An Integrated Approach to Produce a Recombinant Yeast *Pichia Pastoris* For GLA Production

Maryam Sadat Mirbagheri Firoozabad (✉ m.mirbagheri@yazd.ac.ir)

Yazd University <https://orcid.org/0000-0003-4153-0422>

Hamidreza Akhbariyoon

University of Isfahan

Research Article

Keywords: Delta-6 desaturase, gamma-linolenic acid, Mucor rouxii, Pichia pastoris

Posted Date: July 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-706359/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

γ -Linolenic acid (GLA) is an important n-6 polyunsaturated fatty acid (PUFA) used in many nutritional and medicinal applications such as the treatment of cancer, inflammatory disorders, and diabetes. However, GLA level of the total fatty acids in plant seeds and nuts as prominent sources of GLA is not enough to utilize on an industrial scale. The study aimed to improve the expression of delta-6 desaturase, which is one of the important enzymes in GLA production pathway. The expression vector pPICZC was selected for clone *M.rouxii* delta-6 desaturase. The engineered vector was first cloned into *E. coli* DH5 α and after plasmid extraction and confirmation of sequencing was transformed by electroporation into *Pichia pastoris* GS115. The results indicated that the recombinant yeast strain expressed the gene in the presence of methanol 0.5%. The lipids and essential fatty acids especially GLA were extracted to confirm the expression. The results of studies of lipid and fatty acid production by Sudan black and Nile red staining, GC, and flow cytometry revealed that recombinant strain can produce GLA levels up to 19.2% of total fatty acids. The present study may provide an opportunity for the development of an alternative host for manufacturing GLA on an industrial scale.

Introduction

γ -Linolenic acid (GLA 18:3, Δ 6,9,12), is a prominent n-6 polyunsaturated fatty acid (PUFA) that has a structural role in lipid membranes ingredients (Needleman et al. 1986; Vrinten et al. 2007). In humans, GLA is metabolized to produce prostaglandins and eicosanoids such as leukotrienes which have many health and medicinal roles in cardiovascular disorders, atherosclerosis, cancers, inflammatory disorders, diabetes, and some other diseases by regulating the levels of expression in various genes (Fan et al.; Kapoor; Kim et al. 2012; Lu and Zhu 2015).

GLA is rarely found in a small number of nuts and some oil plants seeds such as evening primrose (*Oenothera biennis* L.), borage (*Borago officinalis* L.) and, blackcurrant (*Ribes nigrum* L.) (Gyves et al.; Wan et al.; Zhang et al. 2017). Although up to 22% of the total fatty acids content could be GLA in plant seeds, the quantities and qualities of this PUFA production easily impacted by climate, region, and seasons. Studies have shown that the high proportion of GLA in total fatty acids may be up to 70% in Genetically-engineered safflower seeds (Knauf et al. 2006; Nykiforuk et al. 2011). Nevertheless, there is continued interest in developing alternative source from oleaginous microorganisms with high GLA contents including *Cunninghamella echinulata*, *Mucor rouxii*, *Mortierella alpina*, *Mucor circinelloides*, and *Mortierella isabellina* due to the acceptance uncertainties of genetically modified (GM) materials (Fakas et al.; lipids and 2006; Wan et al., 2011; Zhang et al. 2017)

GLA is synthesized from the conversion of linoleic acid (LA, 18:2, Δ 9,12), an essential omega-6 series of fatty acids, using a delta-6 desaturase. Gene coding for delta-6 desaturase has been previously cloned and characterized by several prokaryotes and higher eukaryotes and is also overexpressed in many hosts, such as microalgae, yeasts, and plants. However, looking for more excellent, safe GLA-producing microorganisms is still one of the current prominent research which considerable interest has focused on oleaginous microorganisms, such as *Thamnidium elegans*, *Cunninghamella echinulata*, *Mortierella alpina*, *Mucor rouxii*, *Pythium irregulare*, *Rhizopus arrhizus*, and *R. nigricans*. ([CSL STYLE ERROR: reference with no printed form.]; Qiao et al.; Zhang et al.; Wang et al. 2007; Nykiforuk et al. 2011; Cui et al. 2016).

Mucor rouxii a typical oleaginous filamentous fungus has been widely used to investigate GLA production. Previous studies have shown that the high proportion of GLA in total fatty acids of *M. rouxii* was up to 39.7% achieved by fed-batch cultivation (Jangbua et al. 2009). Nevertheless, the production of GLA in *M. rouxii* is so far not cost-competitive to the plant sources. Thus, it is necessary to explore another fungus host for GLA production on an industrial scale.

Previous studies have shown that the expression system of *Pichia pastoris* fungus has several advantages, including rapid growth rate along with high cell density fermentation, diverse posttranslational modifications, high levels of productivity, elimination of endotoxin and bacteriophage contamination, and feasible genetic manipulation (Li et al. 2007). These encouraging advantages, make *Pichia pastoris* one of the best hosts for recombinant protein expression.

The present strategy provides an avenue for increasing the GLA level in total fatty acids by overexpression of delta-6 desaturase gene from *M. rouxii* that transformed to *Pichia pastoris*, and may provide an opportunity for the development of the GLA production on an industrial scale.

Materials And Methods

Strains, plasmids, and culture conditions

Mucor rouxii DSM1194 was used as the source of delta-6 desaturase gene. The *Escherichia coli* DH5 α (Stratagene. The USA) and pTZ57R/T (Invitrogen) were used as the host– vector system. *Pichia pastoris* host strain GS115 (Invitrogen. The UK) and pPICZC (Invitrogen) were used for protein expression.

Cultures were grown in Luria Bertani Agar (LB Agar) (Merck) (Green et al.). Media were supplemented with zeocine (25 μ g/ml) when required. The pH was adjusted to 7.5 and 7 for mycelia and colonial growth, respectively.

In vitro assays of delta-6 desaturase gene existence

To verify that the enzyme gene existed in strains of *M.rouxii* and to ensure that mutations were not formed, DNA alignment was carried out. To this end, DNA extraction, PCR with special primers, and DNA sequencing were done using the Yamada et al (Yamada et al. 2002), White et al, and Sanger methods, respectively. The primers used for the PCR are described below.

F primer: 5'-CAAGAATTCAAATGGCTCCCCCAATACTGCGGC3'

R primer: 5'-CAACTCGAGTTCTTTATCATTAGCCCAAATCTC'3

Total RNA isolation

The *M.rouxii* strain was grown at 28 °C in a 250 mL shaker flask containing 50 mL liquid medium in a shaking incubator (250 rpm). After a 48h growth period, the strains were harvested and the total RNA from *M.rouxii* was isolated as described below.

Cells of the digestive gland were disrupted with a sterile homogenizer with liquid nitrogen. Total RNA was extracted using the RNAX-PLUS™ Total RNA Extraction Kit (CinnaGen) according to the manufacturer's instructions.

cDNA synthesis and PCR amplification

The partial cDNA fragment of *M.rouxii* delta-6 desaturase was amplified by RT-PCR. The primers used were Random Hexamer Primers, which were designed according to all kinds of cellular RNAs.

The reactions were performed using RevertAid M-MuLV Reverse Transcriptase. A 10µL reaction contains 4µL 5× PCR buffer, 1µL Reverse Transcriptase, 2 µL of 10mM dNTPs, 1µL of primers, and 1µg of the RNA sample. After mixing and centrifuge for 5sec, samples were incubated at 65°C for 5min and 42°C for 1h. Finally, for deactivation of Reverse Transcriptase, samples were incubated at 70°C for 10min. RT-PCR was performed on the cDNA product using GAPDH primers as a control.

Cloning of PCR product and transformation

The PCR products were characterized by agarose gel electrophoresis and extracted using a DNA extraction kit (Fermentase). Purified products were ligated into pTZ57R/T vector according to the manufacturer's instructions and transformed using the heat shock method into *E. coli* DH5α competent cells prepared by chemical CaCl₂ method (Green et al.). Colony PCR was carried out to verify that the Plasmid DNA had transformed correctly using the below primers.

F primer: 5'-CAAGAATTCAAAATGGCTCCCCAAATACTGCGGC3'

R primer: 5'-CAACTCGAGTTCTTTATCATTAGCCCAAATCTC'3

Construction of the expression plasmid

Plasmid DNA was purified using the plasmid extraction kit (#K0502; Fermentase) and digested using XhoI and EcoRI (Fermentase). Furthermore, pPICZC vector was also digested using XhoI and EcoRI, respectively and after incubation with Alkaline phosphatase (Fermentase), the digested plasmid was cleaned up. Digested pPICZC vector and delta-6 desaturase gene were ligated using DNA T₄ ligase (#EL0014; Fermentase).

The ligation product was transformed into *E. coli* DH5α competent cells. The *E. coli* transformants were selected on plates containing the antibiotic, Zeocin™ at a concentration of 25 µg/ml. Verification of insertion of the PCR fragment into the correct translational reading frame was confirmed by colony PCR, double digestion, and DNA sequencing before the introduction of the chimeric plasmid into *P. pastoris* host cells. Sequencing primers (5'AOX1 and 3'AOX1) were obtained from Invitrogen.

Transformation of *P. pastoris* and expression in shaken flasks

P. pastoris GS115 strain was selected to be used as a host strain. The recombinant plasmid pPICZA-delta-6 desaturase was purified from *E. coli* cells and linearized with the restriction enzyme SacI to allow integration of the vector DNA into the chromosomal DNA.

Pichia transformation was performed using the electroporation (Eppendorf), and the high-level expression transformants were screened in the YPD (1% yeast extract, 2% peptone, 2% dextrose, 1.8% agar) plates supplemented with Zeocin™ (100 µg·mL⁻¹).

For the screening of strains and for the optimization of culture conditions in shaking flasks, methanol 0.5% was used for induction, according to the Invitrogen instructions. GLA-producing *P. pastoris* cells were grown in 200mL YPD with methanol 0.5% at 30 °C for 4 days.

In vitro assays of GLA production

To verify that the delta-6 desaturase had been expressed and to ensure its activity, Sudan black and Nile red staining were carried out. For that experience, samples were obtained from production media after 72 h and stained according to the Zhou method ([CSL STYLE ERROR: reference with no printed form.]).

We also evaluated the amount of GLA production using flow cytometry on recombinant and wild-type spices as a control.

GLA purification and GC analysis

The modified Bligh and Dyer method was performed to extract lipid from *P. pastoris* cells (Pan et al.). After passing *P. pastoris* cells through Whatman No.1 filter paper, mycelia were washed by distilled water three times and once by ethanol, respectively. Samples were incubated at 60°C for 2 h with HCl solution (10 mL of 4M), and. The hydrolyzed solution was shaken with 20 mL of chloroform/methanol (1:1) at room temperature for 3 hours and centrifuged at 2000 ×g for 5 min. After the supernatant phase was separated, the lower phase containing lipids was evaporated under reduced pressure for 10 min. The extracted fatty acids were modified to fatty acid methyl esters (FAMES) according to the Christie method (Christie 1993).

The Gas Chromatography (GC) was performed using Agilent 19091J-413 Series with an FID and the capillary column DB-23 (USA). The injector and detector temperatures were maintained at 260 and 300 °C, and the oven program was 100 °C: 2 min; 160°C: 3 min; 215 °C: 2 min; 217 °C: 2 min; 218 °C: 2 min, and 260 °C for 2 min. The flow rate of nitrogen as the carrier gas was 1.5 mL/min.

Results

Construction of pPICZC - delta-6 desaturase expression plasmid

The delta-6 desaturase gene was amplified from the pTZ57R/T - delta-6 desaturase plasmid and inserted into the expression vector pPICZC. After transformation into *E. coli* DH5 α , positive clones were selected with direct colony PCR screening (Figure. 1A). Finally, pPICZC - delta-6 desaturase plasmid was additionally confirmed by restriction enzyme digestion. The expression plasmid was fragmented, as a result of two EcoRI and XhoI restriction enzyme activities (Figure. 1B).

Lipid production evaluation in recombinant *P. pastoris*

To obtain the recombinant *P. pastoris*, pPICZC - delta-6 desaturase plasmid was linearized by SacI and electro-transformed into *P. pastoris*. After 20h incubation of transformants in YPD, colonies were added to 50ml lipid production culture with methanol 0.5% and linoleic acid 2%.

To evaluate the activity of delta-6 desaturase in recombinant *P. pastoris*, the production of the lipid was studied by Sudan black (Fig. 2) and Nile red staining (Fig. 3) and visualized under the light microscope. It was found that both methods, showed a significant difference in lipid production, compared to the control samples.

Fluorescent assay

To identify the effects of the plasmid on the lipid production of the *P. pastoris*, the FL2 channel of florescent was obtained and the histogram of recombinant *P. pastoris* was compared with wild type (Fig. 4). The results showed that the florescent extension of wild-type *P. pastoris* was lower than that of recombinant species, which suggests that most of the recombinant cells had lipid production capability.

Moreover, SSC dot blots against florescent were studied to evaluate the granules of the recombinant *P. pastoris* along with FL2 studies, which distributed based on FL2=1 and SSC=10 (Fig. 5). It was found that the QA1 group (FI>1 $\square$ SSC<10), revealed a significant difference between species that suggests more lipid manufacturing in recombinant spices compared to wild type. The results of FL2 differentiation against FSC were also in agreement with the previous study, which showed an increase in not only lipid production but also cell size (Fig. 6).

Gas Chromatography (GC) analysis of GLA production

The lipid combination of the recombinant *P. pastoris* was studied by measuring the ester fatty acids using the GC chromatography method. The fungal cells were disrupted, and the lipids were extracted to assay the types and quantities of lipids. The lipid-containing fractions were separated and modified to obtain fatty acid methyl esters (FAMES). The amount had been compared with the wild-type *P. pastoris* GS115, which showed significant differences in lipid components (Fig. 7). Recombinant spices express 19.2% GLA compared to 0% in wild-type spices.

The GC assay of lipid combination was also evaluated on both spices that cultured in restaurant oil waste culture and revealed increasing of GLA manufacturers (Table 1).

Discussion

Studies on harvesting novel and rich resources of PUFAs as food and pharmaceutical additives have been proceeding in various fields. Among them, GLA is of great interest due to its important medicinal and healthy promoting functions, such as kill cancer cells and anti-infection (Zhang et al.; Sakuradani and Shimizu 2003). The main sources of GLA are plant seeds oils such as borage and some fungi, including species of *Mortierella*, and *Mucor* are known. Although, these sources GLA production is inadequate for supplying the expanding market due to such drawbacks as low productivity, insecurity, expensive downstream processing, etc. (Kapoor; Wan et al.; Kenny et al. 2000; Sakuradani et al. 2009). Thus, this is still the hot spot to find more excellent GLA-producing strains.

Generally, the biosynthesis of GLA derives from saturated stearic acid (18:0), which is converted by three desaturases, the delta-9, delta-12, and delta-6, respectively (Wan et al.; Cui et al. 2016). More recently, experiments have been focused on producing recombinant spices with high-level GLA production. In 2012, Cui et al. reported an identification and expression of delta-12 desaturase from *Rhodospiridium kratochvilovae* in *Saccharomyces cerevisiae* (Cui et al. 2016). Huang et al. transformed delta-12 and delta-6 desaturases from *Mortierella alpina* to *Saccharomyces cerevisiae* and yields of GLA reached as high as 8% of total fatty acids (Huang et al. 1999).

Due to the drawbacks of *Saccharomyces cerevisiae* lipid storage, studies have been conducted to find novel hosts for GLA production. In 2011, delta-6 desaturase from *Cunninghamella echinulata* was cloned in *Lipomyces kononenkoae* Wang et al and revealed 1.2% GLA production in the total fatty acids (Wang et al. 2011). Zhu et al. also examined the expression of *mortierella alpina* delta-6 desaturase in *Yarrowia lipolytica* with different vectors, which resulted in 25-35% high-level GLA manufacturing (Zhu and Jackson 2015).

As mentioned above, the expression system of *Pichia pastoris* fungus has several advantages such as diverse posttranslational modifications, high levels of expression with a limited amount of methanol, feasible genetic manipulation, and low-cost medium culture (Li et al. 2007). Because of the lack of delta-6 desaturases gene, wild-type *Pichia pastoris* spores could not produce GLA from their LA and LAL products. Wei et al. identified the existence of delta-12 and delta-15 desaturases in *Pichia pastoris* and transformed these genes to *Saccharomyces cerevisiae* (Wei et al. 2006). In 2005, Zhang et al. worked on the hetero expression of *Rhizopus arrhizus* delta-6 desaturase gene in *Pichia pastoris* (Zhang et al. 2005). Similarly, Wan et al. transformed *Cunninghamella echinulate* delta-6 desaturase gene in *Pichia pastoris*, and produced 3.1% GLA of total fatty acids (Wan et al.).

In this study, after gene cloning and verification tests, a new species of fungus, designated for express delta-6 desaturase, was obtained by insertion of an *M. rouxii* delta-6 desaturase gene into *P. pastoris* GS115 strain using electroporation and the analysis of changes in the level of lipid production was studied. Results from Sudan black and Nile red staining showed a significant difference in the lipid production in recombinant *P. pastoris*, compared to the control wildtype samples. Florescent analysis including, FL2 channel, SSC dot blots, and FL2 differentiation against FSC was also in agreement with previous studies, which showed an increase in lipid production and cell size. GC analysis of the ester fatty acids of recombinant *P. pastoris* revealed 19.2% GLA production compared to 0% in wild-type spores. The GC assay of lipid combination was also evaluated on both spores cultured in restaurant oil waste culture and revealed 46% lipid production with 72.3 mg GLA in recombinant spores, compared to 30% lipid production without any GLA manufacturing in wild type spores. Thus, this host may provide an opportunity for the development of the method for industrial-scale GLA manufacturing.

Declarations

Acknowledgments

The authors would like to thank Dr. Rahman Emamzadeh, Dr. Maryam Fanaei, Zahra fanaei, Dr. Gholamreza Ghezelbash, and Dr. Hossein Ghanavati for their guidance.

Compliance with ethical standards

Conflict of interest: The authors have no conflicts of interest to declare that are relevant to the content of this article

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Authors' contribution: Maryam Sadat Mirbagheri Firoozabad carried out the experiment and supervised the project, Hamidreza Akhbariyoone helped supervise the project and wrote the manuscript.

Funding: Not applicable

References

1. Christie WW (1993) Preparation of ester derivatives of fatty acids for chromatographic analysis. Oily Press
2. Cui J, He S, Ji X, et al (2016) Identification and characterization of a novel bifunctional $\Delta 12/\Delta 15$ -fatty acid desaturase gene from *Rhodospiridium kratochvilovae*. *Biotechnol Lett* 38:1155–1164. <https://doi.org/10.1007/s10529-016-2090-7>
3. Fakas S, Čertík M, Papanikolaou S, et al γ -Linolenic acid production by *Cunninghamella echinulata* growing on complex organic nitrogen sources. Elsevier
4. Fan Y, nutrition RC-TJ of, 1998 undefined Importance of dietary γ -linolenic acid in human health and nutrition. academic.oup.com
5. Green M, Inglis JS-J, 2012 undefined A laboratory manual. seaver-faculty.pepperdine.edu
6. Gyves EM de, ... CS-P, 2004 undefined Genetic manipulation of gamma-linolenic acid (GLA) synthesis in a commercial variety of evening primrose (*Oenothera* sp.). repository.rothamsted.ac.uk
7. Huang YS, Chaudhary S, Thurmond JM, et al (1999) Cloning of $\Delta 12$ - and Δ -desaturases from *Mortierella alpina* and recombinant production of γ -linolenic acid in *Saccharomyces cerevisiae*. *Lipids* 34:649–659. <https://doi.org/10.1007/s11745-999-0410-8>
8. Jangbua P, Laoteng K, Kitsubun P, et al (2009) Gamma-linolenic acid production of *Mucor rouxii* by solid-state fermentation using agricultural by-products. *Lett Appl Microbiol* 49:91–97. <https://doi.org/10.1111/j.1472-765X.2009.02624.x>
9. Kapoor R Gamma Linolenic Acid: An Antiinflammatory Omega-6 Fatty Acid. ingentaconnect.com. <https://doi.org/10.2174/138920106779116874>
10. Kenny FS, Pinder SE, Ellis IO, et al (2000) Gamma linolenic acid with tamoxifen as primary therapy in breast cancer. *Int J Cancer* 85:643–648. [https://doi.org/10.1002/\(SICI\)1097-0215\(20000301\)85:5<643::AID-IJC8>3.0.CO;2-Z](https://doi.org/10.1002/(SICI)1097-0215(20000301)85:5<643::AID-IJC8>3.0.CO;2-Z)
11. Kim D-H, Yoo T-H, Lee SH, et al (2012) Gamma Linolenic Acid Exerts Anti-Inflammatory and Anti-Fibrotic Effects in Diabetic Nephropathy. *Yonsei Med J* 53:1165–1175. <https://doi.org/10.3349/ymj.2012.53.6.1165>
12. Knauf V, Shewmaker C, Flider F, et al (2006) Safflower with elevated gamma-linolenic acid
13. Li P, Anumanthan A, Gao XG, et al (2007) Expression of recombinant proteins in *Pichia pastoris*. *Appl. Biochem. Biotechnol.* 142:105–124
14. lipids CR-H of functional, 2006 undefined Microbial production of γ -linolenic acid. cabdirect.org
15. Lu H, Zhu Y (2015) Screening and molecular identification of overproducing γ -linolenic acid fungi and cloning the delta 6-desaturase gene. *Biotechnol Appl Biochem* 62:316–322. <https://doi.org/10.1002/bab.1281>
16. Needleman P, Truk J, Jakschik BA, et al (1986) Arachidonic Acid Metabolism. *Annu Rev Biochem* 55:69–102. <https://doi.org/10.1146/annurev.bi.55.070186.000441>

17. Nykiforuk CL, Yurchenko O, Fidantsef A, et al (2011) High level accumulation of gamma linolenic acid (C18:3Δ6.9,12 cis) in transgenic safflower (*Carthamus tinctorius*) seeds SemBioSys Technology View project Oilseed DGAT and lipid biosynthesis View project. *Artic Transgenic Res* 21:367–381. <https://doi.org/10.1007/s11248-011-9543-5>
18. Pan L-X, Yang D-F, Shao L, et al Isolation of the Oleaginous Yeasts from the Soil and Studies of Their Lipid-Producing Capacities. hrcak.srce.hr
19. Qiao K, Abidi S, Liu H, et al Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. Elsevier
20. Sakuradani E, Ando A, Ogawa J, Shimizu S (2009) Improved production of various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding. *Appl. Microbiol. Biotechnol.* 84:1–10
21. Sakuradani E, Shimizu S (2003) Gene cloning and functional analysis of a second Δ6-fatty acid desaturase from an arachidonic acid-producing *mortierella* fungus. *Biosci Biotechnol Biochem* 67:704–711. <https://doi.org/10.1271/bbb.67.704>
22. Vrinten P, Wu G, Truksa M, Qiu X (2007) Biotechnology and Genetic Engineering Reviews Production of Polyunsaturated Fatty Acids in Transgenic Plants. *Transgenic Plants, Biotechnol Genet Eng Rev* 24:263–280. <https://doi.org/10.1080/02648725.2007.10648103>
23. Wan X, Zhang Y, Wang P, et al Production of gamma-linolenic acid in *Pichia pastoris* by expression of a delta-6 desaturase gene from *Cunninghamella echinulata*. koreascience.or.kr
24. Wan X, Zhang Y, Wang P, et al Production of gamma-linolenic acid in *Pichia pastoris* by expression of a delta-6 desaturase gene from *Cunninghamella echinulata*. koreascience.or.kr
25. Wan X, Zhang Y, Wang P, Jiang M (2011) Molecular cloning and expression analysis of a delta 6-fatty acid desaturase gene from *Rhizopus stolonifer* strain YF6 which can accumulate high levels of gamma-linolenic acid. *J Microbiol* 49:151–154. <https://doi.org/10.1007/s12275-011-0254-7>
26. Wang D, Li M, Wei D, et al (2007) Identification and functional characterization of the delta 6-fatty acid desaturase gene from *Thamnidium elegans*. *J Eukaryot Microbiol* 54:110–117. <https://doi.org/10.1111/j.1550-7408.2006.00136.x>
27. Wang P, Wan X, Zhang Y, Jiang M (2011) Production of γ-linolenic acid using a novel heterologous expression system in the oleaginous yeast *Lipomyces kononenkoae*. *Biotechnol Lett* 33:1993–1998. <https://doi.org/10.1007/s10529-011-0651-3>
28. Wei DS, Li MC, Zhang XX, et al (2006) A novel Δ12-fatty acid desaturase gene from methylotrophic yeast *Pichia pastoris* GS115. *Acta Biochim Pol* 53:753–759. https://doi.org/10.18388/abp.2006_3303
29. Yamada Y, Makimura K, Mirhendi H, et al (2002) Comparison of Different Methods for Extraction of Mitochondrial DNA from Human Pathogenic Yeasts
30. Zhang Q, Li M, Ma H, et al Identification and characterization of a novel Δ6-fatty acid desaturase gene from *Rhizopus arrhizus*. Elsevier
31. Zhang Q, Li MC, Sun Y, et al (2005) Heteroexpression of *Rhizopus arrhizus* delta6-fatty acid desaturase gene in *Pichia pastoris*. *Sheng Wu Gong Cheng Xue Bao* 21:871–877
32. Zhang Y, Luan X, Zhang H, et al (2017) Improved γ-linolenic acid production in *Mucor circinelloides* by homologous overexpressing of delta-12 and delta-6 desaturases. *Microb Cell Fact* 16:. <https://doi.org/10.1186/s12934-017-0723-8>
33. Zhu Q, Jackson EN (2015) Metabolic engineering of *Yarrowia lipolytica* for industrial applications. *Curr. Opin. Biotechnol.* 36:65–72
34. Heterologous expression of *Mucor rouxii* Δ12-desaturase gene in *Saccharomyces cerevisiae*. Elsevier
35. Zhou DQ. Microbiology Lab Manual. Shanghai Scientific... - Google Scholar. https://scholar.google.com/scholar?hl=en&as_sdt=0%2C5&q=Zhou+DQ.+Microbiology+Lab+Manual.+Shanghai+Scientific+and+Technical+Publishers.+Shanghai.+PR+China.+1983.&btnG=
Accessed 13 Apr 2021b

Tables

Table 1. Comparison of lipid, biomass, yields (%) lipid/ biomass w/w), omega 6 and omega 3 production (mg. L-1) by two strains in medium contains oil waste.

Spices	Biomass (g.L ⁻¹)	Total lipid (g.L ⁻¹)	Yield (w/w %)	C18:2 Linoleate (n-6 %)	C18:3 GLA (n-6 %)	C18:3 (n-3 %)ALA
Recombinant <i>P. pastoris</i>	6.2	3.11	46	174.2	72.3	14.6
wild type <i>P. pastoris</i>	6.4	1.93	30	13.15	-	24.5

Figures

Figure 1

Cloning confirmation using PCR and Double digest techniques. A) lane 1, Gene ruler. Lane 2, PCR products from the pPICZC - delta-6 desaturase plasmid. Lane 3, Negative control. B) lane 1, Gene ruler. Lane 2, Digested plasmid using XhoI. Lane 4, Digested plasmid using EcoRI. Lane 3. 5, Digested plasmid using EcoRI and XhoI.

Figure 2

Evaluation of lipid production by Sudan black staining under 1000x light microscope in A) recombinant *P. pastoris* B) wild type *P. pastoris*

Figure 3

Identifying lipid production by Nile red staining under 1000x light microscope in A) recombinant *P. pastoris* B) wild type *P. pastoris*

Figure 4

FL2 channel fluorescent histogram of Nile red stained A) Auto-fluorescent cells B) wild type *P. pastoris* C) recombinant *P. pastoris*

Figure 5

Evaluation of SSC dot blots against fluorescent of A) Auto-fluorescent cells B) wild type *P. pastoris* C) recombinant *P. pastoris*

Figure 6

Identifying FL2 differentiation against FSC of A) Auto-fluorescent cells B) wild type *P. pastoris* C) recombinant *P. pastoris*

Figure 7

GC lipid combination chromatograph of A) wild type *P. pastoris* B) recombinant *P. pastoris*