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Improved SDA Production in High Lipid Accumulating Strain of *Mucor circinelloides* WJ11 by Genetic Modification

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Abstract: Stearidonic Acid (SDA; 18:4, n-3) is a ω-3 polyunsaturated fatty acid which is nutritionally important and has pharmaceutical applications. Hence, scientists are trying to construct SDA producing oleaginous microorganisms by genetic modification. Two enzymes, Delta-6 Desaturase (D6D) and ω-3 desaturase catalyze the reactions to produce SDA from Linoleic Acid (LA; 18:2, n-6). But the key enzyme, ω-3 desaturase converts LA to ALA and GLA to SDA is absent in high lipid accumulating *Mucor circinelloides* WJ11, retarding its ability to produce SDA. Therefore, in this study, we overexpressed *fad3* gene from *Arabidopsis thaliana* in *M. circinelloides* WJ11 to produce SDA. Overexpression of *fad*3 gene in *M. circinelloides* WJ11 resulted in the production of 340 mg/l SDA. This research opened a new opportunity to make use of this fungus for industrial production of SDA.

Keywords: Fatty Acid Desaturase 3, SDA Production, Homologous Overexpression, *Mucor circinelloides*

Introduction

The fatty acids that have 18 carbon atoms or more and at least two double bonds are known as Polyunsaturated Fatty Acids (PUFAs) (Ander *et al*., 2003). Mammals are unable to synthesize some long chain PUFAs and, therefore, they have to be derived from Linoleic Acid (LA; 18:2, n-6) and Alpha-Linolenic Acid (ALA, 18:3, n-3). The process involves a series of desaturation and elongation reactions (Wang *et al*., 2014). The principle source of dietary ALA is vegetable oils. ALA can be further converted to longer chain ω-3 fatty acids such as Eicosapentaenoic Acid (EPA; 20:5, n-3) and Docosahexaenoic Acid (DHA, 22:5, n-3). But, the biosynthesis of EPA and DHA from ALA in human is inefficient due to the low activity of Delta-6 Desaturase (D6D) which is the rate-limiting enzyme in this process (Wang *et al*., 2006).

Stearidonic Acid (SDA; 18:4; n-3) occurs as a metabolic intermediate in the biosynthetic pathway to produce EPA from ALA. The requirement of D6D

enzyme in this pathway can be bypassed by SDA. So, the oral administration of SDA is considered as an alternative way to raise the EPA level in the body tissues (Baker *et al*., 2016). Thus, dietary SDA was shown to have a superior activity than ALA for EPA production as it increases the EPA level in red blood cells by approximately 17%, while ALA only produced an increase of about 0.1% (Harris *et al*., 2008).

Seafood, fish and several species of seaweed are among the marine sources of SDA where it occurs as a minor ω-3 PUFA (Abeywardena *et al*., 2016). Among the plants, echium oil, blackcurrant seed oil and oil from the plant of *Boraginaceae* family are important where echium oil is the source of both ω-3 and ω-6 PUFA (Petrik *et al*., 2000; Bilgiç and Yeşilçubuk, 2012). In addition, as the yield of plant-based SDA is very low, its utilization for large scale commercial purpose will not be profitable. Nonetheless, the use of oil from fish as a commercial source of ω-3 fatty acids is often controversial because of its safety concerns, shelf life limits, palatability and the possibility of overfishing (Whelan, 2009; Lu and Zhu, 2015). Therefore, due to these limitations, scientists are considering microbial lipids as a promising alternative candidate for producing SDA at commercial scale (Whelan, 2009).

SDA can also be synthesized from Gamma Linolenic Acid (GLA; 18:3, n-6), an ω-6 PUFA synthesized from LA (Fig. 1) that poses significant nutritional value and medicinal benefits due to its effectiveness in treating and preventing several diseases including diabetes, cardiovascular, cancers and inflammatory disorders (Lu and Zhu, 2015; Horrobin, 1992; Fan and Chapkin, 1998; Kim *et al*., 2012). *Mucor circinelloides* (*M. circinelloides*) is an excellent GLA producing filamentous fungus, but unable to produce SDA because it lacks ω-3 desaturase. Fatty acid Desaturase 3 (*FAD3*) from *Arabidopsis thaliana* (*A. thaliana*) is a ω-3 desaturase which converts LA to ALA (Mendes *et al*., 2013) and can also be used to convert GLA to SDA (Fig. 1). Previously, we demonstrated that *M. circinelloides* can accumulated SDA by overexpressing the *Delta*-15 *Desaturase* (*D15D*) gene which was cloned from *Mortierella alpina* (Khan *et al*., 2019c). However, the SDA levels were relatively low probably as a consequence of insufficient gene expression and the use of a low lipid producing strain as recipient. Therefore, in this work we have overexpressed the *A. thaliana fad*3 gene in *M. circinelloides* WJ11, a high lipid producing strain of *M. circinelloides* and constructed an SDA producing cell factory.

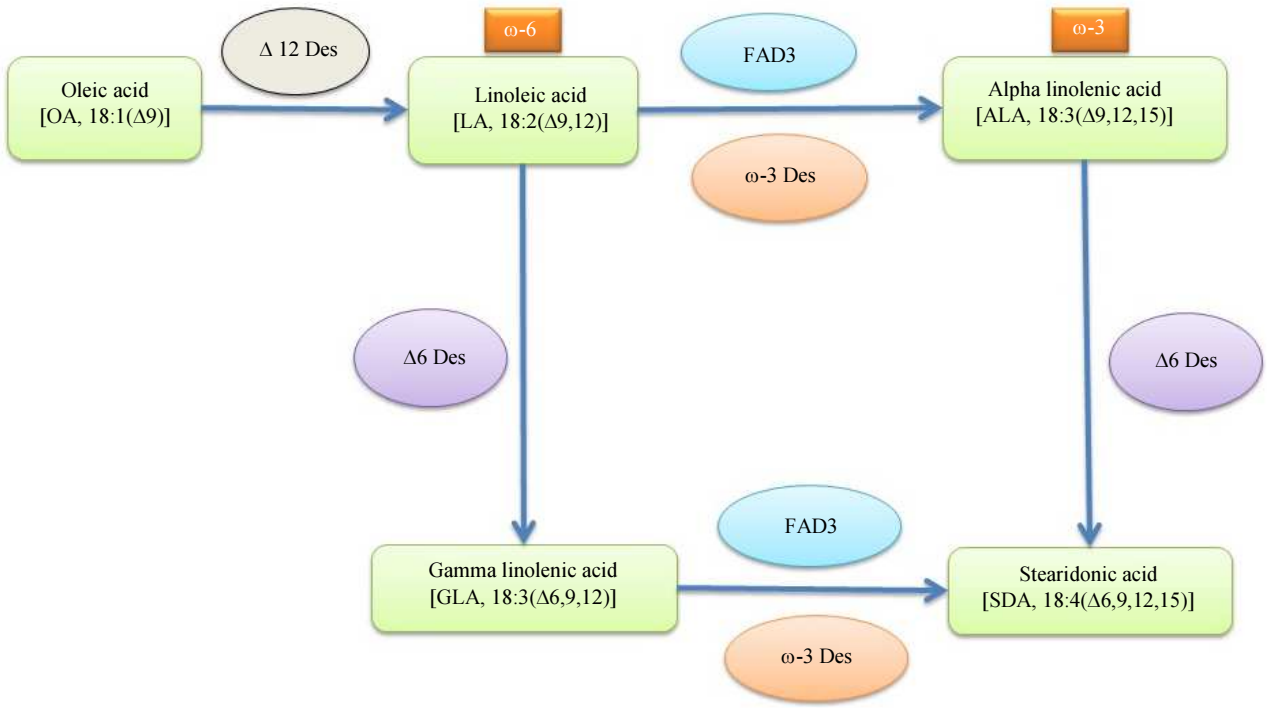

Fig. 1: Metabolic pathway of linoleic acid (LA) flux into ω-6 or ω-3 PUFAs to produce stearidonic acid (SDA)

Methods

Strains, Plasmids and Culture Consditions

According to the genomic data of *Arabidopsis thaliana* in NCBI, the complete coding sequence of the gene *fatty acid desaturase* 3 (*fad*3, Genebank accession number: NM_128552) is 1161 bp long. pUC19-*fad*3 was built by inserting the fragment of *fad*3 gene synthesized according to its gene sequence into pUC19 vector, which was purchased from Polepolar Biotechnology Company, Beijing. The uracil and leucine auxotrophic strain, MU760 of *M. circinelloides* WJ11 was used was used to receive the recombinant plasmid which carried the *fad*3 gene in transformation experiments. Plasmid pMAT2075 was used as the cloning and expression vector. Plasmid pMAT2079 was used as the *fad*3 overexpression host vector generated from pMAT2075. Escherichia coli (E. coli) DH5α was used was used to maintain and propagate the recombinant plasmid. The recombinant strains Mc-2079 (*fad*3 overexpressing strains) and Mc-2075 (control strains) were initially cultivated in 1L flasks containing 150 mL K&R medium for 24 h at 28°C with shaking at 130 rpm. The ingredients of this media are same as previous work (Kendrick and Ratledge, 1992) Production culture was prepared by inoculating 10% (v/v) of the seed culture into a 2 L fermenter (BioFlo/CelliGen115, New Brunswick Scientific, Edison, NJ, USA) containing 1.5 L modified K&R medium. This time the medium was modified because it contained 80 g/L glucose instead of 30 g/L. 0.8 g/L of histidine was supplemented into the cultivation media when it was needed. The temperature in fermenters was maintained at 28°C and pH was maintained at 6.0 by addition of 1 M NaOH automatically, stimulated at 700 rpm with continuous aeration of 1 l/min. Although the recombinant strains Mc-2079 and Mc-2075 were still leucine auxotroph (*leuA*$^-$), it could grow in K&R media supplemented with 0.6 g/L leucine.

Construction of pMAT2075 and pMAT2079 Plasmids and Transformants

To overexpress the target gene in *M. circinelloides* WJ11 a new plasmid was built and named as pMAT2075. The plasmid cloning vector pUC18 was provided by Professor Victoriano Garre (University of Murcia, Spain). The carotenogenic *carRP* DNA fragment which includes up- and down-stream sequences with coding regions, was amplified from the genome of *M. circinelloides* WJ11 and the primers F1/R1 used in this study were list in Table 1. The *carRP* fragment was digested with *SphI* and *SnaBI* and pUC18 vector was digested with *SphI* and *SmaI* restriction endonucleases. Then the two digested DNA fragments were ligated by T4 DNA ligase. From this ligated circular vector, *carRP* coding sequence was deleted by doing reverse PCR using the primers F2/R2 (Additional file 1: Table 1) and linear pUC18 with *carRP* up- and down-stream fragments produced. This linear fragment was digested with *NheI* and *XbaI* restriction endonuclease. The joined *pyrF* and *Pzrt1* fragment was amplified from plasmid PYRF using primers F3/R3 listed in Table 1 and also digested with *NheI* and *XbaI*. Then both the digested fragments were ligated by T4 DNA ligase to make pMAT2075 plasmid. Plasmid, pMAT2075, was used for the construction of recombinant plasmid carrying *fad3* gene. The newly built recombinant plasmid was named as pMAT2079. The DNA fragment of *fad*3 gene was amplified from the plasmid pUC19-*fad*3 which was used for keeping *fad*3 gene. The primers used in this case were *FAD*3-F/R which sequences are mentioned in Table 1. The recombination between linearized plasmid fragment and *fad*3 gene was possible due to the presence of 20 bp homologous fragments on both sides of linearized pMAT2075 and *fad*3 gene fragment. The recombinant plasmid was named as pMAT2079. One step cloning kit from Takara was used to ligate the gene in the restriction endonuclease site. During the experiment of gene cloning, the ligated recombinant plasmid was maintained and propagated in chemically competent cells *E. coli* DH5α. The presence of recombinant plasmid in these transformants was checked by DNA sequencing. The procedure of gene cloning and the complete map of pMAT2075 and pMAT2079 were presented in Fig. 2. Both pMAT2079 and the empty plasmids pMAT2075 were digested with *SmaI* to release the overexpression construct and released construct was used to transform MU760 which was the uridine auxotrophic strain drived from WJ11 and the albino colonies selection was performed out as description of (Rodríguez-Frómeta *et al*., 2013).

Gene Expression Analysis by RT-qPCR

Cultivation of the recombinant strain of *M. circinelloides* WJ11 were done in a 2 L fermenter supplied with 1.5 L modified K&R medium. To carry out reverse transcription-quantitative PCR (RT-qPCR) analysis, mycelium was collected at 3, 24, 48 and 72 h. Trizol was used to extract the total RNA of *M. circinelloides* after grinding the mycelium under liquid N_2. The extracted RNA was reverse transcribed by the PrimeScriptTM RT reagent kit (Takara) according to the manufacturer's instructions. The primer pair *FAD*3qPCR-F/R (Additional file 1: Table 1) was used to carry out RT-qPCR analysis using the in LightCycler 96 Instrument (Roche Diagnostics GmbH, Switzerland) with FastStart Universal SYBR Green Master (ROX) Supermix (Roche) as per the protocol supplied by the manufacture. The mRNA expression levels were normalized to levels of 18S rRNA. The data were calculated and mRNA transcript level was determined by the method of $2^{-\Delta\Delta Ct}$. The results were expressed as relative expression levels.

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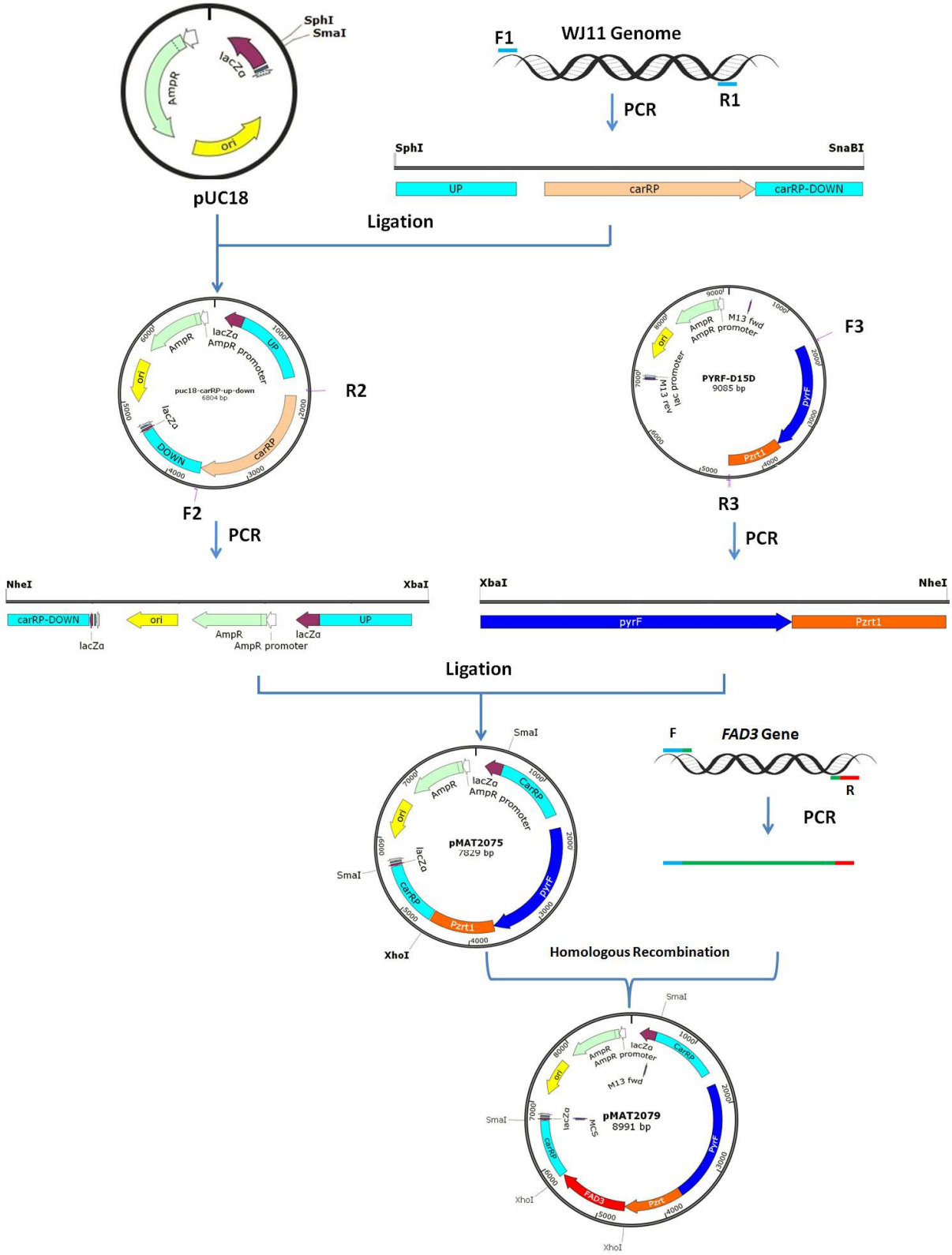

Fig. 2: Structure of plasmids pMAT2075 and pMAT2079 are shown. The *fad3* gene was isolated by PCR amplification with appropriate primers. The PCR fragment was ligated into *XhoI* restriction site to generate plasmid as pMAT2079

Table 1: The fatty acid composition in control and *fad3* overexpressing strains

	Fatty acid composition (relative %, w/w)						
Time (hour)	C(16:0)	C(18:0)	C(18:1) OA	C(18:2) LA	C(18:3) GLA	C(18:3) ALA	C(18:4) SDA
Mc-2075							
12h	20.41±0.55	6.14±0.15	34.32±1.33	16.51±0.65	20.33±1.03	-	-
24h	23.35±0.45	7.22±0.65	34.61±0.42	15.22±0.52	14.91±0.13	-	-
36h	24.03±0.50	7.48±0.35	35.63±0.55	14.72±0.75	13.61±0.60	-	-
48h	23.89±0.40	7.36±0.75	36.25±0.70	14.61±0.65	13.35±0.35	-	-
60h	23.77±1.35	7.03±0.95	37.39±1.12	14.73±0.75	13.36±1.75	-	-
72h	23.12±0.30	6.57±0.25	37.03±0.33	14.91±0.45	13.81±0.40	-	-
84h	22.8±0.38	6.40±0.32	37.4±0.13	15.0±0.35	13.60±0.20	-	-
96h	22.45±0.20	5.9±0.23	37.8±0.45	14.9±0.25	13.40±0.40	-	-
Mc-2079							
12h	22.12±0.47	4.76±2.11	32.04±1.32	14.35±1.71	11.05±1.88	5.76±0.93	8.66±0.43
24h	21.07±0.54	4.88±0.77	33.20±0.64	13.15±0.21	12.11±0.53	5.11±0.88	7.48±0.47
36h	20.06±0.37	4.31±0.28	35.98±0.44	14.23±0.34	13.05±0.28	4.92±0.31	5.55±0.46
48h	20.74±0.99	4.66±1.11	36.25±0.85	13.64±0.35	12.01±0.50	4.88±0.60	5.11±0.75
60h	21.43±0.32	4.87±0.35	37.27±0.30	13.98±0.25	11.16±0.30	3.47±0.25	4.89±0.80
72h	21.96±0.14	5.73±0.75	38.18±0.50	12.27±0.05	10.23±0.22	3.43±0.10	4.66±1.00
84h	22.13±0.32	5.87±0.35	38.11±0.30	12.98±0.25	10.16±0.30	3.35±0.20	4.40±0.50
96h	22.76±0.14	5.95±0.75	37.98±0.50	12.30±0.05	10.12±0.22	3.15±0.30	4.23±0.55

The values represent the mean ± SD of three independent experiments

Measurement of the Glucose Concentration in Culture Medium

Glucose oxidase Perid-test kit was used to measure the glucose concentration in the culture media following the instructions by the manufacturer (Shanghai Rongsheng Biotech Co., Ltd.). Ammonium concentration was determined by the indophenol method (Chaney and Marbach, 1962).

Determination of Cell Dry Weight, Total Lipid and Fatty Acid Profile Analysis During Fermentation Process

The samples of biomass were collected on a pre-weighed filter paper by filtration. And then the biomass was washed with double distilled water for three times and freeze-dried after frozen in -80°C freezer for overnight. The cell dry weight was determined gravimetrically. 40 mg dried biomass was taken for lipid extraction. The final weight of tube with total lipid was taken after extraction of total lipids with chloroform/methanol (2:1, v/v) method.

The determination of total lipid was showed as followed equation (Khan *et al*., 2019a; 2019b):

$$Total\ FA = \frac{T1 - T0}{Wm} \times 100$$

Here:

T_1 = Weight of tube with total lipid
T_0 = Weight of empty tube
Wm = weight of biomass

Analysis of Fatty Acid Profile of Cell Lipid

20 mg of dry mycelia was taken into a fatty acid extraction tube for the purpose of fatty acid analysis. Extraction of total lipid was done by the extraction method with chloroform/methanol (2:1, v/v) mentioned above. 10% (v/v) methanolic HCl was added to the extracted lipid and the samples were kept at 60°C for 3 h. Pentadecanoic acid (15:0) was added as an internal standard. The resultant fatty acid methyl esters, extracted with n-hexane, were analyzed by GC equipped with a DB-Waxetr column with 0.25 μm film thickness (30 m ×0.32 mm). The GC program was set to maintain 120°C for 3 min, then ramp from 120°C to 200°C at the speed of 5°C/min, then ramp from 200°C to 220°C at the speed of 4°C/min and hold for 2 min.

Statistical Analysis

The software SPSS 16.0 was used for statistical analysis. The calculation of mean values and the standard error of the mean were done using the data obtained from three independent experiments. *Student's t* test was used to determine the differences between the means of the test where $P<0.05$ was considered as significantly different.

Results

Construction of fad3-Overexpression Strains of M. Circinelloides WJ11

To overexpress the *A. thaliana fad3* gene in *M. circinelloides* WJ11, an expression vector pMAT2075

was constructed to allow targeting integration of genes in the *carRP* locus. The *pyrF* gene of *M. circinelloides* WJ11 was carried by this vector. This gene encodes orotidine 5'-phosphate decarboxylase and is a selectable marker for *M. circinelloides* WJ11. The strong promoter *zrt*1 worked for *fad*3 gene overexpression. 1-kb sequences upstream and downstream of the carotenogenic *carRP* gene flanked the previous elements to permit chromosomal integration by the whole DNA fragment construct through homologous recombination (Zhang *et al*., 2017) (Fig. 2). Gene replacement in the *carRP* local resulted in albino colonies that are recognizable among the yellow wild-type colonies. The *fad*3 cDNA with codon optimization for *M. circinelloides* was cloned in pMAT2075 producing plasmid pMAT2079. Transformants were generated by transferring the overexpression construct digested with *SmaI* from pMAT2079, recombinant plasmid and pMAT2075, the empty plasmid. After X vegetative cycles the transformants were obtained from plasmid pMAT2075 and pMAT2079 respectively that showed homogenous albino color, suggesting they were homokaryons for the integration in the *carRP* locus. The integration of target gene in transformants were confirmed by doing PCR. The primer pair checking F/R, listed in Table 1, was used to amplify the DNA fragment for this purpose. The primers designed in such a way that it could amplify the fragment from *carRP* 1kb upstream to *carRP* 1kb downstream of genomic DNA, therefore, amplified a 5377- and 6643-bp (including gene) sequence from Mc-2075 and Mc-2079 respectively. The size of the PCR products from the two transformants obtained with pMAT2079 and the one obtained with pMAT2075 showed the expected sizes, confirming that the *fad*3 overexpressing construct had been integrated into the genome of the transformants. The two transformants harboring *fad*3 gene were named as Mc-2079 and Mc-2079-1 and the control transformant without this gene was named as Mc-2075 (Fig. 3). Additional screening was carried out (data was not mentioned) and only the higher lipid accumulation strain (Mc-2079) was selected for further study.

Fad3 gene Expression Analysis in the Overexpressing Transformant

The mycelia were harvested at 3, 24, 48 and 72 h of culture time in 2 L fermenter with 1.5 L K&R medium to perform the Real-time quantitative PCR to analyze the mRNA levels of *fad*3 gene in recombinant strain (Fig. 4). The mRNA expressing levels of *fad*3 gene from Mc-2079 were regarded as 1 at 3 h and in comparison, with it, *fad*3 gene expression levels at other time were determined. It was observed that the expression level increased quickly from 3 to 24 h and gradually decreased with after that time. These results reveal that *fad*3 mRNA expression was found at high levels during the whole fermentation process. These observations indicate the overexpression of *fad*3 gene in the recombinant strain.

Effect on Cell Growth and Lipid Accumulation in fad3 Over-Expression Transformant

In the recombinant strain Mc-2079, the effect of *fad*3 gene over-expressed in transformant on cell growth and lipid accumulation has been investigated and was shown in Fig. 5. This figure also highlights the rate of utilization of ammonium and glucose in the culture medium. During the entire bioprocess, adequate concentrations of glucose remained in excess (Fig. 5a), but ammonium was depleted at approximately 24 h by Mc-2079 (Fig. 5b). CDW was found to be increased drastically from 9 h of cultivation and slowed down gradually after the depletion of nitrogen (Fig. 5c). The fungus started to produce lipids from the culture medium immediately after nitrogen exhaustion. The total lipid increased quickly from 12 h, reached at the peak level at 72 h and then gradually slowed down. It was found that the highest content of TFAs was 35.50% in Mc-2079 (Fig. 5d).

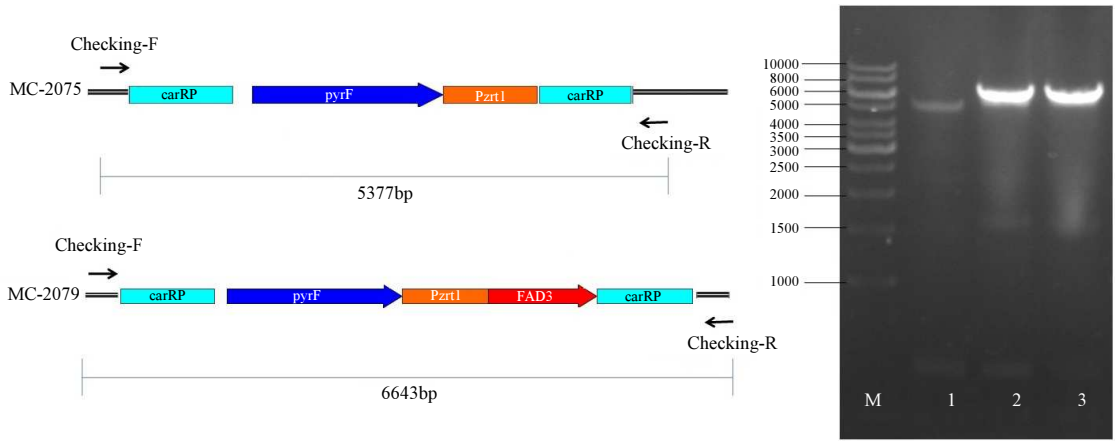

Fig. 3: PCR amplification of genome of control and recombinant strains with the primers checking-F/R. Lane 1 shows the result using DNA from the control strain Mc-2075 and lane 2 and 3 show the results using DNA from the recombinant strains Mc-2079 and

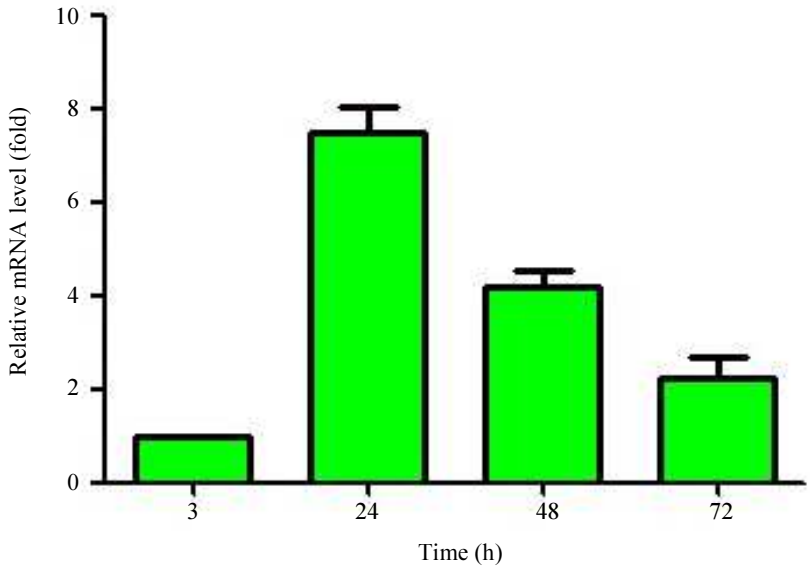

Fig. 4: Determination of expression levels of *fad3* gene by RT-qPCR in the overexpressing strains

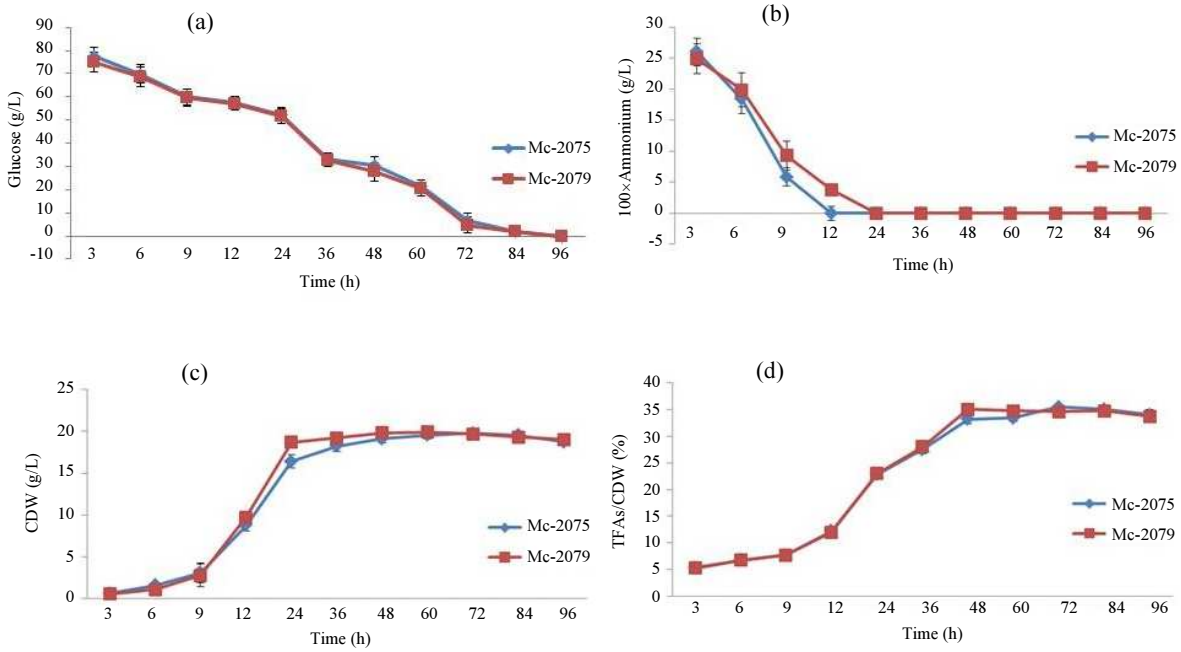

Fig. 5: Cell growth and lipid accumulation of *fad*3 overexpressing strains. Recombinant Mc-2079 and control strain Mc-2075 cultures were grown in 1.5 L modified *K* and *R* medium and (a) Glucose concentration, (b) Ammonium concentration, (c) Cell Dry Weight (CDW) and (d) Lipid content was measured. Samples from the fermenter were taken at the indicated times. The values were mean of three biological replicates. Error bars represented the standard error of the mean

SDA Accumulation in Recombinant M. Circinelloides

In the *fad*3 overexpressing strains, SDA percentages were found higher at the beginning and there was a decreasing trend with increase of time (Table 1).

But the total SDA yield started to increase with progress of time and reached at maximum level at 60 h and then decreased gradually. Total SDA yield was calculated based on the TFAs content (Table 2).

The maximum SDA yields of 340 mg/L were detected in the recombinant strains. Meanwhile, other fatty acid contents had a little change in the recombinant fungi in comparison to the same fatty acids accumulated by the control strain (Table 1).

Table 2: Cell Dry Weight (CDW), Total Fatty Acids (TFA) and SDA yields in control strains

Cultivation time(hour)	CDW (g/l)	TFA (g/l)	SDA (mg/l)
Mc-2079			
12h	9.70±0.77	1.17±0.64	100±0.43
24h	18.7±0.7	4.30±0.44	320±0.47
36h	19.21±0.65	5.39±0.91	300±0.46
48h	19.8±0.4	6.39±0.52	330±0.75
60h	19.9±0.38	6.90±0.67	340±0.80
72h	19.7±0.7	6.81±0.16	320±1.00
84h	19.3±0.43	6.70±0.03	290±0.75
96h	19±0.5	6.40±0.23	270±0.35

The values represent the mean ± SD of three independent experiments

Discussion

In our present study, the high lipid accumulating strain *M. circinelloides* WJ11 was used to construct an SDA producing cell factory by overexpression of *fad*3 (*ω*-3 *desaturase*) gene of *A. thaliana,* which is reported to convert LA to ALA (Mendes *et al*., 2013) and also GLA to SDA. In *M. circinelloides* WJ11 we constructed the complete metabolic pathway by overexpression of *fad*3 gene (Fig. 1). The other gene, *D6D*, was already present in this fungus (Sakuradani *et al*., 2008). The accumulation of preferred products such as the biosynthesis of Very-Long-Chain Polyunsaturated Fatty Acid (VLCPUFA) in recombinant microbes can be further improved by more research (Sakuradani *et al*., 2008). Previously, we overexpressed *Delta*-15 *Desaturase (D15D)* in the low lipid producing *M. circinelloides* CBS 277.49 strain where SDA yield was 64 mg/L. The *D15D* gene was cloned from another filamentous fungus *Mortierella alpina* (Khan *et al*., 2019c). This time after overexpression of *fad*3 gene in *M. circinelloides* WJ11, the SDA yield was 340 mg/L which was the much better from our previous result.

Overexpression of *fad*3 gene did not affect the metabolic pathway to produce major fatty acids by *M. circinelloides* WJ11. The fatty acids, 16:0, 18:0, 18:1, 18:2 (LA) and 18:3 (GLA), were found as the major lipids before and after overexpression of this gene (Khan *et al*., 2019b; Yazawa *et al*., 2007; Lee *et al*., 2019). The natural sources, such as the plants, algae and fungi, produce SDA as a metabolic intermediate during the biosynthesis of long chain ω-3 fatty acids; It was not found to be accumulated substantially in any species (Whelan, 2009). In the recombinant *M. circinelloides* WJ11, we confirmed the recombination and overexpression of *fad*3 gene in three ways: firstly, we checked its DNA, Secondly, by doing RT-qPCR which confirmed that the mRNA transcript level was kept elevated during the whole fermentation process and thirdly, by doing fatty acid profile analysis that showed the accumulation of SDA in this fungus. In Mc-2079, both ALA and SDA accumulated in significant amount which ultimately increased the total yield of ω-3 fatty acids. In this metabolic pathway SDA occurred as the end product. *M. circinelloides* has been used for GLA production industrially for more than 30 years (Yazawa *et al*., 2007), so it can also be used for industrial production of SDA.

SDA production in plant sources is still in progress in comparison to microbial origin. Scientists cloned the *D6D* gene from *Phytophthora citrophthora* and overexpressed in *Perilla frutescens* and the accumulation of GLA and SDA was over 45% (Lee *et al*., 2019). But the unavailability of genomic information kept scientists apart to understand the molecular basis of SDA accumulation in this plant (Sreedhar *et al*., 2017). Because of nutritional value and increased demand, researches are carried out worldwide to construct genetically modified SDA producing microbes. In Japan, *Saccharomyces cerevisiae* which was improved by gene manipulation could produce up to 13% SDA was constructed. But in that process the cultivation media was supplied with 0.8 g/L histidine (Kimura *et al*., 2014; 2009). Our recombinant *M. circinelloides* WJ11 can produce almost 340 mg/L SDA without any supplement. However, for the purpose of using *M. circinelloides* WJ11 as cell factory for industrial production SDA, further upscaling and optimization are required.

Conclusion

The recombinant SDA producing strain of *M. circinelloides* WJ11 constructed successfully by overexpression of *fad*3 gene of *A. thaliana* in our research may have applications for the commercial production of SDA. However, more research can be carried out for further upscaling and optimization of industrial cultivation of this recombinant *M. circinelloides* WJ11.

Abbreviations: *M. circinelloides*: *Mucor circinelloides*, *A. thaliana*: *Arabidopsis thaliana*, SDA: Stearidonic acid, LA: Linoleic acid, ALA: Alpha linolenic acid, GLA: Gamma linolenic acid, ARA: Arachidonic acid, EPA: Eicosapentaenoic acid, DHA: Docosahexaenoic acid, PUFA: Polyunsaturated fatty acid, VLCPUFA: Very-long-chain polyunsaturated fatty acid, D6D: Delta-6 Desaturase, D15D: Delta-15

desaturase, GC: Gas chromatography, CDW: Cell dry weight, SAFA: Saturated fatty acids, MUFA: Mono-unsaturated fatty acids, TFA: Total fatty acids.

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Author Contributions

Junhuan Yang and Md. Ahsanul Kabir Khan: Worked in the experimental design, fermentation testing, manuscript writing and figures and tables arrangement and contributed equally to this paper as first authors.

Sergio López-García: Built the MU760 strain which was the auxotroph of *Mucor circinelloides* WJ11.

Shaista Nosheen, Yusuf Nazir and Huaiyuan Zhang: Was involved in the experimental design.

Victoriano Garre: Carried out results interpretation, manuscript writing and reviewed of final draft.

Yuanda Song: Proposed the project and was involved in data analysis, result interpretation, manuscript writing and review of the final draft.

Ethics Approval and Consent to Participate

The authors did not perform any study with human participants or animals.

Availability of Data and Materials

All data generated and/or analyzed in this research are included in this published article [and its Additional file].

Conflict of Interest

There are no conflicts of interest in relation to this article.

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