates formed were separated from the mixture by centrifugation at 7000 r.p.m. for 10 min. Next, the pH was adjusted to 12 with KOH (5 M) and the suspension filtrated in order to remove suspended debris. Subsequently, the pH of the filtrate was adjusted to 6.8–7.0. Finally, the methanol contained in the solution was removed by autoclaving and the concentration of glycerol quantified by HPLC. Crude glycerol was then added to the medium at a final concentration of 1% w/v.

2.5. Lipid production conditions

Mycelia of each strain was scrapped off agar-solidified MA2 after 2.5 days of growth and treated with 7.5 mg/mL of lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich) until obtaining homogeneously dispersed mycelia (≈ 1 h). After washing and resuspension in culture medium (1.5 mL/plate), 1 mL of this pre-inoculum was used to inoculate 250 mL shake-flasks containing 50 mL of medium. Initial characterization of the generated strains was performed in AFM containing 2% w/v xylose or 1% w/v xylose plus 1% w/v glucose/glycerol as main carbon sources. Medium formulations containing low-cost substrates and AFM-based supplement (10 g/L yeast extract, 10 g/L tryptone and 1 g/L myo-inositol) were further evaluated as production media (Table 1). *A. gossypii* cultures were carried out in an orbital-shaker at 28 °C and 200 r.p.m. and samples were taken at defined time intervals to monitor growth (through CDW determination of lyophilized biomass samples), sugar/glycerol consumption and lipid accumulation.

2.6. Total lipid extraction for quantitative analysis

Mycelial biomass from liquid cultures was collected by filtration, lyophilized and the dry cell weight of each sample was determined. Lipid extraction from 10 to 15 mg of the lyophilized biomass was carried out with chloroform/methanol by applying a modification of the Folch's method (Schneiter and Daum, 2006): equal volumes of methanol and chloroform were added to the mycelium powder and mixed vigorously. Then, 1/2 vol of 1 M NaCl was added, mixed vigorously and centrifuged for 5 min at 2000 r.p.m. The lower organic phase was

Table 1
Low-cost substrate media formulations used for microbial lipid production.

Media	Concentration (g/L)				
	Xylose	Sucrose	Glucose	Fructose	Glycerol
50% CCh[a] plus 4% sugarcane molasses[b]	15.1 ± 1.4	20.3 ± 5.4	2.6 ± 0.7	–	–
50% CCh[a] plus 1% crude glycerol[a]	12.9 ± 0.3	–	–	–	13.5 ± 0.5

(–) not present.
[a] CCh and crude glycerol were prepared as indicated in the Materials and Methods section.
[b] Sugarcane molasses were used without detoxification as kindly provided by RAR: Refinarias de Açúcar Reunidas, S.A. (Porto, Portugal).

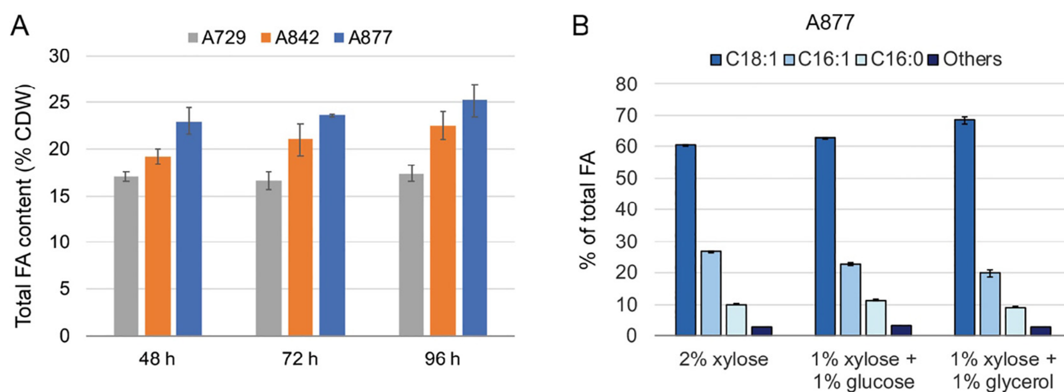

Fig. 2. Lipid production by engineered strains of *A. gossypii* grown in synthetic media. A, total lipid content of the strains A729, A842 and A877 grown in AFM media containing 2% xylose as carbon source. B, lipid profile of the A877 strain grown in AFM media containing different combinations of carbon sources. The results are the means of three independent experiments. The error bars represent the standard deviations.

collected and dehydrated by addition of an excess of anhydrous sodium thiosulfate. Finally, the samples were filtered and the total lipid content was determined gravimetrically after evaporation of organic solvents using a rotary evaporator.

2.7. Lipid extraction for GC–MS analyses

Lyophilized biomass (5–10 mg) was ground in screw-capped glass tubes (Duran) using a glass rod and resuspended in 1 mL of a solution of 2.5% (v/v) sulfuric acid in methanol containing 50 µg of internal standard. The tubes were then closed with Teflon caps and incubated at 80 °C for 100 min, making sure that no evaporation of solvent occurred. The reaction was stopped with 1 mL of 1 M NaCl and 0.5 mL of n-hexane was added to the mixture. Fatty acid methyl esters (FAMES) were extracted into the organic phase by vigorous stirring. The tubes were then centrifuged at room temperature for 5 min at 2500 r.p.m. and approximately 0.3 mL of the upper organic phase was collected into 2 mL glass vials closed with Teflon caps. Just before analysis, the samples were dehydrated by addition of an excess of anhydrous sodium thiosulfate and transferred to new vials containing 200 µL glass inserts.

GC–MS was carried out in a Scion SQ™ 436 GC–MS (Bruker), using a SLB-IL 100 column (30 m long, 0.25 mm internal diameter and 0.20 µm of film; Supelco). For the analyses, helium was used as carrier gas at a flow rate of 1 mL/min, and 1 µL of sample was automatically injected with the following programmed split ratio: initial split ratio of 1:20 for 0.01 min, which was then turned off for 0.49 min, next a split ratio of 1:100 was used for 0.5 min, after which it was switched again to 1:20 indefinitely. The injector temperature was 250 °C and the interface temperature was 200 °C. The oven program was as follows: initial temperature of 90 °C for 5 min, a ramp of 12 °C/min to 190 °C, and a ramp of 4 °C/min to 230 °C. MS detection was from 50 to 500 Da. The FAs were identified by comparison with the FAMES of the commercial standard FAME37 (Supelco). The total quantification of FAs was carried out following the method of standard internal pattern using 50 µg/mL of heptadecanoic acid C17:0 (Sigma).

2.8. HPLC analyses

Samples from liquid cultures were analysed by HPLC for sugars (xylose, glucose, fructose, sucrose) and glycerol determination. Xylose, xylitol and glycerol were quantified using a refractive index detector (Jasco) Aminex HPX-87 (BioRad, USA) column and eluted with 0.005 M H_2SO_4 flow rate 0.6 mL/min at 60 °C. Sucrose, xylose, glucose and fructose were quantified using a refractive index detector (Jasco) and a Prevail Carbohydrate ES column. A mixture of acetonitrile:water (75:25 v/v) pumped at 0.9 mL/min was used as mobile phase at 30 °C.

3. Results and discussion

3.1. Rewiring the regulation of lipid metabolism in A. gossypii

An engineered strain of *A. gossypii* (A729) with improved lipogenic capacity using xylose as carbon source was used as a parental strain (Díaz-Fernández et al., 2017). This strain was previously engineered to overexpress the native xylose-utilizing pathway of *A. gossypii* (*GRE3*, xylose reductase; *XYL2*, xylitol dehydrogenase; *XKS1*, xylulose kinase). In addition, channeling of metabolic flux towards lipid biosynthesis was achieved in the A729 strain by heterologous overexpression of a phosphoketolase pathway (*xpkA*, xylulose-5P phosphoketolase, from *Aspergillus nidulans* and *pta*, phosphotransacetylase, from *Bacillus subtilis*).

Envisioning the further improvement of the lipogenic capacity of this xylose-utilizing strain, three metabolic engineering strategies were carried out to alleviate the feedback inhibition over Acc1 and FAS in *A. gossypii* (Fig. 1). The first strategy involved the overexpression of a mutant allele of *ACC1* to abolish posttranslational regulation of Acc1. It has been shown that substitution of the residues Ser659 and Ser1157 by non-phosphorylatable residues resulted in an enhanced activity of Acc1 in *Saccharomyces cerevisiae* (Shi et al., 2014). Both Ser659 and Ser1157 residues are conserved in the *A. gossypii* Acc1 enzyme. Consequently, an overexpression cassette was constructed for the genomic replacement of the *ACC1* with a mutant *acc1* allele, where the residues Ser659 and Ser1157 were mutated to Ala. The second strategy was aimed at reducing saturated FAs, by increasing the activity of the Δ9 desaturase, to prevent their inhibitory effect over Acc1. It has been shown that the overexpression of the endogenous *OLE1*/*OLE2* genes, coding for Δ9 desaturases, did not enhance the lipid biosynthesis in *A. gossypii* (Lozano-Martínez et al., 2016). However, the expression of a cytosolic C-terminal truncated form of Mga2 (mga2-ΔC-term), which is a positive regulator of *OLE1,2*, resulted in a significant increase of the total lipid content in *A. gossypii* (Lozano-Martínez et al., 2017). Therefore, the expression of the *mga2-ΔC-term* allele was combined with the overexpression of the $acc1^{S659A,\ S1157A}$ in the engineered strain A842. With these modifications, the total lipid content of this strain increase up to 1.3 fold compared to the parental strain A729 when both strains were grown in AFM containing 2% (w/v) xylose (Fig. 2A). A third strategy was further designed as a metabolic sink for the fatty acyl-CoA pool through the overexpression of an additional copy of *DGA1*, which is considered a lipogenic enhancer (Blazeck et al., 2014). Hence, the reduction of the fatty acyl-CoA pool by its redirection towards the biosynthesis of triacylglycerols (TAG) should contribute to diminish the negative regulatory effects of the acyl-CoAs in the engineered strain A877, which combines all the genetic manipulations described (Fig. 1). Accordingly, the total lipid content of this strain reached 25.2 ± 1.7%

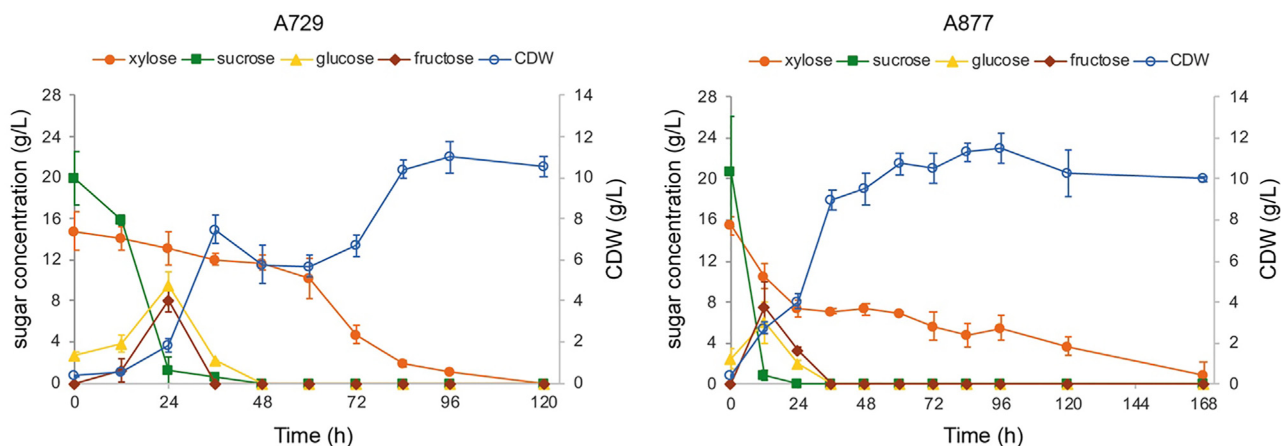

Fig. 3. Utilization of CCh plus sugarcane molasses by engineered strains of *A. gossypii*. Biomass production (CDW) and sugar consumption of the strains A729 (left panel) and A877 (right panel) grown in 50% CCh plus 4% sugarcane molasses. The results are the means of three independent experiments. The error bars represent the standard deviations.

of CDW, which is 1.5 fold the lipid content in the parental strain grown under the same conditions (Fig. 2A). When grown in AFM containing a mixture of 1% w/v xylose plus 1% w/v glucose/glycerol instead of 2% xylose, the maximum amount of lipids accumulated by the strain A877 remained similar (27.9 ± 3.2% in xylose plus glucose and 28 ± 1.9% in xylose plus glycerol), as well as its FA profile (Fig. 2B). However, the mixture of xylose with glucose/glycerol allowed attaining lipid titers higher than those attained with xylose alone (1.4 ± 0.2 g_{Lipids}/L in xylose + glucose and 1.7 ± 0.1 g_{Lipids}/L in xylose + glycerol vs 0.8 ± 0.1 g_{Lipids}/L in xylose).

Simulations performed with the genome-scale metabolic model of *S. cerevisiae* indicated that the combination of xylose metabolism through the phosphoketolase pathway (which is heterologously expressed in the *A. gossypii* strain A877) with glucose metabolism through glycolysis could lead to an increase in the yield of lipids per substrate consumed, as compared to xylose metabolism alone (Caspeta and Nielsen, 2013). In agreement with these predictions, the mixture of xylose with glucose increased the yield of biomass (and lipids), from 42.6 ± 0.7 (and 10.7 ± 0.7) g/mol_{xylose} to 106.3 ± 3.0 (and 26.2 ± 2.8) g/$mol_{xylose+glucose}$, which justifies the observed increase in lipid titer in xylose + glucose cultures as compared to xylose cultures.

On the other hand, the overproduction of microbial lipids mostly depends on both the availability of immediate precursors and the continuous supply of NADPH as reducing power (Ratledge, 2004), which is also required for xylose reduction by xylose reductase (XR) (Fig. 1). Glycerol can be metabolized through glycerol-3-phosphate, which is the first committed precursor of the triglyceride biosynthetic pathway and therefore can be readily utilized for lipid biosynthesis (Wang et al., 2001; Zheng and Zou, 2001) (Fig. 1). The genome of *A. gossypii* encodes syntenologs of the yeast genes that are responsible of glycerol dissimilation (Gomes et al., 2014). Therefore, the combination of xylose with glycerol, a carbon source that besides being a direct source of important precursors for lipid biosynthesis (Fig. 1) also owns a higher degree of reduction than sugars, should positively contribute to lipid production by *A. gossypii* A877. Accordingly, the mixture of xylose with glycerol led to a slight increase in the yield of lipids (from 10.7 ± 0.7 g/mol_{xylose} to 12.9 ± 0.3 g/$mol_{xylose+glycerol}$), whereas the biomass yield remained similar (42.6 ± 0.7 g/mol_{xylose} vs 47.6 ± 3.6 g/$mol_{xylose+glycerol}$).

Altogether, these results suggest that the A877 strain is a promising biocatalyst for the production of microbial lipids from xylose-rich lignocellulosic hydrolysates mixed with other low-cost carbon sources. Multi-wastes valorization approaches have recently been described as a promising strategy for improvement of lignocellulosic-based processes (Cunha et al., 2018). Hence, further characterization of the growth kinetics and the lipogenic capacity of this strain was devised using different formulations of industrial by-products.

3.2. Utilization of CCh and sugarcane molasses for the production of microbial lipids in A. gossypii

Lipid production from agroindustrial wastes has emerged as a promising alternative for the development of sustainable processes. Hence, the valorization of industrial wastes represents an important challenge for the biotechnological production of high-value metabolites. Recently, several low-cost and renewable carbon sources such as lignocellulosic hydrolysates, molasses and crude glycerol have been tested for single cell oil production by oleaginous yeast and filamentous fungi (Chaiyaso et al., 2019; Ganatsios et al., 2017; Gong et al., 2016; Ruan et al., 2012). Therefore, additional experiments focused on the characterization of the engineered strains in culture media formulations containing CCh and sugarcane molasses, which mainly contained xylose and sucrose, respectively.

CCh was prepared and detoxified to minimize the concentration of growth inhibitors such as organic acids and furan compounds (Cunha et al., 2019). Then, flask cultures in medium containing 50% v/v of detoxified CCh plus 4% w/v of sugarcane molasses were carried out with the strains A729 and A877. The initial concentration of carbohydrates in culture media are indicated in Table 1. The biomass production and sugar consumption were recorded until the carbon source was exhausted (Fig. 3). The results show that both strains were able to use the xylose and sucrose from the culture media for cell growth (Fig. 3); with sucrose being consumed more efficiently than xylose by both strains. Remarkably, the strain A729 was able to consume all the sugars after 4–5 days of culture, in contrast to the strain A877 that lasted 7 days until the carbon source was exhausted (Fig. 3). It is worthy to mention that the strain A729 suffered a sporulation and re-growth cycle when the hexose sugars were depleted in the culture media (36–48 h). This effect might be explained by a different utilization rate of hexose and pentose sugars in the A729 and A877 strains. Hence, the absence of sporulation might have contributed to increase the yield of biomass in the strain A877, which was higher than that in the strain A729 (Table 2).

Total lipid production of the two engineered strains was measured at different time-points during the stationary phase. As shown in the Fig. 4A, the strain A877 reached 38.1 ± 1.5% of CDW in lipid content at the end of the culture, which represents an increase of about 2.4-fold with regard to the parental strain. Noteworthy, the lipid titer achieved by strain A877 in this medium was about 2.7-fold higher than that achieved in synthetic AFM containing xylose plus glucose (Table 2).

Table 2
Yields and titers for the engineered strains A729 and A877 in low-cost substrate media formulations.

Strain	Media	Lipid Titer[a] (g_{Lipids}/L)	Lipid Yield (g_{Lipids}/mol)	Biomass Yield (g_{CDW}/mol)
A729	AFM 2% xylose	0.96 ± 0.03	8.8 ± 0.3	51.5 ± 2.8
	50% CCh + 4% molasses	1.8 ± 0.1	12.0 ± 0.8	73.8 ± 5.2
	50% CCh + 1% glycerol	1.7 ± 0.1	7.9 ± 0.4	45.5 ± 5.3
A877	AFM 2% xylose	0.8 ± 0.1	10.7 ± 0.7	42.6 ± 0.7
	AFM 1% xylose + 1% glucose	1.4 ± 0.2	26.2 ± 2.8	106.3 ± 3
	AFM 1% xylose + 1% glycerol	1.7 ± 0.1	13.8 ± 0.9	55.5 ± 2.4
	50% CCh + 4% molasses	3.9 ± 0.1	30.7 ± 0.9	90.7 ± 5.9
	50% CCh + 1% glycerol	3.0 ± 0.2	15.4 ± 0.8	53.2 ± 3.1

[a] For both strains, the maximun lipid titers reached are shown, time at which the presented parameters were calculated. Since the medium formulations contain more than one carbon source, yields were determined per mol of substrate consumed.

The improved lipid titer can be explained by a higher concentration of sucrose/glucose in the CCh + molasses culture media (Table 1) that promoted a higher biomass density during the first 48 h of culture. These results demonstrate that co-fermentation of xylose-rich hydrolysates together with sucrose-rich molasses is a novel and efficient strategy for the production of microbial lipids in *A. gossypii*. Indeed, lipid accumulation is highest when mixed CCh-molasses are used, compared with cultures containing either CCh or molasses alone. In agreement with these results, the co-fermentation of sweet potato vine hydrolysate and molasses by *Trichosporon fermentans* was reported to increase its lipid content, as compared with fermentation of pure molasses (Shen et al., 2015).

In this regard, the mixture of xylose with glucose was shown above to promote the increase in lipid (and biomass) yields in strain A877. Moreover, it has been reported that the utilization of xylose in *A. gossypii* at early stages of culture can be hindered by an insufficient amount of biomass, thus causing an extended lag-phase (Díaz-Fernández et al., 2017). However, during the co-fermentation of xylose-rich and sucrose-rich wastes, the invertase activity of *A. gossypii*, which is responsible for the hydrolysis of sucrose (Aguiar et al., 2014), releases hexose sugars to strongly promote cell growth at early stages of culture, consequently decreasing the lag phase.

The consumption of xylose in the strain A877 at high biomass densities might be compromised by the availability of reducing power. Specifically, NADPH is extensively required for increased production of lipids in the rewired biosynthetic FA metabolism of strain A877, and therefore it might be lacking for the activity of XR, the first enzyme involved in the metabolism of xylose (Fig. 1). In addition, the results might indicate that, once biomass has reached high densities, carbon flux from pentose sugars would be mainly directed for lipid production (Caspeta and Nielsen, 2013).

The co-utilization of composite sugars derived from pentose-rich hydrolysates and molasses have been also introduced for the production of ethanol in *S. cerevisiae* as well as co-cultures of *S. cerevisiae* and *Pichia stipitis* (Brar et al., 2019), thereby further supporting the strategy for the valorization of mixed formulations of industrial by-products.

3.3. Utilization of CCh and waste glycerol for the production of microbial lipids in A. gossypii

The transesterification of vegetable oils and animal fats from different bioprocesses, such as the production of biodiesel, generates crude glycerol as a by-product. Hence, an increasing demand of biodiesel will produce an excess of glycerol that can be considered as a promising feedstock for microbial fermentations (da Silva et al., 2009). In this context, the mixture of lignocellulosic hydrolysates and biodiesel-derived glycerol has been reported as an interesting strategy for the increase of lipid production (Gong et al., 2016). Therefore, considering our promising results in synthetic medium, we decided to characterize the performance of our engineered strains in culture media containing both CCh and crude glycerol.

Analysis of the growth performance of strains A729 and A877 in media containing 50% v/v of detoxified CCh plus 1% w/v of crude glycerol (Fig. 5 and Table 2) revealed that while strain A729 showed a simultaneous co-utilization of glycerol and xylose, the strain A877 exhibited a lower consumption rate for xylose. Simultaneous assimilation of glycerol and sugars at the beginning of the culture was also observed in the co-fermentation of corn stover hydrolysate and molasses for lipid production by *Cryptococcus curvatus* (Gong et al., 2016). This effect concurred with the accumulation of xylitol in the strain A729, which might reflect the saturation of the enzymatic activity of xylitol dehydrogenase (XDH). In contrast, the strain A877, which has a higher lipogenic capacity than A729, did not accumulated xylitol (Fig. 5). These results might be explained in the context of the availability of NADPH as reducing power both for the activity of XR and for the biosynthesis of lipids. A lower demand of NADPH for FA biosynthesis in the A729 strain might promote an increase of the XR enzymatic activity, which is dependent of NAD(P)H, thereby contributing to the saturation of XDH.

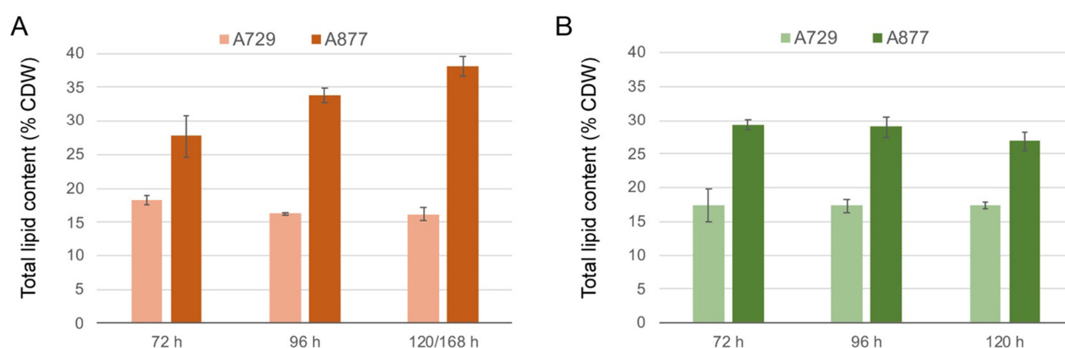

Fig. 4. Lipid production by engineered strains of *A. gossypii* using industrial wastes. A, total lipid content of the strains A729 and A877 grown in 50% CCh plus 4% sugarcane molasses. B, total lipid content of the strains A729 and A877 grown in 50% CCh plus 1% crude glycerol. The results are the means of three independent experiments. The error bars represent the standard deviations.

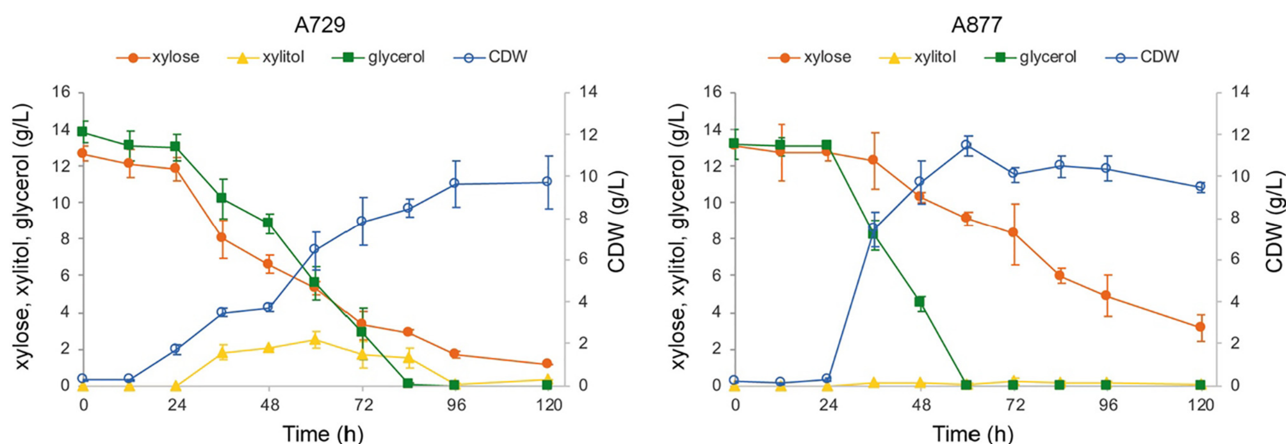

Fig. 5. Utilization of CCh plus crude glycerol by engineered strains of *A. gossypii*. Biomass production (CDW), xylose and glycerol consumption, and xylitol production of the strains A729 (left panel) and A877 (right panel) grown in 50% CCh plus 1% crude glycerol. The results are the means of three independent experiments. The error bars represent the standard deviations.

Conversely, the higher lipogenic capacity of the strain A877 might restrict the supply of NAD(P)H for XR, thus limiting the consumption of xylose and, moreover, preventing the accumulation of xylitol. In this regard, the use of heterologous enzymes for xylose utilization with different cofactor requirements, such as XR and XDH from innate xylose-fermenting yeasts (Cunha et al., 2019), might help to increase the xylose consumption rate of the strain A877.

The production of lipids from CCh and crude glycerol was measured at different time-points of the cultures. In agreement with the previous results, the strain A877 showed higher titer of total lipids than the strain A729 (Table 2 and Fig. 4B). The strain A877 was able to accumulate 29.3 ± 0.8% of CDW in lipid content (lipid titer of 3 g/L) using CCh and crude glycerol, thus demonstrating the efficiency of this strain for the utilization of mixed waste by-products. In this medium, a higher degree of mycelial lysis can contribute to the slight decrease of lipid content at late time-points (Fig. 4B), which may contribute to the generally lower yields obtained in this medium as compared to CCh plus sugarcane molasses (Table 2). The increased mycelial lysis in CCh plus glycerol can be a consequence of a lower content of the carbon source (Table 1). In addition, the concentration of carbon source that was consumed either for biomass or lipid production was significantly lower compared to CCh plus sugarcane molasses.

Similar studies have reported the co-fermentation of crude glycerol together with fresh yeast extract or ammonium sulfate, using the oleaginous *Y. lipolytica* and obtaining lipid titers ranging from 0.8 to 1.3 g/L (Poli et al., 2014). Also, the importance of providing a higher intracellular $NADH/NAD^+$ ratio, through the co-utilization of glycerol and mixed sugars has been described for the production of 1,3-propanediol in *Clostridium diolis* (Xin et al., 2016).

The lipid titers obtained in this work are within the range of previous reports using flask cultures (Donot et al., 2014; Jagtap and Rao, 2018; Lazar et al., 2018). For example, lipid titers ranging from 1.1 g/L to 6.7 g/L have been obtained using *Y. lipolytica* flask cultures [see (Lazar et al., 2018) and references therein]. Moreover, the optimization of culture conditions in a fed-batch bioreactor would increase significantly the lipid production of the *A. gossypii* engineered strains. Indeed, previous works reported the optimization of culture parameters such as the oxygenation conditions and inoculum densities in bioreactors to increase lipid titers over two orders of magnitude, depending of the carbon source and the fermentation conditions [see (Lazar et al., 2018) and references therein]. The results hereby presented show the feasibility of using *A. gossipii* as a cell factory for the valorization of industrial waste by-products. Moreover, these results pave the way for additional valorization processes as alternative genomic manipulations can determine the use of engineered strains for the production of other high-added value compounds.

4. Conclusions

This work reports engineered strains of *A. gossypii* able to accumulate 30–40% of CDW in lipid content using wastes as raw materials. The utilization of CCh, sugarcane molasses and crude glycerol for the production of microbial lipids represents an excellent strategy for the valorization of industrial by-products. Downstream optimization in a controlled bioreactor would increase titer, yield, and productivity of the engineered strains. The use of *A. gossypii* as biocatalyst allows the exploitation of these industrial wastes not only for lipid biosynthesis, but also for the production of other high-added value compounds such as riboflavin, folic acid or nucleosides.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.122054.

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E-supplementary data description:

Supplementary Table 1. *A. gossypii* strains generated in this study.

Supplementary Table 2. Fatty acids profile of the strain A877 strain grown in AFM with different carbon sources (% of total FA).

Supplementary Table 3. Lipid production by oleaginous yeasts and filamentous fungi

Figure S1. Construction of the *DGA1* and the acc1$^{S659A, S1157A}$ overexpression cassettes.

Figure S2. Lipid production of the A877 strain using different formulations of culture media containing corn-cob hydrolysates (CCh) and/or sugarcane molasses (Mol) as carbon sources.

Supplementary Table 1. *A. gossypii* strains generated in this study.

Strain	*Genotype*	*Phenotype*	*Source*
A729	*P_{GPD}-GRE3, P_{GPD}-XKS1, P_{GPD}-XYL2, P_{GPD}-pta, P_{GPD}-xpkA (**GXX-PX strain**)*	xyl^+	our lab stock
A785	*GXX-PX, MGA2-ΔC-term*	$G418^R$, xyl^+	this work
A862	*GXX-PX, P_{GPD}-ACC1$^{(S659A; S1155A)}$*	$G418^R$, xyl^+	"
A789	*GXX-PX, MGA2-ΔC-term*	xyl^+	"
A829	*GXX-PX, MGA2-ΔC-term, P_{GPD}-ACC1$^{(S659A; S1155A)}$*	$G418^R$, xyl^+	"
A842	*GXX-PX, MGA2-ΔC-term, P_{GPD}-ACC1$^{(S659A; S1155A)}$*	xyl^+	"
A877	*GXX-PX, MGA2-ΔC-term, P_{GPD}-ACC1$^{(S659A; S1155A)}$, P_{GPD}-DGA1*	$G418^R$, xyl^+	"

Supplementary Table 2. Fatty acids profile of the strain A877 strain grown in AFM with different carbon sources (% of total FA).

	C14:0	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C24:1	Unsaturated	Saturated
2% xylose	0.2 ± 0.0	10.0 ± 0.1	26.8 ± 0.1	1.0 ± 0.0	0.4 ± 0.0	60.4 ± 0.1	0.1 ± 0.0	0.7 ± 0.02	0.03 ± 0.01	0.1 ± 0.0	89.2 ± 0.2	10.6 ± 0.1
1% xylose + 1% glucose	0.2 ± 0.0	11.2 ± 0.2	22.8 ± 1.2	0.8 ± 0.04	0.7 ± 0.01	62.7 ± 0.4	0.2 ± 0.01	1.0 ± 0.03	0.1 ± 0.0	0.2 ± 0.01	87.7 ± 1.6	12.1 ± 0.2
1% xylose + 1% glycerol	0.1 ± 0.01	9.0 ± 0.3	19.9 ± 1.2	0.7 ± 0.01	0.5 ± 0.1	68.4 ± 1.0	0.2 ± 0.02	0.9 ± 0.1	0.1 ± 0.01	0.1 ± 0.02	90.2 ± 2.3	9.6 ± 0.4

Values are the average of three independent experiments ± standard deviation.

Supplementary Table 3. Lipid production by oleaginous yeasts and filamentous fungi

Microorganism	Carbon source	Lipid titer (g/L)	Lipid content (%)	Culture mode	Ref.
Y. lipolytica	Glucose	2.47	55.8	Flask	(Silverman *et al.*, 2016)
Y. lipolytica	Wheat straw hydrolysate	0.3 – 0.4	4.4 – 4.6	Flask	(Yu *et al.*, 2011)
Y. lipolytica	Agave bagasse hydrolysate	16.5	67	Fed-batch	(Niehus *et al.*, 2018)
Y. lipolytica	Molasses/glycerol	24.2	40	Fed-batch	(Rakicka *et al.*, 2015)
Aspergillus niger	Glycerol	3.4	41.4	Batch	(André *et al.*, 2010)
A. niger	Xylose	0.37	8	Batch	(Zheng *et al.*, 2012)
Mortierella isabellina	Xylose	2.52	50.9	Batch	(Zheng *et al.*, 2012)
M. isabellina	Molasses	5.1	53.6	Batch	(Chatzifragkou *et al.*, 2010)
M. isabellina	Glycerol	1.9	33.9	Batch	(Chatzifragkou *et al.*, 2011)
Cryptococcus curvatus	Wheat straw hydrolysate	4.2 – 5.8	27.1 – 33.5	Flask	(Yu *et al.*, 2011)
C. curvatus	Glycerol	13.9	44.5	Fed-batch	(Liang *et al.*, 2010)
Rhodosporidium toruloides	Wheat straw hydrolysate	2.4	24.6	Flask	(Yu *et al.*, 2011)
R. toruloides	Xylose	6.5-7.5	35.5 – 57	Batch	(Zhang *et al.*, 2016)
Rhodotorula glutinis	Wheat straw hydrolysate	2.4 – 3.5	20.7 – 25	Flask	(Yu *et al.*, 2011)
R. glutinis	Glycerol	6.1	60.4	Fed-batch	(Saenge *et al.*, 2011)

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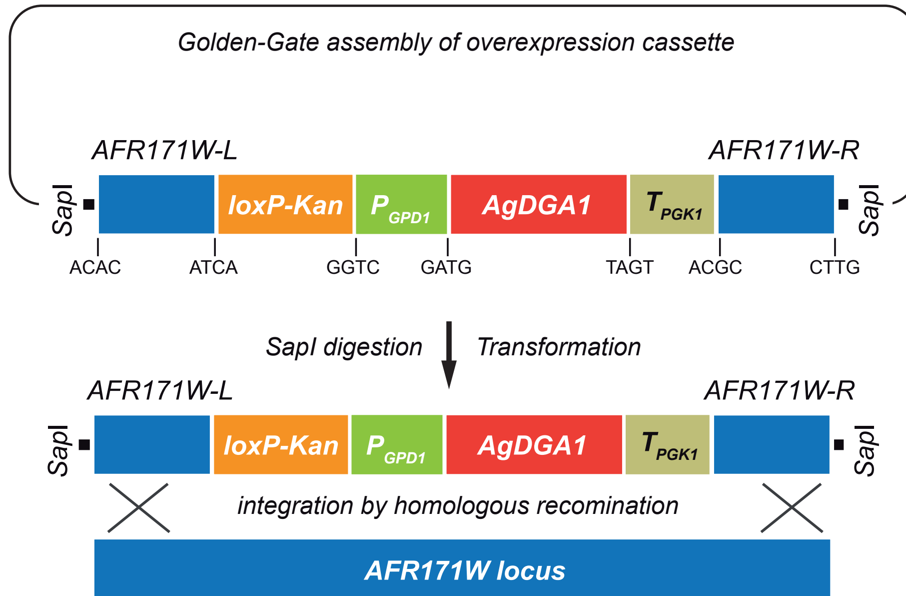
Figure S1. Construction of the *DGA1* overexpression cassette.



Construction of the acc1S659A, S1157A overexpression cassette.

Figure S2. Lipid production of the A877 strain using different formulations of culture media containing corn-cob hydrolysates (CCh) and/or sugarcane molasses (Mol) as carbon sources.