

Engineering and Fermenter Optimization of Fungi GLA Productions in *Pichia Pastoris* GS115 Using Oil Waste

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Research Article

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Abstract

γ -Linolenic acid (GLA) is an important n-6 polyunsaturated fatty acid (PUFA) that has received considerable attention in both levels in human and animal feed. GLA is used in many nutritional and medicinal applications such as the treatment of cancer, inflammatory disorders, and diabetes. Currently, plant seed is the main dietary source of GLA that is not enough to utilize on an industrial scale. To generate a sustainable novel source of GLA, the gene of delta-6 desaturase, which is one of the important enzymes in the GLA production pathway was isolated from *Mucor rouxii* DSM1194 and expressed in *Pichia pastoris* GS115 by pPICZC vector. The recombinant yeast expressed the GLA up to 19.2% (72 mg/g) of total fatty acids. GLA production of recombinant yeast was studied in fermenter by oil waste along 5 days and results detected 6.3 g / l lipid and 103 mg/g GLA was produced in 72 hours. 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 expression levels of various genes (Kim et al. 2012; Lu and Zhu 2015).

GLA is rarely found in some nuts and oil plants seeds such as evening primrose (*Oenothera biennis* L.), borage (*Borago officinalis* L.) and, blackcurrant (*Ribes nigrum* L.) (De Gyves et al. 2004; Wan et al. 2009a; Zhang et al. 2017). Although up to 22% of the total fatty acid content can be GLA in plant seeds, the quantities and qualities of this PUFA production are 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. 2012). Nevertheless, there is continued interest in developing alternative sources 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 (lipids and 2006; Fakas et al. 2008; Wan et al. 2009a, 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. The gene for delta-6 desaturase has been previously cloned and characterized for several prokaryotes and higher eukaryotes. It was also overexpressed in many hosts, such as microalgae, yeasts, and plants. However, looking for more developed GLA-producing microorganisms is still one of the current prominent researches, 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*. (Passorn et al. 1999; Wang et al. 2007; Lu et al. 2009; Nykiforuk et al. 2012; Qiao et al. 2015; Cui et al. 2016).

Mucor rouxii a typical oleaginous filamentous fungus has been widely used to investigate GLA production. Previous studies have shown that a 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 not cost-competitive compared with plants up to now. Thus, it is necessary to explore another fungus host for GLA production on an industrial scale.

Previous studies have shown the gene expression in *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.

Material 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. All chemicals were obtained from Merck with analytical grade and the restaurant oil wastes sample were collected from Naz plant oil factory.

Cultures were grown in Luria Bertani Agar (LB Agar) (Opperhuizen 1982). 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.

Preparation of oil wastes media was carried out according to Papanikolaou method contained (per liter of distilled water) 7.0 g KH₂PO₄, 2.5 g Na₂HPO₄, 1.5 g MgSO₄, 0.06 g MnSO₄, 0.15 g CaCl₂, 0.15 g FeCl₃, 0.5 g yeast extract, 0.5 g (NH₄)₂SO₄, pH 6.0, and 2% (V/V) oil waste as a carbon source (Papanikolaou et al. 2007).

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`-CAAGAATTCAAATGGCTCCCCCAAATACTGCGGC3`

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 48 h 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 10 µL reaction containing 4 µL 5× PCR buffer, 1 µL Reverse Transcriptase, 2 µL of 10 mM dNTPs, 1 µL of primers, and 1 µg of the RNA sample was used. After mixing and centrifuge for 5 sec, 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 10 min. 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 (Opperhuizen 1982). Colony PCR was carried out to verify that the Plasmid DNA had transformed correctly using the below primers.

F primer: 5`-CAAGAATTCAAATGGCTCCCCCAAATACTGCGGC3`

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 50ml YPD with methanol 0.5% and linoleic acid 2% 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 experiment, samples were obtained from production media after 72 h and stained.

We also evaluated the amount of GLA production using flow cytometry on recombinant and wild-type species as a control. First, the FL2 channel of fluorescent recombinant *P. pastoris* lipid production was compared with wild type. Subsequently, SSC dot blots of the recombinant *P. pastoris* along with FL2 studies (FL2=1, SSC=10) against fluorescent were studied.

Gla Purification And Gc Analysis

The modified Bligh and Dyer method was performed to extract lipid from *P. pastoris* cells (Pan et al. 2009). 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 4 M), 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.

Optimization in fermenter by using of oil waste

Under optimal conditions, recombinant yeast was cultured in a 3-liter fermenter (with 1.5 liters of culture fluid) and the results of lipid production and dry weight, and GLA production were obtained during 5 days of growth. It should be noted that oil waste was used as a carbon source and the experiment was done three times.

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* DH5α, positive clones were selected with direct colony PCR screening. 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.

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 50 mL 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 and Nile red staining (Fig. 1) 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 fluorescent was obtained and the histogram of recombinant *P. pastoris* was compared with wild type (Fig. 2). The results showed that the fluorescent 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 fluorescent were studied to evaluate the granules of the recombinant *P. pastoris* along with FL2 studies, which were distributed based on FL2=1 and SSC=10 (Fig. 3). It was found that the QA1 group (FI>1 & SSC<10), revealed a significant difference between species that suggests more lipid manufacturing in recombinant species 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. 4).

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. 5). Recombinant species express 19.2% GLA compared to 0% in wild-type species.

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

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 ALA (n-3%)
Recombinant <i>P. pastoris</i>	6.2±0.12	3.11±0.1	46±0.05	174.2±0.13	72.3±0.23	14.6±0.09
wild type <i>P. pastoris</i>	6.4±0.06	1.93±0.09	30±0.2	13.15±0.1	-	24.5±0.12

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 killing cancer cells and anti-infection (Sakuradani and Shimizu 2003; Lu et al. 2009). The main sources of GLA are plant seed oils such as borage and some fungi, including species of *Mortierella*, and *Mucor* are known. However, these sources for GLA production are inadequate for supplying the expanding market due to such drawbacks as low productivity, insecurity,

expensive downstream processing, etc. (Kenny et al. 2000; Kapoor and Huang 2006; Wan et al. 2009a; Sakuradani et al. 2009). Thus, it is still important to find higher 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. 2009a; Cui et al. 2016). More recently, experiments have been focused on producing recombinant species 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* 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* species 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. 2009b).

In this study, after gene cloning and verification tests, a new transformed strain 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 wild-type samples. Florescent analysis including, FL2 channel, SSC dot blots, and FL2 differentiation against FSC were 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 spices. The GC assay of lipid combination was also evaluated on both species cultured in the restaurant oil waste culture and revealed 46% lipid production with 72.3 mg GLA in recombinant spices, compared to 30% lipid production without any GLA manufacturing in wild-type spices. Thus, this host may provide an opportunity for the development of the method for industrial-scale GLA manufacturing.

Declarations

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

Consent to Participate: The authors are consent to participate in this article.

Consent to Publish: All authors are agree to publish the article in the journal of Applied biochemistry and biotechnology

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

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

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Figures

Figure 1

Evaluation of lipid production by Sudan black staining of A) recombinant *P. pastoris* B) wild type *P. pastoris* and also Nile red staining of C) recombinant *P. pastoris* D) wild type *P. pastoris* under 1000x light microscope.

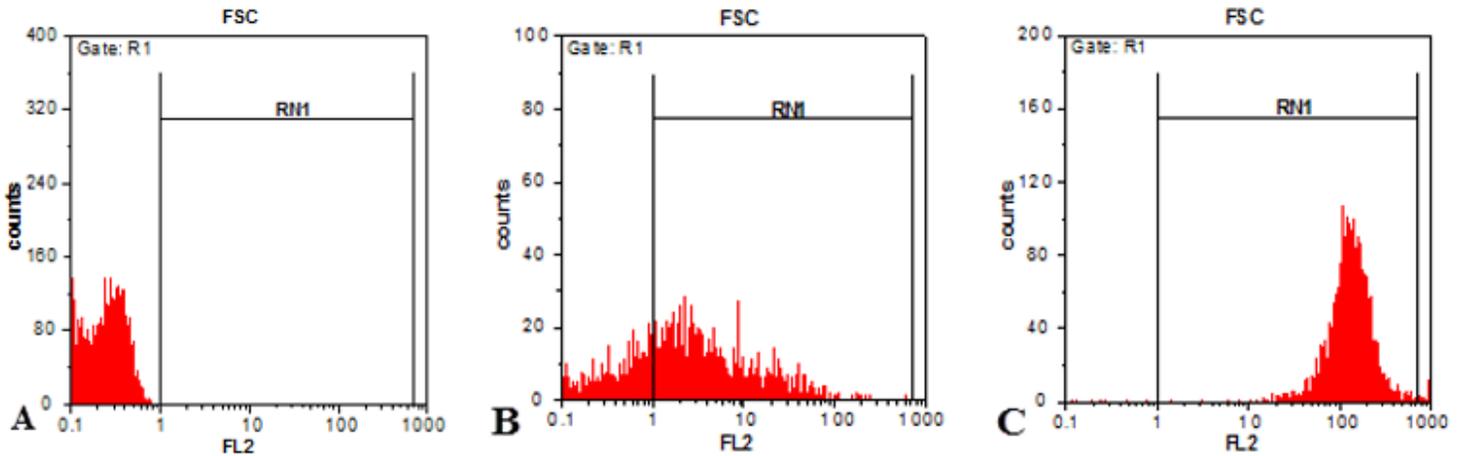


Figure 2

FL2 channel florescent histogram of Nile red stained A) Auto-florescent cells B) wild type *P. pastoris* C) recombinant *P. pastoris*. All experiments described were performed at least three times and statistical significance p value was below 5%.

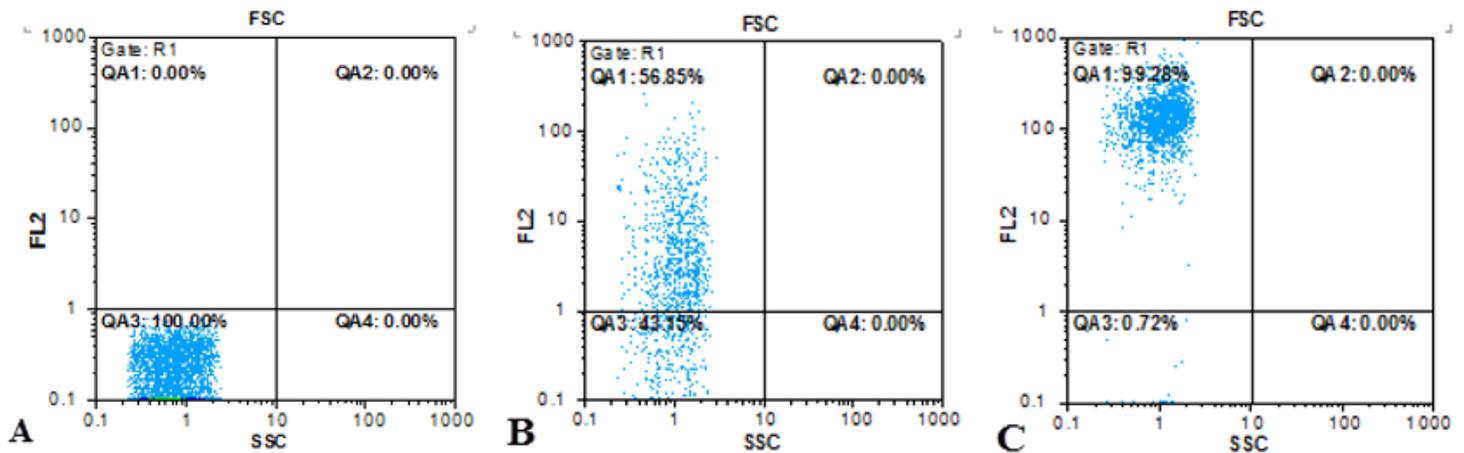


Figure 3

Evaluation of SSC dot blots against florescent of A) Auto-florescent cells B) wild type *P. pastoris* C) recombinant *P. pastoris*. All experiments described were performed at least three times and statistical significance p value was below 5%.

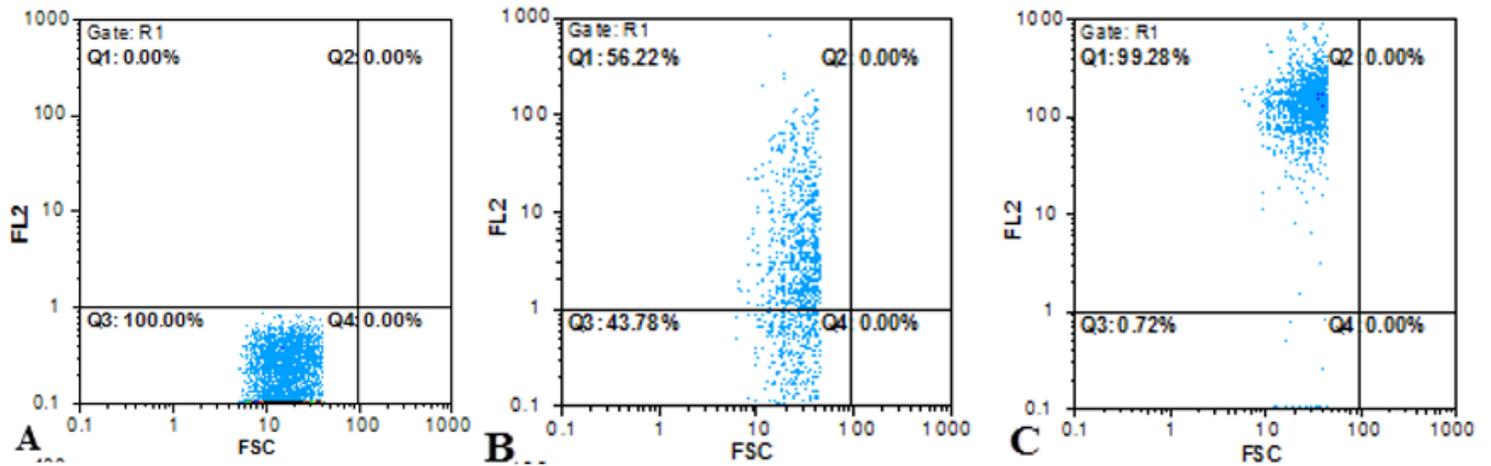


Figure 4

Identifying FL2 differentiation against FSC of A) Auto-florescent cells B) wild type *P. pastoris* C) recombinant *P. pastoris*. All experiments described were performed at least three times and statistical significance p value was below 5%.

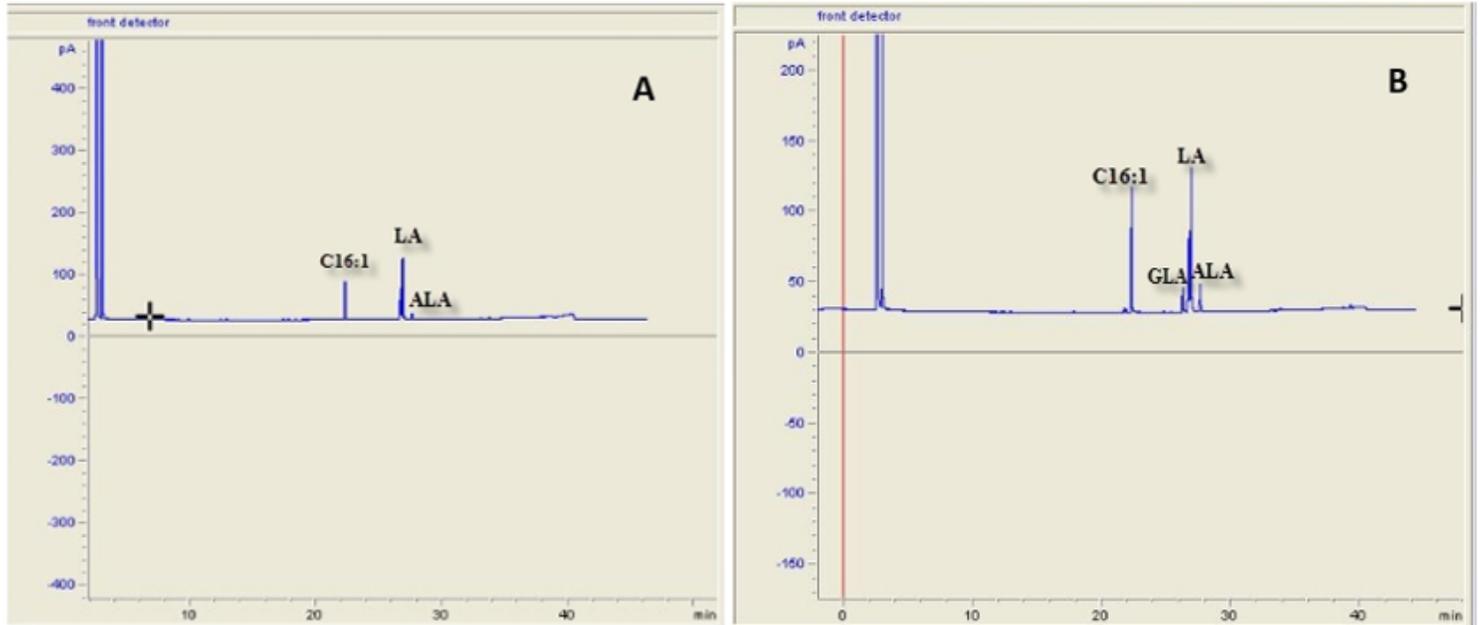


Figure 5

GC lipid combination chromatograph of A) wild type *P. pastoris* B) recombinant *P. pastoris*. All experiments described were performed at least three times and statistical significance p value was below 5%.

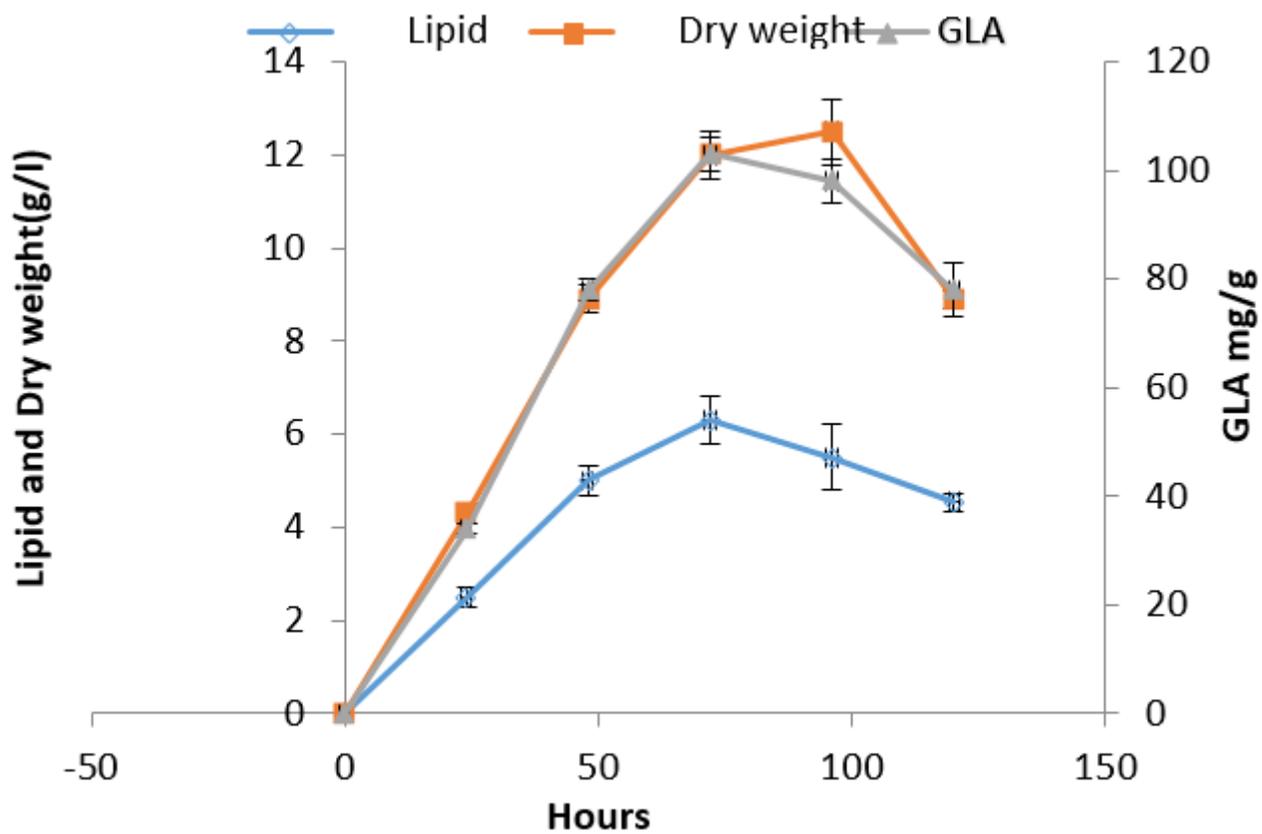


Figure 6

Diagram of cell dry weight, lipid production, and GLA production related recombinant strain in production medium containing 3-liter fermenter by oil waste in batch culture. All experiments described were performed at least three times and the statistical significance p value was below 5%.