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Increased Production of Free Fatty Acids and Triglycerides in Aspergillus carbonarius by Metabolic Engineering of Fatty Acid Biosynthesis and Degradation Pathways

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- Fara

Abstract

Microbial conversion of biomass into fatty acid-derived advanced biofuels has gained a lot of attention recently. Fatty acids and triglycerides can be used as source materials for the production of biodiesel fuel. Here, we increased internal free fatty acid and triglyceride levels in *Aspergillus carbonarius*, by manipulating two key enzymes of the fatty acid metabolism of the fungus. Our strategy involved the replacement of the native promoters of the ATP-citrate lyase, which catalyzes the conversion of cytosolic citrate to acetyl-CoA, and fatty acid synthase, which is responsible for the biosynthesis of fatty acyl-ACP in the fatty acid metabolic pathway, with strong, constitutive promoters. With the enhanced expression of ATP-citrate lyase and fatty acid synthase, we managed to achieve a significant increase in the internal free fatty acid levels and triglyceride levels of the fungus, thus showing the potential of *A. carbonarius* as a producer of fatty acid-derived biofuels. In addition, we also increased the intracellular free fatty acid and triglyceride levels of the fungus by identifying and deleting the gene for FaaA fatty acyl synthetase, responsible for the conversion of fatty acid to fatty acyl-CoA which mediates the feedback inhibition of the fatty acid biosynthesis. Finally, we have studied the effect of the deletion of FarA regulatory protein which acts as a transcription factor for genes related to fatty acid degradation in fungi.

ABBREVIATIONS

ACL: ATP- Citrate Lyase; FAS: Fatty Acid Synthase; FAAA: Fatty Acyl-Coa Synthetase; DCW: Dry Cell Weight;

INTRODUCTION

Due to the concerning situation with decreasing global fossil fuel supplies and increasing greenhouse gas emissions, a lot of interest has been drawn to the production of renewable liquid transportation fuels. Ethanol is the most prominent biofuel which is produced on a worldwide scale [1]. However, ethanol has a low energy density and it is not an optimal fuel for the currently existing petroleum-based transport infrastructure [2]. New types of biofuels, such as fatty acids, fatty alcohols, and medium and long chain alkanes/alkenes [collectively referred to as advanced or drop-in biofuels], are on the other hand a more promising alternative, as they can be used for substituting gasoline, diesel, and kerosene components [3]. Biodiesel is currently produced by the transesterification of vegetable oils or animal fats with methanol. The use of first generation feedstock's, such as vegetable oil, creates a competition between food and fuel, resulting in a non-sustainable process from economical and ethical point of view [4]. Other feed stocks used for biodiesel production are used vegetable oils. However, triglycerides from this feedstock contain a high diversity of fatty acyl side chains which may exhibit undesirable characteristics for the biodiesel produced [5].

Instead of using food crops, the potential of employing second generation feedstocks, such as lignocellulosic biomass, for the production of fatty acid based biofuels by engineered microbial factories are currently being explored [1]. In fungi, fatty acid synthesis is essential for the production of membrane lipids. Fungi show a great promise for biodiesel production due to their suitable lipid profiles and the consistency in the quality of the accumulated fatty acids and triglycerides [6]. Furthermore, fuel characteristics of the fungal lipids and secretion of the products could be potentially further improved by genetic engineering.

Recent development on increasing lipid production in *Aspergillus oryzae* has been achieved by identifying and

manipulating fatty acid metabolism related genes [7]. Fatty acid metabolism of fungi starts in the cytosol with ATP-citrate lyase [ACL]. This enzyme has the function of cleaving cytoplasmic citrate to yield acetyl-CoA [and oxaloacetate] [8,9] which stimulates the production of fatty acids and triglycerides by being further converted to free fatty acids in three consecutive steps catalyzed by acetyl-CoA carboxylase [ACC], fatty acid synthase [FAS] and palmitoyl-ACP thioesterase [PAT]. The resulting free fatty acids are then further converted to fatty acyl-CoA molecules by different acyl-CoA synthetases [10], which are channeled into desaturation, elongation, degradation and lipid synthesis pathways [11,12]. The degradation of long chain fatty acids takes place in the peroxisomes, where fatty acids are degraded to acetyl-CoA through the Beta-oxidation pathway [10,13]. Cytosolic fatty-acyl CoA molecules, including palmitoyl-CoA, are also channeled back into the peroxisomes where they are degraded [11]. An overall illustration of the fungal fatty acid metabolism is presented in Figure 1.

In *Aspergillus nidulans* and *A. oryzae*, the ACL and FAS enzymes are reported to be composed of two subunits, each subunit being encoded by separate genes. For both enzymes, the genes encoding the two subunits are situated next to each other in the genome, in a reverse orientation sharing a common, bidirectional promoter [7,8,14]. There are a number of phylogenetically distinct FAS enzymes in fungi with different roles, ranging from the primary metabolism to the synthesis of secondary metabolites [e.g. alfatoxin] [14]. The FAS enzyme, involved in the fatty acid metabolism is under tight regulation on translational and transcriptional levels alike, thus genetic engineering of such an essential enzyme can prove to be difficult [15].

Six different acyl-CoA synthetase genes have been identified in *Aspergillus nidulans*, namely FatA, FatB, FatC, FatD, FaaA and FaaB, with different functions and cellular localizations [10]. The FaaA protein is considered to be responsible for the generation of cytosolic palmitoyl-CoA from palmitic acid [11], which can prevent excess fatty acid production by binding to the acyl-CoA binding site of the acetyl-CoA, in this way mediating a feedback inhibition [16]. In addition, the fatty acyl-CoA can enter the Beta-oxidation pathway where it is degraded. Deletion of the *faaA* gene led to increased levels of intracellular fatty acids in *A. oryzae* [11].

In addition, two regulatory proteins [FarA, FarB], highly conserved among ascomycetes, have been shown by Hynes et al.[17], to act as transcription factors for genes related to Beta-oxidation of lipids [17]. Deletion of these genes in *A. nidulans* resulted in the loss of ability of the fungus to utilize long- and short chain fatty acids, respectively, due to loss of induction of a number of genes involved in shuttling acetyl-CoA from the peroxisomes to the mitochondria [by the carnitine acetyltransferase], as well as genes of the Beta-oxidation in the presence of fatty acids.

The filamentous fungus *Aspergillus carbonarius* has been in focus in several studies as a potential cell factory for the production of industrially relevant enzymes [18], organic acids [19,20] and also advanced biofuels, such as hydrocarbons [21]. In this study, we carried out enhanced expression of the putative ACL and FAS genes of *A. carbonarius* based on previously identified sequences from other *Aspergilli* [7,8] and examined the fatty acid metabolism of these enhanced expression strains. We hypothesized that choosing a fungus capable of elevated production of citrate, such as *A. carbonarius*, is a good candidate for the constitutive expression of key fatty acid enzymes, as the cytoplasmic citrate is a substrate for acyl-CoA formation catalyzed by ACL; increased availability of acyl-CoA triggers lipogenesis which in combination with an active FAS could lead to the increased accumulation of fatty acids and triglycerides. In addition, we have deleted putative genes encoding FarA and FaaA proteins in *A. carbonarius* ITEM 5010 in order to examine the effects of these genetic modifications on the fatty acid metabolism of the fungus and to potentially increase the free fatty acid and triglyceride levels of the fungus.

MATERIALS AND METHODS

Strains and media

Aspergillus carbonarius strain KB1039 [ITEM 5010 *ΔkusAΔpyrG*, kindly donated by K. S. Bruno, PNNL, USA], deficient in the non-homologous end joining mechanism [22] was used as parent strain for constructing ACL and FAS over expression strains as well as the *ΔfarA* and *ΔfaaA* deletion strains. The parent strain was chosen in order to promote homologous integration events. The orotidine-5′-monophosphate decarboxylase gene [*pyrG*] complemented strain of *A. carbonarius* [named KB1039_ *pyrG*$^{+}$, donated by KS. Bruno, PNNL, USA] was used as the control strain for all analysis. The control strain was maintained on minimal medium agar [MMA] [23], supplemented with 10 mM Uracil and 10 mM Uridine to maintain the *pyrG*- deficient strain.

Conidial suspension was prepared from the MMA [or MMA plus Uracil/Uridine] plates by adding sterile double distilled water and filtering the suspension through sterile Mirracloth [Millipore, Billerica MA, USA] followed by counting with a haemocytometer.

The *A. nidulans* A851 genomic DNA (provided by K. S. Bruno PNNL, USA] or *Aspergillus carbonarius* ITEM 5010 genomic DNA, isolated by Cetyl trimethylammonium bromide (CTAB) and phenol-chloroform extraction method [24], was used to amplify the promoter region of translation elongation factor 1α [*tef1*] by PCR.

E. coli DH5α or STBL2 served as a host for all plasmid construction.

All chemicals were purchased from Sigma-Aldrich [St. Louis, MO, USA] unless otherwise stated. Modified Czapek-Dox broth [10% glucose, 2% $NaNO_3$, 1% K_2HPO_4, 0.5% $MgSO_4 \cdot 7H_20$, 0.5% KCl, 0.01% $FeSO_4 \cdot 7$ H2O, pH 7.3] and Yeast malt medium [YM, 3% yeast extract, 3% malt extract, 10% glucose] was used for fungal biomass growth for the analysis of fatty acids and triglycerides.

Construction of ACL and FAS enhanced expression strains

Genes encoding Fas1 [Beta subunit], Protein ID [PID] 208134, and Fas2 [alpha subunit], PID 208136, as well as the genes for both Acl subunits [subunit 1 and 2], PID 204896 and PID 204897, were identified in *A. carbonarius* based on sequence homology to the previously identified Fas and Acl genes in *A. nidulans.*

Enhanced expression of the Acl and Fas genes was obtained by

the replacement of their original promoters, in a similar manner, to the strong, constitutive *tef1* promoter. Both genes have a similar arrangement in the genome, having their two subunits situated next to each other in a reverse orientation, sharing a common [bidirectional] promoter. This native promoter was replaced by gene targeting techniques using two *tef1* promoters from two different species [*A. nidulans* and *A. carbonarius*], each oriented towards a subunit of the gene. The use of different *tef1* promoters was chosen instead of using identical *tef1* promoters from one species, due to the possible stability issues with having similar DNA sequences in close proximity, previously reported in *A. oryzae* [7].

Enhanced expression vectors were constructed by inserting *tef1* promoters and targeting sequences homologous [previously amplified by PCR using oligonucleotides presented in Supplementary Table S1], to either Acl or Fas, into plasmid *pSB415* which contained *pyrG* fungal selection marker from *A. fumigatus* by Gibson Assembly [New England Biolabs, Ipswich, MA, USA] according to manufacturer's protocol. Prior to assembly, plasmid *pSB415* was linearized with restriction enzyme *PacI* and *AsiSI* [Thermo Scientific]. Enhanced expression vectors *pSB415ACLoe* and *pSB415FASoe* are presented in Supplementary Figure S1-A,B, respectively.

DNA for the generation of the ACL transformant (Figure 2A) was obtained by restriction enzyme digestion of the gene targeting fragment from plasmid *pSB415ACLOE* with *EcoRI* and *NotI* (both from Thermo Scientific, Pittsburgh, PA, USA), followed by the gel purification (NucleoSpin Gel and PCR Clean-up kit, Clontech Laboratories Inc., Mountain View, California, USA) of the ~7,5 kilo base pairs (kb) DNA fragment. Bipartite gene targeting fragments *A* and *B* (3.5 kb and 3.4 kb, respectively) for generating the FAS transformants were obtained by PCR amplification from plasmid *pSB415FASOE* using the oligonucleotides presented in Supplementary Table S1. The fragments were then purified using commercial PCR purification kit [NucleoSpin Gel and PCR Clean-up kit, Clontech Laboratories Inc., Mountain View, CA, USA], mixed in a total volume of 10 µl [in equal molar ratio] and used for the transformation of protoplasts (Figure 2B). All PCR reactions were carried out using Phusion polymerase [New England Biolabs, Ipswich, MA, USA], following the manufacturers protocol.

Construction of farA and faaA deletion strains

Genes encoding FaaA and FarA proteins were identified in *A. carbonarius* based on sequence homology to previously identified orthologs in *A. oryzae* [11] and *A. nidulans* [17], respectively. 1.5 kb fragments of the flanking regions of the candidate genes were amplified by PCR using the oligonucleotides presented in Supplementary Table S2 and cloned into different plasmids. The up- and downstream regions of the gene encoding FaaA was cloned into plasmid *pSB415*, so that it flanks the *pyrG* selection marker, resulting in knock out vector *pSB415F3AKO* [Supplementary Figure S2A]. The upstream and downstream regions of the putative FARA were cloned into separate plasmids, *pSBi415A* and *pSBi415B*, respectively, yielding *pSB415iAFarAKO* and *pSB415iBFarAKO* [Supplementary Figure S2B]. These plasmids were designed to complement each other by containing only a partial [but overlapping] sequence of the *pyrG* selection marker, in addition to two similar direct repeat sequences, one before the start of the *pyrG* sequence and one at the end, which facilitate the counter-selection of the marker gene. The necessity to use two separate plasmids instead of one is due to stability problems observed during *E. coli* propagation of single plasmids containing two similar direct repeats.

Bipartite gene targeting fragments were amplified from plasmid *pSB415F3AKO* by PCR. One fragment consisted of upstream region and partial sequence of the *pyrG* marker, while the other fragment included a partial *pyrG* sequence (overlapping with the *pyrG* sequence of the first fragment) and the downstream fragment. The two fragments were mixed in equal molar ratio and used for the protoplast transformation of the parent strain to generate *ΔfaaA* mutant (Figure 3A). The correct mutants were confirmed by PCR on their genomic DNA. All the oligonucleotides used for this purpose are presented in Supplementary Table S2.

The generation of the *ΔfarA* mutant was carried out by using bipartite gene targeting fragments that were amplified from plasmids *pSB415iAFarAKO* and *pSB415iBFarAKO* by PCR and used in equal molar ratios for the protoplast transformation of the parent strain [Figure 3B]. Oligonucleotides that were used for this purpose are presented in Supplementary Table S2. All PCR reactions were carried out using Phusion polymerase (New England Biolabs, Ipswich, MA, USA), and cloning was carried out using Gibson Assembly (New England Biolabs, Ipswich, MA, USA), following the manufacturer's protocols.

Fungal transformation

Protoplasting of *A. carbonarius* KB1039 was carried out by inoculating conidial suspension of the strain to 100 ml Yeast malt broth [Sigma-Aldrich, St. Louis, MO, USA] containing 10 mM Uracil and 10 mM Uridine, in a final concentration of 10^6 conidia/ml medium, followed by cultivation at 30°C at 140 rpm for 18-24 hours. The cell walls of the harvested mycelia were then digested by a solution of 60 mg/L Vino Taste Pro [Novozymes, Bagsvaerd, Denmark] prepared in 50 ml protoplasting buffer [1.2 M $MgSO_4$, 50 mM Phosphate Buffer, pH 5.0] for 4-6 hours until most of the mycelia were digested. The protoplasts were filtered through sterile Mira cloth, overlaid with 5 ml of 0.4 M ST buffer [0.4M Sorbitol, 100 mM Tris pH 8.0] and centrifuged for 15 minutes at 800 g. Protoplasts were collected from the interface between the two layers and further purified with 1M ST buffer [1M sorbitol, 50 mM Tris pH 8.0] in 3 consecutive steps of washing and centrifuging for 10 minutes at 800 x g. The protoplasts were then re-suspended in STC buffer [1 M sorbitol, 50 mM Tris, 50 mM $CaCl_2$ pH 8.0] in a final concentration of at least $2x10^7$ protoplasts/ml STC.

The ACL and FAS overexpression strains and the *ΔfarA* and *ΔfaaA* deletions strains were generated by transforming protoplasts of the KB1039 strain, while the AF and FF double transformants were generated by transforming protoplasts of the *ΔfarApyrG*$^-$ strain, in a similar manner, as follows. 1-10 µg of transforming DNA in a maximum volume of 10 µl was added to 100 µl freshly prepared protoplasts and incubated on ice for 15 minutes, followed by the addition of 1 ml of PEG solution [40 % m/v Polyethylen Glycol 4000 in STC] and incubation at room temperature for 15 minutes. The protoplast mixture was

transferred to a Falcon tube containing 25 ml minimal medium agar with 1M sorbitol, mixed and then poured into a Petri dish, followed by incubation at 30°C until the transformants appeared [3-5 days].

Homokaryotic transformants were obtained by several successive rounds of plating conidia of the transformants on minimal medium agar and isolating single colonies.

Verification of the enhanced expression transformants and the *farA* and *faaA* knock-out transformants was carried out by PCR amplifying the target gene regions with oligonucleotides presented in Supplementary Table S1, from the genomic DNA of the transformants. In addition, the enhanced expression strains were verified by sequencing of the PCR products.

Production of spore inoculum and growth conditions

Concentrated spore inoculum was prepared by the "rice bag method" [David E. Culley, PNNL, USA, personal communication, December 5, 2014]. Briefly, 75 grams of short grain brown rice and 75 milliliters of 1% Yeast Extract solution were added to autoclavable sun bags [Sigma-Aldrich, St. Louis, MO, USA]. The bags were sealed with tape and autoclaved for 15 minutes on the liquid cycle. After cooling, the bags were opened under sterile conditions and inoculated with 10 milliliter of conidial suspension [10^4 conidia per ml], spread evenly over the entire surface of the rice. The bags were re-sealed and incubated at 30°C for 5-7 days until the rice was fully covered with fungal spores. After incubation, the spores were harvested by adding 150 ml of sterile 0.9% NaCl solution to the bags, firmly massaging the solution all over the rice and finally filtering the spore suspension through Mirracloth [Millipore, Billerica MA, USA] into centrifuge tubes. The spore suspension was then centrifuged at 800 x g for 10 minutes. After centrifugation, some of the liquid supernatant was removed to get a final concentration of around 10^9 spores per milliliter.

Cultures for biomass growth were prepared by inoculating conidial suspension of each *A. carbonarius* strain [in triplicates] into 100 ml modified CD broth or YM broth to a final concentration of 10^6 conidia per milliliter media, in 500 ml flasks, followed by incubation at 30°C at 140 rpm for 120 h. After incubation, liquid samples were taken from the cultures for HPLC analysis and the fungal hyphae was collected, lyophilized and used for analysis with the internal fatty acid and triglyceride assays as described below. The AF and FF double transformants were only analyzed on modified CD broth.

Intracellular free fatty acid and triglyceride assays

Lyophilized hyphae of the enhanced expression strains, the deletion strains and the control strain, were prepared for analysis using a method previously described by Tamano et al [7]. To determine fatty acid and triglyceride concentrations a commercial free fatty acid kit [Free fatty acids, Half-micro test kit; Roche Applied Science, Mannheim, Germany] and a triglyceride kit [Triglyceride colorimetric assay kit; Cayman Chemical Company, Ann Arbor, MI, USA] was used, respectively, with the prepared fungal hyphae, according to the manufacturer's protocol.

Growth assay for single transformants ACL, FAS, ΔfarA and ΔfaaA

In order to assess the radial growth rate of the transformants and the control strain, the selected transformants were grown on minimal medium agar. Equal amount of conidia [10^5] was inoculated in the middle of the agar plate from each strain. The plates were incubated on 30°C for 3 days.

Growth assay of ΔfarA mutant using different fatty acid carbon sources

The growth of the *ΔfarA* mutant was studied on minimal medium agar containing glucose, acetate, hexanoic acid, lauric acid, myristic acid, oleic acid, tween 20, or tween 80 as sole carbon source. Conidia [10^5] were inoculated in one spot in the middle of the plate, followed by incubation at 30°C for 5 days. The control strain was used as reference for this growth assay.

Fatty acid methyl ester [FAME] reaction and analysis of ΔfarA and ΔfaaA strains

FAMEs reaction was performed on lyophilized hyphae, following a method described by O'Fallon et al.[25], using tridecanoic acid as internal standard. The FAMEs were detected by gas chromatography [GC]/ Flame ionization detector [FID] [GC-system model # 6890N [G1540N], Agilent Technologies, Wilmington, DE, USA] equipped with and DB-WAX column [30 m x 0.53 mm x 1.00 µm, Agilent Technologies, Wilmington, DE, USA]. Retention times of the detected peaks were compared to authentic standards [FAME mix C8-C22, Sigma-Aldrich, St. Louis, MO, USA]

RESULTS

The generation of ACL and FAS enhanced expression strains was achieved by replacing the native promoters of the genes to strong, constitutive promoters, using the described DNA recombinant techniques, followed by confirmation by PCR. The deletion of the *faaA* or *farA* genes was also verified by PCR on the gDNA of randomly selected transformants. One verified strain from each transformation was selected for fermentation and further analyzed for internal free fatty acid and triglyceride levels.

The growth assay on minimal medium agar showed the radial growth rate for the single transformants and the control strain [Figure 4]. The radial growth of the ACL transformant was significantly reduced, while the FAS strain showed only slight decrease, compared with the control strain. There was no significant difference observed between the growth of the *ΔfarA* and *ΔfaaA* mutants and the control strain.

To confirm the function of the putative gene, coding for transcription factor FarA, in the fatty acid degradation of *A. carbonarius*, the *ΔfarA* mutant was grown on minimal medium agar containing different short and long chain free fatty acids as sole carbon source [Figure 5]. On basic minimal medium agar containing glucose, there was no significant difference in growth between the control strain and the *ΔfarA* mutant. However, on the different fatty acid media, many differences were observed. The growth of the *ΔfarA* mutant was completely inhibited on

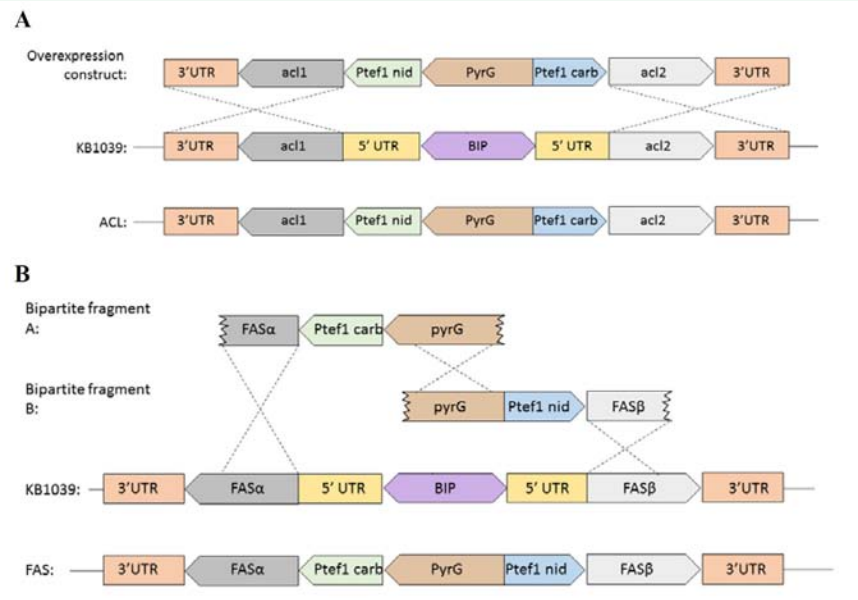

Figure 1 Illustration of the fatty acid metabolic pathway in fungi. The synthesis of fatty acids and triglycerides starts with the conversion of cytosolic citrate to acetyl-CoA (and oxaloacetate) by the ATP-citrate lyase (ACL, EC 2.3.3.8). Acetyl-CoA is carboxylated to malonyl-CoA by the acetyl CoA carboxylase (ACC, EC 6.4.1.2). These two substrates are further used for the synthesis of long chain fatty acyl-ACP by the fatty acid synthase (FAS, EC 2.3.1.85). Finally, the fatty-acyl is hydrolyzed to fatty acid by the palmitoyl protein thioesterase (PAT, EC 3.1.2.14). Triglyceride synthesis, as well as elongation and desaturation steps for the production of C16-C18 fatty acids takes place in the endoplasmic reticulum. Part of the synthesized fatty acids is converted to fatty acyl-CoA by the acyl-CoA synthetase (FAAA, EC 6.2.1.3) which prevents the further synthesis of fatty acids by feedback inhibition. The transcriptional regulator farA (FARA) regulates genes involved in the degradation of fatty acids by the Beta-oxidation pathway in the peroxisome.

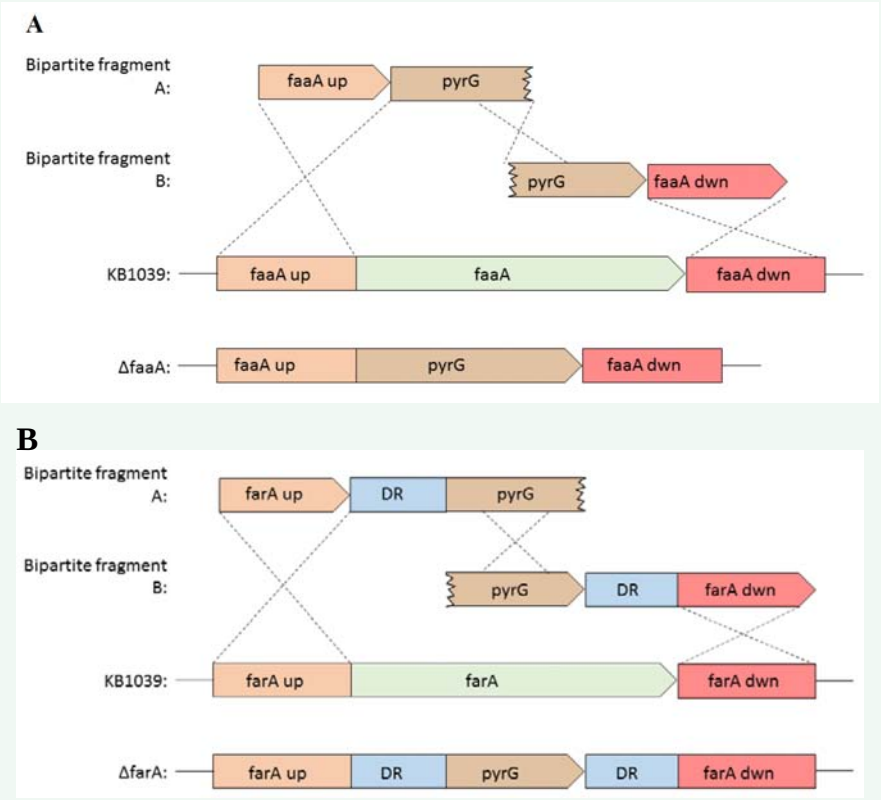

Figure 2 Genetic engineering strategy for the construction of ACL and FAS enhanced expression strains. (A) ACL enhanced expression strain. (B) FAS enhanced expression strain. BIP denotes the native bidirectional promoter of the genes.

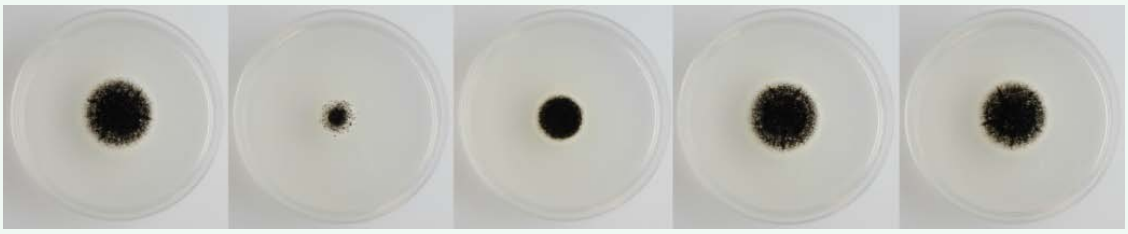

Figure 3 Genetic engineering strategies for the construction of ΔfarA and ΔfaaA deletions strains. (A)ΔfaaA deletion strain(B)ΔfarA deletion strain.

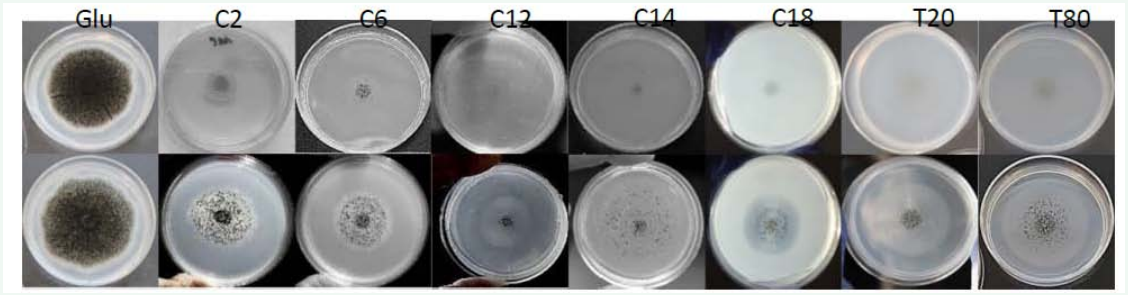

Figure 4 Growth assays of the single transformants and control strain on minimal medium agar. The picture was taken after 3 days of incubation. From left to right: control, FAS, ACL, ΔfarA, ΔfaaA strains.

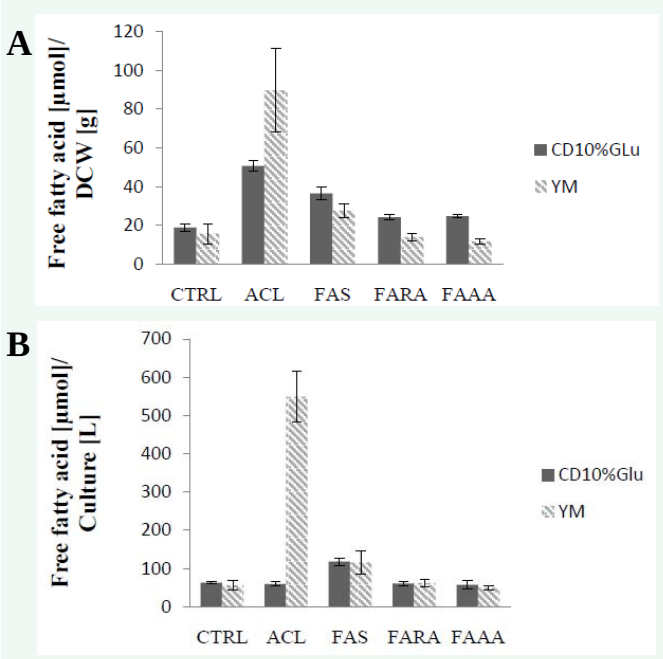

Figure 5 Growth assay of ΔfarA mutant (upper row) and control strain (lower row) on media containing glucose (Glu) or different chain length fatty acids as sole carbon source. C2, C6, C12, C14, and C18 denote acetate, hexanoic acid, lauric acid, myristic acid and oleic acid, respectively. T20 and T80 indicate Tween 20 and Tween 80 carbon sources, respectively.

media containing lauric acid, myristic acid, oleic acid, tween 20, tween 80, lauric acid, myristic acid, oleic acid, and erucic acid as sole carbon sources, while it was partially reduced on acetate and hexanoate.

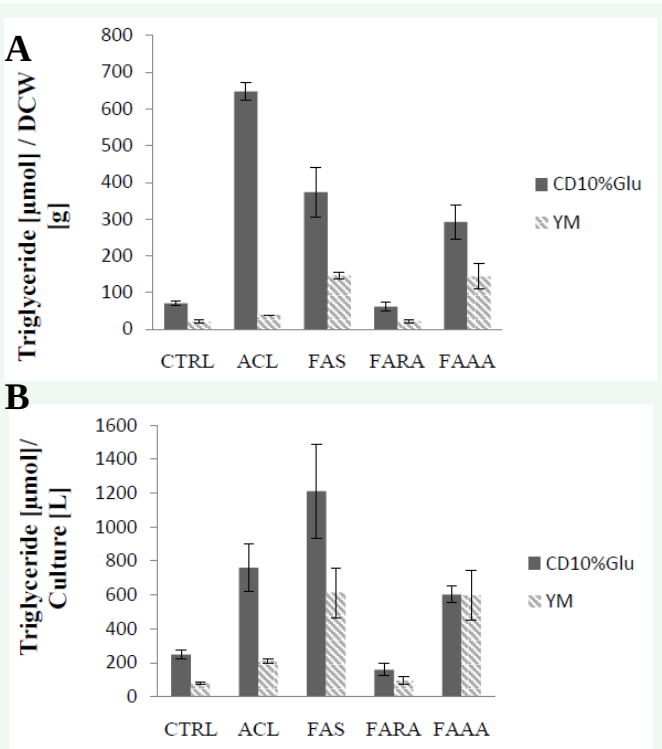

Figure 6 Results of assay for measuring internal free fatty acids. A. carbonarius strain KB1039_pyrG+ used as control strain (CTRL). ACL, FAS, FARA, FAAA denote the enhanced expression strains of ATP-citrate lyase and fatty acid synthase and the knock-out strains of farA and faaA, respectively. Results derive from fermentation on modified CD medium (CD10%Glu) and YM medium (YM). Amounts are presented per gram of lyophilized hyphae (DCW) and those per liter of culture in panels (A, B), respectively. Graphs show the averages and standard deviations of triplicate samples.

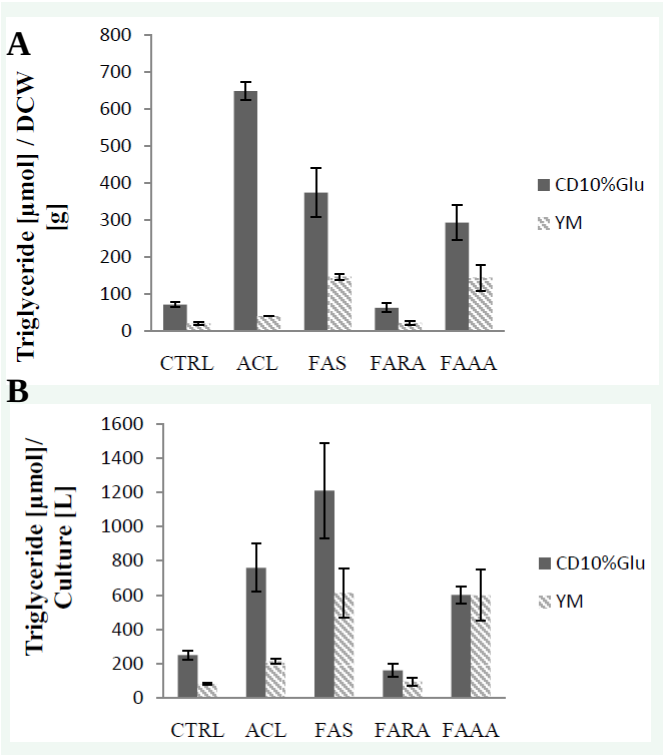

Figure 7 Results of assay measuring internal triglycerides. A. carbonarius strain KB1039_pyrG+ used as control strain (CTRL). ACL, FAS, FARA, FAAA denote the enhanced expression strains of ATP-citrate lyase and fatty acid synthase and the knock-out strains of farA and faaA, respectively. Results derive from fermentation on modified CD medium (CD10%Glu) and YM medium (YM). Amounts are presented per gram of lyophilized hyphae (DCW) and those per liter of culture in panels (A, B), respectively. Graphs show the averages and standard deviations of triplicate samples.

Glucose and citric acid concentrations were measured at the end [120 h] of the flask cultivation of all transformants with the modified CD medium [Table 1]. The ACL strain showed similar glucose consumption to the control strain, while FAS, *ΔfarA* and *ΔfaaA* strains showed higher glucose consumption after 120 h of fermentation. However, citric acid yields were reduced in both the enhanced expression and the deletion strains, with 2-fold, 4-fold, 6 fold and 1.3 fold reductions in ACL, FAS, *ΔfarA* and *ΔfaaA* strains, respectively, compared with the control strain. In addition, the collected fungal biomass of the ACL strain was significantly lower, with only 119 ± 13 mg of biomass produced after 5 days of cultivation, compared with 353 ± 1 mg by the control strain. The other strains also show slight reduction in biomass growth.

The fungal biomass of the transformants was collected for analysis, lyophilized and weighed at the end of the flask cultivation with YM medium as well. All strains showed elevated biomass production on this medium: 409 mg [± 58], 415 mg [± 53] and 587 mg [± 78], 445 mg [± 8] and 417 mg [± 13] mycelial dry weight for the control strain, ACL, FAS, *ΔfarA* and *ΔfaaA,* respectively.

The quantification of internal free fatty acids [FFA] and triglycerides [TG] were carried out by using commercial assay kits on the lyophilized hyphae obtained after flask cultivation using modified CD or YM medium [Figure 6 and 7]. Significantly increased internal FFA levels were detected in both, ACL and FAS enhanced expression strains. However, the assays showed only a slight increase with the *ΔfarA* and *ΔfaaA* deletion strains. In general, FFA levels of the ACL strain were notably higher on YM medium, than on the modified CD, while other transformants showed a better ability to accumulate FFAs on the modified CD-medium. Compared with other transformants, the ACL strain showed the highest level of increase of FFAs per gram of mycelial dry weight on both media, 2.5 and 5 fold, respectively [Figure 6A and Figure 7A]. However, when expressed as production per liter of flask culture, only the FAS strain shows increased [2-folds] FFA levels, on both media. All other transformants show reduced levels of FFAs on the modified CD medium, compared with the control strain [Figure 6B and Figure 7B], but not on the YM medium. On YM, the ACL strain had significantly increased FFA levels, while *ΔfarA* showed a slight increase and *ΔfaaA* a slight decrease.

In general, the results showed significantly lower levels of TGs in all the strains on the YM medium, than on the modified CD medium [Figure 7A]. There was a 9-fold, 5-fold and 4-fold increase in the TG levels of the ACL, FAS and *ΔfaaA* strains, respectively, expressed relative to dry mycelial weight, compared with the control strain, on the CD medium. These increases amounted for 2-fold, 7-fold and 7-fold, respectively, on YM medium. A slight decrease of TG levels was detected with the *ΔfarA* strain on the modified CD medium. Expressed in liter of culture volume, the highest extent of increase was detected with the FAS strains [5-fold on modified CD and 7-fold on YM medium]. On the modified CD medium, the second highest increase was detected with the ACL strain [3-fold], followed by the *ΔfaaA* strain [2.5 fold]. However, on YM medium, the *ΔfaaA* strain showed a higher extent of increase [7 fold] of TG levels than the ACL strain [2.5-fold], when compared with the control strain.

FAMEs analysis results of the *ΔfarA* and *ΔfaaA* mutants reveals that the major fatty acids present in the strain are palmitic acid [PA], stearic acid [SA], oleic acid [OA] and linoleic acid [LA] [Table 2]. The FarA deletion strain shows reduced levels of PA, SA and OA and increased amounts of LA, compared with the control strain. The *Δfaa*A mutant showed increased levels of all the detected fatty acids, compared with the control strain.

DISCUSSION

In the present study, we have successfully increased the free fatty acid and triglyceride levels in *A. carbonarius* by the enhanced expression of putative genes encoding ATP-citrate lyase and fatty acid synthase. The prediction of these genes was based on sequence homology to previously identified Acl enzymes and Fas enzymes of primary metabolism in other fungi, such as *A. nidulans* and *A. oryzae*. During the genome wide search using the sequences of the homolog genes, the reported tandem arrangement of the two subunits had also been observed with the selected, putative ACL and FAS genes of *A. carbonarius.*

In addition to the ACL and FAS enhanced expression strains, we have identified and deleted the faaA gene encoding acyl-CoA synthetase in *A. carbonarius*, which is responsible for catalyzing

Table 1: Parameters of the flask cultivation for the different transformants using CD medium.

Strain	Initial glucose (g/ L)	Final glucose (g/ L)	Cell mass (g 10^{-3}), lyophilized	Citric acid yield (g/ g DCW)
Control strain	101 ± 0.4	93.98 ± 2.38	353 ± 1	0.73 ± 0.04
ACL	101 ± 0.4	91.12 ± 0.47	119 ± 13	0.34 ± 0.03
FAS	101 ± 0.4	87.01 ± 1.24	323 ± 19	0.17 ± 0.03
ΔfarA	101 ± 0.4	84.32 ± 3.45	253 ± 37	0.12 ± 0.02
ΔfaaA	101 ± 0.4	84.82 ± 1.68	230 ± 41	0.56 ± 0.06

Abbreviations: ACL- ATP- citrate lyase overexpression strain;FAS- Fatty acid synthase overexpression strain; ΔfarA- FarA deletion strain; ΔfaaA- Fatty acyl-CoA synthetase deletion strain; DCW- Dry cell weight

Table 2: FAMEs analysis results for *ΔfarA* and *ΔfaaA* deletion strains cultivated in modified CD broth.

Strain	Palmitic acid (C16:0), mg/ g DCW	Stearic acid (C18:0), mg/ g DCW	Oleic acid (C18:1), mg/ g DCW	Linoleic acid (C18:2), mg/ g DCW
CTRL	43.42 (± 3.66)	20.21 (± 1.8)	69.02 (± 3.74)	52.98 (± 6.53)
ΔfarA	32.27 (± 1.33)	15.57 (± 0.79)	52.98 (± 2.03)	81.45 (± 21.23)
ΔfaaA	54.22 (± 2.61)	25.35 (± 1.45)	88.91 (± 1.47)	58.64 (± 3.49)

Abbreviations: CTRL- control strain; ΔfarA- FarA deletion strain; ΔfaaA- Fatty acyl-CoA synthetase deletion strain; DCW- Dry cell weight

the conversion of free fatty acids to fatty acyl-CoA, in this way eliminating the feedback inhibition to the fatty acid biosynthesis and reducing the degradation of free fatty acids. Furthermore, the deletion of the FarA, regulatory protein for the peroxisomal Beta-oxidation pathway genes, was also carried out in this study. As a result, the *ΔfarA* strain lost its ability to utilize medium and long chain length [>12] fatty acids, which confirms the function of this gene in *A. carbonarius*.

A. carbonarius is a natural citric acid producing fungus [19,26]. Initially, citric acid is formed in the mitochondria and then transported to the cytosol before being secreted by the cell. The Acl has a role in cleaving cytosolic citrate to acetyl-CoA and oxaloacetate. The ACL enhanced expression strain showed elevated free fatty acid levels and a 2-fold reduction of citric acid compared to the control strain. These results indicate that the genes coding for Acl were correctly identified and the *tef1* promoters replacing the native promoter were efficiently expressing the two subunits of the enzyme, since cytosolic citrate was being shuffled into the fatty acid metabolism, instead of being secreted. However, citric acid secretion by the FAS strain was even lower than the ACL strain. This might be an indication for the fact that the activity of the natively expressed *Acl* might not be the limiting factor when it comes to providing substrate for the initiation of the fatty acid biosynthesis by the Fas. In fact, it seems the increased flow of citric acid into the fatty acid metabolism would take place without the enhanced expression of the Acl, once the Fas enzyme is over expressed.

For the enhanced expression strains FAS and ACL, a correlation between colony size and increase in free fatty acid and triglyceride levels was observed. A similar observation was reported by Tamano et al., in *A. oryzae* [7], where the correlation was attributed to the possible diversion of acetyl-CoA to fatty acid and triglyceride synthesis from sterol biosynthesis, which is essential for normal hyphae development [27]. However, only the ACL transformant showed a significant reduction in growth, as observed from the plate assay results on radial growth and the cell mass yield after flask cultivation on the modified CD medium. On the other hand, growth of this strain on YM medium was positively affected, yielding a biomass amount comparable to the FAS and the control strains. YM medium provides nitrogenous compounds, carbon and other important growth factors, which are essential for the formation of hyphae. The increased availability of these compounds could be the reason for the increased cell mass yield of the ACL strain observed on the YM medium. The reduced growth of the ACL strain was accompanied with low sporulation, when the strain was cultivated on agar minimal medium prior to preparation of conidial suspension. Similar, reduced radial growth and reduced spore production was observed by Tamano et al. [7], with the enhanced Acl expression strain of *A. oryzae*. In order to obtain highly concentrated conidial suspension in relatively high volumes, required for the flask cultivation, the strains were grown on brown rice and yeast extract. By using this rich solid medium, the ACL strain showed a higher rate of growth and sporulation, corresponding to that observed during the flask cultivation of the strain using YM medium. This characteristic of poor cell mass growth of the ACL strain could be a major drawback for potential industrial use in the future. Compared with the control strain, the growth of the FAS and *ΔfarA* strains is not as dramatically reduced as with the ACL strain. Reduced biomass growth might also bea result of the accumulation of certain toxic intermediates in these transformants, such as acyl-CoA esters, including malonyl-CoA and palmitoyl-CoA, and free fatty acids. Somewhat reduced biomass yield was observed with the *faaA* strain after the fermentation, although the radial growth of the strain was similar to the growth of the control strain on the plate assay. Similar observation was made by Tamano et al. [11], on the radial growth of *ΔfaaA* mutant of *A. oryzae*. However, the *A. oryzae* strain also showed reduced sporulation, generating only 14 percent of the number of spores produced by the parent strain, which was associated with the decreased ratio of linoleic acid [from 50 to 27 percent] compared with the parent strain. Interestingly, low sporulation was not observed with the *ΔfaaA* strain of *A. carbonarius*, although the linoleic acid ratio was also

reduced in the strain, but to a smaller extent [from 29 to 25 percent].

Nevertheless, increased yields of fungal biomass were observed when the strains were grown on YM medium. Once again, the increased availability of compounds in this medium, that positively affect hyphal growth and development, might be the reason why all the strains resulted in similar, higher biomass yields.

Significantly elevated levels of FFAs and TGs were observed with ACL and FAS enhanced expression strains. These results suggest that the *tef1* promoters successfully increased the expression of both genes. Previous observations made by Tamano et al. [7], in *A. oryzae* revealed that, amongst the enhanced expression of the four key enzymes of the FAB [*Acl*, *Acc*, *Fas* and *Pat*], the FAS strain showed the greatest increase of FFAs and TGs, followed by the ACL. However, in this study with *A. carbonarius*, the enhanced expression of Acl resulted in higher levels of FFAs and TGs, than the enhanced expression of Fas. The reason for this contrast between the two studies might be due to the differences in the cytosolic citrate availability of the two fungi. *A. carbonarius* is a natural producer of citric acid and there could be a higher availability of cytosolic citrate, which serves as substrate for the Acl. Therefore, the over expression of the Acl gene might have a stronger effect on FFA and TG production in *A. carbonarius* compared with *A. oryzae*, in which significantly less citrate is exported from the TCA cycle to the cytosol. For the FAS strain, the increase in FFA and TG levels are not as high as for the ACL transformant, which supports the previous observation regarding the correlation between reduced colony size and increased fatty acid production in the enhanced expression strains.

Using a medium with high nutrient content, such as YM, the growth of the ACL transformant remained normal [at similar level with the parent strain] while the FFA levels got increased even further. However, the TG levels were significantly reduced compared to when this strain was grown on modified CD-medium. It has previously been shown that the composition of the culture medium influences not only the metabolism of fungi, but also sporulation, hyphal development, growth rate and other characteristics of the mycelium [28,29]. Therefore, it might be possible that because of the difference in composition between the YM medium and the modified CD broth, at the time of sampling, the biosynthesis of triglycerides was at an early stage in the strain. The fact that all the other strains, including the parent strain, showed reduced TG levels when grown on YM medium as well, also indicates that longer fermentation time might have been necessary to obtain higher TG levels. However, it is possible that with prolonged fermentation, the FFA levels would decrease while the TG levels increase in the strains. Nevertheless, the fact that YM medium restores the biomass growth of all the strains to similar levels indicates that, with further optimization of the production medium, the productivity of the strains, especially the ACL, could be brought to a feasible level.

Deletion of the *faaA* gene resulted in an increase in the intracellular levels of FFAs, at a smaller extent, and TGs at a greater extent. These results correspond with other findings [15], when similar approaches were taken to increase FFAs and TGs in bacterial, yeast, and fungal strains. However, in *A. carbonarius* the increase in FFA was not as significant as reported in other microorganisms. Interestingly, the increase in TG levels was significant, in contrast to what was reported for *A. oryzae* by Tamano et al. [11], where the deletion of this gene did not significantly affect TG levels while the FFAs got increased 9.2-folds. The fact that these results do not concur with the results from *A. oryzae* might be attributed to the time of sampling, due to differences in the metabolism and the metabolic rate of the two species. It is presumed that FFA and TG levels significantly change during the growth of the fungus, therefore samples taken at different time points during the fermentation might show different results than the ones observed after 120 hours, that might be in accordance with the results observed by Tamano et al. [11]. However, the fact that the deletion of the *faaA* gene has a positive effect on the accumulation of FFAs and TGs remains certain.

The deletion of FarA resulted in reduced levels of TGs and did not significantly increase FFAs in *A. carbonarius*. It has been proposed by Hynes et al.[17], that the FarA has not only an activator, but also a repressor function, as the *ΔfarA* mutant of *A. nidulans* showed increased activity for the carnitine acetyltransferase, which is involved in the shuttling of acetyl-CoA from the peroxisomes to the mitochondria. Furthermore, FarA having this repressor function could also be the case with other enzymes that are regulated by it. In this case, the reduced TG levels in the *ΔfarA* mutant of *A. carbonarius* could be explained by the fact, that in the absence of FarA, the fatty acid degradation enzymes are more active than in the control strain, and although FFAs are being synthesized at a constant rate, increased accumulation does not take place as the precursor FFAs for the biosynthesis of TGs are shuttled into the degradation pathways. In fact, FAMEs analysis showed that the quantities of all the fatty acids were decreased in the strain, compared with the control strain, except linoleic acid, which was slightly increased.

In this study, we showed the increased production off fatty acids and triglycerides by over expressing key enzymes of the fatty acid metabolism, namely Acl and Fas, and by deleting the *faaA* gene, thus confirming the potential of *A. carbonarius* as candidate for further research on the production of biodiesel and other, fatty acid-derived advanced biofuels. However, additional improvements on the fatty acid composition of the fungus are required, as high content of unsaturated fatty acid results in lower oxidative stability and thermal efficiency in biodiesel, which negatively affects the operation of the diesel engine.

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