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Ph. D. In Food Engineering

DERYA DURSUN

REPUBLIC OF TURKEY

GAZİANTEP UNIVERSITY

GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES

PRODUCTION OF ASTAXANTHIN PIGMENT FROM CEREAL AND LEGUME WASTES BY SOLID STATE FERMENTATION AND TECHNO-ECONOMIC ANALYSIS BY SIMULATION

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BY

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Supervisor

Assoc. Prof. Dr. Ali Coşkun DALGIÇ

By

Derya DURSUN

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Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. A. Necmeddin YAZICI
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Medeni MASKAN
Head of Department

This is to certify that we have read this thesis and that in our consensus opinion it is fully adequate, as a thesis for the degree of Doctor of Philosophy.

Assoc. Prof. Dr. Ali Coşkun DALGIÇ
Supervisor

Examining Committee Members:	Signature
Prof. Dr. Filiz ÖZÇELİK	.....
Prof. Dr. Canan CAN	.....
Prof. Dr. Mustafa BAYRAM	.....
Prof. Dr. Hasan VARDİN	.....
Assoc. Prof. Dr. Ali Coşkun DALGIÇ	.....

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Derya DURSUN

ABSTRACT

PRODUCTION OF ASTAXANTHIN PIGMENT FROM CEREAL AND LEGUME WASTES BY SOLID STATE FERMENTATION AND TECHNO-ECONOMIC ANALYSIS BY SIMULATION

DURSUN, Derya
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In this thesis, it was aimed to produce astaxanthin pigment from wheat, rice and lentil wastes by applying solid state fermentation with *Yamadazyma guilliermondii* ATCC 90197, *Yarrowia lipolytica* ATCC 24060, *Xanthophyllomyces dendrorhous* ATCC 24202 and *Sporidiobolus salmonicolor* ATCC 24259 yeasts. Box-Behnken experimental design matrix consisting 17 runs was created with temperature, pH and moisture content parameters for each waste and yeast fermentation system. The experimental results were modeled and optimized to obtain the highest astaxanthin amount by using response surface methodology.

Maximum astaxanthin yield as 109.23 μg astaxanthin/g dry waste with a 96.71 % antioxidant capacity was produced using wheat bran with *X. dendrorhous* at the temperature of 20 °C, pH of 3.5 and 90 % of moisture content. The results of the optimum fermentation conditions were verified by the experimental and statistical analyses.

Kinetic study was performed for the optimum system by the measurements of product formation, substrate consumption and microbial growth parameters. The yield coefficients in batch systems were calculated to determine the productivity.

A plant design for the optimum system by using SuperPro Designer simulation program was proposed and economic parameters were analyzed. For the base case, the total capital investment for the plant was estimated at $22,308,000. The payback period of astaxanthin production was calculated as 4.92 years. The capacity of 4,799.91 kg/batch was calculated as the break-even point for the astaxanthin production. Economic evaluations demonstrated that the process was a feasible alternative to produce astaxanthin from wheat bran by using *X. dendrorhous.*

Key words: astaxanthin, solid state fermentation, response surface methodology, kinetic, simulation

ÖZET

HUBUBAT VE BAKLİYAT ATIKLARINDAN ASTAKSANTİN PİGMENTİNİN KATI HAL FERMENTASYONU İLE ÜRETİMİ VE SİMULASYON İLE TEKNO-EKONOMİK ANALİZİ

DURSUN, Derya
Doktora Tezi, Gıda Mühendisliği
Tez Danışmanı: Doç. Dr. Ali Coşkun DALGIÇ
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Bu tezde, buğday, pirinç ve mercimek atıklarından *Yamadazyma guilliermondii* ATCC 90197, *Yarrowia lipolytica* ATCC 24060, *Xanthophyllomyces dendrorhous* ATCC 24202 ve *Sporidiobolus salmonicolor* ATCC 24259 mayaları ile katı hal fermantasyonu uygulayarak astaksantin pigmentinin üretimi amaçlandı. Atık ve maya fermantasyon sistemi için sıcaklık, pH ve nem içeriği parametreleriyle 17 deney içeren Box-Behnken deneysel tasarım matrisi oluşturuldu. Deneysel sonuçlar, yüzey cevap metodolojisiyle en yüksek astaksantin miktarını elde etmek için modellendi ve optimize edildi.

Buğday kepeği kullanılarak *X. dendrorhous* tarafından 20 ° C sıcaklık, pH 3.5 ve % 90 nem içeriğinde 109.23 µg astaksantin/g kuru atık üretildi. Optimum fermantasyon koşullarının sonuçları deneysel ve istatistiksel analizlerle doğrulandı.

Optimum sistem için ürün oluşumu, substrat tüketimi ve mikrobiyal büyüme ölçümleri ile kinetik çalışmalar gerçekleştirildi. Verimliliği belirlemek için batch sistemlerinde verim katsayıları hesaplandı.

SuperPro Designer simülasyon programı ile optimum sistem için bir tesis tasarımı önerildi ve ekonomik parametreler analiz edildi. Temel alınan durum için tesisin toplam sermaye yatırımı 22.308.000 $ olarak belirlendi. Astaksantin üretiminin geri ödeme süresi 4,92 yıl olarak hesaplandı. Astaksantin üretmek için kırılma noktası 4.799,91 kg/batch olarak hesaplandı. Ekonomik değerlendirmeler sonucunda, prosesin *X. dendrorhous* ile buğday kepeğinden astaksantin üretimi için uygulanabilir bir alternatif olduğu ortaya çıktı.

Anahtar kelimeler: astaksantin, katı hal fermantasyonu, yüzey cevap metodu, kinetik, simülasyon

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TABLE OF CONTENT

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

NOMENCULATURE

ATCC	American Type Culture Collection
AX	Astaxanthin
BBD	Box-Behnken Design
C.N.	Cell Number
DCM	Dichloromethane
DFC	Direct Fixed Cost
DPPH	1,1-diphenyl-2-picrylhydrazyl
gdw	gram dry waste
IR	Inoculation Ratio
IRR	Investment Rate of Return
MC	Moisture Content
MP	Main Product
MPC	Main Product Cost
NPV	Net Present Value
O.D.	Optical Density
OLC	Operating Labor Cost
PAX	Powdered Astaxanthin
PBT	Payback Time
PC	Purchased Equipment Cost
PSS	Physiological Salt Solution
Q	Production Capacity
RM	Raw Material
RMC	Raw Material Cost
ROI	Return on Investment
RSM	Response Surface Methodology
S.A.	Scavenging Activity
SAM	Steepest Ascent Method
SmF	Submerged Fermentation

SSF	Solid State Fermentation
TCI	Total Production Cost
UIC	Unit Investment Cost
UPC	Unit Production Cost
V.C.	Viable Cell

CHAPTER 1

LITERATURE REVIEW

1.1 Fermentation Technology

Fermentation is a process where, when and how started is unknown as written record. Fermentation has been applied as a food preservation technique since ancient times. Products obtained after fermentation and their properties such as nutrition, organoleptic, functionality, uniqueness and textural etc. has created another purpose for fermentation; transforming/converting or producing a new product in terms of a definition and/or property. Characteristics of raw material, technique and last product lead to varied technological applications of fermentation. Particularly, physical form of raw material brings about solid and liquid systems. These systems take a major place in those applications. Solid state fermentation (SSF) and submerged fermentation (SmF) are declarative names of solid and liquid systems, respectively. These biotechnological systems require microorganisms in order to obtain a target product.

SmF is applied to liquid based media such as synthetic and waste sources. Molasses, fruit-vegetable juices, waste water, liquefied or soluble media are common SmF sources (Nigam and Pandey, 2009; Panesar et al., 2015). In the event that product requires a liquid medium or microorganism needs high moisture amount, SmF system is used (Panesar et al., 2015).

SSF is a fermentation technique which is carried out with microorganism (s) growing on nutrient colonized or impregnated solid substrate with very little or no free water (Pandey et al., 2000; Krishna, 2005; Couto, 2008; Nigam and Pandey, 2009). As it understood from the definition, there are two materials to be used in SSF system. Inert or synthetic material is used by microorganisms to hold on. Polyurethane,

amberlite, inert fibers, resins, sugar cane bagasse and vermiculate are several examples for inert materials. They are impregnated with liquid nutrients such as synthetic media, sugar and organic solutions. Whereas performing easier experimental and kinetic studies, providing improvements for controlling and monitoring of the system and presenting easier and cheaper downstream processes are among various advantages of using inert materials, they have economical disadvantages. The other one, non-inert or organic material acts as support supplies and nutrient source why it is also called as support-substrate (Pandey et al., 2000; Pandey, 2003; Pérez-Guerra et al., 2003; Couto, 2008). Bhargav et al. (2008) have indicated that inert materials are rarely practiced. Agricultural products (cereals, potato, soybean, cassava and grains etc.) and agro-industrial wastes (agricultural wastes or biological wastes) coming from industries of cereal, fruit-vegetable, agricultural residues, dairy and miscellaneous could be used as support-substrate (Pandey, 2003; Pérez-Guerra et al., 2003; Panesar et al., 2015). Advantages of the support-substrates are; (i) they may be used as alternative substrates, (ii) agro-industrial wastes may solve environmental and disposal problems of the wastes, (iii) they -particularly the wastes- are cheap and present a cost effective process. Agro-industrial wastes are more useful and sufficient sources considering the handling pollution issues, low process investments and costs and simple treatments (Pandey et al., 2000; Nigam and Pandey, 2009; Panesar et al., 2015).

Because of low productivity, high operation and downstream processing costs, SmF is not appropriate industrially (Uyar and Baysal, 2004; Couto and Sanromán, 2006). SSF has low contamination risk and higher yield for the same product produced by SmF, requires low-cost materials, low energetic issues and labor, needs minimal or no pretreatment and provides a suitable environment for growth and metabolic activities of microorganisms. Additionally, SSF does not require complex mechanical and controlling systems (Pérez-Guerra et al., 2003; Couto and Sanromán, 2006; Couto, 2008; Yang, 2007, Nigam and Pandey, 2009). Differences according to some features in SSF and SmF are shown in Table 1.1 (Rao, 2010).

However, SSF has several disadvantages such as difficulty of: heat and mass transfer; biomass determination; monitoring fermentation parameters; agitation, controlling of the reaction; operation in large-scales, requirement of high inoculum

volume and fermentation time (Pérez-Guerra et al., 2003; Couto and Sanromán, 2006; Couto, 2008).

Table 1.1 Basic differences between SSF and SmF

Characteristic features	**SSF**	**SmF**
Condition of microorganisms and substrate	Static	Agitated
Water availability	Limited	High
Oxygen supplementation	Diffusion	Bubbling
Contact with oxygen	Direct	Dissolved
Energy requirements	Low	High
Pollution problem	Low	High

There are many studies on SSF, which focusing on progressing the system (Pandey, 2003). Appropriate substrate and microorganism selection, optimization of fermentation parameters, purification of product and genetic modifications are the main subjects for developing SSF (Pandey et al., 2000; Pandey, 2003; Vissier et al., 2003).

Substrate selection

Although it is hard to decide what kind of a material to be used for fermentation system, substrate should have suitable properties in terms of size, shape, anchorage, technical, nutrition, disposability and economics to be utilized by the selected microorganism and for the production of target product. Support-substrate materials are the first and most used substrate types for SSF systems. Table 1.2 shows the reasons of fermentation material selection (Pérez-Guerra et al., 2003; Pandey, 2003; Nigam and Pandey, 2009).

Table 1.2 Considerations of fermentation materials

Availability	Consistency of nutritional quality
Cost per unit of nutrient	Flexibility in application
Transportation cost	Rheological properties
Price stability	Surface tension factors
Pre-treatment costs	Product recovery impact
Stabilization costs	Process yield
Storage costs	Product concentration and type
Safety factors	Overall productivity

Microorganism selection

SSF processes can be placed in two main classes based on the type of microorganism involved for targeted product: *Natural (Indigenous) SSF*: carried out by mixed cultures in which several microorganisms show symbiotic cooperation, *Pure culture SSF*: known purified microorganisms are used in such processes either singly or in mixed culture. A pure culture is necessary in industrial SSF processes for improved rate of substrate utilization and controlled product formation (Bhargav et al., 2008; Nigam and Pandey, 2009).

Optimization of fermentation parameters

Temperature, moisture content, particle size and composition of the substrate, agitation, aeration rate, pH, nutrient supplementation, inoculation rate and pre-treatments are fundamental parameters for SSF technique. Among the parameters, temperature, moisture content and pH are the most important parameters for the technique (Laufenberg et al., 2003; Pérez-Guerra et al., 2003; Pandey, 2003; Bellon-Maurel et al., 2003; Longo and Sanromán, 2006, Nigam and Pandey, 2009). The parameters have crucial importance to provide the growth of microorganism and biosynthesis of the product. Optimization of the fermentation parameters and their levels numerically is necessary to have a standard process and unique product, to improve the fermentation conditions economically, to develop the system, to enable scaling-up industrially and moreover, to provide convenience to study kinetics of the process and simulation.

Purification of product

Isolation and purification treatments are fundamentally depended on substrate type. The structure of the substrate material used in SSF system affects cost, simplicity, time, method selection of the treatments. The material which used only for microorganism anchorage provides cheaper and easier purification. Organic materials may cause some difficulties due to their possessive of soluble items (Pandey, 2003). In addition to substrate type, target product is also determinative for the treatments. Sensitivity, importance, specific method/equipment/supplement requirement and physicochemical properties of the product should be considered.

Genetic modifications

The topic is directly related to microorganism to be selected for SSF systems which use pure culture. The modifications are operated to improve skill of microorganism to produce target product, to provide adaptation of it to medium and finally to increase productivity.

1.2 Cereal and Legume Wastes

Wheat, corn (maize), rice, barley, oats, rye and sorghum as major cereals; in order of wheat, corn and rice as three largest crops are cultivated in the world. 50 % of world cereal production belongs to wheat and rice which are the most important crops in whole world with regards to easy cultivable, taking a very big part of human and animal diets, containing number of essential nutrients and potentially for producing many types of products etc. (Arvanitoyannis, 2010). Lentil is one of the world's oldest cultivated plants and also one of the most edible legumes. It plays an important role in human diet and animal feed due to its rich nutrition content, especially in terms of protein (Togay and Engin, 2000; Erskine et al., 2009). When it is mixed with rice and wheat, it forms a complete protein diet (Erskine et al., 2009).

The wastes of cereals and legumes are obtained after post-harvesting operations and processing of them industrially. Straw, husk, bran, grits and meals are qualified as agricultural wastes (or crop residues) come out from cereal industry which is an important column of agro-industrial wastes. There is less known about legume

wastes (López et al., 2005; Panesar et al., 2015). Pandey et al. (2000) have reported that wheat bran is on the first rank among all the agro-industrial wastes.

Waste treatment

There are several methods for the agro-industrial waste treatment including composting, pyrolysis, combustion, gasification, landfilling and animal feed etc. (Arvanitoyannis, 2010). Combustion, animal feed and leaving on fields for decay are the most applied treatment methods. Green-house gases causing global warming are formed after the combustion. Just in Spain, combustion of the cereal and legume wastes causes the formation of 11 Tg CO_2, 23 Gg nitrogenous compounds and 80 Gg particles (1 Teragram=10^9 kg, 1 Gigagram=10^6 kg) and releasing of them to the atmosphere (Lopez et al., 2005; Zárate et al., 2005; Couto, 2008). Instead of using rich nutrition content (fatty acids, vitamins, diet fiber and amino acids etc.) of the wastes for animal feed, it can be used for the production of useful, new and natural characterized products. Bio-processes are alternative and good way of utilization of the wastes, not just owing to their rich organic content but also for good anchorage feature, easy accessibility, abundantly available and low cost what kind of properties SSF system requires (Pandey et al., 2000; Couto and Sanromán, 2006; Couto, 2008). SSF is uniquely suited to produce value-added products from the wastes such as organic acids, food pigments, stabilizers, antibiotics, polymers, enzymes, aroma compounds, bio-fuels, bio-surfactants, preservatives and filing materials. (Pandey et al., 2000; Laufenberg et al., 2003; Mitchell et al., 2006; Couto, 2008; Nigam and Pandey, 2009). Solid state fermentation of the wastes is a highly attractive process to study due to providing environmental solutions, technique performance, wide usage and application areas, feasibility, low cost and labor and product manifoldness.

1.3 Pigments

Color of food products as a result of their natural structure, processing and addition as an ingredient is a main and significant factor that appeals to visual and chemical sense of humans. Color of food products are related to the product quality and acceptability by the consumers. On the other hand, natural characterization of color may cover the demand of healthy and safe food products. Color of food products may arise from a chemical compound which is called ‘colorant’ or ‘pigment’

indicated by cells or tissues. Pigment concept is exhibited by plant, animal and microorganisms which are sources of natural pigments (Table 1.3). Toxic and carcinogenic effects of pigments produced synthetically reveal that the importance of natural raw materials and production techniques (Joshi et al., 2003; Dufossé et al., 2005; Gupta et al., 2011; http://dravyagunatvpm.files.wordpress.com).

Table 1.3 Colors of several natural pigments

Colors	**Pigment name**
Blue	indigoidine
Blue-reddish	anthocyanin
Bluish-red	beet powder, carmine
Orange	annatto, β-carotene, canthoxanthin, paprika, cochineal, sandal wood, monascus pigments, torularhodin
Yellow	β-carotene, saffron, crocin, lucin, turmeric, riboflavin, safflower, zeaxanthin, xanthomonadin
Red	canthoxanthin, paprika, astaxanthin, prodigiosin, monascus pigments, anthraquinone, lycopene, rubrolone, torularhodin
Green	pyocyanin blue
Black	melanin
Pink	astaxanthin, monascus pigments, canthoxanthin

1.3.1 Production Methods

Chemical synthesis, extraction from plants (flowers, seeds, roots etc.) and animals (female insects like *Coccus cacti*) and bio-production (biotechnological production by microorganisms) are the methods for pigment production. Synthetic and unnatural products are obtained from chemical synthesizing. However, the chemical method could be resulted in undesirable products, needs selective substrates or components and is unsuitable for natural and/or functional labeling. Pigments extracted from plants and animals are qualified as natural. Amount of the pigments depends on seasonal, climatic, geographical and diseases which are not controllable. Additionally, limited color range are obtained, physical properties (like stability

against heat and oxygen) of the pigments could not be appropriate for all applications and the process could have high purification costs and low productivity (Joshi et al., 2003; Nigam and Pandey, 2009; Gupta et al., 2011).

Microbial pigments are characterized as ‘natural’ and both the pigments itself and the production method submit many advantages that the interest of manufacturers and scientists has been rising by these advantages (Joshi et al., 2003; Nigam and Pandey, 2009; Gupta et al., 2011). The pigments produced biotechnologically:

- provide a wide color range to beverages and foods,
- present convenience to all process applications,
- resist to chemical and physical conditions,
- present favorable and beneficial effects on health,
- enable products to be labeled natural,
- have a wide usage area.

Bio-production method:

- is independent from weather conditions,
- is simple and efficient operation,
- provides safe, easy and fast production,
- propounds controllable and predictable production model,
- enables the agro-industrial wastes to be utilized,
- permits a viable medium for a large microorganism group.

1.3.2 Usage Area and Marketing

In addition to supplying main food necessities for consumers, food industry should reply to expectations of quality, price and other demands. Healthy, natural, safe food choice of consumers, thinking ‘natural is best’ causes the awareness of them about ‘natural’ inscription on the labels of food products. Increasing consuming consciousness provides an increasing on the demand of natural food products and concentrating of the industry on the food manufacturing by this way. Food ingredients may be used for many aims such as stabilization, deodorizing, color or

odor enhancement, acidification, preservative, gelation and sweetening, or for a necessity of food processing. Natural characteristic of food ingredients has an important place in food safety and quality, and is directly related production method and raw material used. Pressure of consumers on food industry in order to manufacture natural characterized products brings the market about an expansion internationally. It is reported that there was $4 billion market in 2000 just in USA (Nigam and Pandey, 2009).

Natural pigments have a wide industrial usage area like food, feed, cosmetic, pharmacy, textile and printing. Additionally, they are used in processed food product such as Eastern Asian foods like rice dishes, breakfast cereals, fishes, pastas, sauces, canned foods, candies, confectionary products, snacks, soups, salad dressings, baby foods, cheeses, bakery goods, fruit juices, ice-creams, desserts, margarines and milk products (Joshi et al., 2003; Dufossé et al., 2005; Nigam and Pandey, 2009; Gupta et al., 2011, Carle and Schweiggert, 2015; http://dravyagunatvpm.files.wordpress.com). There is a remarkable progress about natural pigments in global food coloring agent market, even if the market is mostly based on synthetic ones. In 2000, $400 million (mn) of $940 mn market belonged to synthetic pigments whereas $250 mn for natural pigments were. $35 mn market in 1987 of natural pigments reached to $250 mn in 2000 by a 200 % increasing in the global color market consisting of USA and Europe as the biggest participants (Nigam and Pandey, 2009). Moreover, Gupta et al. (2011) have recently informed about $1.2 billion global food color market which has a 31 % proportion of natural pigments. The boom in the global market and numerous researches point out that there is great economic potential of natural pigments, particularly of produced by microorganisms (Joshi et al., 2003; Nigam and Pandey, 2009; Gupta et al., 2011; Panesar et al., 2015).

1.3.3 Pigment Producer Microorganisms

Fungi, yeasts, bacteria and algae, primarily *Monascus, Blakeslea, Mucor, Dunaliella, Xanthophyllomyces, Flavobacterium*, are used for bio-production of the pigments (Joshi et al., 2003; Nigam and Pandey, 2009). Table 1.4 shows the pigment colors produced by different types of microorganism groups (Joshi et al., 2003; Dufossé et al., 2005; Dufossé, 2006; Gupta et al., 2011). Fungi and yeasts are the most suitable

microorganism groups for SSF system whereas bacteria are not because of their high water activity requirement (Pandey, 2003; Panesar et al., 2015). It is concluded that yeasts are good sources for the production of the pigments due to their physiological properties such as being unicellular and rapid growth rate on media comparing with fungi and bacteria (Joshi et al., 2003; Panesar et al., 2015). Notably focused on yellow and red, and blue are the major pigments which are produced by the microorganisms (Gupta et al., 2011). Different species of yeasts such as *Rhodotorula* spp., *Sporidobolus* spp., *Xanthophyllomyces dendrorhous* (anamorph of *Phaffia rhodozyma*) are capable of accumulating yellow and red pigments (Nelis and De Leenheer, 1991; Joshi et al., 2003; Nigam and Pandey, 2009; Gupta et al., 2011; Panesar et al., 2015).

Table 1.4 Colors of pigments produced by microorganisms

	Microorganisms			
Pigments	**Bacteria**	**Molds**	**Yeasts**	**Algae**
Black			*Saccharomyces neoformans* var. *nigricans*	
Blue	*Corynebacterium insidiosum*, *Streptomyces* sp.			*Spirulina*
Bluish red	*Rhodococcus maris*			
Bronze		*Helminthosporium* sp.		
Brown	*Bacillus* sp.		*Yarrowia lipolytica*	
Creamy	*Achromobacter*	*Blakeslea trispora*		
Dark red	*Bradyrhizobium* sp.	*Cordyceps unilateralis*, *Aspergillus glaucus*		
Deep blood		*Cordyceps unilateralis*		
Grayish	*Pseudomonas aeruginosa*			
Grayish-creamish	*Corynebacterium michigannise*			
Green	*Pseudomonas aeruginosa*			
Orange	*Brevibacterium* sp.,	*Blakeslea trispora*,	*Rhodotorula* spp.	*Dunaliella*

	Bradyrhizobium sp.	*Aspergillus* sp., *Monascus* sp., *Penicillium cyclopium, Mucor circinelloides, Neurospora crassa, Fusarium sporotrichioides, Phycomyces blakesleeanus*		*salina*
Pink	*Paracoccus carotinifaciens, Agrobacterium aurantiacum*	*Monascus roseus*	*Xanthophyllomyces dendrorhous*	
Purple	*Janthinobacterium lividum*			
Red	*Agrobacterium aurantiacum, Serratia marcescens, S. rubidaea, Rugamonas rubra, Streptoverticillium rubrirericuli, Vibrio gaogenes, Alteromonas rubra, Streptomyces* sp., *Paracoccus carotinifaciens*	*Pacilomyces farinosus, Blakeslea trispora, Aspergillus* sp., *Helminthosporium* sp., *Monascus* sp., *Penicillium oxalicum, Fusarium sporotrichioides*	*X. dendrorhous, Rhodotorula* spp., *Cryptococus* sp.	*Haematococcus pluvialis, Dunaliella salina, Porphyra, Porphyridium*
Yellow	*Staphylococcus aureus, Pseudomonas* sp., *Xanthomonas oryzae, Streptomyces* sp., *Flavobacterium* sp., *Brevibacterium* sp., *Paracoccus zeaxanthinifaciens*	*Blakeslea trispora, Ashbya gossypii, Monascus* sp., *Penicillium nalgeovensis, Phycomyces blakesleeanus, Mucor circinelloides, Neurospora crassa, Fusarium sporotrichioides*		

1.3.4 Carotenoids

Carotenoids introduce an extensive hue from yellow to red and very important one of the major pigments (Nelis and De Leenheer, 1991; http://foodwaste-fruit.tripod.com/id3.html). Carotenoids chemically belong to C_{40} tetraterpenoid compounds that includes of 8 isoprene molecules and 40 carbon atoms. Xanthophylls (oxygen carrier like lutein) and carotenes (no oxygen like lycopene) are two main subgroups of carotenoids whether owing a functional component or not (Saini et al., 2015). Xanthophylls containing epoxy, hydroxyl, keto, methoxy and carbonyl groups are substituted derivatives whereas carotenes are un-substituted derivatives of carotenoids (Nelis and De Leenheer, 1991). Polyene (poly un-saturated organic compounds) system builds their molecular structure and is determinative for chemical and physiological properties which are related with type and functionality (Higuere-Ciapara et al., 2006; Saini et al., 2015). Biological functionality of carotenoids are stated by depending on including a polar molecule (eg., epoxy, keto and hydroxyl) (Saini et al., 2015) and provides their addition to food products (Valduga et al., 2009a). Bioaccessibility (a part of bioavailability) and bioavailability could be named the most critical functions of carotenoids and affected by several factors; carotenoid type, amount of carotenoid, linkages at molecular levels, location in source matrix, effectors, nutrient status, genetics, host-related factors and interactions. Action mechanisms of carotenoids are mainly about antioxidant capacity and the others cell cycle, cell-cell communication, cytotoxicity and apoptosis, which have become an important investigation subject due to their effects on cell life (Amorim-Carrilho et al., 2014). Biological and functional properties of carotenoids indicate the usage as a food coloring agent and/or nutritional supplement which have a $935 mn global market during 2005 (Nelis and De Leenheer, 1991; Hernández-Almanza et al., 2014). β-carotene, astaxanthin, lycopene and canthaxanthin are examples permitted for food colorant and animal feed (Nelis and De Leenheer, 1991). On the other hand, lycopene, β-carotene, astaxanthin, fucoxanthin, zeaxanthin, lutein and β-crptoxanthin are the most important ones among 700 carotenoids exist in nature (plants, roots, seeds etc.) (Amorim-Carrilho et al., 2014; Saini et al., 2015).

The effects on metabolic and cardiovascular diseases, eye and skin, tumors, nutrition supporting, teeth and bones, immune system, cancer (e.g., breast, lung and gastrointestinal), infections and oxidative stress are remarkable benefits of carotenoids for human health declared in many searches (Nelis and De Leenheer, 1991; Valduga et al., 2009a; Dufossé et al., 2005; Jomova and Valko, 2013; Hernández-Almanza et al., 2014; Amorim-Carrilho et al., 2014; Saini et al., 2015).

1.3.5 Astaxanthin

Chemistry

Orange-red carotenoids which are more valuable than yellow ones arouse interest of researchers and industry, although yellow carotenoids are the most popular, investigated, produced industrially and used in varied areas (Nelis and De Leenheer, 1991). Astaxanthin is a red color xanthophyll carotenoid which has $C_{40}H_{22}O_4$ formula and 596.34 g/mol molecular weight. Fat soluble astaxanthin is both lipophilic and hydrophilic. It can dissolve in solvents such as acetone, methanol, dichloromethane, oils and acids. Figure 2.1 depicts that astaxanthin has two terminal rings that each ring consists one oxygen and hydroxyl molecules. Physically, astaxanthin may be in four different forms; free, esterified, stereoisomer and geometric isomer. All forms exist in nature. Esterified form is consisting of mono- and di- esters which are formed by reaction of hydroxyl and fatty acids. Stereoisomers, [*3S, 3'S*] and [*3R, 3'R*] are configurationally isomers and the most abundant astaxanthin form in nature. Position of double bonds in polyene chain is determinative for geometric isomers which have *cis* and *trans* forms. On the other hand, depending on the origin of astaxanthin there are three main groups (Higuere-Ciapara et al., 2006; Ambati et al., 2014):

- free,
- esterified, with fatty acids (oleic, estearic etc.), eg., astaxanthin produced by algae,
- complex, with a chemical molecule, eg., lipoprotein (carotenolipoprotein) and protein (carotenoprotein).

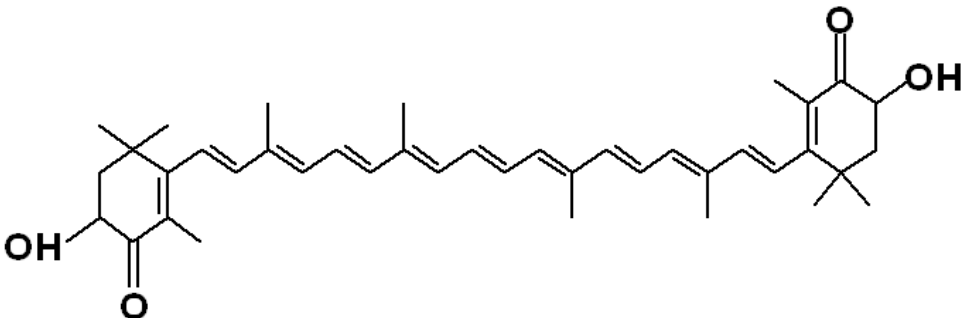

Figure 1.1 Structure of astaxanthin *(3,3'-dihydroxy-β,β-carotene-4,4'-dione)*

Benefits

Astaxanthin contains double bonds, hydroxyl and keto groups that settle the biological functions of it. For example, centered position of double bonds constitutes very strong antioxidant property of astaxanthin. Thus, it neutralizes free radical compounds and transforms them to more stable form (Higuere-Ciapara et al., 2006; Ambati et al., 2014). Antioxidant effect is strikingly bigger than other antioxidant compounds like vitamin E (500 times) and several carotenoids such as β-carotene, lutein and zeaxanthin (10 times) (Naguib, 2000; Dufossé et al., 2005; Higuere-Ciapara et al., 2006). Miki (1991) has entitled astaxanthin as 'super vitamin E'. Localization of astaxanthin in cell membrane system increases its antioxidant activity that is higher than the other carotenoids. As a polar compound, astaxanthin takes position from inside to outside of the membrane, thus it strengthens the membrane (Jomova and Valko, 2013; Ambati et al., 2014). Studies reveal that astaxanthin has numerous health benefits due to its primary property, antioxidant activity; a) molecular degeneration, b) ischemic diseases, c) diabetes, *d) cardiovascular diseases, e) neurodegenerative disorders, *f) cancer, *g) immune system, h) infectious diseases, *i) oxidative damages of cells and tissues, j) muscle diseases, k) inflammation, l) skin and eye disorders, m) brain functions, n) blood pressure, o) gastrointestinal disorders, p) macular degeneration that [*] refers the most important ones (Naguib, 2000; Higuere-Ciapara et al., 2006; Ambati et al., 2014).

Usage and importance

Astaxanthin is found most widely in marine animals (e.g., salmonids, crustaceans, trout), algae (*Haematococcus pluvialis* as the best source), yeasts (*Xanthophyllomyces dendrorhous,* as the best source) and plants (e.g., fruits and

vegetables) (Miki, 1991; Yamane et al., 1997; Ambati et al., 2014; Saini et al., 2015). It is commercially produced in the form of tablet, capsule, extract, syrups, oil, soft gel, cream, powder and biomass, and used in preferentially food, feed, aquaculture, nutraceutical, pharmaceutical and poultry areas (Ambati et al., 2014). There is a great commercial interest to astaxanthin because of its antioxidant power, economic value, wide usage and healthy benefits for human (Yamane et al., 1997; Naguib, 2000; Ramírez et al., 2001; Visser et al., 2003; Higuere-Ciapara et al., 2006; Amorim-Carrilho et al., 2014).

1.4 Response Surface Methodology

1.4.1 Experimental Design

Experimental design, introduced by Sir R. R. Fisher firstly in 1920s, aims to investigate and minimize problems in a system or process, and to submit deterministic (not random) results. First step of creating an experimental design is to assign the problem. The second one is to determine experimental variables (independent variables/factors/parameters) to be studied and response(s) (dependent variables) to be measured. The questions of 'How many parameters affect the response(s)? Can the effects of the parameters on response(s) be calculated? What are the interactions of the parameters? and What kind of a data analysis should be used?' should be answered for the determination of the variables. The last step is to plan the experiments and carry out. Consequently, maximum information about the problem is gained by minimum experiments (Lundstedt et al., 1998; Araujo and Brereton, 1996).

Classical/passive methods or active/statistical methods may be used to specify experimental design. It has been known that classical/passive methods are not enough to understand and solve the problem, especially while studying with variables more than two and multiple responses. Thus, active/statistical methods are more preferable and currently used. In the active methodologies, fractional factorial designs provide the opportunity of decreasing cost, energy, effort, labor and time by reducing the number of experiments. Central composite, face centered cube, rotatable central composite, Doehlert, mixture and Box-Behnken designs are the most dwelled on fractional designs because they allow to use more than two factors at different

levels (Lundstedt et al., 1998; Araujo and Brereton, 1996; Baş and Boyacı, 2007; Ferreira et al., 2007; Bezerra et al., 2008). Among these, central composite and Box-Behnken (Figure 1.2) are the response surface designs.

The factorial designs differ from each other based on experimental points, number of factors; levels, runs and blocks. The one that suits scientific area and experimental domain is chosen and implemented. Box-Behnken design (BBD) aims to make prediction of 2^{nd} order model, construction of sequential design, permission of blocks and analysis of regression. BBD is rotatable and uses minimum 3 factors and 3 levels at each factor. The experiment (run) number is determined by $[2k^x(k–1)+c_p]$ formula where k is factor number and c_p is center point. (Aslan and Cebeci, 2007; Ferreira et al., 2007). Run points are settled at mid points of a cube as seen in Figure 1.2. It generates 17 runs with 5 center points for 3 factor–3 level design. The extreme points (axial points) in BBD are avoided due to the probability of their unsatisfactory results. Also, this keeps the runs in the safe zone, experimental domain. Central composite design presents experiment points out of the domain which may be irrelevant with the interest, meaningless as result and impossible to carry out (http://support.minitab.com; Ferreira et al., 2007). In terms of number of experiments and coefficients, BBD is more efficient than central composite design and much more than 3 level factorial design. BBD is generally employed for extraction and separation processes (Ferreira et al., 2007).

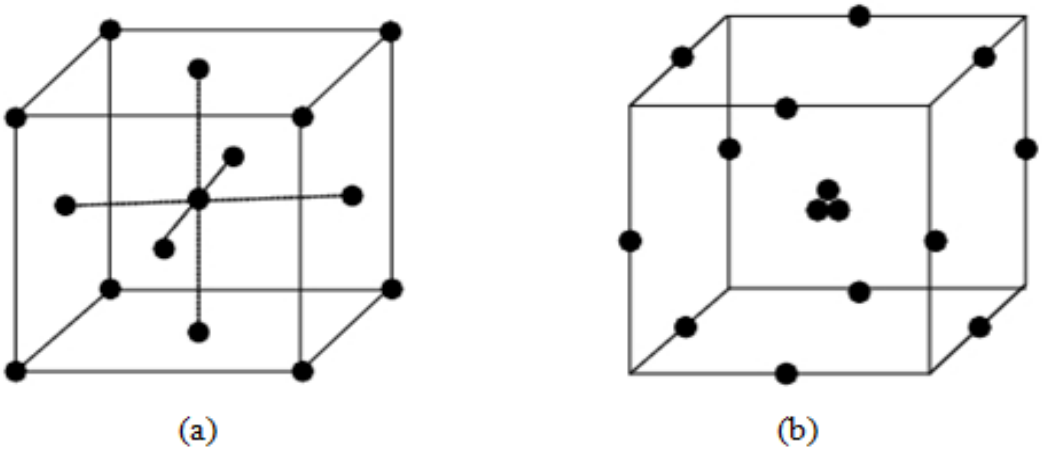

Figure 1.2 Schematic presentation of the response surface designs. (a): central composite, (b): Box-Behnken

1.4.2 Modeling

Model equation is defined following selection of the experimental design. Modeling as mathematical description of the experimental design reveals that function of the variables on the response(s). The fractional designs use second order models with polynomial equations. Quadratic models are able to explain not just the individual effects of the variables on the response(s) but also the interactions of the variables which may have significant effects on the response(s). Besides, the quadratic models exhibit a critical point which may be maximum, saddle or minimum depended on the desired result (Lundstedt et al., 1998; Baş and Boyacı, 2007; Bezerra et al., 2008).

1.4.3 Optimization

Optimization is used to serve some aims such as reducing costs, getting maximum benefit or profit, increasing capacity and productivity in a system or along a process by efficient utilization of the sources. Furthermore, optimization seeks to investigate optimum conditions by some strategies. '1-variable at 1-time ' optimization technique uses keeping one factor constant and changing the other strategy to obtain a result, which does not answer the purpose of observing the effects of all the variables and the interactions of them on the response(s). Additionally, time and material requirement in terms of amount, diversity and expenses increase as a result of increasing the number of the experiments by this strategy (Baş and Boyacı, 2007; Bezerra et al., 2008). Multivariate statistic techniques should be used to remove these disadvantages and have exact optimum points. Response surface methodology (RSM) as a popular and widely applied technique fits the purposes. RSM is a methodology which uses the collection of numerical and statistical analyses by fitting the experimental data to a polynomial equation. RSM by doing this describes the single and combination relations of the variables and responses mathematically and graphically as a result. Main aim of RSM is to obtain best performance by optimizing the variables simultaneously, thus developing and improving of the systems and processes are explicit goals. RSM is purposely used in chemical and biochemical processes and analytical procedures for optimization. Beside optimization, RSM may determine some sources/terms for kinetic studies (Lundstedt et al., 1998; Araujo and

Brereton, 1996; Aslan and Cebeci, 2007; Baş and Boyacı, 2007; Bezerra et al., 2008).

Baş and Boyacı (2007) detail the steps of RSM. *The first step* is to determine the factors and the levels of them. Preliminary studies are necessary for this objective and crucial not to face primary mistake of RSM results which have been done in many searches. *The second step* is decision of what kind of an experimental design - which is done by many computer packages- and model is carried out, which is the collateral mistake of RSM results. Because, the design should construct a good experiment matrix and the model equation should explain the experimental and predicted data well. The designs may use 1^{st} and 2^{nd} order model equations. 1^{st} order model equations (linear functions) run experimental data that does not require a curvature presentation, so they can not present a right response function. Eventually, 2^{nd} order model equations are used. The equation is solved by a multiple regression technique; method of least squares. Central composite and derivations, Doehlert, 3-level factorial and Box-Behnken designs put 2^{nd} order model equations to use. *The third step* is decisive step by introducing visualized results (three dimensional and contour plots) and optimized points. In addition to those steps, evaluation and verification of model fitting are necessary when RSM is performed (Baş and Boyacı, 2007; Bezerra et al., 2008).

1.5 Kinetic Study of Fermentation System

Kinetic studies should be done after optimal medium and environment conditions are identified. Modeling of kinetic studies is a powerful tool to determine and optimize the performance of SSF system. Kinetic modeling describes primarily growth kinetics and secondary energy, mass balances and transport phenomena. Empirical equations are used for kinetic models as long as kinetic measurements are carried out at appropriate units. Biomass measurement is generally used for definition of growth kinetics. However, biomass determination is mostly impossible in SSF systems, not as easy as in SmF systems. Thus, different methods or applications are done such as protein and glucosamine measurements. Besides, cell number (as viable or overall) determination may also present growth kinetics (Mitchell et al., 2006).

Different kinetic profiles; linear, exponential, logistic and deceleration (Figure 2.3) are used to define the kinetics in SSF systems. Stoichiometric parameters are used to determine the productivity in SSF systems (Mitchell et al., 2006; Bhargav et al., 2008; Valduga et al. 2009b). It is emphasized that logistic equation is the equation that mostly used and strongly suggested (Mitchell et al., 2004; Bhargav et al., 2008). Additionally, it is indicated that logistic equation fits experimental data of SSF systems around 75 % (Mitchell et al., 2006).

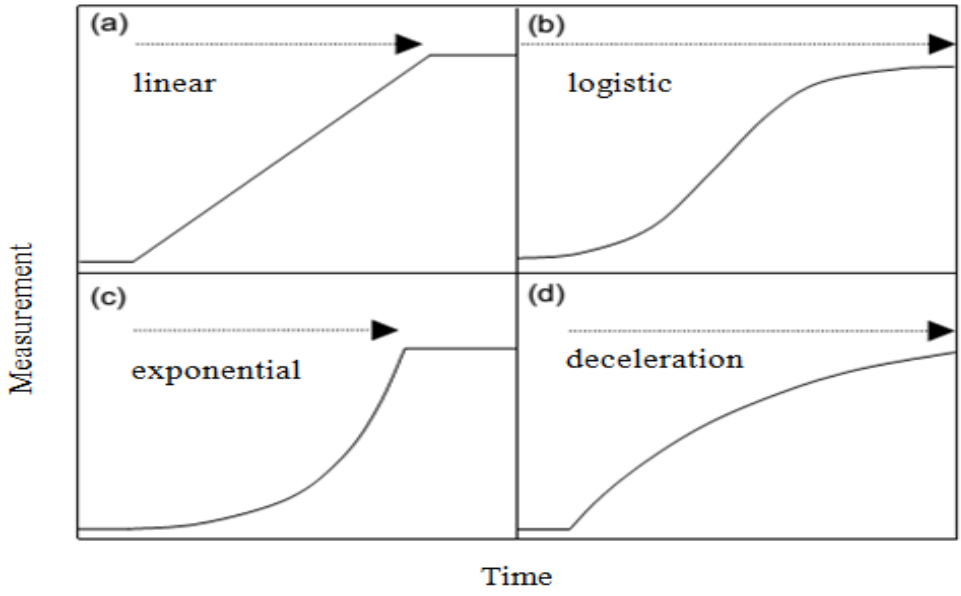

Figure 1.3 Kinetic profiles found and used in SSF systems

It is stated that there is not enough information about kinetics of SSF systems (Mitchell et al., 2006). Moreover, kinetic studies are needed to understand and develop the fermentation, to pursue the reactions and to carry out scale-up studies (Pandey, 2003; Mitchell et al., 2004).

1.6 Process Simulation

The simulation of bioprocesses has recently gained great attention for use as an optimization tool for operations. Industrial development of commercially important products like astaxanthin has seen growth with increasing demand. Progressing with large scale production that follows economic reports is crucial in developing simulation for processes, making investment decisions, and improving the quality of

the products and system. Before the simulation application, optimization data must be analyzed and verified statistically. These measures are very important in application, especially for systems like fermentation systems, which can be affected by many factors such as the environment, labor, cost, time and management (Rouf et al., 2001; Petrides et al., 2014; Torres-Acosta et al., 2016).

Simulators were first designed in the 1960s to model processes in the chemical and petrochemical industries. As simulation application progresses, use in industries like pharmacy, food products and fine chemicals has increased. Simulator' features for use modeling, visual presentation, optimization and material energy balances have been developed for use processing as in bioprocesses. Production type has also progressed into continuous, batch and semi-batch processing (Petrides et al., 2014). Computer aided simulators may detail all phases of a process so that the material energy balances, productivity and economic analysis of the process can be reported. Main advantages of simulator use include shortening process time, defining the economic goals of the entire system and accelerating the processing time. Simulators also provide a good integration or relationship between up and down stream processes (Rouf et al., 2001; Petrides et al., 2014). Software programs for simulation include Batches, Aspen Batch Plus, SuperPro Designer, Simnon, Bioestim, Promax, Cambio and ChemCad. SuperPro Designer is a comprehensive and popular simulator for bioprocessing. It allows engineers to design the unit operations of processing. Process design and development are performed easier when the unit operation conditions are optimized based on the target product of the bioprocess. Performance and economic analyses can be more focused with bioprocess simulation and are considered in the following research study (Groep et al., 2000; Araujo et al., 2002; Taraş and Woinaroschy, 2011).

The most important aspect of designing and operating a plant is profitability which is calculated by gross and net profits. The system inputs which determine profitability are production cost, revenues, cost of supplies, taxes and labor cost. Profitability is analyzed by return on investment (ROI), payback time (PBT), internal rate of return (IRR) and net present value (NPV) terms (Peters and Timmerhaus, 1991). These economic considerations are outputs or responses of the system. Profitability can also be evaluated by a sensitivity analysis that investigates the relationship between

parameters and outputs. A sensitivity analysis examines the system inputs themselves, their intervals, compatibility with the system and effects on the responses individually and interactively (Norton, 2015).

SuperPro Designer finds a wide application area to model, evaluate and optimize the processes in such as pharmaceutical, biotechnology, food, mineral processing, microelectronics and wastewater treatment industries (Table 1.5) (Groep et al., 2000; Araujo et al., 2002). SuperPro Designer is also a teaching tool used by scientists, engineers and academicians, particularly in Process and Plant Design, Pharmaceutical Engineering, Food Engineering, Unit Operations, Water Purification and Air Pollution Control.

Table 1.5 Unit operation models in SuperPro Designer

Reaction Well-Mixed Reactor Batch/Continuous Fermentor Air-Lift Fermentor Plug Flow Reactor Equilibrium Reactor Heat Sterilizer Aeration Basin Plug Flow Aeration Basin Trickling Filter Anoxic Reactor Anaerobic Digester Neutralizer Wet Oxidation Incinerator UV Radiation **Phase Separation** Centrifugal Extractor Differential Extractor Mixer-Settler Extractor Batch Extractor Crystallizer Short-Cut Distillation Batch Distillation Flash Condenser Absorber/Stripper Adsorber Decanter Tank **Homogenization** High Pressure Homogenizer Bead Mill Nano-Mill	**Chromatography** Gel Filtration Ion Exchange Reverse Phase Affinity Expanded Bed Adsorption **Drying/Evaporation** Freeze Dryer Tray Dryer Fluid Bed Dryer Rotary Dryer Flash Evaporator Rotary Evaporator Sludge Dryer **Pressure Change** Pumps Compressors Fan/Blower **General Unit Operations** Generic Boxes for Reaction and Separation Flow Mixers/Splitters Component Splitter Heater/Cooler Heat Exchanger **Product Formulation** Dryers & Granulator Tablet Press Tablet Coater Fillers Packaging Machines	**Solid/Liquid Separation** Microfilter Ultrafilter Diafilter Reverse Osmosis Dead-End Filter Basket Centrifuge Nutsche Filter Bowl Centrifuge Disk-Stack Centrifuge Decanter Centrifuge Centritech Centrifuge Hydrocyclone Clarifier / Thickener Flotation Oil Separator Plate & Frame Filter Rotary Vacuum Filter Granular Media Filter Belt Filter **Solid / Gas Separation** Electrostatic Precipitator Baghouse Filter Gas Cyclone Air Filter **Tanks** Various Storage Tanks Various Blending Tanks Equalization Tank Junction Box

1.7 Studies on Astaxanthin

It is known that optimization and response surface methodology analyses in a scope of an experimental design has been extensively used in biotechnological searches in recent years. Bioprocess subjects such as optimization of medium composition and conditions, and determination of optimum parameter levels are considered by these

applications. However, there is no information available about optimization, mathematical modeling, simulation and large scale evaluation studies for astaxanthin production by yeasts biotechnologically.

Astaxanthin production has been searched in various purposes such as extracting from marine animals (like lobster and crustaceans), and producing and extracting from *H. pluvialis* alga and *X. dendrorhous* yeast since it was first mentioned in 1876 by Pouchet (Kläui and Bauernfeind, 1981) and structurally described in 1975 by Basil Weedon. As a result of the searches, it is an approved pigment as a food coloring agent by FDA and EU (Ushakumari and Ravi Ramanujan, 2013).

Astaxanthin production has been carried out by using synthetic or waste media with yeasts in submerged fermentation system. Most of the studies are depend on synthetic media with *Phaffia rhodozyma* and *Sporidiobolus salmonicolor* yeasts in literature. In 1979, Johnson and Lewis produced 387 μg/g yeast as maximum astaxanthin by *P. rhodozyma* UCD 67-210 in YM (yeast extract/malt extract) medium at 20-22 °C temperature and 4.5 pH in fermentor. In flask system, they obtained 510 μg/g yeast as maximum astaxanthin at 22 °C temperature and 5.0 pH. They also indicated that astaxanthin was produced in the time of exponential phase of the growth. Yamane et al. (1997) obtained 16 mg/L astaxanthin concentration by *P. rhodozyma* ATCC 24202 using enhanced synthetic medium in a batch fermentor. Kusdiyantini et al. (1998) studied with glycerol to produce astaxanthin by *P. rhodozyma* PR 190 and obtained 1.8 mg astaxanthin/g yeast at 37.8 g/L glycerol concentration.

Ramírez et al. (2001) performed an optimization study for astaxanthin production using wild type *P. rhodozyma* ATCC 24202 in YM medium and Yucca medium (based on date juice) within the scope of an experimental design. They reported that 8100 μg/L as the highest astaxanthin production was reached at 19.7 °C temperature, 6.0 pH and 5 % inoculum ratio as optimal conditions. Moriel et al. (2005) produced 383.73 μg astaxanthin/g biomass by *P. rhodozyma* ATCC 24202 using synthetic medium which was supported with sugar cane juice and urea as carbon and nitrogen sources in continuous fed-batch process at 6.0 pH and 24 °C temperature by 3-4 % inoculation ratio in 96 h conditions. 1333.96 μg/L astaxanthin concentration was

obtained by addition of agro products in fermentation medium in the study of Bhatt et al. (2013). They designed the experiments by BBD with *P. rhodozyma* MTCC 7536.

Parajó et al. (1997) presented a work including 10.4 mg/L astaxanthin production from concentrated eucalyptus wood using *P. rhodozyma* NRRL Y-17268. Ananda and Vadlani (2011) studied with 8 different solid wastes to produce astaxanthin from *P. rhodozyma* ATCC 24202. They stated that 66.75 μg astaxanthin per gram wheat bran, 80.42 μg astaxanthin per full-fat rice bran and 16.94 μg astaxanthin per defatted rice bran were produced at the end of 11^{th} day of the process.

Sporidiobolus salmonicolor yeast has been studied mostly for carotenoid production. Valduga et al. (2009b) indicated that a total carotenoid content of 590.4 μg/L was produced by *S. salmonicolor* CBS 2636 (ATCC 24259) using agro-industrial substrate, pretreated cheese whey, at the conditions of 4.0 pH and 25 °C temperature.

1.8 The Objectives of the Thesis

Fermentation is rapid and productive biotechnological method for production of pigments. Pigments produced biotechnologically are defined as natural, microbial, food grade pigments or biocolorants. Fermentation medium in terms of nutrient content and fermentation conditions are the most important factors for fermentation systems. Solid state fermentation is suggested due to its providing natural characterization for the pigment production. A clear process which provides large scale production of microbial pigments has not been described yet. There are crucial items for the large scale production of microbial pigments used in processed food products due to their great economical potential; experimental design, medium composition, optimization of fermentation medium and conditions and evaluation of studies statistically which is revealed by response surface methodology to screen and optimize the pigment production (Chattopadhyay et al., 2008; Gupta et al., 2011; Nigam and Luke, 2016).

Production of astaxanthin pigment biotechnologically, which has unquestionable biological and commercial importance is the point to be emphasized to be studied

and developed. Solid state fermentation as a sub-technology subject of Food Engineering discipline becomes more of an issue to produce astaxanthin using agro-industrial wastes since strategy of fermentation has serious effects on production of astaxanthin pigment (Duffosé, 2006).

The variety and quantity of carbon and nitrogen sources of the growth medium and water activity are fundamental parameters for the growth of microorganisms. In the manufacturing of industrial products, there is a great deal of scientific research on the characteristics and conditions of the growth medium. These studies are mainly carried out for realization, development and recently improvement of a scale-up production. Raw material is a significant parameter for the scale-up production because it directly affects the production efficiency with the variety and nutritive content of it. It also plays an important role in the process and environmental issues. All these statements influence the production economy which is the most important point for industrial production. The use of natural raw material resources has been used for a very long time in industrial production worldwide. Trying to produce target products using any kind of waste material has a significant place; particularly in branches of science such as chemistry, food, biology, microbiology and biotechnology. Compared to synthetic media, the products manufactured by the utilization of wastes are qualified and labeled as natural. Many food additives such as organic acids, pigments, gelling agents and flavors can be produced by applying the SSF technique for solid wastes, which is referred to bio-processing. The pigments produced by microorganisms are exhibited as natural pigments. The toxic and carcinogenic effects of the pigments produced synthetically reveal the importance of natural raw materials and production techniques (Joshi et al, 2003; Duffosé et al, 2005; Gupta et al, 2011). Turkey has an important status by producing and processing agricultural crops and coming out of agro-industrial wastes. The wastes are needed to be utilized as value-added products. Cereal and legume wastes in Gaziantep province as research region of the study have advantages to be use as raw material for solid state fermentation system in terms of easy accessibility, cheapness, great amount and nutrition content.

Microorganisms selected in solid state fermentation system are tools to be used to produce target product. Number and variety of microorganisms are kept high in order

to investigate the abilities and behaviors of them, to obtain new knowledge about them in the studied specific area, to diversify and enhance the study. *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) and *Sporidiobolus salmonicolor* are the yeasts known as carotenoid pigments producers. *X. dendrorhous* is one of the best commercial astaxanthin producers. Although there have been lots of academic studies and production methods tried by commercial firms about *X. dendrorhous*, an economically efficient process has not been built up yet (Duffosé, 2006). Chattopadhyay et al. (2008) and Gupta et al. (2011) have reported that *X. dendrorhous* is the only yeast to be used for the large scale production of astaxanthin biotechnologically. *Candida guilliermondii* and *Yarrowia lipolytica* are the other pigment producers, mainly yellow and brown respectively. Other species of the main genus could have the ability to produce various pigments, which should be investigated.

There has not been reached a search or study about producing astaxanthin from cereal and legume wastes by solid state fermentation technology using the yeasts in the scope of an experimental design and evaluation of the results by response surface methodology. Additionally, there is no literature knowledge of solid state fermentation kinetics which is difficult to determine because of medium solidity, homogeneity and separation problems. Modeling and optimization of the fermentation systems should be revealed by statistical analyses with verification for certain fermentation conditions and productivity. Kinetic studies of the fermentation systems are based on the growth of microorganisms and their activities in medium. It is important to present rate parameters such as growth rate and substrate consumption rate by kinetic studies after determination of the certain conditions of the fermentation systems.

Simulation of biotechnological processes has been recently gained a great interest to optimize the processes which are related to each other and to present economics, particularly for the products like astaxanthin which have a commercial importance industrially. Progressing with large scale production and detailing economic reports are crucial points of simulation in terms of developing the processes, giving right decisions about investments, improving quality of the products and system. Besides, it is the best approach to have a data which is optimized and verified statistically

before simulation application. Thus, background of simulation should be created correctly. Especially it could be significant for the fermentation systems that affected by many factors such as environment, labor, cost and time management.

The main aims of this thesis depending on the problem statements explained above are to investigate the most appropriate waste and microorganism for the astaxanthin bio-production, to determine fermentation conditions, to maximize the astaxanthin yield by modeling, optimization and kinetic study, to reveal operation economy by process simulation.

Hereby, the works were performed in this thesis;

i. The bran parts of wheat and rice as cereal wastes and lentil residue as legume waste were used as raw material of solid state fermentation system in order to produce astaxanthin pigment by *Xanthophyllomyces dendrorhous, Sporidiobolus salmonicolor, Candida guilliermondii* and *Yarrowia lipolytica* yeasts.

ii. The growth of the yeasts in synthetic media was investigated to determine; the growth parameters such as cell concentration and biomass, fermentation period, the best growth and productivity in different sugar types and concentrations.

iii. Nutrition content of the wastes was determined for raw and fermented materials.

iv. Box-Behnken experimental design was generated for each waste and yeast combination with temperature, moisture content and pH as independent variables at three levels.

v. Astaxanthin amount as the response of the design was measured for each waste and yeast combination at the end of the fermentation period. Response surface methodology was used to determine the optimum conditions of the fermentation systems.

vi. The optimized fermentation conditions and the inoculation ratio selected for the optimized system were verified experimentally and statistically.

vii. Kinetic studies were carried out depending on the optimized waste, yeast and fermentation conditions. Substrate consumption, yeast growth and product formation as kinetic parameters were followed out.

viii. Antioxidant capacity of the produced astaxanthin was determined.

ix. Simulation by SuperPro Designer was carried out for the optimized fermentation process to report an economic analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Wastes

Bran parts of wheat and rice as cereal wastes and lentil residue (consisting of germ, seed coat, plumule and aleurone powder mixture) as legume waste were supplied from Gaziantep, Turkey and stored at cold storage (+10 °C) in polyethylene packages. They were sieved to size 0.85 mm in order to obtain a uniform material for the fermentation system.

2.1.2 Microorganism Cultures

Freeze-dried yeasts, ATCC 90197 (*Yamadazyma guilliermondii*), ATCC 24060 (*Yarrowia lipolytica*), ATCC 24202 (*Xanthophyllomyces dendrorhous*) and ATCC 24259 (*Sporidiobolus salmonicolor*) were purchased from the American Type Culture Collection (ATCC) (Manassas, USA). The microorganisms were transformed in yeast extract-malt extract (YM) broth and maintained both in YM broth and YM agar. The compositions of the media were presented in **A1.1.**

2.1.3 Sterilization of the Supplies

Media, solvents, solutions, chemicals and consumables were sterilized by using an autoclave (HMC HV-85L, GERMANY) at 121 °C for 15 min (Anonymous, 2005).

2.2 Methods

2.2.1 Analytic Analyses

The analytic analyses were carried out for raw material and fermented content. The experiments were performed as duplicate and the results were presented as average.

pH analysis

The sample was homogenized with bi-distilled water. The homogenized sample was mixed with bi-distilled water at 1:9 ratio and analyzed by using a pH-meter (NEL pH890, TURKEY) (AOAC, 1990).

Titratability acidity analysis

The sample was homogenized with a specific volume of bi-distilled water. The homogenized sample was titrated with 0.1 N NaOH solution according to 1 % phenolphthalein indicator. The results were expressed in terms of sulphuric acid (H_2SO_4). The equation below was used for calculation (AOAC, 1990):

$$\text{Titratability Acidity (TA) \%} = (N^x V^x SF^x E^x 100)/m \quad (2.1)$$

N = NaOH concentration (0.093 N)

V = used NaOH (mL)

SF = dilution factor (1000 mL water/25 mL filtrate)

E = 0.0049 = milliequivalent value of H_2SO_4 for 1 mL 0.1 N NaOH solution

m = weight of sample (g)

Moisture content analysis

Five grams sample was dried in a drying oven (RT 500 W. C. Heraeus Hanau, GERMANY) at 105±5 °C temperature. The equation below was used for calculation (AOAC, 1990):

$$\text{Moisture Content (MC) \%} = [(M_1 - M_2)/m]^x 100 \quad (2.2)$$

M_1 = wet weight of sample (g)

M_2 = dried weight of sample (g)

m = wet weight of sample (g)

Ash analysis

Five grams sample placed in thimble and combusted in an ash oven (MF 120 Nüve, TURKEY) till constant weight. The results were expressed in terms of dry basis. The equation below was used for calculation (AOAC, 1990):

$$\text{Ash \%} = [(M_2-M_1)/m]^x100 \quad (2.3)$$

M_1 = weight of thimble (g)
M_2 = [weight of ash (g)+weight of thimble (g)]
m = dry weight of sample (g)

Protein analysis

One gram sample was analyzed according to Kjeldahl method by digestion and distillation apparatuses (DK6 Velp Scientifica, EUROPE; Kjeltec 2200 Foss, SWEEDEN). The results were expressed in terms of dry basis. The equation below was used for calculation (AOAC, 1990):

$$\text{Nitrogen \%} = NT = (N^xV^xF^x100)/m \quad (2.4)$$

$$\text{Protein \%} = (NT)^x(PF) \quad (2.5)$$

N = adjusted HCl concentration (0.0983 Normality)
V = used HCl (mL)
F = nitrogen factor (0.014)
PF = protein factor*
m = dry weight of sample (g)

*: Protein factors were used as 6.31, 5.95 and 6.25 for wheat bran, rice bran and lentil residue, respectively.

Fat analysis

Five grams sample was analyzed by using Soxhlet device (SER 148 Velp Scientifica, ITALY). The results were expressed in terms of dry basis. The equation below was used for calculation (AOAC, 1990):

$$\text{Fat \%} = [(M_2 - M_1)/m]^x 100 \quad (2.6)$$

M_1 = weight of balloon (g)

M_2 = [weight of fat (g)+weight of balloon (g)]

m = dry weight of sample (g)

Sugar analysis

Phenol-sulfuric acid **(**Masuko et al., 2005) method was applied. Stock solution was prepared with 1 gram glucose (Merck, Germany) and 1 L bi-distilled water as first step. Stock solution was exposed to a chemical reaction. 2 mL stock solution was mixed with 5 mL concentrated (98 %) H_2SO_4 solvent (Sigma-Aldrich, Germany) and 1 mL phenol solution (**A1.2**). The mixture was waited for 10 minutes and vortexed (Grant Bio, PV-1, ENGLAND) for 30 seconds. 20 minutes was waited again and spectrophotometric wavelength scan was performed by double beam UV/VIS Spectrophotometer (Lambda 25 UV/VIS Spectrophotometer, USA). Bi-distilled water instead of stock solution was used for blank and the chemical reaction was applied.

Dilution series from the stock solution were prepared with bi-distilled water at different concentrations. The series were exposed to the chemical reaction and absorbance values of the series were measured against the blank at the determined wavelength. The absorbance values versus concentrations were plotted and modeled by linear model using SigmaPlot Version 11.0 (Systat Software GmbH, Erkrath, GERMANY) program and standard curve equation ($y=y_0+a^x x$) was obtained.

Five grams sample was mixed with 100 mL bi-distilled water and shaken by a rotary shaker (Innova 40R New Brunswick Scientific, USA) for 2 hours. The mixture was filtered by Whatman No: 1 filter paper and centrifugated at 6000 rpm for 5 min. The chemical reaction was applied to the supernatant and spectral analysis was performed. Glucose amount in the sample was calculated according to the standard curve equation.

2.2.2 Growth Curve Determination

Standard curve

Cell counting was implemented using fresh culture by Thoma lam method (Anonymous, 2005). Thus, probable cell number per milliliter in the culture could be estimated. The same fresh culture was diluted with sterile YM broth six times at 1:1 ratio. The cell numbers in the dilution series were calculated depending on the Thoma lam counting. Optical density (O. D.) values (absorbance values) of the dilution series were measured by double beam UV/VIS Spectrophotometer at 540 nm (Aber et al., 2012). Absorbance values versus cell numbers were plotted and modeled by linear model using the SigmaPlot program and curve equation ($y=y_0+a^x x$) was obtained. Same procedure was applied for each yeast under aseptic conditions.

Growth curve

Growth curves of the yeasts were obtained to observe the phases of the growth and to determine the fermentation period. Same fresh culture used in standard curve was used for three measurements. Each measurement (Anonymous, 2005) was carried out daily during 20 days. Experimental data were modeled by the SigmaPlot program using Gompertz-4 Parameter equation; $y=y_0+a^x \exp(-\exp(-(x-x_0)/b))$.

a. Optical density measurement

   Spectrophotometric analysis was performed for the sample taken from the culture at 540 nm. If the culture was needed, it was diluted with sterile YM broth at the same volume and the measurement was followed out.

b. Plate counting

   Dilution series were obtained by mixing 1 mL culture and 9 mL sterile physiological salt solution (PSS) (**A1.1**). 0.1 mL inoculation from the mixture to YM agar plate was achieved at the aseptic conditions. Colony (viable cell) counting was carried out after 2 days incubation and cell number was calculated by the equation below:

$$N=C/[V^x d^x(n_1+(0.1^x n_2))] \quad (2.7)$$

N: colony number in 1 g or 1 mL sample (cfu/mL or cfu/g)

C: total colony number at all plates

V: inoculation volume (mL)

d: dilution ratio of more concentrated dilution series

n_1: plate number counted of first dilution

n_2: plate number counted of second dilution

c. Biomass analysis

Five mL culture was washed with sterile bi-distilled water twice. Cell suspension was dried in drying oven at 105 °C for 4-5 hours and weight of the dried cell was determined.

2.2.3 Fermentation Process

2.2.3.1 Fresh Culture Preparation

The fresh cultures of the microorganisms were prepared by growing at the optimum temperature values of them for 24 hours. One colony from the Petri dish was taken and placed in 10 mL sterile YM broth under aseptic conditions. Optimum growth conditions of the each yeast with regard to ATCC protocol are shown in Table 2.1.

Table 2.1 Optimum temperature and pH values for growth of the yeasts

Microorganisms	**ATCC code**	**Optimum growth conditions**	
		T (°C)	**pH**
Yamadazyma guilliermondii	ATCC 90197	25.0	5.0
Yarrowia lipolytica	ATCC 24060	24.0	5.6
Xanthophyllomyces dendrorhous	ATCC 24202	20.0	4.5
Sporidiobolus salmonicolor	ATCC 24259	18.0	6.0

2.2.3.2 Inoculum Preparation

The fresh cultures of the yeasts at 2 % inoculation ratio with known cell number (by Thoma lam) were used for inoculation.

2.2.3.3 Pigmentation Examination

Astaxanthin production ability of the yeasts on the wastes was examined at the optimum growth conditions during the determined fermentation period. 100 gram fermentation content in Erlenmeyer flask was prepared for each waste and yeast combination. The flasks were inoculated (2 %) and incubated at the optimum growth temperature values of the microorganisms. When the pigmentation was accomplished, the experimental design procedure was applied.

2.2.3.4 Experimental Design

Box-Behnken design (BBD) was generated for all waste and yeast combination using three independent variables; temperature (x_1), moisture content (x_2) and pH (x_3), which are the most effective parameters for the solid systems (Bellon-Maurel et al., 2003; Norliza and Ibrahim, 2005), at three levels. The depended variable, response, was astaxanthin yield for the design**.** The levels of temperature (°C) and pH variables were based on the optimum growth conditions of the yeasts. Optimum moisture content (%) was selected based on water activity requirement (0.60-0.88) for growth of yeasts (Anonymous, 2005). The level intervals of the independent variables were adjusted by a design program, Design-Expert Version 7.1.5 (Stat-Ease, Inc., Minneapolis, USA). High (+1), middle (0) and low (-1) codes of the independent variables were presented in Table 2.2.

There were 17 runs with 5 center points conducted through the design for each waste and yeast combination (Table 2.3).

Table 2.2 Levels of the independent variables for the yeasts

Yeasts	Coded Levels								
	x_1			x_2			x_3		
	-1	**0**	**+1**	**-1**	**0**	**+1**	**-1**	**0**	**+1**
ATCC 90197	20.0	25.0	30.0	70.0	80.0	90.0	4.0	5.0	6.0
ATCC 24060	19.0	24.0	29.0	70.0	80.0	90.0	4.6	5.6	6.6
ATCC 24202	15.0	20.0	25.0	70.0	80.0	90.0	3.5	4.5	5.5
ATCC 24259	13.0	18.0	23.0	70.0	80.0	90.0	5.0	6.0	7.0

Table 2.3 BBD matrix for production of astaxanthin by four yeasts

Run	ATCC 90197			ATCC 24260			ATCC 24202			ATCC 24259		
	x_1	x_2	x_3	x_1	x_2	x_3	x_1	x_2	x_3	x_1	x_2	x_3
1	30.0	70.0	5.0	24.0	80.0	5.6	15.0	80.0	3.5	23.0	90.0	6.0
2	25.0	80.0	5.0	24.0	90.0	4.6	15.0	80.0	5.5	13.0	80.0	7.0
3	20.0	70.0	5.0	19.0	70.0	5.6	15.0	70.0	4.5	18.0	80.0	6.0
4	25.0	80.0	5.0	24.0	90.0	6.6	15.0	90.0	4.5	18.0	70.0	7.0
5	20.0	80.0	4.0	29.0	70.0	5.6	20.0	70.0	3.5	13.0	80.0	5.0
6	25.0	90.0	4.0	19.0	80.0	6.6	20.0	80.0	4.5	13.0	70.0	6.0
7	30.0	80.0	4.0	24.0	80.0	5.6	25.0	80.0	5.5	23.0	80.0	5.0
8	20.0	90.0	5.0	24.0	80.0	5.6	20.0	80.0	4.5	18.0	80.0	6.0
9	25.0	80.0	5.0	19.0	80.0	4.6	20.0	80.0	4.5	18.0	90.0	7.0
10	30.0	80.0	6.0	29.0	80.0	4.6	25.0	90.0	4.5	18.0	80.0	6.0
11	30.0	90.0	5.0	24.0	70.0	4.6	25.0	80.0	3.5	18.0	90.0	5.0
12	25.0	80.0	5.0	19.0	90.0	5.6	20.0	70.0	5.5	13.0	90.0	6.0
13	25.0	80.0	5.0	24.0	80.0	5.6	20.0	90.0	5.5	23.0	70.0	6.0
14	20.0	80.0	6.0	29.0	90.0	5.6	20.0	90.0	3.5	23.0	80.0	7.0
15	25.0	70.0	4.0	24.0	80.0	5.6	20.0	80.0	4.5	18.0	70.0	5.0
16	25.0	70.0	6.0	29.0	80.0	6.6	25.0	70.0	4.5	18.0	80.0	6.0
17	25.0	90.0	6.0	24.0	70.0	6.6	20.0	80.0	4.5	18.0	80.0	6.0

2.2.3.5 Fermentation

The fermentation was carried out in 250 mL Erlenmeyer flasks containing 100 gram total amount of the wastes and water. The amount of water to be added was determined depending on initial and final moisture content of the solid waste. pH value of bi-distilled water to become according to the experimental design was adjusted by HCl and NaOH solutions. Water and solid waste was mixed in flasks and the flasks were sterilized. After cooling, they were inoculated with 2 % fresh culture. Incubation was maintained at the design temperature levels during the determined fermentation period.

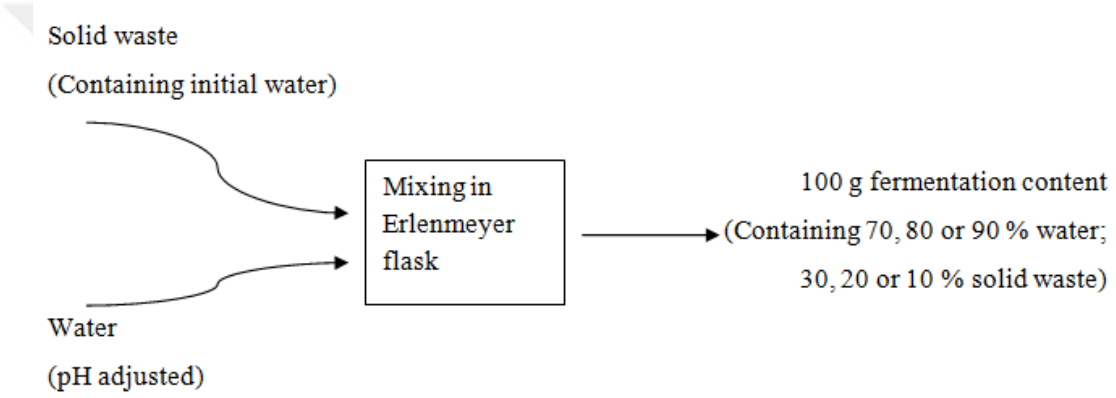

Figure 2.1 Preparation of fermentation content

2.2.4 Pigment Analysis

2.2.4.1 Standard Curve Preparation

A spectral scanning was done for astaxanthin (AX) standard (Chromadex, USA) which was purchased dissolved form in methanol and the wavelength of the maximum pick point was determined.

Different concentrations (dilution series) of the AX standard were prepared with pure methanol (Sigma-Aldrich, Germany). Spectrophotometric measurements of the series were carried out against pure methanol at the maximum wavelength. Standard curve of AX pigment was obtained using absorbance values versus the concentrations of

the standard. The data was modeled by linear model using the SigmaPlot program and curve equation ($y=y_0+a^x x$).

2.2.4.2 Extraction and Spectral Analysis

Raw material and fermented content were subjected to pigment analysis. The sample was mixed with pure methanol at 1:4 ratio. The mixture was waited for 2 hours, 5 mL of the liquid phase of the mixture was taken and centrifugated at 6000 rpm for 10 min. The supernatant was analyzed by using UV/VIS spectrophotometer against the pure methanol blank (Babitha et al., 2007). AX concentration was calculated with regard to the equation of the standard curve. The results were explained as the mean of triplicate measurements.

2.2.5 Productivity in Synthetic Media

Three different sugar types (glucose, fructose and sucrose) at 4 different concentrations (5, 10, 20 and 40 g/L) and no sugar (0 g/L) synthetic media were prepared to measure the growth and product formation capabilities of the yeasts by keeping malt extract, yeast extract and peptone ingredients constant. The optical density values of the media during the fermentation period and astaxanthin pigment produced in synthetic media were measured at the last day of the fermentation period.

2.2.6 Modeling and Optimization

Quadratic model, which uses second order polynomial equation (Montgomery, 2001), was used for modeling of the fermentation systems. The equation of the model is:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x^2{}_j + \sum_{i=1}^{j-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j \qquad 2.8$$

where y is the predicted response or dependent variable, β_0 is model constant, β_j, β_{ij} and β_{jj} are the regression coefficients (linear, interaction, quadratic) and x_j and x_i are the levels of the independent variables.

The optimal conditions of each fermentation system were determined according to maximum astaxanthin yield. Optimization for the astaxanthin production was disclosed in terms of waste, microorganism and fermentation conditions. The experimental data of each fermentation system were evaluated by regression and variance analyses (ANOVA) in RSM.

2.2.7 Verification Studies

2.2.7.1 Inoculation Ratio

Five different inoculation ratios (1.0, 1.5, 2.0, 3.0 and 4.0 %) were studied for the optimized waste and yeast combination in order to verify the inoculation ratio (2 %) which is selected in most fermentation processes and was used for this study.

Viable cell (V. C.) of the fermented medium by plate counting method and pigment analysis were performed at the end of the fermentation period for each inoculation ratio. The results were modeled by SigmaPlot program with the equation [$y=a^x\exp(-0.5^x(\ln(x/x_0)/b)^2)/x)$] and optimized by SAM (Steepest Ascent Method) and MS Excel 2010 Solver tool (**A4**).

2.2.7.2 Parameter Levels

Another BBD was generated with same parameters (temperature, moisture content and pH) at different levels for the optimized waste and yeast combination. The levels of temperature (17.0, 22.0 and 27.0) and moisture content (60.0, 70.0 and 80.0) were increased and pH range (4.0, 4.5 and 5.0) was narrowed in order to observe whether or not the astaxanthin yield would change.

2.2.8 Kinetic Studies

Product formation (astaxanthin production), yeast growth (cell number formation) and sugar (glucose) consumption as kinetic parameters were followed for the optimized waste during the fermentation period. The measurements were carried out daily during the fermentation period after the optimized waste, microorganism and fermentation conditions were determined. Fermentation system was set up for the optimized waste and yeast combination at the optimized conditions. Astaxanthin production and glucose consumption measurements were performed according to pigment and sugar analyses respectively. For cell number analysis; 25 g sample was taken from the fermented content and 225 mL sterile PSS was mixed under aseptic conditions. A dilution series was prepared and plate counting was performed for the determination.

The kinetic parameters were modeled by SigmaPlot program defining logistic equation (Mitchell et al., 2004) for yeast growth and product formation, using Logistic-3 Parameter equation for glucose consumption.

$$y = a/(1 + ((a/b)-1) * exp(-m * x)) \quad (2.9)$$

where *y* is response; *a* and *b* indicate cell number or astaxanthin amount; *m* is slope; *x* is time.

$$y = a/(1 + (g/m)b) \quad (2.10)$$

where *y* is response; *g* is glucose amount; *a* and *b* are coefficients.

Quantitative descriptions of bio-conversions were determined by yield coefficients −$Y_{p/s}$, product/substrate; $Y_{x/s}$, cell number/ substrate; $Y_{p/x}$, product/cell number−, which were referred difference between initial and final values of the kinetic parameters mathematically.

2.2.9 Antioxidant Capacity by DPPH Radical Scavenging Activity Assay

Extracts of both fermented and un-fermented content were prepared for DDPH (1,1-diphenyl-2-picrylhydrazyl) analysis. Five grams content was filled up to 50 mL with pure methanol. The mixture was shaken at 250 rpm and 30 °C for 2 hours. The extract was waited for 30 minutes and then filtered. The filtrate was centrifugated at 6000 rpm for 10 minutes. The mixture of 3 mL of 60 µM DPPH radical (Sigma-Aldrich, Germany) (**A1.3**) in methanol and 250 µL extract were left at dark and room temperature (20 °C) for the reaction. The control sample was prepared with bi-distilled water instead of the extract. Remaining purple color was measured at 517 nm after 25 minutes (Kwon et al., 2006). The scavenging activity (S. A.) of the DPPH radical (or inhibition of DPPH radical) was calculated by the following equation:

$$S.A.\,\% = \frac{(\text{absorbance of control} - \text{absorbance of extract})}{\text{absorbance of control}} \times 100 \qquad (2.11)$$

2.2.10 Simulation Studies

SuperPro Designer 9.0 (Intelligen, Inc., Scotch Plains, NJ, USA) was used to evaluate the economic feasibility of the astaxanthin production from the optimized fermentation process. The amount of wheat bran used as feed was varied from 1,000 to 50,000 kg/batch to produce astaxanthin from the SSF process. A base case plant capacity (batch size) was assumed; then, the effect of varying the batch size on the unit investment cost and the unit production cost was investigated. Material balance, economic evaluations and profitability analysis were carried out for the astaxanthin bio-production. It was planned for the construction of the plant in one year in Gaziantep, Turkey. The operating life time of the plant was assumed to be 25 years; the plant was operated for 330 days a year in batch mode. Implementation of the simulation was discussed at various intervals which were presented as minimum, base and maximum in Table 2.4. Reaction stoichiometry and coefficients of the each constituent [$(a=0.24)^x$Carbohydrate + $(b=0.08)^x$Fat + $(c=0.02)^x$Oxygen → $(d=0.0011)^x$AX + $(e=0.34)^x$Biomass] was entered in the model. The optimal fermentation period was estimated at 7 days.

Table 2.4 Simulation parameter variations for AX production from wheat bran

Variations	Minimum	Base	Maximum
Raw material cost, RMC ($/kg waste)	0.08	0.1	0.12
Main Product Cost, MPC ($/kg MP*)	8,000	10,000	12,000
Operating Labor Cost, OLC ($/h)	1.84	2.30	2.76

*MP: Main Product

Process flow diagram was depicted in Figure 2.2 to exhibit the whole production. The upstream processes were grinding, sieving and leaching unit operations. A drying process was carried out to reduce the moisture content after fermentation in order to increase the leaching yield. The leaching process was performed with dichloromethane (DCM) as the extraction solvent. The leached mixture was encapsulated by starch during batch distillation. The solvent was recovered in the batch distillation and recycled back to the extractor. Finally, the encapsulated mixture was dried by a spray drier. The encapsulation and spray drying were conducted to obtain a powder product. There were numbers (P-#) and names of each process in the figure, which were described in terms of base case in Table 2.5. The additional processes to lab-scale production were: oil leaching with hexane (P-21), air supply (P-17, P-6), tray drier (P-1), batch distillation (P-11, P-12), spray drier (P-15) and design specification (P-13).

Cost studies are based on preliminary economic analysis (accuracy up to ±20 %) conducted for the estimation of the total capital investment and operating cost for the process flow sheet producing AX (Peters and Timmerhaus, 1991; Green and Perry, 2008). The total capital investment for any process consists of the fixed-capital investments for the physical equipment and facilities in the plant and the working capital to pay salaries, keep raw materials and products on hand, and handle other items requiring a direct cash outlay. The direct fixed capital (DFC) investment was estimated based on the total purchasing equipment cost (PC). In this study, the equipment purchasing cost for most processes was obtained by SuperPro Designer's built-in cost models. The cost of fermentors (which are the most expensive

equipment units) was obtained by a local manufacturer. All other components of the capital investment cost were calculated with the use of PC-based factors.

The major components of the total production cost include raw materials like agro-industrial wastes and other necessary chemicals for astaxanthin production, utilities, and operation labor. For the purposes of the sensitivity analysis, a range of values was considered for the raw material and labor cost as shown in Table 2.4. The AX selling price was also varied in the range shown also in Table 2.4 (Panis and Carreon, 2016). The selling price relates to AX in its pure form and, therefore, applies to the product for the proposed process.

The annual profit is the difference between the revenue and the total production cost. The payback time of the project is estimated using the annual cash flow and refers to the period of time required to recover the capital investment. The effect of the discount rate on plant economics is found by calculating the overall net present value (NPV) generated from the investment and plant operation (Michailos et al., 2016). The internal rate of return (IRR) was determined by setting the overall NPV to zero. The profitability analysis and projection of the revenues for the AX production on the maximization of the IRR for the given initial capital investment was evaluated by analyzing operating costs and annual cash flow over its 25 year lifespan.

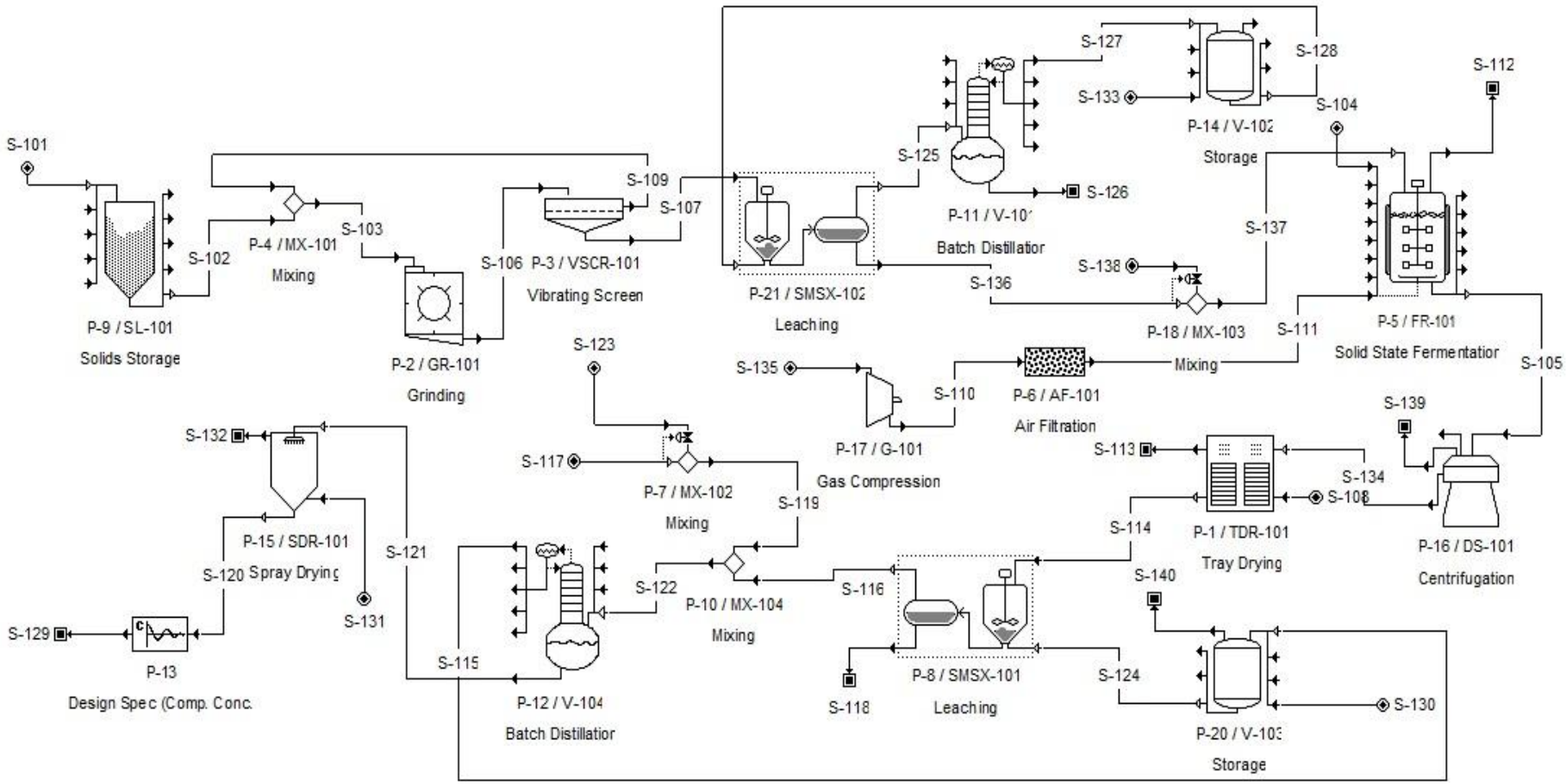

Figure 2.2 Flow diagram of astaxanthin production by simulation program

Table 2.5 Descriptions of all processes at base case

ID[a]	Processes	Operational parameters		Equipment properties		Equipment cost ($)
P-9	Solids storage	Temperature (°C) = Time (h) =	25 24	Volume (m^3) = Number =	45.6 1	78,000
P-2	Grinding					78,000
P-3	Vibrating screen	Mesh size (mm) =	0.8			7,500
P-21	Leaching (Oil extraction with hexane)	Mixer residence time (min) = Settler residence time (min) =	2 10	Number of stage = Mixer volume (L) = Settler volume (L) =	1 54 264	5,000
P-11	Batch distillation	Volatile components =	Hexane	Volume (m^3) = Number of trays =	25 5	911,000
P14	Hexane storage			Volume (m^3) = H/D =	25 1.5	171,000
P-18	Custom mixing	Set output composition of water =	90 %			
P-17	Air compression	E (%) = Time (day) = Exit temperature (°C) =	70 7 25			107,000
P-6	Air filtration	Time (day) =	7			7,500
P-5	Fermentation	Time (day) = Temperature (°C) = Aeration rate (V air/V liq.min) = Target product conc. (mg/L) =	7 20 0.001 11	Volume (m^3) = H/D = Stagger mode extra vessel =	120 3 7	250,000
P-16	Centrifuge	Solids removal		Number of units =	3	519,000

		Time (min) =	360			
P-1	Tray dryer	Drying time (min) = Final LOD = Air flow rate (wt gas/wt evaporated) = Final solids temperature (°C) =	360 10 76.92 70	Number of units = Tray area (m^2) =	2 250	187,000
P-20	DCM storage			Volume (m^3) = H/D =	5 1.5	52,000
P-8	Leaching (AX extraction)	Temperature (°C) = Mixer residence time (min) = Settler residence time (min) =	35 5 10	Number of stage = Mixer volume (L) = Settler volume (L) =	3 180 360	26,000
P-7	Custom mixing	Set output mass ratio of water =	10			
P-12	Batch distillation	Volatile components =	DCM	Volume (m^3) = Number of trays =	5 5	711,000
P-15	Spray dryer	Drying time (min) = Final LOD = Air flow rate (wt gas/wt evaporated) = Final solids temperature (°C) =	60 3.03 5 70	Volume (L) = H/D =	89 3	105,000
P-13	Design Specification (CP)	Manipulated stream = Control component = Set point mass fraction =	S-117 starch 0.07			

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Analytic Analyses

3.1.1 Standard Curve for Sugar Analysis

Maximum peak was obtained at 490 nm wavelength for the stock glucose solution. Absorbance values of 4 different glucose dilutions were measured at 490 nm. Modeling results of the data in terms of curve, equation and simple linear regression (r^2) are presented in Figure 3.1.

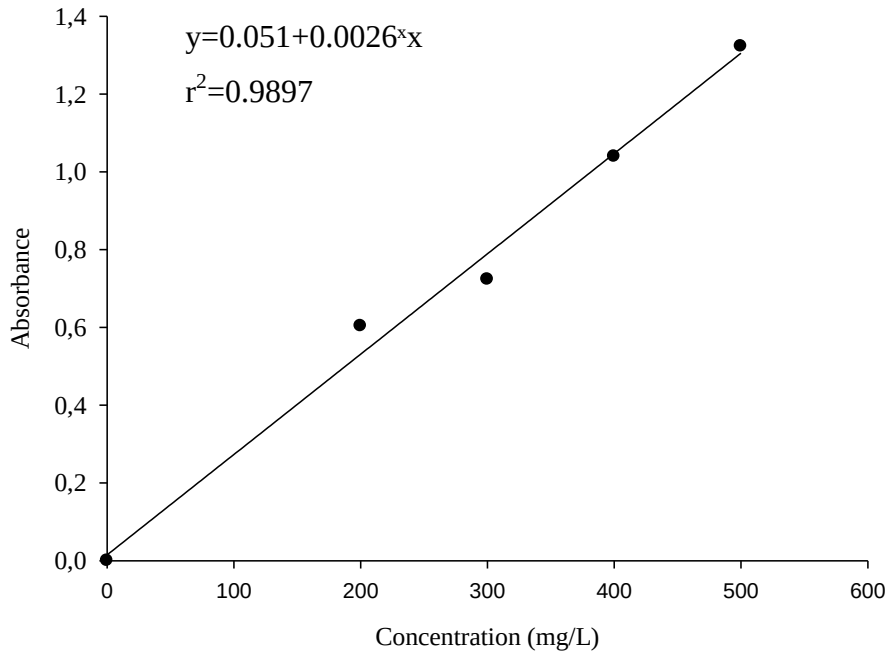

Figure 3.1 Standard curve of glucose at 490 nm

3.1.2 Analytic Analyses

The organic and inorganic content of the wastes according to dry basis calculation were determined and the results are presented in Table 3.1. The protein amount increased why the yeast formed biomass. The reason of fat amount decreasing might be the utilization of it by the yeast.

Table 3.1 The results of analytic analyses of the raw materials and the fermented contents

Content		pH	TA %	MC %	Protein %	Fat %	Ash %
Wheat bran	**r**	6.73±0.02	0.40±0.06	11.34±0.00	17.29±0.02	3.99±0.09	5.28±0.13
	f	5.90±0.05	0.25±0.06	78.85±13.50	25.10±0.01	1.32±0.03	5.95±0.02
Lentil residue	**r**	6.28±0.02	0.25±0.08	7.04±0.03	43.60±0.11	22.94±0.17	5.17±0.64
	f	7.24±0.02	0.29±1.05	71.76±15.03	47.46±1.11	1.77±0.03	4.27±0.08
Rice bran	**r**	6.93±0.00	0.44±0.02	9.79±0.01	15.52±0.31	18.08±1.90	9.73±0.09
	f	6.75±0.06	0.40±0.11	71.35±17.04	20.02±0.11	9.84±1.09	11.17±0.02

r: raw material, f: fermented content, *: not percent

3.2. Growth Curve Determination

3.2.1 Standard Curve for the Yeast Growth

Absorbance values of the dilution series versus cell number (C. N., cell/mL) for each yeast are presented in Table **A2.1**. Simple linear regression values and coefficients of the model equation ($y=y_0+a^x x$) are submitted in Table 3.2. Linear standard curves are depicted in Figures 3.2-3.5.

Table 3.2 Modeling results of standard curves of the yeasts

Sources	ATCC 90197	ATCC 24060	ATCC 24202	ATCC 24259
r^2	0.9728	0.9504	0.9945	0.9946
y_0	0.0216	0.0540	0.0291	0.0374
a	7.7519^{x}e-009	6.0267^{x}e-009	2.2775^{x}e-009	1.5766^{x}e-009

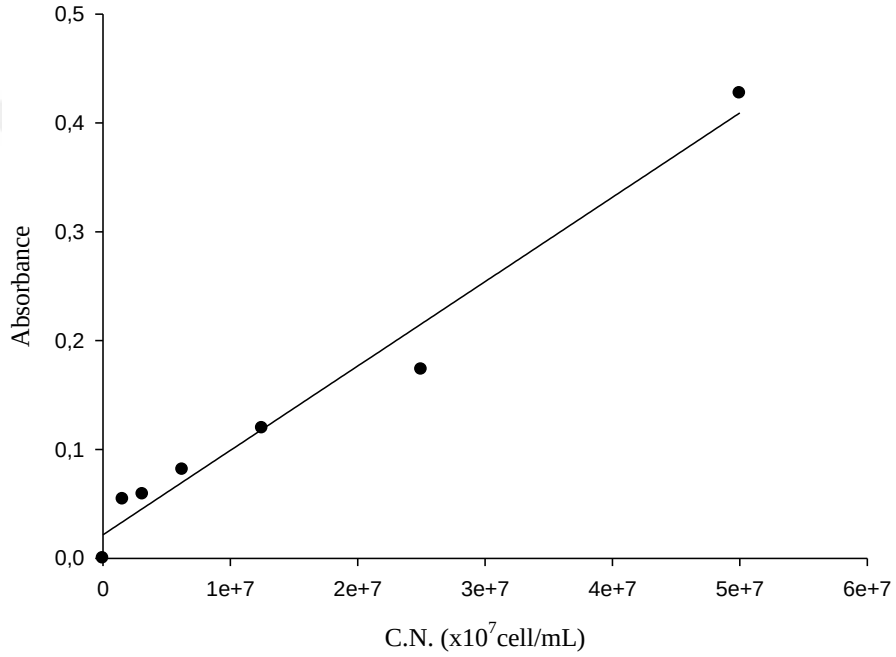

Figure 3.2 Standard curve of ATCC 90197 yeast

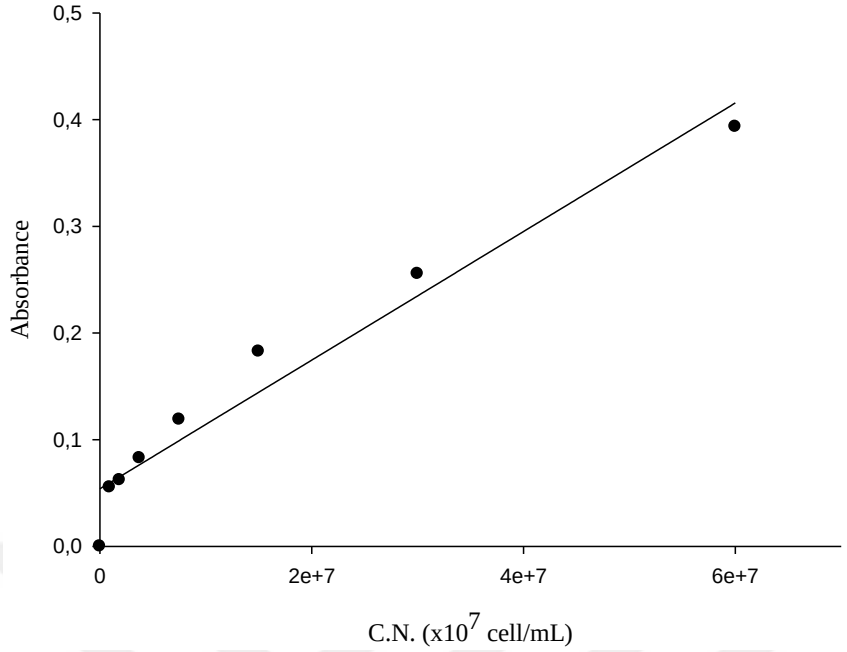

Figure 3.3 Standard curve of ATCC 24060 yeast

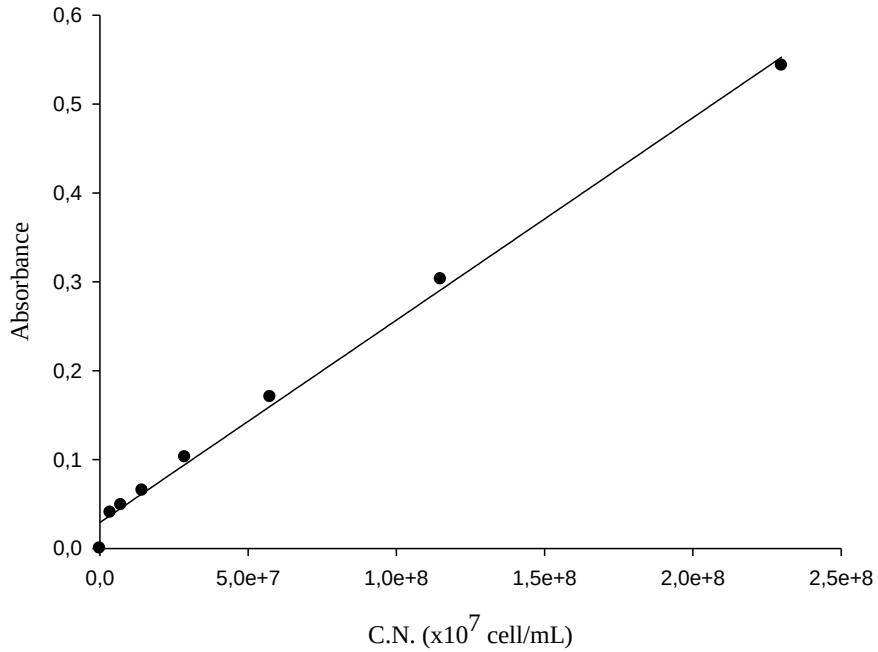

Figure 3.4 Standard curve of ATCC 24202 yeast

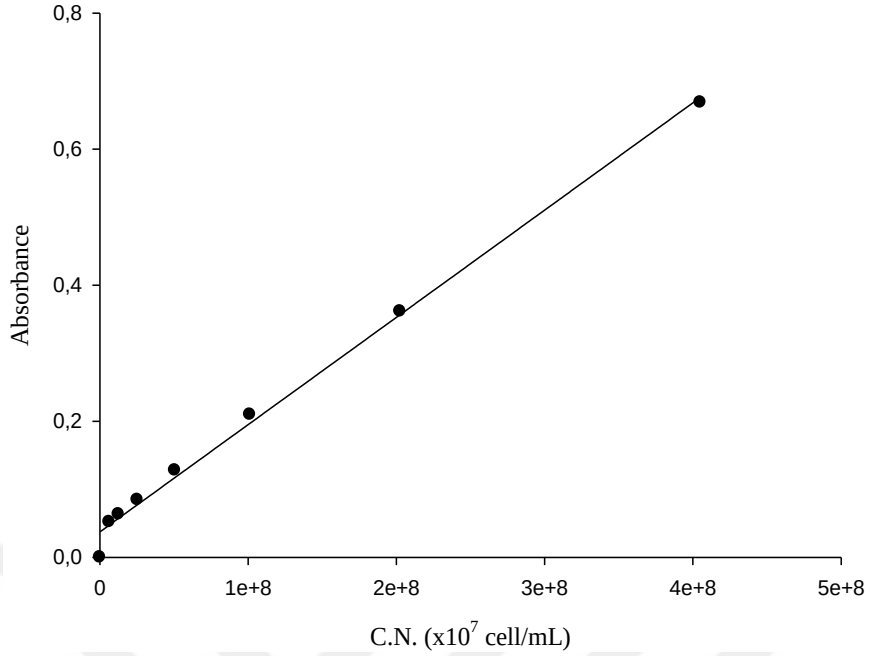

Figure 3.5 Standard curve of ATCC 24259 yeast

3.2.2 Growth Curve

Optical density (O. D.), number of viable cell (V. C.), biomass and natural logarithm of the O. D. values (ln [O. D.]) for each yeast are presented in Tables **A2.2-A2.5.** ln [O. D.] values were used for modeling in SigmaPlot program. Thirteen days for ATCC 90197 and ATCC 24060 and ten days for ATCC 24202 and ATCC 24259 were discussed as fermentation period depending on the growth deceleration. Growth curves and modeling report for each yeast are presented in Figures 3.6-3.9 and Table 3.3, respectively.

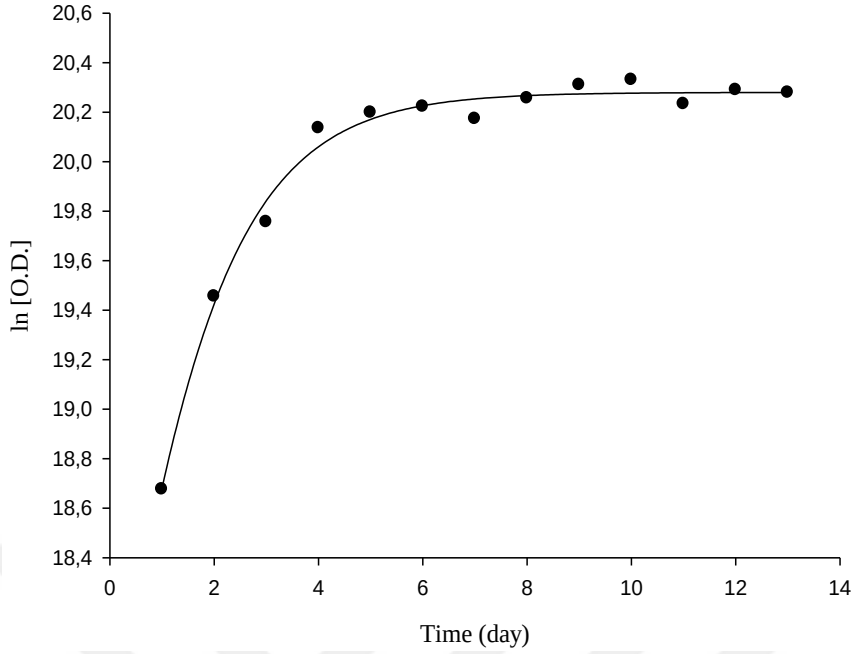

Figure 3.6 Growth curve of ATCC 90197

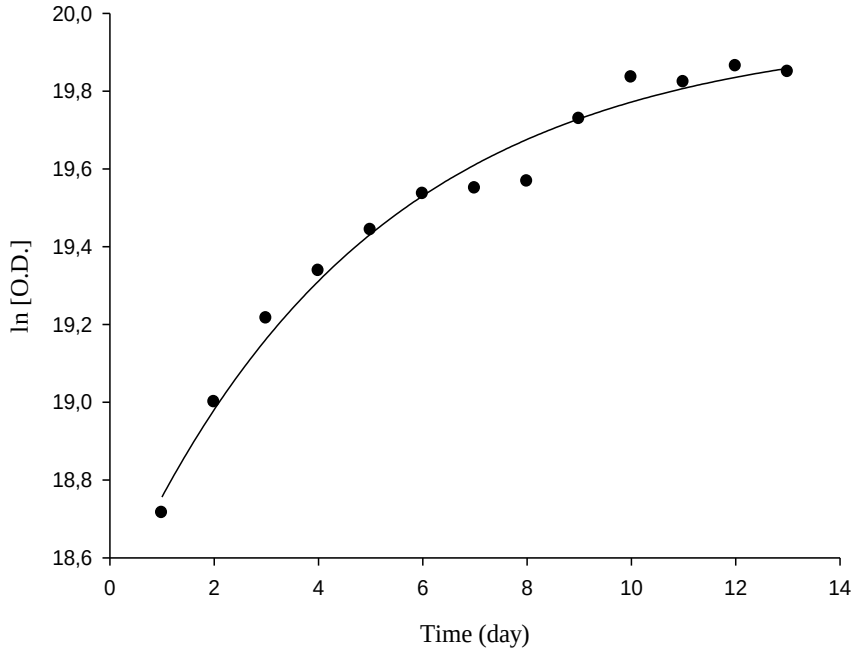

Figure 3.7 Growth curve of ATCC 24060

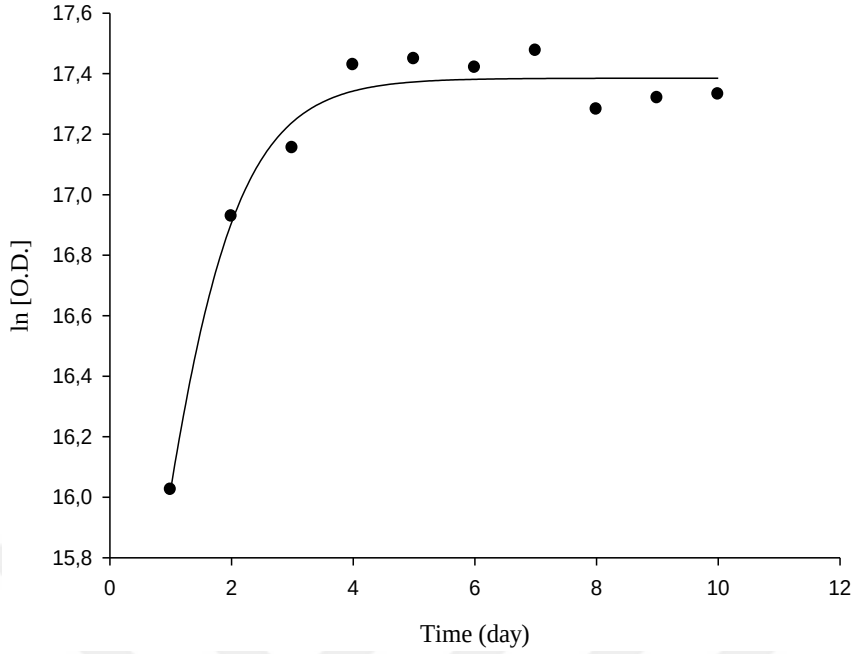

Figure 3.8 Growth curve of ATCC 24202

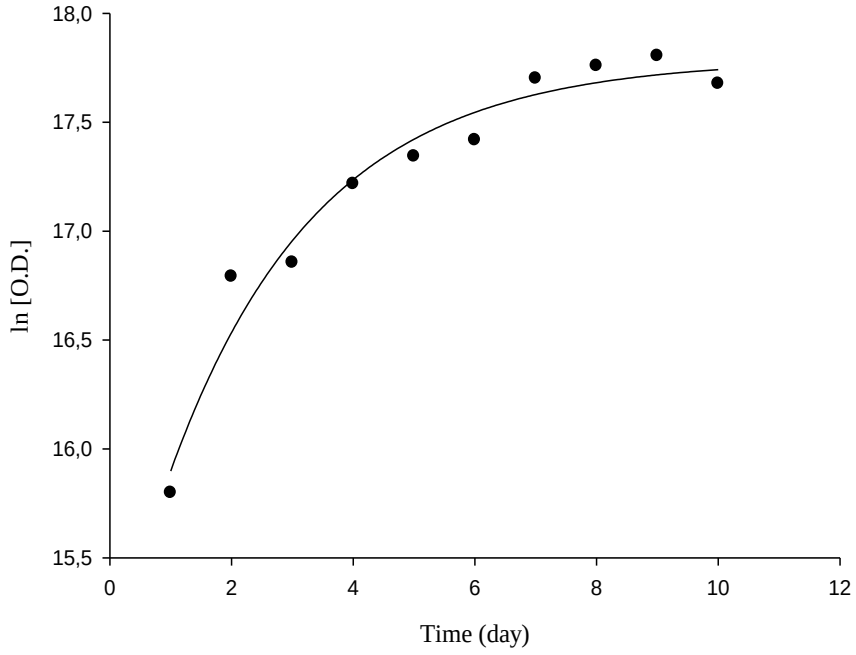

Figure 3.9 Growth curve of ATCC 24259

Table 3.3 Modeling report of the growth curve determination

Sources	ATCC 90197	ATCC 24060	ATCC 24202	ATCC 24259
a	5.4026	2179.4996	2.9690	4282.1518
b	1.4054	4.8503	0.7998	2.4397
x_0	–0.4706	–35.3826	0.6075	–17.8474
y_0	14.8773	–2159.5395	14.4159	–4264.3628
R^2	0.9898	0.9826	0.9721	0.9618
Adj. R^2	0.9864	0.9769	0.9581	0.9427
Normality test*	0.5376	0.2470	0.3128	0.1285

*: passed probability value at $p<0.05$

3.3 Pigment Analysis

3.3.1 Standard Curve for Astaxanthin

Maximum peak was obtained at 474 nm wavelength for the astaxanthin standard. Absorbance values of 4 different astaxanthin dilutions were measured. Modeling results of the data in terms of curve, equation and simple linear regression are also presented in Figure 3.10.

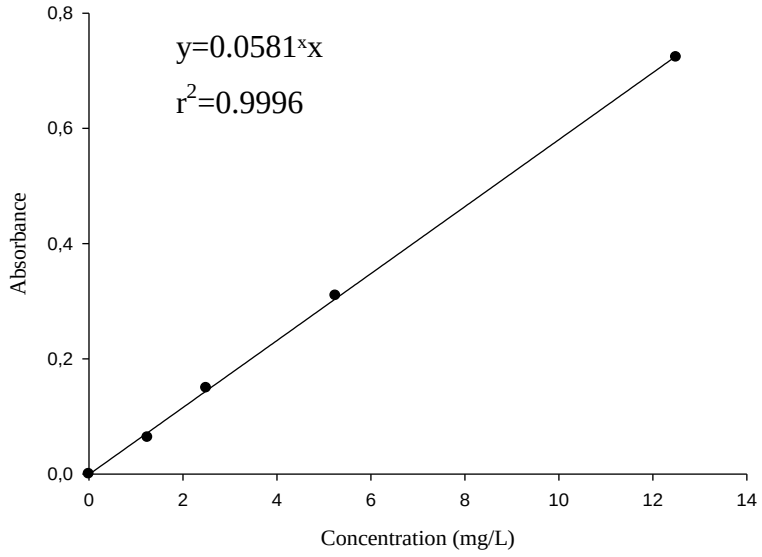

Figure 3.10 Standard curve of astaxanthin

3.3.2 Astaxanthin Analysis of Raw Material

Solid and water mixture of the fermentation flask (Table 3.4) was prepared according to the moisture content levels by doing a simple material balance.

Table 3.4 Content of the fermentation flask

MC (%)	Wheat bran		Lentil residue		Rice bran	
	Solid (g)	Water (mL)	Solid (g)	Water (mL)	Solid (g)	Water (mL)
70.0	33.84	66.16	32.27	67.73	33.26	66.75
80.0	22.56	77.44	21.52	78.49	22.17	77.83
90.0	11.28	88.72	10.76	89.24	11.09	88.92

Astaxanthin analysis was performed for raw material before SSF procedure applied. The amounts (Table 3.5) were calculated by using the standard curve equation of astaxanthin.

Table 3.5 Astaxanthin amount of the raw materials

Raw material	Astaxanthin amount (µg AX/gdw)
Wheat bran	3.93±0.08
Lentil residue	28.5±0.13
Rice bran	9.39±0.18

µg AX/gdw: microgram astaxanthin/gram dry waste

3.3.3 Pigment Examination

Pigment examination was performed in order to investigate whether the yeasts could grow on the wastes. Thus, SSF procedure would be applied depending on the results. Positive pigmentation results of the yeasts (Table 3.6) were obtained on the wastes at the optimum growth conditions.

Table 3.6 The results of pigmentation

Yeasts	Temperature (°C)	Moisture content (%)	pH	Pigmentation
ATCC 90197	25.0	80.0	5.0	+
ATCC 24060	24.0	80.0	5.6	+
ATCC 24202	20.0	80.0	4.5	+
ATCC 24259	18.0	80.0	6.0	+

3.4 Effect of Sugar Types and Concentrations

It is a well-known phenomenon for the growth of microorganisms that a carbon source is essential, glucose is in particular fundamental, and the concentration of the source determines the growth phases and period, metabolite production, and limitation of the growth. Besides, glucose and fructose are directly utilized by yeasts in their metabolic mechanisms. Sucrose may be then metabolized (Carlson, 1987). The concentration of the fermentable sugars is a significant parameter for the growth rate of the microorganisms (Arroyo-López et al, 2009). The media prepared by the glucose (G), fructose (F) and sucrose (S) sugars at 4 different concentrations were investigated to see the effects of the sugar type and concentration on the growth and product formation abilities of the selected yeasts during 10 days. Data of cell concentration versus time for each yeast in different sugar types and concentrations are presented in Tables **A3.1**-**A3.3**.

The optical density values (including viable cells, death cells and metabolites) of the yeasts at different sugar types and concentrations are shown in Figures 3.11-3.14. ATCC 90197 yeast submitted approximately the same growth trend at all sugar concentrations (Figure 3.11). The optical density values at 10 g/L concentration of all sugar types were the lowest. The O. D. changes after 150^{th} and 175^{th} hour seem that they are not significant. The growth of ATCC 24060 yeast at any sugar type and concentration did not follow any trend as seen in Figure 3.12, except a demonstration of general increasing trend after 125^{th} hour. A decrease in cell concentration was seen from the beginning of about 100^{th} to 125^{th} hours for ATCC 24202 yeast. There seems to be a few non-significant data points such as the increase in S5 after 150^{th}

hour in Figure 3.13, which is apprehensible because of the optical values including any form of cells and metabolites. It can be stated that glucose at any concentration presents a high cell concentration for ATCC 24202 yeast. The O. D. values of ATCC 24259 yeast showed significant changes from the 75th hour in any concentration of glucose and sucrose sugars in Figure 3.14. The cell concentrations at low fructose concentrations (5 and 10 g/L) were quite high for ATCC 24259 yeast. It is thought that it took time for the ATCC 24259 yeast to adapt to the F20 medium and there was no active growth at F40.

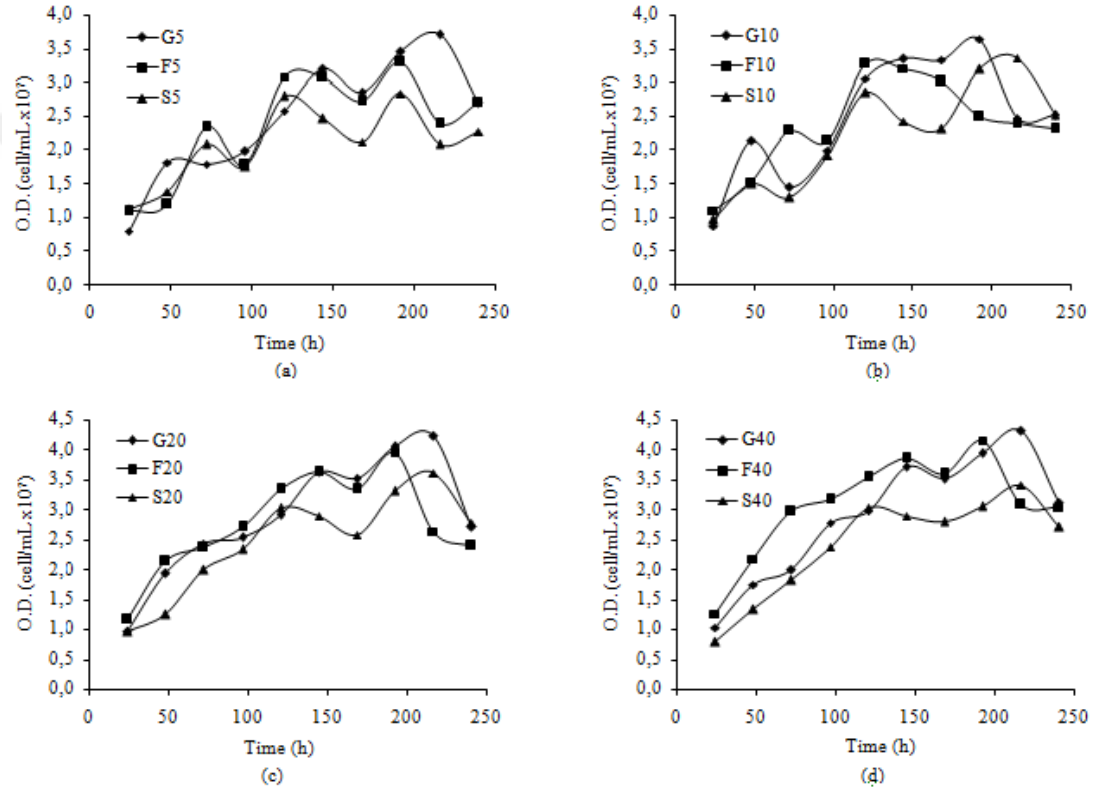

Figure 3.11 Time versus optical density values for ATCC 90197 yeast at different sugar concentrations; a: 5 g/L, b: 10 g/L, c: 20 g/L, d: 40 g/L

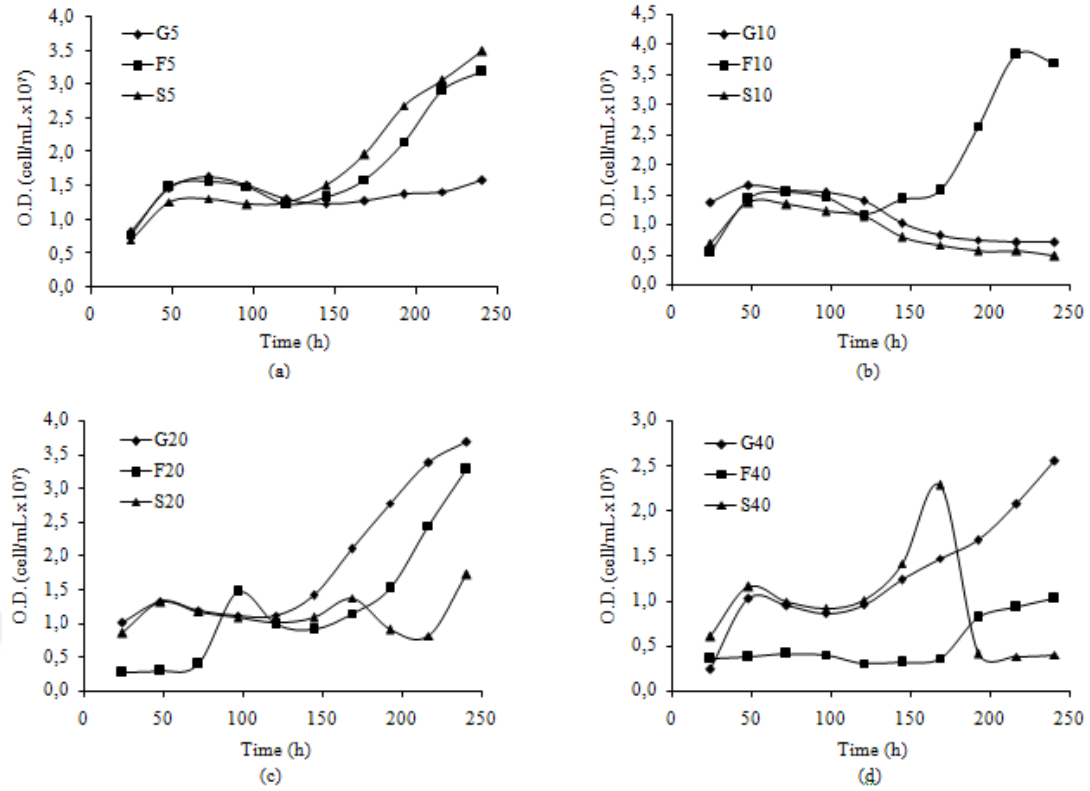

Figure 3.12 Time versus optical density values for ATCC 24060 yeast at different sugar concentrations; a: 5 g/L, b: 10 g/L, c: 20 g/L, d: 40 g/L

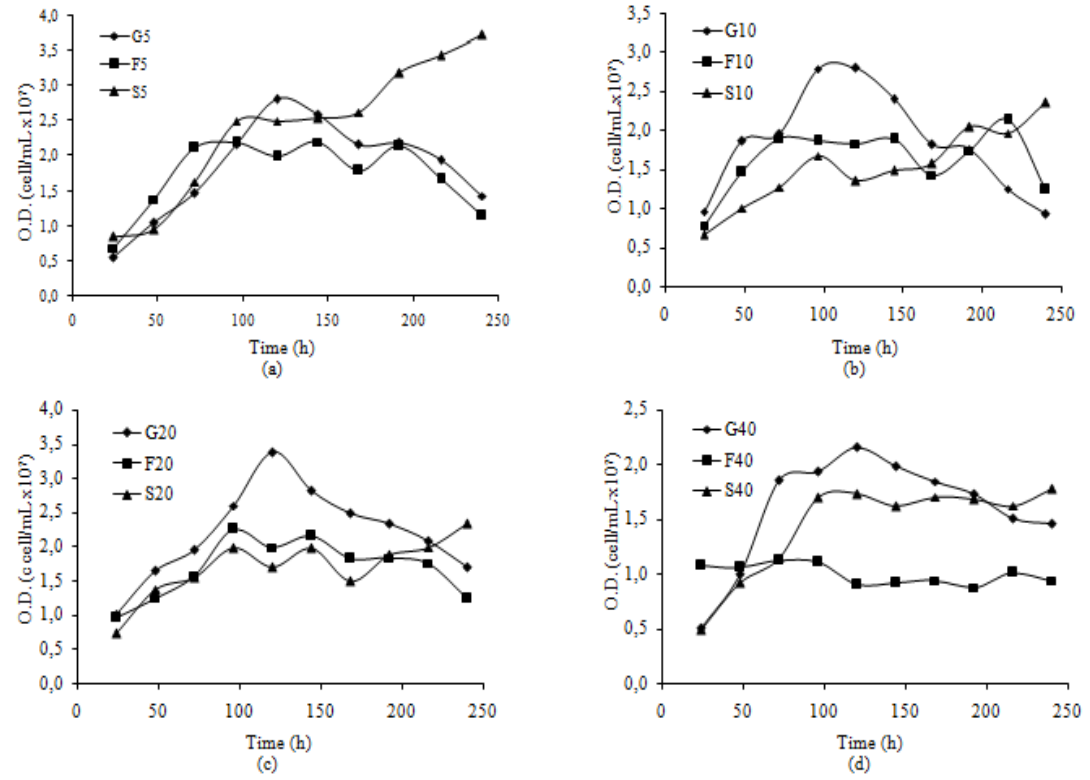

Figure 3.13 Time versus optical density values for ATCC 24202 yeast at different sugar concentrations; a: 5 g/L, b: 10 g/L, c: 20 g/L, d: 40 g/L

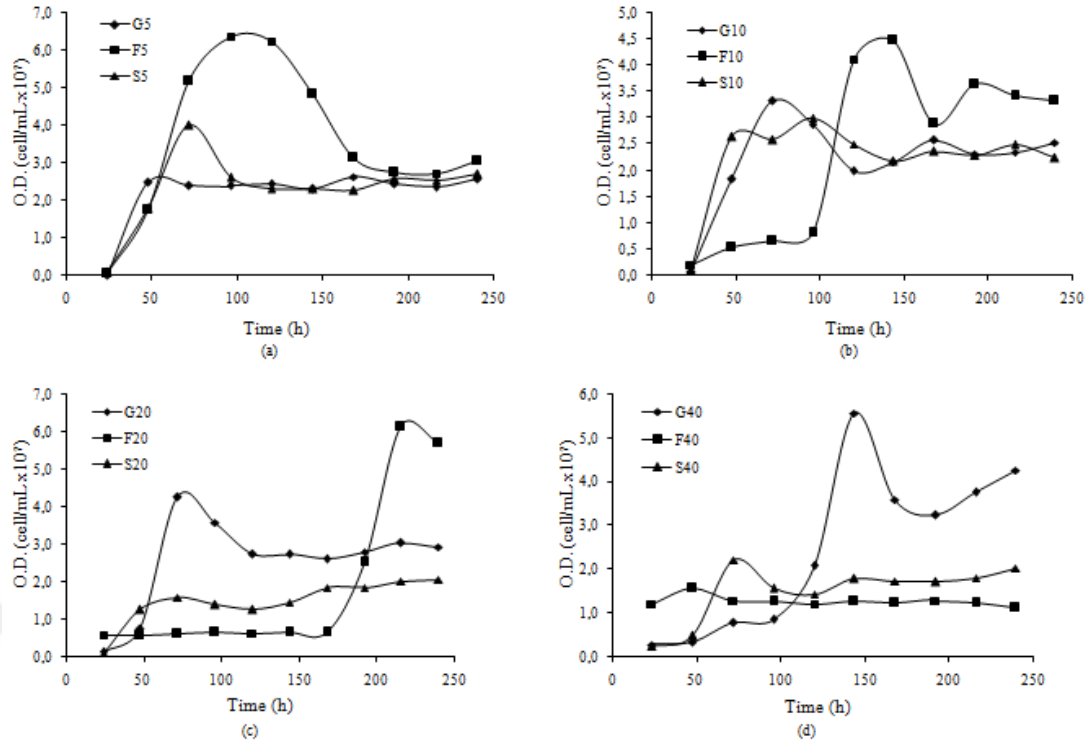

Figure 3.14 Time versus optical density values for ATCC 24259 yeast at different sugar concentrations; a: 5 g/L, b: 10 g/L, c: 20 g/L, d: 40 g/L

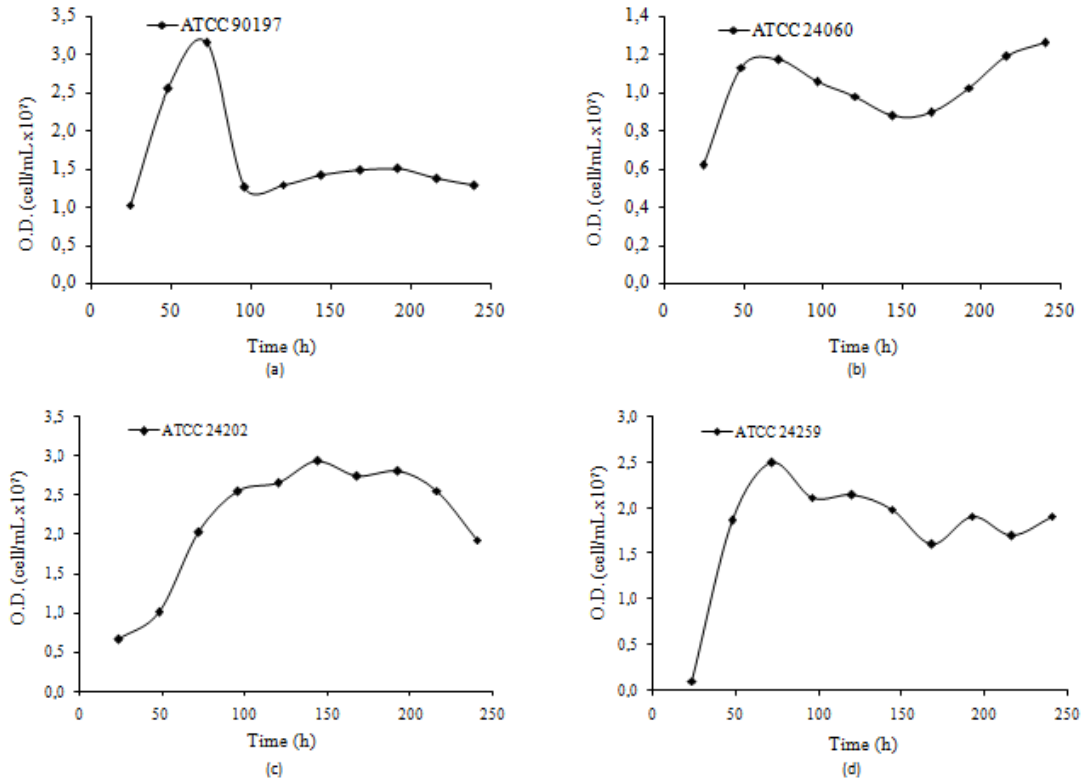

Figure 3.15 Time versus optical density values at no sugar medium for all yeasts

The increase after the 50th hour and the decrease after in no sugar medium for ATCC 90197 are not similar to any of the sugar including medium. It is thought that the growth without fermentable sugar cannot continue actively. It is obvious that the cell number of ATCC 24060 did not increase in the sugar-free medium. The growth trend resembled that of high glucose containing media and fructose containing media. ATCC 24202 yeast at a no sugar medium in Figure 15c showed a curve similar to the G40's in Figure 3.13d. The growth trend of the ATCC 24259 yeast at a no sugar medium in Figure 15d resembles the trend obtained from the glucose and sucrose sugars.

The product formation of the yeasts according to the sugar type and concentration as given in Table 3.7 showed that it was not necessary to supply more substrate for the microorganism in order to reach a high product amount and promote the cell growth, which is an explanation supported by Bailey and Ollis (1986). Glucose as the sugar type and the lowest concentration of it resulted in the highest astaxanthin produced by *X. dendrorhous.* Johnson and Lewis (1979) studied with different sugar types including glucose and sucrose to produce astaxanthin by *X. dendrorhous* (formerly *P. rhodozyma*), and indicated that the concentration of astaxanthin was higher in sucrose medium than glucose medium. Guo et al. (2010) studied with different strain of *X. dendrorhous* and found that astaxanthin yield is higher in sucrose medium that glucose and fructose. Any literature knowledge of sugar consumption has not reached for ATCC 90197 and ATCC24060 yeasts. The increasing sugar concentration revealed that the substrate inhibition on astaxanthin production for all yeasts and there was no similar effect on cell concentration in the study. It has been reported that high glucose concentration inhibits the pigment production for *X. dendrorhous* by Hu et al. (2005). No sugar medium showed that other nutrients are required for the product formation. Cells may grow in no sugar medium, but product formation requires sugar. However, it was reported that nitrogen source and concentration are important parameters for astaxanthin production and yield by Hui et al (2007) and Guo et al (2010).

Table 3.7 Astaxanthin yield and cell concentration in synthetic media

Media	Astaxanthin concentration (mg AX/L YMB)[a]				Astaxanthin amount (µg AX/g sugar)[b]				Cell concentration (cell/mL$^{x}10^{7}$)[c]			
	ATCC* 90197	ATCC* 24060	ATCC 24202	ATCC 24259	ATCC 90197	ATCC 24060	ATCC 24202	ATCC 24259	ATCC 90197	ATCC 24060	ATCC 24202	ATCC 24259
G5	0.0006	0.0028	2.08±0.02	0.51±0.02	3.15±0.00	13.92±0.93	51.88±0.57	12.72±0.44	2.69	1.59	1.42	2.57
G10	0.0016	0.003	1.96±0.02	0.60±0.01	3.96±0.00	7.38±0.34	24.44±0.22	7.53±0.11	2.69	3.19	1.14	3.05
G20	0.0024	0.0033	1.87±0.02	0.81±0.00	2.95±0.00	4.14±0.17	11.71±0.13	5.04±0.02	2.27	3.50	3.72	2. 69
G40	0.0032	0.0142	1.76±0.02	0.75±0.02	1.99±0.01	8.9±8.95	5.50±0.05	2.34±0.06	2.51	0.70	0.93	2.52
F5	0.0011	0.0034	1.88±0.02	0.72±0.01	5.26±0.71	17.1±0.65	46.93±0.59	17.98±0.23	2.31	3.70	1.24	3.32
F10	0.0024	0.0045	2.08±0.03	0.68±0.04	5.97±0.15	11.22±0.24	26.02±0.34	8.49±0.50	2.51	0.48	2.35	2.23
F20	0.0033	0.0064	2.42±0.02	1.38±0.02	4.18±0.11	8.02±0.11	15.13±0.11	8.61±0.16	2.70	3.68	1.69	2.94
F40	0.0062	0.0059	0.70±0.02	1.19±0.01	3.88±0.06	3.66±0.08	2.20±0.07	3.73±0.04	2.40	3.27	1.23	5.67
S 5	0.0003	0.0017	1.91±0.02	0.34±0.01	1.42±0.00	8.71±0.33	47.67±0.49	8.39±0.28	2.78	1.72	2.34	2.07
S10	0.0003	0.0017	1.90±0.01	0.41±0.02	0.8±0.00	4.16±0.01	23.71±0.07	5.10±0.29	3.11	2.55	1.46	4.26
S20	0.0003	0.0024	2.72±0.01	0.37±0.01	0.39±0.00	2.97±0.04	16.98±0.05	2.33±0.09	3.04	1.02	0.94	1.12
S40	0.0002	0.001	1.82±0.02	0.33±0.02	0.15±0.03	0.63±0.01	5.70±0.06	1.03±0.05	2.71	0.40	1. 8	2.03
No sugar	0.0049	0.0019	1.60±0.01	0.27±0.02	0.0	0.0	0.0	0.0	1.28	1.26	1.92	1.91

[a]: Astaxanthin concentration: milligram astaxanthin/Liter yeast malt extract broth as mean value of duplicate results

[b]: Astaxanthin amount: microgram astaxanthin/gram sugar as mean value of duplicate results

[c]: Optical density values at 540 nm

*: 4th digit of standard deviation (±) was not written due its ineffective

The production of the astaxanthin pigment from the wheat, lentil and rice wastes was achieved at the optimal growth conditions of the yeasts (Table 3.8). Based on the sugar content of the wastes, the wheat bran provided the highest astaxanthin yield (201.49 µgAX/g glucose) by ATCC 24202 yeast. The solid waste medium with low sugar content as it is in the synthetic environment has provided the highest productivity. It was seen that the selected yeasts were inhibited in the presence of excess sugar. After the consideration of the astaxanthin yield obtained from the wastes, an optimization study for all yeasts by SSF technology without any substrate addition was conducted in the scope of Box-Behnken (BBD) experimental design.

Table 3.8 Astaxanthin yield produced in waste medium at optimum growth conditions of the yeasts

Sugar content (g glu/g w)[a]		**Yeast**	**Wheat bran**	**Lentil residue**	**Rice bran**
			0.44±0.01	0.75±0.00	0.88±0.00
AX amount	**(µg AX/g w)[b]**	**ATCC 90197**	11.33±0.35	5.73±0.40	10.08±0.39
		ATCC 24060	8.83±0.44	6.22±0.08	11.39±1.28
		ATCC 24202	87.83±5.67	59.33±4.57	50.17±2.54
		ATCC 24259	35.55±1.19	21.70±0.51	13.34±0.36
AX amount	**(µg AX/g glu)[c]**	**ATCC 90197**	25.99±0.80	7.63±0.53	11.39±0.44
		ATCC 24060	20.26±1.01	8.28±0.11	12.87±1.45
		ATCC 24202	201.49±13.02	79.02±6.09	56.69±2.87
		ATCC 24259	81.56±2.72	28.9±0.67	15.08±0.40

[a]: Sugar content: gram glucose/gram waste as mean value of duplicate results

[b]: Astaxanthin amount: microgram astaxanthin/gram waste as mean value of triplicate results

[c]: Astaxanthin amount: microgram astaxanthin/gram glucose as mean value of triplicate results

3.5 Solid State Fermentation Systems

The fermentation systems of ATCC 90197, ATCC 24060, ATCC 24202 and ATCC 24259 with wheat, lentil and rice wastes were performed according to the BBD matrixes. Astaxanthin amount as response of each yeast fermentation system was measured daily during the fermentation period and the results of the response surface methodology containing ANOVA, model coefficients, optimized conditions and 3-dimensional plots were presented for each waste.

3.5.1 Wheat Bran Fermentation Systems

The data of astaxanthin amount response for each yeast according to the BBD matrix was presented in Table 3.9. Significant status (+) of 'model' is good, but not good for 'lack of fit', which means model chosen at the determined probability represents a fitting between experimental and predicted data (Montgomery, 2001). It is seen in Table 3.10 that the quadratic model was significant at $p<0.1$ probability for 'model' and 'lack of fit' tools for all yeasts, except ATCC 24060. R^2 (determination coefficient, multiple correlation coefficient) and adjusted R^2 are used to show the verification of the model whether it is sufficient and fit for the data. The fitted proportion of the total variation for the response is revealed by the R^2 value. High R^2 value indicates high satisfaction of the fitting. The adjustment of sample size and term number in the model is done by the adjusted R^2 that analyzes the adequacy and fitness of the model (Swaym et al., 2014). R^2 and adjusted R^2 values of ATCC 24259; 0.89 and 0.74 respectively presented more satisfied results. Low standard deviation (square root of the pure experimental error) and coefficient of the variance (C.V. %) values are better results for precision and reliability of the experimental data (Montgomery, 2001; Zou et al., 2013). ATCC 90197 demonstrated the lowest standard deviation and C.V. % values.

The model coefficients (Table 3.11) and response surface plots (Figures 3.16-3.19) depicted that the effect of each variable and the interactions of them on the response. Temperature was more effective and significant parameter for ATCC 90197 and ATCC 24060 fermentation systems. Temperature levels close to the optimum growth temperature values of ATCC 90197, ATCC 24060 and ATCC 24202 induced high

AX production. Moisture content was the most effective parameter for ATCC 24202 fermentation system. The AX yield increased when the level of the moisture content increased. High moisture content and pH levels influenced the AX yield positively for ATCC 24259 fermentation. When the interactions of temperature and moisture content, and also moisture content and pH increased, the yield increased for ATCC 24259 and ATCC 24202 fermentation systems. All the yeasts were affected by increasing of the interaction of temperature and pH parameters.

Table 3.9 Astaxanthin results of wheat bran fermentation systems

Run	Astaxanthin amount (µg AX/gdw)			
	ATCC 90197	**ATCC 24060**	**ATCC 24202**	**ATCC 24259**
1	14.35±0.61	11.56±0.20	95.00±2.45	60.54±1.83
2	20.33±0.26	17.47±3.24	55.90±1.21	41.81±2.47
3	18.96±1.36	15.49±0.23	34.98±2.90	37.26±0.87
4	22.29±0.04	10.17±0.39	66.31±1.75	11.49±0.32
5	14.26±0.89	12.20±0.67	28.96±2.89	30.50±0.16
6	15.67±0.72	17.28±1.53	79.64±5.17	17.21±0.48
7	9.61±0.87	10.45±0.16	70.76±4.53	20.76±0.71
8	16.19±1.57	9.76±0.57	86.80±8.65	36.62±1.78
9	16.53±0.45	15.80±0.33	60.85±1.55	51.83±1.68
10	11.16±0.75	9.08±0.28	60.84±2.20	32.78±0.92
11	9.79±0.67	10.48±0.50	72.84±1.26	35.32±1.19
12	15.43±0.42	13.28±1.11	33.40±5.27	51.56±2.18
13	17.47±0.17	9.74±0.58	109.23±12.08	16.76±1.79
14	13.13±0.73	4.92±0.31	84.46±0.00	35.37±0.38
15	15.46±0.21	12.18±0.48	88.99±0.17	11.60±0.20
16	16.65±0.38	16.57±0.70	26.82±2.76	21.42±0.56
17	15.21±0.18	9.34±0.33	87.70±8.20	19.90±0.62

Table 3.10 Quantitative model assessment tools for wheat bran fermentation systems

Tools	p<	ATCC 90197	ATCC 24060	ATCC 24202	ATCC 24259
Model	0.10	+	–	+	+
	0.05	–0.0901	–0.4986	–0.0507	+0.0136
	0.01	–	–	–	–
Lack of fit	0.10	–	+	–	–
	0.05	–0.8285	+0.0062	–0.1701	–0.6817
	0.01	–	+	–	–
Standard deviation		2.3639	3.4309	15.6699	7.4771
Mean		15.4398	12.1038	67.2639	31.3367
C.V. %		15.3104	28.3455	23.2961	23.8604
PRESS		163.2748	1249.1195	19561.2229	2233.4014
R^2		0.7860	0.5686	0.8246	0.8858
Adj. R^2		0.5109	0.0139	0.5991	0.7389

+: significant, –: not significant

Table 3.11 Coefficients of the model equations of each optimized wheat bran fermentation systems

Coefficients	ATCC 90197		ATCC 24060		ATCC 24202		ATCC 24259	
	Coded	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**
β_0	18.4101	–135.9611	10.7375	–11.5758	80.7968	–1203.4599	29.5950	178.3606
β_1	–2.2032	6.9057	–2.3843	–3.5432	–2.6167	9.2277	–0.9554	–13.2096
β_2	–1.0704	0.2298	–0.2080	3.1171	24.5856	34.4392	17.7734	–3.4578
β_3	0.1416	27.3178	0.0674	–19.1022	–1.4973	–122.3691	5.2886	7.7306
β_{12}	–0.4495	–0.0090	–1.2670	–0.0253	0.6725	0.0135	2.3546	0.0471
β_{13}	0.6686	0.1337	1.5040	0.3008	9.2562	1.8512	0.8253	0.1651
β_{23}	–0.4117	–0.0412	–1.5426	–0.1543	5.0800	0.5080	4.1536	0.4154
β_{11}	–3.6479	–0.1459	1.7756	0.0710	–11.9735	–0.4789	5.7367	0.2295
β_{22}	0.0586	0.0006	–1.0412	–0.0104	–21.5848	–0.2158	1.1846	0.0118
β_{33}	–2.7226	–2.7226	2.1689	2.1689	4.8008	4.8008	–3.2201	–3.2201

The parameters were optimized based on the maximal yield for each yeast as shown in Table 3.12. The maximum yield, 109.23 μg AX/gdw, by ATCC 24202 and wheat bran fermentation was obtained at the conditions; 20.0 °C temperature, 5.5 pH and 90.0 % moisture content. Johnson and Lewis (1979) reported that higher astaxanthin amount was obtained at 4.5 pH than the amount at 5.5 pH. However, Yuanshuai (2006) indicated that 5.0 pH is selected in most recent studies and a temperature range, 15-20 °C is recommended and for astaxanthin production. Maximum astaxanthin concentration from ATCC 24202 was reached at temperature of 19.7 °C and 6.0 pH using a synthetic medium in the scope of an experimental design generated by Ramírez et al. (2001). Ananda and Vadlani (2011) studied with wheat bran to produce astaxanthin by ATCC 24202. At the end of 11 fermentation days, they managed to produce 66.75 μg AX/g substrate from wheat bran.

Table 3.12 Experimental and predicted values of the optimized conditions for wheat bran fermentation systems

Optimized conditions	**ATCC 90197**		**ATCC 24060**		**ATCC 24202**		**ATCC 24259**	
	Coded	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**
x_1 (°C)	23.83	25.0	19.0	24.0	21.48	20.0	23.0	23.0
x_2 (%)	70.0	80.0	90.0	90.0	86.92	90.0	90.0	90.0
x_3	5.07	5.0	4.6	4.6	5.5	5.5	7.0	6.0
AX amount (μg AX/gdw)	19.76	22.29	20.06	17.47	95.35	109.23	62.74	60.54

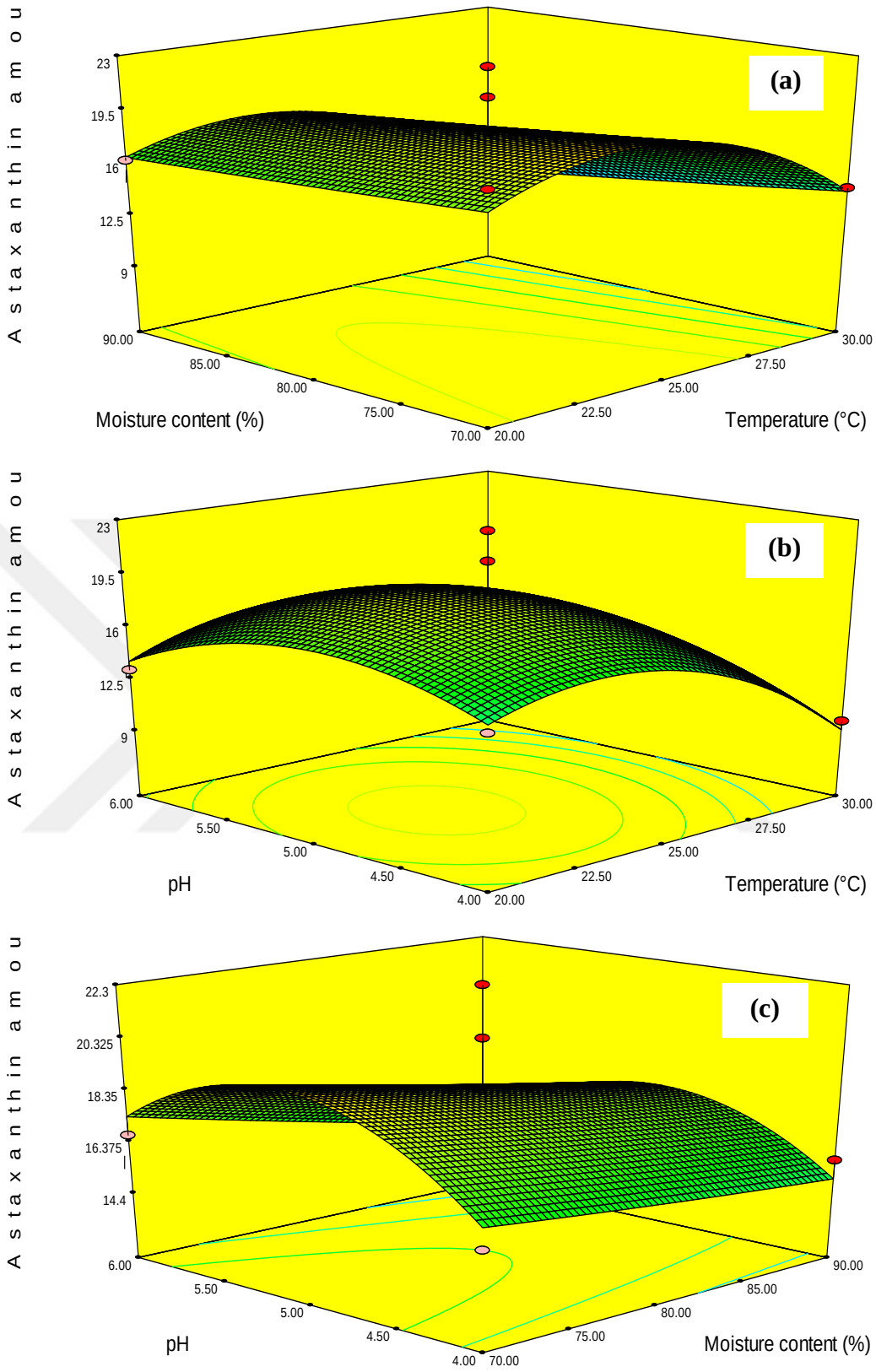

Figure 3.16 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 90197 wheat bran fermentation system

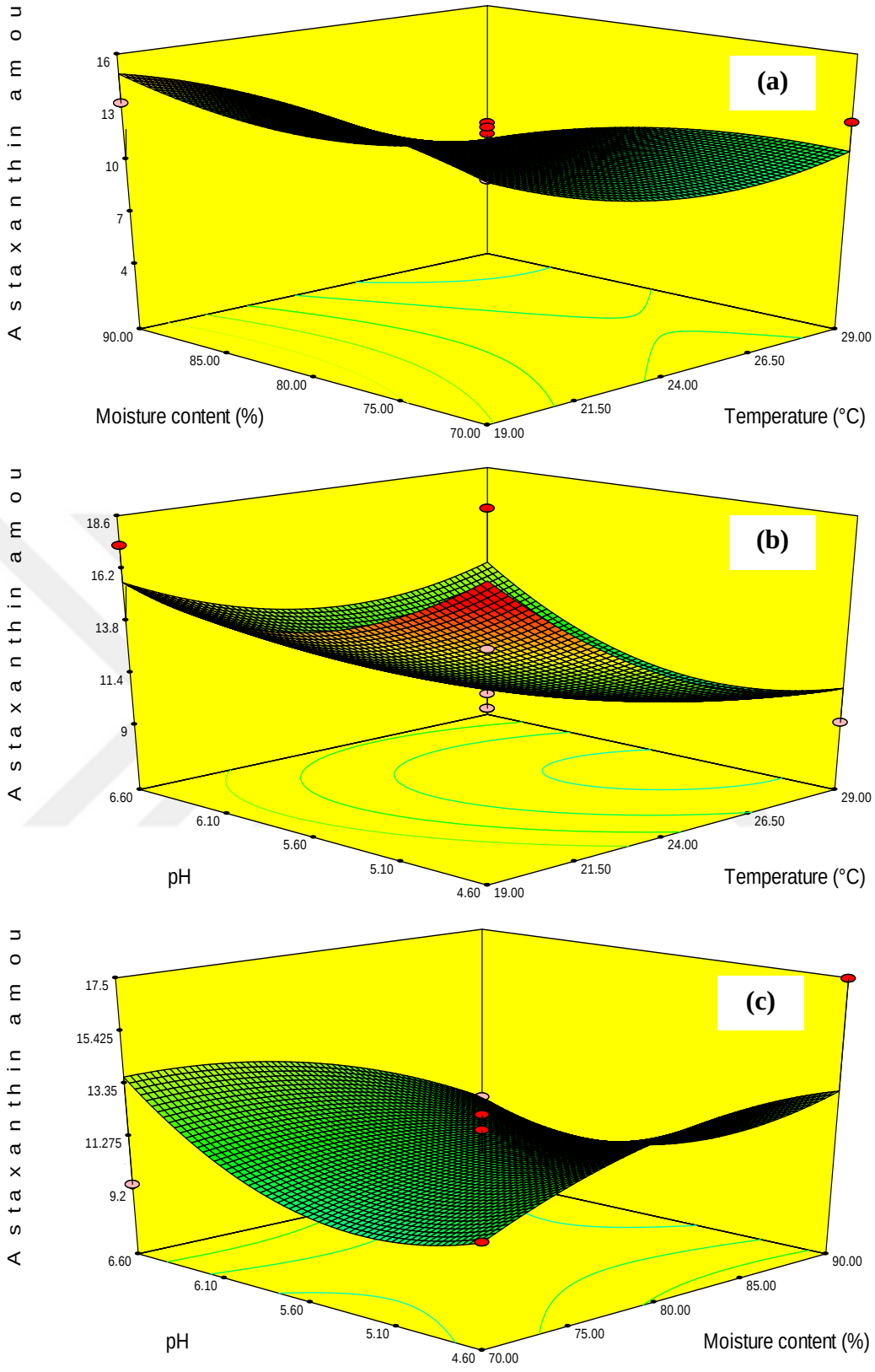

Figure 3.17 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24060 wheat bran fermentation system

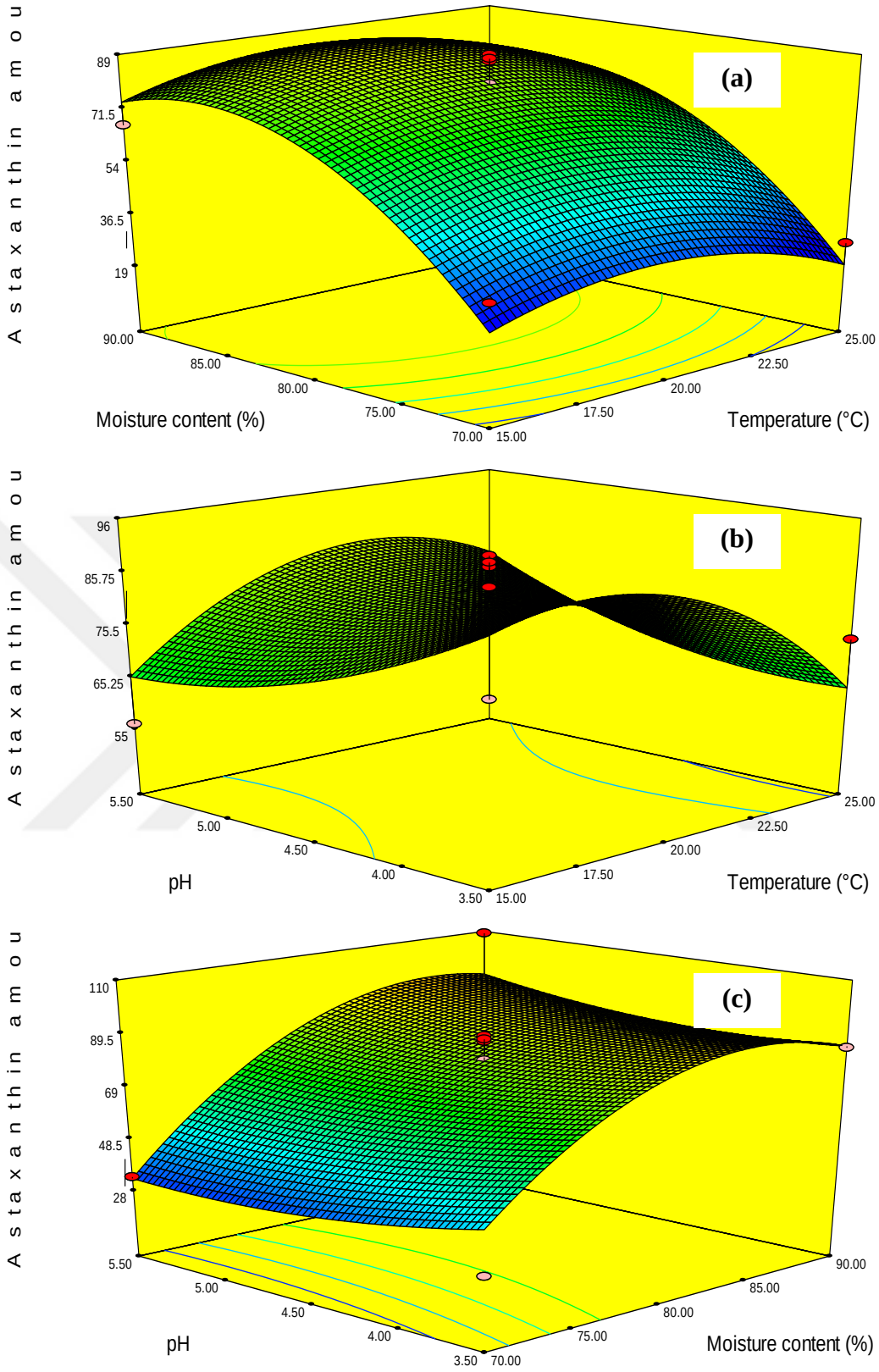

Figure 3.18 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24202 wheat bran fermentation system

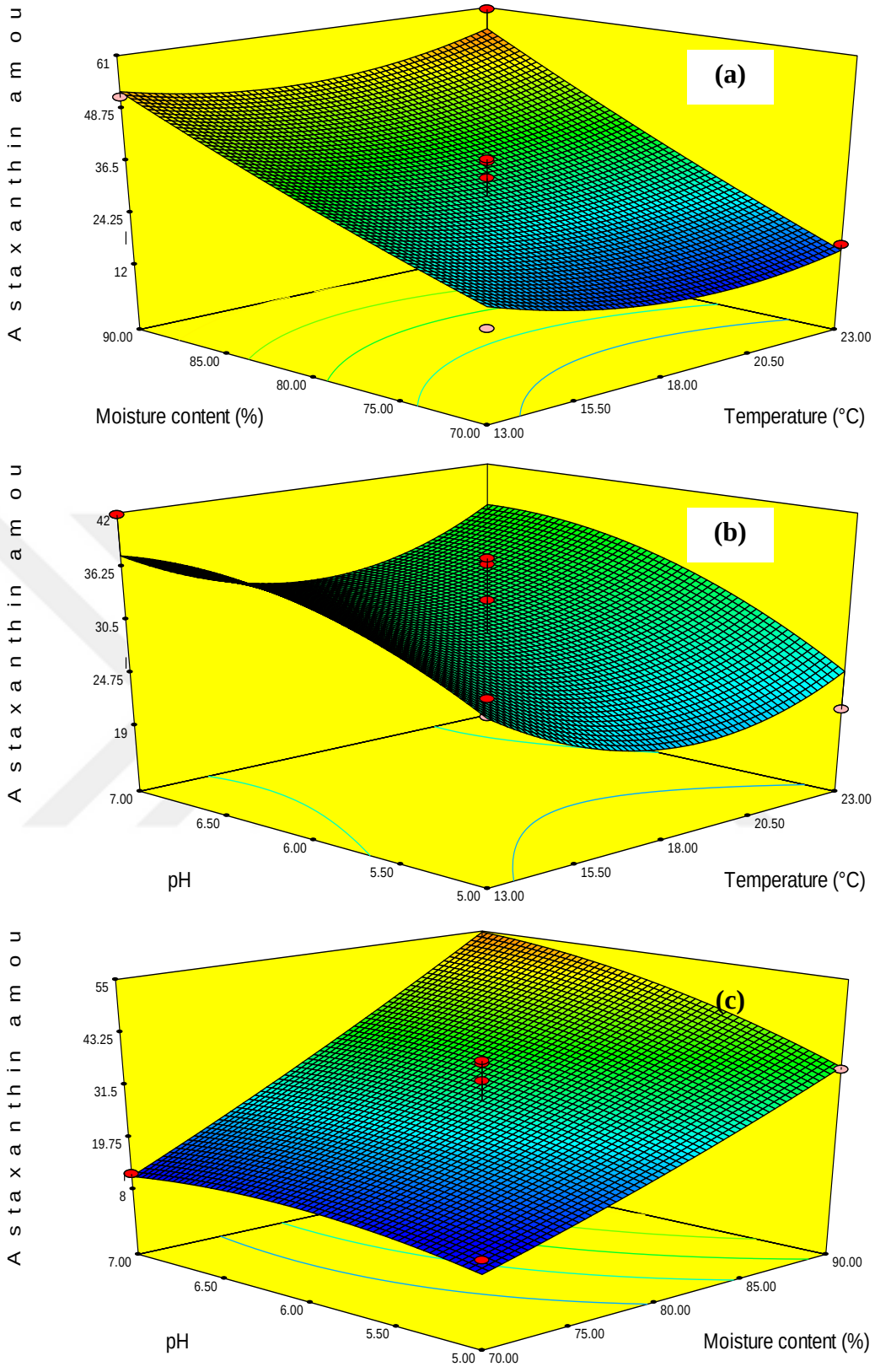

Figure 3.19 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24259 wheat bran fermentation system

3.5.2 Lentil Residue Fermentation Systems

Astaxanthin amount produced by four yeasts using lentil residue was introduced in Table 3.13. The model was significant at $p<0.1$ probability and lack of fit was significant just for ATCC 90197 and ATCC 24060 yeasts as seen in Table 3.14. The highest R^2 (0.95) and adjusted R^2 (0.89) values and the other tools' values in the table denoted ATCC 90197 yeast for good statistical results. However, maximum astaxanthin amount, 100.25 µg AX/gdw, was obtained from the lentil residue and ATCC 24202 fermentation system at 20.0 °C temperature, 5.5 pH and 90.0 % moisture content, which the second highest astaxanthin amount had been produced at the same conditions with the wheat bran fermentation system. The model coefficients and optimum conditions of all the yeasts are presented in Table 3.15 and Table 3.16 respectively.

Table 3.13 Astaxanthin results of lentil residue fermentation systems

Run	Astaxanthin amount (µg AX/gdw)			
	ATCC 90197	**ATCC 24060**	**ATCC 24202**	**ATCC 24259**
1	10.68±0.19	10.87±0.85	37.40±0.94	36.11±3.13
2	3.54±0.14	0.00±0.00	31.50±1.08	47.73±6.29
3	11.15±0.24	12.46±0.06	11.53±0.95	20.06±0.56
4	7.54±0.60	6.42±0.28	38.02±3.14	16.04±1.16
5	10.76±0.92	4.40±0.09	19.17±0.00	14.75±0.34
6	24.75±1.59	10.68±0.09	49.44±0.00	14.16±0.74
7	7.06±0.53	6.37±0.05	51.35±5.55	15.65±0.45
8	25.87±1.98	6.70±0.06	59.59±3.07	14.05±0.42
9	6.83±0.42	10.19±0.23	57.28±8.56	35.82±1.28
10	9.27±0.26	6.47±0.12	90.51±0.96	25.30±0.28
11	15.05±2.67	0.28±0.05	64.22±2.62	58.15±1.41
12	3.12±0.12	5.17±0.15	70.2±52.36	36.85±1.46
13	7.24±0.67	5.60±0.13	100.25±0.00	13.05±0.04
14	6.92±0.64	5.48±0.50	75.15±0.00	16.22±1.24
15	10.97±0.40	7.36±0.14	61.12±2.09	9.44±1.13
16	11.06±0.70	9.78±0.64	16.70±0.78	20.89±0.56
17	22.41±3.80	2.75±0.22	64.72±5.40	20.54±0.63

Table 3.14 Quantitative model assessment tools for lentil residue fermentation systems

Tools	p<	ATCC 90197	ATCC 24060	ATCC 24202	ATCC 24259
Model	0.10	+	+	+	+
	0.05	+0.0007	+0.0414	+0.0206	–0.0523
	0.01	+	–	–	–
Lack of fit	0.10	–	–	+	+
	0.05	–0.4071	–0.3987	+0.0183	+0.0260
	0.01	–	–	–	–
Standard deviation		2.2471	2.1658	13.6106	8.7492
Mean		11.4247	6.5275	52.8316	24.3995
C.V. %		19.6684	33.1803	25.7623	35.8580
PRESS		300.5112	282.1503	18867.0789	7642.6583
R^2		0.9529	0.8361	0.8696	0.8228
Adj. R^2		0.8923	0.6253	0.7019	0.5949

Table 3.15 Coefficients of the model equations of each optimized lentil residue fermentation systems

Coefficients	ATCC 90197		ATCC 24060		ATCC 24202		ATCC 24259	
	Coded	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**
β_0	5.6535	555.6022	7.3790	–52.1781	58.4286	–237.4092	20.1651	–116.2402
β_1	–1.5803	1.0736	–1.5459	–10.6050	13.0403	16.6989	–4.0569	9.7712
β_2	5.5263	–12.9152	–0.3528	4.3414	23.2897	–0.1568	14.2797	–3.1850
β_3	–0.4875	–25.4786	1.5880	4.9418	7.1687	–0.1106	2.2263	39.7894
β_{12}	–2.5877	–0.0518	2.0936	0.0419	11.8316	0.2366	0.0906	0.0018
β_{13}	1.5117	0.3023	0.7050	0.1410	–1.7421	–0.3484	–8.1016	–1.6203
β_{23}	–0.6068	–0.0607	0.9873	0.0987	–6.4830	–0.6483	–7.2321	–0.7232
β_{11}	0.6195	0.0248	3.2065	0.1283	–19.6584	–0.7863	–0.6983	–0.0279
β_{22}	9.4157	0.0942	–3.7090	–0.0371	0.4190	0.0042	5.5748	0.0557
β_{33}	2.2287	2.2287	–1.3068	–1.3068	7.3457	7.3457	4.1216	4.1216

Table 3.16 Experimental and predicted values of the optimized conditions for lentil residue fermentation systems

Optimized conditions	ATCC 90197		ATCC 24060		ATCC 24202		ATCC 24259	
	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual
x_1 (°C)	20.11	20.0	19.0	19.0	23.39	20.0	23.0	18.0
x_2 (%)	89.99	90.0	77.0	70.0	90.0	90.0	90.0	90.0
x_3	4.0	5.0	5.83	5.6	3.5	5.5	5.0	5.0
AX amount (μg AX/gdw)	30.04	25.87	12.60	12.46	97.81	100.25	52.60	58.15

Figures 3.20-3.23 as the response surface plots demonstrated that temperature had slight effect for all fermentation systems. Generally low and middle temperature values affected astaxanthin production in a positive way. Moisture content was the inducer parameter for all fermentation systems. The AX yield increased when the level of the moisture content was at its maximum value (90 %). High pH value caused increasing of astaxanthin amount except ATCC 90197 yeast.

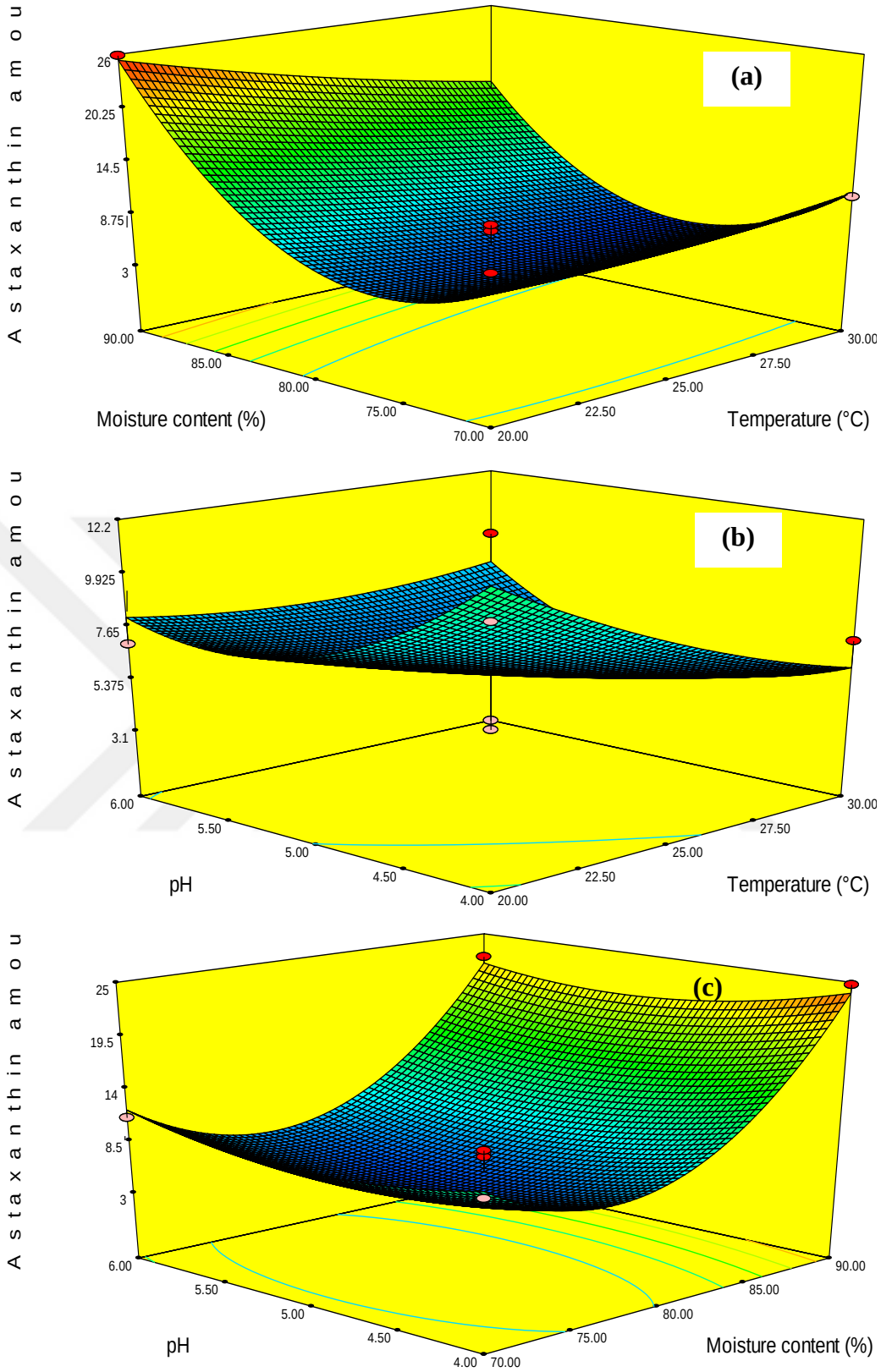

Figure 3.20 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 90197 lentil residue fermentation system

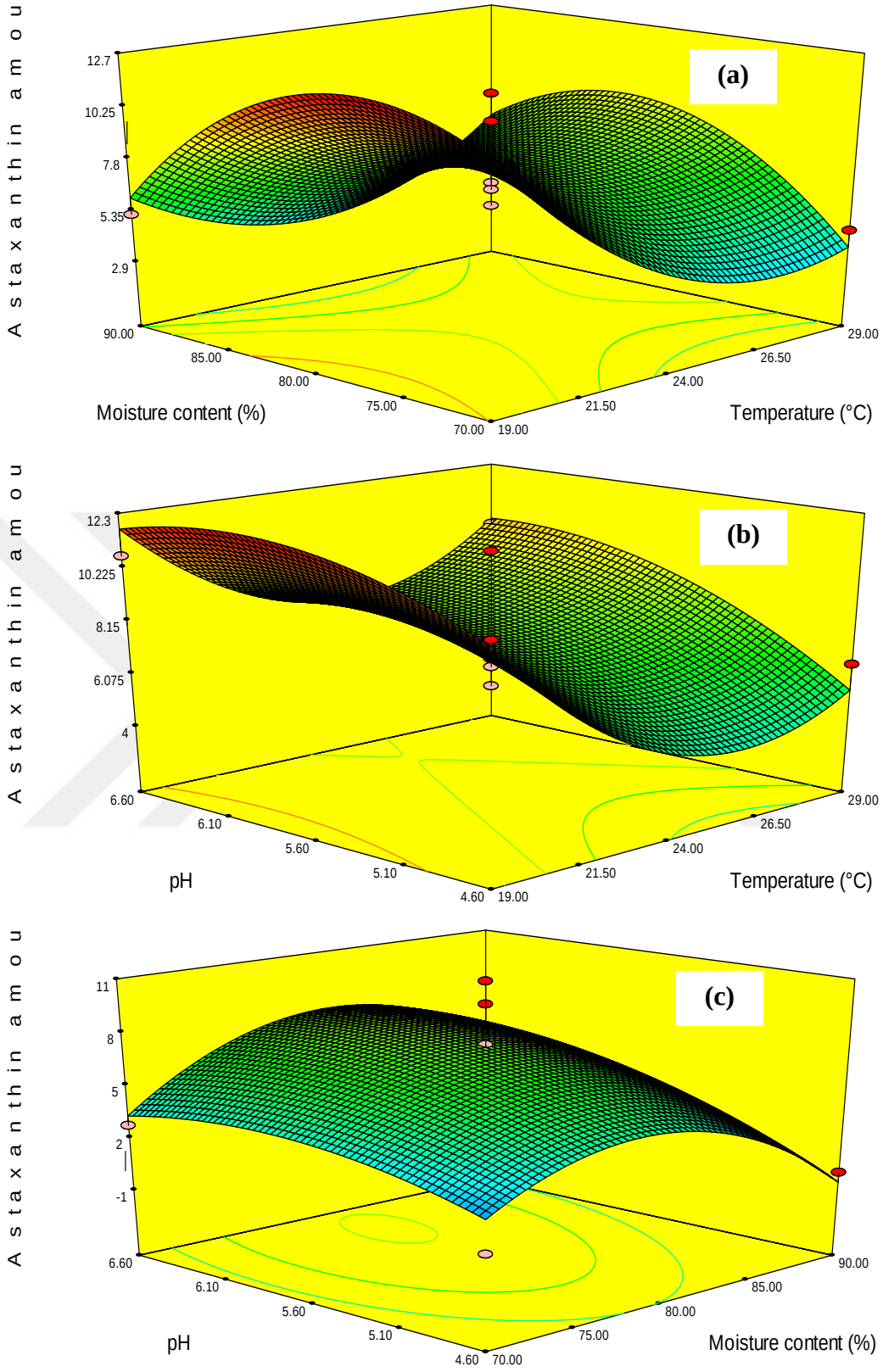

Figure 3.21 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24060 lentil residue fermentation system

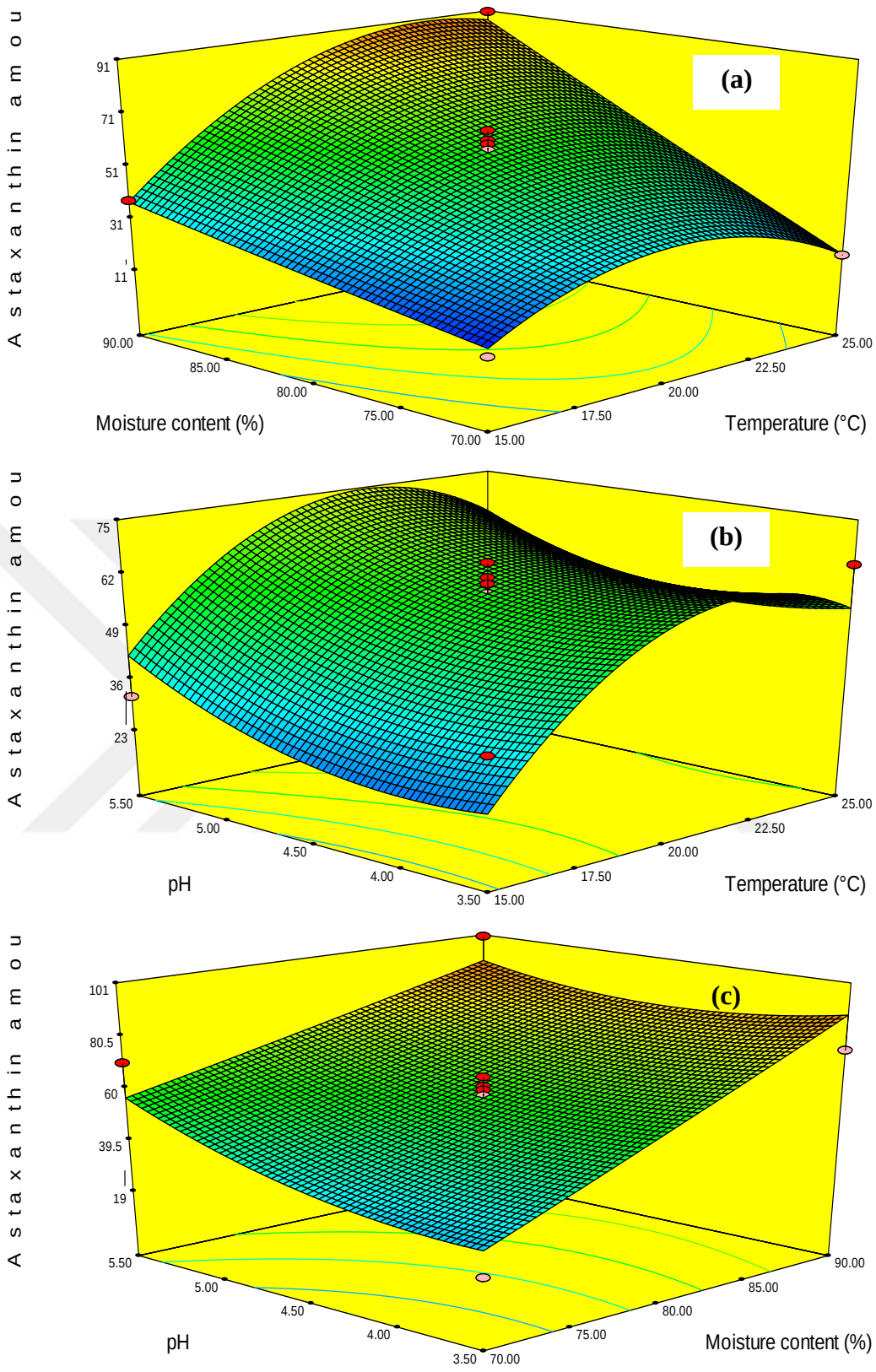

Figure 3.22 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24202 lentil residue fermentation system

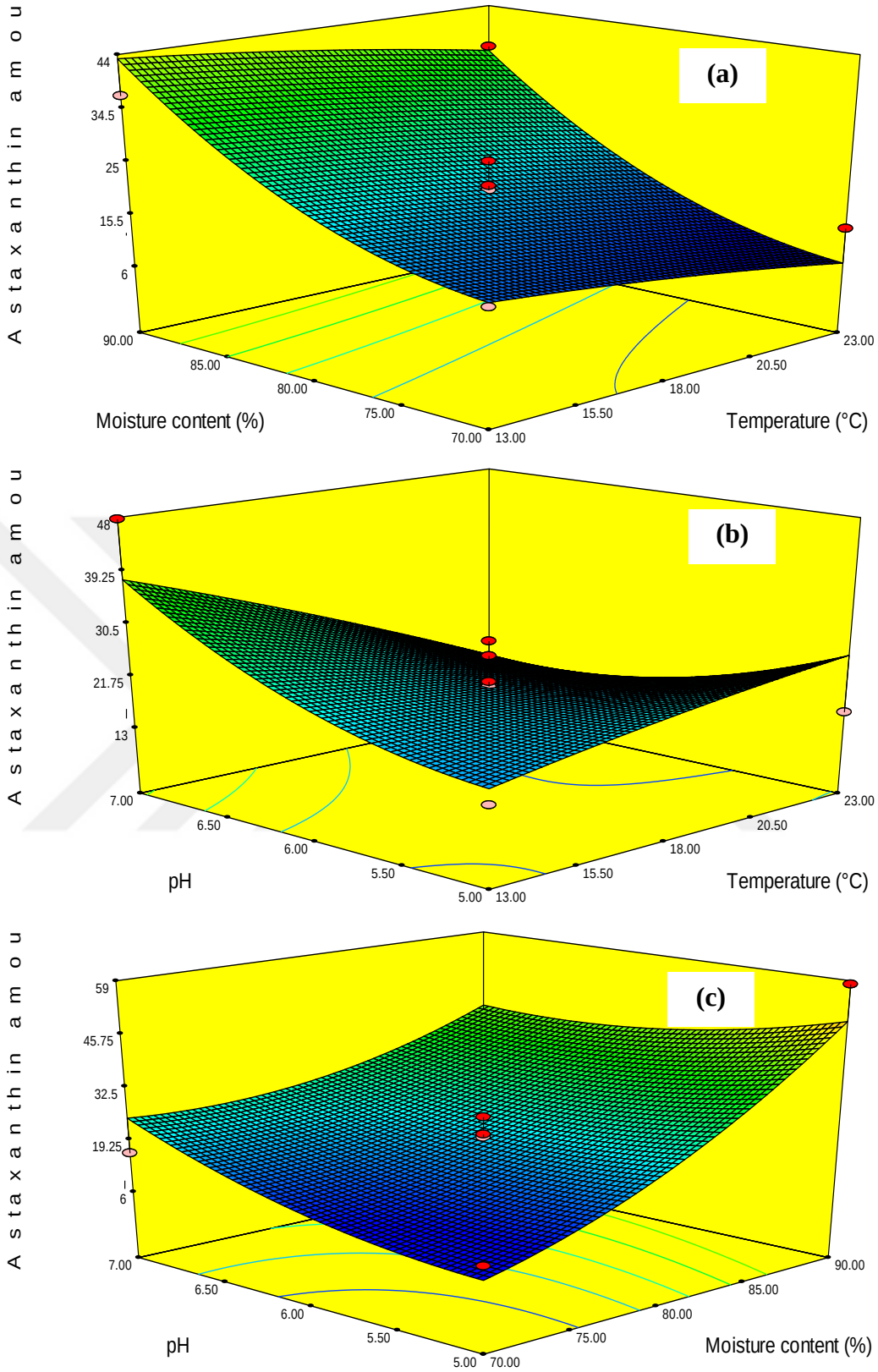

Figure 3.23 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24259 lentil residue fermentation system

3.5.3 Rice Bran Fermentation Systems

Maximum astaxanthin amount, 66.03 µg AX/gdw was produced by utilizing rice bran as seen in Table 3.17. The model was significant at $p<0.1$ probability; lack of fit was significant at $p<0.05$ and $p<0.01$ probability values for all fermentation systems of the yeasts (Table 3.18). The highest fitting satisfaction of the model was obtained from ATCC 24060 yeast fermentation when R^2 value (0.88) was consulted. The model coefficients were presented in Table 3.19. It was showed in Table 3.20 that maximum astaxanthin production was obtained from ATCC 24202 fermentation system at 15.0 °C temperature, 3.5 pH and 80.0 % moisture content conditions.

Table 3.17 Astaxanthin results of rice bran fermentation systems

	Astaxanthin amount (µg AX/gdw)			
Run	**ATCC 90197**	**ATCC 24060**	**ATCC 24202**	**ATCC 24259**
1	27.26±1.26	9.04±0.13	66.03±10.2	25.10±0.77
2	4.08±0.59	10.57±1.42	51.00±1.89	8.92±0.85
3	18.22±0.00	20.31±0.79	21.71±0.00	10.83±0.28
4	4.73±0.60	10.19±0.00	49.67±0.00	4.28±0.73
5	12.24±0.24	10.88±0.82	17.04±0.96	9.73±0.29
6	0.00±0.00	14.33±1.25	42.35±2.54	9.69±0.28
7	9.84±0.54	12.93±0.97	34.68±0.44	11.75±1.28
8	3.29±0.09	13.23±2.23	55.16±1.55	9.86±0.96
9	10.35±0.67	14.21±1.11	46.13±5.85	27.29±0.42
10	17.16±0.48	5.31±0.58	26.19±2.06	25.71±0.70
11	5.96±0.00	11.38±0.14	43.57±3.84	33.41±1.74
12	10.12±0.24	29.66±2.60	47.49±1.55	17.15±0.06
13	9.76±0.25	8.00±0.63	32.73±0.85	7.95±0.55
14	18.58±0.29	3.60±1.36	38.33±1.61	9.86±1.68
15	4.23±0.12	10.63±0.77	50.47±1.27	5.23±0.73
16	9.43±0.13	6.76±0.34	32.47±8.76	13.41±0.14
17	5.11±2.56	10.04±0.34	48.90±1.47	15.79±0.65

Ananda and Vadlani (2011) used full-fat rice bran (21 % fat content) and defatted rice bran (2 % fat content) to produce astaxanthin by ATCC 24202. They managed to produce 80.42 µg AX/g substrate from full-fat rice bran and 16.94 µg AX/g substrate from defatted rice bran at the 11th day of fermentation period. The rice bran used in this thesis had 16.31 % fat content and 66.03 µg AX/gdw was produced at the end of 10th days. As it was understood, AX amount increased when the amount of fat increased in rice bran.

Table 3.18 Quantitative model assessment tools for rice bran fermentation systems

Tools	**p<**	**ATCC 90197**	**ATCC 24060**	**ATCC 24202**	**ATCC 24259**
Model	0.10	+	+	+	+
	0.05	–0.0901	+0.0160	–0.0688	–0.0995
	0.01	–	–	–	–
Lack of fit	0.10	+	–	+	–
	0.05	–0.0946	–0.1611	–0.0585	–0.5726
	0.01	–	–	–	–
Standard deviation		4.8749	3.1380	8.4737	6.0164
Mean		10.0201	11.8269	41.4067	14.4678
C.V. %		48.6514	26.5325	20.4646	41.5846
PRESS		2098.5790	793.5998	6717.2565	1723.0647
R^2		0.7860	0.8798	0.8053	0.7784
Adj. R^2		0.5109	0.7252	0.5550	0.4934

Table 3.19 Coefficients of the model equations of each optimized rice bran fermentation systems

Coefficients	ATCC 90197		ATCC 24060		ATCC 24202		ATCC 24259	
	Coded	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**
β_0	7.8081	15.8639	10.7649	44.3852	48.6025	–1510.6939	15.1201	85.3044
β_1	0.9879	–13.1327	–6.4941	0.2217	–6.4361	11.9675	1.1456	2.1782
β_2	–5.5980	3.3601	0.1775	–2.7096	3.5249	32.6439	9.4745	–5.0212
β_3	2.9964	13.6526	–0.0187	31.0829	0.1169	58.8337	–1.2215	24.9207
β_{12}	–1.5947	–0.0319	–4.1571	–0.0831	–8.5609	–0.1712	2.4226	0.0485
β_{13}	0.2476	0.0495	0.3315	0.0663	1.5343	0.3069	–0.2715	–0.0543
β_{23}	–0.0219	–0.0022	0.2396	0.0240	–9.0102	–0.9010	–1.2909	–0.1291
β_{11}	7.8171	0.3127	2.4789	0.0992	–0.5864	–0.0235	–3.8191	–0.1528
β_{22}	–1.9447	–0.0194	2.8679	0.0287	–15.5078	–0.1551	3.6694	0.0367
β_{33}	–1.1719	–1.1719	–3.0901	–3.0901	0.8030	0.8030	–1.2364	–1.2364

Table 3.20 Experimental and predicted values of the optimized conditions for rice bran fermentation systems

Optimized conditions	ATCC 90197		ATCC 24060		ATCC 24202		ATCC 24259	
	Coded	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**
x_1 (°C)	30.0	30.0	19.0	19.0	15.0	15.0	20.52	18.0
x_2 (%)	70.0	70.0	90.0	90.0	86.8	80.0	90.0	90.0
x_3	6.0	5.0	5.58	5.6	3.5	3.5	5.0	5.0
AX amount (μg AX/gdw)	22.40	27.26	26.94	29.66	63.85	66.03	30.51	33.41

3-D plots (Figures 3.19-3.22) indicated that high moisture content levels induced the astaxanthin production, except ATCC 90197 yeast; high astaxanthin amount was obtained at high pH levels for ATCC 90197 and ATCC 24060 yeasts; pH parameter has no effect on ATCC 24259 yeast; the interaction of low pH with low temperature showed better results for ATCC 24202 yeast; high temperature level revealed a significant effect on ATCC 90197 yeast.

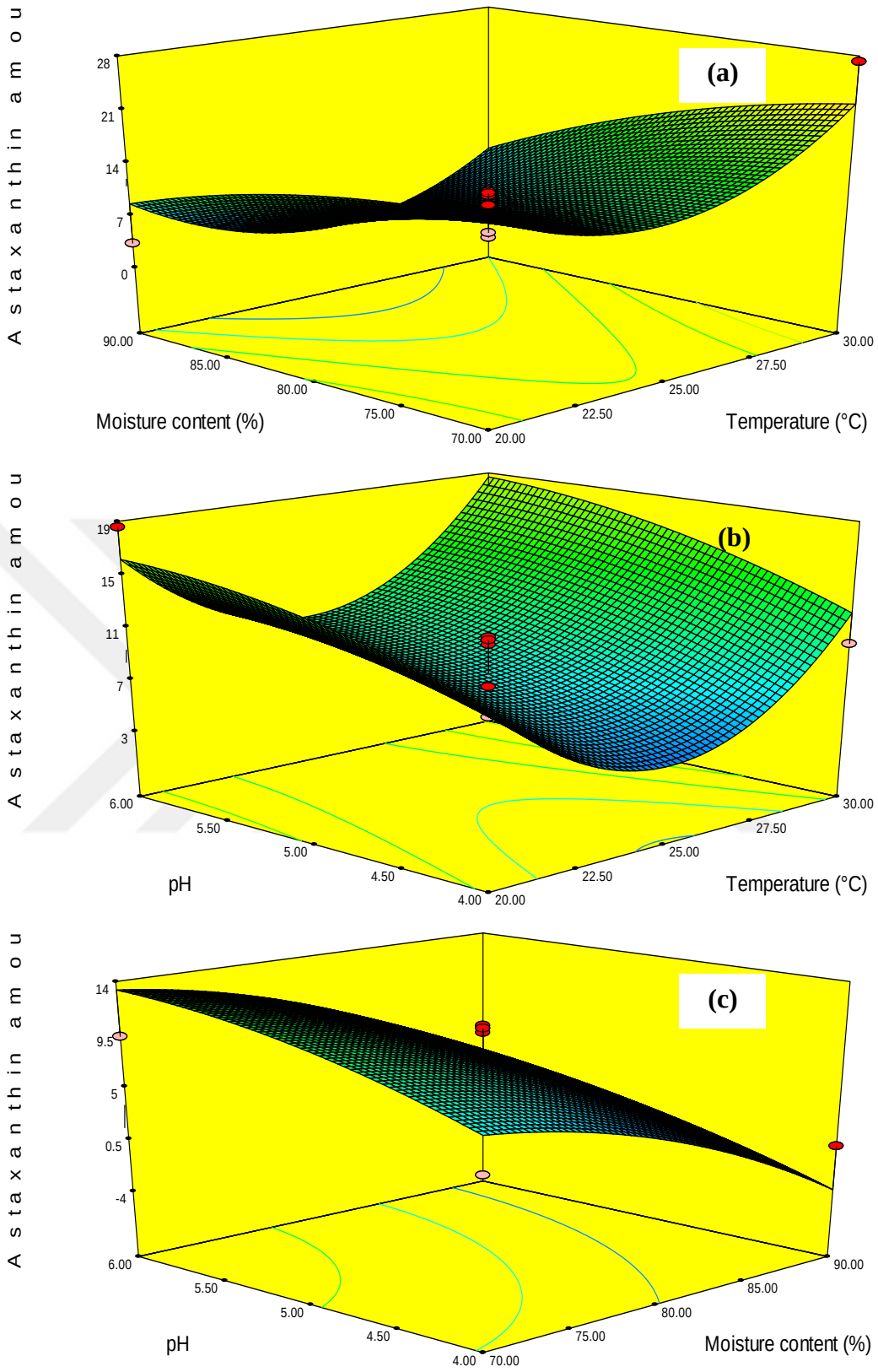

Figure 3.24 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 90197 rice bran fermentation system

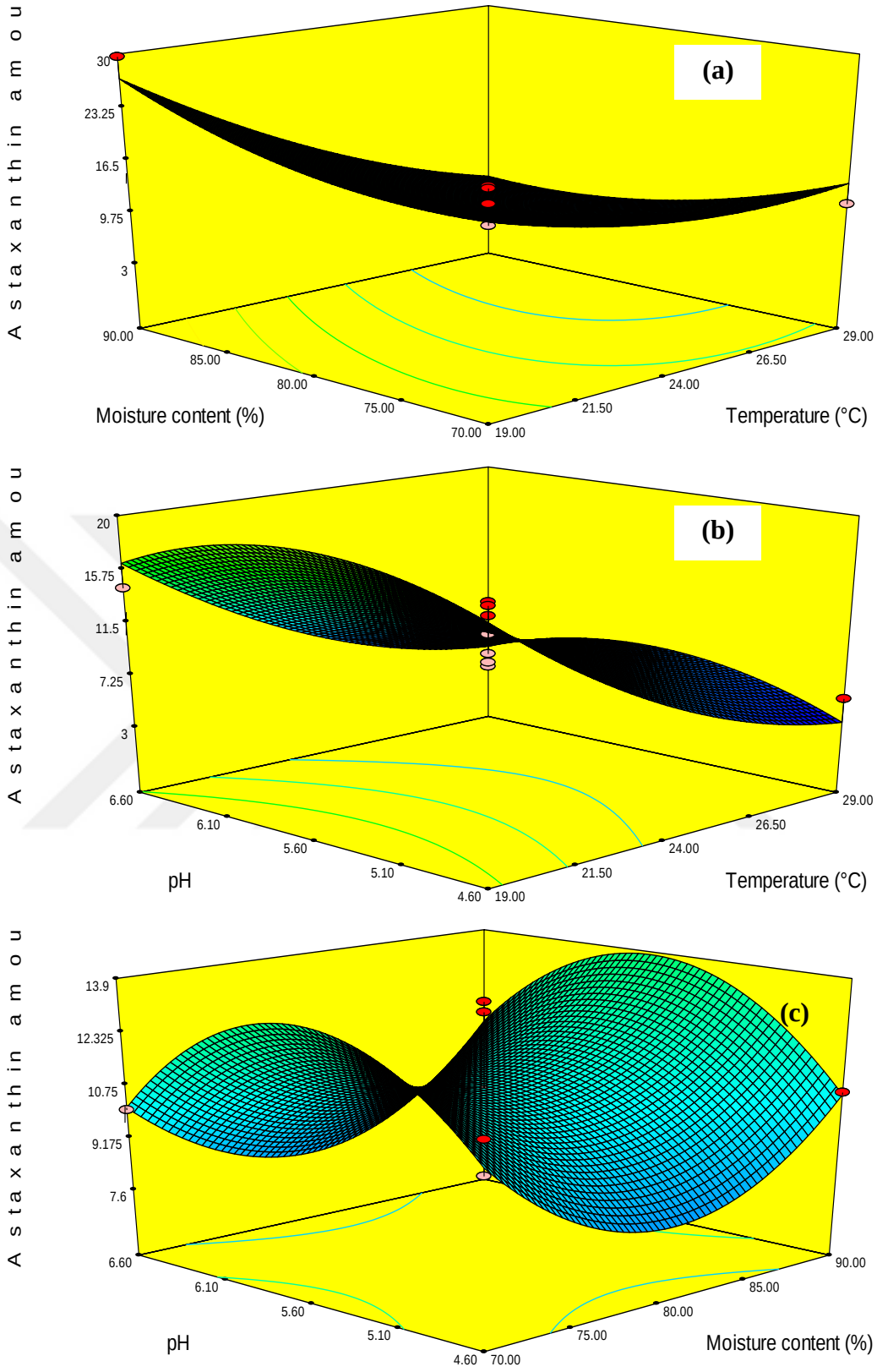

Figure 3.25 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24060 rice bran fermentation system

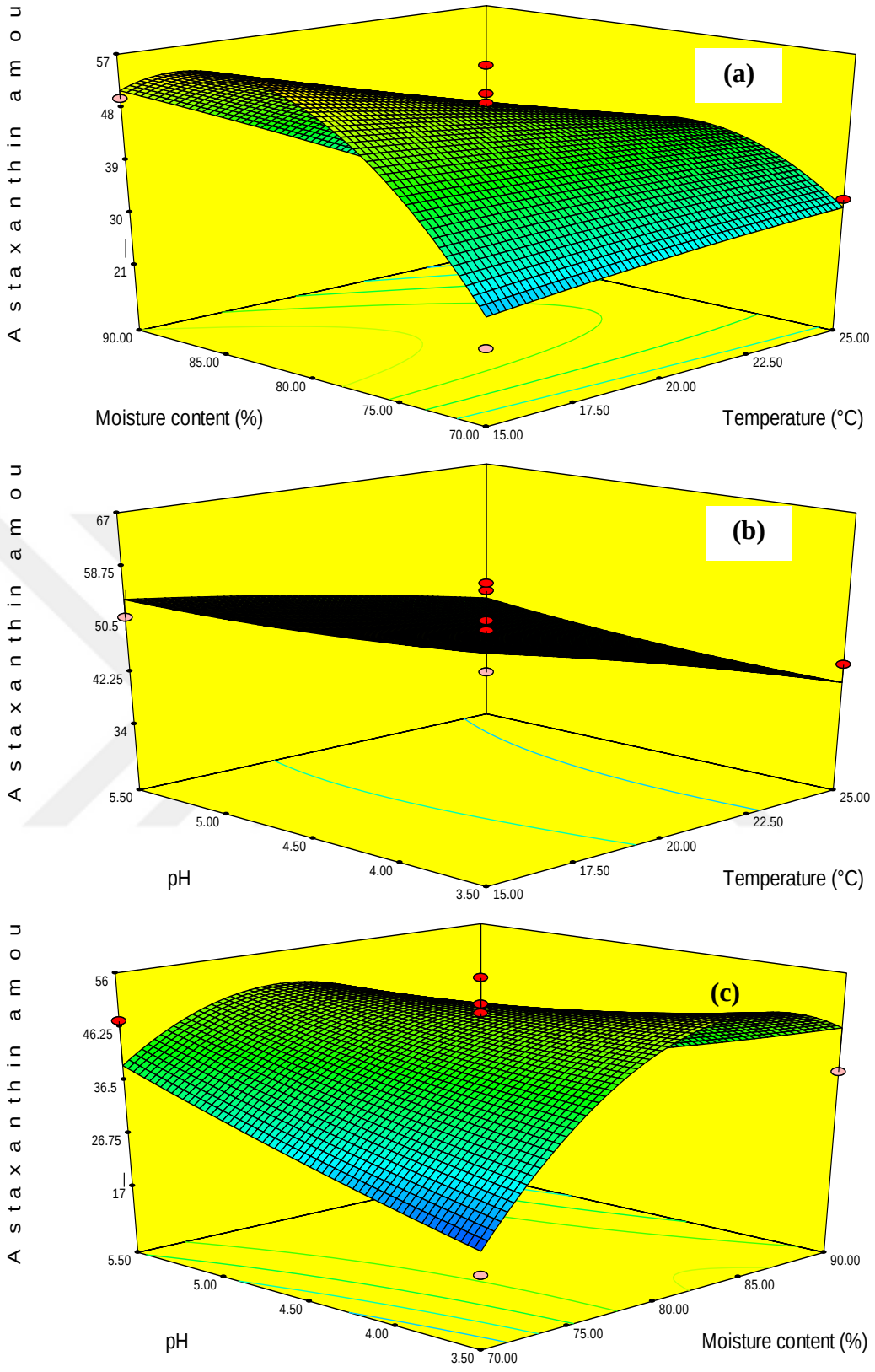

Figure 3.26 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24202 rice bran fermentation system

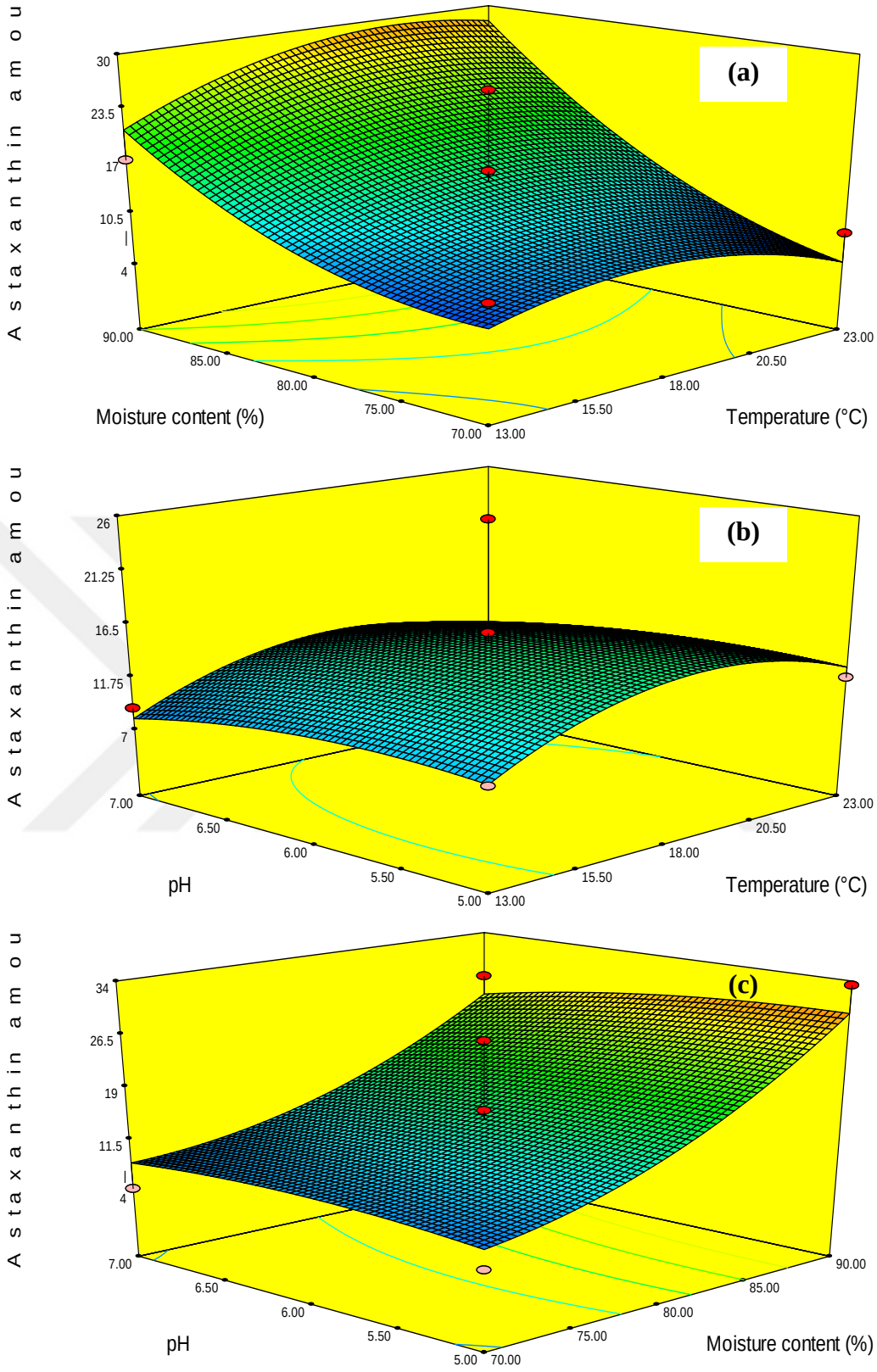

Figure 3.27 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24259 rice bran fermentation system

Optimum fermentation conditions were tabularized for all wastes, thus the results are summarized in Table 3.21. Depending on the maximum astaxanthin yield, ATCC 24202 and the wheat bran were determined as the optimum microorganism and waste, respectively. Although the conditions and the yeast were same with the wheat bran for the lentil residue, more astaxanthin amount was obtained from the wheat bran fermentation experimentally. However, according to the predictive model results, max yield (97.81 µg AX/gdw) was obtained from lentil residue and ATCC 24202 fermentation system. The significancy of the difference between the predictive yield results of wheat and lentil fermentation systems was investigated statistically by independent t-test. The result ($p<0.05$) showed that they are different. When all the experimental and statistical results are evaluated together, the fermentation system of ATCC 24202 and the wheat bran was discussed as the optimized system.

Table 3.21 Optimized conditions for each waste

Optimized conditions	**Wheat bran**	**Lentil residue**	**Rice bran**
Run number	13	13	1
Yeast	ATCC 24202	ATCC 24202	ATCC 24202
Yield (µg AX/gdw)	109.23	100.25	66.03
Temperature (°C)	20.0	20.0	15.0
Moisture content (%)	90.0	90.0	80.0
pH	5.5	5.5	3.5

3.6 Verification Studies

Nowadays, it has become important to confirm the experimental data and the results in order to reveal the reliability of the scientific results and evaluations, to lead the studies which require the same pattern and progressing correctly and finally to prevent scientific pollution. By the way, some studies which have not been worked on so much or specifically may need to be verified. At this point, several computer programs may be used for reconsideration and verification. Experimental data and optimized results obtained from an experimental design and RSM respectively may be re-modeled and re-optimized by the programs performing mathematical and statistical analyses.

3.6.1 Inoculation Ratio

Inoculation rate study was carried out for ATCC 24202 yeast at the optimized conditions by BBD. Astaxanthin yield and number of viable cell (V. C.) with regard to different inoculation ratios changed in a same pattern as seen in Figure 3.28. Generally it is expected to observe a parallel increasing in product yield and inoculation ratio. An increasing was determined till 2 % ratio, but a decline observed after this value. The chosen model in SigmaPlot program showed a quite high R^2 value (0.93) for the AX yield. Optimization results for yield and viable cell obtained by SAM method and MS Excel Solver Tool (goal seek) referred similar inoculation rate as seen in Table 3.22. Hence, the inoculation ratio used for the fermentation systems (2 %) was confirmed. Ananda and Vadlani (2011) reported that 10 % (v/v) inoculum was used for SSF system; 11.11 % (v/v) by Yamane et al. (1997) and 20 % (v/v) ratio by Ramírez et al. (2001) were used for SmF systems.

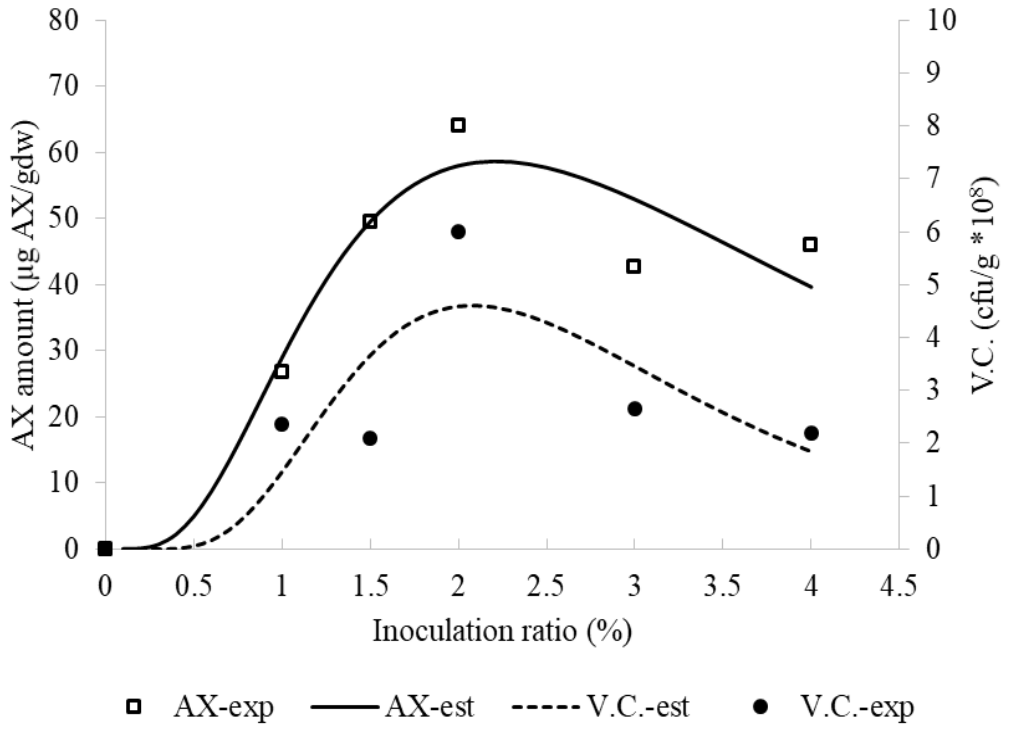

Figure 3.28 Experimental data points (AX-exp, V. C.-exp) and estimated model (AX-est, V. C.-est) graphs of the relation between the inoculation ratio and astaxanthin amount and viable cell

Table 3.22 Modeling and optimization results of inoculation ratio

Sources	Modeling				Optimization (inoculation ratio, %)	
	a	b	x_0	R^2	SAM	Goal seek
Yield	162.33	0.6684	3.46	0.93	2.2132	2.2134
V. C.	10.75	0.4835	2.63	0.68	2.0863	2.0889

3.6.2 Parameter Levels

The first fermentation systems were designed according to the growth conditions of the yeasts indicated in ATCC protocols. In literature, there have been submitted different growth and astaxanthin production conditions of *Xanthophyllomyces dendrorhous* yeast. A new experimental design was discussed and applied to test and verify the conditions. Various references (Johnson and Lewis, 1979; Ramírez et al., 2001) indicated that 19-22 °C and 4.5-6.0 pH are the optimum growth temperature and pH values respectively for *X. dendrorhous* yeast. Since moisture content as the most important parameter for the SSF systems has been determined, the pigmentation ability of *X. dendrorhous* was investigated at reduced moisture content levels. The lowest level, 60 %, was chosen due to keep homogenized form of fermentation medium and to provide the growth of the yeast. Temperature levels were increased and intervals of pH were narrowed.

Table 3.23 shows that astaxanthin amount determined at the regenerated BBD runs. The maximum yield, 93.17 µg AX/gd wheat bran was obtained at temperature of 22 °C, 70 % moisture content and 4.5 pH conditions that did not match with the previous optimum conditions (Table 3.24). Different astaxanthin amount was obtained with same microorganism at different fermentation conditions. Temperature and pH intervals provided consistent result with the literature. Besides, lower optimum moisture content was surprising result. Difference between coded and actual optimum conditions was explained by very low R^2 (0.52) value (Table 3.25). The model coefficients were presented in Table 3.26.

Table 3.23 Regenerated BBD for the wheat bran and ATCC 24202 yeast and astaxanthin results

Run	Temperature (°C)	Moisture content (%)	pH	Astaxanthin amount (µg AX/gdw)
1	22.0	70.0	4.5	11.61±0.54
2	22.0	60.0	4.0	45.15±0.25
3	27.0	60.0	4.5	93.11±0.00
4	22.0	60.0	5.0	40.72±1.45
5	27.0	80.0	4.5	17.57±0.27
6	17.0	80.0	4.5	19.93±2.27
7	17.0	60.0	4.5	46.55±0.41
8	22.0	70.0	4.5	29.21±0.01
9	22.0	70.0	4.5	93.17±0.24
10	17.0	70.0	5.0	13.85±0.49
11	22.0	70.0	4.5	32.37±0.38
12	22.0	80.0	5.0	19.77±1.24
13	27.0	70.0	4.0	28.77±0.08
14	17.0	70.0	4.0	13.41±0.01
15	27.0	70.0	5.0	27.62±0.01
16	22.0	80.0	4.0	36.10±3.08
17	22.0	70.0	4.5	54.63±0.13

Table 3.24 Parameter levels and optimum conditions comparison

BBD levels	**First fermentation**			**Second fermentation**		
T (°C)	15.0	20.0	25.0	17.0	22.0	27.0
MC (%)	70.0	80.0	90.0	60.0	70.0	80.0
pH	3.5	4.5	5.5	4.0	4.5	5.0
Optimum conditions	**Actual**	**Coded**		**Actual**	**Coded**	
T (°C)	20.0	21.48		22.0	27.0	
MC (%)	90.0	86.92		70.0	60.0	
pH	5.5	5.5		4.5	4.5	
Yield (µg AX/gdw)	109.23	95.35		93.17	82.21	

Table 3.25 Quantitative model assessment tools for the wheat bran and ATCC 24202 yeast fermentation system

Tools	**p<**	**ATCC 24202**
Model	0.1	–
	0.05	–0.6029
	0.01	–
Lack of fit	0.1	–
	0.05	–0.8598
	0.01	–
Standard deviation		25.81
Mean		36.68
C.V. %		70.37
PRESS		17819.64
R^2		0.5205
Adj. R^2		–0.0959

Table 3.26 Coefficients of the model equations of the wheat bran and ATCC 24202 yeast fermentation system

Coefficients	ATCC 24202	
	Coded	**Actual**
β_0	+44.20	–1519.75675
β_1	+9.17	+32.37471
β_2	–16.52	–3.82227
β_3	–2.68	+618.37446
β_{12}	–12.23	–0.24464
β_{13}	–0.40	–0.15931
β_{23}	–2.98	–0.59523
β11	–7.22	–0.28863
β_{22}	+7.31	+0.073078
β_{33}	16.07	–64.28586

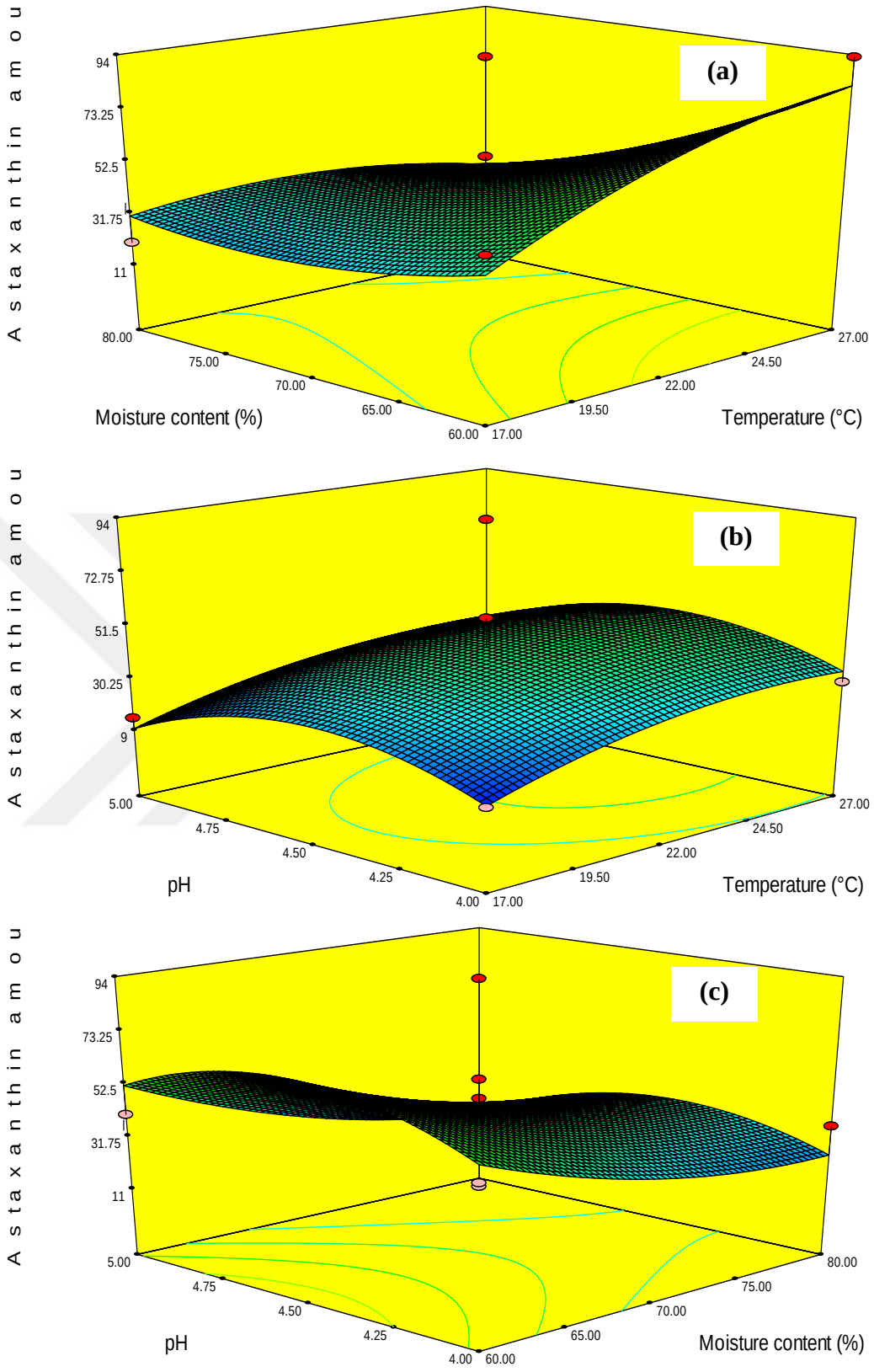

Figure 3.29 Response surface plots of; (a) moisture content and temperature, (b) pH and temperature, (c) pH and moisture content for ATCC 24202 and wheat bran combination fermentation system

3.7 Kinetic Study of Optimized Fermentation System

Three kinetic parameters; cell number, astaxanthin amount and glucose amount for the optimized waste were followed for 12 days (Table 3.27). The results of the model coefficients (Equations 2.9 and 2.10) and statistical analyses were presented in Table 3.28. Quite high regression values were obtained for cell number and glucose amount. Normality test explains whether the data is well-modeled. All the parameters passed the test at $p<0.05$, which meant that sample distributions were normal or the correlation between the data and the corresponding scored normal. Specific growth rate (μ) as *m* value and maximum cell number as *a* value were represented in the table. Maximum specific growth rate, 3.48 day^{-} (0.145 h^{-}) which calculated for each day interval was obtained at the forth day. Figure 3.30 was generated to introduce the graphs of both experimental and estimated data for each parameter. So long as glucose was consumed, the yeast grew up and produced astaxanthin. The accordance of the shapes demonstrated this phenomenon.

Yield coefficients in batch systems may refer productivity. $Y_{p/s}=0.1057$ (μg AX/g glucose), $Y_{x/s}=0.25^{x}10^{8}$ (number of cells/g glucose), $Y_{p/x}=3.68^{x}10^{-9}$ (μg AX/number of cells) were calculated.

Table 3.27 Kinetic parameters during the fermentation of wheat bran

Days	Astaxanthin amount (µg AX/gdw)	Glucose amount (mg G/gdw)	Cell number (cfu/gdw$^{x}10^{8}$)
1	66.25±1.36	435.91±6.68	0.09
2	nd	nd	0.01
3	58.35±0.68	328.82±1.67	0.03
4	70.07±2.38	196.02±3.34	0.97
5	65.74±1.53	158.38±2.86	0.51
6	nd	186.00±2.63	3.91
7	nd	66.71±0.00	18.55
8	72.36±0.85	52.38±1.43	14.00
9	66.5.±2.55	28.65±2.86	25.45
10	90.20±1.53	74.00±0.95	113.64
11	103.19±0.51	nd	nd
12	102.04±0.38	97.16±3.10	97.27

nd: not detectable

Table 3.28 Regression tools, statistical tests, equation coefficients

Sources	Product formation	Cell formation	Glucose consumption
R^2	0.84	0.93	0.92
Adjusted R^2	0.78	0.92	0.90
Normality test (Shapiro–Wilk)	0.8935 (p)	0.3056 (p)	0.0978 (p)
a	59.00	$108.04^{x}10^{8}$	462.33
b	61.60	2.27	2.23
m	–0.20	2.42	3.94

p: passed at $p<0.05$

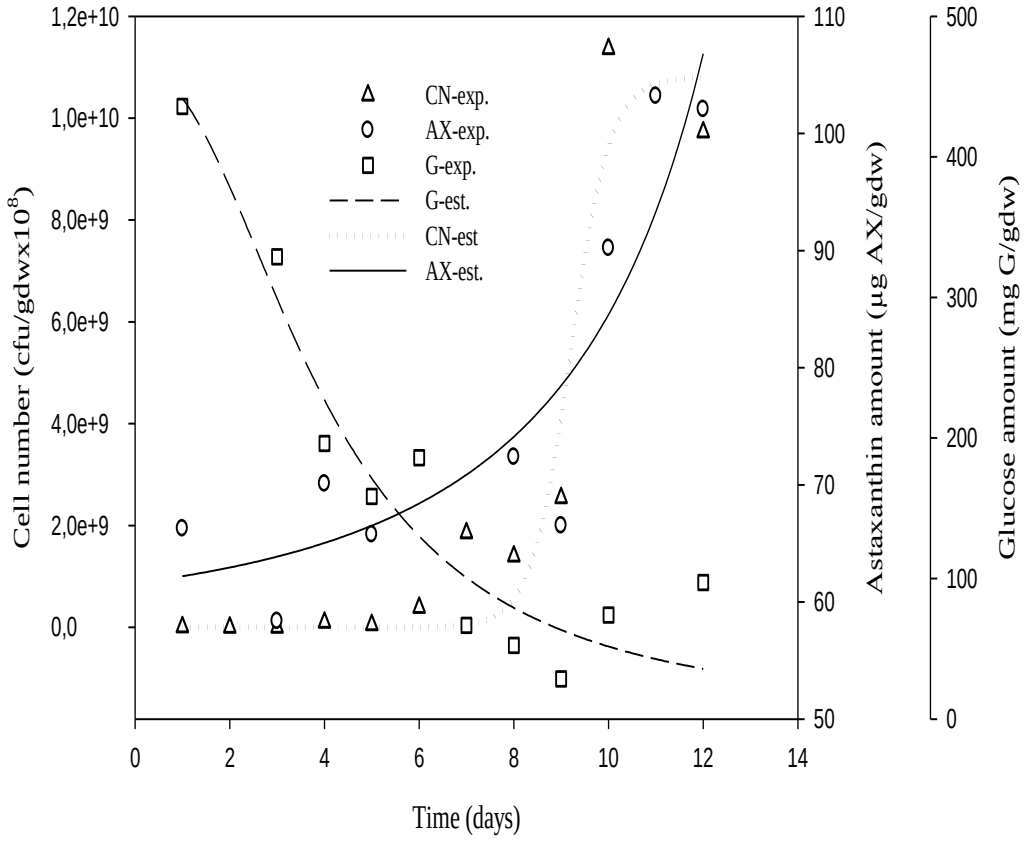

Figure 3.30 Experimental and estimated plots of kinetic parameters; CN-exp.: Cell Number experimental, AX-exp.: Astaxanthin experimental, G-exp.: Glucose experimental, CN-est.: Cell Number estimated, AX-est: Astaxanthin estimated, G-exp.: Glucose estimated

3.8 Antioxidant Activity Determination

The scavenging activity of AX produced by ATCC 24202 at the optimized conditions and un-fermented wheat bran was measured using DPPH radical. Scavenging activity values of the astaxanthin in un-fermented and fermented wheat bran were attained as 66.39 % and 96.71 %, respectively. The difference between the results revealed that the antioxidant capacity of AX produced. Gramza-Michałowska and Stachowiak (2010) has stated that 90-95 % antioxidant potential resulted by DPPH analysis of *Xanthophyllomyces dendrorhous* extracts.

The importance of antioxidant power of astaxanthin pigment is obvious to reveal in studies. It is another explanation of astaxanthin presence in the SSF systems of the wastes for this study by investigating before and after fermentation S. A. values. The highest S. A. value (96.71 %) related with astaxanthin amount was determined from the fermented wheat bran (Table 3.29).

Table 3.29 Antioxidant capacities of the wastes

Wastes	**Scavenging activity (%)**	
	r	**f**
Wheat bran	66.39	96.71
Lentil residue	14.94	82.03
Rice bran	57.99	77.25

3.9 Simulation Study of Optimized Fermentation System

Economic and process analyses should be performed in order to carry out the studies through industrial extent. Computer aided process simulation systems, known as the imitation of the operations and/or characteristics of system(s) to test or perform, could be used for this purpose; to reveal the feasibility of the studies. They provide the estimation of processing characteristics like mass and energy balances, physical and environmental conditions, equipment properties like capacity, power etc., economic properties like investments, operating costs etc. Thus, the costs can be reduced, product quality can be improved, efficiency of the system can be increased, process and marketing duration can be decreased. Optimization and validation of the process and its conditions are the important criterions before the simulation. Empirical studies and statistic evaluations of them prepare a substructure for the simulation.

3.9.1 Material Balance

A detailed stream summary for the powdered astaxanthin (PAX) process was shown in Table 3.30. The mass flow and composition of the raw material, the main outputs

from the basic unit operations, and the final product were presented in the table. For the process, the final product was in powder form with 7 % starch. The simulation predicted that 1.1 kg powdered AX with 89.97 % AX content could be obtained from 10,000 kg of wheat bran for each batch.

The fermentation step (with duration of 7 days) was the process bottleneck. To remove the bottleneck and decrease the cycle times, the process was designed assuming the availability of 8 fermentors operating in stagger mode (alternating fermentors among batches). This resulted in a cycle time of approximately one day, which, in turn, allows the facilities to perform 367 batches for wheat bran fermentation annually. So, for base case capacity, this was equivalent to an annual production of 453.25 kg PAX/year (Table 3.31).

Table 3.30 Stream summary of the production for base case

Content	*S-101 (RM)	S-136	S-105	S-134	S-114	S-116	S-129 (MP)
Total	10,000.00[a] (100.00%)[b]	11,251.74 (100.00%)	101,262.17 (100.00%)	19,250.65 (100.00%)	9,747.49 (100.00%)	8,257.99 (100.00%)	1.24 (100.00%)
Ash	468.00 (4.68%)	468.00 (4.16%)	468.00 (0.46%)	468.00 (2.43%)	468.00 (4.80%)	-	-
PAX	-	-	1.12 (0.00%)	1.12 (0.01%)	1.12 (0.01%)	1.11 (0.01%)	1.11 (89.97%)
Biomass	-	-	346.92 (0.34%)	346.92 (1.80%)	346.92 (3.56%)	-	-
Carbohydrate	6,659.00 (66.59%)	6,659.00 (59.18%)	6,413.10 (6.33%)	6,413.10 (33.31%)	6,413.10 (65.79%)	-	-
DCM	-	-	-	-	-	8,256.88 (99.99%)	-
Fat	354.00 (3.54%)	88.50 (0.79%)	6.87 (0.01%)	6.87 (0.04%)	6.87 (0.07%)	-	-
Hexane	-	1,517.24 (13.48%)	1,517.24 (1.50%)	1517.24 (7.88%)	-	-	-
Protein	1,385.00 (13.85%)	1,385.00 (12.31%)	1,385.00 (1.37%)	1,385.00 (7.19%)	1,385.00 (14.21%)	-	-
Starch	-	-	-	-	-	-	0.09 (7.00%)
Water	1,134.00 (11.34%)	1,134.00 (10.08%)	91,123.92 (89.99%)	9,112.39 (47.34%)	1,126.47 (11.56%)	-	0.04 (3.03%)

[a]: Mass flows in kg/batch

[b]: The data in parentheses are the mass % compositions in streams

*:S refers stream

3.9.2 Total Capital Investment and Total Production Cost

The estimation of the total capital investment was based on the required equipment. Table 2.4 provided the details of the major equipment costs for the proposed process. The total equipment cost of the PAX processing at baseline capacity (10,000 tones/batch) was $7.1 million. The percentage of the associated costs comprising the total direct fixed capital was determined based on the distributed set of PC-Factors (Peters and Timmerhaus, 1991). The direct fixed capital for the PAX plant at this capacity was $21.05 million. The total capital investment including working capital, direct fixed cost, and start-up cost was $22.31 million. For the case, the total capital investment was equivalent to around three times that of the bare equipment cost. The working capital, which includes the raw materials required for initializing the production and the operation training, was calculated at 5 % of the total fixed capital investment for the process. All economic parameters used in the analysis of the base case were shown in Table 3.31. Astaxanthin production with a production capacity (Q) of 10,000 kg per batch has an annual operating cost of $6.287 million and unit production cost of $13,870 for base case operation.

Raw material is nearly 14.0 % of the total operating cost of processing. Operation labor costs are calculated using the minimum wage in Turkey of $2.3 an hour for base case operations. Labor costs are nearly 10 % of the operating cost. Figure 3.31 depicts the relationship of unit investment cost (UIC) and production capacity. It is seen that UIC decreases when the capacity increases. However, the rate of decrease slows down significantly after the capacity reaches 10,000 kg per batch. The effect of production capacity on unit production cost (UPC) is notable. There is an inverse relationship between the capacity and production costs. Similar studies were shown that the unit production cost decreases with an increase in capacity (Cheng et al., 2017; Wei et al., 2017).

Table 3.31 Overview of economic considerations for base case

Consideration parameters	Unit	Result
Simulation set parameters		
Raw material processing size (QRM)	kg RM/batch	10,000
Main Product Cost (MPC)	$/kg MP	10,000
Raw Material Cost (RMC)	$/kg RM	0.10
Service Life (L)	y	25
Operating labor cost - basic rate (LC)	$/h	2.3
Material balance calculations		
Unit Production Ref. Rate	kg MP/y	453.25
Batch Size (QMP)	kg MP/batch	1.235
Number of Batches per Year (N)	#/y	367
Economic evaluations		
Total Capital Investment (TCI)	$	22,308,000.00
Purchased Equipment Cost (PC)	$	7,098,000.00
Direct Fix Capital (DFC)[a]	$	21,084,000.00
Working Capital (30 days Operation) and Start-up Cost	$	1,224,000.00
Annual Operating Cost (AOC)	$/y	6,287,000.00
Total Revenues (TR=MR+OR)	$/y	10,187,000.00
Main Revenues (MR)	$/y	4,533,000.00
Other Revenues (OR)	$/y	5,654,649.00
Unit Production Cost (UPC=AOC/N^{x}QMP)	$/kg MP	13,870.25
Gross Profit (GP=TR-OC)	$/y	3,900,000.00
Income Taxes (25%) (IT)	$/y	1,365,000.00
Annual depreciation charge (D)	$/y	2,003,000.00
Net Profit (NP=GP-T+D)	$/y	4,538,000.00
Unit investment cost (UIC=TCI/L^{x}N^{x}QMP)	$/kg MP	1,968.74
Profitability analysis		
Return On Investment (ROI=NP^{x}100/TCI)	%	20.34
Payback Time (PBT=TCI/NP)	years	4.92
IRR (After Taxes)	%	18.98
NPV (at 0.0 % Interest)	$	74,417,000.00

[a]: DFC=Direct Cost (DC) + Indirect Cost (IC) + Other Cost (OC). Distributed set of PC-Factors: DC=[1+0.25(piping)+0.1(instrumentation)+0.2(building)+0.1(electricity)+0.15(auxiliary)+0.1(others)]^{x}PC; IC = 0.15^{x}DC; OC=0.15^{x} (DC+IC)

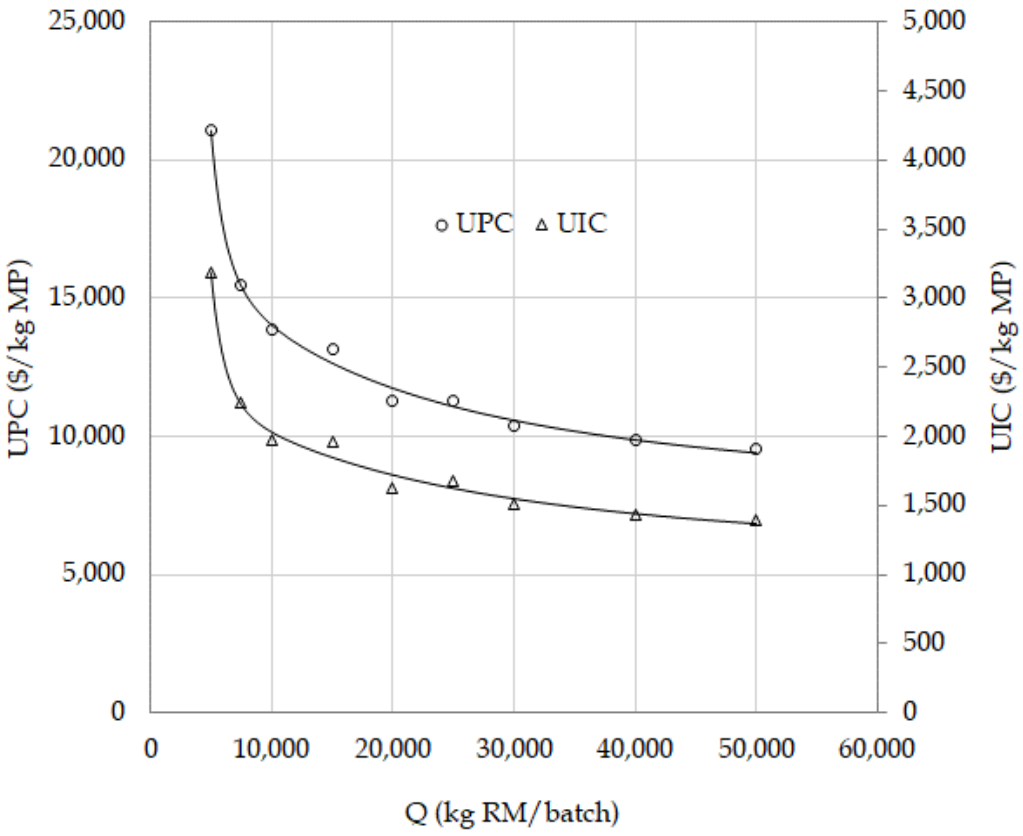

Figure 3.31 Distribution of unit production and investment costs of the astaxanthin production

3.9.3 Profitability

The main revenues of the proposed processing plant resulted from the sales of the powdered AX and the by-products. Table 3.31 shows that 1.235 kg PAX was obtained from wheat bran at a feed capacity of 10,000 kg/batch. The calculated total annual revenue generated from the facility is $10.187 million for the powdered AX. As shown earlier, the annual production cost was $6.287 million. The gross profit calculated for the production was $3.9 million. The amount of depreciation (considered as expense in the operating cost calculation) is needed to be added back for the net profit calculation (Peters and Timmerhaus, 1991). On an annual basis and assuming a straight-line calculation method over a period of 10 years, the annual depreciation was calculated at $2.3 million for the astaxanthin production. Therefore, the net profit after taxes (assuming a 35 % tax rate) was calculated at $4,538,000 (Table 3.31).

The economic viability of the production processes analyzed in this study could be deducted from the indices presented in Table 3.31. The impact study suggests that the process studied is economically viable due to the low PBT break-even point, as well as the high NPV, ROI and IRR. The economic analysis indicates a positive NPV at $74,417,000 for astaxanthin processing. According to the PBT values, the investment expenses may be paid back in 4.92. The higher the ROI value, the more economically advantageous is the project. In many cases, the economically acceptable ROI limit for a project is 10-15 % (Osorio-Tobón et al., 2016). In this study, the ROI value was calculated at 20.34 for the process. Considering the above ROI limits, the astaxanthin bioprocessing qualifies as economically acceptable.

Figure 3.32 illustrates how IRR and PBT vary in relation to the production capacity of astaxanthin. In the production model, the PBT value decreases and IRR value increases. The break-even point is the point at which total operating cost and revenues are equal resulting in no net loss or gain. Figure 3.33 plots the total operating cost and revenues versus the capacity of production. The total production cost consists of the variable cost and fixed cost. Fixed costs do not change when production capacity change. The study indicates that the plant has negative profit when the production capacity is less than 4799.91 kg/batch for the production.

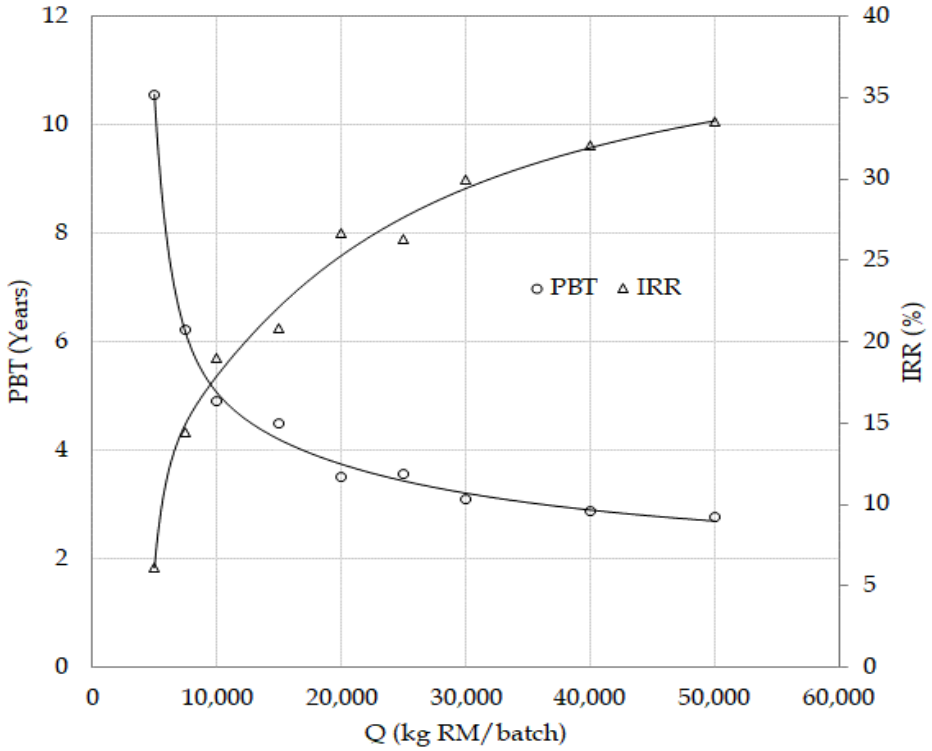

Figure 3.32 Variation of the payback time as well as the IRR when the inlet wheat bran capacity varies

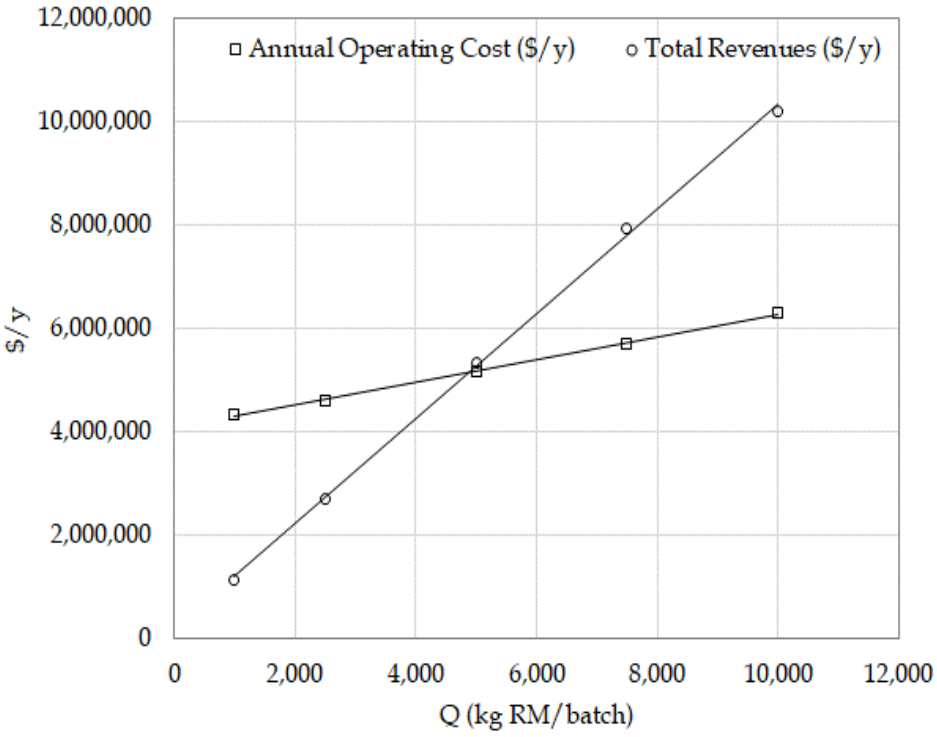

Figure 3.33 Breakeven chart of the astaxanthin production

3.9.4 Sensitivity Analysis

Risks associated with the variability of economic and technical parameters of the plant during production must be avoided. In this study, a sensitivity analysis of parameters like raw material cost, operating labor cost, and product cost affecting the economic performance of PAX production was conducted. The effects of varying the selected parameters by a 20 % deviation from the base case values on the NPV index were calculated (Vlysidis et al., 2011; Han et al., 2016). From the results, it was concluded that the raw material price and the labor cost generally have no significant effect on NPV. The effect of the product selling prices is more pronounced than the raw material price and labor cost, as shown in Figure 3.34. In general, the profitability of biotechnological production processes depends on raw material prices. Studies that examine the effect of raw material prices on profitability show a significant negative effect when the raw material prices are high (Misailidis et al., 2009; Marchetti, 2013; Wood et al., 2014). Keeping low cost is a primary objective in the sensitivity analysis evaluation of agricultural waste. In this study, however, due to the low costs of agricultural waste, sensitivity analysis did not show any effect of raw material price deviations on profitability.

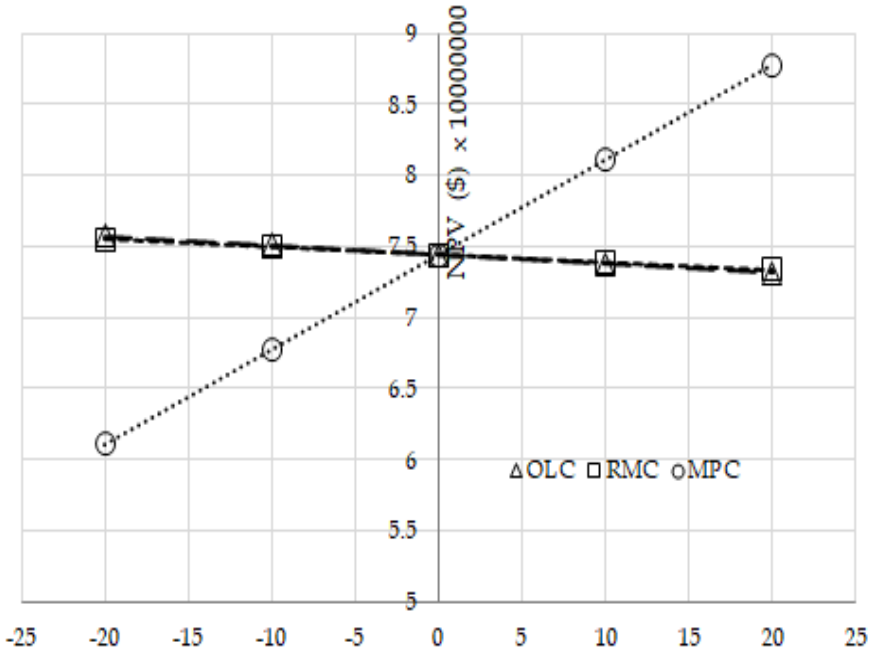

Figure 3.34 Sensitivity analysis of the astaxanthin production

CONCLUSIONS

This study has disclosed the bioprocessing of the wastes as economic and environmentally; developing SSF system for agro-industrial wastes by different microorganisms; modeling and optimizing the fermentation parameters and inoculation rate; determining the significant parameters of each fermentation system; producing AX carotenoid as an important and powerful antioxidant bio-product.

The modeling, optimization and kinetic studies of a solid state fermentation for astaxanthin production, which were accomplished in this study was fulfilled the requirements in this area. Key factors such as levels and effects of parameters, type of waste, fermentation period and significance of numerical analysis for SSF of astaxanthin production were constituted by modeling and optimization of the fermentation parameters for three different wastes within an experimental design.

The study might be basic and explicit to perform other studies and developments. It was deduced from the RSM results that the most effective independent variable was moisture content. High moisture content might provide homogeneous distribution of mass and heat, thus easy accessibility of the yeast to the nutrients occurred and the growth and pigmentation were supported. Organic and inorganic content alteration of the wastes and nutrition value of the product as antioxidant capacity were also manifested. Modeling of kinetic parameters and determination of the productivity for the process has been attributed as difficult and must been worked about to understand and improve the SSF.

The main results of the thesis were summarized below.

i. It was revealed that Box-Behnken design can be used successfully for a solid state fermentation.

ii. It was presented that modeling by polynomial quadratic equation can explain the relationship of the experimental and estimated data of a solid state fermentation system and provide the evaluation to reach maximum yield.

iii. Solid state fermentation kinetics, which is difficult to determine because of their homogeneity, solidity and separation problems, could be achieved in this study.

iv. It was understood that high sugar amounts has no effect on the astaxanthin bioproduction.

v. The highest astaxanthin yield 109.23 μg AX/gdw at temperature of 20 °C, 90 % moisture content, pH of 5.5 was obtained from wheat bran and *Xanthophyllomyces dendrorhous* ATCC 24202 fermentation system which was discussed as the optimized system.

vi. The moisture content was determined as the most effective parameter for all fermentation systems. pH parameter showed slightly effects on the astaxanthin yield.

vii. *Xanthophyllomyces dendrorhous* ATCC 24202 was confirmed as the sole astaxanthin producer for large scale production as it is indicated in literature.

viii. The verification studies for inoculation ratio and parameter levels in BBD matrix for the optimized fermentation system were showed that 2 % inoculation ratio provided the maximum astaxanthin yield and a lower yield (93.17 μg AX/gdw at the conditions of 22 °C temperature, 70 % moisture content and 4.5 pH) was obtained from the re-performed fermentation system.

ix. The antioxidant activity of the produced astaxanthin pigment was measured as 96.71 % from the optimized fermentation system.

x. Plant design for the astaxanthin bioproduction from wheat bran by *X. dendrorhous* ATCC 24202 yeast was resulted in a feasible and promising technology depending on the techno-economic analysis by the SuperPro Designer program.

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APPENDIXES

A1. Preparation of Media, Solutions and Chemicals

A1.1 Media Preparation for Maintenance and Growth of Microorganisms

YM agar medium

The composition:

3 g/L yeast extract (Merck, Germany),
3 g/L malt extract (Merck, Germany),
5 g/L peptone (Merck, Germany),
10 g/L dextrose (Sigma-Aldrich, Germany),
20 g/L agar (Merck, Germany)

of the medium is mixed and filled up with bi-distilled water.

YM broth medium

The composition:

3 g/L yeast extract (Merck, Germany),
3 g/L malt extract (Merck, Germany),
5 g/L peptone (Merck, Germany),
10 g/L dextrose (Sigma-Aldrich, Germany)

of the medium is mixed and filled up with bi-distilled water.

0.85 % (w/v) Physiological salt solution (PSS)

0.85 g NaCl (Sigma-Aldrich, Germany) is dissolved with bi-distilled water and filled up to 100 mL.

A1.2 Solution Preparation for Sugar Analysis

5 % (w/w) Phenol solution

5 gram solid phenol (Sigma-Aldrich, Germany) is dissolved with bi-distilled water and filled up to 100 mL.

A1.3 Solution Preparation for S. A. % Analysis

60 µM DDPH (1,1-diphenyl-2-picrylhydrazyl) solution

Approximately 4 mg DPPH, which has a molecular weight, 394.0 g/mole (Sigma-Aldrich, Germany), is mixed with pure methanol and filled up to 100 mL in a flask that is covered with a folio. The mixture is shaken at 200 rpm, 25 °C for 1 hour. The mixture is diluted by addition of methanol to obtain 0.8±0.005 absorbance value at 517 nm.

A2. Curves of the Yeasts

A2.1 Standard Curves of the Yeasts

Table A2.1 Data of cell number versus absorbance

ATCC 90197		ATCC 24060		ATCC 24202		ATCC 24259	
Abs	**C. N.**	**Abs**	**C. N.**	**Abs**	**C. N.**	**Abs**	**C. N.**
0.4271	5.0	0.3932	6.0	0.5432	23.0	0.6685	40.5
0.1734	2.5	0.2553	3.0	0.3029	11.5	0.3613	20.25
0.1195	1.25	0.1825	1.5	0.1702	5.75	0.2096	10.125
0.0813	0.625	0.1187	0.75	0.1025	2.875	0.1278	5.0625
0.0588	0.3125	0.0827	0.375	0.0650	1.4375	0.0843	2.5313
0.0543	0.1563	0.0620	0.1875	0.0486	0.7188	0.0632	1.2656
–	–	0.0553	0.0938	0.0402	0.3594	0.0519	0.6328

Abs: Absorbance, C. N.: Cell number ($^{x}10^{7}$ cell/mL)

A2.2 Growth Curves of the Yeasts

Table A2.2 Data of the growth curve measurements of ATCC 90197

Days	ATCC 90197			
	O. D. (cell/mL)	**V. C. (cfu/mL)**	**Biomass (g/L)**	**ln [O. D.]**
1	129103439	1300000	0.36	18.68
2	281448907	4454546	0.48	19.46
3	379990482	7027273	0.54	19.76
4	555312920	9272727	0.59	20.14
5	591289209	8018182	0.64	20.20
6	605790327	5945455	0.62	20.22
7	576480865	3800000	0.81	20.17
8	626374540	3881818	0.77	20.26
9	661398425	2118182	0.83	20.31
10	674916416	600000	0.92	20.33
11	612242095	1709091	0.88	20.23
12	647880435	1318182	0.88	20.29
13	641121439	1309091	0.90	20.28

O. D.: Optical Density, V. C.: Viable Cell, In: natural logarithm

Table A2.3 Data of the growth curve measurements of ATCC 24060

Days	ATCC 24060			
	O. D. (cell/mL)	V. C. (cfu/mL)	Biomass (g/L)	ln [O. D.]
1	134220568	12363636	0.42	18.71
2	178488658	28909091	0.35	19.00
3	221373437	18727273	0.49	19.22
4	250121306	19000000	0.37	19.34
5	277879575	28090909	0.30	19.44
6	304879152	29818182	0.57	19.54
7	309282870	71818182	0.45	19.55
8	314865861	15545455	0.41	19.57
9	369681434	23090909	0.38	19.73
10	411426045	62000000	0.44	19.84
11	406379087	48727273	0.49	19.82
12	423466174	39636364	0.40	19.86
13	417314163	85090909	0.42	19.85

Table A2.4 Data of the growth curve measurements of ATCC 24202

Days	ATCC 24202			
	O. D. (cell/mL)	V. C. (cfu/mL)	Biomass (g/L)	ln [O. D.]
1	9103542	24090909	0.12	16.02
2	22469529	9363636	0.15	16.93
3	28174524	7018182	0.16	17.15
4	37058016	15000000	0.18	17.43
5	37791515	15909091	0.21	17.45
6	36732016	12818182	0.35	17.42
7	38851014	10727273	0.16	17.48
8	32005020	9272727	0.25	17.28
9	33227519	5363636	0.25	17.32
10	33635019	6200000	0.15	17.33

Table A2.5 Data of the growth curve measurements of ATCC 24259

Days	ATCC 24259			
	O. D. (cell/mL)	**V. C. (cfu/mL)**	**Biomass (g/L)**	**ln [O. D.]**
1	7260469	nd	0.13	15.80
2	19596981	96364	0.16	16.79
3	20903316	462727	0.18	16.86
4	30000511	8099182	0.26	17.22
5	34027557	15909091	0.45	17.34
6	36659870	18545455	0.47	17.42
7	48662431	76818182	0.44	17.70
8	51577620	26545455	0.44	17.76
9	54001705	17363636	0.50	17.80
10	47511286	21363636	0.51	17.68

A3. Cell Concentrations of the Yeasts in Different Sugar Types and Concentrations

Table A3.1 Data of cell concentrations in terms of optical density ($^{x}10^{7}$ cell/mL) versus time (h) for ATCC 90197

Time (h)	Sugar types												
	G5	**F5**	**S5**	**G10**	**F10**	**S10**	**G20**	**F20**	**S20**	**G40**	**F40**	**S40**	**N. S.**
24	0.79	1.09	1.11	0.87	1.10	0.96	0.97	1.16	0.96	1.03	1.26	0.79	1.03
48	1.81	1.18	1.38	2.13	1.53	1.50	1.93	2.13	1.25	1.75	2.17	1.33	2.55
72	1.79	2.33	2.07	1.45	2.27	1.30	2.44	2.37	1.99	1.99	2.96	1.82	3.15
96	1.98	1.78	1.75	1.99	2.13	1.91	2.53	2.71	2.33	2.77	3.16	2.38	1.26
120	2.58	3.08	2.79	3.06	3.27	2.84	2.90	3.33	3.03	2.98	3.53	3.03	1.29
144	3.20	3.07	2.46	3.35	3.21	2.43	3.62	3.62	2.89	3.72	3.85	2.89	1.41
168	2.84	2.71	2.12	3.33	3.02	2.31	3.52	3.34	2.58	3.51	3.60	2.79	1.49
192	3.46	3.31	2.83	3.64	2.49	3.20	4.05	3.93	3.31	3.95	4.15	3.05	1.50
216	3.70	2.39	2.09	2.47	2.39	3.36	4.22	2.64	3.61	4.31	3.08	3.41	1.36
240	2.69	2.69	2.27	2.51	2.31	2.51	2.70	2.40	2.78	3.11	3.04	2.71	1.28

G: Glucose, F: Fructose, S: Sucrose, N. S.: No Sugar

Table A3.2 Data of cell concentrations in terms of optical density ($^{x}10^{7}$ cell/mL) versus time (h) for ATCC 24060

Time (h)	Sugar types												
	G5	**F5**	**S5**	**G10**	**F10**	**S10**	**G20**	**F20**	**S20**	**G40**	**F40**	**S40**	**N. S.**
24	0.83	0.78	0.71	1.36	0.52	0.67	1.01	0.26	0.84	0.23	0.36	0.60	0.62
48	1.47	1.48	1.27	1.65	1.42	1.36	1.32	0.29	1.30	1.02	0.38	1.16	1.13
72	1.64	1.57	1.32	1.58	1.52	1.33	1.19	0.39	1.17	0.94	0.41	0.99	1.17
96	1.52	1.50	1.23	1.53	1.44	1.21	1.12	1.46	1.08	0.85	0.40	0.91	1.05
120	1.31	1.22	1.25	1.39	1.17	1.13	1.12	0.97	1.01	0.96	0.30	1.01	0.98
144	1.24	1.34	1.51	1.01	1.41	0.78	1.42	0.90	1.08	1.24	0.31	1.41	0.88
168	1.28	1.60	1.98	0.81	1.56	0.66	2.10	1.14	1.37	1.47	0.36	2.28	0.89
192	1.39	2.16	2.69	0.74	2.64	0.56	2.77	1.52	0.90	1.67	0.81	0.41	1.03
216	1.42	2.90	3.06	0.70	3.83	0.55	3.38	2.44	0.81	2.08	0.93	0.38	1.19
240	1.59	3.19	3.50	0.70	3.70	0.48	3.68	3.27	1.72	2.55	1.02	0.40	1.26

Table A3.3 Data of cell concentrations in terms of optical density ($^{x}10^{7}$ cell/mL) versus time (h) for ATCC 24202

Time (h)	Sugar types												
	G5	**F5**	**S5**	**G10**	**F10**	**S10**	**G20**	**F20**	**S20**	**G40**	**F40**	**S40**	**N. S.**
24	0.55	0.68	0.84	0.97	0.79	0.66	1.02	0.95	0.73	0.50	1.08	0.49	0.66
48	1.05	1.38	0.95	1.87	1.46	1.01	1.64	1.25	1.38	0.99	1.06	0.92	1.00
72	1.46	2.11	1.62	1.95	1.90	1.28	1.95	1.58	1.55	1.86	1.13	1.15	2.02
96	2.16	2.18	2.49	2.79	1.86	1.68	2.60	2.25	1.98	1.94	1.11	1.70	2.54
120	2.80	1.98	2.49	2.79	1.84	1.36	3.38	1.99	1.69	2.16	0.90	1.73	2.65
144	2.57	2.20	2.53	2.40	1.89	1.49	2.82	2.16	1.98	1.99	0.92	1.62	2.93
168	2.16	1.78	2.61	1.82	1.43	1.57	2.49	1.82	1.50	1.85	0.94	1.70	2.73
192	2.18	2.14	3.17	1.77	1.74	2.04	2.33	1.84	1.87	1.73	0.88	1.68	2.80
216	1.93	1.66	3.42	1.25	2.13	1.97	2.08	1.76	1.98	1.50	1.01	1.63	2.55
240	1.42	1.14	3.72	0.93	1.24	2.35	1.69	1.23	2.34	1.46	0.94	1.78	1.92

Table A3.4 Data of cell concentrations in terms of optical density ($^{x}10^{7}$ cell/mL) versus time (h) for ATCC 24259

Time (h)	Sugar types												
	G5	**F5**	**S5**	**G10**	**F10**	**S10**	**G20**	**F20**	**S20**	**G40**	**F40**	**S40**	**N. S.**
24	0.01	0.03	0.08	0.08	0.20	0.09	0.15	0.59	0.12	0.29	1.21	0.24	0.11
48	2.46	1.72	1.79	1.83	0.52	2.63	0.76	0.58	1.27	0.33	1.57	0.49	1.86
72	2.40	5.16	3.99	3.31	0.65	2.58	4.26	0.61	1.59	0.78	1.28	2.21	2.49
96	2.38	6.35	2.61	2.87	0.82	2.98	3.59	0.65	1.41	0.86	1.26	1.57	2.11
120	2.44	6.22	2.31	1.98	4.10	2.49	2.74	0.63	1.27	2.08	1.21	1.44	2.14
144	2.30	4.82	2.31	2.16	4.47	2.17	2.72	0.66	1.47	5.56	1.26	1.79	1.98
168	2.63	3.11	2.26	2.56	2.88	2.35	2.61	0.67	1.85	3.59	1.23	1.73	1.60
192	2.42	2.75	2.59	2.31	3.62	2.28	2.80	2.52	1.85	3.23	1.29	1.72	1.91
216	2.35	2.71	2.52	2.34	3.41	2.48	3.03	6.12	2.02	3.76	1.23	1.81	1.69
240	2.57	3.05	2.69	2.52	3.32	2.23	2.94	5.67	2.07	4.26	1.12	2.03	1.91

A4. SAM and Goal Seek Numeric Analysis

A4.1 Modeling and Optimization Studies Using MS Excel

A4.1.1 Modeling of IR-V. C. and AX Data Using MS Excel's Goal Seek Tool

To perform nonlinear least-squares curve fitting using the Solver Tool, spreadsheet model must contain *a* column of known *y* values and *a* column of calculated *y* values, so that the sum of squares of residuals can be calculated. The calculated *y* values must be spreadsheet formulas depending on the curve fitting coefficients that will be varied by the Solver Tool.

Table A4.1 Formula view of modeling spreadsheet study on optimization of inoculation ratio (IR) for astaxanthin yield (AX) with MS-Excel

Steps/Cells	A	B	C	D
1	a	1		
2	b	1		
3	x_0	1		
4				
5	IR	AXexp	AXest	(AXexp-AXest)^2
6	1	26.6731	=($B$2/A6)*ÜS(-0.5*(LN(A6/$B$4)/$B$3)^2)	=(C6-B6)^2
7	1.5	49.4822	=($B$2/A7)*ÜS(-0.5*(LN(A7/$B$4)/$B$3)^2)	=(C7-B7)^2
8	2	64.1106	:	:
9	3	42.6945	:	:
10	4	45.9431	:	:
11			Sum=	=SUM(A6:A11)

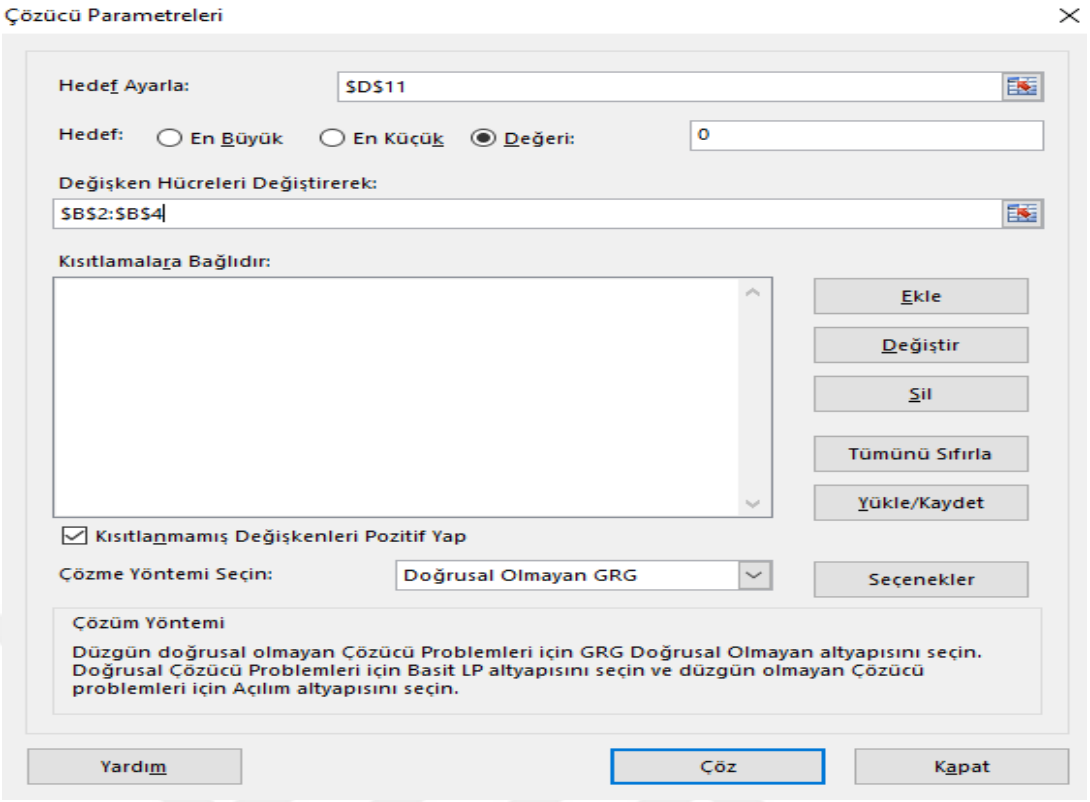

Figure A4.1 Excel Solver menu (Data→Solver)

Table A4.2 Modeling results on optimization of IR for AX with MS-Excel

Steps/Cells	A	B	C	D
1	a	162.3308		
2	b	0.6684		
3	x_0	3.4604		
4				
5	IR	AXexp	AXest	(AXexp-AXest^2
6	1	26.6731	28.9250	5.0708
7	1.5	49.4822	49.5030	0.0004
8	2	64.1106	57.9782	37.6067
9	3	42.6945	52.8900	103.9450
10	4	45.9431	39.6398	39.7322
11			Sum=	186.3551

Table A4.3 Modeling results on optimization of IR for V. C. with MS-Excel

Steps/Cells	A	B	C	D
1	a	10.7455		
2	b	0.4835		
3	x_0	2.6321		
4				
5	IR	CFUexp	CFUest	(CFUexp-CFUest)^2
6	1	2.36	1.4494	0.8292
7	1.5	2.09	3.6426	2.4104
8	2	6	4.5723	2.0384
9	3	2.64	3.4531	0.6611
10	4	2.18	1.8470	0.1109
11			Sum=	6.0500

A4.2 Numerical differentiation by Finite Difference Method with MS Excel

Finite-difference methods (FDM) are numerical methods for solving differential equations by approximating them with difference equations, in which finite differences approximate the derivatives. The main concept behind any finite difference scheme is related to the definition of the derivative of a smooth 'f' function. The derivative of a function 'f' at *a* point *x* is defined by the limit (Eq. A4.1):

$$f'(x) = \lim_{h \to 0} \frac{f(x+h) - f(x)}{h} \qquad \text{A4.1}$$

and to the fact that when *h* tends to zero, the quotient on the right-hand side provides a 'good' approximation of the derivative.

A4.3 Optimization with Steepest Ascent Method (SAM) with MS Excel

The method of steepest ascent is a method whereby the experimenter proceeds sequentially along the path of steepest ascent, that is, along the path of maximum increase in the predicted response. The procedure is iterative in that it starts at a base point. Then the following iterative process is used to move from the base point across to a point near the optimum:

- Compute the derivative of the objective function (by numerical differentiation-FDM)
- Set intervals (x_i and x_{i-1}), calculate $f(x_i)$ and $f(x_{i-1})$ (step 6)
- Calculate (step 7):

$$x_i = x_{i-1} + p * f' \qquad \text{A4.2}$$

Where +*p* constant, for ascent method and -*p* for descent method for minimization.

Check for convergence (step 7):

$$If\ f(xi) > f(xi-1), xi-1 = xi, otherwise\ xi-1(7) = xi-1(6) \qquad \text{A4.3}$$

If the maximum is equal to the previous value, terminate the computation.

Table A4.4 Formula view of SAM study on optimization of IR for AX with MS-Excel

Steps/Cells	A	B	C	D	E	F	G	H	I
1	**Model coefficients**			p=	0.1		h=	0.001	
2	a	162.33							
3	b	0.6684	**SAM**				**FDM**		
4	x_0	3.46							
5			x_i	x_i-1	fx_i	fx_i-1	x+h	f(x+h)	f'
6			1	5	=(a/C6)*exp(-0.5*(LN(C6/x_0)/b)^2)	=(a/D6)*exp(-0.5*(LN(D6/x_0)/b)^2)	=D6+h	=(a/G6)*exp(-0.5*(LN(G6/x_0)/b)^2)	=(H6-F6)/h
7			=D6+p*I6	=IF(E6>F6;C7;D6)	=(a/C7)*exp(-0.5*(LN(C7/x_0)/b)^2)	=(a/D7)*exp(-0.5*(LN(D7/x_0)/b)^2)	=D7+h	=(a/G7)*exp(-0.5*(LN(G7/x_0)/b)^2)	=(H7-F7)/h

Table A4.5 SAM study results on optimization of IR for AX with MS-Excel

Steps/Cells	A	B	C	D	E	F	G	H	I
1	**Model coefficients**			alpha=	0.001		h=	0.01	
2	a	162.33							
3	b	0.6684							
4	x_0	3.46	**SAM**				**FDM**		
5			x_i	x_i-1	fx_i	fx_i-1	x+h	f(x+h)	f'
6			1	4	28.9412	39.6383	4.01	39.5072	-13.116
7			3.9868	4	39.8107	39.6383	4.01	39.5072	-13.116
			:	:	:	:	:	:	:
			:	:	:	:	:	:	:
576			2.2135	2.2135	58.6590	58.6590	2.2235	58.6576	-0.1374
577			2.2133	2.2135	58.6590	58.6590	2.2235	58.6576	-0.1374
578			2.2133	2.2133	58.6590	58.6590	2.2233	58.6577	-0.1338
579			2.2132	2.2133	58.6590	58.6590	2.2233	58.6577	-0.1338
580			2.2138	2.2133	58.6590	58.6590	2.2233	58.6577	-0.1338

Table A4.6 SAM study results on optimization of IR for VC with MS-Excel

Steps/Cells	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
1	a	10.75		p=	0.1		h=	0.001	
2	b	0.48							
3	x_0	2.63	**SAM**				**FDM**		
4			x_i	x_i-1	fx_i	fx_i-1	x+h	f(x+h)	f'
5			1	5	1.4129	0.8778	5.001	0.8772	-0.6649
6			4.9335	4.9335	0.9231	0.9231	4.9345	0.9224	-0.6977
			:	:	:	:	:	:	:
			:	:	:	:	:	:	:
70			2.0886	2.0889	4.5865	4.5865	2.0899	4.5865	-0.0031
71			2.0886	2.0886	4.5865	4.5865	2.0896	4.5865	-0.0017
72			2.0884	2.0886	4.5865	4.5865	2.0896	4.5865	-0.0017
73			2.0884	2.0886	4.5865	4.5865	2.0896	4.5865	-0.0017

CIRRUCULUM VITAE

Personal Information

Name Surname: Derya DURSUN
Date of Birth: 16.01.1984
Nationality: Turkey
Marriage Status: Single
E-mail: derya_dursun_@hotmail.com
Mobile: +905558068131

Academic Qualifications

Bachelor Degree:
Department of Food Engineering, Ankara University, GPA: 3.19/4 (2002-2006)

Graduation Project:
Microbial Cellulose Production and Usage in the Food Industry, 1001 TUBITAK Project (2005-2008).

Master Degree:
Department of Food Engineering, Ankara University, GPA: 3.50/4 (2007-2010)

Master Thesis Study:
Identification of Lactic Acid Bacteria Isolated from Pickles in Ankara Çubuk Region and Determination of Their Total Cell Protein Profiles

Master project:

Identification of Lactic Acid Bacteria Isolated from Ankara Çubuk Region, Determination of Their Technological and Functional Properties and Their Potential for Using as a Starter Culture, 1001 TUBITAK Project (2008-2011).

Doctorate Degree:

Department of Food Engineering, Gaziantep University, GPA: 3.86/4 (2010-2018)

Doctorate Thesis Study:

Production of Astaxanthin Pigment from Cereal and Legume Wastes by Solid State Fermentation and Techno-Economic Analysis by Simulation

Doctorate Projects:

1. Production of Lactones from Fruit and Vegetable Wastes by Solid State Fermentation, 1002 TUBITAK Project (2011-2012).
2. Production of Carotenoid Pigments from Cereal and Legume Wastes by Solid State Fermentation, Scientific Research Projects Governing Unit Project (2011-2013).
3. Production of Pigment and Aroma Compounds from Pomegranate Wastes using by S. *salmonicolor* Yeast Solid State Fermentation, Scientific Research Projects Governing Unit Project (2015-2017).
4. Computer Aided Simulations of Biotechnological Processes: Carotenoid Production by Solid State Fermentation (2016-2017).

Education Mobility:

1. An education seminar was attended for one week in Aurel Vlaicu University in Romania in 2013.
2. Erasmus program in Lithuania with a research group headed by Prof. Dr. P. *Rimantas* Venskutonis for 5 months in 2014.

Academic Awards and Patents

1. Izmir Institute of Technology, X. "What Produce?" Activities and Project Competition, 19 October 2012, Championship Award.

Subject of the project: Omega fatty acids production from apple pomace and rice bran by solid state fermentation.

2. Oral Presentation at EuroBiotechnology 2013 Congress, Italy, 2nd Prize.
   Subject of the project: Astaxanthin pigment production by ATCC 24202 from fruit wastes using solid state fermentation method.
3. Patent about 'Astaxanthin pigment production from wheat wastes by solid state fermentation method' with 2592-510 P reference number in Turkey.

Working Skills and Experiences

Languages:

1. Turkish: mother tongue
2. English: advanced level (English Language Exam (YDS) result: 83.75, March 2017)
3. Spanish: beginner level (continuing course)

Computer Literacy:

Excellent level at:

1. All Microsoft Office programs
2. SigmaPlot (graphing and statistical analysis program)
3. SPSS (statistical analysis program)
4. Design of Expert (experimental design and data analysis program)
5. SuperPro Design (simulation and economic analysis program)

Research Assistant:

Nine years (2009-2018) at Department of Food Engineering, Gaziantep University and responsible for:

1. Instructing general chemistry, organic chemistry, analytic chemistry, food chemistry, fermentation technology, biotechnology, operation, general microbiology, and food microbiology laboratory classes.
2. Training and mentoring students throughout laboratory classes.
3. Assessment and evaluation of the courses with oral and written exams.

4. Organizing scientific and technical tours to institutions, workshops and factories for students.
5. Consulting at least 4 students in each semester to find scientific topics, do literature searches, make analyzes in laboratory, and report their results as a scientific document.
6. Attending and organizing meetings such as congresses, symposiums, accreditation commissions, academic activities within and between departments and universities.
7. Researching and developing; project management; financial management and documentation; planning and organizing activities with limited supervision and in hardship environment; preparing scientific documents such as article, poster and oral presentations for academic and social meetings.
8. Doing all analysis of food products and their by-products which are about determining nutrition content, defining chemical composition, data analyzing statistically and economically, mass and energy calculations, preference mapping instrumentally and sensory, preparing feasibility report of a plant, and etc.
9. Obtaining and providing knowledge and experience in engineering tools, systems and operational standards, including governmental concept.

Project Management:

Gains during six projects accomplished in the period of high education:

1. Project writing, budgeting, managing, and reporting.
2. Budget transfer and auditing requirements.
3. Delivering the assignments and project outputs in time.
4. Full-scale laboratory installation with consumables, chemicals, equipments purchase.
5. Instructing and supervising scholarship holders.
6. Working with people having professional backgrounds.
7. Improving personal commitment and efficiency.

Publications

International and National Presentations:

1. Dursun D, Yakar N, Çakır İ, Çakmakçı ML. Microbial cellulose production and usage in the food industry (poster presentation). Turkey 9th Food Congress, p. 981-984, 24-26 May 2006, Bolu-Turkey.
2. Tokatlı M, Dursun D, Bağder S, Şanlıbaba P, Özçelik F. Identification of lactic acid bacteria isolated from pickles in Ankara Çubuk region (poster presentation). Biotech METU 2009 International Symposium on Biotechnology: Developments and Trends, p. 141, 27-30 September 2009, Ankara-Turkey.
3. Şanlibaba P, Tokatli M, Bağder S, Dursun D, Özçelik F. Characterization of lactic acid bacteria based on genotypic analyses. New Biotechnology, Volume 29, Supplement, 23–26 September 2012, Page S125.
4. Bağder S, Tokatli M, Dursun D, Arslankoz N, Şanlibaba P, Özçelik, F. Turşudan İzole Edilen Laktik Asit Bakterilerinin Hidrojen Peroksit Üretim Yeteneklerinin Belirlenmesi (poster presentation). 2. Ulusal Biyoloji ve Biyoteknoloji Kongresi. 15-18 Kasım 2012, Antalya-Turkey.
5. Şanlibaba P, Tokatli M, Bağder S, Dursun D, Özçelik F. Characterization of lactic acid bacteria based on genotypic analyses (poster presentation, abstract publishing). 15. European Congress on Biotechnology, 23-26 September 2012, Antalya-Turkey.
6. Dursun D, Dalgıç AC. Production of natural aroma compounds from apple pomace by using solid state fermentation (poster presentation). NAFI 2011 Congress on Novel Approaches in Food Industry, p. 1040, 26-29 May 2011, Izmir-Turkey.
7. Dursun D, Dalgıç AC. Aroma and pigment compounds produced by *Sporidiobolus salmonicolor*. Turkey 11th Food Congress, 10-12 October 2012, Hatay-Turkey.
8. Eryılmaz EB, Dursun D, Dalgıç AC. Evaluation of pomegranate wastes biotechnologically (poster presentation). 4th Traditional Foods Symposium, p. 381-384, 17-19 April 2014, Adana-Turkey.

9. Dursun D, Işık E, Dalgıç AC. Natural pigment production from agricultural wastes (poster presentation). EuroBiotechnology 2012 Congress, 12-14 April 2012, Kayseri-Turkey.
10. Dursun D, Altınçiçek EA, Boğusoğlu M, Dalgıç AC. Astaxanthin pigment production by ATCC 24202 from fruit wastes using solid state fermentation method (Oral presentation). EuroBiotechnology 2014 Congress, 15-18 May 2014, Lecce-Italy.
11. Dursun D, Evaluation of lentil wastes biotechnologically (poster presentation). The 2nd International Symposium on "Traditional Foods from Adriatic to Caucasus", 24-26 October 2013, Struga-Ohrid/Macedonia.
12. Dursun D, Dalgıç AC. Optimization of pigment production from lentil wastes using solid state fermentation. EWMS 2016, EurAsia Waste Management Symposium, 02-04 May 2016**,** Yildiz Technical University, İstanbul-Turkey.

Papers:

1. Dursun D, Işık E, Dalgıç AC. Natural pigment production from agricultural wastes (conference paper). Journal of Biotechnology, 161 (2012) 42. DOI: 10.1016/j.jbiotec.2012.07.136.
2. Şanlibaba P, Tokatli M, Bağder S, Dursun D, Özçelik F. Characterization of lactic acid bacteria based on genotypic analyses (conference paper). New Biotechnology, 29 (2012) S125.
3. Tokatlı M, Dursun D, Arslankoz N, Şanlıbaba P, Özçelik F. The importance of lactic acid bacteria in pickles production. Academic Food Journal, 10 (1); 2012, 70-76.
4. Dursun D, Altınçiçek EA, Boğusoğlu M, Dalgıç AC. Astaxanthin pigment production by ATCC 24202 from fruit wastes using solid state fermentation method (conference paper). Journal of Biotechnology, 185S (2014) 24. DOI: 10.1016/j.jbiotec.2014.07.080
5. Bağder S, Tokatli M, Dursun D, Özçelik F, Şanlibaba P. phenotypic and genotypic identification of lactic acid bacteria isolated from traditional pickles of Çubuk region in Turkey. Folia Microbiologica, 60 (2015) 241–251.
6. Dursun D, Dalgıç AC. Optimization of astaxanthin pigment bioprocessing by four different yeast species using wheat wastes. Biocatalysis and Agricultural Biotechnology, 7 (2016) 1–6.

7. Eryılmaz EB, Dursun D, Dalgıç AC. Multiple optimization and statistical evaluation of astaxanthin production utilizing olive pomace. Biocatalysis and Agricultural Biotechnology, 7 (2016) 224–227.
8. Dursun D, Dalgıç AC. Rice bran or apple pomace? Comparative data analysis of astaxanthin bioproduction. Journal of Agricultural Sciences. 24:3 (2018). *In press.*
9. Dursun D, Dalgıç AC. Bioproduction of high value-added pigments from agro-industrial wastes. Academic Food Journal. *Accepted, In press.*

Hobbies and Interests

In personal life, I always care about humanity feeling which compromises helping people, even a tree cultivating or giving water to an animal. I have always believed in that being with people will come up with brilliant, helpful, merciful, and useful results firstly for yourself and then for other people. It also resulted in good communication skills with your circle, national and international. The humanity feeling serve the purposes of helping people who need peace and safety.

Most of my spare time is taken up with watching documentaries, reading books and cooking, and socializing with close friends. Travelling is my great interest. I have been learning Spanish.