**An-Najah National University**

**Faculty of Graduate Studies**

# Optimal Conditions of Astaxanthin Production by *Phaffia rhodozyma* Yeast using Sesame Meal

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# Dedication

To my mother and family with all love and respect

To everyone who loves science and does the impossible to achieve it.

I did this work

# Acknowledgement

The people who are successful are those who are grateful to others. All praises to Allah for his blessings that helped me to reach to complete my research. Also my gratitude to my supervisors, Dr. Mohammad Altamimi and Dr. Samer Mudalal, the words have nothing to say and describe how much you were helpful to me, thank you for your guidance, direct supervision, reading and approval of my thesis. Moreover, all thanks for the laboratory technician for tips, continuous follow up and hard work they showed during the course of this study.

Finally, I am especially thankful to my family who encourages me all the way, they always inspire me to follow my dreams and for their support and patience.

# الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

**Optimal Conditions of Astaxanthin Production by *Phaffia rhodozyma* Yeast using Sesame Meal**

أقر بأن ما اشتملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الإشارة إليه، حيث ان هذه الرسالة كاملة، أو أي جزء منها لم يقدم من قبل لنيل أي درجة أو لقب علمي أو بحث لدى أي مؤسسة تعليمية أو بحثية أخرى.

# Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

**اسم الطالب:** **Student's name:**

**التوقيع:** **Signature:**

**التاريخ:** **Date:**

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# List of Abbreviations

| | |
|---|---|
| % | Percentage |
| *P. rhodozyma* | *Phaffia rhodozyma* |
| *X. dendrorhous* | *Xanthophyllomyces dendrorhous* |
| *H. pluvialis* | *Haematococcus Pluvialis* |
| NCYC | National Collection of Yeast Cultures |
| AOAC | Association of Official Analytical Chemists |
| YM | Yeast Malt media |
| PDB | Potato Dextrose Broth |
| PDA | Potato Dextrose Agar |
| SOC | Sesame Oil Cake |
| HCl | Hydrochloric Acid |
| °C | Celsius |
| Psi | Pound per square inch |
| UV | Ultraviolet |
| H | Hour |
| V | Volume |
| W | Weight |
| Min | Minute |
| V/V | Volume per Volume |
| W/V | Weight per Volume |
| Nm | Nanometer |
| g/ml | gram per milliliter |
| mg/ml | milligram per milliliter |
| mg/g | milligram per gram |
| rpm | Revolution per minute |

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## Abstract

**Background**: There is a growing trend to employ different by-products from the food industries as a sustainable source of feed in the production of biomass of probiotics and other beneficial microorganisms. Accordingly, this trend contributes effectively to reduce the cost of production and alleviates the environmental burdens. Sesame cake (SOC) is one of a by-product of local sesame oil extraction industries.

**Material and Methods**: *P. rhodozyma* yeast was used to inoculate sesame cake using two growing systems; batch and fed-batch. Sesame cake (SOC) content of moisture protein, fat, fiber, ash carbohydrate were determined. Biomass, astaxanthin production and pH were measured and compered with potato dextrose (PDB, a control and selective medium).

**Results**: The results showed that the average (± SD) moisture content for sesame cake was (30.82± 0.74%) on wet basis. Protein, fat, ash, fiber and carbohydrate (calculated by difference) based on dry basis were 49.44% ± 3.75, 8.89± 1.62%, 6.458± 0.143%, 33.382± 2.174% and 1.829% respectively. Biomass production using PDB and SOC (2% W/V) in batch culture after 72 h was 5.9± 0.29 g/L and 4± 0.26 g/L respectively.

Production of astaxanthin after 72 h in batch culture was 1.131± 0.039 mg/L and 0.854± 0.039 mg/L respectively. While using the fed-batch culture SOC (2% W/V) produced 12.3± 0.53 g/L biomass and 7.031± 0.285 mg/L astaxanthin after 72 h. The pH of PDB and SOC (2% W/V) in batch culture after 72 h dropped by 0.89 and 1.24 respectively. While the pH of SOC (2% W/V) after 72 h dropped by 0.91 in fed-batch culture.

**Conclusion**: Findings of this study showed that chemical composition of sesame cake is promising not only to provide adequate nutrients to support the growth of *phaffia rhodozyma* but also to produce astaxanthin in effective and low cost way. This can be added value to plenty of by-products of sesame industries in Palestine.

# Chapter One
# Introduction and Literature Review

## 1.1 Pigment

Materials called pigments could change the color of transmitted and reflected light. Chromophore is a structure known to absorb energy and helps in excitation process and elevate electrons from one orbital to another higher orbital [1]. The reflected non-absorbed energy is seen by eyes through a neural transmission to brain areas and seen as colors [2].

These pigments can be classified depending on their origin into; organic (natural and synthetic) and inorganic. Microorganisms, plants, fungi, and animals are used to generate natural pigments while synthetic ones are produced in laboratories. Inorganic pigments either occur in nature or synthesized in laboratories [3].

Moreover, pigments classified by their chlorophore chemical structure. Anthocyanins, carotenoids, caramel, betalains, and lakes are chlorophores with the conjugated system while chlorophyll, myoglobin and their subtypes are Metal-coordinated porphyrins [4].

Another classification is founded according to a characteristic of pigments structure like Tetrapyrrole, Isoprenoid, and Benzopyran derivatives [2].

## 1.2 Carotenoids

The most popular group of pigments are carotenoids. There are around 108 tons produced annually of carotenoids. They are synthesized in plants and microorganisms, they are founde in both non-photosynthetic like bacteria, fungi and photosynthetic organisms like plants, algae respectively [1]. The color of many foods, fruits, flowers, trout, insects, crustaceans, and vegetables are due to the presence of carotenoids [5]. There are about 600 carotenoids identified until 1992, this number is increasing because carotenoids can be extracted from marine organisms. Most of the produced carotenoids are neoxanthin, in green leaves, fucoxanthin, in marine algae, lutein, and violaxanthin [1]. Carotenoids present in two forms Cis and Trans, most of them are trans isomers [6]. These forms are changeable by heat, chemical energy and light [7].

Carotenoids are divided depending on chemical structure into the ones that contain hydrogen, carbon, and oxygen are called oxy-carotenoids or xanthophylls and those that contain hydrogen and carbon. Additionally, carotenoid is categorized into primary and secondary [1]. The primary carotenoid is used in photosynthesis by plants (neoxanthin, β-carotene, and violaxanthin) while secondary carotenoids are found in flowers and plants (capsanthin, zeaxanthin, antheraxanthin, capsorubin, α-carotene and β-cryptoxanthin) [8].

Generally, carotenoids contains eight units of isoprenoid and all carotenoids are lycopene ($C_{40}H_{56}$) subtypes by the following: (1) hydrogenation, (2) dehydrogenation, (3) cyclization, (4) oxygen insertion, (5) double bond migration, (6) methyl migration, (7) chain elongation, (8) chain shortening. [8].

### 1.3 Astaxanthin

Astaxanthin (3, 3’-dihydroxy-β, β'-carotene-4, 4'-dione) is classified as xanthophylls which are the carotenoid contain oxygen that comes from lycopene [9]. It has a $C_{40}H_{55}O_4$ molecular formula and 596.86 molecular weight. In crystalline form, astaxanthin exists as dark, fine, brown violet powder with 224 °C melting temperature. Its lipophilic moiety does not dissolve in an organic or aqueous solvent but it is soluble in solvents that are non-polar like acetone and chloroform (~ 0.2 g/L) and  (~ 10 g/L), respectively at room temperature [10]. There are four astaxanthin configurations including meso forms (3'R, 3S, and 3R, 3'S) and identical enantiomers (3R, 3'R, and 3S, 3'S). The configurations of 3R, 3'R are found in *Phaffia rhodozyma* red yeast [11] and krill *Euphatisiasuperba* [12], while the configurations of 3S,3'S  isolated from natural sources (aquatic and algal) like alga *Haemotococcus Pluvialis* [13] and lobster Homanisgammarus.

Moreover, Astaxanthin is a major pigment found in fish feed, salmon, and crustacean. Its function is to give orange-red color to these organisms because they have not internal carotenoids source. Astaxanthin

role has been improved in the industry, not due to consumer preference of natural pigments but also it has an important nutrient utilized in reproduction and growth. In addition, it has an antioxidant characteristic that surpasses α-tocopherol and β-carotene [9].

Astaxanthin has many health effects and properties, for example, it is considered as anti-cancer, decrease inflammation and stress. In addition, it plays important role in reperfusion of ischemia, hypertension and hyperlipidemia treatment. It is also 54 fold more potent than β-carotene as anti-oxidant and 10 folds better than lutein and zeaxanthin [14, 15] as well as 65 folds stronger than vitamin C [16].

### 1.4 Microorganisms contain astaxanthin

Astaxanthin is a constitute of many microorganisms including the heterobasidomycetous yeast *Xanthophyllomyces dendrorhous* [11], the bacteria using hydrocarbon *brevibacterium* and *Mycobacterium lacticola* [17], also in many green algae including *Haematococcus species*, *Chlamydomonas nivalis* and *Neochloris wimmeri* [18]. In addition, the basidiomyceteous fungus *Peniophora* [19]. *H. pluvialis* green alga and their species in addition to *X. dendrorhous* yeast are the most biologically important sources for astaxanthin production in industrial scales. On the other hand, pigment production by other microorganisms is less popular because of low rates of growth and low content of carotenoid.

*H. pluvialis* characterize as motile, unicellular, one of the chlorophyceae green class. Under suitable conditions, *H. Pluvialis* present as biflagellate unicellular organism able to grow by the photosynthetic autotropic way [18]. While in unsuitable conditions, cells become immobile cysts. The cyst is usually generated along with lipid globules synthesis and precipitation of large amounts of astaxanthin. This formation starts in the perinuclear area and exceeds to the peripheral cytoplasm. Thirty-five percent of cell mass is accounted by astaxanthin, which represents the maximum level of astaxanthin in the organisms.

### 1.5 *Phaffia rhodozyma* Yeast

Another promising important source for astaxanthin is *X. dendrorhous* yeast known formerly as *Phaffia rhodozyma*. It is well known that all strains of *Phaffia rhodozyma* are designated to be *X. dendrorhous*. However, some strains of teleomorphic *X. dendrorhous* and anamorphic *P. rhodozyma* are unable to form basidiospores and basidia distincts. The first isolation of *X. dendrorhous* has happened in 1970 by Herman J. Phaff. This was isolated from deciduous tree exudates in Alaskaand Japan [21]. Later 67 strains were isolated near Moscow through birch fluxes in 1977 [22].

Depending on properties of the cell wall, bud formation mode, morphology, metabolic properties and pigmentation, yeast are categorized. Despite many attempts had been done to study this yeast, its sexual cycle still not well known [23]. There are many main properties of *X.*

*dendrorhous* like coenzyme Q-10, xylose component of the cell wall, carotenoid pigment synthesis and single site for repetitive budding.

One of the most important characteristics of *X. dendrorhous* is its carotenoid content and its ability to do sugar metabolism [23]. The carotenoid that present in *X. dendrorhous* differs from that present in *H. pluvialis*. *H. pluvialis* contains, violaxanthin, lutein, astaxanthin, and β-carotene which found in the configuration of 3S, 3'S stereo [24]. While, *X. dendrorhous* generate astaxanthin in a configuration of 3R, 3'R and many ketocarotenoids like phoenicoxanthin, canthaxanthin, and echinenone [11].

This configuration difference indicates the difference in enzyme mechanism and pathways of biosynthesis [25]. Figure 1 illustrates vegetative cells budding, jagged collar elongated cells with bud scars, and spheroidal chlamydospores [26].

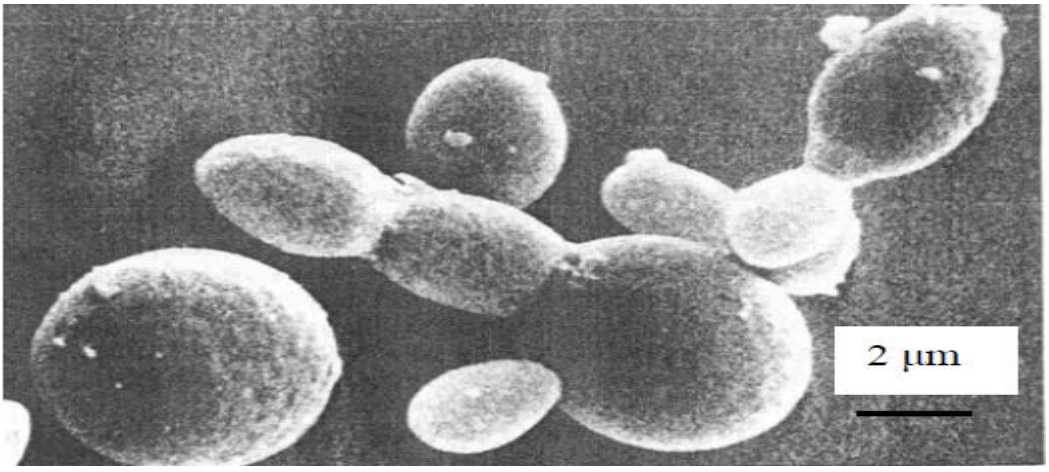


**Figure (1):** *Phaffia rhodozyma* micrograph by scanning electron microscope.

## 1.6 By- products as growth substrates

There has been a trend in using low cost substrates from different origins for production of astaxanthin by fermentation of *X. dendrorhous*. Materials like juices, hydrolysates, molasses and agro-industrial by-products were studied at fermenter and flask scales in liquid phase media. In order to have flavoring substances, mustard seeds have been treated and its waste products were used as media to produce astaxanthin and biomass of 25.8 mg/L and 19.6 g/L, respectively. Therefore, astaxanthin production improved by 11 times compared to yeast malt medium. [27].

Nowadays, agricultural wastes are used in bioconversion to chemicals and fuels at industrial scales. For example, it was reported that 25 kg of processed maize were required to produce 7 kg of residue rich in fiber and protein. Three main types of corn fiber sugar (xylose, arabinose, and glucose) and five different strain *of X. dendrorhous* were studied to investigate the production of astaxanthin. This result in the metabolism of three sugars giving that arabinose produces the maximum yield of astaxanthin while the high concentration of glucose inhibits the use of another two sugars. Dilution of hydrolysate-corn fibers increased astaxanthin production as 0.8 mg/g of consumed sugar [28]. Additionally, there are about 6 types of corn-wet milling co-products that are used as a cheap fuel for production of ethanol were also used as a source of carbon for production of astaxanthin by *X. dendrorhous*. Three of these co-products (corn-condensed distiller's solubles, corn gluten, and thin stillage)

generate up to 148%, 104%, and 65%, respectively of carotenoids per ml of the culture of YM. Demonstrating that at correct concentration, they can be used as an efficient source of the substrate of *X. dendrorhous* growth and production of astaxanthin [29].

Residual juice of alfalfa encourages the growth of *X. dendrorhous* while inhibition of astaxanthin and biomass formation have more autolysis susceptibility. This inhibition is not reserved by nutrient supplement, indicating that is an inhibitor of astaxanthin synthesis found in the juice [30]. There is a stimulator of astaxanthin synthesis in *X. dendrorhous* found in carbon natural sources like grape juice, coconut milk, cane sugar molasses and coconut pulp. Astaxanthin 1.8 mg/g was yielded by fermentation, which was more than other sources of carbon [31]. While 1.1 mg/g of astaxanthin (15.3 μg/ml) is produced when *X. dendrorhous* was grown in carbon source of cane molasses 10% which is 3 folds more than with glucose and 2 folds more than molasses as sugar blend [32]. There was 40 mg/L of carotenoid produced when blackstrap molass was used for fermentation.

Glucose, fructose, and sucrose with its component, which are the 3 major molasses sugar, are assimilated but slower consumption of fructose was found. In molasses use, Atomic emission spectrometry shows that P and Na are the determinant nutrient. There were 40 mg/L carotenoid and 36 g/L biomass yield when sodium phosphate and urea-formulated medium were used in a fed-batch pilot- scale 100 L cultivation [33].

A successful result has been shown when pineapple diluted juice was studied as a medium for the production of carotenoid. At the dilution of 10% pineapple juice, the production of *X. dendrorhous* mutant increased by 66% compared to YM medium [34]. When the medium was supplemented with carbon source as *Yucca filifera* date juice yeast growth was enhanced and astaxanthin production has been documented as 2.5 times better than YM medium [35]. Three forms of experimental designs were applied to maximize production of astaxanthin in the medium of yucca, this gave astaxanthin of 8.1mg/L, which was 92% better than the production of initial conditions [36].

A medium of grape juice was used for *X. dendrorhous* fermentation [37]. Although glucose presence inhibited utilization of fructose, consumption of fructose occurred before depletion of glucose. Production of astaxanthin and pigment reached values of 1.3 and 2.1 mg/g, respectively. The highest rate of growth, pigment production and cell content of astaxanthin significantly increased when an extract of yeast was added to the medium of grape juice. When peat hydrolysate was used as the substrate in medium (liquid-phase), total production of carotenoid reaches 1.6 mg/g of dry yeast [38]. Culture medium based on the extraction of peat for production of astaxanthin showed the same concentration in both continuous and batch processes [39].

A good concentration of *X. dendrorhous* biomass (23.2 g/L), pigment (12.9 mg/L carotenoids, 10.4 mg/L astaxanthin) and good rates of production of carotenoid (0.079 mg/L-h) were achieved by using media containing xylose and supplemented with Eucalyptus wood neutralized hydrolysates [40].

Production of carotenoid was improved by intermittent feeding reaching the carotenoid yield of 33.5 mg/L, 30.5 mg/L of astaxanthin and 30.6 g/L biomass values [41].

For *X. dendrorhous* growth, a nutrient source with a low cost basal medium like sodium phosphate, urea and sugar cane diluted juice was developed. A good yield of cells and astaxanthin have been found (>5 g/L and >1.3 mg/g, respectively) [42]. The liquor of corn steep has been demonstrated as a rich supplement for production of astaxanthin (1.3 mg/g) and yield of biomass (9.2 g/L). Despite Distillery effluent, has a minimum positive effect on the growth of yeast, it has high production of pigment (1.9 mg/g) [43].

As well as the replacement of carbon sources or other nutrients by inexpensive substrates, an addition of astaxanthin inducers was reported to improve astaxanthin yield. The addition of plant extracts from *Perilla frutescens* or *Allium fistulosum* enhanced the pigment production to about 32 mg/L without changing cell growth, suggesting that both extracts act not only as a nutrient, but they may contain carotenogenesis inducers. In fact,

the addition of *P. frutescens* extract in a batch fermenter reduced the cultivation time by 2 days [44].

Inducers of astaxanthin were recommended to improve the yield of astaxanthin in addition to the use of other cheap substrates [45 46]. Moreover, added extracts of plants from *Allium fistulosum* or *Perilla frutescens* improve production of pigments up to 32 mg/L with no cell growth changing indicating that extract work both nutrient and inducers of carotenogenesis. In addition, extract of *P. frutescens* in batch fermenter decrease the time of cultivation by two days [44]. Table 1. Illustrate different substrate was used as alternatives carbon source for growth different strains of *P. rhodozyma* using different fermentation system for biomass and astaxanthin [47].

**Table (1) Comparison of biomass and astaxanthin production by *P. rhodozyma* strains grown on different low cost substrates [64].**

| Strain | Carbon source | Fermentation process | Cell mass (g/l) | Astaxanthin (mg/g dry cells) | Astaxanthin (mg/l culture fluid) |
|---|---|---|---|---|---|
| *P. rhodozyma* ATCC 24202 | Peat hydrolysate | Continuous | 4.95 | 0.544 | 2.69 |
| *P. rhodozyma* UCD-FST 484 | Grape juice | Batch | 38.0 | 0.300 | 11.4 |
| *P. rhodozyma* 25-2 | Date juice of Yucca fillifera | Fed-batch | 39.0 | 0.618 | 23.81 |
| *P. rhodozyma* 2A2 N | Corn hydralysate | Fed-batch | 32.0 | 1.64[a] | 52.40[a] |
| *P. rhodozyma* CBS 215-88 | Molasses | Fed-batch | 24.0 | 1.18<br>1.36[a] | 29.9<br>43.4[a] |
| *P. rhodozyma* UBV-AX | Hydrolyzed corn syrup | Fed-batch | 79.0 | 7.2 | 561.0 |
| *P. rhodozyma* | Sugar-cane juice | Batch | 9.2 | 1.9 | 17.5 |
| *P. rhodozyma* ATCC 24202 | Sugar-cane juice | Fed-batch | 19.35 | 0.384 | 7.44 |
| *X. dendrorhous* 2A2 N | Sugar-beet molasses | Fed-batch | 36.0 | 1.11[a] | 40.0[a] |
| *X. dendrorhous* TISTR 5730 | Hydrolyzed mustard waste isolate | Batch | 19.6 | 1.31 | 25.8 |

[a] Total carotenoid content (mg/g or mg/l)

## 1.7 Sesame seed (*Sesamum indicm* L.)

One of the most important seed crops for oil production is sesame seed (*Sesamum indicm* L.). Tropical and temperate zones are considered good zones for sesame growth. From early centuries, sesame was cultivated particularly in Africa and Asia. By 2009, the total sesame seed production was around 4 million tons with Asia and Africa were the main contributors to this production representing 62.6% and 33.1%, respectively [48, 49]. In addition to its importance as edible oil, it is also used in the preparation of bakery, confectionery and sweet products [49]. Sesame is considered as a valuable source of nutrition because it contains about 50% protein. And of its high oil production as it contains 48% to 55% [50].

Oil cake is a by-product of oil extraction process. It can be used in animal feeding as a protein source [51].

## 1.8 Objectives

The main objectives of this study are:

1. Reuse sesame cake as a growth medium for *P. rhodozyma* (NCYC874).
2. Evaluate the possibility of astaxanthin production as natural color by using *P. rhodozyma* (NCYC874) grown on sesame cake.
3. Compare astaxanthin production using two systems; batch and fed-batch fermentation.

# Chapter Two
# Materials and Methods

*P. rhodozyma* yeast (NCYC874) was obtained from the National Collection of Yeast Cultures (Norwich, UK) as freeze-dried ampoule. Several sesame cake samples have been collected from local industries. PDB (HIMEDIA, India) and PDA (HIMEDIA, India) were used as control medium. In addition to acetone (Sigma, USA) was used as astaxanthin dissolving solution.

**Table (2) Name of devices used in the study, brand name, model, and country.**

| Instrument | Brand name | Model | Country |
|---|---|---|---|
| Drying oven | Blnder | E 28 | Germany |
| Drying oven digit | Raypa | DAF-78 | Spain |
| Lipid content | ANKOM | XT10 | USA |
| Fiber analyzer | ANKOM | A200 | USA |
| Digestion | Gerhardt | TT152 | Germany |
| Distillation | Gerhardt | 20S | Germany |
| Tissue homogenizer | Heidolph | DIX 900 | Germany |
| Refrigerated incubator | Sanyo | MIR-553 | Japan |
| Shaker water bath | Stuart scientific | SBS30 | UK |
| pH- meter | Jenway | 3310 | UK |
| Balance | Gibertini | EU-500 | Italia |
| Stirring magnetic stirrers | RAYPA | AG-2 | Spain |
| UV/visible spectrophotometer | Jenway | 6705 | UK |
| Microscope | Olympus | CH20 | Japan |

## 2.1 Sesame cake preparation

Sesame oil cake was obtained from the local market of Nablus. Sesame oil cake was macerated by hands, then a cheese cloth was used to remove oil residue by gravitational force overnight. Then, the sesame oil

cake was pressed into 0.5 cm thickness paste using kitchen roller. All pastes were kept at 4 °C for further analysis.

Further characterization of sesame oil cake was done in an average of 10 samples based on procedures of Association of Official Analytical Chemists (AOAC, 1990).

### 2.1.1 Determination of moisture content of Sesame Oil Cake

Metal dishes were placed in the oven (Blnder, Germany) for complete drying at 105 °C for 2 hours. After that, they were transferred by the tong to the desiccator for cooling at room temperature for 15-20 min. Then the dishes were weighed by (Gibertini, Italia) analytical balance as $W_1$. Sesame oil cake samples were placed evenly in the dishes, 5 g of the sample was accurately weighted and recorded as $W_2$. The samples have been dried at 105°C for 16 h. After that, the samples were left for cooling in a desiccators until they reached the room temperature. Eventually, the samples were weighted on analytical balance and the weights were recorded as $W_3$. Moisture content was calculated according to the following equation:

$$\boldsymbol{Moisture\ content}\% = \left\{\frac{\{\boldsymbol{W2} - (\boldsymbol{W3} - \boldsymbol{W1})\}}{\boldsymbol{W2}}\right\}.\boldsymbol{100}$$

### 2.1.2 Determination of Ash content of Sesame Oil Cake

The crucibles were precleaned with 2M HCl and rinsed with distilled water then placed in the muffle furnace (Raypa, Spain) for one hour at 600 °C. After that, the muffle furnace was turned off and crucibles were left in the furnace until the temperature has reached 250 °C. By the tong, the crucibles were transferred to the desiccator to cool at room temperature, and then crucibles were weighed as $W_1$. Five grams of the dry sesame oil cake sample was weighed in the crucibles as $W_2$ and placed in the muffle furnace for 5 hours at 550 °C. The furnace turned off and crucibles were left in the furnace until the temperature reached 250 °C. By the tong, the crucibles were transferred to the desiccator to cool at room temperature, and then crucibles were weighed as $W_3$. Ash content was calculated as follows:

$$\boldsymbol{Ash\ content}\% = \left\{\frac{\{\boldsymbol{W3 - W1}\}}{\boldsymbol{W2}}\right\}.\boldsymbol{100}$$

### 2.1.3 Determination of Lipid content in Sesame Oil Cake

1.5 gram of air dry sesame oil cake sample $W_1$ was used for analysis using filter bag. After that, the filter bags were sealed to encapsulate the sample and were placed in the extraction vessel (ANKOM, USA). Petroleum ether (350 ml) was added to the extraction vessel and the extractor was run for 30 min. At the end of the process, the samples were dried at 102 °C for few minutes to remove the extraction solvent and the

weight of samples was taken as $W_2$. Lipid content was calculated as follows:

$$\boldsymbol{Lipid\ content}\% = \left\{\frac{\{\boldsymbol{W2 - W1}\}}{\boldsymbol{W1}}\right\}.\boldsymbol{100}$$

### 2.1.4 Determination of Fiber content in Sesame Oil Cake

The filter bags were weighed as $W_1$. Then approximately 0.5 gram of oven dried sesame oil cake sample was placed in the filter bags and re-weighed as $W_2$. After that, the filter bags were sealed to encapsulate the sample and placed in a fiber analyzer (ANKOM, USA) in the suspender tray. The suspender tray was lowered in the acid detergent solution; a lightweight was placed on suspender tray to make sure the samples were immersed in the solution. Time was set for one hour and the heat was turned on. The samples were weighed after extraction as $W_3$. Fiber content was calculated as:

$$\boldsymbol{Fibre\ content}\% = \left\{\frac{\{\boldsymbol{W3 - W1}\}}{\boldsymbol{W2}}\right\}.\boldsymbol{100}$$

### 2.1.5 Determination of protein contents in Sesame Oil Cake

Protein content was determined according to the procedure reported by Kjeldahl method (AOAC, 1990). A digestion tube, which contains 0.5 gram of air dry sesame oil cake sample, a little spoon of catalyst and boiling chips, were added to the digestion unit (Gerhardt, Germany) for three hours. In case if the sample was not clear, the tube was returned to the digestion unit for additional one hour. After completing the process, the

sample was left to cool at room temperature. A 250 ml flask containing 25.5 ml of boric acid in addition to digestion tube was placed in the distillation unit (Gerhardt, Germany). The solution was titrated with 0.1M HCl. Protein content was calculated as:

$$Protein\ content\% = \left(\left[\frac{\{\langle ml\ standard\ acid - ml\ blank\rangle \times N.acid \times 1.4007\}}{weight\ of\ sample\ (g)}\right] \times 6.25\right) \times 100$$

## 2.2 *Phaffia rhodozyma* yeast

### 2.2.1 Culture handling and Maintenance

*P. rhodozyma* yeast (NCYC874) was obtained from the National Collection of Yeast Cultures (Norwich, UK) as freeze-dried ampoule. The culture was rehydrated with potato dextrose broth (PDB) in test tubes as selective media and loopfulls were streaked on plates of potato dextrose agar. All tubes and plates were incubated at optimal temperature, which is 20 °C in a refrigerated incubator (Sanyo, Japan) for three days. Then stored at 4 °C after the growth was apparent on the plates. This process was performed 10 times to insure culture purity and cell activity. Maintenance of the culture was carried out every three days using PD broth and every week using PD agar. Purity was further assessed by microscopic examination (Olympus, Japan).

### 2.2.2 Preparation Sesame Oil Cake for yeast growth

Sesame oil cake was prepared as mentioned in section 2.1. SOC was dried in oven (Blnder, Germany) for 16 h at 105 °C. Then, it was ground to fine particles. Mixing percentages of SOC in distilled water were used as follows: 1%, 2% and 5%. The mixtures then were homogenized by tissue homogenizer (Heidolph, Germany) for 6 min at 3 X 100 rpm.

### 2.2.3 Batch culture

*P. rhodozyma* was grown on two types of media using batch culture system. Namely PDB and SOC (2% W/V), PDB was used as control medium. Growth conditions were as the following: 400 ml medium was inoculated by 5% (V/V) ($12*10^6$ c.f.u/mL). Shaker water bath (Stuart scientific, UK) at (100 rpm) was maintained at (20 °C) to control the culture condition. Each run of batch culture contained 4 bottles and lasted for 120 h.

#### 2.2.3.1 Inoculums preparation for batch culture

For every batch, a new inoculum of yeast was prepared as follows: 5 ml of stock yeast culture was transferred to 95 ml fresh medium of PDB and incubated as mentioned in section 2.2.3. Four Duran bottles (500 ml capacity), containing 380 ml either PDB or SOC (2% W/V) were inoculated by 5% (V/V) of the freshly pre-prepared culture.

### 2.2.3.2 Batch culture sampling

Two samples of 25 ml each were taken from the batch culture every 24 h for 5 days to assess growth, pigment production and to insure the purity of the culture.

### 2.2.4 Preparation of Fed-batch culture

A 100 ml capacity fermenter was set up to assess lab scale production of astaxanthin. The growth conditions used for pigment production were based on previous results; temperature was maintained at 20 °C, agitation was conducted by magnetic stirrer, and working volume was 40 ml.

### 2.2.4.1 Inoculums Preparation of Fed-batch culture

A 100 ml containing SOC (2% W/V) was prepared as previously mentioned. Then inoculated by 5% yeast and maintained as mentioned in section 2.2.3. After 72 h 5% (v/v) were transferred to the fermenter, which contained 40 ml.

### 2.2.4.2 Sampling procedure of Fed-batch culture

24 ml represent sixty percent of the fermenter working volume was sampled every 72 h to study growth, pigment production, pH and to examine the culture purity. Then a fresh sterile medium 24 ml of SOC (2% W/V) has been added.

### 2.2.5 pH measurement

pH was measured for all samples using pH meter (Jenway, UK). The pH electrode was washed with distilled water between each sample and dried by tissue for accuracy measurements.

### 2.2.6 Biomass measurement

The biomass was measured as the weight of the difference between Eppendorf tube containing 1ml of PDB or SOC media ($W_1$g), and 1 ml of the sample ($W_2$g). Biomass (g/ml) = ($W_2$g) - ($W_1$g).

### 2.2.7 Cell disruption

Cell disruption was carried out mechanically using magnetic stirrer. To assess the best time and cells to solvent ratio a preliminary test was conducted. Briefly, cells were grown on PDA as previously mentioned then 1g of the cells was scraped out using microscopic slide. The cells were suspended in 10 ml acetone and glass beads (0.5 mm) were added to ease the breakage of cells. The suspension was placed on magnetic stirrer and the stirrer was set at medium speed. A sample of 1ml was taken every 5 min to assess cell disruption under the microscope. After 20 min the suspension was centrifuged at 4100 rpm and 5° C for 15 min. Supernatant containing astaxanthin was measured against blank (acetone) using UV/Visible spectrophotometer (Jenway, UK) at 480 nm wavelength. The result of measurement was calculated according to the following formula:

$Y = (A480 \times \text{ Volume of acetone} \times 10\text{^}6)/(100 \times \text{Volume of culture})*$ ($E_{1\%}$)

Where Y is the astaxanthin per volume of culture (mg/L);

A480 is an absorbance at 480 nm;

$E_{1\%}$ is specific extinction coefficient of astaxanthin in acetone (which is 1600) [52].

# Chapter Three
# Results

## 3.1 Approximate analysis of sesame oil cake

The approximate composition analysis (wet and dry basis) of sesame oil cake is shown in Table 3. Protein represented almost half of SOC, while fiber was 33%, and other competent were minor constituents.

**Table (3) Approximate analysis of sesame oil cake according to the Association of Official Analytical Chemists (AOAC, 1990).**

| Sesame oil cake content | Wet basis (%) ± Standard deviation | Dry basis (%) ± Standard deviation |
|---|---|---|
| Moisture | 30.82 ± 0.749 | - |
| Ash | - | 6.458 ± 0.143 |
| Protein | - | 49.44 ± 3.755 |
| Cured Fat | - | 8.890 ± 1.622 |
| Cured Fiber | - | 33.382 ± 2.174 |
| Carbohydrate by difference | - | 1.829 |

## 3.2 Determination of yeast growth on SOC plates

Growth of yeast using SOC plates is shown on Fig 2. SOC (2% W/V) has shown similar growth to standard medium, hence was chosen for further experiments.

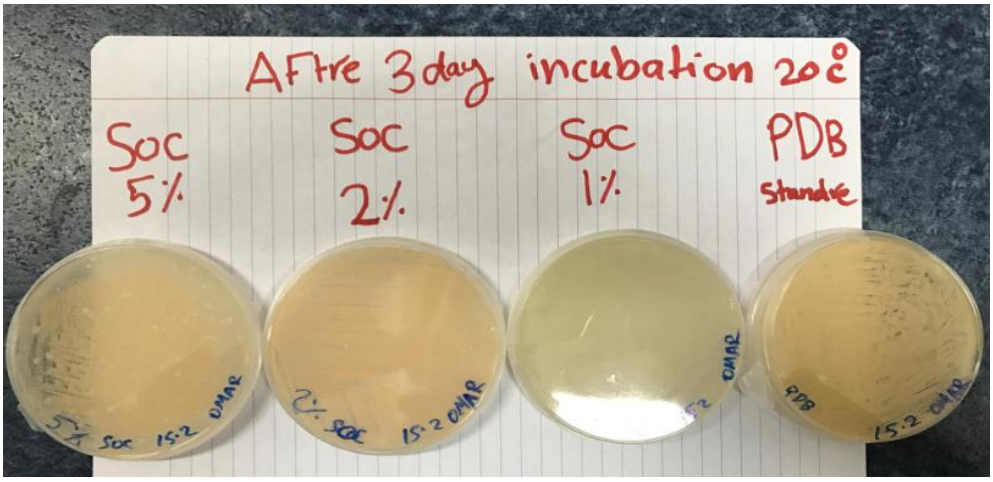


**Figure (2):** Growth of P. rhodozyma on 1, 2 and 5% (W/V) of SOC plates after 3 days of incubation at 20 °C. PDA was used as control for comparison.

## 3.3 Assessing cell disruption

Pictures of cell disruption under the microscope over a 20 min period are shown in Fig 3. Twenty minutes has shown best disruption time with more than 80% cells breakage.

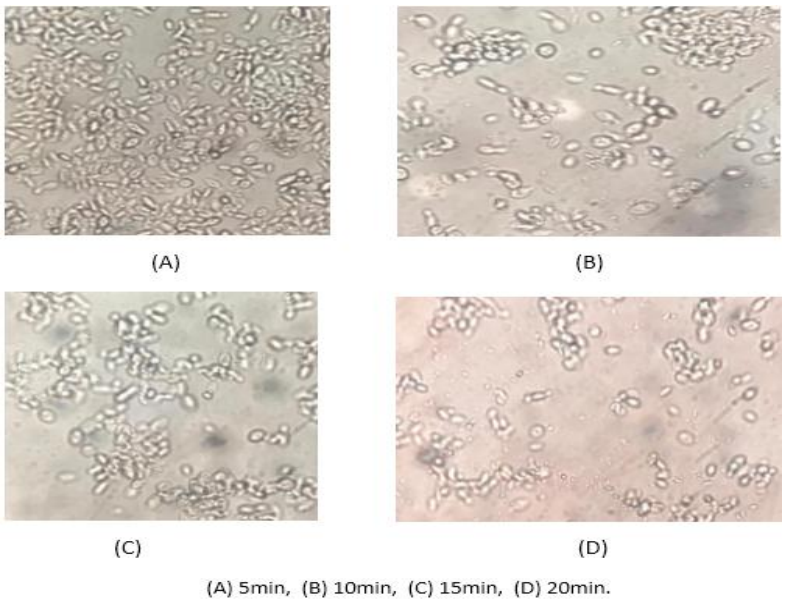


**Figure (3):** Photos of mechanically disrupted cells under the microscope. A: control, B: 10 min, C: 15 min and D: 20 min.

## 3.4 Astaxanthin production and biomass result using PDB and SOC (2% W/V) as a growth media for parent strain *P. rhodozyma* (NCYC874)

### 3.4.1 Biomass of *P. rhodozyma* in PDB and SOC (2% W/V) using batch culture

Total biomass production using both growth media is shown in Fig 4. PDB and SOC (2% W/V) supported production of biomass with maximum production of 5.9± 0.290 and 4± 0.268 g/L respectively. The maximum growth rate was achieved after 72 h for both media. PDB showed higher quantity of cell mass than SOC (2% W/V) by 47%.

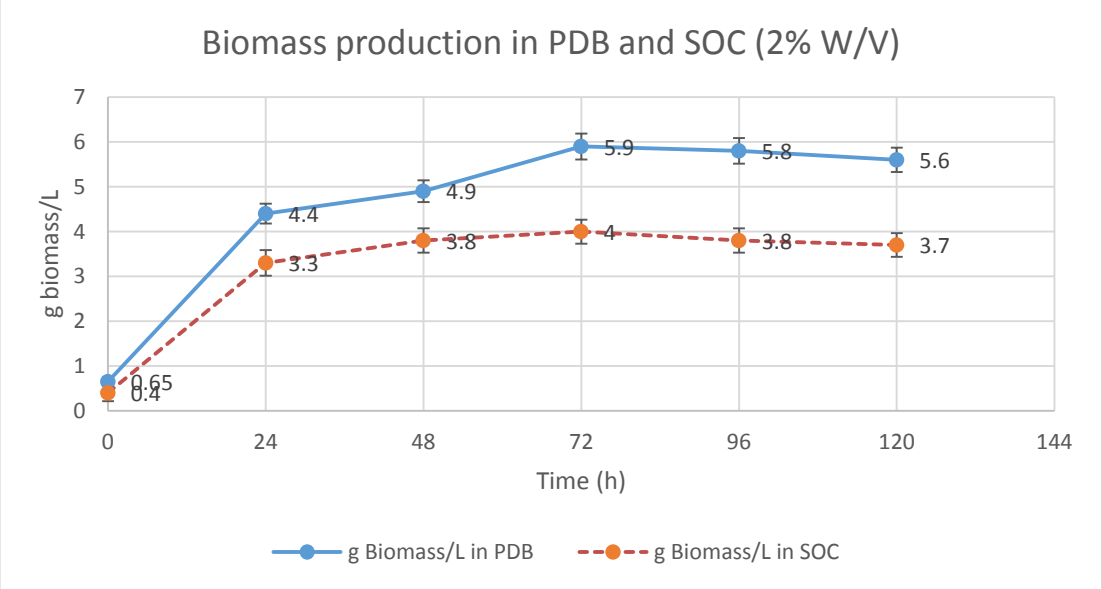


**Figure (4):** Biomass production of *P. rhodozyma* (NCYC874) on batch culture containing either PDB or SOC (2% W/V) for 5 days incubated at 20 °C.

### 3.4.2 Astaxanthin production by *P. rhodozyma* in PDB and SOC (2% W/V) using batch culture

Production of astaxanthin by *P. rhodozyma* is shown in Fig 5. PDB and SOC (2% W/V) supported production of astaxanthin with maximum production of 1.131± 0.039 and 0.854± 0.039 mg/L respectively. *P. rhodozyma* reached such a level after 72 hour in both media. PDB showed higher quantity of pigment than SOC by 32%.

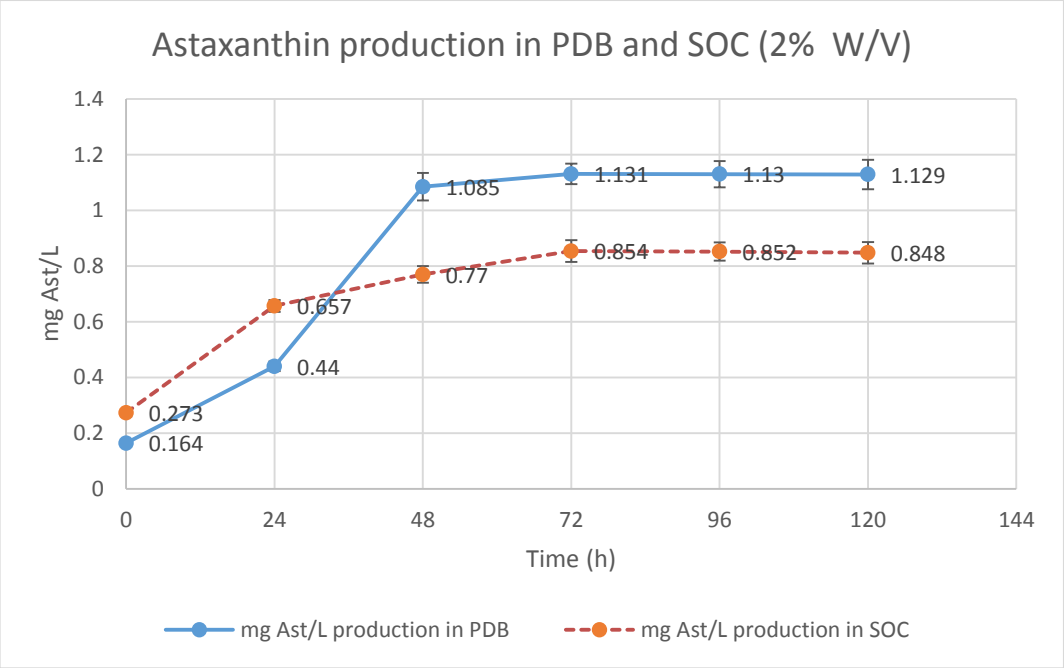


**Figure (5):** Astaxanthin production of parent strain *P. rhodozyma* (NCYC874) on batch culture containing either PDB or SOC (2% W/V) for 5 days incubated at 20 °C.

### 3.5 pH measurement of batch culture

Change in pH in PDB and SOC (2% W/V) batch culture of *P. rhodozyma* are shown in Fig 6. *P. rhodozyma* in PDB decreased pH to 4.13± 0.20 while it was 6.19± 0.29 in SOC (2% W/V).

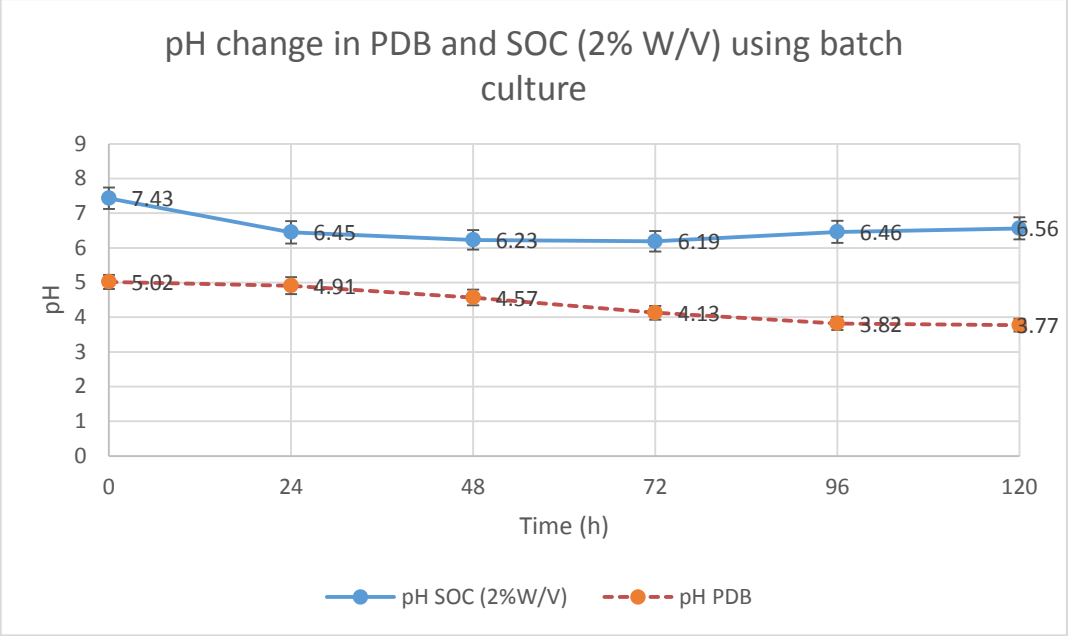


**Figure (6):** pH change in batch culture of *P. rhodozyma* (NCYC874) grown on PDB and SOC (2% W/V) medium.

## 3.6 Cell mass and astaxanthin production by *P. rhodozyma* (NCYC874) on SOC (2% W/V) using fed-batch culture

Production of biomass and astaxanthin using fed-batch system and SOC is shown in Fig 7. The system was left for 72 hours to reach the maximum and feeding procedure was carried out every 72 hours thereafter. An average of production of 12.3± 0.53 g/L and 7.031± 0.285 mg/L was maintained for biomass and astaxanthin respectively.

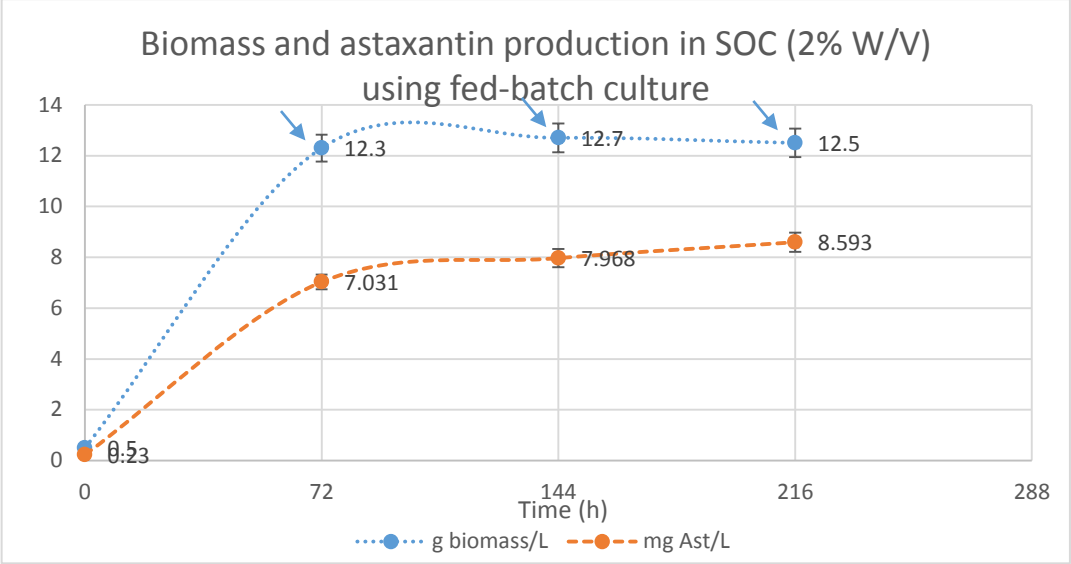


**Figure (7):** Biomass and astaxanthin production by *P. rhodozyma* (NCYC874) in fed-batch culture. SOC (2% W/V) used as growth medium. The arrows indicate the feeding times.

## 3.7 pH measurement of fed-batch culture

Changes of fed-batch culture of *P. rhodozyma* over time are shown in Fig 8. pH has decreased 7.4± 0.25 to 6.11± 0.27 after 144 h and maintained to this value.

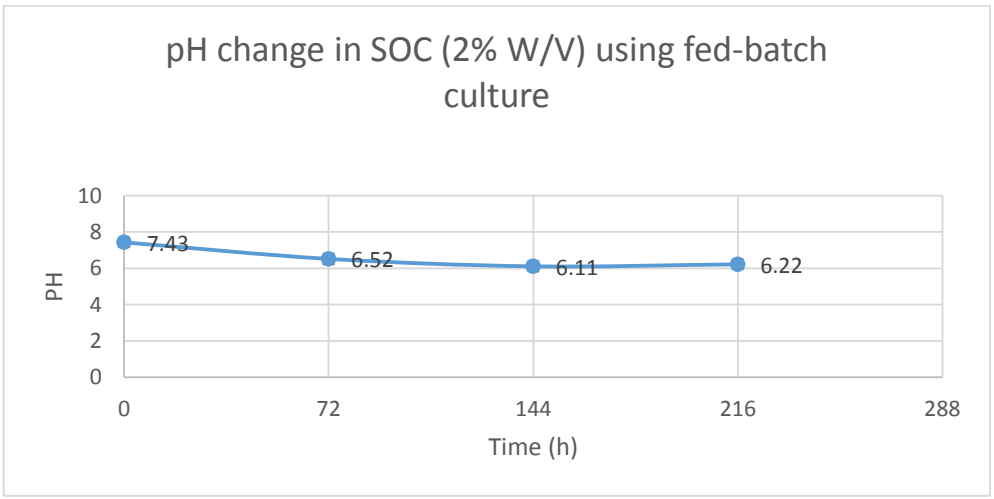


**Figure (8):** pH change by *P. rhodozyma* (NCYC874) grown in Fed-batch culture using SOC (2% W/V) media.

### 3.8 Productivity of astaxanthin under different systems

Productivity of astaxanthin (mg/g biomass) by *P. rhodozyma* (NCYC874) is shown in Table 4. PDB and SOC (2% W/V) at time 72 h have produced 0.19 and 0.21 mg/g biomass respectively under batch culture while it was 0.57 mg/g biomass using SOC (2% W/V) in fed-batch culture, productivity of astaxanthin increases 2.7 folds than the batch culture.

**Table (4) Productivity of Ast (mg/g dry cell mass) after 72 h by P. rhodozyma (NCYC874) in different fermentation systems.**

| Fermentation system | Biomass (g/L) | Astaxanthin (mg/L) | Productivity (mg/g biomass) |
|---|---|---|---|
| Batch culture PDB | 5.9 | 1.131 | 0.19 |
| Batch culture SOC (2% W/V) | 4 | 0.854 | 0.21 |
| Fed- batch culture SOC (2% W/V) | 12.3 | 7.031 | 0.57 |

# Chapter Four
# Discussion

In general, oil cakes contain high amounts of protein, energy and fiber. They produce many benefits when they act as substrate for production of biochemical and organic chemicals by bioprocesses [53].

One of these by-products is sesame oil cake. It has been chosen for many reasons such as availability all year around, price stability, flexibility in application, storage cost, safety factors and transportation cost [54].

The composition of edible oil cake and its nutritional value vary depending on many factors like, extraction method and growing conditions [53]. Sesame oil cake rich in protein which is an important source of nitrogen that is significant for yeast growth and which act as a buffering system while other previously used substrate such as cane molasses, sugar cane juice, corn wet-milling co-products, alfalfa residual juice, grape juice, hydrolyzed peat and raw coconut milk were deficient in protein [31].

However, according to this study, sesame oil cake is poor in simple carbohydrate, rich in complex carbohydrate in the form of fibers while other used substrates were rich in simple carbohydrate and poor in complex carbohydrate. This different composition between our substrates and others allow us to bypass carbtree effect, which inhibit the production of carotenoid and biomass by affecting the pathway of metabolism and producing ethanol and organic acid instead via tricarboxylic acid (TCA) cycle.

The yield of biomass is affected by pH ranging from 3.8 to 7.5 [55]. Fang and Cheng demonstrated that the optimum pH for astaxanthin production was 5 and many other studies founded that the maximum growth rate of the cells also occur at pH 5.8 [56]. Moreover, other studies suggested that the best pH for *X. dendrorhous* strains to grow and produce astaxanthin is 5 [58] [59], while another higher pH like 6.9 is also has been reported for maximum *X. dendrorhous* ATCC 24202 growth in continuous culture [41] while in batch culture the pH was 6 [36]. The difference in yeast strains and basal media have led to difference in the optimum pH.

In this study, pH has an effect on the production of astaxanthin and biomass [59]. End point pH of SOC was higher than pH of PDB, this difference in pH partly due to the buffering capacity of SOC which is more than PDB due to higher protein content. When PDB was used as growth medium for *P. rhodozyma*, pH has reached (4.13± 0.20) after 72 h of incubation and this value of pH encouraged production of astaxanthin (1.131± 0.039 mg/L) more than the production of biomass (5.9± 0.29 g/L). Furthermore, when SOC (2% W/V) was used as growth medium for *P. rhodozyma*, pH has reached (6.19± 0.29) after time 72 h of incubation and this value of pH encouraged production of biomass (4± 0.26 g/L) more than production of astaxanthin (0.854± 0.039 mg/L).

In batch culture, the astaxanthin production in PDB was 32% more than SOC (2% W/V), while the biomass production in PDB growth media was 47% more than SOC (2% W/V). A higher production of biomass in

PDB can be explained by that is a selective media for yeast growth while the SOC contains some inhibitory agents such as oxalate and phytate.

It was observed that when 0.8 g of SOC (2% W/V) was used in fed-batch culture after 72 h, the astaxanthin production was 8.23 folds than 8g of SOC (2% W/V) in batch culture, while the biomass production in fed-batch culture was 3 folds than batch culture. In addition, productivity of astaxanthin (mg/g biomass) in fed-batch after 72 h was 2.7 folds than batch culture. This higher production of biomass and astaxanthin in fed-batch culture referred to better control on temperature and agitation with adding fresh media every 72 h and aeration, which led to better mass conversion efficiency.

The results of this study showed that the productivity of astaxanthin (mg/g biomass) was comparable with other studies that use different strain of *P. rhodozyma* grown on different substrate in fed batch culture. The result of this study was (0.57 mg/g biomass) in *P. rhodozyma* (NCYC874) strain by using sesame oil cake. While the astaxanthin productivity (mg/g biomass) of other strain such as UBV-AX, 2A2N, CBS215-88, 2A2N, 25-2, ATCC24202 were 7.2, 1.64, 1.18, 1.11, 0.618, 0.384 (mg/g biomass) respectively when hydrolyzed corn syrup, corn hydralysate, molasses, sugar peat molasses, date juice of yucca fillifera, sugar cane juice were used as low cost substrate respectively.

To get preliminary view about astaxanthin production, mechanical cell disruption by magnetic stirrer was applied in this study, though it was not the ideal method. Acetone was also used as apolar solvent that showed better ability to dissolve astaxanthin than polar and organic solvents [60].

For improvement of cell disruption process, it is recommended to use French press rather there than an enzymatic process to produce a pure astaxanthin. Moreover, French press method is better than enzymatic process and safer as enzymatic process uses an organic solvent such as dimethyl sulfoxide which is considered as a toxic substance [61].

This research has faced some difficulties and limitations that may influence the results. For example, temperature in the city of Tulkarm was difficult to be at 20 °C especially during summer time. Furthermore, cell disruption process was not very efficient due to unavailability of specialized mechanical cell disruption equipment.

## Conclusion

In conclusion, sesame oil cake has shown a promising natural source to produce biomass and valuable product such as astaxanthin. In addition, its chemical composition is promising not only to provide adequate nutrients to support the growth of *phaffia rhodozyma* but also to produce astaxanthin in effective and low cost way. This can be added value to the sesame oil industries in Palestine.

Furthermore, this study showed that to optimize the condition for astaxanthin production it is recommended to have pH = 4.5 ± 0.2 , temperature 20.0 ±2 °C, Nitrogen concentration 9.8 g\L, carbon concentration 10.17 g\L and inoculum rate of 5% (V/V) and using fed-batch as commercial way of production.

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# تهيئة الظروف لإنتاج الاستازانثن من خميرة فافيا رودوزايما باستخدام كسبة السمسم

**إعداد**

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**د. سامر مدلل**

**قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في التغذية وتكنولوجيا الغذاء، بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.**

**2018**

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## الملخص

**خلفية:** في هذه الأيام هناك ميول واضح ومتزايد نحو استخدام مخلفات الصناعات المختلفة كمصدر قابل للاستعمال في إنتاج الكتلة الحيوية من البروبيوتك وغيرها من الكائنات الحية الدقيقة المفيدة. بناء على ذلك، فقد ساهم هذا الميول الى التقليل من تكلفة الإنتاج والعبء البيئي بفعالية. كسبة السمسم هي واحدة من المنتجات الثانوية لصناعة استخراج زيت السمسم المحلية الناتج من عملية الكبس الميكانيكي على البذور لإنتاج الزيت.

**المواد والطرق:** تم استخدام خميرة فافيا رودوزايما لحقن كسبة السمسم باستخدام نظامين نمو الأول نظام الدفعة الواحدة والثاني نظام عديد الدفعات. تم حساب مكونات كسبة السمسم كل من الرطوبة، البروتين، الدهون، الألياف، الرماد والكربوهيدرات. وتم قياس الكتلة الحيوية، الاستازانثن ودرجة الحموضة ومقارنتها مع محلول دكستروز البطاطا (وسط انتقائي مرجعي).

**النتائج:** أظهرت نتائج الدراسة أن وجود متوسط محتوى الرطوبة لكسبة السمسم في الوضع الرطب 0.749±30.82 % بينما كان متوسط محتوى كسبة السمسم من كل من (البروتين، الدهون، الألياف، الرماد، الكربوهيدرات) في الوضع الجاف 49.44% ±3.75، % 8.890 ±1.622، % 33.382±2.174، 6.458% ±0.143 ، 1.829% على الترتيب.

علاوة على ذلك، كانت نتائج الكتلة الحيوية في كل من محلول دكستروز البطاطا وكسبة السمسم (2% وزن/حجم) في نظام نمو الدفعة الواحدة بعد مرور 72 ساعة 5.9± 0.2غم/لتر، 4± 0.268 غم/لتر على الترتيب. كان إنتاج الاستازانثن في كل من محلول دكستروز البطاطا

وكسبة السمسم (2% وزن/حجم) بعد مرور 72 ساعة 1.131± 0.039 ملغم/لتر، 0.854± 0.039 ملغم/لتر على الترتيب. لكن عند استخدام كسبة السمسم (2%وزن/حجم) في نظام نمو عديد الدفعات كانت نتائج كل من الكتلة الحيوية والاستازانثن بعد مرور 72 ساعة 12.3±0.53 غم/لتر، 7.031± 0.285 ملغم/لتر على الترتيب. انخفضت درجة الحموضة بعد مرور 72 ساعة في نظام نمو الدفعة الواحدة كل من محلول دكستروز البطاطا وكسبة السمسم (2% وزن/حجم) بقيمة 0.89، 1.24 على الترتيب. لكن انخفضت في نظام نمو عديد الدفعات عند استخدام كسبة السمسم (2% وزن/حجم) بقيمة 0.91.

في الختام، أوضحت نتائج الدراسة أن التركيب الكيميائي لكسبة السمسم يعد بإيجاد ظروف مثالية لنمو خميرة فافيا رودوزايما لإنتاج الكتلة الحيوية وصبغة الاستازانثن كبديل طبيعي للصبغات الصناعية التي لها مخاوف صحية كبيرة.