NANYANG TECHNOLOGICAL UNIVERSITY

CAROTENOIDS SYNTHESIS IN RHODOSPORIDIUM TORULOIDES AND THEIR EXTRACTION BY PULSED ELECTRIC FIELD TECHNOLOGY

TOH SHI HUI
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Abstract

This research demonstrates the potential of *R. Toruloides* as carotenoid producer grown on low-cost crude glycerol, a by-product of biodiesel plant, and applications of PEF treatment on to extract proteins and fatty acids biomass as an alternative, mild extraction technique. The conversion rate of crude glycerol to carotenoids by *R. Toruloides* was optimized and found that a concentration of 60g/L of glycerol was optimal for production. The carotenoids profile of *R. Toruloides* analyzed using High Performance Liquid Chromatography (HPLC). The later part of the research focuses on the investigation of PEF treatment on *R. Toruloides* to isolate the carotenoids, proteins and fatty acids. The compositions of fatty acids and carotenoids remained unchanged by PEF treatment. In both PEF treated and PEF untreated samples, the predominant fatty acids present analyzed by GC/MS were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). Use of PEF treatment to extract proteins and fatty acids were also investigated. The PEF treatment allowed little protein extraction yields (9.7 % of total protein at electric field = 10 kV/cm), however application of PEF treatment allowed higher lipid extraction yield (up to 50% of total lipids). When PEF treatment is applied, followed by heat drying at 60°C, the decanting and drying process of *R. Toruloides* was enhanced. There were no negative influences on the fatty acid and carotenoids distribution pattern caused by the higher temperature during drying process. Since carotenoids are present as stable intact oleosomes in *R. Toruloides*, this suggests the possibility of isolating carotenoids from the chromoplasts within a whole dehydrated *R. Toruloides* cell after PEF treatment.

1. Introduction and Objectives

There has been a significant increase in commercial biodiesel production as a clean alternative fuel to replace crude oil used in diesel engine [1]. Biodiesel is produced via transesterification of oils, with crude glycerol as the main by-product (approximately 10 wt%) [2]. By 2016, biodiesel production is estimated to reach 37 billion gallons, implying a production of more than 4 billion gallons of crude glycerol each year [3]. However, it is too expensive and environmentally damaging to refine crude glycerol into pure glycerol, which can only then be used in industries such as food, cosmetics and pharmaceuticals [4]. Therefore, there exist a need to define alternative uses for the crude glycerol in order to make biodiesel production less expensive and more sustainable [5]. Little attention has been paid till now on using oleaginous yeast in bioconversion of crude glycerol. Biomass production by yeast has many advantages attributed to its high growth rate and its ability for rapid lipids accumulation [6].

The red yeast *R. toruloides* is an oleaginous species that can carry out many biochemical reactions such as biodegradation of epoxides, biphenyls and oxiranes [7]. Such oleaginous yeast is of great interest to the food technology industry, because they can up to 70 % of useful storage lipids within their cell [8]. These storage lipids include polyunsaturated fatty acids, omega fatty acids [9] that can be use as food supplements. However, a major biotechnological potential of *R. toruloides* is its ability to use glycerol as its food source to produce carotenoids. Thus *R. toruloides* was chosen as the model organism to be studied in this research due to its potential for resource recovery. Yeast produces carotenoids as part of cell stress response. The production of carotenoids increases under stress conditions, such as light and oxidative stress [10]. The inherent biosynthetic capacity of the R. toruloides as a biological source to produce valuable β-carotene commercially reemphasizes the importance to develop systems that can maximize the full potential of such biological hosts.

Pulsed Electric Field (PEF) is a non-thermal technology able to permeabilize various types of cell membrane reversibly and irreversibly via electroporation of its phospholipid bilayers [10]. Various applications in the use of PEF treatment

have been described, most notably for microbial inactivation, enhancing cell biomass mass transfer during drying and extraction processes, and to induce stress response in cells, via the high-voltage electrical pulses applied [12]. PEF treatment is a promising environmental friendly and energy saving processing method [13].

The objectives of my work are (a) to study and optimize rate of conversion of crude glycerol to carotenoids by *R. toruloides*, and (b) to investigating the possible use of PEF treatment in mass transfer of proteins and lipophilic substances. This work would also look into a heat drying process after PEF pretreatment, which can mechanically decant *R. toruloides,* allowing the isolation of carotenoids as intact (and very stable) oleosomes as product in its own "natural encapsulation". The influence of PEF, and the effect of heat drying process on the fatty acid distribution of *R. toruloides* will also be looked into.

2. Literature Reviews

The literature review will give a detailed background on the research areas covered by this thesis. An overview of the oleaginous yeast *R. toruloides,* the physiology, morphology and taxonomy will be addressed. The lipid metabolism, carotenoid production pathway, and its biomass potential in industrial applications using crude glycerol as substrate for *R. toruloides* will also be discussed (chapter 2.1). The various extraction and analysis techniques for yeast lipids and proteins will be looked into (chapter 2.2). The last section will focus on the introduction of PEF treatment mechanism, the applications of PEF and the influence of the electric field on cell membrane permeabilizability (chapter 2.3).

2.1 Oleaginous yeasts

Oleaginous microorganisms such as yeasts, microalgae, and fungi, can accumulate huge amounts of neutral storage lipids under optimal cultivation conditions. Microorganisms are classified as "oleaginous" when they can accumulate lipids exceeding 25 % of their dry cell weight [8]. Yeast strains such as *Candida curvata, Cryptococcus terricolus, Lipomyces starkeyi, Waltomyces lipofer, Yarrowia lipolytica*, bacteria species such as *Rhodococcus opacus, Thraustochytrium sp,* fungi species such as *Aspergillus terreus, Fusarium bulbigenum* and microalgae species such as *Thalassiosira pseudonana, Amphidinium cateri* are considered as oleaginous. Yeasts are considered as a promising source of microbial oil, as they can accumulate lipids up to 70% of their dry weight [14]. The increasing interests in microbial lipid compounds, single cell oils (SCO), have the structure and composition similar to high-value fats. A possible large scale production of such SCO would present an enormous financial interest to the highly demand market [9]. Furthermore, some oleaginous yeast has been identified to show promising growth and SCO production when cultured in by-products of the argoindustrial sector as their substrate [15]. Therefore, the ability of oleaginous yeast to valorize such waste residues, along with its high value lipid production could be translated into various potential biotechnological applications, for example in biodiesel production, food ingredients and even for antimicrobial activities [16].

2.1.1 *Rhodosporidium Toruloides* - physiology, morphology and taxonomy

Yeasts belong to the Fungi kingdom, the largest group of eukaryotic microorganisms. These species grow mainly as single-cell yeasts and they reproduce via the mode of budding or binary fission. The yeast cell is made of mainly of glucans and chitin. Fungi produce secondary metabolites close in structure almost to those produced by the plants species. They can grow in a very diverse degree of condition, even under very extreme conditions such as in high salt concentrations or under intense radiation environment. There has been around 100,000 species of fungi officially identified, however the actual diversity of our fungus kingdom has yet been fully comprehended [17]. Quite a number of red yeasts species, such as *Rhodotorula, Rhodosporidium, Sporidiobolus, Sporobolomyces, Cystofilobasidium, Kockovaella* and *Phaffia* are known to be able to produce carotenoids naturally. *Rhodosporidium toruloides* (*R. toruloides*), is a non-pathogenic, orange colored fungus due to the presence of carotenoids. It belongs to the anamorphic genus *Rhodotorula,* which have been commonly and very popularly used for the transformation of renewable resources into microbial oils [18]. There are 3 main species in *Rhodotorula* genus; namely the *Rhodotorula glutinis*, *Rhodotorula mucilaginosa* and *Rhodotorula minuta* [19]. These red yeast colonies grow rapidly and have a smooth outlook. The color can range from pink, orange to red in color. *R. toruloides* is a non-fermenting, unicellular yeast found naturally and well due to its carotenoids production (torulene, torularhodin and β-carotene) and lipids accumulation capability [20].

R. toruloides can accumulate up to 70% lipids of its dry cell weight, higher level than other types of oleaginous species[21]. *R. toruloides* exist as haploids in two mating types, a and A. This makes it as an attractive and easier host for genetic and molecular analysis [22]. This red yeast is also a good producer of biotechnologically important enzymes, and shows great tolerance towards inhibitory compounds in biomass hydrolysates [23]. However, a major biotechnological potential is their ability to utilize glycerol as their carbon source to accumulate both lipids and β-carotene as the main carotenoids. Biomass production by yeast has many advantages due to its unicellular high growth rate and rapid rate of accumulating lipids [6]. This makes *R. toruloides* a unique yeast strain of interest focus in this research. However to our

knowledge, very little work has been done on the study of conversion of and crude glycerol into carotenoids by yeasts. Also, the inadequate genetic background of oleaginous species also limits the basis molecular understanding of microorganism oleaginicity and the possibility for strain engineering work. *R. toruloides* ability in accumulating large volume of fatty acids brings our focus in its lipid metabolism. The main lipids produced by *Rhodotorula* yeast are mainly 16 and 18 carbon fatty acids.

2.1.2 Crude glycerol – A biodiesel by-product as carbon source

The main by-product produced by biodiesel plant during the transesterification process of oils into biodiesel is Glycerol (1,2,3-propanetriol) [2]. Glycerol is a colorless, viscous and sweet tasting molecule widely used in the pharmaceutical, cosmetics, and food and beverage industry. The worldwide consumption of glycerol in 1998 was estimated to be 750,000 tons. Glycerol is produced synthetically, or purified from waste products generated from biodiesal and soap-making industries. The high-pressure splitting, saponification, and trans-esterification processes used in these industries results in a waste or "crude" glycerol ranging from 15%-90% purity [24]. There has been a significant increase in commercial biodiesel production as a clean alternative fuel to replace crude oil used in diesel engine [1]. By 2016, biodiesel production is estimated to reach 37 billion gallons, implying more than 4 billion gallons of crude glycerol could be produced annually [3].

However, it is too expensive and environmentally damaging to refine crude glycerol into pure glycerol, which can only then be used in industries such as food, cosmetics and pharmaceuticals [25]. Therefore, there is a pending need to define effective uses for these crude glycerol in order to make biodiesel production a cheaper and more sustainable process [26]. Little attention has been paid till now on using oleaginous yeast in bioconversion of crude glycerol. We need a cost and time efficient method to convert these crude glycerol into value-added products, for example as animal feeds and single cell oil [27]. The compositions of crude glycerol produced by biodiesel production have got valuable elements such as calcium, magnesium, potassium, sulfur and sodium. There have already been papers exploring the conversion process of glycerol and crude glycerol into high value products, for example to hydrogen gas, propanediols, single cell oil (SCO), organic acids, polyunsaturated fatty acids for

health supplements and biosurfactants [28]. There have been reports on oleaginous yeasts' capability to accumulate lipids solely with glycerol as growth medium [29]. It is also documented that such oleaginous yeasts have short generation times and with very basic nutrition requirements [30]. Hence, if using crude glycerol for yeast cultures growth would be an additional bonus to reduce the costs of biodiesel production. Since glycerol can be the precursors for growth and lipids synthesis in oleaginous yeasts [8], this idea will be explored in this study using crude glycerol as the sole carbon source for the production of carotenes by *R. toruloides*. The growth rate of *R. toruloides* and the rate of its lipid production will also be optimized.

2.1.3 Lipid metabolism in yeast

Metabolism is the chemical and physical processes occurring within a living cell that are necessary for the maintenance of life. The chemical processes goes through regulated pathways, utilizing reactants and releases metabolites as products. The de novo lipid synthesis for yeast is governed by fatty acid synthase (FAS) system in the cytosol via an enzymatic process, with acetyl-CoA and malonyl-CoA as crucial starting reactants. Acetyl-CoA acts as the initial biosynthetic unit and the carbon chain lengthening is done through malonyl-CoA addition where 2 carbon atoms are added at each step during the fatty acid chain synthesis. Precursor of the Acetyl-CoA is the excess citrate that will be exported out the mitochondrion from the Krebs cycle. Citrate is cleaved in the cytosol by the action of ATP:citrate lyase (ACL) in the reaction as shown below [31]:

$$\text{Citrate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{Pi}$$

ACL is a two subunits molecule that is coded by gene ACL1 and ACL2. They require an ammonium ion to be activated, and hence lipid accumulations are favored in high nitrogen or low C/N conditions [9]. These precursors are fed into the lipid storage pathway, and acytyl-CoA is assembled to give us the triacylglycerols (TAG) and steryl esters (SE). TAGs are classified as neutral lipids, which are produced naturally during in the fatty acid biosynthetic pathways. ACL has been reported to be one of the essential key enzymes present in all eukaryotic microbial cells that are capable of accumulating TAG. It has been reported that no organisms without ACL activity were

able to accumulate lipid higher than 20% of their biomass [9], even though some lipid accumulations in some oleaginous yeast without ACL activity was still possible. This shows there exist some direct correlation in the ACL activity and the yeast lipid accumulation abilities.

Generation of malonyl-CoA occurs during when an acetyl-CoA unit undergoes condensation with a bicarbonate anion, catalyze by the acetyl-CoA carboxylase (ACC) enzyme as shown in the equation below:

$$\text{acetyl-CoA} + HCO_3^- + \text{ATP} \rightarrow \text{malonyl-CoA} + \text{ADP} + \text{Pi}$$

Fatty acid synthesis is catalyzed by an enzyme called the fatty acid synthase (FAS). During each carbon chain elongation reaction in fatty acid synthesis, the FAS makes use of 2 molecules of NADPH, which is produced by the malic enzyme (malate dehydrogenase carboxylase):

$$\text{malate} + NADP^+ \rightarrow \text{pyruvate} + \text{NADPH}$$

The NADPH can also be produced by the pentose phosphate pathway occurring in the cytoplasm, through glucose-6-dehydrogenase, 6-phosphogluconate de-hydrogenase, and NADPH isocitrate dehydrogenase (Figure 1).

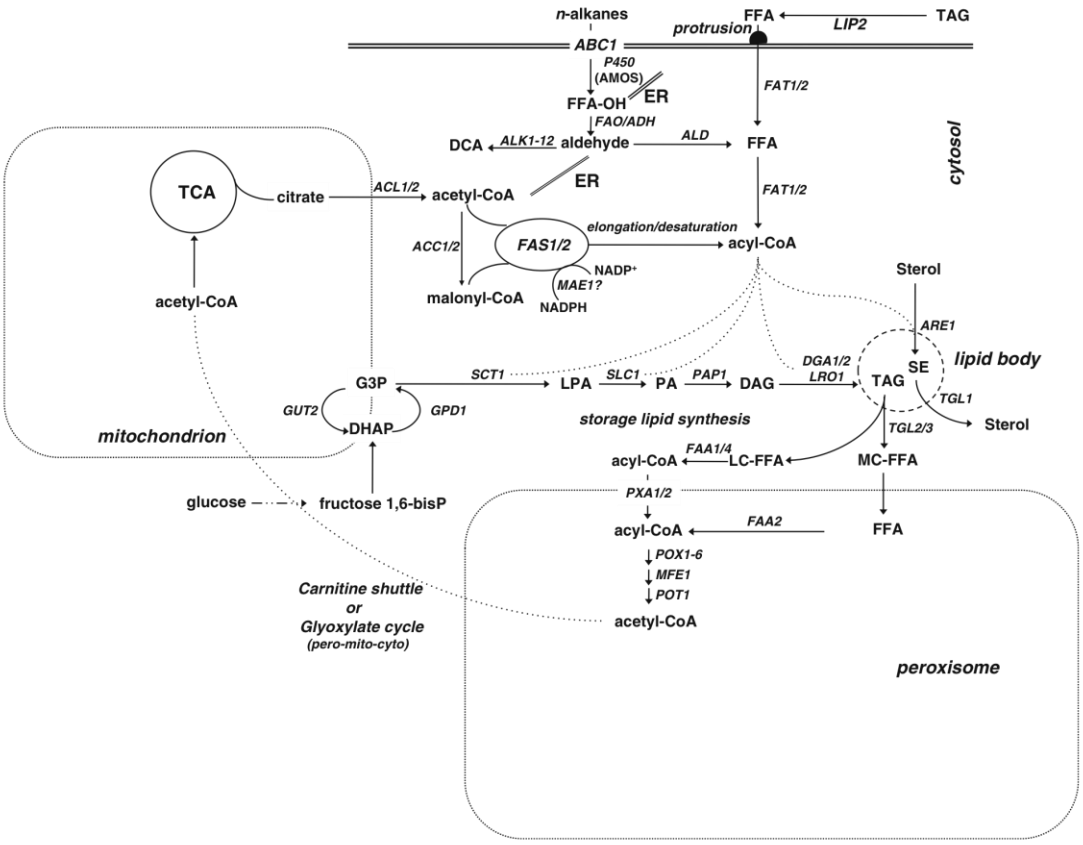

Figure 1 Overview of the lipid metabolism in yeasts.

Abbreviations for metabolic intermediates: DAG *diacylglycerol*, DCA *dicarboxylic acid*, DHAP *dihydroxyacetone phosphate*, FFA *free fatty acids*, FFA-OH *hydroxylated fatty acid*, G3P *glycerol-3- phosphate*, LC-FFA *long-chain fatty acids*, LPA *lysophosphatidic acid*, MC-FFA *medium-chain fatty acid*, PA *phosphatidic acid*, TAG *triacylglycerol*, SE *steryl ester*, TCA *tricarboxylic acid cycle*. Enzymes names: ACC *acetyl-CoA carboxylase*; ACL: *ATP citrate lyase*; ADH: *alcohol dehydrogenase*; ALK: *cytochrome P450 oxidase*; ARE: *acyl-CoA:cholesterol acyltransferase*; DGA: *acyl-CoA:DAG acyl- transferase*; FAA: *fatty acyl-CoA synthetase*; FAD: *fatty aldehyde dehydrogenase*; FAO: *fatty alcohol oxidase*; FAS: *fatty acid synthetase*; FAT: *fatty acid import and activation*; GUT: *G-3-P dehydrogenase/ glycerol kinase*; LIP: *lipase*; LRO: *phospholipid:diacylglycerol acyl-transferase*; MAE: *malic enzyme*; MFE: *multifunctional enzyme*; PAP: *phosphatidate phosphatase*; POT: *thiolase*; POX: *acyl-CoA oxidase*; PXA: *peroxisomal acyl-CoA transporter*; TGL: triacylglycerol lipase; SCT: *glycerol-3-phosphate acyltransferase*; SLC: LPA *acyltransferase* [32].

2.1.3.1 Lipid metabolic pathways in *R. toruloides*

R. toruloides contains ACL, mitochondrial β-oxidation (MBO) and the carotenoid biosynthetic pathways. The major source for lipid synthesis is the acetyl-CoA. The MBO pathway in *R. toruloides* produces ATP via the degrading of fatty acids through using flavinadenine dinucleotide (FAD) cofactor with the respiratory chain [33]. The carotenoid synthesis pathway is governed by two gene, namely phytoene synthase (PSY1) and phytoene dehydrogenase (CRTI). A novel fatty acid synthase (FAS) system has been recently discovered by Zhao *et al*. in their full genome and transcriptome sequencing of *R. toruloides*.

The FAS biosynthetic complex found in yeasts comprises of two subunits structure, integrated type I multi-enzyme Fas1 (β-subunit) and dissociated type II system Fas2 (α-subunit), in a hexameric α6β6 complex structure. The FAS in *R. toruloides* consists of two subunits: the β-subunit (Fas1) (acyltransferase and enoyl reductase domains), and α-subunit (Fas2) (all other remaining domains). In the Fas2 of *R. toruloides*, its two acyl carrier protein (ACP) domains were reported to share 76% sequence identity, and also shared share a high degree of similarity with the other ACPs of other yeats species.

2.1.3.2 Carotenoid biosynthesis

Carotenoids are a group of pigment molecules found naturally in different life forms. They play several important roles in nature, ranging from as photosynthetic pigments, as a visual attraction to animals and insects for pollination and mating purposes, to health benefits of antioxidants and as precursors of vitamin A [34]. Many types of microorganisms produce carotenoids naturally, such as bacteria, algae, molds and yeast. The structure of carotenoids is derived from phytoene, a 40-carbon intermediate during its biosynthesis (Britton, 1995). The carotenoid structure takes the form of polyene hydrocarbon chain, joined with 2 terminal ring structures. Carotenoids are split into two classes: Carotenes, which composes only of carbon and hydrogen atoms, and Xanthophylls, the oxygenated derivatives of carotenes.

There are over 700 different carotenoids in nature [35], of which many are widely utilized as a source of food colorants suitable in human food source, as well as

constituents in vitamins and dietary supplements. A diet high in carotenoid content was found to reduce risks of degenerative diseases (e.g., Alzheimer and Parkinson), and cardiovascular diseases [36]. Carotenoids also are antioxidants, owing to their long conjugated system of C-C double bonds. Only 10% of all known carotenes in nature possess vitamin A precursor ability in human [35] with β-carotene being the best known. Vitamin A is a crucial element important for human health and the body's immunity. Vitamin A deficiency (hypo-vitaminosis A) causes impairment in the human vision and is also related to lungs and oral pathologies. Hypo-vitaminosis A is major nutritional problem worldwide, with up to 500,000 malnourished children in the developing world going blind each year, approximately half of whom die within a year [37]. Hypo-vitaminosis A is also the cause of maternal mortality and other poor outcomes in pregnancy and lactation [38]. However, we rely on our food for the carotenoids that are the precursor for vitamin A because animals cannot synthesize carotenoids on their own.

The growing evidence of carotenoid potential and benefits in human health is leading to increasing commercial interest for alternative natural biological sources. Microorganisms that are capable of producing carotenoids naturally using low-cost carbohydrate sources (e.g. Crude Glycerol) have been viewed in light as potential pigment sources. This success could mean solutions to not only global malnutrition problem of hypo-vitaminosis A, but also allows biodiesel production to be more sustainable and profitable. The oleaginous red yeast of interest, *R. toruloides*, belongs to the genus *Rhodotorula*, widely known carotenoid producers. The carotenoid produced by *R. toruloides* in majority consist of β-carotene, torulene (3',4'-didehydro- β-ψ-carotene), and torularhodin (3',4'-didehydro- β-ψ-caroten-16'-oic acid) (Figure 2).

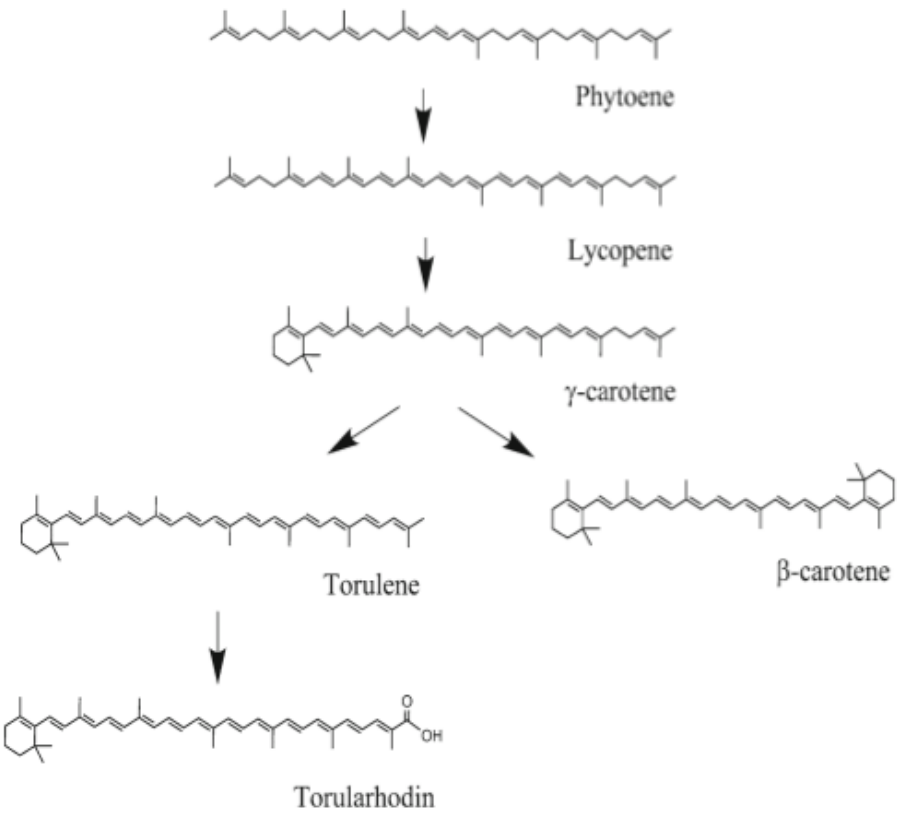

Figure 2 Metabolic pathway of carotenoids biosynthesis in *R. toruloides* (Moliné, 2012)

The carotenoid biosynthesis pathway occurs in three main steps. The acetyl-CoA first goes through the mevalonate pathway; it is first converted into 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA) with the aid of HMG-CoA synthase enzyme. This is followed by the conversion of HMG-CoA into mevalonic acid (MVA), a precursor of the terpenoid synthetic route. MVA is phosphorylated by the enzyme, MVA kinase, and further decarboxylated to give isopentenyl pyrophosphate (IPP), which goes into the isoprene biosynthesis pathway. In the isoprene biosynthesis pathway, three IPP is added and isomerized to dimethylallyl pyrophosphate (DMAPP). DMAPP is converted into a C20 compound geranyl geranyl pyrophosphate (GGPP), catalyzed by the enzyme, prenyl transferase. The first C40 carotene, phytoene is produced when two molecules of GGPP is condensed together. Phytoene would then undergo further desaturation to form lycopene, the precursor of all cyclic carotenoids. Lycopene goes though further metabolic reactions to form β-carotene, γ-carotene, torulene and torularhodin. γ-Carotene is the precursor for the production of β-carotene and torulene. Torularhodinis formation is via hydroxylation and oxidation of torulene (Figure 3).

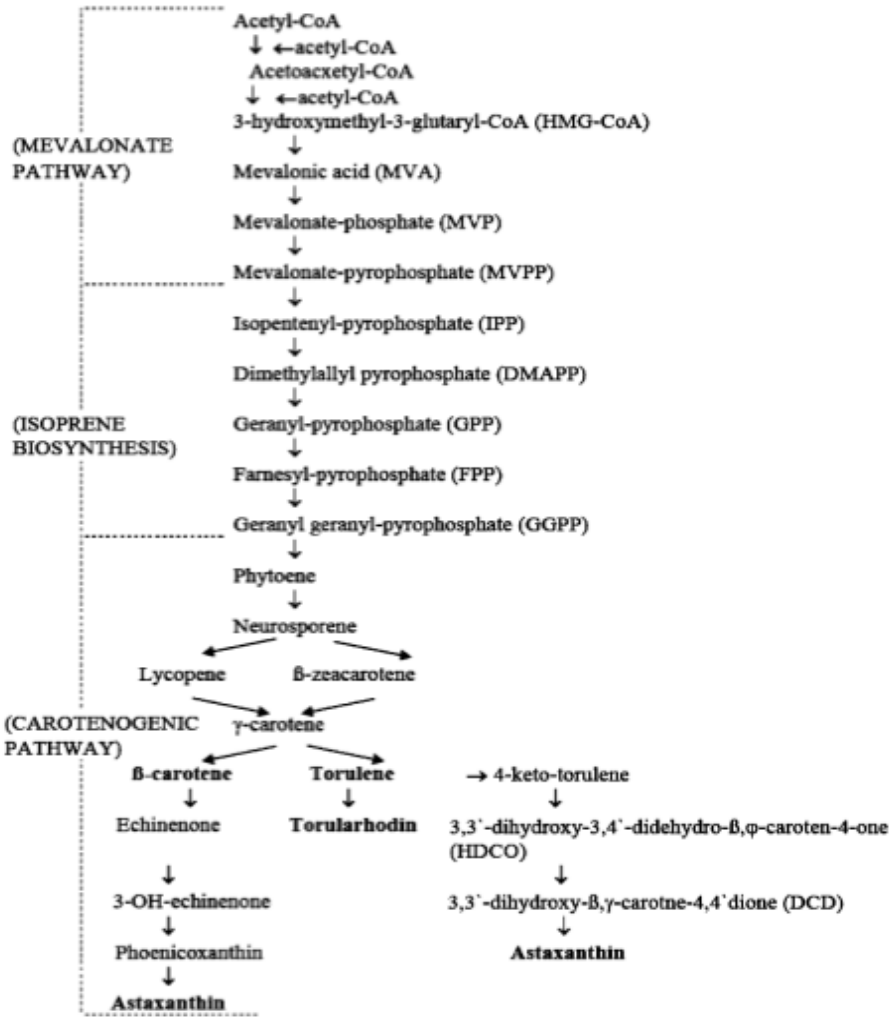

Figure 3 Carotenoid biosynthesis pathways of *R. toruloides*; Acetyl-CoA to β-carotene, torulene and torularhodin [39].

2.2 Extraction and analysis of yeast biomass

One of the main obstacles when trying to take full advantage of the valuable biomass that microorganism produces is to be able to efficiently extract all its cell biomass. In particular, the yeast cell wall is tough and it has to be opened to gain access to the intracellular products. Traditional mechanical and thermal methods for cell opening are effective, but costly and destroy much of the native functionality of extracted components. For example, lipohilic components are generally protected in the cells in oleosomes, and are very stable in this state. Cell opening destroys the natural protection, and initiates degradation processes such as (aut)oxidation, polymerisation and hydrolysis [40]. In addition, the cell debris complicates the downstream

purification. However, extraction of yeast biomass without prior cell disruption is not successful [41]. A mild method that opens up the cells, but keeps the organelles (such as olesomes, chromotplasts, etc) intact could greatly facilitate downstream processing while at the same time improving the quality and stability of the carotenoids from *R. toruloides*. In this section, the various extraction methods (2.2.1) and the methods for analysis of respective yeast biomass (2.2.2) will be discussed.

2.2.1 Methods for yeast extraction

There are many types of yeast biomass extraction techniques which are applied depending on the type of biomass one desires to collect. To consider which method to use for cell lysis, a few variables have to be taken into consideration before selecting a technique. Factors such as the cell sample type, size or volume, the stability of the sample and the extractant desired and future downstream purification processes would have to be able to complement the extraction technique. In this section, methods ranging from the traditional mechanical extraction, to extraction using different solvent will be briefly discussed.

2.2.1.1 Supercritical carbon dioxide (CO_2) extraction

Supercritical fluid extraction by using carbon dioxide solvent (SC-CO_2), is an environmental friendly method for lipid extraction. Carbon dioxide is a readily available substance that is non-toxic and non-explosive, and a major benefit is that it is easily removable from the desired extractant and thus its purity is the greatest advantage over all other solvents [42]. The supercritical state is reached when the combination of temperature-pressure reaches the critical point of the substance. For CO_2, its critical point is reached when the temperature exceeds 31°C and 73.8 bar [43]. A supercritical phase diagram is elaborated in Figure 4 [43]. Upon reaching the critical point, the supercritical solvent would be able to selectively extract the fats from the sample matrix dissolved into the solvent. The dissolved fats can be easily separated away from the supercritical solvent by reducing the solvent pressure drastically [44].

The properties of SC-CO_2 are governed by a couple of rules and solvent preferences. Its dissolving power favors non-polar to slightly polar compounds, and lower

molecular weight compounds. This means that heavier polar molecules such as polysaccharides, proteins and salts will be insoluble, unless at higher pressure [45]. Supercritical Fluid Extraction (SFE) allows the isolation of fatty acids, volatiles and flavors by extraction and fractionation. Today, SC–CO_2 is used extensively in extracting useful oil from nuts with high fat contents, such as almonds [46], peanuts [47] and walnuts. Food grade oil such as grape seed oil [48] and pumpkin seed oil [49] could also be extracted using SC–CO_2. Carotenoids and chlorophyll extraction was also possible, as done by Macías-Sánchez et al., using SC–CO_2 with methanol from Nanochloropsis gaditana, an aqua microalgae. Comparing SFE against other conventional extraction processes, the extracted yield have been reported comparable with the conventional Bligh and Dyer extraction procedure [50].

SFE has been used in not just food, but also in pharmaceutical and sterilization process. The use of SC–CO_2 for sterilization of bacteria have been studied intensively and it was found to play a key role in not just able to inactive microorganisms, but the process could be performed at lower temperature and time of exposure [51].

In summary, the advantages of SFE are: quick extraction and separation, high purity and quality extractants, selectivity and low separating cost. However, main shortcomings in this method are the more expensive and technically demanding equipments, and inability to extract polar substances, as compared to other extraction techniques [52].

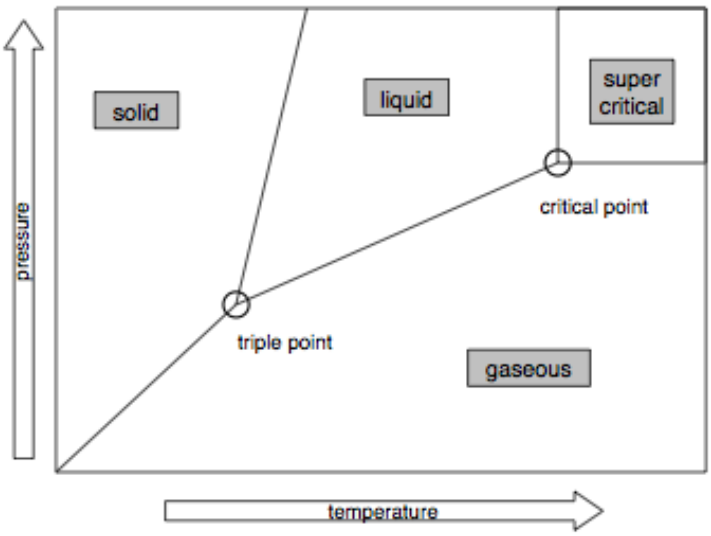

Figure 4 Generalized phase diagram of supercritical substances with critical point [43]

2.2.1.2 Chemical lysis – Detergent method

Detergent based chemical lysis method can be an alternative to mechanical cell disruption method, or can also be used in conjunction with the mechanical lysis. The detergents are mild surfactants that disrupt the cell lipid layer by interrupting and destroying the protein-protein, protein-lipid and lipid-lipid interactions. The chemical composition of detergents comprises of organic compounds, with a hydrophobic tail and a hydrophilic head (Figure 5). In solution, these detergent molecules rearranges their orientations such that the hydrophobic tail faces inwards and forms the interior, and the hydrophilic head faces outwards to form the exterior. This structure formed is called the micelle (Figure 6). The aggregation number is total number of detergent molecules that forms a micelle, and this number helps to determine the solubility of the membrane protein [53]. Some of the common detergents can be categorized into three main groups: Ionic (anionic or cationic), Zwitterionic and Non-ionic (Table. 1).

Table 1 Classification of different types of detergent [54].

Types of detergent	**Chemicals present**
Ionic	Sodium Dodecyl Sulfate (SDS), Deoxycholate, Cholate, Sarkosyl
Non-ionic	Triton X-100, DDM, Digitonin, Tween 20, Tween 80
Zwitterionic	CHAPS
Chaotropes	Urea

Ionic detergents have a hydrophobic chain and a charged anion or cation head. It works by disrupting all non-covalent bonds within the proteins, which effectively denatures and results in a total loss of their 3-D structure and functions. Anionic SDS is an extremely effective surfactant that can solubilize most proteins. Nonionic detergents have an uncharged and hydrophilic head. They are mild surfactants that only disrupt protein-lipid, lipid-lipid interactions, but they are not strong enough to break the stronger protein-protein interactions. Due to their milder nature, nonionic detergents are more preferred during isolation of membrane proteins. Zwitterionic detergents have amphoteric and hydrophilic head, with a zero net charge. They are a much more harsher surfactants as compared to nonionic detergents.

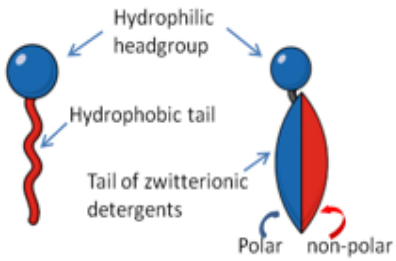

Figure 5 Structure of a detergent molecule [54]

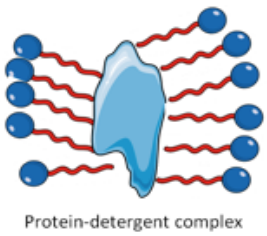

Figure 6 Structure of micelle when detergent are in solution [54]

2.2.1.3 Mechanical cell disruption (Beads method)

Mechanical cell disruption using glass beads at high level of agitation is one of the simplest and cheapest extraction techniques. The glass beads are added into the cell suspension and the sample is agitated at high speed on a vortex mixer. The vigorous agitation of the beads against the cell breaks the cell walls and causes the intracellular organelles to be released. However, several cycles of agitation (per minute) must be interspersed with a short period of cooling on ice to avoid sample heating up significantly due to the extreme agitation. Multiple samples can be conveniently processed simultaneously as well without any cross contamination issues. However, the disadvantage of this method includes: Limitation in terms of upscaling to larger samples, variability in terms of extracted product yield and purity, and most importantly, the problem with overheating of the cell suspension due to the vigorous agitation. All beadbeating machines can warm the samples up to 10°C per minute, due to the frictional collisions of the glass beads during homogenization. Some extractants, such as proteins, are temperature sensitive. Hence overheating during extraction process can cause undesired modification and degradation of quality of the extraction products. It is often that denaturing of proteins occurs due to the resulting high temperature and excessive shear force during the mechanical disruption process.

2.2.1.4 Ultrasound/sonication

Cell disruption is performed by applying ultrasound ranging from 20–50 kHz to the sample during sonication. The high frequency ultrasound is electronically generated, and the resultant mechanical energy is being transmitted to the cell suspension sample via a metal probe that will oscillate at a high frequency. The metal probe is placed in contact with the cell suspension sample, and the high frequency oscillation would proceed to result in cavitations and impaction within the cell suspension, and the pressure built up would the cause the cells to break open. One advantage of this method is that it is applicable for most cell types, and up-scaling to larger sample volume is possible. However, some disadvantage using the ultrasound method includes also the large amount of heat and noise levels produced. In most of the ultrasound systems, it is required to wear hearing protection and sonic enclosures. The extracted yield of the cell sample can vary time to time too. The free radicals that are

generated during the sonication process might also react unspecifically with other molecules.

2.2.2 Methods for yeast biomass analysis

There are various analytical methods for different yeast biomass, such as proteins and lipids. The analysis method varies for the different biomass, thus the analytical methods specific for our biomass of interest found in *R. toruloides* will be outline in this section.

2.2.2.1 Fatty acids analysis

There are numerous methods to analyze fatty acids, such as gas chromatographic-mass spectrometry (GC-MS), GC linked to Fourier-transform infrared spectroscopy (FTIR) and reversed-phase high performance liquid chromatography (HPLC).
The most common method for analyzing fatty acid content is done via GC-MS. The fatty acids are non-volatile and cannot be analyzed directly by GC, and they have to be first hydrolyzed and derivatized. The esterification of fatty acids (after hydrolyzation of the oil) with methanol results in the formation of fatty acid methyl esters (FAMEs). FAMEs will then be sufficiently volatile to be analyzed via GC. The fatty acids after vaporized are then ionized, forming charged fragments that are then analyzed by MS based on its mass to charge ratio. The results are then compared against the library of known mass spectra database to identify the fatty acids present in the sample. Many approaches of FAMEs analysis have been performed for the past few years [55]. Other derivatization methods other then FAME were developed to identify and analyze different nature of the various fatty acids. Derivatization at the carboxyl group, such as 4,4-dimethyloxazoline (DMOX) derivatives and picolinyl (3-hydroxymethyl pyridinyl) esters formed with 2-amino-2-methyl-1-propanol (AMP), just to name a few [56].

2.2.2.2 Protein analysis

Yeast protein is a good source of some of the nutritionally essential amino acids, but the greatest challenges in this post-genomic era is to how to identify and quantify all expressed protein in the cells. The quantification of overall protein concentration is way easier then to qualify them, as there are many common spectrophotometric

methods of quantifying protein concentration (e.g. Coomassie dye, protein catalyzed reduction of (Cu2+) ion).

For protein qualification, the one-dimensional (1D) gels allow a low-resolution separation of proteins based on their respective molecular mass. More complex mixtures that cannot be sufficiently resolved in a 1D gel can use two-dimensional (2D) gels. In 2D gel, the proteins are separated based on their isoelectric (pI) and their molecular mass. The position of the protein on the 2D gel represents its approximate pI and molecular mass. Complex protein mixtures are sometimes first separated by gel electrophoresis, and the desired protein spot is excised from the gel and digested with a protease enzyme (e.g. trypsin).

Another more sensitive qualitative analysis of protein profiles is using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS-MS). It is an extremely powerful technology for complex protein characterization and identification. In LC-MS-MS, the complex proteins are first reduced to simpler peptides prior to loading into the separating column. The columns are usually made of fused silica reverse-phase C18 material. The peptides mixture is eluted from the column and directly into ionization source of mass spectrometer, running on a low gradient flow rate. A stable electrospray is produced at the heated capillary and the selected peptides (charge state or m/z) are fragmented using CID. The MS/MS spectrum that is generated allows distinguishing between individual proteins in complex mixtures even without prior purification [57].

2.2.2.3 Carotenoids analysis

The quantification of total carotenoid is most commonly done through measuring the absorption at the maximum visible absorption (λ_{max}) of carotenes. The identification and quantification of carotenoids is possible via its visible absorption spectrum due to the double bond conjugation present in its structure. Most of the carotenoids absorbs at three wavelengths to give a three-peak spectra (Figure 7). Their λ_{max} values depend on total number of double bonds conjugations present in their structure; the higher the number, the higher its λ_{max} value.

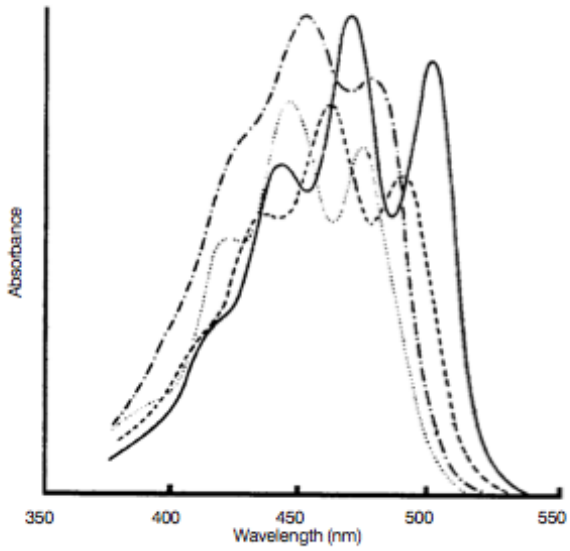

Figure 7 Visible absorption spectra of lycopene (__), γ-carotene (---), β-carotene (-.-.-.) and α-carotene (....) [58].

The various quantification methods have been done by several authors [59; 60] using UV-Vis detector and LC-MS/MS followed by comparing the MS mass spectra data with the mass spectral libraries database, or using Reverse Phase High Pressure Liquid Chromatogram (RP-HPLC) coupled with diode array (DAD) detector [61]. Other alternative analysis methods include UV/Vis Spectrophotometric analyses [61], Capillary Electrochromatography [62] and supercritical fluid chromatography [60]. Non-destructive techniques that are based using infrared (IR) [64], Fourier transform infrared spectroscopy (FTIR) [62], Raman spectroscopy [63] and Attenuated total reflectance (ATR-IR) [65] were promising methods explored to be able to provide excellent data quality with high reproducibility.

2.3 Pulsed electric field treatment (PEF)

Pulsed electric field (PEF) is a non-thermal technology that applies short pulses of electric fields to permeabilize cell membrane [13]. It is environmental friendly, energy saving, and a very gentle method commonly used for food pasteurization, microbial inactivation and extraction processes. The research on PEF treatment started as early as in the 1960s [66]. In this section, the principles and mechanisms of electroporation (2.3.1), PEF equipment system (2.3.2), PEF processing parameters (2.3.3) and the different applications of PEF treatment (2.3.4) will be discussed.

2.3.1 Principles and mechanisms of Electroporation

The principle of PEF is by applying short pulses of high electric fields (kV/cm), ranging from μs to ms. The pulsed electrical current is applied to the samples that is located between a pair of electrodes (the treatment chamber), and the high voltage result in an electric field that will be conducted to the samples. The electric field can be in exponentially decaying, square wave, bipolar, or oscillatory pulses shape. This phenomenon is also known as electroporation which causes permeabilizing of cell membrane. Extend of membrane permeabilization would depend on the strength of electric field that have been applied. According to Zimmermann [67], this observation was proposed as the "dielectric breakdown theory".

Cell membrane consists of a phospholipid bilayers; the hydrophobic fatty acid chains face inside the double layer, and the hydrophilic head faces outwards to the cytoplasm. The electrochemical gradient is maintained within and outside the cell, with a transmembrane potential of ~0.1V (resting potential). The lipid bilayer behaves like a capacitor because of the low conductance of the cell membrane [67]. The presence of an applied electric field leads to an increased in attraction of charges inside and outside of the cell menbrane, which results in an increased in the potential difference across the cell wall. Free charges starts to accumulate between both sides of the cell membrane, causing an induced additional potential ($\Delta\varphi$) larger than the cell membrane's resting potential ($\Delta\varphi_r$). A compression pressure arises, causing a decrease of cell membrane thickness.

Induced membrane breakdown and the degree of permeabilization of membrane are determined by the strength of the electric field, the duration, type and the number of pulses applied. If the critical electric field strength (E_{crit}) is not exceeded, cell membrane would not be permeabilized. Reversibly permeabilization (formation of hydrophobic pores) of the membrane is possible if E_{crit} has been slightly exceeded. If the E_{crit} has been greatly exceeded, the cell membrane will be irreversibly permeabilized (Figure 8).

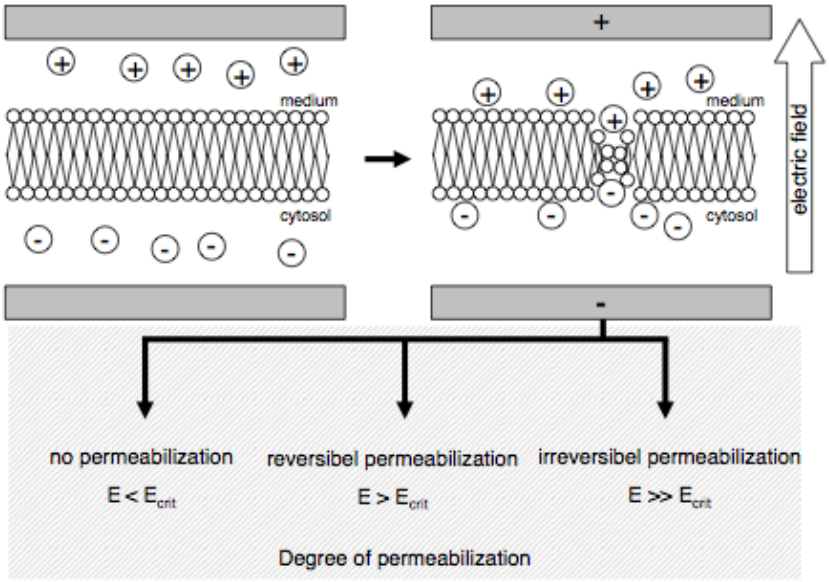

Figure 8 Schematic diagram of the mechanism of electroporation and degree of permeabilization of cell membrane due to the E_{crit} and the applied electric field.

2.3.2 PEF equipment system

The general PEF equipment comprises of three main components: a high volt pulse generator, a treatment chamber and a control system (Figure 9). Exponential decay pulses (Figure 10) and square wave pulses (Figure 11) can be generated, based on the design of the equipment. The capacitor is charged by the high voltage generator to the desired voltage, and the electric field is generated in the treatment chamber. For exponential decay pulses, the intensity of the pulse drops sharply after the maximum voltage is reached. It is different for square wave pulses as the intensity of the pulse is maintained at high density throughout the treatment. Hence square wave pulses have higher efficiency and is more energy saving [13].

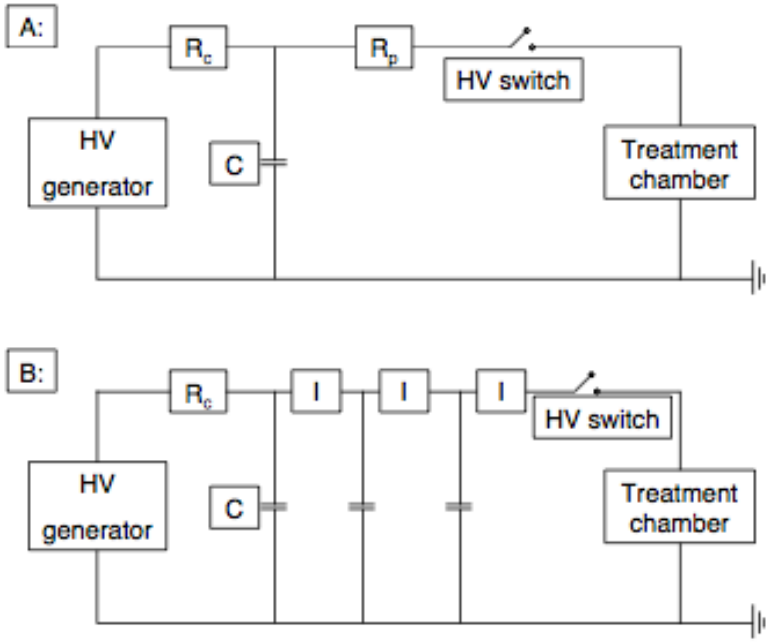

Figure 9 A general PEF system generating A: Exponential decay pulses, B: Square wave pulses. HV: High voltage, R_c: Charging resistor, R_p: Protective resistor, C: Capacitor, I: Inductor [13].

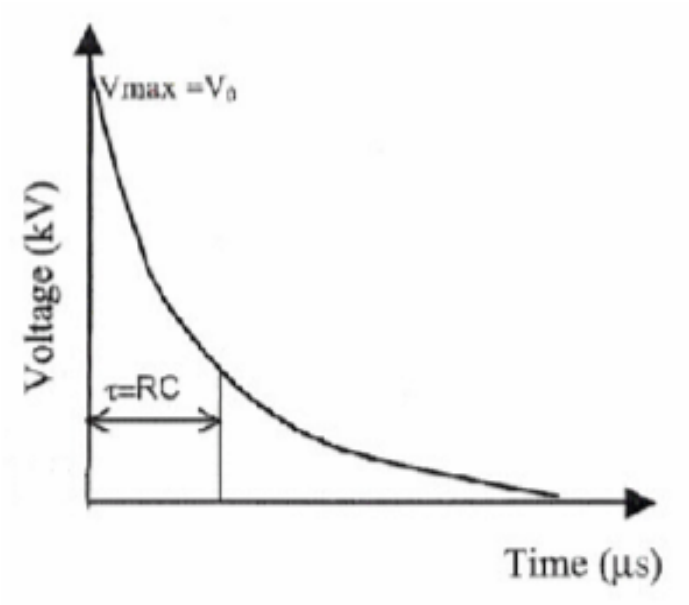

Figure 10 Exponential decaying pulse [68]

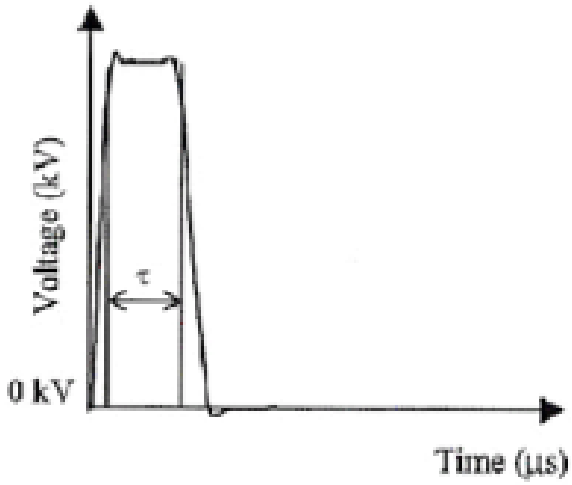

Figure 11 Square pulse [68]

2.3.3 PEF processing parameters

There are other parameters that can influence the PEF treatment other then the pulse shape, number of pulse, treatment time and temperature. Parameters such as the treatment media composition (2.3.3.1) and electric field strength (2.3.3.2) play a significant role too, and it will be discussed briefly below.

2.3.3.1 Treatment media

Treatment media are important to guarantee the efficiency of PEF treatment. Factors for example its conductivity, ionic strength, resistance, pH and dielectric properties can affect the result. The media conductivity cannot be too low, as it will hinder the generation of the pulses. Too high a conductivity media is not good either, as no electric field could be generated, which will also result in arcing. Small particles in the media such as air bubbles, lipids or cell agglomerates are undesired, because it leads to an inhomogeneous electric field and will drastically reduce the PEF treatment efficiency [69].

2.3.3.2 Electric field strength (E)

The electric field strength parameters is important when we discuss about PEF, because the critical electric field strength (E_{crit}) value decides how much voltage we need to apply for different purposes. The E_{crit} value will depend on our cell type, size

and its geometry. The electrical field strengths used for the inactivation of yeast and bacteria can be from < 1 kV/cm for root tuber tissue, to more then 5 kV/cm for *S. Cerevisiae*, and up to > 20 kV/cm for *E.coli* [70]. The average electric field strength formula is as follows:

$$E = V / d$$

where V is the voltage applied, and d is distance between the electrodes gap in the treatment chamber.

2.3.4 Applications of PEF treatment

Application of PEF technology in the field of food technology and biotechnology has been demonstrated successfully as a cost effective technique that improves cell disintegration, mass transfer and microbial inactivation processes. The various applications will be further discussed in this section.

2.3.4.1 Molecular biology (Gene transfer)

Gene transfer requires efficient delivery of desired gene into the host cell, and electroporation delivery using pulsed electric field treatment is an effective delivery approach. The application of PEF helps to introduce various molecules, such as DNA and drug molecules with the presence of electric field [71]. This generates competent cells which is used for transformation process. The use of electropermeabilization in transformation was first studied by Neumann et al., [72].

2.3.4.2 Microbial inactivation (Food technology)

PEF treatment beyond the E_{crit} strength of the microbial can be used to inactivate microbial. When the E_{crit} of the microbial is strongly exceeded, it leads to an irreversible permeabilization and the breakdown of the plasma membrane [67]. The potential applications using PEF treatment in food technology for food preservation and decontamination have been thoroughly reviewed extensively [70, 73, 74]. PEF is also an advantage over the conventional food pasteurization process, but without involving any thermal factor at temperatures below 30-40°C. *E. coli* that inoculated in skim milk was treated with PEF at an electric field of 45 kV/cm at 35°C, and the

microbial inactivation was reduced by 2D [75]. The food treatment and pasteurization was successfully achieved for a variety of food, such as fruit juice, liquid eggs, and pea soup at PEF treatment of 25–45 kV/cm in Washington State University. The main interests besides achieving microbiological safety in the food products using PEF are to also maintain the food flavor, freshness and shelf lives [76].

2.3.4.3 Induction of stress response

Another possible application of PEF treatment that has been explored is to induce stress response in cells. Since the reversible permeabilization of cells when exposed to electric field lower then its E_{crit} without impairing the vitality and metabolism of the cells has been proven [11], the potiential application of using PEF as a stress inducer in cells has been explored by Ja¨ger , Balasa & Knorr [77]. The metabolite profiling of potato tissues after being treated with PEF at low electric field intensity (0.4 kV/cm) had resulted in stress induced changes [78].

2.3.4.4 Enhancement of mass transfer processes

A major application of PEF treatment is focused on enhancing the mass transfer processes. This is great interest for the food industries as it can significantly enhance the extraction of substances during food processing step. Use of PEF to increase juice yield was demonstrated, and it was shown that pressing yield of apple mash increased by 12%, and up to 25% increment was observed for extraction of carrot juice [79, 80]. Up to 90% of the red pigments in beetroot was released after treated with PEF at 1 kV/cm [81]. Oil yield extraction is also improved via PEF treatment for olives and maize germs. Other then extraction, PEF can also assist in drying and speeding up of water removal from cells. It was demonstrated in red peppers and carrots a faster dehydration process [82, 83].

2.3.4.5 Induction of structural changes

There have been reports of plant cells tissues structural changes after PEF treatment. The alterations of the tissue structures in sugar beets [84], potato tissues and carrots [85] have been observed, and the softening of the tissues results in a reduced cutting résistance after PEF treatment.

3. Material and Methods

3.1 Mircoorganisms

The natural red yeast strains *R. toruloides* CBS 5490 was obtained from Central Bureau voor Schimmelcultures (CBS-KNAW, The Netherlands).

3.2 Media

3.2.1 YED Media

The YED media (per litre) was prepared with the composition as shown. All prepared media were sterilized at 121ºC for 15 minutes before use.

Table 2 Composition of YED media

YED Medium	**(L^{-1})**
Dextrose	20g
Peptone	20g
Yeast extract	10g

3.2.2 YEG Media

The YEG media (per litre) was prepared with the composition as shown.

Table 3 Composition of YEG media

YEG Medium	**(L^{-1})**
Crude glycerol	20, 60, 120g
Peptone	20g
Yeast extract	10g

3.2.3 Crude Glycerol

The impurities in the crude glycerol vary differently depending on the industrial source of origin. Hence in this research, a general composition of synthetic crude glycerol based on the average concentration of impurities similar to bio-diesel industries derived crude glycerol was prepared for this research [86]. The composition

is as follows: 65% glycerol, 4% potassium and sodium salts (w/w), 4% methanol (w/w), and 27% water (w/w).

3.3 Cultivation Methods

3.3.1 Maintenance and Storage

Yeast cells are stored in -80ºC freezer. The cells were maintained on YED and YEG agar plates and stored in 4ºC. The yeast cells are transferred to fresh agar plates every 2 months.

The YED agar plates were prepared with the composition as shown. All prepared agar were sterilized at 121ºC for 15 minutes before use.

Table 4 Composition of YED agar

YED agar	**(L^{-1})**
Dextrose	20g
Peptone	20g
Yeast extract	10g
Agar	20g

The YEG agar plates were prepared with the composition as shown.

Table 5 Composition of YEG agar

YEG agar	**(L^{-1})**
Glycerol	20g
Peptone	20g
Yeast extract	10g
Agar	20g

3.3.2 Fermentations

Batch cultures fermentations were carried out in 250ml conical flasks after inoculating with 48hr old inoculums to a starting optical density of 0.2. The flasks were incubated at 30ºC with agitation rate of 250rpm on a rotary shaker in minimal light for 8 days.

3.4 Cell disintegration and extraction methods

3.4.1 Mechanical cell disintegration

The destruction of the yeast cell membranes and walls by collision forces is done by vortexing the yeast samples in the presence of glass beads. The yeast samples and containers are pre-chilled prior to vortexing. Glass beads, acid-washed, 425-600 μm diameter (Sigma G8772) are added in a 1:3 ratio to the sample volume, and vortex for 3 minutes using FastPrep®-24 Instrument (MP Biomedicals, 116004500). The vortexing is conducted at 20 seconds intervals, and paused after each interval for another 20 seconds to re-chill the samples on ice to combat the increased in temperature due to the beads mechanical disruption process. This mechanical method provides complete disruption of the cell wall due to the shear forces and collisions with the glass beads, and all intracellular content are released into the surrounding medium. Temperature control is crucial for mechanical cell disintegration method to prevent any denaturation of the intracellular contents.

3.4.2 PEF treatment

3.4.2.1 PEF generation

The PEF treatment was performed using exponential decay wave pulses, generated using Gene Pulser Xcell^{TM} Electroporation System (Bio-Rad, 165-2660). The powerful exponential decay waves generate an electrical pulse into the sample and the voltage is increased rapidly to the maximum, before declining over time. The following parameters were used.

Table 6 PEF treatment parameters employed

Voltage (V)	250 - 3000
Number of pulses	10
Pulse length (msec)	4
Pulse interval (sec)	0.1
Cuvette (mm)	2
Capacitance (uF)	25
Resistance (ohms)	200

3.4.2.2 PEF sample preparation

Before PEF treatment, the yeast samples are first transferred into a 1.5mL reaction tube, centrifuged and cell pellet is washed 3x with ddH_2O. The washed yeast suspension is then transferred into the electroporation cuvettes, and treatment at desired voltage condition. The PEF treatment yeast suspension is collected and stored at 4°C for further protein and lipid extraction analysis.

3.4.3 Extraction

To quantify extraction yield of the proteins and lipids after treated with PEF, protein and lipid extraction techniques are established and described as follows.

3.4.3.1 Proteins extraction

For protein extraction, the lysis buffer was prepared as shown below.

Table 7 Composition of lysis buffer

Components	**Final concentration in lysis buffer**
Urea	8M
Triton™ X-100 (Sigma, 9002-93-1)	0.1% (w/v)
Tris-Cl, pH 7.5	50mM
NaCl	100mM
DTT	50mM
EGTA	1mM
EDTA	1mM
PMSF	1mM

The yeast cultures are grown to typically 5 – 6 days old, and an appropriate volume of yeast culture (OD_{600} = 25) is isolated based on its OD value measured using a spectrophotometer (see chapter 3.5.1.1). The yeast culture is transfer into a 1.5mL microcentrifuge tube and centrifuged at 16,800rpm for 5 minutes, and the supernatant is discarded. The cell pellet is washed 3x with ddH_2O, and resuspended with 600uL of the lysis buffer, along with addition of glass beads a 1:3 ratio to the sample volume. The microcentrifuge tube is sealed tightly with sealing film (Parafilm® M, 01852-AB), and it is subjected to the mechanical cell disintegration treatment (see chapter 3.4.1). The microcentrifuge tube and its content is centrifuged at 4°C, 16,800rpm for 15 minutes, and the supernatant is collected and stored in 4°C for further protein quantification.

3.4.3.2. Fatty acid extraction

The fatty acid extraction was performed according to Bligh & Dyer (1959) method. The yeast cultures are grown to typically 5 – 6 days old, and an appropriate volume of yeast culture (OD_{600} = 20) is isolated based on its OD value measured using a spectrophotometer (see chapter 3.5.1.1). The yeast culture is transfer into a 2mL microcentrifuge tube and centrifuged at 16,800rpm for 5 minutes, and the supernatant is discarded. The cell pellet is washed 3x with ddH_2O, and resuspended with 400uL of methanol, along with addition of glass beads a 1:3 ratio to the sample volume. To prepare for analytical analysis using GC/MS (see chapter 3.5.2.1), spike each sample with 50ug of Heptadecanoic acid, C17 (Sigma, 506-12-7) as internal standard. Heptadecanoic acid should be dissolved in ethanol with a stock concentration of 5mg/mL. The microcentrifuge tube is sealed tightly with sealing film (Parafilm® M, 01852-AB), and it is subjected to the mechanical cell disintegration treatment (see chapter 3.4.1). After which 200uL of chloroform is added into the suspension and the microcentrifuge tube is sealed tightly with sealing film and further extracted on a shaker for 1 hour. Following that, another 200uL of chloroform and ddH_2O is added into the suspension, continued by further extracted on a shaker for 10 minutes. The microcentrifuge tube and its content are centrifuged at 16,800rpm for 10 minutes, and the 2-phase separation of methanol/H_2O layer should be obvious. Transfer the lower methanol phase into a 2mL screw cap microcentrifuge tube (VWR® SuperClear™, 16466-060) and evaporate dry the methanol completely at 30°C for further GC-MS pretreatment before fatty acid quantification (see chapter 3.5.2.2).

3.5 Analytical methods

3.5.1 Yeast growth measurement

The growth monitoring and measurement of the *R. toruloides* yeast cultures were conducted using photometric methods. The various methods and devices used will be discussed below.

3.5.1.1 Cuvette Spectrophotometer

The *R. toruloides* yeast growth is monitored by the measurement of cell suspension optical density (OD) at 600nm wavelength spectrophotometrically using NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). One millimeter of the yeast culture sample is taken from culture flask every 24hr interval, and the measurement of the OD were performed in cuvettes after appropriate dilutions (typically 100x). The growth medium without yeast cultures was used as the blank and as the diluting solution. The final OD is calculated by multiplying the measured OD values with the dilution factors.

3.5.1.2 Dry cell mass

Dry cell weight were measured by washing one millimeter of yeast cultures with dd$H_2$0 three times and decanted. The wet cell pellet is collected after centrifuged at top speed for 5 minutes, and left to dry at 70ºC until constant mass. The mass of the dry cell pellet is recorded for every 24hr interval.

3.5.2 Fatty acid analysis

Gas chromatography/Mass Spectrometer (GC/MS) with mass selective detector was used to determine the fatty acid distribution in *R. toruloides*.

3.5.2.1 GC/MS instrument parameters

Table 8 GC/MS instrument parameter and setting

GC/MS Instrument	The Agilent 5975C Series GC/MSD
Mode	Electron Ionization (EI)
Ion source temperature	230°C
Quadrupole temperature	150°C
Oven temperature	Ambient +4 – 450 °C
Column	HP-INNOWAX, 50 m × 0.2 mm id × 0.4 μm film
Scan range	33 to 320 AMU
Software	Agilent MSD Productivity ChemStation
Data analysis	MassHunter software
Detector	Triple-Axis Detector with long life EM

The GC/MS fatty acid identification is performed using Agilent's FAME methods. The peaks are identified based on the retention time alignment to the Agilent's FAME application note (5988-5871EN) and spectral interpretation using the database. Quantification is done by comparing the integral of the TIC of our internal standard, 50μg C17 (see chapter 3.4.3.2) against the TIC integral of the individual fatty acid methyl ester. The following formula is used to calculate the total mass of the respective fatty acids in the sample:

X fatty acid in sample (μg) = (TIC integral of X fatty acid peak/TIC integral of internal standard) x (Molecular mass of X fatty acid/Molecular mass of internal standard) x 50

3.5.2.2 Sample preparation for GC/MS

The fatty acids extracted have to be derivatized before being analyzed by GC/MS. A continuation after the fatty acid extraction step (see chapter 3.4.3.2), 500uL of 14% Boron trifluoride-methanol solution (BF_3) (Sigma, B1127) is added into each 2mL screw cap microcentrifuge tube containing the extracted fatty acids. The microcentrifuge tube are screwed tightly and heat at 95°C for 30 minutes before leaving to cool. Next, 300uL of saturated NaCl solvent and Hexane are added respectively. The suspension is then vortex for 30 minutes and centrifuged at

16,800rpm for 15 minutes. The upper phase (Hexane layer) that contains the derivertizd fatty acid is collected, and 30uL is transferred into a glass insert vial for GC/MS analysis.

3.5.3 Carotenoid analysis

3.5.3.1 High-performance liquid chromatography (RP-HPLC)

To accurately measure qualitatively and quantitatively the carotenoids present in the yeast samples, the highly sensitive analytical instrument reversed-phase high-performance liquid chromatography (RP-HPLC) with UV/vis diode-array detection is employed in this study. All solvents used in our HPLC analysis are of HPLC grade. The parameter used in the HPLC are as follows.

Table 9 HPLC instrument parameter and setting

Column	C18
Mobile phase	(A) ddH_2O; (B) Acetone (30:70, A:B), Isocratic elution
Flow rate	1.0 mL/min
Column temperature	30 °C
Injection	10 μL
Detector	UV-VIS diode-array detection, 450 nm

3.5.3.2 Sample preparation for HPLC

The yeast cultures are grown to typically 5 – 6 days old, and an appropriate volume of yeast culture (OD_{600} = 20) is isolated based on its OD value measured using a spectrophotometer (see chapter 3.5.1.1). The yeast culture is transfer into a 2mL microcentrifuge tube and centrifuged at 16,800rpm for 5 minutes, and the supernatant are discarded. Cell pellet is washed 3x with ddH_2O, and resuspended with 1mL of ice-cold acetone containing 0.01% (w/v) butylated hydroxytoluene (BHT). The yeast cells are subjected to the mechanical cell disintegration treatment (see chapter 3.4.1), and centrifuged at 16,800rpm for 5 minutes to bring down the cell debris. The acetone extract containing the carotenoids were collected and fresh ice-cold acetone with 0.01% (w/v) BHT are added again to the cell pellet and mixed until colourless. The total acetone extracts were collected and measured using HPLC.

3.5.3.3 Carotenoid internal standards

It is of absolute necessity to include internal standards measurements and plot standard curves for the quantitative measurement of the unknown carotenoids during HPLC. All carotenoid standards (torulene, torularhodin and β-carotene) were purchased from Sigma-Aldrich.

3.5.4 Florescence microscopy

3.5.4.1 Florescence microscopy setting

Samples were observed through Olympus IX71 Inverted Microscope, with 4x-60x air objectives and magnification ranging from 4X to 100X oil immersion objectives. Come equipped with Roper Cool Snap CCD camera, phase contrast, transmission or reflectance bright field, and epifluorescence options. The microscopy image is analyzed by ImagePro Insight software.

3.5.4.2 Lipid droplet staining (Nile Red)

Nile Red (9-diethylamino-5H-benzo [α] phenoxa-phenoxazine-5-one) is a lipid selective stain that is used specifically to illuminate lipophilic substances under florescence microscopy. Nile Red (Sigma, 7385-67-3) is weighed out and dissolved in acetone to achieve a final concentration of 0.1 mg/ml. To prepare our yeast sample for staining, isolate 200μL of the yeast cultures that is grown to 5 – 6 days old. The yeast culture is transfer into a 1.5mL microcentrifuge tube and centrifuged at 16,800rpm for 5 minutes, and the supernatant is discarded. The cell pellet is then washed 2x with 1mL of PBS buffer (10mM Potassium phosphate, 0.15M KCl, pH 7.0). Resuspend the cell pellet in 1mL of PBS buffer, and treat the yeast suspension with 6μL of the Nile red solution prepared earlier. Incubate the suspension in dark at room temperature for 5 minutes for the yeast cells to be fully stained, and it is ready to be observed through the fluorescence microscope (see chapter 3.5.4.1).

3.5.5 Protein analysis

3.5.5.1 2-D Quant Kit

The protein extracts (see chapter 3.4.3.1) are quantitatively analyzed by employing the 2-D Quant Kit (GE Healthcare, 80-6483-56). For each protein sample, the

following setup is prepared: 500µl of the precipitant (provided) is added to the 2mL microcentrifuge tube, followed by the addition of 20uL of the protein extract. The solutions are vortex for 10 seconds and incubate at room temperature for 2 minutes. 500µl of the co-precipitant (provided) is next added into the microcentrifuge tube and vortex for 10 seconds. Next, to sediment the precipitated protein, the microcentrifuge tube is centrifuged at 13000 × g for 5 minutes. The supernatant is decanted quickly and a small pellet of the protein is visible. Any remaining liquid is removed completely by using a P10 (0 – 10µl) micropipette. The protein pellet is resuspended by adding 100µl of copper solution (provided) and 400µl of ddH_2O, and vortex for 10 seconds. 1mL of color reagent A and B (provided) mixture (100:1) is added and instant mixing is achieved by inversion of the microcentrifuge tube. The mixture is incubated at room temperature for 15 minutes, and the absorbance (A) of the sample is measured at 480 nm using a NanoDrop 2000c Spectrophotometer. A standard curve is generated as the reference by substituting the protein sample with the Bovine serum albumin (BSA) standard (provided). The standard curve of A_{480} vs known BSA concentration is used to determine the protein concentration of the unknown samples.

3.5.5.2 SDS-PAGE (Sodium dodecyl sulfte polyacrylamide gel electrophoresis)

SDS-PAGE is a protein separation technique based on the respective length of the protein polypeptide chains or based on their molecular weight. The SDS is a detergent that is added to remove the higher orders secondary and tertiary protein structures, such that the protein stays as linear polypeptide chains, by coating the proteins and conferring the same negative electrical charges across all proteins. This results in a uniform mass:charge ratio for the proteins as the charges differences due to isoelectric points are avoided. Hence the migration speed of the proteins is only directly related to the size of the protein.

A polymer of acrylamide monomers, called polyacrylamide is used as the environment for protein migration. A polyacrylamide gel is made up of a laberynth of tunnels and a meshwork of fibers, which will then allow the different molecular weight proteins to travel down at different speed. Electricity is then employed to pull the proteins through the gel. The tris-glycine gel comprises of a stacking gel (focus proteins into sharp bands at beginning of electrophoretic run) and resolving gel

(varying acrylamide gel percentages) to separate the proteins based on their respective molecular mass. The SDS-PAGE gel is prepared as shown below, with the separating gel at the bottom, and the stacking gel on top.

Table 10 SDS-PAGE separating gel (10%) composition

	mL
H_2O	4.0
30% acryl-bisacrylamide mix	3.3
1.5MTris (pH8.8)	2.5
10% SDS	0.1
10% ammonium persulfate	0.1
TEMED	0.004

Table 11 SDS-PAGE stacking gel (5%) composition

	mL
H_2O	3.4
30% acryl-bisacrylamide mix	0.83
1.5MTris (pH8.8)	0.63
10% SDS	0.05
10% ammonium persulfate	0.05
TEMED	0.005

The separating gel is first prepared, followed by the stacking gel after the separating gel has set. A 1.5mm thick comb is used based on the desired thickness of the gel. The comb is removed after the stacking gel has set, and the wells are ready to be load with the sample protein. The protein samples before loading are first pretreated with 2x SDS loading buffer and boiled at 95°C for l0 minutes, to fully linearised the tertiary structures.

Novex® Sharp Pre-stained Protein Standard (Invitrogen, USA) were loaded into the wells as an indicator of the size of the proteins present in the samples. The loaded gels are placed in the electrophoresis buffer chamber and filled with running buffer (0.025M Tris, 0.192 M glycin, pH 8.3, 0.1 % SDS). A total of 20uL of pretreated

protein samples are loaded into each well, and the gel is run at 100V for 30 minutes. The gel is removed from the buffer chamber after the run is completed, and stained with Coomassie® blue (50 % (v/v) methanol, 40 % (v/v) distilled water, 10 % (v/v) glacial acetic acid, 0.05 % (w/v) Coomassie® brilliant blue G-250) by shaking for 1 hour. This is followed by destaining of the gel by incubating the gel in the destaining buffer (50 % (v/v) methanol, 40 % (v/v) distilled water, 10 % (v/v) glacial acetic acid) by shaking for 1 hour.

4. Results and discussion

This section describes the results and discussion of the research experiments and is further divided into the following sub-sections.

4.1 Growth monitoring and optimization of *R. toruloides* on glucose and crude glycerol media

In order to achieve a successful fermentation strategy, an optimal growth condition for *R. toruloides* has to be established. In this section, the effects of different concentrations of crude glycerol on *R. toruloides* will be investigated. The growth and carotenoid production of *R. toruloides* at various concentrations crude glycerol will also be explored and discussed.

4.1.1 Biomass of *R. toruloides* cultured in glucose and crude glycerol media

The composition of crude glycerol can vary greatly because of the numerous different methods and ingredients that can be used to produce biodiesel. Hence in this section, the effect of the various different concentrations of crude glycerol as compared to the traditional glucose carbon source on *R. toruloides* is explored. 3 crude glycerol concentration (20g/L, 60g/L and 120g/L) were investigated respectively, and compared against the growth of *R. toruloides* in glucose media. The concentration of glucose media used throughout this study is fixed at 20g/L; which is the established culture media concentration for optimized yeast biomass production. The biomass of the *R. toruloides* cultures were harvested at every 24-hour time point, their total dry weight mass and their optical density at 600 nm absorbance wavelength (OD_{600}) were measured respectively (Figure 12, 13).

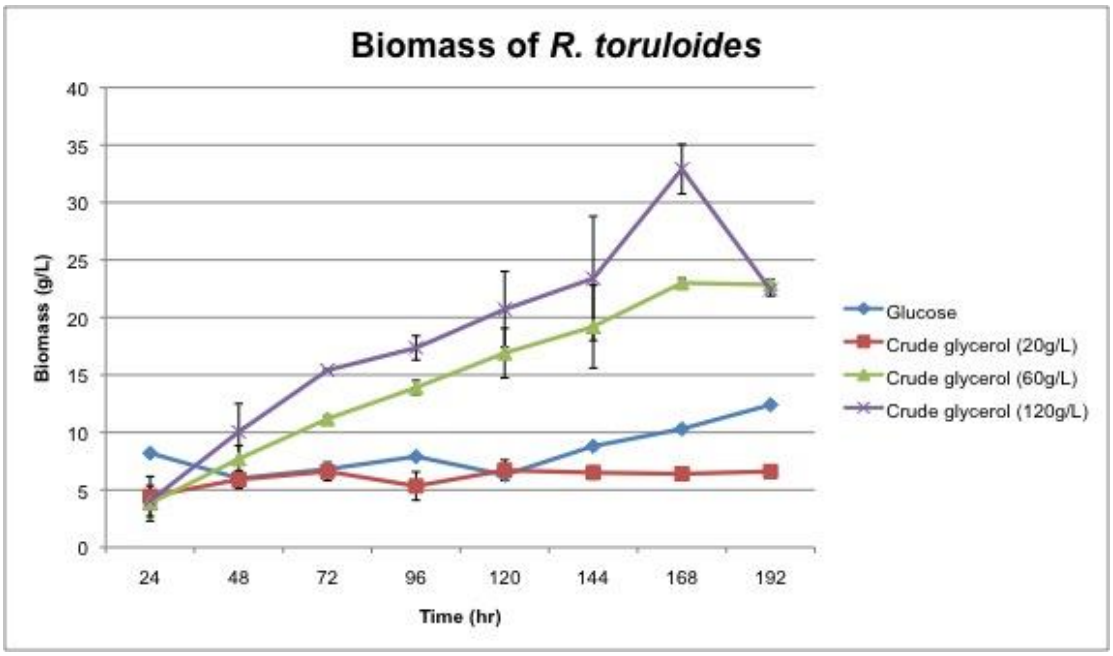

Figure 12 Biomass of *R. toruloides* after 192 hours of culture in glucose and varying concentrations of crude glycerol media.

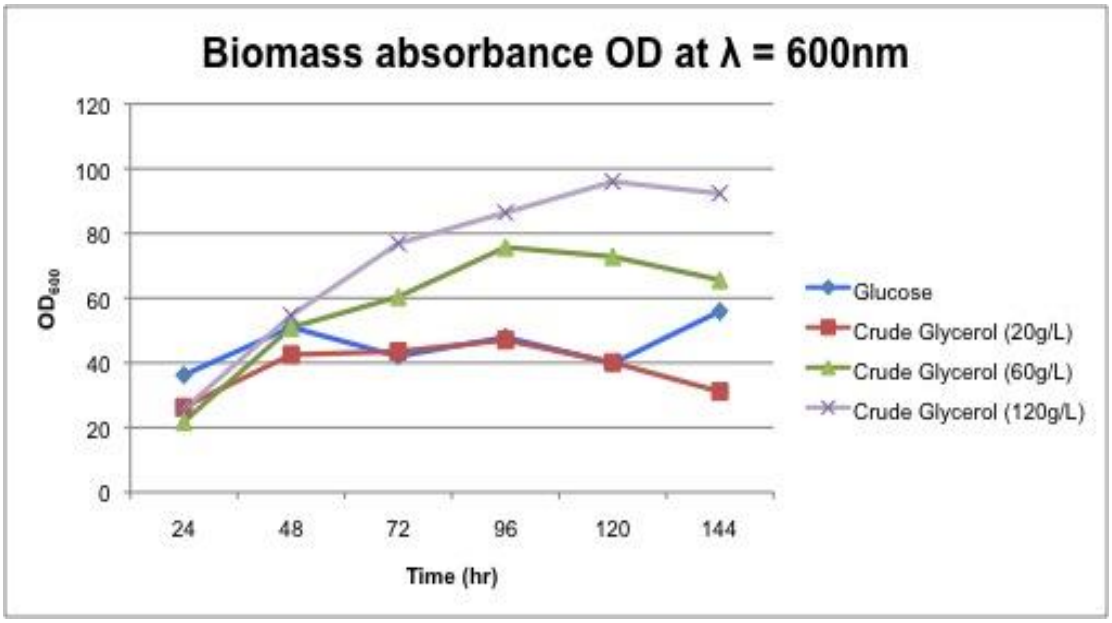

Figure 13 OD_{600} of *R. toruloides* after 144 hours of culture in glucose and varying concentrations of crude glycerol media.

The biomass accumulation for *R. toruloides* was observed to increase with increasing concentration of crude glycerol in the culture. The total biomass was the highest at 120g/L of crude glycerol, with a total biomass yield of more than 80g/L. The total biomass yield (at ±40g/L) was comparable for 20g/L of glucose and 20g/L of crude

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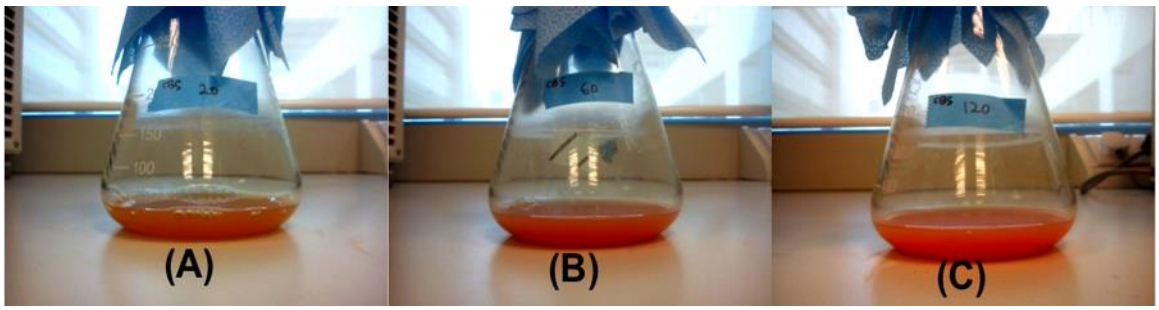
glycerol concentration culture media, and this is a good indication of the robustness of our *R. toruloides* strain to survive under impure culture media conditions. The lag phase of the cultures were reached approximately after 168 hr, for all 3 crude glycerol cultures growth pleautued in their biomass accumulation even with further growth time.

4.1.2 Carotenoid production by *R. toruloides* cultured in glucose and crude glycerol media

The *R. toruloides* are also investigated in their ability to grow and produce carotenoids on glucose and varying concentration of crude glycerol. The result below shows that the *R.toruloides* was able to tolerate the impurities in the crude glycerol, and continue to accumulate biomass and carotenoids. The effect of different concentrations of crude glycerol vs the traditional glucose carbon source on *R. toruloides* carotenoids production is explored. Similar to the biomass study, the 3 different crude glycerol concentration (20g/L, 60g/L and 120g/L) were investigated, and compared against the total carotenoids production of *R. toruloides* in glucose media. The concentration of glucose media used throughout this study is fixed at 20g/L for consistence with the biomass study. The flask cultures of *R.toruloides* in glucose and various concentration of crude glycerol at 144 hr are shown in Figure 14.



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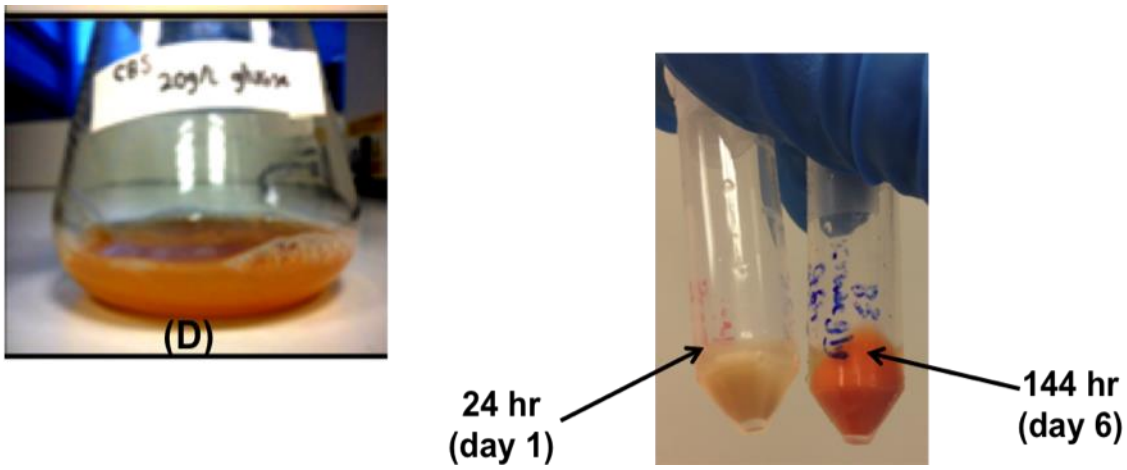

Figure 14 Flask cultures of *R.toruloides* grown in crude glycerol concentration (A) 20g/L, (B) 60g/L, (C) 120g/L. Flask cultures of *R.toruloides* grown in 20g/L of glucose is shown in (D). The spin-down cell pellet of *R.toruloides* (24 hr v.s. 144 hr) color contrast is seen in the bottom right.

The *R. toruloides* cultures were harvested at every 24-hour time point, the optical density of total carotenoid at 452 nm absorbance wavelength (OD_{452}) were measured (Figure 15). The total carotenoids mass (Figure 16) in solution obeys the Beer-Lambert law, and final total carotenoid yield was calculated using the proposed absorption coefficient $A^{1\%}1cm = 2500$ using the following equation:

$$\frac{\text{Abs X volume (mL) X 10,000}}{\text{AC X sample weight (g)}}$$

where A_{total} is absorbance at 452nm and AC (absorption coefficient $A^{1\%}1cm$) = 2500 for a mixture of carotenoids.

Clearly, the results have shown that carotenoid accumulation with lower glycerol concentrations was more favorable for accumulating higher carotenoids, at crude glycerol concentration of 60g/L. When a higher crude glycerol concentration at 120g/L was employed, it revealed a lower in carotenoid production (Figure 16). There seemed to be no relationship between higher biomass to higher carotenoid production correlation. The maximum carotenoid yield was 45mg/L on 60g/L crude glycerol at 144hr. Even though the highest crude glycerol concentration of 120g/L produced the highest biomass production at 23g/L at 144h, followed by crude glycerol concentration of 60g/L with biomass production of 19g/L, however a lower crude

glycerol media of 60g/L was the optimum for total carotenoid yield of 45mg/L at 144hr, vs crude glycerol media of 120g/L with a total carotenoid yield of 27mg/L at 144hr. By decreasing the crude glycerol media concentration would decrease the yeast biomass production, but increase a total carotenoid production. Hence the media concentration should be altered strategically depending on what's the desired product one wish to harvest.

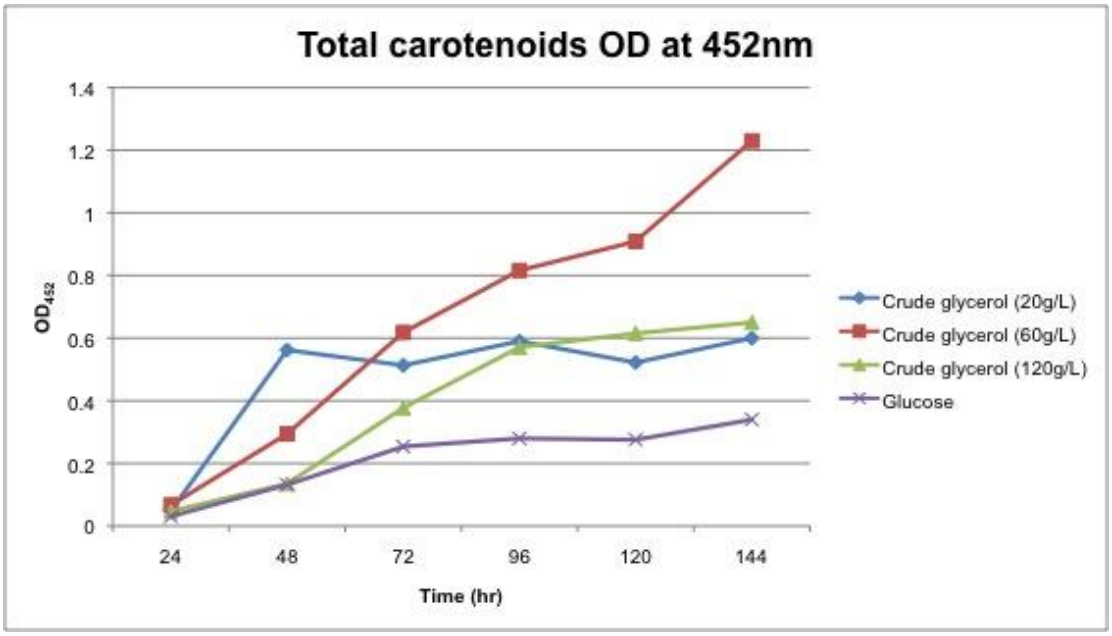

Figure 15 OD_{452} of *R. toruloides* after 144 hours of culture in glucose and varying concentrations of crude glycerol media.

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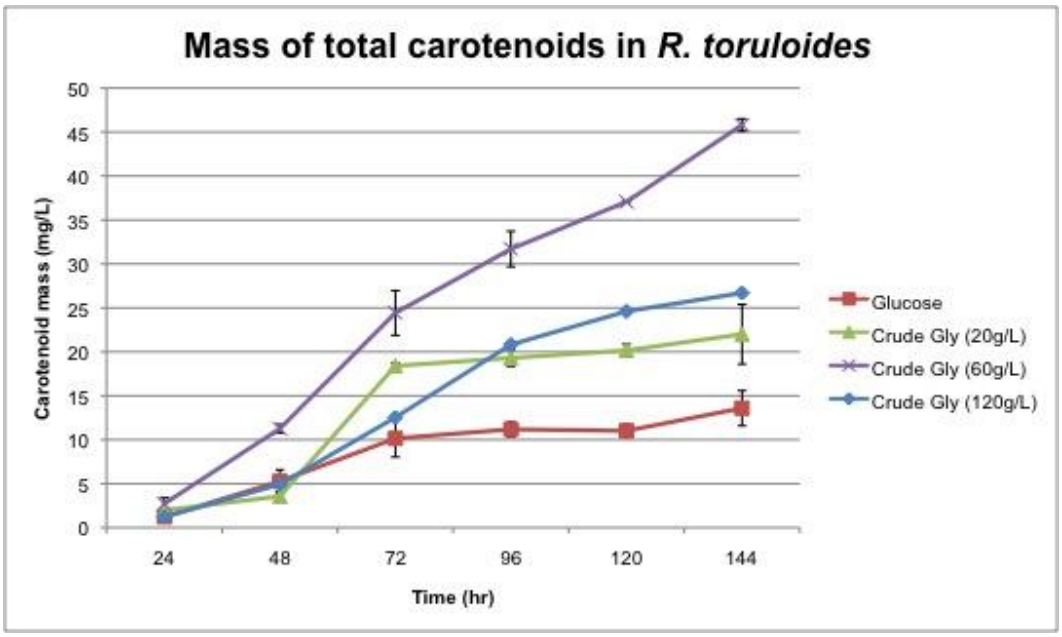

Figure 16 Total carotenoid of *R. toruloides* after 144 hours of culture in glucose and varying concentrations of crude glycerol media.

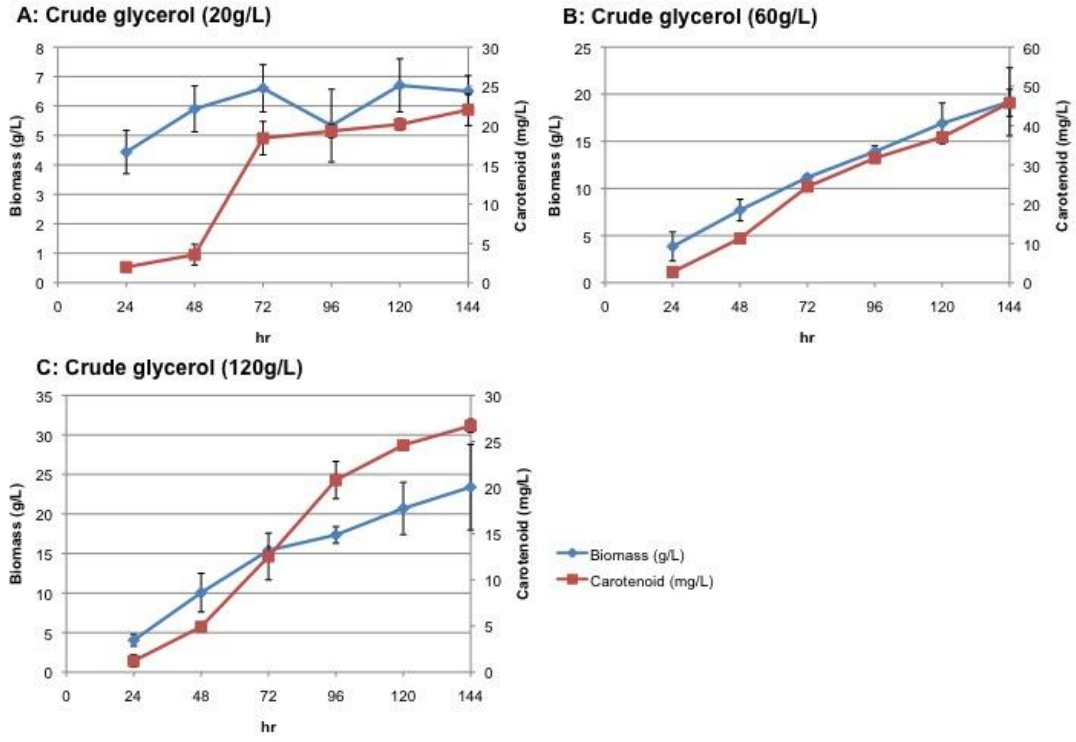

Figure 17 Plot showing the biomass growth (diamonds) and carotenoids production (squares) profile of *R. toruloides* at various crude glycerol concentrations.

The summary of the total biomass and carotenoids yield after 6 days batch fermentation for *R. toruloides* is displayed in (Figure 17). The outcome of this finding

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proves the robustness of the *R. toruloides* strains to successfully withstand varying concentrations of crude glycerol as their culture media, and that carotenoids can be successfully produced by *R. toruloides* in crude glycerol media as their sole carbon source.

4.2 Characterization and analysis of carotenoids profile in *R. toruloides*

The *R. toruloides* cultures were harvested and subjected to mechanical beads extraction method. The extracted carotenoids were analyzed using high-performance liquid chromatography with photodiode array detection (HPLC-DAD). The identification of carotenoids was based on the retention time value and the absorption peak spectra (λ_{max}), as the absorption spectrum will be able to provide clues for the identification of the carotenoids. The concentration of the carotenoids present was quantified by comparing against standard curves of carotenoids that were purchased commercially. The commercially brought carotenoid standards of various concentrations were run on HPLC-DAD, and standard curves (Figure 18, 19, 20) were plotted to quantify the carotenoids present in *R. toruloides* cultures grown in glucose and crude glycerol media

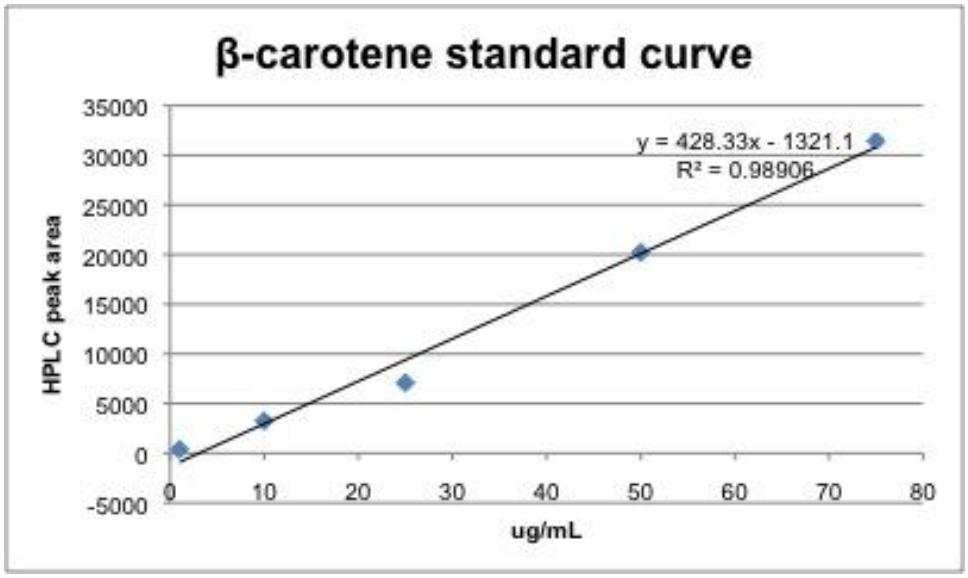

Figure 18 Standard curve of commercial β-carotene standards.

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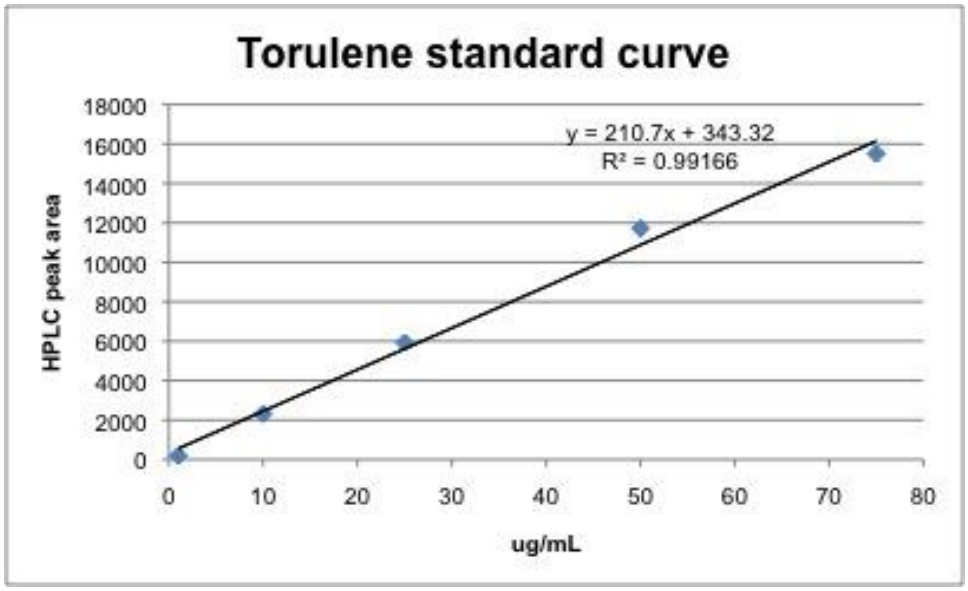

Figure 19 Standard curve of commercial Torulene standards.

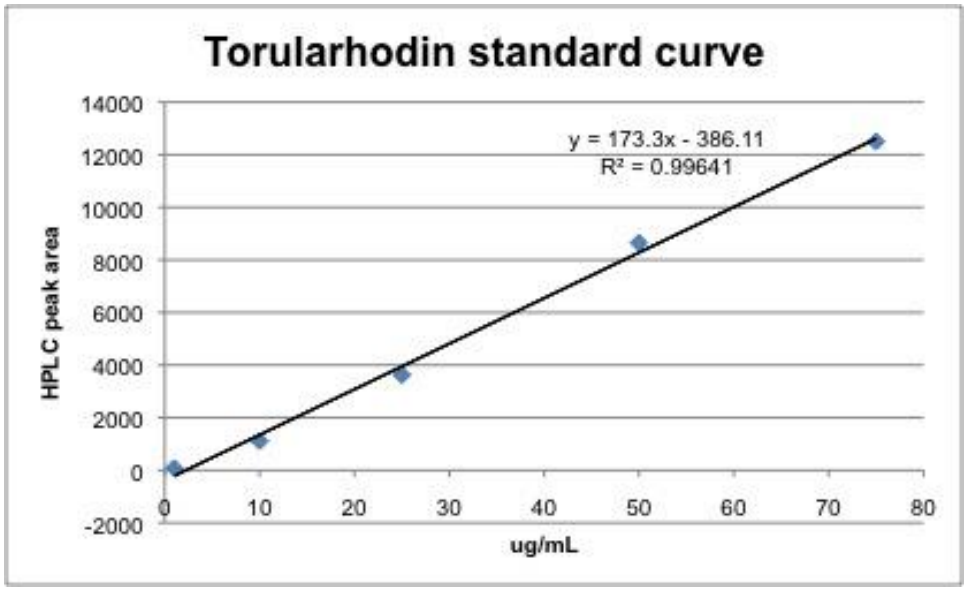

Figure 20 Standard curve of commercial Torularhodin standards.

The carotenoids present in *R. toruloides* identified by the HPLC-DAD chromatogram results (Figure 21) revealed the presence of β-carotene, Torulene and Torularhodin (Table 12), which is inline with the literature reviews. It can been seen that *R. toruloides* cultured in glucose as sole carbon source has the same carotenoid profile as compared in crude glycerol culture media, with β-carotene and Torularhodin as the major carotenoid composition in *R. toruloides,* and Torulene present in minute percentage (Figure 22).

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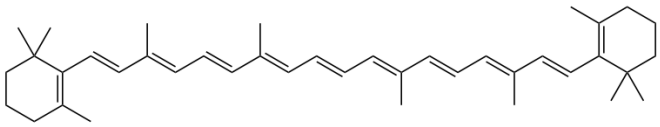
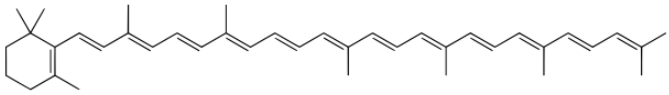
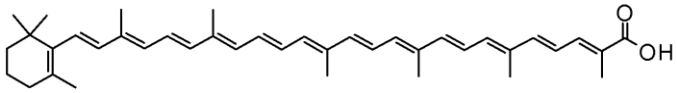
Table 12 Summary of the major carotenoid produced by *R. toruloides*.

Carotenoid	Molecular formula	Carotenoid structure
β-carotene	$C_{40}H_{56}$	
Torulene	$C_{40}H_{54}$	
Torularhodin	$C_{40}H_{52}O_2$	

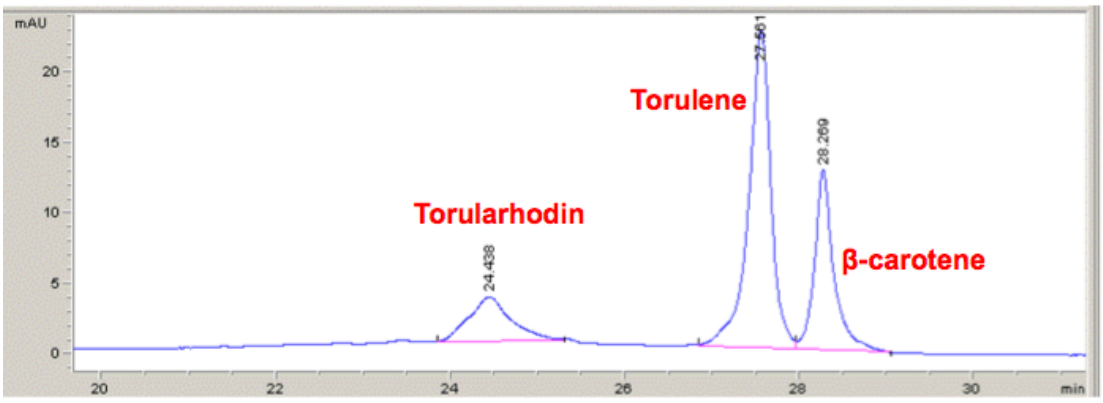


Figure 21 HPLC-DAD chromatogram analysis of *R. toruloides* cultured in glucose culture media.

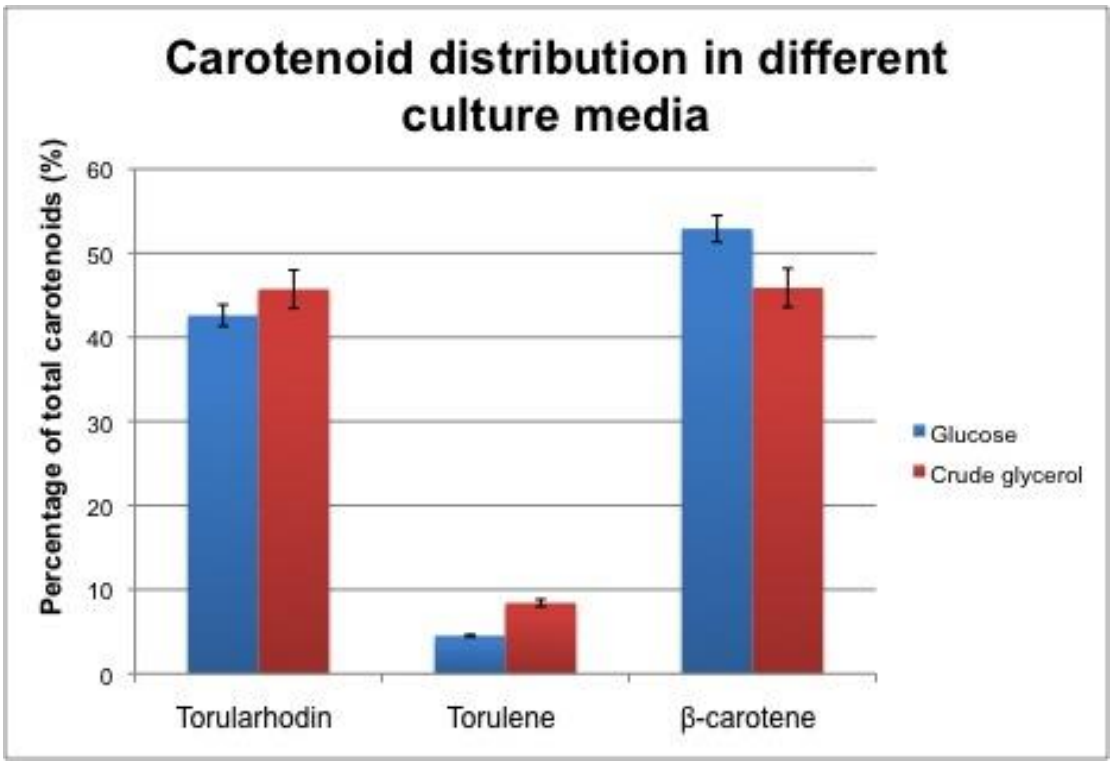


Figure 22 The carotenoids profile of *R. toruloides* in both glucose and crude glycerol culture media.

4.3 PEF treatment on extraction processes of *R. toruloides*

The PEF treatment influence on extraction of hydrophilic and hydrophobic intracellular biomass of *R. toruloides* was explored in this section. Various electric field strengths was tested to demonstrate the effect of membrane permeabilization due to PEF treatment and how it will affect the mass transfer processes of proteins and fatty acids. The degree of mass transfer processes of biomass in *R. toruloides* from a higher concentration inside the cell toward a lower concentration will be discussed, and the correlation to finding the E_{crit} value of *R. toruloides* will be determined.

4.3.1 Extraction of carotenoids

To tap on the naturally produced carotenoids in oleaginous yeasts, which can be potentially produced at a lower cost using crude glycerol and at reduced production time, we need to have a good knowledge how to isolate these carotenoids from the yeast cells by an efficient method without affecting its quality. This section will be discussing the study done in attempting to extract carotenoids from *R. toruloides* using PEF treatment.

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The *R. toruloides* cultures were harvested and subjected to PEF extraction treatment. The extractants were analyzed using HPLC-DAD and the identification of carotenoids was based on the retention time value and the absorption peak spectra (λ_{max}). However, as seen in the HPLC results, no carotenoids were detected from the PEF extracted *R. toruloides* samples. PEF treatment does not allow carotenoid extraction from *R. toruloides,* and this could be due to the inefficient mass transfer process by the huge C40 bulky structure of the carotenoids (MW>500).

Carotenoids are membrane-bound lipid-soluble pigments, and are accumulated in a particular cell organelles. It is not clear so far, whether carotenoids are present in plasma membrane only or in other inner membrane systems as well as in cell wall. As the cells are not broken during PEF treatment, no free carotenoids are available in the red yeast cells. Carotenoids are present in the plasma membrane where they protect membrane components from photo-oxidation, associated with membrane lipid. As PEF-assisted extraction depends on the accessibility of intracellular compound, the carotenoids which are strongly trapped in membrane-bound chromoplast and not freely situated in the cytosol are thus challenging to be extracted via PEF treatment.

Also, another plausible explanation of the inefficient transfer of carotenoids out of the cell walls could be because of the pore size on the cell wall. The irreversible membrane pores created by the PEF treatment might not be large enough for these bulky carotenoids to come out successfully.

Hence, we need to look at an alternative method to isolate these valuable carotenoids from *R. toruloides.* As discuss previously, since PEF treatment can allow a more efficient decanting of the *R. toruloides,* we would like to look into the possibility of isolating the carotenoids within an intact dehydrated *R. toruloides* cell. Its natural cell wall is stable, and hence could serve as a ”natural encapsulation” for the dehydrated cells. These dehydrated *R. toruloides* cell containing the valuable carotenoids can be harvested and supplied to the food industries, where they can be used as coloring agents and food supplements, or to the pharmaceutical industries as additive to cosmetics products. The stability of the carotenoids after PEF and heat drying treatment will be discuss further in the following chapter (4.4.2.1).

4.3.2 Extraction of Proteins

The extraction of amino acids and proteins using PEF was attempted by various research groups. Amino acids was extracted from *Corynebacterium glutamicum* [87] and yeast cells *S.cerevisiae* [88]. However, no attempt has been made to explore the extraction feasibility of proteins using PEF treatment on our yeast of focus in this research, *R. toruloides*. Hence in this section of the work, the yeast *R. toruloides* was exposed and treated with different PEF condition. The 5 days old yeast culture of *R. toruloides* in glucose and crude glycerol media were harvested and exposed to different electric field strength (1.25, 3, 5, 10, 12.5 kV/cm) PEF treatment conditions (Figure 22).

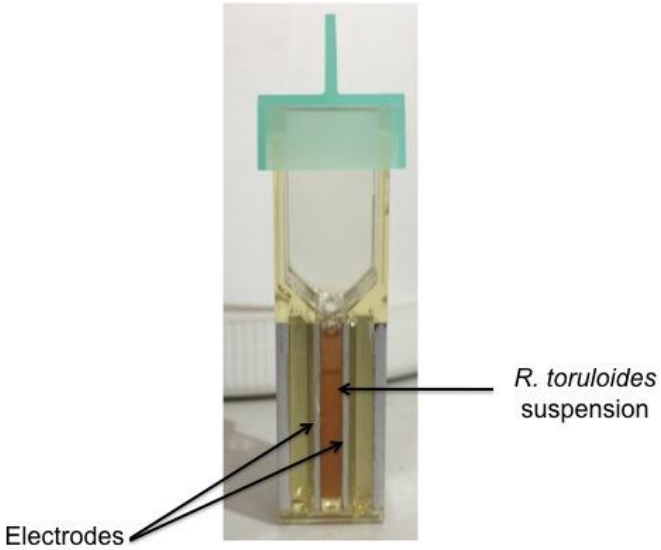


Figure 23 Yeast extracts in electroporation treatment cuvette before PEF treatment.

The control set of experiments was using mechanical disintegration method, rupturing the yeast cell wall via glass beads agitation. Both the PEF treated and control samples were spin down at top speed and the supernatant was collected. The protein concentration in the extractant was determined using 2D Quant kit and spectrophotometric measurement. Due to the lower protein concentration present in the extractant of PEF treated samples, the supernatant collected was concentrated 10 times before protein spectrophotometic measurement. The protein content measured in the supernatant of untreated cells was subtracted from all our yeast samples. The percentage of the total amount of extracted proteins for the various PEF treated

samples and treatment conditions were determined by taking the mechanical disintegrated method samples as the total protein concentration (Figure 23, 24).

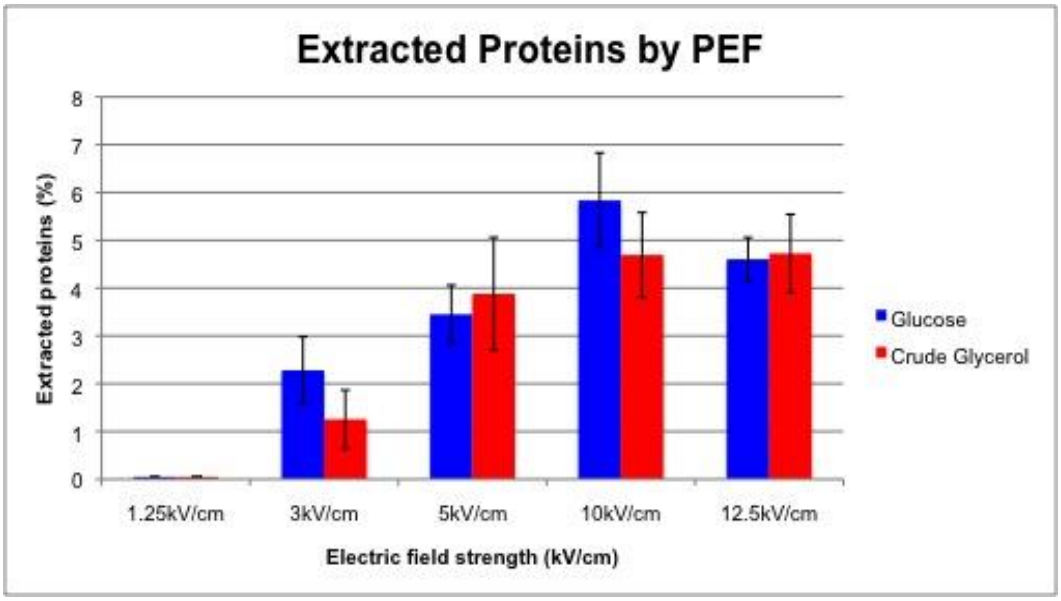


Figure 24 Comparison of the total protein extract from *R. toruloides* after the PEF treatment. *R. toruloides* samples were cultured in Glucose and Crude Glycerol media respectively.

It can be seen that at 1.25kV/cm, no proteins were extracted and small amounts were extracted at higher PEF treatment conditions of 3kV/cm and above. Based on the following observations, we can conclude that the E_{crit} of *R. toruloides* for both glucose and crude glycerol cultures lies between 3 – 5kV/cm, and the lowest electric field condition for protein extraction is at 3kV/cm. This observation is in agreement with the literature review that the E_{crit} of yeast cells lies between 2 – 4kV/cm [12]. The optimal electric field strength for PEF treatment for both glucose and crude glycerol cultures was observed to be at 10kV/cm, as there was a notable increase in the percentage of protein extracted and not much meaningful increase observed at higher electric field treatment at 12.5kV/cm. The maximum amount of proteins extractable via PEF treatment was around 6% for *R. toruloides* grown in glucose culture, and around 5% for *R. toruloides* grown in crude glycerol culture.

The low protein yield extracted seemed to suggest that proteins in *R. toruloides* requires larger sized pores and also greater number of pores for more efficient mass transfer process of the complex protein content in the yeast cell. The mass transfer process is greatly dependant on the size, molecular weight, charge and the structure of

proteins. Also, one important point to note is that some bounded intracellular proteins cannot be extracted easily without the disruption of the intracellular organelles. More work would need to be done in this area to modify this extraction method using PEF treatment, such as using a different buffer solution that can better induce mass transfer of proteins out of the cell when applying the PEF treatment.

4.3.3 Extraction of fatty acids

The application of using PEF to extract valuable fatty acids was looked into, and will be discussed in this section. These microbial lipids, also commonly known as single cell oil (SCO), are of great interest to the food and healthcare industries as a valuable source of lipid products. Oleaginous yeasts are excellent model for such a study, because of its high lipid content. Some of the polyunsaturated fatty acids produced by microbial are essential fatty acids that are not naturally synthesized by human, and have to be uptake in our everyday diet. For instance, linoleic acid is an essential omega-6 fatty acid found in food such as pine nuts, pistachio nuts and chicken, and oleic acid is an omega-9 fatty acid, which can only be made by our own body in the presence of the essential fatty acids.

Using microbial source for SCO production have numerous advantages versus using plant sources, as it requires lesser labor, production time and is not limited by space constrain issues. Microbial syntheses method is also easier to scale up for mass production. Lipid production by the *R. toruloides* studied here, uses both glucose and crude glycerol as the sole carbon source. The *R. toruloides* cultures were harvested and subjected to both mechanical beads extraction method and PEF extraction treatment. The extracted lipids for both treatments were measured and compared using Gas Chromatograph/Mass Selective Detector (GC/MSD) system.

Gas chromatography analysis have shown that the fatty acids produced by *R. toruloides* consist of mainly long-chain 16 and 18 carbon atoms (Figure 25). The four major constituent fatty acids were Palmitic acid (Hexadecanoic acid), Stearic acid (Octadecanoic acid), Oleic acid (cis-9-Octadecenoic acid) and Linoleic acid (cis-9,12-Octadecadienoic acid). The analysis of the major fatty acids present in *R. toruloides* is summarized in Table 13.

It was also revealed that *R. toruoides* could grow a similar fatty acid profile in the crude glycerol media. This indicates that it has the ability to handle osmotic stress in presence of impurities in the growth media. Hence, this study amplifies the feasibility of using *R. toruloides* as a natural alternative source for essential fatty acid production, using waste crude glycerol as carbon source.

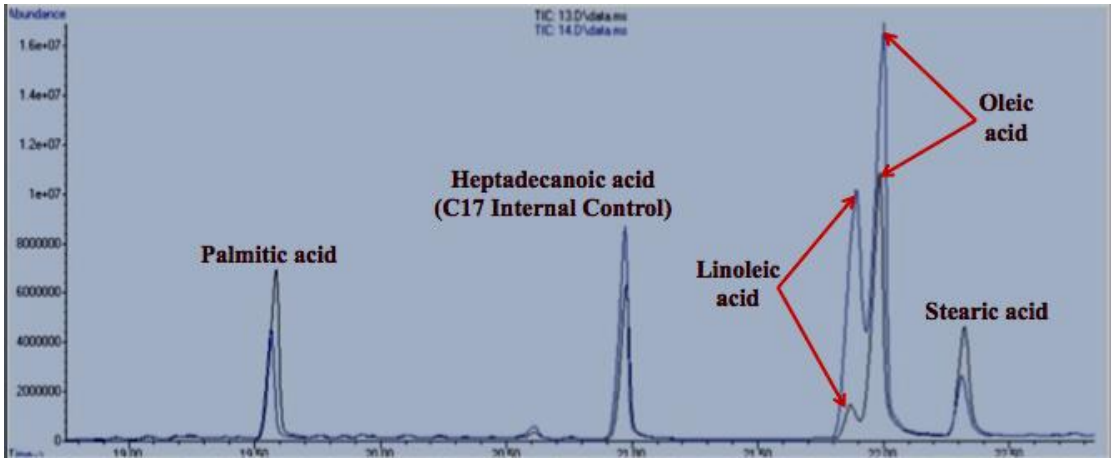
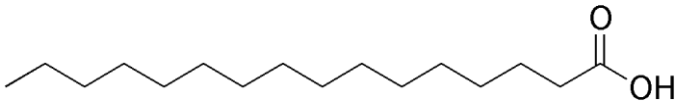
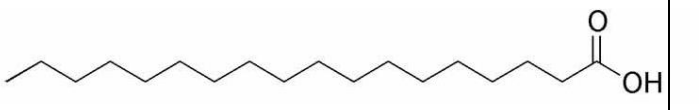
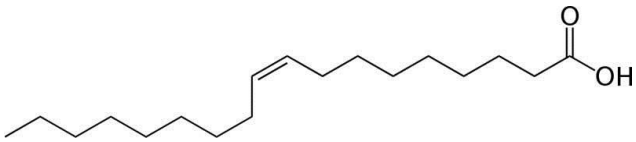
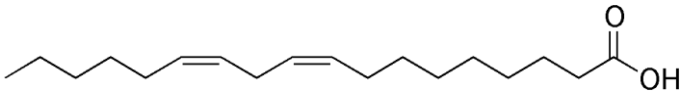


Figure 25 Fatty acid gas chromatography analysis of *R. toruloides* cultured in glucose (black) and crude glycerol (blue) culture media.

Table 13 Summary of the major fatty acids present in *R. toruloides.*

Fatty acids	Lipid number	Fatty acids structure
Palmitic acid (Hexadecanoic acid)	C16:0	
Stearic acid (Octadecanoic acid)	C18:0	
Oleic acid ((9Z)-Octadec-9-enoic acid)	C18:1	
Linoleic acid ((9Z,12Z)-9,12-Octadecadienoic acid)	C18:2	

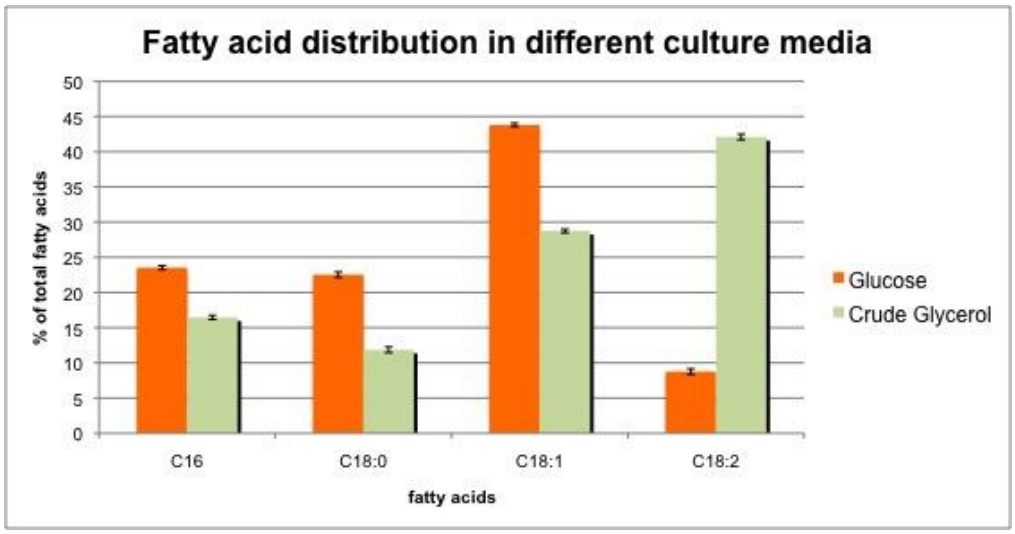


Figure 26 The fatty acids distribution in *R. toruloides* cultured in glucose or crude glycerol as sole carbon source.

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One interesting observation is that the *R. toruloides* cultured in glucose as sole carbon source has a slightly different fatty acid percentage profile when compared against when cultured in crude glycerol (Figure 26). The major fatty acid present in *R. toruloides* that was cultured in glucose was oleic acid, however for *R. toruloides* cultured in crude glycerol, the major fatty acid present was linoleic acid. This is quite an exciting discovery because as discussed previously, linoleic acid is an essential omega-6 fatty acid which cannot be naturally synthesized by human and is only found in external food sources.

An attempt to explain this interesting observation is by looking at the fatty acid biosynthesis pathway of the oleaginous yeast. The basic pathway of fatty acid synthase (FAS) has identified the main enzymes that are involved during the biosynthesis and catabolism of the fatty acids in *R. toruloides* (Figure 27). Based on the FAS pathway, conversion of oleic acid to linoleic acid is by the activity of $\Delta^{12}(\omega^{6})$-desaturase (EC: 1.4.19.6) enzyme [89]. The fatty acid desaturase enzyme requires cofactors for its enzymatic activities, and since potassium ions are present as impurities in the crude glycerol media but not for pure glucose media, it provides a source of cofactors for delta-12-desaturase activity. The enzymatic reaction of enzymes possessing cofactors is affected by the availability of the cofactors. This seemed to be the most reasonable explanation for our observation of a higher production level of linoleic acid seen in *R. toruloides* cultured in crude glycerol, and not the case in pure glucose cultures. It is proven that different culture media composition imposes different stress and condition for yeast culture, which would in turn affect the metabolite pathway of the yeast. However, more studies have to be done in this area to confirm our postulation.

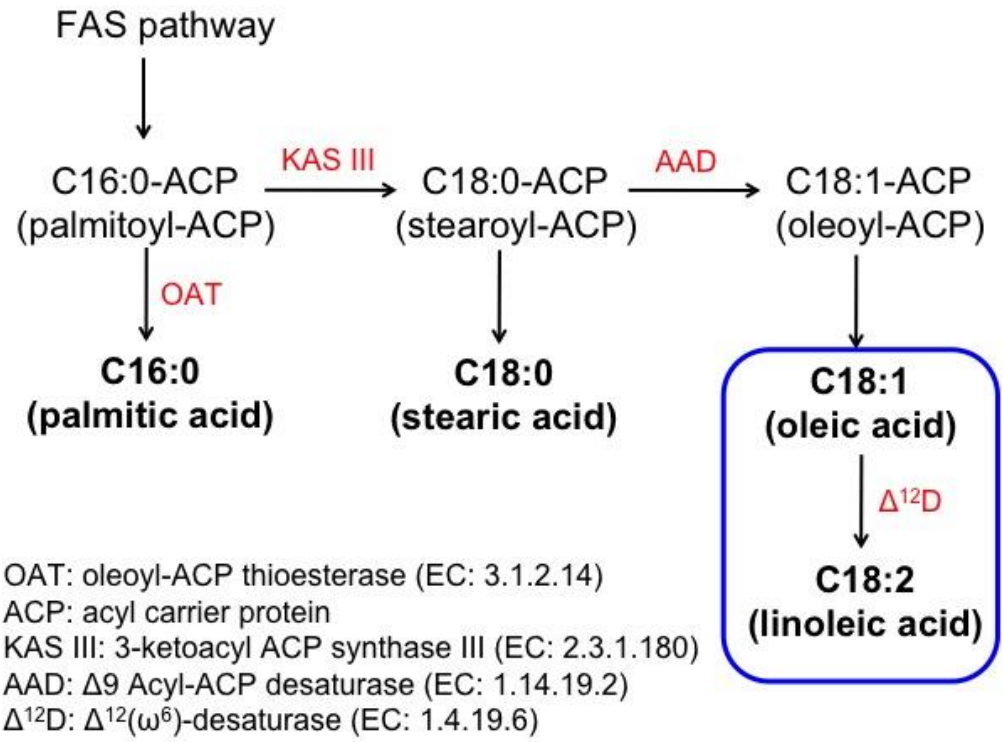

Figure 27 Fatty acid biosynthesis pathway reconstructed of *R. toruloides*.
The enzymes are shown in red, and the blue box represents the destauration reaction converting oleic acid (C18:1) to linoleic acid (C18:2), catalyzed by $\Delta^{12}(\omega^{6})$-desaturase.

PEF treatment was applied to the *R. toruloides* cultures to explore the possible application of using PEF to extract these valuable fatty acids. The maximum electric field strength of 12.5kV/cm, which greatly exceeds the critical electric field strength of *R. toruloides* (i.e. 3 - 5kV/cm) was applied to the cell samples cultured in glucose and crude glycerol. The fatty acids yielded from PEF extraction treatment was compared against the fatty acids yielded from the beads mechanical extraction method, which serves as the control (Figure 28, 29). The maximum total lipid extraction yield for *R. toruloides* cultures grown in glucose and crude glycerol using PEF treatment were 41.2% and 49.4% respectively.

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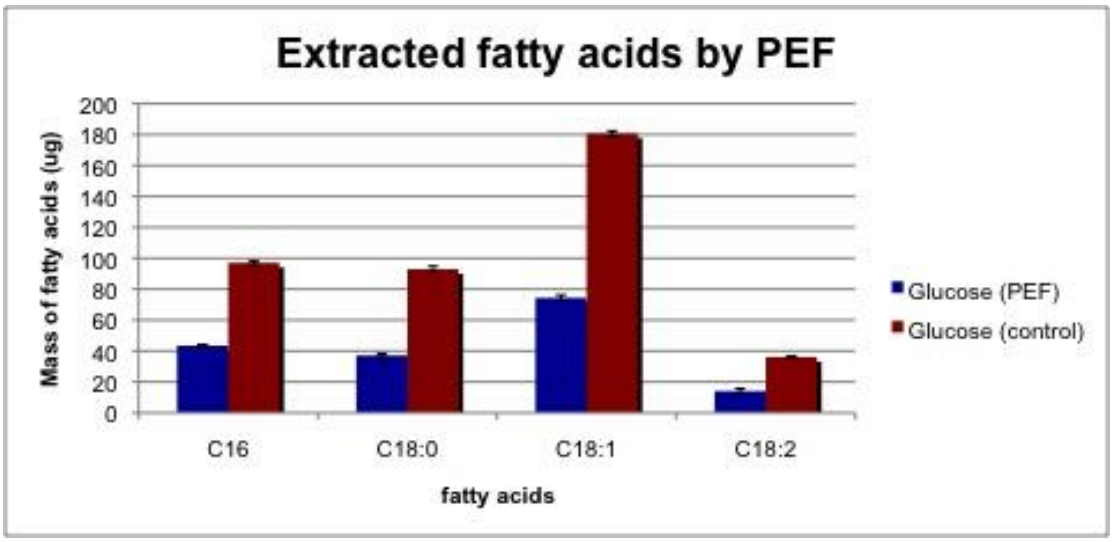

Figure 28 Mass of fatty acids extracted via PEF treatment for *R. toruloides* cultured in glucose as sole carbon source. Total lipid extraction yield was 41.2% of the control (beads mechanical method).

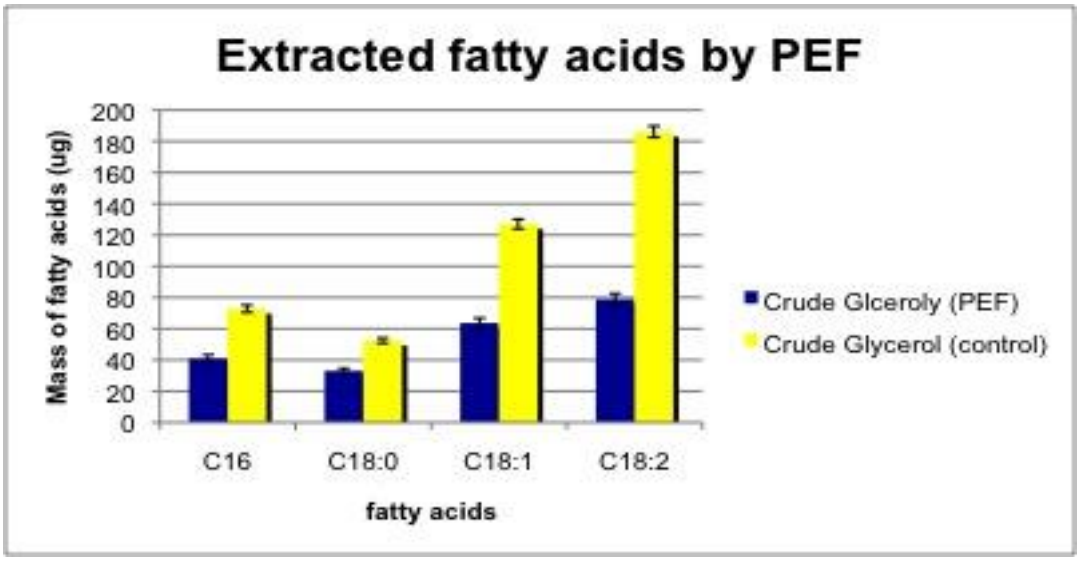

Figure 29 Mass of fatty acids extracted via PEF treatment for *R. toruloides* cultured in crude glycerol as sole carbon source. Total lipid extraction yield was 49.4% of the control (beads mechanical method).

4.3.3.1 The influence on the fatty acid pattern after PEF treatment

As previous results suggest, up to 50% of the total fatty acid content is extractable from both *R. toruloides* cultures under PEF extraction treatment. It is important to analyze whether PEF extraction treatment would modify the fatty acid profile during the lipid extraction of *R. toruloides*. Further analysis of the gas chromatography

results revealed that the PEF extraction treatment do not modify the fatty acid profile in *R. toruoides* for both cultures grown in glucose and crude glycerol media (Figure 30, 31).

For *R. toruoides* cultured in glucose media, the main fatty acids extracted by PEF treatment is also Oleic acid (C18:1). This is also the case for *R. toruoides* cultured in crude glycerol media as seen below, the main fatty acids extracted by PEF treatment is also Linoleic acid (C18:2). This is important because one of the crucial factors that influence the viability of a particular extraction technique is its ability to still retain the functional properties and nutritive values of the biomass. These results shine an optimistic light on the potential application of utilizing *R. toruloides* as "fungi factories" for valuable fatty acids production, which can be used for food supplements or food ingredients in the food and health industries.

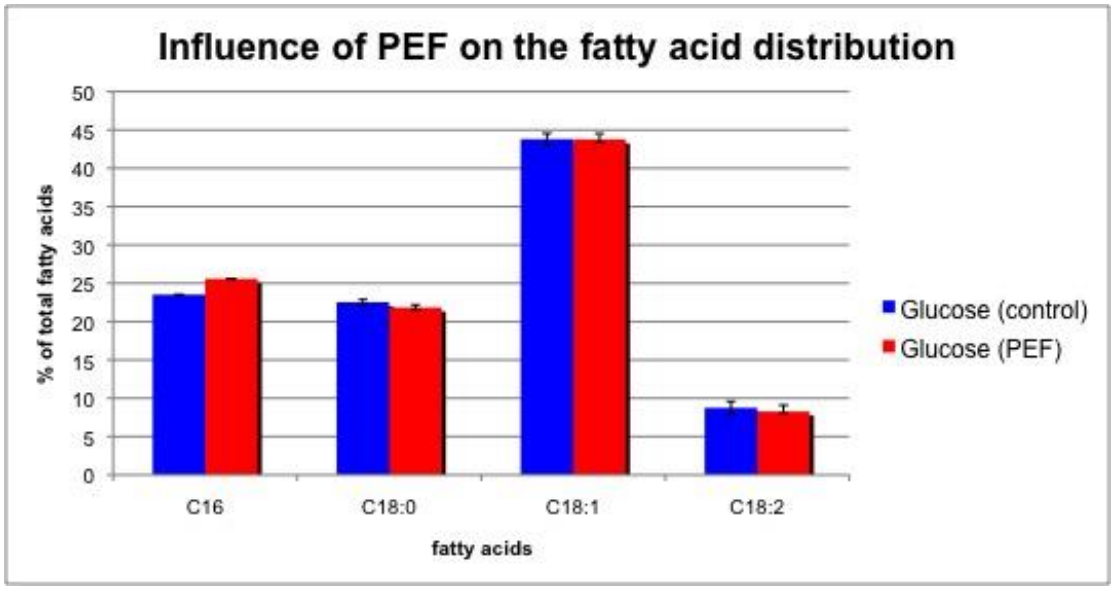

Figure 30 Fatty acids profile of *R. toruloides* cultured in glucose media, extracted via PEF treatment.

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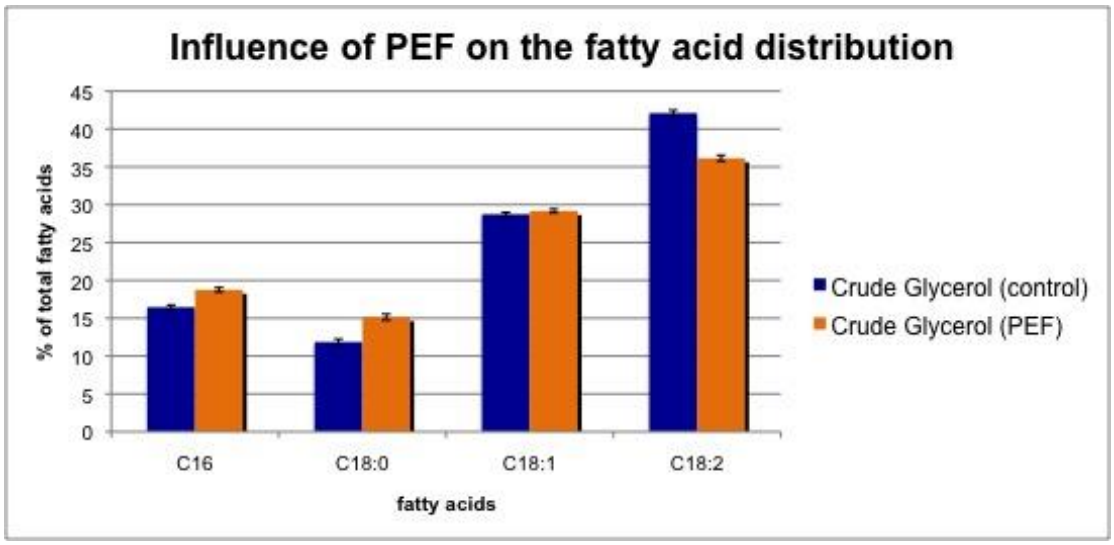

Figure 31 Fatty acids profile of *R. toruloides* cultured in crude glycerol media, extracted via PEF treatment.

4.4 PEF treatment for drying processes

From the previous study, since PEF treatment did not allow much significant extractability of proteins and does not allow extraction of carotenoids, hence an alternative process is attempted in order to isolate the valuable carotenoids in *R. toruloides*. Hence, the effect of heat drying after PEF treated *R. toruloides* will be discussed in this section. The fatty acids stability and carotenoids composition of *R. toruloides* after the influence of heat drying will also be looked into.

4.4.1 Heat drying effect after PEF treatment

PEF treatment allows the formation of pores on the cell membrane surface, hence it is studied in this segment whether the PEF treatment is capable of enhancing the mechanical decanting of *R. toruloides*. Gentle heat drying was shown to allow water and moisture to evaporate faster from the *R. toruloides* cell content, as shown in the heat drying curve of *R. toruloides* cells after PEF treatment (Figure 32, 33). The *R. toruloides* cultures for both glucose and crude glycerol media was subjected to maximum electric field PEF treatment of 12.5kV/cm, and the cell pellets were subjected to heat drying at 60°C until constant mass was achieved after 80 minutes. The mass of the *R. toruloides* cell pellets was measured and recorded every 5 minutes.

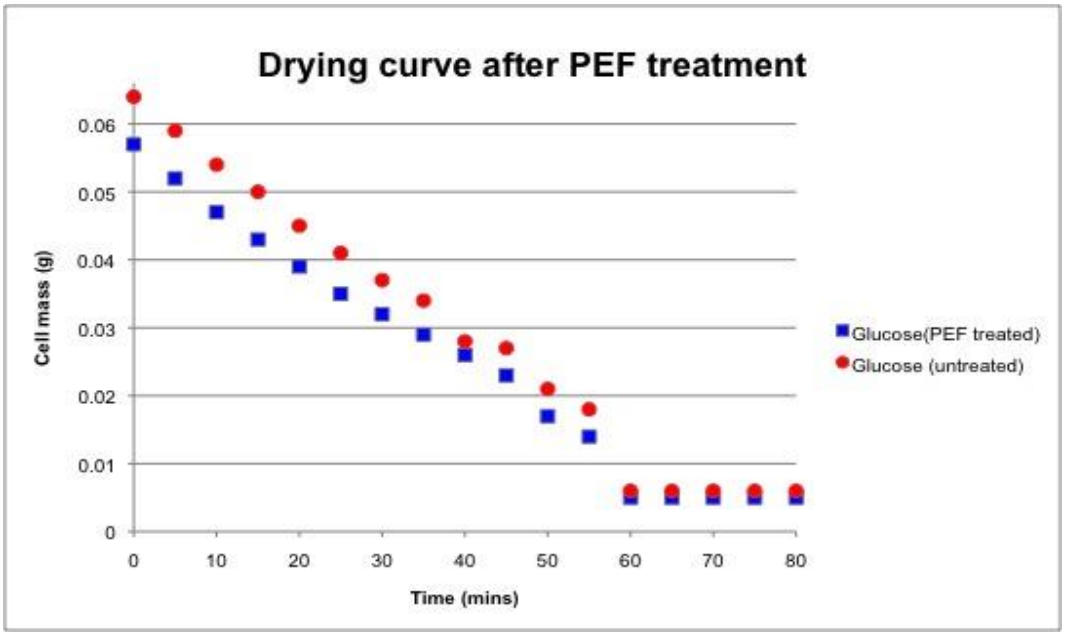

Figure 32 Drying curves of PEF treated and untreated *R. toruloides* cells cultured in glucose media at 60°C.

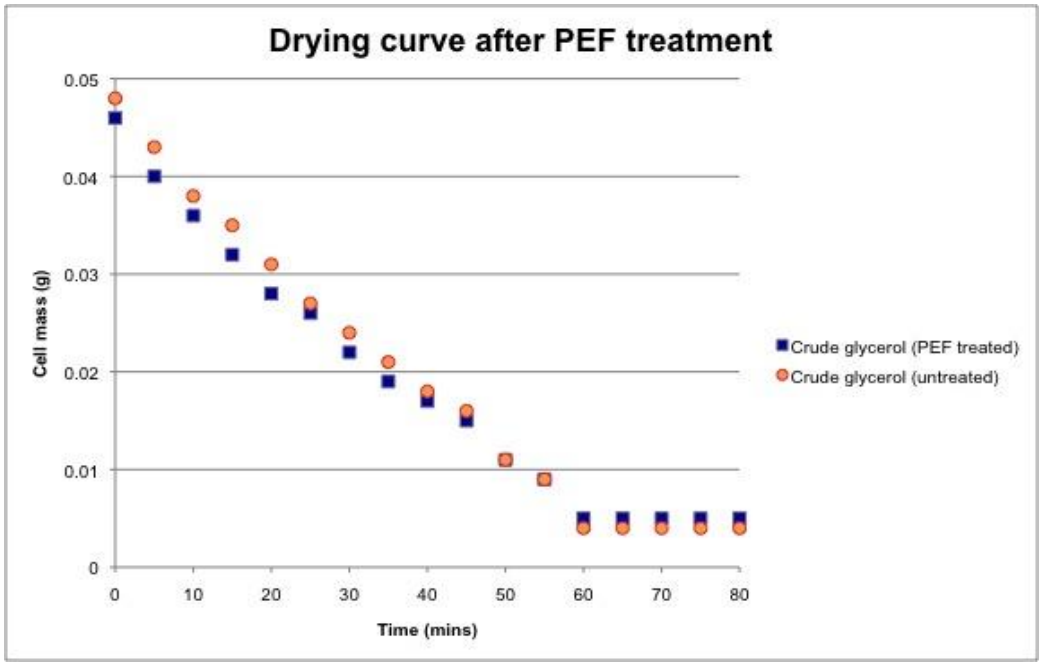

Figure 33 Drying curves of PEF treated and untreated *R. toruloides* cells cultured in crude glycerol media at 60°C.

From the heat drying curves, it is apparent that the PEF treated *R. toruloides* cells have enhanced drying efficiency as compared to the non-treated cells. The cell mass for both glucose and crude glycerol PEF treated *R. toruloides* cultures was already lower as compared to the non-treated cells, and this could be attributed to the fact that

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water loss has already begun immediately after the permeabilization of the cell membrane. The higher water loss rate can be related easily to the presence of the non-reversible pores on the *R. toruloides* cell membrane after being subjected the maximum electric field intensity during PEF treatment. After the dehydration of the cells, the mechanical decanting PEF technique can result in the lipids, cytoplasm and cellular organelles encapsulated by cell membrane. Since cell wall compositions of *R. toruloides* consist of different carbohydrates, this stable barrier could serve as a ”natural encapsulation” for the dehydrated *R. toruloides* cells. Since the carotenoids will remain in the *R. toruloides* cells after PEF treatment and mechanical decanting, the carotenoids can be isolated chromoplasts within a whole dehydrated *R. toruloides* cell. These dehydrated *R. toruloides* cell containing valuable carotenoids and fatty acids would be of great interest to the food and health industries as food/health supplements.

4.4.2 Influence of heat drying on *R. toruloides* biomass

The stability of the dried *R. toruloides* biomass is a paramount parameter to evaluate, because it is definitely not desirable if the heat drying process is detrimental to the quality and the fatty acids and carotenoid pattern. The effect of PEF treatment and heat drying on *R. toruloides* will be looked into in this section.

4.4.2.1 Influence of heat drying on *R. toruloides* carotenoids pattern

Due to the highly unsaturated chemical structure of carotenoids, they are very susceptible towards isomerization and oxidation process in the presence of heat, light or acids. Carotenoids isomerization swoop its original *trans* configuration into a *cis* configuration. The oxidation process, also known as oxidative degradation, is the main cause for the losses of carotenoids. All these chemical process due to heat results in the unwanted consequences of color loss and the lost of provitamin A activity in carotenoids. Hence, it is crucial to verify whether such a heat drying process during the mechanical decanting process of *R. toruloides* would alter its carotenoid profile. It is important to keep in close consideration the quality of these naturally occurring carotenoids.

Hence, it is important to find out whether the heat drying process to decant *R. toruloides* would alter its natural carotenoid profile. The *R. toruloides* cultures were harvested and subjected to PEF treatment at 12.5kV/cm, and centrifuged at high speed. The cell pellets collected were oven dried at 60°C and weighted consistently until it reaches a constant mass. Carotenoid extraction was performed on these samples and the extracted carotenoids were analyzed using HPLC for a quantitative and qualitative determination of the carotenoid composition. The carotenoid profile extracted from *R. toruloides* for both glucose and crude glycerol culture media after PEF treatment and heat drying was compared (Figure 34, 35). From the HPLC chromatogram data, no difference was observed in the carotenoid profile of the different treated samples (i.e. PEF, PEF + Drying) for *R. toruloides* cultured in glucose and crude glycerol. The carotenoids detected (β-carotene, Torulene and Torularhodin) were constant with previous findings even after being subjected to PEF treatment and heat drying. This proves that heat drying do not cause any detrimental effect on the carotenoids present in *R. toruloides*, and hence further supports the promising application of PEF treatment for enhancing drying and isolating of carotenoids from *R. toruloides*.

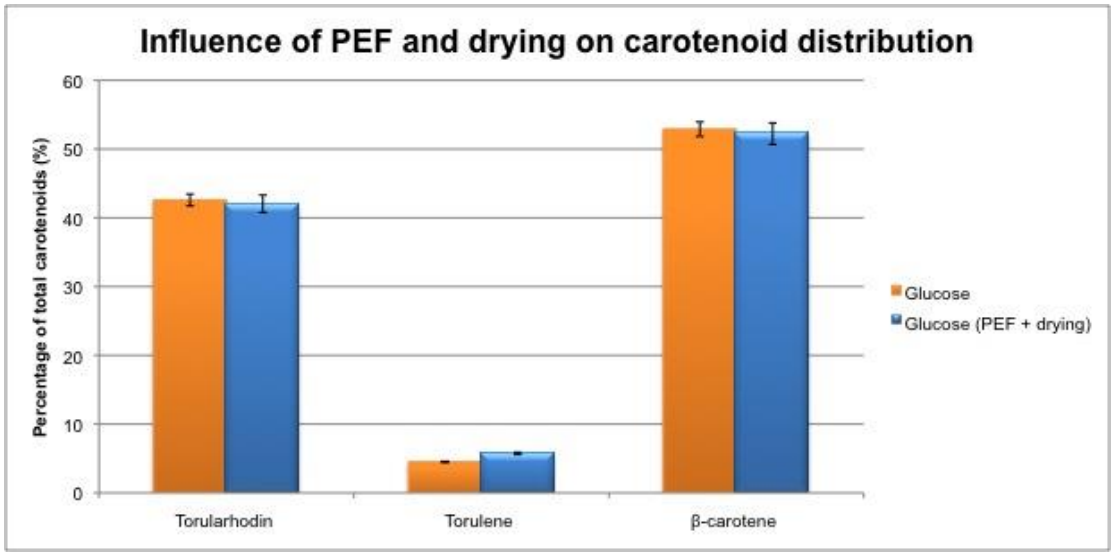

Figure 34 Influence of heat drying and PEF treatment on the carotenoids of *R. toruloides* cultured in glucose.

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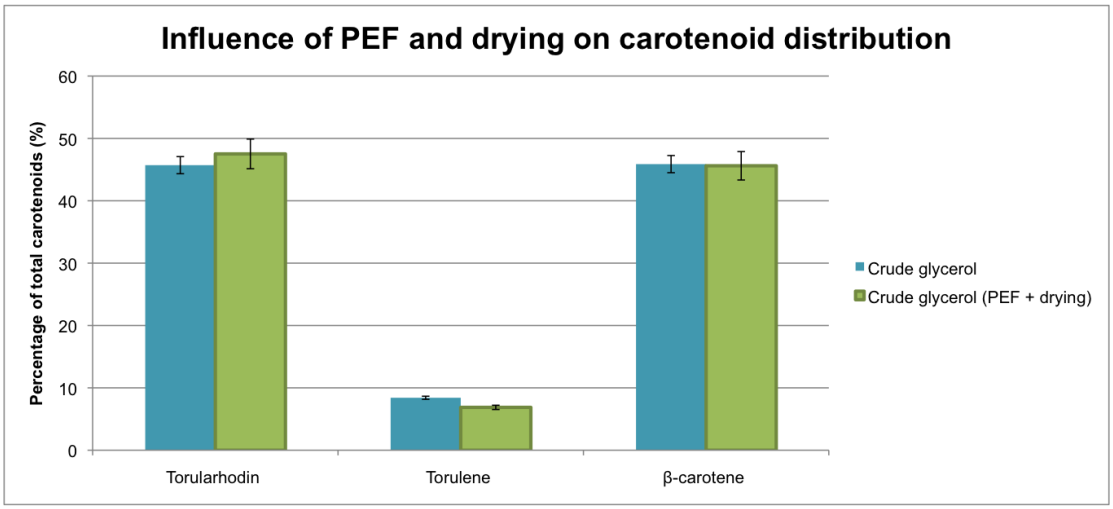

Figure 35 Influence of heat drying and PEF treatment on the carotenoids of *R. toruloides* cultured in crude glycerol.

4.4.2.2 Influence of heat drying on *R. toruloides* fatty acid distribution

The fatty acid composition of storage lipids in cells could be modified due to exposure to higher temperature, as unsaturated fatty acids can undergo chemical reactions such as oxidation, polymerization and reduction depending on the temperature applied. Hence, it is crucial to verify whether such a heat drying process during the mechanical decanting process of *R. toruloides* would alter its natural fatty acid profile.

The 5 days old *R. toruloides* cultures were harvested and subjected to PEF treatment at 12.5kV/cm, before being centrifuged at high speed and the cell pellets were collected. The cell pellets were dried at 60°C until a constant mass is achieved. Lipid extraction was performed on these samples and the extracted lipids were analyzed using GC/MSD for a quantitative and qualitative determination of the fatty acid composition. The fatty acid profile of the lipid extracted from *R. toruloides* for both glucose and crude glycerol culture media after PEF treatment and heat drying were compared (Figure 36, 37).

From the GC/MSD data analysis, no difference was observed in the fatty acid profile of the different treated samples (i.e. control, PEF + Drying) for *R. toruloides* cultured in both glucose and crude glycerol. The main fatty acids that were detected after PEF and heat drying treatment of *R. toruloides* were C16:0, C18:0, C18:1 and C18:2,

which is similar to the previous findings. This proves that heat drying does not cause any detrimental effect or alter the fatty acid patterns in *R. toruloides*, and hence further supports the promising application of PEF treatment for enhancing drying processes.

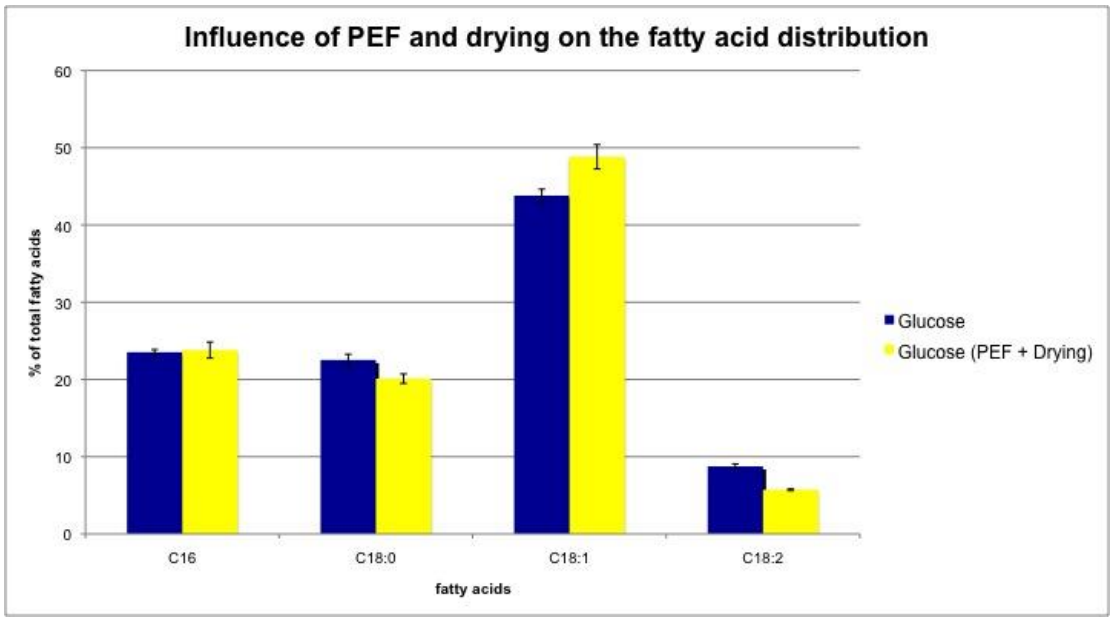

Figure 36 Influence of PEF treatment and heat drying on the fatty acid distribution pattern of *R. toruloides* cultured in glucose.

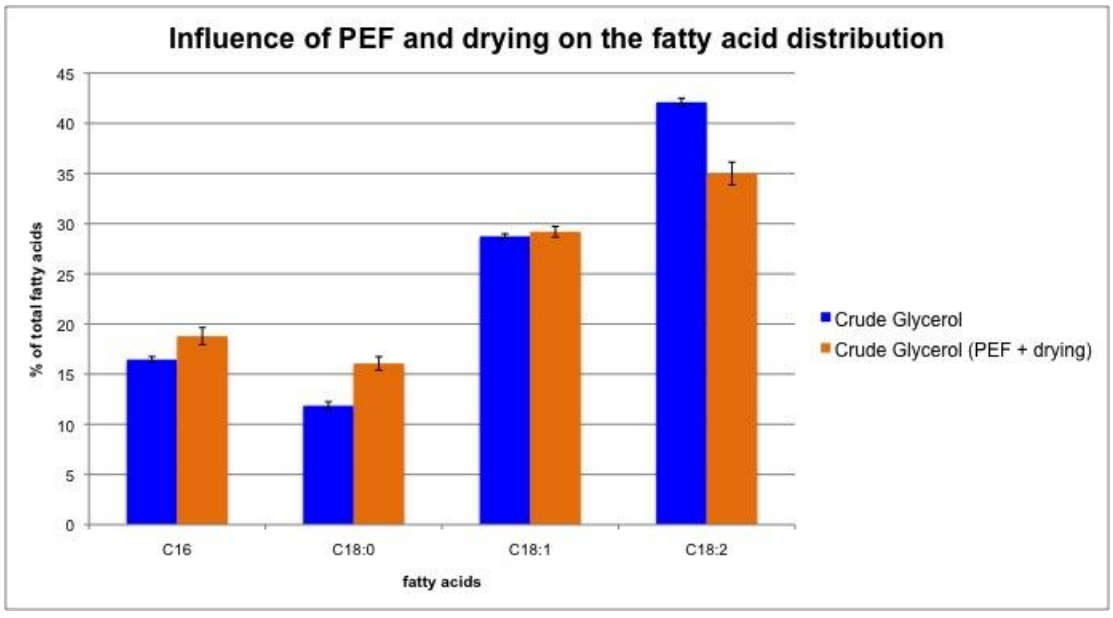

Figure 37 Influence of PEF treatment and heat drying on the fatty acid distribution pattern of *R. toruloides* cultured in crude glycerol.

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5. Summary

In summary, this work has successfully demonstrated the ability of oleaginous red yeast *R. Toruloides*, a natural carotenoid producer, to grown on crude glycerol. This shows that the *R. Toruloides* has great potential for an efficient approach towards bioresources utilization. Media optimization is performed for the crude glycerol culture and the concentration of 60g/L of glycerol was found to be optimal for carotenoid production. The carotenoids profiles of *R. Toruloides* were studied using HPLC analysis, and were found that the main carotenoids present are Torulene, β-carotene and Torularhodin.

Later part of the research concentrates on investigating the potential of using PEF treatment on *R. Toruloides* to isolate the carotenoids. PEF is a non-thermal and mild technique that can reversibly permeabilize cell membranes, hence is an attractive alternative to mechanical beads extraction methods. However unfortunately, no yield was possible when attempting to extract carotenoids from *R. Toruloides*. Hence alternative strategies were taken and the influence of PEF treatment to extract proteins and fatty acids were investigated. The PEF treatment allowed poor protein extraction yields (9.7 % of total protein at electric field = 10 kV/cm), this could be due to the difficulties in extracting the intercellular proteins.

The application of PEF treatment allowed higher lipid extraction yield, up to 50% of total lipids in *R. Toruloides* were extracted. The extracted fatty acids were analyzed by GC-MS and the predominant fatty acids present in *R. Toruloides* were Palmitic acid (C16:0), Stearic acid (C18:0), Oleic acid (C18:1) and Linoleic acid (C18:2). Linoleic acid is an essential omega-6 fatty acid that cannot be naturally synthesized by human, which is only found in external food sources. Hence, we can apply PEF extraction of these valuable fatty acids from *R. Toruloides* as food supplements or feedstock.

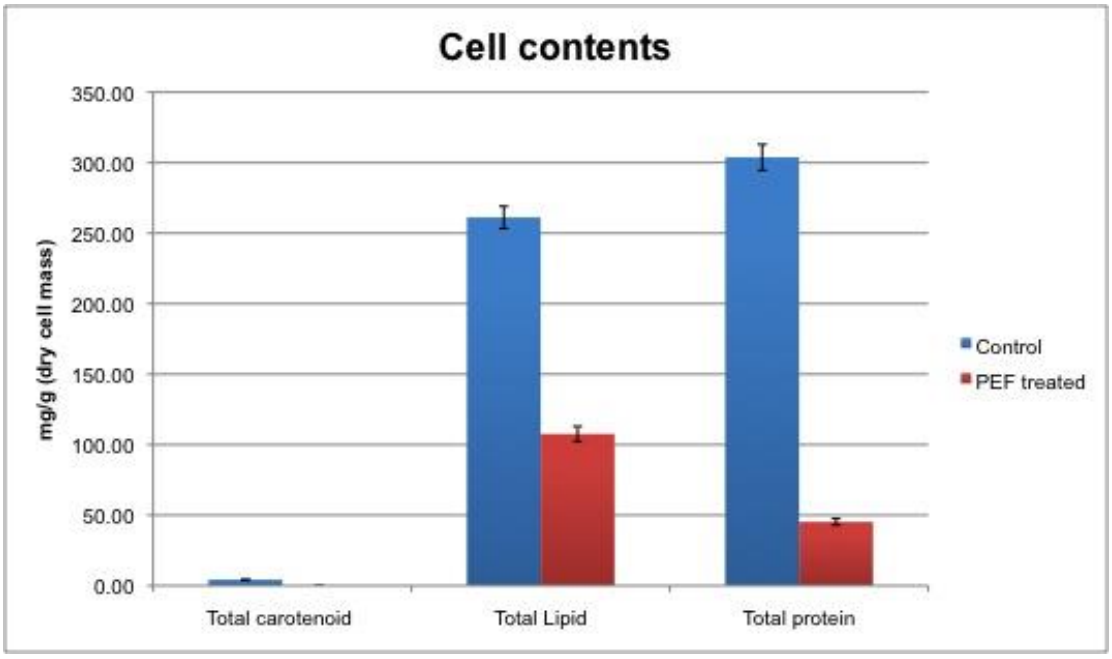

Figure 38 Summary of the extraction efficiency of *R. Toruloides* using control (beads) v.s. PEF technology.

Due to the poor extraction yield for carotenoids, it was tested whether it was possible to apply PEF treatment followed by gentle heat drying at 60°C to enhance the mechanical de-watering and drying process. The drying curve revealed that the *R. Toruloides* cells dried quicker after being subjected to PEF treatment. Since carotenoids are present as stable intact oleosomes in *R. Toruloides*, this suggests the possibility of isolating carotenoids from the chromoplasts within a whole dehydrated *R. Toruloides* cell after PEF treatment. Its natural cell wall is stable, and hence could serve as a ”natural encapsulation” for the dehydrated cells. The fatty acid and carotenoids distribution pattern were analyzed after the PEF treatment and heat drying process, and no negative influences were observed due to the higher temperature exposure.

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6. Future work

The future work can look into improving the efficiency of proteins extraction by PEF treatment from *R. Toruloides.* The extraction condition can be further optimized to have a better mass transfer process of the proteins from the cell, by using higher pulse strength or longer treatment time. Another possible suggestion is to incorporate additional assisting method, such as ultrasound treatment to improve the degree of mixing during extraction. Increased kinetic motion can increase the surface area for better mass transfer processes in yeast extractants.

Another area to further investigate stress induction by the influence of PEF treatment on *R. Toruloides*. Stress induction through PEF treatment below its E_{crit} value could trigger stress respond, and it can potentially lead to a stimulated increased in carotenoid production if the *R. Toruloides* are allowed to continue to grow. The culture of PEF treated *R. Toruloides* culture could be harvest and have its carotenoid content quantified, so it can be determined whether PEF induced stress response can result in an increased in carotenoid production.

Initial studies are on going by our group to study the unique phenomena when PEF-treated *R. Toruloides* were cultured in media containing grape seed oil after 120 hr, and the natural secretion of carotenoids from the yeast cells into grape seed oil was observed (Figure 39). This is however not observed in the control culture, where non-treated cells were cultured in the same media containing grape seed oil. More work are required to be done to explain why such a phenomena is seen, and to explore what kind of stress response did the PEF treatment induced in the cells. This is a very exciting discovery due to the potential applications associated; such as the direct enrichment of grape seed oil with carotenoids to improve its nutritional quality.

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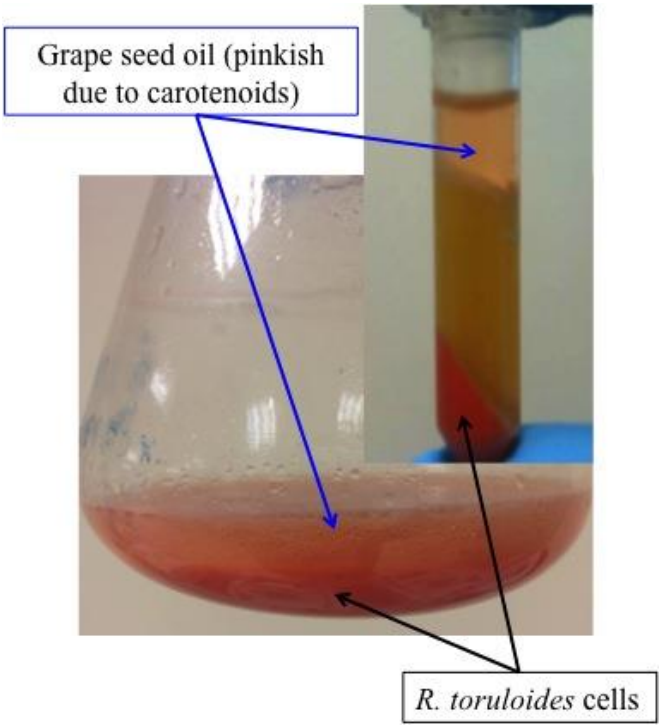

Figure 39 Secretion of carotenoids from *R. toruloides* into grape seed oil observed when PEF-treated cells are continuously cultured.

Lastly, to look into the possibility of isolating the carotenoids from the chromoplasts within a whole dehydrated *R. Toruloides* cell, a further optimization of the drying method is required. Different drying method such a freeze drying or spray drying can be explored. Also, very importantly, we need to establish the storage stability of the carotenoids in the dehydrated *R. Toruloides* cells, so that its can be truly useful in achieving the target goal of utilizing *R. Toruloides* as the cheap and natural source of microbial carotenoids producer.

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Supplementary data

S 1 Biomass and carotenoids yield after 6 days batch fermentation for *R. toruloides.*

	Initial substrate concentration (g/L)	Final biomass concentration (g/L)	Biomass yield (g/gram)	Final carotenoid concentration (mg/L)	Carotenoid yield (mg/gram)
Glucose	20	8.8	0.4	13.6	0.7
Crude glycerol	20	6.5±0.5	0.3±0.03	22.0±2	1.1±0.1
Crude glycerol	60	19.2±3.6	0.3±0.06	45.8±3.4	0.8±0.01
Crude glycerol	120	23.4±5.4	0.2±0.04	26.7±0.7	0.2±0.01

S 2 Biomass and carotenoids average yield for every 24 hr during the batch fermentation of *R. toruloides.* The means and ± standard deviation of the triplicates are shown.

		24 hr	48 hr	72 hr	96 hr	120 hr	144 hr
Biomass (g/L)	Crude glycerol (20g/L)	4.4±1.7	5.9±0.8	6.6±0.8	5.3±1.2	6.7±0.9	6.5±0.5
	Crude glycerol (60g/L)	3.8±1.5	7.7±1.2	11.2±0.27	13.9±0.6	16.9±2.2	19.2±3.6
	Crude glycerol (120g/L)	4.1±0.8	10.1±2.5	15.4±0.1	17.6±1.1	20.7±3.3	23.4±5.4
	Glucose	8.20	6.00	6.80	7.90	6.30	8.80
Carotenoid (mg/L)	Crude glycerol (20g/L)	2.0±0.02	3.6±1.3	18.4±2.1	19.3±0.8	20.2±0.7	22.0±2
	Crude glycerol (60g/L)	3.0±0.04	11.3±0.5	24.4±0.3	31.7±0.9	37.1±0.7	45.8±3.4
	Crude glycerol (120g/L)	1.0±0.7	4.9±0.5	12.5±2.5	20.8±2	24.6±0.0	26.7±0.7
	Glucose	1.2	5.3	10.1	11.2	11.0	13.6

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S 3 Protein quantification of PEF treated *R. toruloides* cultured in glucose and crude glycerol media, at various electric fields respectively.

Protein quantification (Glucose)

	1.25kV/cm	3kV/cm	5kV/cm	10 kV/cm	12.5kV/cm
Mass of protein (µg)	2.3±0.3	3.9±0.5	3.8±0.3	4.5±0.4	4.2±0.6
Percentage of protein (%)	3.7±0.4	5.9±0.7	7.1±0.6	9.4±1.1	8.2±0.9

Protein quantification (Crude glycerol)

	1.25kV/cm	3kV/cm	5kV/cm	10 kV/cm	12.5kV/cm
Mass of protein (µg)	3.4±0.2	4.2±0.4	3.1±0.4	4.0±0.3	2.8±0.4
Percentage of protein (%)	5.3±0.3	6.3±0.6	8.9±1.3	9.7±0.9	7.2±0.9

S 4 Lipid quantification of PEF treated *R. toruloides* cultured in glucose and crude glycerol media using GCMS.

Lipid quantification (Glucose)

	C16	C18:0	C18:1	C18:2
Mass of lipids (µg)	43.5±0.3	37.1±0.5	74.4±0.3	14.0±0.4
Percentage of lipids (%)	25.6±0.4	21.8±0.7	43.8±0.8	8.3±0.3

Lipid quantification (Crude glycerol)

	C16	C18:0	C18:1	C18:2
Mass of lipids (µg)	40.9±0.2	33.0±0.4	63.7±0.4	78.9±0.3
Percentage of lipids (%)	18.8±0.3	15.1±0.6	29.2±1.3	36.1±0.9

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S 5 Carotenoid quantification of PEF treated *R. toruloides* cultured in glucose and crude glycerol media using HPLC.

Carotenoid quantification (Glucose)

	Torularhodin	Torulene	β-carotene
Mass of carotenoids (μg/mL)	2.9	0.3	3.6
Percentage of carotenoids (%)	42.6	4.5	52.9

Carotenoid quantification (Crude glycerol)

	Torularhodin	Torulene	β-carotene
Mass of carotenoids (μg/mL)	3.7	0.7	3.7
Percentage of carotenoids (%)	45.7	8.4	45.9

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