Mechanism study on the enhanced DHA synthesis in the mutant *Thraustochytriidae* sp. through comparative transcriptomic analysis

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Research

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Abstract

Background: Docosahexaenoic acid (DHA) is an essential omega-3 fatty acid for the human retina, skin, and cerebral cortex. Marine eukaryote *Thraustochytriidae* sp. was considered as a promising source for the n -3 LC-PUFAs production. However, the mechanism how the LC-PUFAs was synthesized in *Thraustochytriidae* sp. still remained unclarified. To explore the vital genes responsible for the DHA enrichment, the functional transcriptomic annotation was compared between the wild type and preeminent mutant of *Thraustochytriidae* sp. X2. Results: After the UV irradiation (50 W, 30 s), the mutant X2 showed enhanced lipid (78.88 % more) and DHA (23.77 % more) production compared with the wild type. Instead of EPA, 9.07 % of DPA was observed in the mutant X2. The comparative transcriptomic analysis showed that in both wild type and mutant strain, FAS was incomplete and lacked key desaturases, but genes related to the PKS pathway were observed. It was oberved that mRNA expression levels of CoA-transferase (CoAT) , acyltransferase (AT), enoyl reductase (ER) , dehydratase (DH) and methyltransferase (MT) down-regulated in wild type but up-regulated in mutant X2, corresponding to the increased intercellular DHA accumulation. Conclusion: These findings indicated the potential genes that can be exploited for high DHA yields in *Thraustochytriidae* sp..

1. Background

Owing to the importance of cell membrane function and numerous cellular processes for maintaining health, long-chain polyunsaturated fatty acids (LC-PUFAs) have attracted much attention. LC-PUFAs can be classified into two principal families, namely, omega-3 (n-3) and omega-6 (n-6) fatty acids (FAs) [1]. The typical n-3 LC-PUFAs are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which can strongly influence monocyte physiology. Previous studies have reported that DHA could potently inhibit platelet aggregation [2] and reduce haemoglobin formation [3] and can be used to treat cardiovascular diseases [4] and prevent osteoporosis [5]. Currently, the primary commercial source of DHA is floating fish [6]; however, the industry is severely limited by the low original levels and the instability of n-3 LC-PUFAs due to differences among fish, the climate and high concentrations of cholesterol [7]. Microalgae, such as Thraustochytrium and Schizochytrium, with abundant FA content, have emerged as promising producers of n-3 LC-PUFAs [8]. The fermentation process of the Schizochytrium sp. SR 21 was optimized with bioreactor cultivation, with the DHA content doubled up to 66.72 ± 0.31% w/w total lipids [9]. The FA content required in the industry is currently 40–45 g l^{-1}, and the biomass required is 200 g l^{-1} [10]. Nevertheless, it is difficult for the general wild-type (WT) strain to meet the requirements of industrial production due to the low biomass and n-3 LC-PUFA content, which account for the high cost of the downstream process [11].

To obtain high-yield DHA-producing strains for microbial industrial fermentation, artificial mutagenesis has been applied. Ultraviolet (UV) radiation, a kind of non-ionizing radiation, causes gene mutation via maximum absorption by purines and pyrimidines present in DNA [12]. With UV irradiation, the DHA percentage of the total fatty acids up to 56.22% was achieved using the mutant, which was 38.88% higher than the parent strain [13]. Therefore, UV radiation was used as method for mutagenesis to obtain

a Schizochytrium strain with a high yield of DHA. De novo assembly of RNA-seq data serves as an important tool for studying the transcriptomes of “non-model” organisms without existing genome sequences [14]. Recently, transcriptome analysis has emerged as an essential method for the genes identification involved in the secondary metabolites biosynthesis [15], such as those involved in fatty acids accumulation in the microalgae Nannochloropsis sp. [16], Schizochytrium mangrovei PQ6 [17], Neochloris oleoabundans [18], Euglena gracilis [19], and Rhodomonas sp. [20]. There is an abundance of research on the optimization of fermentation parameters in terms of salinity, pH, temperature, and cultivation medium for high DHA production [21]. In addition, metabolic engineering is also used as a promising approach to promote DHA productivity. Recent research has indicated that DHA is synthesized by two distinct pathways in thraustochytrid: the fatty acid synthase (FAS) pathway and the polyketide synthase (PKS) pathway [22]. The standard FAS pathway synthesizes fatty acids through a series of elongase- and desaturase-catalyzed reactions. Delta-4 desaturase, delta-5 desaturase, and delta-12 desaturase have been reported in Thraustochytrium aureum ATCC 34304 [23], and delta-5 elongase, delta-6 elongase, and delta-9 elongase have also been successfully identified in some thraustochytrid strains [24]. Fatty acids are synthesized through the PKS pathway via highly repetitive cycles of four reactions, including condensation by ketoacyl synthase (KS), ketoreduction (KR), dehydration, and enoyl reduction (ER). Nevertheless, to date, the exact biosynthetic mechanism of DHA in Thraustochytrid species remains unknown.

In this study, UV mutagenesis was utilized to obtain competitive Thraustochytriidae sp. strain with enhanced biomass and DHA production. By comparing the transcriptome between the mutant and the parent strain, the key genes related to the increasing DHA accumulation were explored.

2. Results

2.1 Cell mutagenesis

To obtain a DHA-rich mutant with a relatively high biomass yield, Thraustochytriidae sp. was subjected to random mutagenesis with UV irradiation. As shown in Fig. 1, the fatality rate of microalgal cells was sensitive to both UV treatment time and power. With a UV treatment time of 30 s, as the UV power increased from 10 W to 50 W, the survival rate decreased from 92.15% to 8.29%. The mutant treated with UV power of 50 W showed a rapid decrease in survival rate after 15 s (survival rate of 83.67%). The survival rate was 2.67% when the microalgal cells were exposed to UV (power of 50 W) for 33 s. The results showed that both the UV exposure time and UV power contributed to the severity of DNA damage in microalgal cells. UV radiation has been widely utilized as a physical mutagen in microorganism breeding, but few studies have reported its effect on the enhancement of DHA accumulation in Thraustochytriidae sp microalgae. Under the UV irradiation, the DHA content (0.20 g/g dry biomass) of Schizochytrium sp. increased by 38.88% compared with the parent strain [25]. Thus, based on the results, UV irradiation at 50 W for 30 s was chosen as the mutagenesis condition for breeding the DHA-overproducing mutant strain.

2.2 Screening of the mutant Thraustochytriidae sp.

After UV irradiation, 135 colonies were obtained from the surviving cells. Due to the requirements for industrial production, the first round of mutant screening was based on dry cell weight (DCW) enhancement (Table 1). As shown in Fig. 2A, 14 colonies (X1 to X14) exhibited significantly enhanced cell growth compared with the parent strain. Notably, the biomass yield of mutants X2 and X4 increased by 53.22% and 52.49%, respectively. The lipid and DHA contents of the mutants were also analysed (Fig. 2A). Among the 14 mutants, eight independent colonies (X1, X2, X3, X4, X5, X9, X11, and X13) exhibited increased lipid yields (0.528 g/g, 0.817 g/g, 0.596 g/g, 0.573 g/g, 0.59 g/g, 0.548 g/g, 0.443 g/g, and 0.492 g/g, respectively). The lipid production of the mutant strains X2 and X14 was 78.88% and 63.75% more than that of WT. Four mutants (X2, X3, X5, X14) showed an improved ability of DHA production. In particular, mutant strain X2 showed a marked improvement of 28.61% for DHA production. According to the cell dry mass, lipid and DHA content, mutant strain X2 was chosen as the preferable DHA-producing candidate for the following experiments. To verify the hereditary stability of mutant X2, the strain was cultivated continuously in a shake flask for ten generations (Table 1). There was no significant difference for the DHA production observed among the ten generations. The DHA, lipid and biomass yields of the tenth generation were 214.02 mg l^{-1}, 485.33 mg l^{-1}, and 2920.60 mg l^{-1}, respectively. The results showed that UV irradiation (50 W, 30 s) could be utilized as a breeding strategy to screen for high-yield DHA-producing Thraustochytriidae sp.

Table 1
The total fatty acids (TFAs) and docosahexaenoic acid (DHA) contents of mutant strain Thraustochytriidae sp. X2 during ten-generations subculture.

Generation	TFAs (% DCW)	DHA (% TFAs)	DHA content (mg/L)
WT	41.07 ± 2.05	40.55 ± 2.05	321.37 ± 14.98
X2-2nd	50.00 ± 3.87	44.39 ± 3.87	624.34 ± 2.95
X2-6th	48.10 ± 0.74	44.98 ± 0.74	623.40 ± 7.14
X2-10th	48.53 ± 1.40	44.09 ± 1.40	624.93 ± 13.32
The fermentation conditions during continuous subculture remained the same and were set as below: inoculum size 10% (v/v) ,culture temperature 23 °C, initial PH 6.5 and fermentation medium volume 1000 ml. WT = wild type strain, X2-2nd = the second-generation subculture of mutant strain X2, X2-6th = the sixth-generation subculture of mutant strain X2, X2-10th = the tenth-generation subculture of mutant strain X2. All data were collected from three independent experiments.			

2.3 PUFAs production by the mutant Thraustochytriidae sp.

Significant differences in FAs production were observed between the mutant and WT. As shown in Fig. 2B, the amounts of LC-PUFAs (DHA (22:6, n-3) and EPA (20:5, n-3)) and saturated fatty acids (SFAs; hexadecanoic acid (HDA, 16:0) and pentadecanoic acid (PDA, 15:0)) were markedly different after UV mutation. The HDA and PDA levels decreased from 36.28% to 30.21% and 6.18% to 2.44%, respectively, whereas the DHA levels increased from 40.55% to 50.19%. The production of DHA in the mutant strain X2 increased 23.77% compared with the WT. Interestingly, 9.07% of DPA was observed instead of EPA in the mutant X2. The results indicated that the mutation led to the transformation of SFAs to PUFAs, reflecting the mutated genes responsible for FA carbon chain lengthening and unsaturation. Previous studies have confirmed that the accumulation of LC-PUFAs can be improved by increasing the SFA levels in the

substrate. The culture conditions of mutant X2 were also studied to explore the appropriate conditions for DHA production. Figure 3 showed that pH 6.5, a fermentation volume of 200 ml, a culture temperature of 27 °C, and an inoculum size of 5% were suitable conditions for DHA accumulation in mutant X2.

2.4 Sequence analysis and assembly

For a comprehensive understanding of the molecular mechanism underlying FA improvement in the collection, a comparative transcriptomic study was conducted between the WT and the mutant X2. The Q20 base value with a base quality greater than 20 and an error rate ≤ 0.01% made up more than 96.82% and 96.67% of the WT and X2 reads, respectively, which indicates that the raw sequence reads were very reliable and of high quality (Table 2).

Table 2

Summary of sequencing data for wild type Thraustochytriidae sp. PKU#Mn16 and mutant Thraustochytriidae sp. X2

Sample	Total Raw	Total Clean	Total Clean	Clean Reads	Clean Reads	Clean Reads
	Reads(Mb)	Reads(Mb)	Bases(Gb)	Q20(%)	Q30(%)	Ratio(%)
Mn16	50.94	44.69	6.70	96.82	88.53	87.72
X2	47.43	43.14	6.47	96.67	87.97	90.94
Total Raw Reads(Mb):The reads amount before filtering						
Total Clean Reads(Mb):The reads amount after filtering						
Total Clean Bases(Gb):The total base amount after filtering						
Clean Reads Q20(%):The rate of bases which quality is greater than 20 value in clean reads						
Clean Reads Q30(%):The rate of bases which quality is greater than 30 value in clean reads						
Clean Reads Ratio(%):The ratio of the amount of clean reads						

2.5 Differentially expressed gene analysis by RNA-Seq

In the comparison between Mn16 and X2, a total of 39,826 differentially expressed genes (DEGs) existed. Of these total DEGs, 1,350 were downregulated and 1,945 were upregulated in Mn 16 WT and X2 mutant Thraustochytriidae sp.. Further elucidation of DEGs with different expression arrays was performed with hierarchical DEG clustering through Euclidean distance associated with complete linkage (Fig. 4).

GO analysis of the transcriptome was based on three main categories: biological processes, cellular components and molecular functions (Fig. 5A). In many cases, several GO terms were assigned to the same unigene. Biological processes, molecular functions, and cellular components related to functional subgroups were used to categorize all DEGs. DEGs in both WT and mutant X2 were implicated in cellular and metabolic processes, which are plentiful in biological processes. One of the proteins involved in catalytic and binding processes was the foremost protein in molecular function, and other cellular components included cell and cellular part. KEGG is a database of metabolic pathways that is used to identify the gene products and functions associated with a cellular process. This pathway analysis provides a logical understanding of the complex biological performance of different genes in a network, and the analysis is performed by using BLAST software against the KEGG database.

DEG-associated pathways were analysed by using KEGG pathway tools in Thraustochytriidae sp., with 0.05 < P-value as the threshold. Nineteen significantly enriched pathways were found in WT and mutant X2 (Fig. 5B). These enriched pathways were associated with lipid metabolism, carbohydrate metabolism, biosynthesis and translation, and secondary metabolite biosynthesis. KEGG pathways involved in lipid metabolism include fatty acid metabolism, glycerophospholipid metabolism, glycerolipid metabolism,

fatty acid biosynthesis and secondary metabolite biosynthesis, which probably play an important role in PUFA biosynthesis.

2.6 Identification and characterization of genes involved in DHA biosynthesis

Through this study, we determined the expression of related genes in the PKS system in mutant Thraustochytriidae sp. X2. Key genes of the PKS pathway, such as CoA-transferase (CoAT), acyltransferase (AT), enoyl reductase (ER), dehydratase (DH) and methyltransferase (MT), are mentioned in Table 1. Using transcriptomic sequencing, we identified only one fatty acid synthesis (FAS) desaturase encoded by a unigene. In addition, RNA sequence analysis was used to investigate key biosynthetic enzymes of PKS pathway genes (Table 4). This analysis reported that Unigene4591_All, Unigene2419_All, Unigene10491_All, CL663.Contig2_All and CL555.Contig2_All were involved in synthesizing DHA, which was downregulated in Mn16 and upregulated in mutant X2.

Table 4

The key candidate genes related with PKS Pathway wild type Thraustochytriidae sp. PKU#Mn16 and mutant Thraustochytriidae sp. X2

Gene ID	Protein name	Gene Name	Control FPKM	Treat FPKM	log2
CL555.Contig2_All	Enoyl-(Acyl carrier protein) reductase	ER	3.85	10.1	1.400242079
CL663.Contig2_All	CoA-transferase	CoAT	1.14	3.04	1.394908036
Unigene10491_All	Acyltransferase	AT	74.10	149.53	1.006525578
Unigene2419_All	Methyltransferase domain	MT	8.46	18.11	1.083865306
Unigene4591_All	Dehydratase family	DH	0.01	1.66	7.211944308
CL94.Contig2_All	Fatty acid desaturase	FAD	2.02	4.34	1.101442064

2.7 mRNA expression level of the mutant X2 and WT

qRT-PCR was used to check the DEG expression profiles associated with PKS pathways. mRNA expression levels were checked for different genes, such as CoAT, ER, DH, MT, and AT, in comparison with the levels in Mn16, showing downregulation in Mn16 but upregulation in X2 samples. The qRT-PCR results were consistent with the RNA-seq results (Fig. 6) and validated the DEG expression profile.

3. Discussion

3.1 DHA synthesis enhancement

Recently, the DHA produced by microalgae in the ocean has received increased attention (da Silva 2019). Although industrial DHA production has been accomplished, certain approaches have been applied to enhance the synthesis of DHA via intrinsic [26, 27] or extrinsic parameters [28]. Conversely, several shared features, including low adaptability, degeneration, and low production, continue to hinder significant production by strains [29]. An effective approach such as mutagenesis is broadly useful for selecting high-yield strains [30]. Alonso reported that microalgae produced high yields of DHA and EPA after mutagenesis, and increased EPA content was also observed in Phaeodactylum tricornutum mutated by UV light, similar to our results [31].

3.2 PKS pathway

The DHA biosynthesis pathway in Thraustochytriu has not been fully elucidated. It has been reported that the two pathways, i.e., The PKS system and FAS pathway are likely to be present [32, 33]. The commonly found extended products of FAS in nearly all organisms are long-chain saturated FAs of either C16 or C18 [34, 35]. The FAS pathway comprised 7 or more kinds of desaturases, including delta-12, delta-9, delta-8, delta-6, delta-5, delta-4, and n-3 (e.g., delta-17 and delta 15). The transcriptomic study of the mutant X2 and WT (PKU#Mn16) Thraustochytriu revealed that only one gene encoding desaturase was involved in the FAS pathway; however, the Δ4, Δ6, and Δ12 desaturase genes are important for DHA production but were not observed in the present study. Similar to previous studies, expressed sequence tag (EST) sequencing or PCR-based detection failed to identify the probable desaturases in the FAS pathway [36]. The modification of FAs is performed to produce long-chain DHA (C22:6) by an enzyme-dependent continuous process [37]. PKS pathway domains are likely involved in the production of DHA, such as AT, DH, MT and ER [38, 39]. As a probable source of high-value DHA, the PKS pathway is important in DHA biosynthesis, as genes related to the PKS pathway were mined in the transcriptome study of wild Thraustochytriidae sp. (PKU#Mn 16) and mutated (X2) (Table 4). These unigenes were homologs to MT, AT, ER and DH, which are crucial in polyketide synthesis. These findings suggest that DHA synthesis is likely to occur via the PKS pathway in Thraustochytriidae sp. PKU#Mn 16 (wild type) and Thraustochytriidae sp. X2 (mutant). Currently, no evidence supports this hypothesis that DHA biosynthesis occurs via either of the two hypothetical pathways [40]. Furthermore, the formation of PUFAs of > C22 (e.g., 28:8n-3 and 28:7n-6) also occurs via the PKS pathway, which has been described in some species of oceanic dinoflagellates by Mansour M.P. (1999) [41].

3.3 Transcriptional responses of the PKS pathway

The omega-3 PUFAs, including EPA, DPA and DHA, are produced by certain strains, e.g., thraustochytrids [42]. Biochemical studies have been performed to characterize the distinct enzymes from the standard PKS pathways, which is ultimately helpful for understanding the underlying biosynthetic mechanisms [43]. These findings revealed that the FAS pathway does not participate in the biosynthesis of DHA in the Thraustochytriidae sp. strain (Table 3). PUFA synthesis is carried out by ACP in the PKS pathway. ACP acts as a covalent joint for chain elongation during many cycles. The synthesis of lengthy (unsaturated) fatty acids includes several enzymes in the PKS system, e.g., AT, MT, ER, and DH. A vital role is played by AT domains and their allies, i.e., ACPs. AT loads the building units onto ACP (substrate acceptor). Therefore, AT decides which building blocks will be incorporated into polyketide assembly [44]. AT plays significant roles in the PKS pathway; here, AT showed down-regulation in WT and up-regulation in X2 at the transcription level. The data showed increased the gene expression of the PKS pathway in the mutant. PKS-linked genes contained DH, AT, ER, and MT domains, as revealed by the transcriptomic study of Thraustochytriidae sp. X2 (Table 4). The mutation led to the formation of ORFC, which contains two DH domains and one ER domain, with up-regulation similar to that observed by Zhi-Qian Bi (2018) [45]. The mutation also improved the expression of DH and ER in Thraustochytriu, which suggests increased production of DHA. It is believed that mutagenesis is a valuable strategy for enhancement of PUFA biosynthesis. The PKS anchor gene up-regulation suggests that the PKS system is actively involved in PUFA biosynthesis, which is supported by [40].

Table 3

Quality metrics of transcriptome and unigenes assembly for wild type Thraustochytriidae sp. PKU#Mn16 and mutant Thraustochytriidae sp. X2

	Unigenes	
	Mn16	X2
Total Number	20,874	18,952
Total Length	23,991,758	22,844,185
Mean Length	1,149	1,205
N50	1,880	2,032
N70	1,264	1,328
N90	480	504
GC(%)	48.36	48.75
N50: The N50 length is used to determine the assembly continuity, the higher the better.N50 is a weighted median statistic that 50% of the total length is contained in transcripts that are equal to or larger than this value.		
N70:Similar to N50		
N90:Similar to N50		
GC(%):the percentage of G and C bases		

4. Conclusions

UV mutagenesis enhanced the ability of DHA production and led to the generation of competitive industrial strain from Thraustochytriu sp.. The transcriptome of the WT and the mutant strain were compared to investigate the vital genes responsible for the DHA enrichment. Results showed that in both WT and mutant strain, FAS was incomplete and lacked key desaturases, but genes related to the PKS pathway were observed. The qPCR revealed that the upregulation of key PKS pathway genes (CoAT, DH, AT, ER, MT) involved in the high yield of DHA for the mutant strain X2.

5. Materials And Methods

5.1 Microbial cultivation

Thraustochytriidae sp. PKU#Mn16 (CGMCC 8095) was inoculated into M4 liquid medium made with 100% filtered natural seawater (from Mirs Bay in Shenzhen, China) containing glucose (2.00%), yeast extract (0.10%), peptone (0.15%), and KH_2PO_4 (0.025%). The seed inoculum of Thraustochytriidae sp. PKU#Mn16 was cultured in a shaking incubator (LYZ-123CD, Shanghai Longyue Equipment Co., China) at 23 °C and 180 rpm for 48 h. One hundred millilitres of medium in a 250-ml flask was inoculated with 5 ml (5% (v/v) inoculation ratio) of the above culture. Three biological replicates of each sample were examined.

5.2 UV-mediated mutagenesis

The microalgae solution was diluted 10^5 times and applied to the plate and then mutagenized after 24 h of incubation in a constant-temperature incubator (LR-250, Shanghai Yiheng Technology Co., Ltd., China) at 23 °C. Before UV mutagenesis, the UV crosslinker (SZ03-2, Shanghai Netcom Business Development Co., Ltd., China) was turned on for 30 min to stabilize the light waves. The plates were placed in 0 W,

10 W, 20 W, 30 W, 40 W, 50 W, and 60 W UV crosslinkers and irradiated for 0 s, 6 s, 12 s, 18 s, and 24 s. After mutagenesis, the plates were incubated for 48 h in the dark, and then, the number of colonies was counted, and the lethality was calculated. Three biological replicates for each sample were examined.

5.3 Microalgal biomass determination

The mutagenized strain was cultivated as described in Chap. 2.1 for 48 h. The culture was then centrifuged (Z366K, HERMLE, Germany) at 10,000 rpm for 5 min to obtain the precipitate. After washing three times with deionized water, the precipitate was collected as the microalgal biomass and then lyophilized in a freeze dryer (Triad 2.51, LABCONCO, USA) for 72 h. Three biological replicates for each sample were examined in the experiment.

5.4 Fatty acid extraction

Before the experiment, filter paper bags (Hanjiang Road, Shuncheng District, Fushun City, Liaoning Province, China) were pretreated with a solvent mixture (chloroform:methanol = 2:1 (v/v)) for 48 h and dried at 50 °C. Five hundred milligrams of dried cells was placed in a pretreated filter paper bag as a filter paper package and extracted in a Soxhlet extractor at 70 °C for 48 h (solvent as described above). Then, the filter paper package was dried and weighed. The difference between the weights before and after was the weight of the FAs. The remaining liquid was evaporated to dryness at 70 °C by a rotary evaporator. The FAs were completely rinsed with 5 ml of n-hexane and placed in a 10-ml glass tube. Three biological replicates for each sample were examined.

5.5 Fatty acid structure and composition analysis

5.5.1 Fourier transform infrared (FTIR) spectrometer analysis

KBr powder was uniformly mixed with the dried cells and compressed into a sheet (KBr to dried cell ratio of approximately 100:1). KBr was used as a background and detected using a Fourier transform infrared (FTIR) spectrometer (Thermo Fisher Scientific, USA). The infrared spectrometer had a spectral range of 7800 – 350 cm^{-1}, and its scanning frequency was 65 spectras (16 cm^{-1} resolution). Three biological replicates of each sample were prepared.

5.5.2 Gas chromatography and mass spectrometry (GC/MS) analysis

The FAs obtained in Sect. 2.4 were added to 5 ml of a 4% sulfuric acid-methanol solution (v/v), and 100 µl of a nonanecene-methylene chloride solution (500 µg ml^{-1}) was used as an internal standard. After the tube was allowed to stand in a 65 °C water bath for 1 h, 2 ml of n-hexane and 2 ml of deionized water were added, and the mixture was shaken for 30 s. Three biological replicates for the extraction were examined. Then, the upper organic layer was transferred to a new test tube, and the organic solvent was

thoroughly dried with nitrogen. Finally, 1 ml of dichloromethane was added to each tube to dissolve the FAs, and the solution was then transferred to a chromatography bottle.

The FAs in the chromatography bottle were diluted 100-fold and analysed by gas chromatography-mass spectrometry (GC-MS,7890 – 5975 Agilent USA). The GC column for FA determination was HP-5MS (19091S-433) with a stationary phase of (5%)-diphenyl (95%)-dimethylpolysiloxane, constituting a weakly polar capillary column. The column had a maximum temperature of 350 °C and dimensions of 30.0 m × 250 μm × 0.25 μm. The GC inlet temperature was 250 °C, the carrier gas was high-purity He, constant pressure mode was used, the head pressure was 1.2 psi, the split ratio was 10:1, and the injection volume was 1 μl. The column temperature rise program was determined by 37 FA mixing standards. The steps for selecting the peaks for the separation of the 37 FAs in the sequence were as follows. First, the temperature was raised to 180 °C at a rate of 25 °C min^{-1} from 60 °C, increased to 240 °C at a rate of 3 °C min^{-1}, maintained for 1 min, and then heated to 250 °C at a rate of 5 °C min^{-1}. The GC-MS transfer line temperature was 250 °C, and the mass spectrometer detector selected the full scan mode. Three physical replicates for each sample were prepared.

5.6 Comparative transcriptomic analysis

5.6.1 RNA extraction and cDNA library construction

cDNA library construction, RNA extraction, RNA sequence analysis, mRNA sequence selection and library preparation were performed after total RNA extraction with TRIzol (Life Technologies, Thermo Fisher Scientific Inc., USA) using the Illumina HiSeq 4000 system. To assess the integrity of the total extracted RNA, an Agilent 2100 bioanalyzer was used. The preparation of two libraries of cDNA constructs and transcriptome sequencing were conducted by Huada Gene Technology Co., Ltd. (Shenzhen, China). Oligo (dt) magnetic beads were utilised for enrichment and purification of mRNAs from the total RNA of each sample. The purified mRNAs enriched were short fragments, which were reverse transcribed for first-strand synthesis, and the second strand was used for cDNA synthesis. Then, these obtained double-stranded fragments were ligated with adapters, and appropriate DNA fragments were used as PCR amplification templates.

5.6.2 Illumina sequencing, assembly and annotation

cDNA library sequencing was carried out by an Illumina HiSeq™ 4000, with 100-nt paired-end reads generated. The obtained reads were then filtered based on quality parameters of GC content, sequence duplication level, Q20 and Q30. High-quality clean reads were chosen from raw reads, and reads with adapters and poly-N sequences were eliminated. De novo transcriptome assembly of clean reads was implemented through the Trinity assembly database program using default parameters (Grabherr et al., 2011). Trinity software consists of three modules, namely, Chrysalis, Inchworm and Butterfly. (http://trinityrnaseq.sourceforge.net/). Initially, the Inchworm module formed a k-mer dictionary by breaking sequence reads (k-mer fixed-length sequence of k nucleotides, in repetition k = 25 bp). For contig assembly, the most recurrent k-mers were selected by removing low-complexity, error-containing and

singleton k-mers. Contigs were obtained until both side sequences could not protract with k-1 overlap. Then, the Chrysalis module was used to make the de Bruijn graph and gather the linear contigs. Finally, the Butterfly module was constructed to analyse de Bruijn graphs and produce transcript sequences. Transcript assembly was performed by using all generated contigs. The main transcripts that contained more than 200 bp were selected as unigenes. BLASTX (Altschul et al., 1997) alignment was performed against public protein databases such as the non-redundant (Nr) protein (Deng et al., 2006), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2004), Clusters of Orthologous Groups (COG), Gene Ontology (GO), and Swiss-Prot (Ashburner et al., 2000) databases and the generated database of unisequences.

5.6.3 qRT-PCR analysis

Total RNA was extracted using the TRIzol (Life Technologies, Thermo Fisher Scientific Inc., USA) method. For quantitative real-time PCR (qRT-PCR), primers were first designed according to transcriptomic sequence data using Primer Premier 5 software (Supplementary Table 1). Then, the SYBR Taq™ Ex Premix (Tli RNaseH Plus) Kit (TaKaRa Japan) was used with the following thermocycler protocol: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The entire process was performed in a CFX96 BioRad RT-PCR detection system. Actin was used as a housekeeping gene, which helped us check for standard and normal gene expression. qRT-PCR was performed in 3 replicates, and relative gene expression was quantified using the 2- ΔΔCt method (Livak and Schmittgen, 2001).

5.6.4 Statistical analysis

Analysis of variance (ANOVA) was utilized for the statistical analysis of the data. The least significant difference (LSD) test was applied to determine the significant differences among the group means at $P < 0.05$.

Abbreviations

ACP for substrate acceptor

AT for acyltransferase

CoAT for CoA-transferase

COG for clusters of orthologous groups

DCW for dry cell weight

DEGs for differentially expressed genes

DH for dehydratase

DHA for docosahexaenoic acid

DPA for Docosapentaenoic acid

EPA for eicosapentaenoic acid

ER for enoyl reductase

FA for fatty acid

GC-MS for gas chromatography-mass spectrometry

GO for Gene Ontology

HDA for hexadecanoic acid

KEGG for kyoto encyclopedia of genes and genomes

LC-PUFAs for long-chain polyunsaturated fatty acids

MT for methyltransferase

MUT for mutant

Nr for non-redundant

n-3 for omega-3

n-6 for omega-6

PDA for pentadecanoic acid

PKS for polyketide synthase

PUFAs for Polyunsaturated fatty acids

qRT-PCR for quantitative real-time PCR

SFAs for saturated fatty acids

UV for ultraviolet

WT for wild type

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Liangxu Liu, Hao Yang Siting Li and Chuhan Lv analyzed the data and wrote the paper; Xuewei Yang, Zhangli Hu, and Shuangfei Li designed the concept of experiments; Christopher H.K. Cheng, Huapu Chen and Madiha Zaynab checked and revised the detail of manuscript; Xuewei Yang, as the corresponding author, responsible for critical reading and finalization of the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

The datasets generated and/or analysed during the current study are available in the NCBI] repository, with the Accession No. PRJNA604511 and ID No. 604511.

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Figures

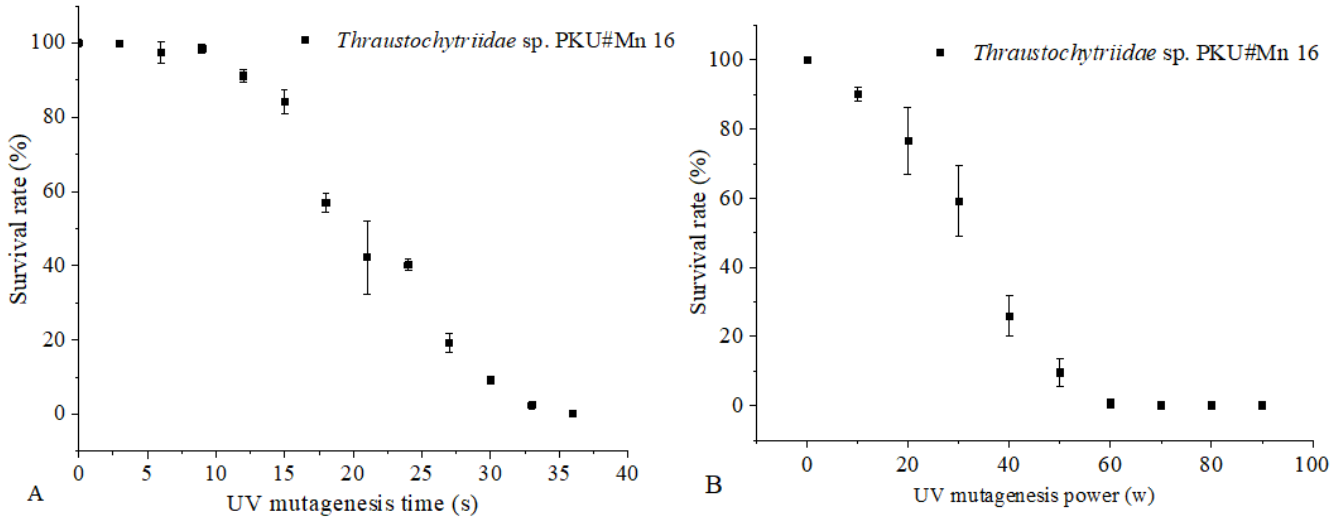

Figure 1

The survival rate of Thraustochytriidae sp. PKU#Mn 16 under various UV mutagenesis time (A) and UV mutagenesis power (B). All data were collected from three independent experiments. Error bars were the standard deviation.

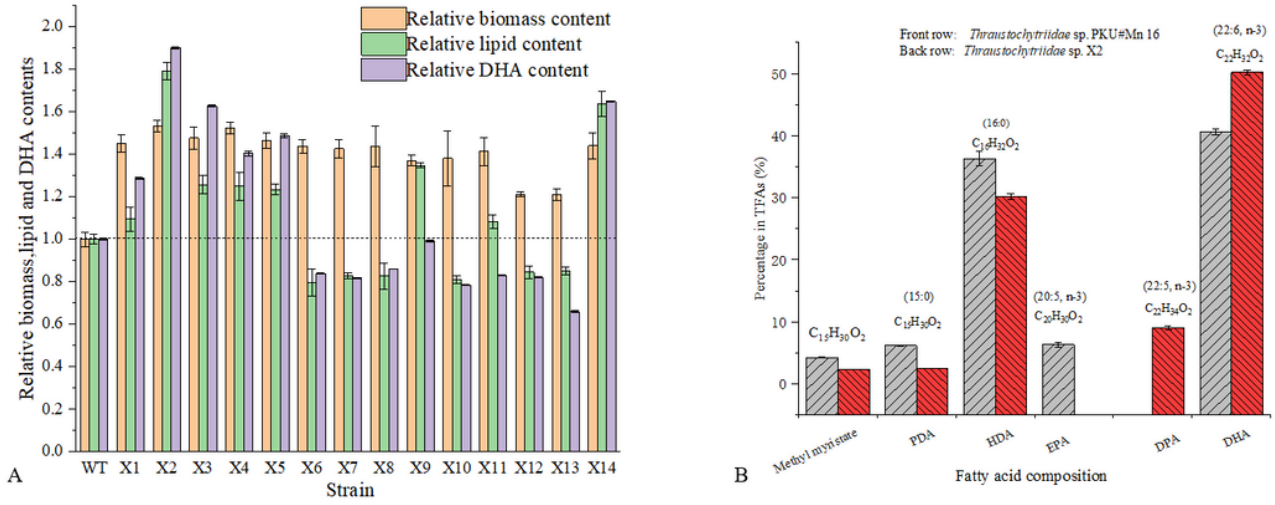

Figure 2

(A)The biomass, lipid and DHA (docosahexaenoic acid) contents of wild-type strain (WT) Thraustochytriidae sp. PKU#Mn 16 and 14 isolated mutant strains (X1~X14), and (B)the fatty acid component in TFAs (total fatty acids) of wild type strain Mn16 and mutant strain X2. PDA=Pentadecanoic acid, HDA=Hexadecanoic acid, EPA=Eicosapentaenoic acid

DPA=Docosapentaenoic acid, DHA=Docosahexaenoic acid. All data were collected from three independent experiments. Error bars were the standard deviation. The values of biomass, lipid and DHA contents of the wild-type strain were set to 1.0. All data were collected from three independent experiments. Error bars were the standard deviation.

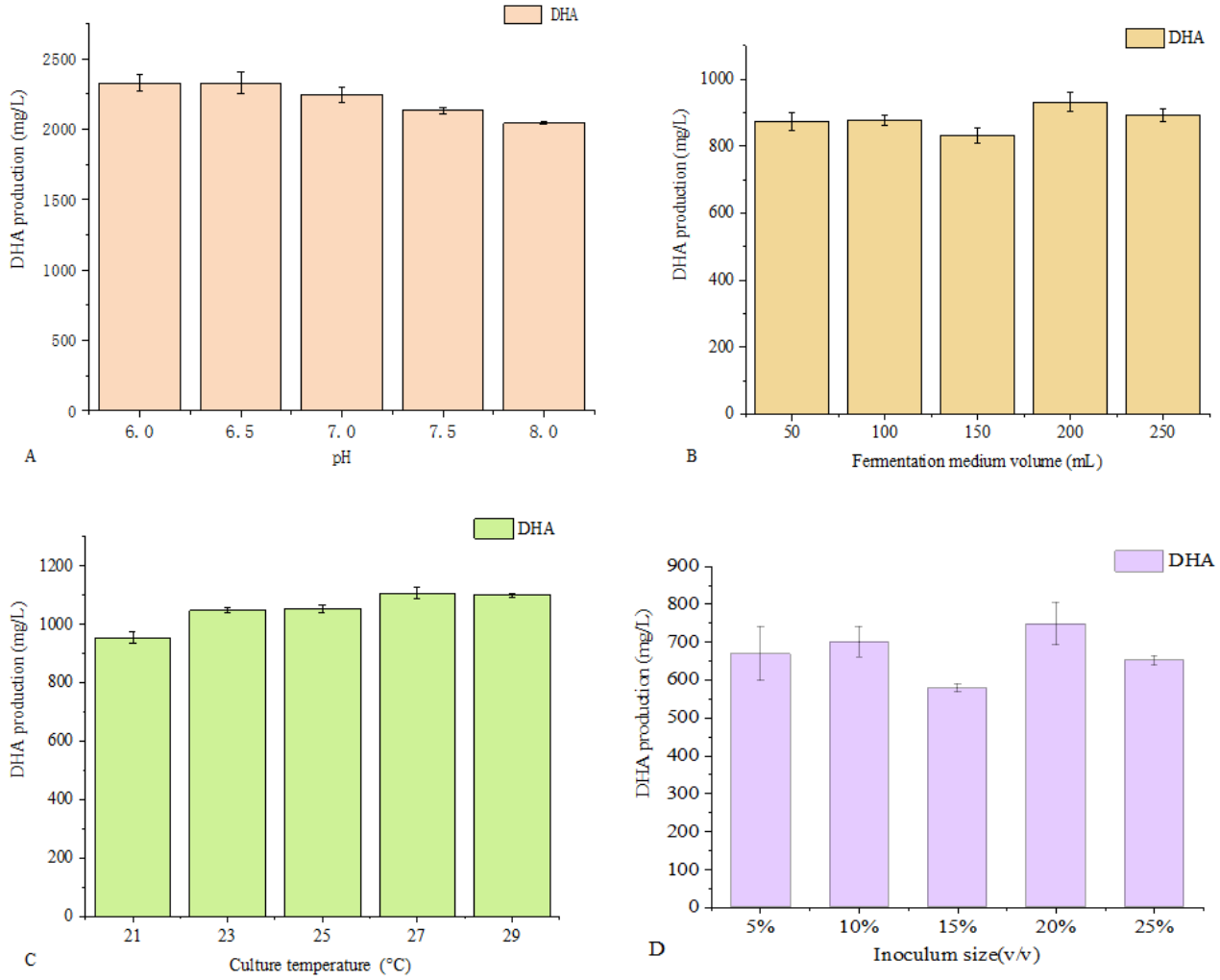

Figure 3

The effect of pH (A), cultivation temperature (B), fermentation medium volume (C), and inoculum size on the DHA production of mutant strain X2. The cultivation conditions were described as followed: (A) initial pH 6.0-8.0, culture temperature 23 °C, fermentation medium 100 ml, inoculum size 5 %; (B) initial pH 6.5, culture temperature 23-29 °C, fermentation medium 100 ml, inoculum size 5 %; (C) initial pH 6.5, culture temperature 23 °C, fermentation medium 50-250 ml, inoculum size 5 %; (D) initial pH 6.5, culture temperature 23 °C, fermentation medium 100 ml, inoculum size 5-25 %. All data were collected from three independent experiments. Error bars were the standard deviation.

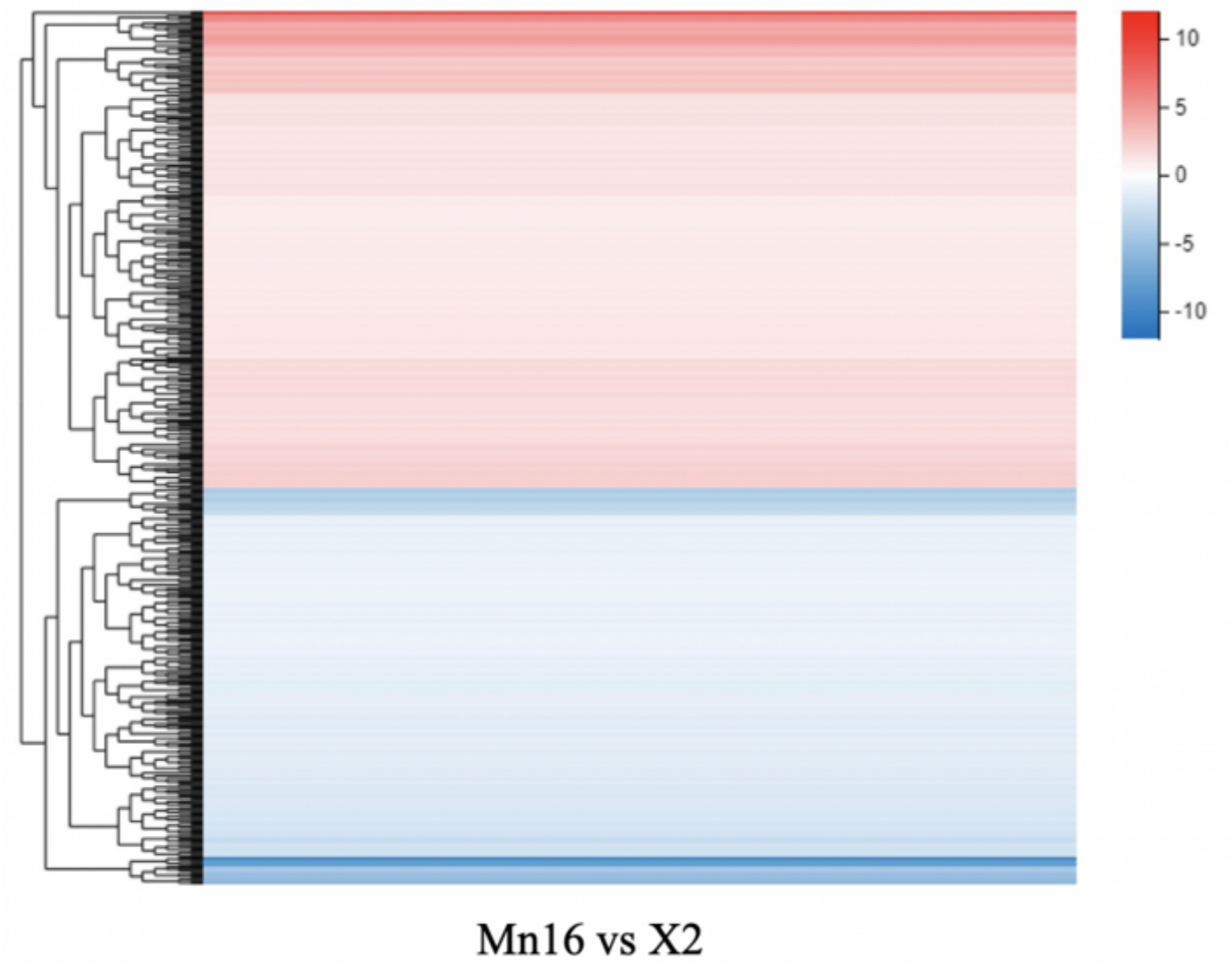

Figure 4

Heat map of the differentially expressed genes identified in Thraustochytriidae sp. PKU#Mn 16 (wild type) and Thraustochytriidae sp. X2 (mutant).

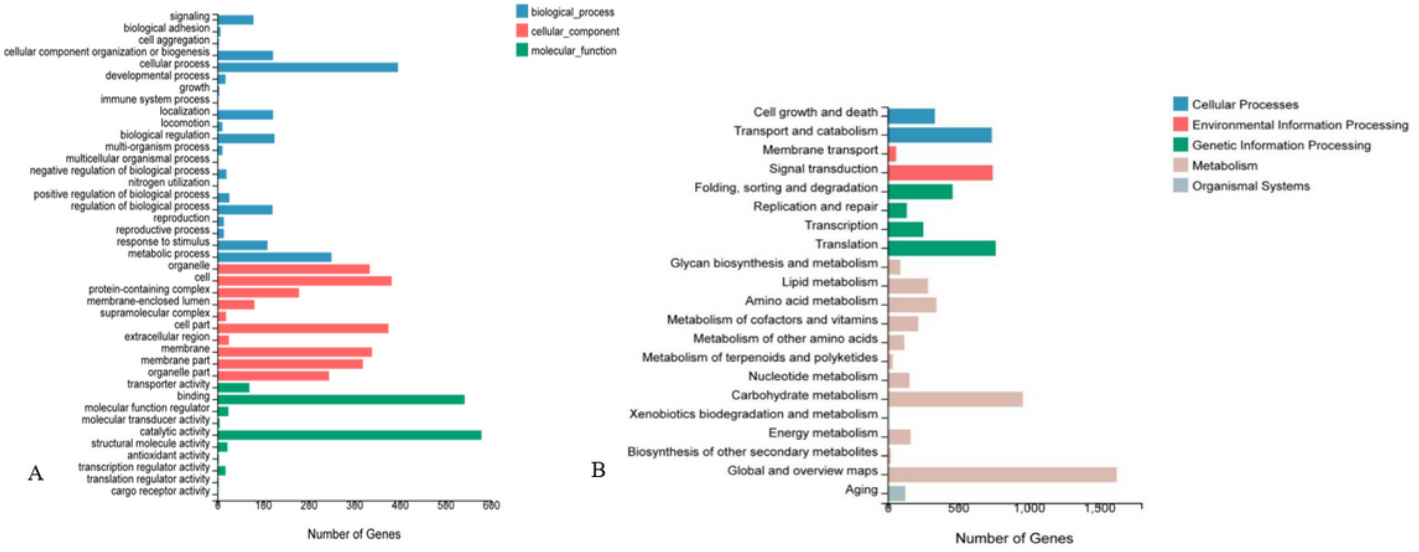

Figure 5

GO classification of the differentially expressed genes (A) and Enriched Kyoto Encyclopedia of Genes and Genomes pathway (B) identified in Thraustochytriidae sp. PKU#Mn 16 (wild type) and Thraustochytriidae sp. X2 (mutant).

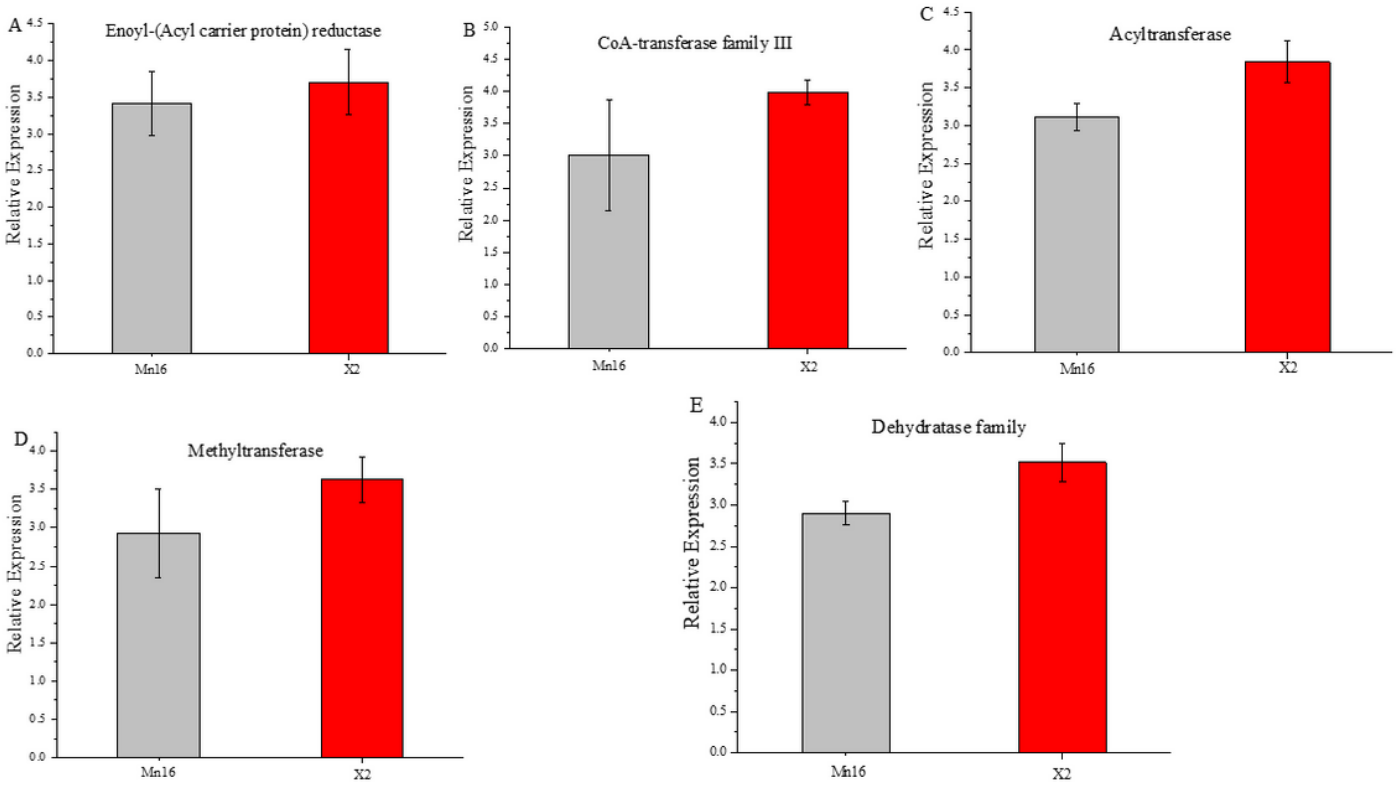

Figure 6

qPCR relative expression of genes annotated for enoyl-(acyl carrier protein)reductase (A), coA-transferase family Ⅲ (B), acyltransferase (C), methyltransferase (D), and dehydratase family (E) of wile type

(Mn16=Thraustochytriidae sp. PKU#Mn16) and mutant strain (X2=Thraustochytriidae sp. X2). All data were collected from three independent experiments. Error bars were the standard deviation.

Supplementary Files

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- 6.2Table1S2020.2.2.docx