LUTEIN PRODUCTION BY *Chlorella vulgaris* USING POULTRY LITTER ANAEROBIC DIGESTATE AND ITS POTENTIAL APPLICATIONS

A Thesis

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

by

SURJITH RAMASAMY

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

GUWAHATI - 781039, ASSAM, INDIA

December 2021

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES & BIOENGINEERING

DECLARATION

I, hereby declare that the content embodied in this thesis entitled "**Lutein production by *Chlorella vulgaris* using poultry litter anaerobic digestate and its potential applications**" is the result of investigations carried out by me at the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of **Prof. Kannan Pakshirajan.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: **Surjith Ramasamy**

Place: IIT Guwahati

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES & BIOENGINEERING

CERTIFICATE

It is certified that the work described in this thesis entitled **"Lutein production by *Chlorella vulgaris* using poultry litter anaerobic digestate and its potential applications"** by **Surjith Ramasamy** for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, and this work has not been submitted either in whole or in part elsewhere for a degree.

_____

(Signature of Thesis Supervisor)

Date:

Place: IIT Guwahati

Prof. Kannan Pakshirajan
Professor
Indian Institute of Technology Guwahati
Department of Biosciences &
Bioengineering
Indian Institute of Technology Guwahati
Guwahati-781039, Assam, India

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Indian government, judiciary system, election commission, army, and farmers for maintaining peace, harmony and health of 1.38 billion Indian people. Secondly, I would like to thank and acknowledge the scientists, teachers and individuals who are trying their best to save the environment and ecosystem of the earth.

I would like to thank my supervisor, Prof. Kannan Pakshirajan, Professor, Department of Biosciences and Bioengineering, IIT Guwahati for his consistent guidance and valuable time he has spent on materialising this thesis work. Once again, I would like to thank my supervisor for fine tuning my academic, research and management skills.

I take this opportunity to express my gratitude to my Doctoral committee members, Prof. Debasish Das, Prof. Senthilkumar Sivaprakasam and Prof. G. Pugazhenthi for their constructive criticism and precious suggestions throughout this work.

I extend my gratitude to the Department of Biosciences and Bioengineering, Centre for the Environment, Department of Chemical Engineering, Centre for Energy and Central Instruments Facility, IIT Guwahati for providing technical and instrumental support to this work. I also take this opportunity to thank all the non teaching staff and teaching assistants who helped me. I would gratefully acknowledge the fellowship and infrastructure provided to me by Institute during all these years for this work.

I am grateful to all my former and current lab mates Dr. Vibha Sinha, Dr. Madhavi Singh, Dr. M. Gopi Kiran, Dr. Lalit Goswami, Dr. M.M Tejas Namboodiri, Dr. Arul Manikandan, Dr. Arun Sakthivel, Dr. Tanushree Paul, Manoj Kumar, and Dipak Kumar Kanaujiya, for their help and support during my research work.

At last but not the least, I am thankful to my family and friends for their patience, understanding and encouragement. This would not have been possible without their unwavering and unconditional love and support given to me at all times for which I shall ever remain indebted.

December 2021 *Surjith Ramasamy*

IIT Guwahati

ABSTRACT

Lutein is an alpha carotenoid, and it is used as an antioxidant, colouring agent, and for treating age related macular degeneration. Lutein is currently produced from petals of marigold grown on agricultural land. Production and harvest of marigold flowers involves huge amount of land, man power and fresh water. Besides, utilization of fertilizers, and pesticides for its cultivation are drawbacks of lutein production from marigold flower petals. This study aimed to produce lutein from marine microalgae using anaerobic digestate and explore its potential applications. Microalgae are known to utilize nutrients and other pollutants from wastewater. Anaerobic digestate is rich in nutrients and discharged from anaerobic digestion reactors.

Four different marine halophilic microalgae strains, namely *Chlorella vulgaris* 92001, *Chlorella vulgaris* 52091, *Chlorella vulgaris* 10241 and *Tetraselmis indica* were evaluated for their biomass and lutein production using three different synthetic digestates (municipal, dairy litter and poultry litter). Lutein production and biomass production values were high using diluted poultry and dairy digestate, respectively, for *Chlorella vulgaris* 92001 as compared to the other microalgal strains.

Using poultry litter anaerobic digestate as the substrate, the effect of different parameters such as dilution factor, pH, $MgCl_2$ (g/L), NaCl (g/L), $NaHCO_3$ (g/L) and inoculum size (cells/l) on lutein production by *C. vulgaris* was studied by employing Plackett-Burman screening design. Dilution factor, high salinity, $NaHCO_3$ and inoculum size significantly influenced the lutein production by *C. vulgaris*, and a maximum lutein production of 2.86 mg/L was obtained. The level of the parameters on the lutein production were further optimized by response surface methodology. Under the optimum conditions of 22.12 dilution factor, 27 g/L NaCl, 4.5 g/L $NaHCO_3$ and 4.82×10^9 cells/L inoculum size, maximum lutein production of 3.412 mg/L was obtained in the screening study.

Kinetics of substrate utilization ($NaHCO_3$ and CH_3COONa), biomass growth and lutein production by *C. vulgaris* was studied and evaluated using different bio kinetic models. Experiments were carried out at various initial $NaHCO_3$ (autotrophic mode) and CH_3COONa (heterotrophic mode) concentrations in the range 1000 – 5000 mg/L and 500 – 2500 mg/L, respectively. Complete utilization of $NaHCO_3$ and CH_3COONa was observed at 3000 mg/L of initial concentration on 14^{th} day and 1500 mg/L of initial concentration on 9^{th} day. Among the different models employed in this study, modified Gompertz model for substrate utilization, logistic model for biomass growth and Gompertz model for lutein production were found suitable. In the case of $NaHCO_3$ as the substrate, its uptake rate was unaffected even at a high initial concentration, whereas in the case of CH_3COONa (heterotrophic condition) a reduction in its specific uptake rate was observed above 1000 mg/L of initial concentration. Model predicted specific uptake values of CH_3COONa due to Edward and that of $NaHCO_3$ due to Monod model matched well with the experimental data. Static carbon flux distribution in the microalgae was carried out on 7^{th} day of its culture for both heterotrophic and autotropic conditions, which showed that the flux towards chlorophyll biosynthesis in autotrophic mode was 4 times higher than that in the heterotrophic mode.

Lutein production *C. vulgaris* 92001 was carried out in indigenously designed and fabricated split column photobioreactor operated under batch and continuous modes. In the batch mode operation, maximum biomass growth of 0.96 g/L and lutein production of 4.379 mg/L was attained at 336 h. The substrate utilization were as follows 4.29 g/L (95.33 %) of $NaHCO_3$, 82.38 mg/L (73.29 %) of NH_4^+, 8.41 mg/L (86.45 %) of PO_4^{3-}, 11.16 mg/L (11.6 %) of K^+. The effect of different HRT (150, 130, 100, 90 h) was studied in continuous operation mode which revealed maximum biomass growth of 0.89 g/L and lutein production of 4.25 mg/L at 150 h HRT. The biomass and lutein productivity values were 0.0058 $gL^{-1}h^{-1}$ and 0.026 $mgL^{-1}h^{-1}$. In

the steady state at 150 h HRT, substrate utilization efficiency was 79.53 % for $NaHCO_3$, 71.71 % for NH_4^+, 84.32 % for PO_4^{3-} and 13 % for K^+ from the feed containing poultry litter digestate.

Lutein production by *C. vulgaris* 92001 using synthetic anaerobic digestate in continuously stirred tank photobioreactor in batch and continuous operation mode was further examined. In batch mode, maximum biomass growth of 0.8160 g/L and lutein production of 3.128 mg/L was obtained at 504 h. The substrate consumption profile showed that 2.926 g/L (65.02 %) of $NaHCO_3$, 63.26 mg/L (56.22 %) of NH_4^+, 7.695 mg/L (78.92 %) of PO_4^{3-}, 10.443 mg/L (10.91 %) of K^+ were utilized from the wastewater. In continuous operation mode, maximum biomass growth of 0.7870 g/L and lutein production of 2.7860 mg/L was obtained at 200 h HRT. The substrate consumption profile showed that 44.44 % of $NaHCO_3$, 50.28 % oNH_4^+, 62.35 % of PO_4^{3-} and 10.79 % of K^+ were utilized from the feed containing poultry litter digestate. The lutein production in the continuously stirred tank photobioreactor was however lower as compared to that using the split column photobioreactor.

Downstream processing strategies, namely electrocoagulation flocculation (ECF), sonication and nanofiltration were employed for cell separation, cell disruption and lutein purification, respectively. The influence of different electrodes (aluminium, copper, zinc and iron), current density (1.5 to 3.5 mA/cm^2) and recovery time (10 to 30 min) on the recovery of microalgal biomass and lutein by ECF was studied. *C. vulgaris* biomass recovery was maximum with copper and aluminium electrodes at the current density values 1.5 and 2.5 mA/cm^2. Lutein recovery was higher with aluminium (92.05 %) and copper electrode (87.98 %) at 10 min recovery time and at a current density of 1.5 mA/cm^2. At the current density 1.5 mA/cm^2 and 10 min recovery time, the energy consumption was 0.27 KW/kg of biomass, which was the lowest among all the experimental values using aluminium as the electrode.

Cell disruption by sonication was carried out for different time (2.5, 4.5, 6.5, 8.5 min) and biomass concentration (0.125, 0.250, 0.50, 0.75 g/15mL) for lutein recovery from *C. vulgaris*. The maximum

lutein recovery was 95.42% for biomass concentration of 0.5 g/15 mL and 4.5 min treatment time. Energy consumption for the maximum lutein recovery by cell disruption was 5351 J/15 mL. Effect of different concentration of lutein in the range 20 -100 mg/L and different filter cutoff (600 - 800 and 800 -1000 Da) on lutein recovery and purification by nanofiltration was studied under different applied membrane pressure. Maximum amount of lutein obtained in the permeate was 89.34% using 800-1000 Da filter. Lutein purity was 83.36, 84. 84, 84.32 and 72.02 % for the applied pressures 2, 3, 4 and 5 kg/cm^2 respectively. FETEM analysis of pure lutein sample revealed its uniform particle size (~5 nm) obtained due to nanofiltration.

Cytotoxicity of the lutein extract obtained from *C. vulgaris* cultivated on synthetic anaerobic poultry litter digestate was assessed employing MTT assay. Lutein concentration ranging from 0.15µg/mL to 0.9 µg/mL showed no effect on the viability of SF9 cell line, whereas an increase in lutein concertation from 1.6 to 6 µg/mL drastically reduced the cell viability. The toxicity effect was found to be dose dependent but not due to presence of any pyrogens. Insecticidal activity of lutein produced by *C. vulgaris* was investigated against *Spodoptera litura* (army worm), which showed that mortality was zero at the low concentrations of 62.5 and 125 µg/mL after 72h exposure, whereas the mortality was 100% at 187.5, and 250 µg/mL of lutein after 48 h exposure.

Lutein enhancement in egg yolk was finally demonstrated by supplementing lutein, lutein ester and microalgae at different concentrations (75 to 450 mg/kg) in the basal diet of hen. Lutein content of the eggs increased significantly($p<0.001$) with the increase in lutein concentration with lutein, lutein ester and microalgae containing basal diet. Increase in egg yolk colour was observed in all the diets for different lutein concentrations. However, maximum Roche's colour score of 12 was observed for the eggs obtained by feeding the hens with diet containing 300 mg/kg lutein ester.

This study demonstrated sustainable production of lutein by the marine microalgae *C. vulgaris* using poultry litter anaerobic digestate and its potential applications in poultry and in agriculture as a biopesticide.

CONTENTS

List of figures

List of Tables

Abbreviations

ANOVA: Analysis of variance

APHA: American public health association

ATP: Adenosine triphosphate

CH_3COONa: Sodium acetate

CO_2: Carbon dioxide

COD: Chemical oxygen demand

CSTPBR: Continuous stirred tank photobioreactor

DD: Dairy digestate

Df: Degree of freedom

ECF: Electro coagulation flocculation

F: Test static

FETEM: Field emission transmission electron microscopy energy dispersive X-ray spectroscopy

MD: Municipal digestate (MD)

$NaHCO_3$: Sodium bicarbonate

PD: Poultry litter digestate

PO_4^{3-}: Phosphate

RSM: Response surface methodology

SCALPBR: Split column airlift photobioreactor

E_r: Axial dispersion coefficient

K^+: Potassium

NaCl: Sodium chloride

HRT: Hydraulic retention time

H_2: Hydrogen

pH : Potential of hydrogen ion

pOH : Potential of hydroxide ion

P: Probability 'P' value

MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

PRESS: Predicted residual error sum of squares

FBA: Flux balance analysis

LED: Light emitting diode

B_0: Bodenstein number

SS: Sum of square

MW: Molecular weight

R^2 Adj: adjusted R^2

UV-vis: ultra violet-visible spectrophotometer

Fr : Froude number

Notations

°C: Degree centigrade	µM: Micro mol
d: Day	I : Ampere
g/L: Gram per liter	S/m : Siemens per meter
g: Gram	R^2: Regression coefficient
h: Hour	rpm: Rotational per minute
kg: Kilogram	µ: Specific growth rate
mg/L: Milligram per liter	w/v: Weight/volume
min: Minute	J/mL: Joule per milliliter
ml/min: Milliliter per minute	v/v: Volume/volume
µ/m^2s: Micromole of photons per square meter per second	V : Volt
µl: Micro liter	s: Second

Chapter - 1

INTRODUCTION

1 INTRODUCTION

Lutein is an essential carotenoid present in rod cells and also in the retinal outer layer of the eye which filters blue light to safeguard the underlying cells from photoxoidative damage. Lutein plays a vital role in inhibiting free radicals during cellular oxidative damage. Combined with its coloring property it finds wide application in fishery, poultry and dairy industries. Lutein helps in prevention of age related macular degeneration. Currently, marigold flowers petals are the main source for lutein production. Also, as the land use pattern changes to supply more food to the ever growing population, the production capability of lutein from marigold might fall (Yaxuan et al., 2016).

Carotenoids are made up of 40 carbon atoms. Oxygenated carotenoids are called xanthophyll and remaining carotenoids as carotene. Carotenoids absorb light at a wavelength that chlorophyll cannot and protects light harvesting system from excess heat and light induced damages. Carotenoids have different colours like red, yellow (or) orange; lutein is yellow in colour. Lutein in nature is found in plants, vegetables, fruits and microalgae in its mono and di ester forms and, therefore, alkaline treatment is required to extract the free lutein. Light and oxygen can damage the lutein easily. It is estimated that lutein can hit the market value up to 3.18 billion U.S. Dollars (Qiao et al., 2016).

Green microalgae are an attractive alternative source of carotenoids compared to conventional plants that utilizes huge amount of land, water, pesticides and labour. Compared with fresh water microalgae, marine microalgae in halophilic and in extreme conditions are an everlasting source for lutein production (Acien et al., 2012). It is therefore important to explore novel sources for lutein production. Moreover, for a sustainable production of lutein it is important to utilize cheaply available raw materials, such as waste resources for microalgae cultivation, which would also reduce the pollutant load on the environment.

Anaerobic digestate from anaerobic digester (i.e., biogas plant, anaerobic wastewater treatment plant) is one such cheaply available resource. Anaerobic digestate consists of organic components including carbohydrates, lipids, proteins, etc. and inorganic components such as nitrogen, phosphorus and other ions. A high concentration of salts present in anaerobic digestate can cause sodicity which can clog the pores in drip irrigation system, whereas ions such as ammonium, nitrate and phosphate can cause nutrient pollution if applied excess in agricultural fields. Phosphate in anaerobic digestate can cause eutrophication in water bodies, which can reduce dissolved oxygen in the pond, thereby posing a serious risk to aquatic and other organisms that rely on water bodies. Unionized form of ammonia can be converted to nitric acid which can also reduce the dissolved oxygen. Marine algae can grow effectively in high concentration of salts and can also utilize all the nutrients in the anaerobic digestate at very low dilution over a wide range of physical parameters such as temperature and pH (Naveed et al., 2016; Maria et al., 2015).

In addition to its major application as a nutraceutical supplement, lutein can serve as a potential biopesticide due to its antioxidant property(Chan et al., 2013). *Spodoptera litura* is a prominent pest in Africa and Asia, which damages more than 100 agricultural plant varieties. This insect has acquired resistance against commonly used pesticides such as organophosphates, benzoylurea, Spinosyn and pyrethrins (Murugan & Saini, 2019; Sundström et al., 2014). It is high time to find a potential biopesticide to control this pest without causing any environmental and occupational hazard for nature and farmers. Hence, this work focused on utilizing cheaply available anaerobic digestate as a substrate for lutein production by marine microalgae and utilizing the same as a biopesticide and poultry feed supplement.

1.1 Aim and Objectives

The main aim of this study is focused on utilizing cheaply available anaerobic digestate for lutein production from microalgae and development of the bioprocess. This study also aims to explore suitable application potential of lutein as a biopesticide and poultry feed supplement.

- Screening of halophilic microalgal strains capable of lutein production using synthetic anaerobic digestate and optimization of process parameters
- Biokinetics of growth, substrate utilization and lutein production using sodium acetate and sodium bicarbonate as the substrates and metabolic flux analysis
- Lutein production in a split column photobioreactor and continuously stirred tank photobioreactor under batch and continuous operation mode
- Development of an effective downstream processing strategy for lutein extraction and purification from microalgae
- Cyto-toxicity and insecticidal activity of lutein extract obtained from microalgae grown on anaerobic digestate
- Application potential of microalgal lutein in poultry to increase lutein content in eggs

1.2 Presentation and layout of the thesis

The thesis of this work consist of five chapters with subsections and references.

Chapter 1 contains the introduction to this work which includes lutein, anaerobic digestate and microalgae. **Chapter 2** involves detailed literature review carried out for this work. **Chapter 3** details the materials and methodology followed for selection of microalgae, screening and optimization of parameters involved in lutein production. Further, it details the different reactor systems used in this study and their operation modes with different biochemical assays. Methods employed for cell separation, cell disruption, cytotoxicity, insecticidal activity and poultry investigations are elaborated. **Chapter 4** describes the

results obtained in this research work along with discussions on the results. The results of screening, optimization, biokinetics and carbon flux distribution are discussed. Details of the biomass and lutein productivity in split column photobioreactor and stirred tank photobioreactors are discussed in detail. Results of the downstream process for cell separation, cell disruption and lutein purification from *Chlorella vulgaris* are discussed**.**

Chapter 5 presents the summary and conclusions based on this study along with some future perspective

Chapter - 2

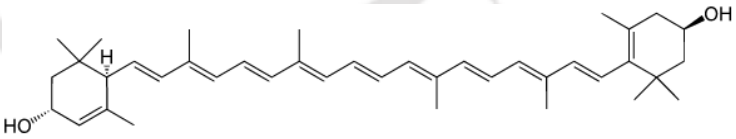
LITERATURE REVIEW

2 LITERATURE REVIEW

2.1 Lutein properties

Lutein is a non polar compound, and hence, it is readily soluble in non-polar and amphipolar solvents. Solubility of lutein varies from one solvent to another, for example lutein solubility is high in tetrahydrofuran (8000 mg/L), whereas in hexane the solubility is only 20 mg/L. Maximum light absorption of lutein is in 444 – 460 nm wavelength, which depends on the solvent used. Lutein consists of one beta and one epsilon – ionone ring with conjugated double bond system. Table 2.1 provides the various physicochemical properties of lutein. Lutein exist either in cis or trans form, of which the latter is predominant. Human body can uptake trans isomer effectively compared to cis form. Mono or poly ester reacts with OH groups of lutein to form lutein esters (Chureeporn et al., 2004).

Table 2.1 Physicochemical properties of lutein (Neal et al., 1992)

IUPAC ID	β,ε-Carotene-3,3'-diol
Molecular Formula	$C_{40}H_{56}O_2$
Molecular weight	568.88 g/mol
Colour	Yellow and Orange
Property	Antioxidant
Structure	
Sources	Marigold, microalgae and vegetables

2.2 Uses and application of lutein

Aesthetic look is one of the important parameters for selecting food from the market by consumers. Modern poultry, fisheries and diary industries utilize lutein from marigold flower

to improve the colour, nutrition value and the stability of biochemicals in egg yolk, fish and dairy products. Lutein is known to increase the egg shell strength which is important for long term storage as well as for safe transportation of eggs. Also, it is reported that uptake of lutein enriched egg increases lutein content in blood plasma (Englmaierova et al., 2013) and the bioavailability of lutein is higher from hen eggs than from lutein, lutein ester and spinach supplements (Yun et al., 2004). In a study, it has been proved that oral intake of lutein inhibits expression of m-RNAs such as HO-1, ICAM-1 and MMP1, which are responsible for UVA1, UVA, and UVB induced skin irritation and inflammation (Beck et al., 2016).

Table 2.2 Activity of lutein against various diseases/effects and their mode of actions

Diseases/Disorder	**Mode of lutein action**	**References**
Arthrosclerosis	Reduces oxidative stress and inflammation	Jueun et al., 2008
Lipopolysaccharide induced skin inflammation	Reduces protein and cytokine expression	Jueun et al., 2013
Ischemic injury in small intestine and kidney	Antioxidant effect	Guo et al., 2015
Carbon tetrachloride, ethanol and paracetamol induced liver damage	Antioxidant effect	Edakkadath et al., 2010
Osteoarthritis	Reduces oxidative stress and apoptosis	Qiao et al., 2016

The hydroxyl group in lutein acts against reactive oxygen species formed from oxidative stress and inflammation. Lutein has shown various beneficial effects against different diseases and age related damages (Table 2.2). Age related macular degeneration (AMD) and cataract are avoided by supplementing with lutein. Risk of age related macular degeneration is inversely proportional to lutein concentration in blood serum and macular region of the eye. A typical

diet should contain 6 mg/day of lutein and zeaxanthin to reduce the risk of AMD (Eisenhauer et al. 2017).

Feeding 9 gm of *Chlorella* for one month as a dietary supplementary to humans is found to increase lutein content from 14.3 to 54.1 (pmol/mL of packed cell) in erythrocytes and 442 to 1256 (pmol/mL) in blood plasma (Taiki et al., 2013). Lutein ester based sunscreen lotion has sun protecting factor (SPF) 1.08 with a boot star rating of 4, which is a basic requirement for sunscreen lotion (Kale et al., 2011; Zheng et al., 2011). Utilization of lutein is however, based on its purity (Table 2.3)**.**

Table 2.3 Application of lutein based on its purity (Lin et al., 2014)

Purity percentage (w/w)	**Application**
3-80	Oleoresin or capsule
5-20	Fodder additives

2.3 Sources of Lutein

2.3.1 Lutein production from marigold flower

Marigold is a herbaceous plant and it is native to North and South America. There are more than 40 varieties of marigold species, among which *Tagetes erecta* and *Tagetes patula* are cultivated in large quantity throughout the world. Commercial production of lutein is based on petals of marigold flowers. Table 2.4 provides some basic requirements for marigold cultivation in order to produce lutein. Following manual collection of petals from marigold flower, ensiling method (anaerobic fermentation) is applied to remove water content from the petals so as to enable its long term storage, which removes 10% water content. Dewatering (compression) and drying are then applied to reduce the total water content to 10% of dry biomass. Dry biomass is powdered and desterified with the help of KOH. Liquid – liquid

extraction is finally carried out to extract lutein followed by rotary vacuum distillation or solvent evaporation with the help of nitrogen (Panatpong et al., 2012; Lin et al., 2015).

Table 2.4 Requirements and yield of lutein production from marigold flower (Acién et al.,2012)

Labor requirements	30-60 per hectare
Cultivation period	200 days (15-20 days in nursery + 30 days for yielding buds)
Fresh flowers	30-60 tonnes
Lutein content	20-30 g/kg of marigold dry biomass
Water and nutrients requirement	
Open irrigation	9000- 10500 m^3
Drip irrigation	5250 – 6750 m^3
NPK requirements	200-400 kg, 150-300 kg(P_2O_4), 200-400 kg(K_2O)
NPK = Ratio numbers of three plant nutrients: nitrogen (N), phosphorus (P) and potassium (K).	

2.3.2 Lutein from vegetables

Green colour of plants and microalgae are due to their light harvesting complexes that can absorb light from red and blue spectra leaving green color. A light harvesting complex is generally comprised of seven chlorophyll a, five chlorophyll b and two lutein pigments. Vegetables, green leaves and fruits are other sources of lutein. Concentration of lutein in vegetables is 40-200 times lower than in marigold and microalgae. Lutein content in vegetables and other sources ranges from 0.32 μg/g to 0.126 mg/g with spinach containing the maximum amount of lutein (0.126 mg/g) followed by kale (0.088 mg/g) (Alisha et al., 2009). Vegetables are important sources for macronutrients and their very low lutein content is undesirable for large scale lutein production.

2.3.3 Lutein production from microalgae

Microalgae are either unicellular or multicellular, and are classified into *Cyanophyta, Prochlorophyta, Rhodophyta* and *Chlorophyta*. Red algae (*Rhodophyta),* eg. *Rhodella reticulata* and green algae (*Chlorophyta*), eg. *Chlorella vulgaris* are lutein producers. Majority of microalgae are cultivated commercially for lipids, proteins and carbohydrates. *Dunaliella salina* and *Haematococcus pluvialis* are cultivated commercially for beta carotenoid production, but commercial production of lutein from such microalgae is not reported yet. Some algal species are able to accumulate lutein under a variety of culture conditions, such as high irradiance, nitrogen content, temperature, pH and mineral concentration (Olivier et al., 2013). *Scenedesmus obliquus, Chlorella sorokiniana, Chlorella vulgaris* and *Chlorella zofingiensis* are some of the lutein producing microorganisms. Lutein concentration in microalgae ranges from 0.2 to 4.85 mg/g, which is 4.2 times lower than in marigold flowers.

2.3.3.1 Advantages of microalgae cultivation over marigold

Compared with seasonal and limited cultivation of marigold flowers, microalgae cultivation can be made continuous with easy harvesting. This is mainly because growth rate of microalgae is higher than that of marigold. Microalgae can grow even in saline or nutrient rich wastewater. Annual production of microalgae per hectare is 6 tonnes whereas microalgae is 70-150 tonnes which is 11 times higher than marigold. Yield of lutein is 5 g/kg from microalgae whereas it is 20g/kg from marigold. All these data revealed that lutein productivity is 3-6 times higher in microalgae than in marigold. Besides microalgae require 2-10 times less water than marigold plant (Lin et al., 2015; Acien et al., 2012). Table 2.5 provides NPK requirement and lutein content in marigold and microalgae.

Table 2.5 Marigold Vs algae for lutein and lutein ester production (Lin et al., 2015)

NPK requirement to produce 1kg of lutein		
Marigold	2.5, 0.8, 2.1 kg	
Microalgae	15.1, 6.6, 2.4 kg	
Free lutein content marigold species(mg/g)	Lutein	Lutein ester
Tagetes erecta	0.44	0.57
Tagetes patula	0.15	19.4
Lutein content in *Chlorella* species (mg/g)		
Chlorella pyrenoidosa	2.39	2.72
Scenedesmus obliquus	1.26	1.55

2.3.3.2 Lutein biosynthesis pathway in microalgae

Microalgae can grow effectively under photoautotrophic, heterotrophic and mixotrophic conditions to produce energy and biochemical for its biological functions. The overview of biochemical pathway and enzymes involved in lutein synthesis are presented in Figure. 2.1. In autotrophic condition, algae use Calvin cycle to synthesize NADPH, ATP and then utilize CO_2 to synthesize 2-3 carbon containing compounds, followed by glucose synthesis with the help of other biological pathways.

Pyruvate and acetyl CoA from glycolysis are utilized in mevalonate pathway to synthesize 5 carbon containing compound, isopentenyl pyrophosphate (IPP), which is the main precursor for carotenoid synthesis. In 1-deoxy-D-xylulose-5-phosphate pathway, glyceraldehyde -3-phosphate and pyruvate are the precursors for IPP synthesis. Three IPP are utilized to synthesize farnesyl pyrophosphate, followed by the addition of one IPP to synthesize geranylgeranyl pyrophosphate with the help of geranylgeranyl pyrophosphate synthase enzyme. Phytoene synthase condenses two geranylgeranyl pyrophosphate with the help of ATP to synthesize first carotenoid compound. Lycopene is produced from phytoene with the help of phytoene desaturase, δ-carotene desaturase and cis-carotene isomerase. Lycopene β *and* ε

cyclase and hydroxylase help in converting lycopene to α-carotene and then to lutein (Shinichi et al., 2011).

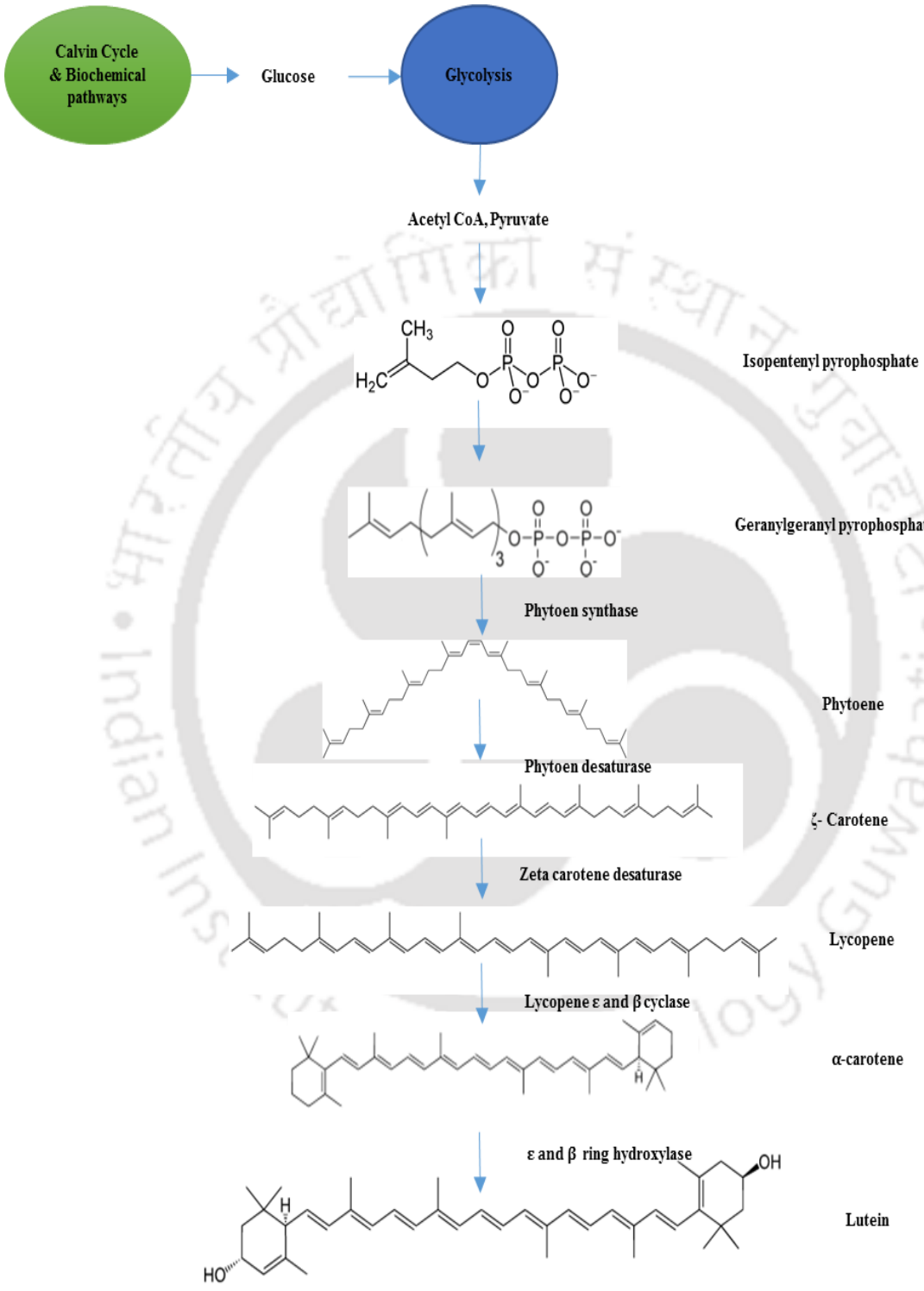


Fig. 2.1 Biochemical pathway involved in lutein synthesis by microalgae

Enzymes involved in lutein synthesis were reported in *Chlorella, Chlamydomonas, Dunaliella and Haematococcus*. Alpha carotene and its derivatives are found only in specific algal families, such as *Rhodophyta, Cryptophyta, Euglenophyta, Chlorarachniophyta* and *Chlorophyta*. In a photosystem, lutein protects chlorophyll a and chlorophyll b against oxidation caused due to temperature and light (Jin et al., 2003).

2.4 Lutein production media and strategies

Scenedesmus obliquus, Chlorella species and *Dunaliella salina* have been reported for lutein production. Table 2.6 provides the literature on different microalgal strains, culture media used, and culture condition followed for lutein production. Algae are cultivated in photoautotrophic, heterotrophic and mixotrophic mode. In autotrophic mode, CO_2 is used as the carbon source and light acts as the energy source. Light intensity for algal cultivation is in the range of 50 – 690 μmol photon $m^{-2}s^{-1}$ for algal cultivation. Microalgae fixes 1.8–1.9 mg CO_2/g of biomass in autotrophic condition when air containing 0.5 - 5% of CO_2 is passed into the culture media, at a flow rate of 90 – 190 mL/min/L (Oilgae, 2010). Media used for photoautotrophic mode of cultivation are Detmer's, Arnon and BG 11 medium (Table 2.7). Lutein content is higher in photographically grown *Scenedesmus obliquus* with a yield value of 4.61 mg/g compared to the other strains.

Heterotrophic mode of algal cultivation uses organic carbon sources such as acetate, glycerol, ethanol and glucose. Energy provided by glycolysis of glucose is 600% higher than the energy provided by light and CO_2. *Chlorella protothecoides* yielded 83.8 mg/L of lutein in heterotrophic mode in batch fermentation and production reached 225.3 mg/L in fed batch condition in shake flask (Shi et al., 2002). Increasing acetate concentration to 20 mM in *Chlorella sorokiniana* increased lutein content by 20 % (Shi et al., 1997). High cost of media

and contamination are main problem in commercialization of heterotrophic mode of microalgae cultivation

Mixotrophic condition provides dual benefit of autotrophic and heterotrophic mode of microalgal cultivation. In mixotrophic mode, specific growth rate and biomass production were enhanced along with lutein produced by 33 to 22% when compared to photoautotrophic culture. In *Chlorella sorokiniana* 5.2 mg/g of lutein was obtained in Arnon medium containing 40 mM concentration of acetate. Lutein content was 5.2 mg/g with *Chlorella sorokiniana MB-1* grown in BG 11 medium containing sodium acetate in mixotrophic condition (Baldo et al., 2011).

Table 2.6 Literature report on lutein production from different microalgae, culture condition and extraction method

Microalgae	Source	Cultivation Condition	Media used	Extraction method	Lutein content (mg/g)	Lutein Productivity (mg/L/day)	Reference
Scenedesmus obliquus FSP-3	Fresh water	Autotrophic, Batch and Semi-batch	Modified Detmer's Medium	Alkaline treatment, Liquid- Liquid extraction (Acetone)	4.61 4.6	4.35 6.01	Hsin et al. (2014)
Chlorella pyrenoidoa ***Scenedes musobliquus***	Fresh water	Autotrophic, Batch	Modified Detmer's Medium	Alkaline treatment , Liquid - liquid extraction(Acetone)	1.55 2.72	NA	Lin et al. (2014)
Chlorella sorokiniana	Fresh water	Autotrophic and Mixotrophic, Batch	Arnon medium	Liquid-Liquid Extraction (Methanol)	3 5.2	NA	Cordero et al. (2011)
Desmodesmus sp	Fresh water	Autotrophic, Fed batch	Modified Bold 3N medium and nitrate	Alkaline Treatment, Liquid-Liquid extraction (Ethyl acetate)	5.05	3.56	Xie et al. (2013)

Chlorella minutissima	Fresh water	Autotrophic, Air lift photo bioreactor - batch	BBM	Alkaline treatment, Liquid-Liquid extraction (Dichloromethane, methanol)	6.05	3.45	Kumar et al. (2015)
Scenedesmus obliquus FSP-3	Fresh water	Autotrophic, Continuous	Detmer's Medium	Alkaline treatment, Diethyl ether	4.52	4.15	Ho et al. (2014)
Chlorella sorokiniana MB-1	Fresh water	Mixotrophic (Sodium acetate), Fed batch	BG-11	Alkaline Treatment, high pressure pretreatment, Liquid –Liquid extraction (Tetrahydrofuran and ethanol)	5.21	5.78	Chen et al. (2016)
Coccomyxa onubensis	Fresh water with high metal concentration	Autotrophic, Semi-continuous	K9 medium with 0.2 M Cu(II) at pH 2.5	Heat treatment and extraction with methanol	0.80	6.485	Vaquero et al. (2012)

Dunaliella salina	Sea water	Autotrophic, Batch	Gg-8	Liquid-Liquid extraction(Ethanol and Hexane(containing butylated hydroxytoluene)	0.75	3.45	Weiqui et al. (2014)
Scenedesmus obliquus FSP-3	Fresh water	Autotrophic, Continuous	Detmer's Medium	Lipid hydrolysis 60% (w/w) KOH, Liquid-Liquid extraction (diethyl ether)	4.85	1.45	Ho et al. (2014)
Chlorella vulgaris ***Chlorella zofingiensis*** ***Chlorella protothecoides***	Fresh water	Autotrophic, Batch	Modified BG11	Cell disruption, 6% (w/v) KOH for lipid hydrolysis, liquid – liquid extraction (Diethyl ether)	3.86 4.38 3.59	0.131 0.122 0.103	Arya et al. (2014)

Table 2.7 Components of synthetic media used for cultivating microalgae for lutein production

S. no			Blue-Green medium (BG-11)	Artificial seawater nutrient-III (ASN –III)	Conway (mg/L)	F/2 (Guillard medium) (mg/L)	F/2
		Macronutrients (mg/L)					
1	Nitrogen as	$NaNO_3$ (or)	1500	750		75	
		KNO_3			100		
2	Phosphorus as	K_2HPO_4	40	750	20	5	
3	Mg as	$MgSO_4.7H_2O$ (or)	75	3500	NIL	NIL	
		$MgCl_2.6H_2O$		2000	NIL	NIL	
4	Calcium as	$CaCl_2.2H_2O$	36	500	NIL	NIL	
5	Carbon as	Citric acid (or)	6	3	NIL	NIL	
		Ferric ammonium Citrate	6	3	NIL	NIL	
6	EDTA as	Disodium EDTA (or)	1	NIL	NIL	NIL	
		Mg EDTA (or)		0.5	NIL	NIL	
		$Na_2H_2EDTA.2H_2O$		NIL	45	4.36	
7	Carbonate as	Na_2CO_3 (or)	20	NIL	NIL	NIL	
		$NaCO_3$		0.02	NIL	NIL	
		Micronutrients (µg/L)					
8	Boric acid as	H_3BO_3	2860	2860	33400		
9	Iron as	$FeCl_2.6H_2O$			1300	3150	
10	Manganese as	$MnCl_2.4H_2O$	1810	1810	360	180	
11	Zinc as	$ZnSO_4.7H_2O$ (or)	222	222		22	
		$ZnCl_2$			4200		
12	Molybdate as	$NaMnO_4.2H_2O$ (or)	390	390		63	
		$(NH_4)_6MO_7O_{24}.4H_2O$			1800		
13	Copper as	$CuSO_4.5H_2O$	79	79	4000	9.8	
14	Cobalt as	$Co(NO_3).6H_2.O$	49.4	49.4			
		$CoCl_2.6H_2O$	NIL	NIL	4000	10	
15	Silica as	Na_2SiO_3	NIL	NIL		30 (g/lit)	
16	Vitamins	Vitamin B_{12}(cyanocobalamin) (or)	10	10	10	10	
		Thiamin (or)	NIL	NIL	200	200	
		Biotin	NIL	NIL		100	

2.4.1 Effect of media components on lutein production

Algal cell is composed of 7% nitrogen and 1% phosphorus of the total dry biomass. Nitrogen is the important constituent for enzyme synthesis. Campo et al. (2000) reported that irradiance and high concentration of nitrogen could increase lutein accumulation in microalgae. Type of nitrogen sources can also influence the lutein production. Compared to ammonia and urea, nitrate is found to increase productivity and content of lutein in *Scenedesmus obliquus FSP-3*, whereas *ellipsoidion* has a high growth rate on ammonia (Nianjun, et al., 2001). Microalgae have less urease activity, and, therefore, utilization of urea is very less than other nitrogen source (Luveshan et al.,2014).

Biomass production and growth rate declined in case of *Chlorella sorokiniana* when nitrate content exceeded more than 40 mM in modified Arnon medium, but lutein content in the biomass remained constant (Baldo et al., 2011). *Scenedesmus species* and other microalgae utilize carbon containing carotenoid for synthesizing lipids and carbohydrates during nitrogen starvation period; hence, harvesting time is an important factor for the production of any carotenoids (Campo et al., 2000). *Chlorella protothecoides* produced 0.27 mg/g higher lutein under nitrogen starvation condition than under nitrogen rich condition (Shi, 2002). In general, phosphorus is also the important nutrient for growth and metabolism in algal cell. A lower concentration of phosphorus is known to increase lutein and biomass yield, and at a concentration more than 372 µM, lutein productivity decreases (Karemore et al., 2013).

2.4.2 Abiotic factors influencing lutein accumulation

Abiotic factors such as light intensity and salinity tends to sharply influence lutein productivity. Light harvesting antenna and lutein increases simultaneously when there is enough light energy (Solovhenko et al., 2008). Minerals and their concentrations act as an inducer of lutein production in microalgae, which can also influence nitrogen, phosphorus and CO_2 uptake. Mn^{2+}

is a cofactor in phytoene synthase in carotenoid synthesis pathway, and it prevents the copper induced oxidative damages by non-enzymatic reactions (Mallick et al., 2004).

Coccomyxa onubensis, an extremophile, produced 6.48 mg/g of lutein in 0.2 mM Cu(II) containing media and survived till 0.4 mM Cu(II), whereas other microalgae could not grow even at a lower concentration of copper (Isabel, et al., 2012). Hyper (1.5 M to 2.5 M) osmotic and hypo-osmotic (1.5 to 0.5 M) condition cause stress in microalgae due to its impact on the algal photosystem (Weiqi et al., 2014). Chlorophyll-a, b and carotenoid absorb light in the ranges of 630-675, 450-475 and 400-550 wavelength, which are very important for ATP and NADPH production during photosynthesis. Kim et al. (2013) reported that a specific wavelength of light improves nutrient uptake, growth rate, primary and secondary product formation in algae.

2.5 Anaerobic digestion and digestate

Anaerobic digestate is obtained from biogas plant and anaerobic wastewater treatment plant. The wastewater consists of high amounts of ammonium, phosphorus, sodium and other minerals. Anaerobic digestion is a biological process in which complex feedstock is converted into compost, anaerobic digestate, methane and CO_2 with the help of anaerobic microorganisms. Anaerobic digestion process involves hydrolysis, acidogenesis and methanogenesis (Fig 2.2), in which most of the minerals remain in the anaerobic digestion process without any change (Chanakya et al., 2011; Desloover et al., 2012).

Feed stock for biogas plant comprises of food, plant and agricultural wastes with a large amount of lignocellulose, cellulose, pectin, proteins and fats. In the hydrolysis step, complex organics are converted to simple sugars, amino acids and long chain fatty acids. During acidogenesis, acetate, CO_2 and H_2 are produced with lowering of the pH. In the methanogenesis step, acetate, CO_2 and hydrogen are utilized to produce methane. During hydrolysis, amino acids are

converted by deammination into ammonium or ammonia, and nitrification further converts ammonia to nitrite and then to nitrate. Phosphate, potassium and other micronutrients are consumed in minor quantities and major quantities of these compounds remain in the anaerobic digestate.

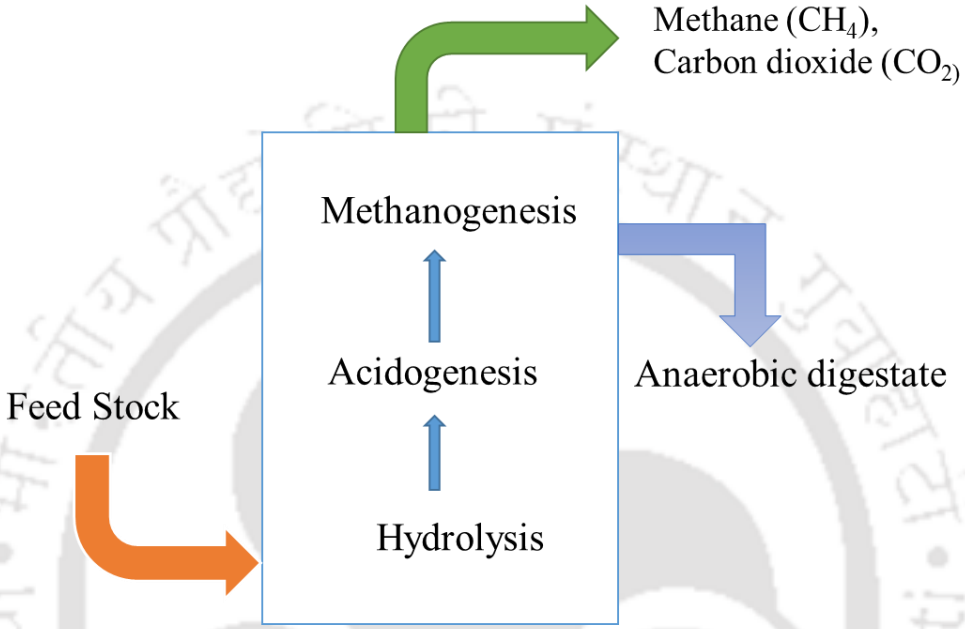


Fig. 2.2 Anaerobic digestion process in biogas production plant

Salinity in anaerobic digestate is due to Na, K, Ca, Mg and Fe, which inhibits the anaerobic digestion process. It is observed that 8 g/L of NaCl can inhibit maximum methane production (154 mL/g of VS) by 10%, whereas 16g/L NaCl can inhibit production of methane upto 80%. Growth inhibition and cell lysis can occur when the concentration of sodium salt is high (5-8 g/L), and, therefore, dilution of anaerobic digestate is required for the growth of fresh water microalgae and bacteria (Naveed, et al., 2016). Digestate obtained from food waste are found to increase the sodicity (sodium content) of the soil, which is a potential threat to sustainable agriculture practice (Pawlett et al., 2016; Abubakar et al., 2011; Moller et al., 2012). Variation

in chemical composition of anaerobic digestate depends on the feed stock, biotic and abiotic factors during the digestion process, as presented in Table 2.8.

Table 2.8 Composition of anaerobic digestate obtained from different sources

S. No	Feed	Treatment unit	Total COD (mg/L)	NH_4^+-N (mg/L)	PO_4^{3-} - P(mg/L)	Na^+ (mg/L)	References
1	Wastewater	Wastewater treatment plant	210	950	415	126	Ugatti et al., (2014)
2	Diary manure	Plug flow anaerobic digester	23760	2232	249.7	Nil	Liang et al.,(2010)
3	Wastewater	Waste water treatment plant	2304	82.5	212	Nil	Yecong et al., (2011)
4	Wastewater	Anaerobic wastewater treatment plant	2180	2120	Nil	2580	Joachim et al., (2012)
5	Wastewater	Anaerobic wastewater treatment plant	134	406.2	35.3	214	Maria et al., (2015)

2.5.1 Anaerobic digestate for algal cultivation

Lin et al. (2015) reported that 1kg of microalgae needs two kilograms of carbon dioxide, 0.05 kg of nitrogen and 0.01 kg of phosphorus based on mass balance. Due to the high demand for NPK fertilizers in agriculture, their supply for scale algal cultivation seem to be limited. Hence, it is essential to utilize easily available low cost substrates for algal cultivation to keep the lutein production cost low. In this regard, utilization of anaerobic digestate for algal cultivation is already reported, but there is no study reported so far for carotenoid or lutein production by microalgae using anaerobc digestate. Fresh water *Muriellopsis sp*. and *Pseudokirchneriella subcapitata* grown in 40 -50 % anaerobic digestate showed 90% removal of COD, phosphorus and nitrogen along with biomass productivity of 1.13 and 1.02 g/L day^{-1} respectively (Maria et al., 2015). Mixed algal culture predominantly *Scenedesmus species,* were grown in anaerobic digestate obtained from wastewater treatment plant, which yielded biomass concentration of

2.6 g/L. An increase in concentration of the digestate reduced the algal specific growth rate from 0.9 to 0.04 d^{-1} (Tran et al., 2014). Digestate obtained from a digester fed with dairy waste was filtered using glass micro filter to remove indigenous bacteria and then used as a feed stock for *Chlorella species* cultivation. Total fatty acid content improved from 9 to 13.7 % in *Chlorella species* when it was fed with the diluted anaerobic digestate (10-25 times) (Li et al., 2011). Biomass production by *Chroocous sp.* was 1.29 g/L, when cultivated in anaerobic digestate which is closer to that obtained from BG11 medium. Subsequent anaerobic digestion of *Chroocous sp.* biomass obtained from anaerobic digestate yielded 317.3 ml of methane per gram of VS_{fed} (Kumar et al., 2014).

2.6 Photobioreactors for algal cultivation

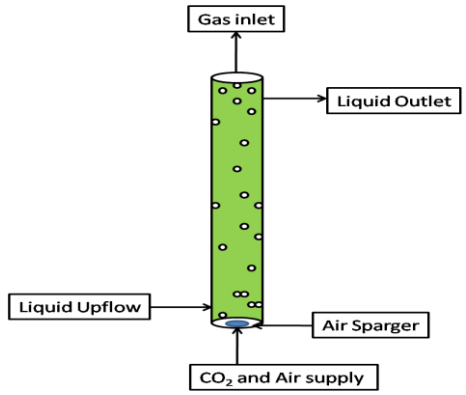
Closed photobioreactors systems have an edge over other passive system such as raceway pond for production due to the control system for controlling the various parameters such as pH, temperature, airflow rate and agitation. Table 2.9 represents the different photobioreactors used for cultivating microalgae using different kinds of wastewater.

2.6.1 Column photobioreactor

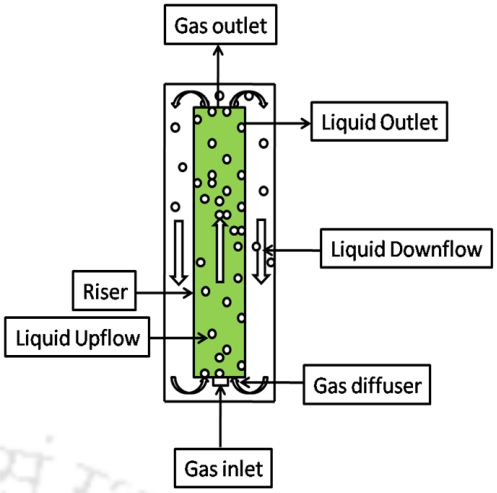
Column reactor includes bubble column and airlift reactors, and these reactors are the most prominent reactors used for algal cultivation. Column photobioreactors have a maximum radius and height of up to 20 cm and 400 cm respectively. In order to achieve a high surface area, it is desired to keep the ratio of diameter to height of the reactor vessel small. Increasing the column height beyond 400 cm leads to nutrient gradient in the reactor and, hence leads to starvation condition for the organism. Moreover, long residence time of O_2 can be detrimental to algae in such reactors (Xu et al., 2009). These reactors are suitable for attaining very good mass transfer coefficient for liquid - gas system.

2.6.2 Bubble column reactor

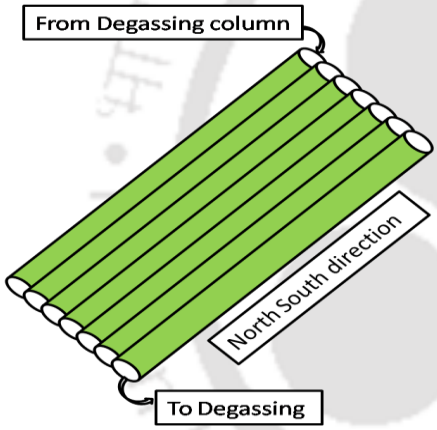
Bubble column reactor is a tubular reactor with a sparger at the bottom, which supplies gas for mixing the contents without the need for any internal mixing units e.g. impeller. A High mass transfer of CO_2 and nutrient can be achieved with an increase in retention time and number of small sized bubbles. Flow rheology of the fluid and geometry of the reactor can affect the light availability. In this type of reactor, diameter exceeding 20 cm reduces the permeability of light whereas the height is limited to 400 cm to avoid shading effect in sequential reactor system. Air bubbles can cause cloud effect in the middle of the reactor which increases the path length of light inside. Cloud effect is defined as group of bubbles which reduces the intensity of light reaching the microalgae. Placing perforated plates along the reactor height increases bubble dispersion for an enhanced mixing efficiency. Bubble column reactor is most suitable for high density culture. Qiang and Richmond (1994) cultivated *Isochrysis galbana* in outdoor condition during summer and achieved 4.6 g L^{-1} biomass with highest productivity of 1.6 g L^{-1} d^{-1}. Cell density directly influenced algal productivity and a, slight change in optimal cell density affects the productivity. During photoadaption productivity is reduced by 5-35%, which signifies that higher the cell density faster is the adaptation. Cell density affects both productivity and dissolved oxygen in this reactor. Damage to light is prominent in bubble column reactor due to smaller path length as compared to that in raceway ponds. Photinhibition of algae is reported when its initial cell density is below an optimal level (2.8-3.8 gL^{-1}), as cells undergo photooxidative death in short span of full strength natural light. Hulatt and Thomas (2011) varied the power input for sparging energy (10, 20 and 50 Wm^{-3}) to study its effect on algal biomass production. Biomass productivity was high with 50 Wm^{-3}, whereas energy obtained from this biomass is very low as compared to the energy input. However, biomass obtained by applying 10 Wm^{-3} energy showed a 39 % higher net energy.



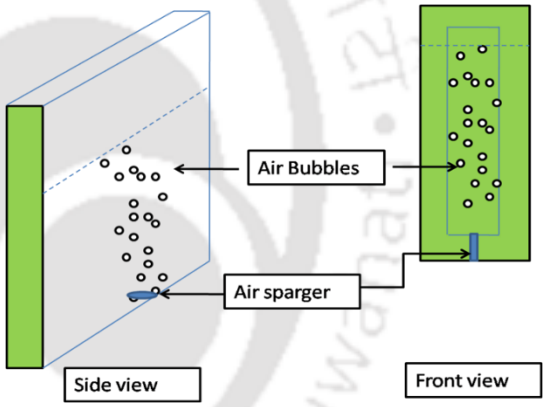
Bubble column photobioreactor



Airlift photobioreactor



Tubular photobioreactor



Flat panel photobioreactor

Fig. 2.3 Pictorial representation of different photobioreactors for microalgal cultivation

2.6.3 Airlift photobioreactor

Airlift reactors consist of a tubular vessel with more than one interconnecting zones comprising of a riser, where gas mixture is sparged and a down comer without any sparger. Blenke in 1979 introduced the airlift loop reactor (ALR), which is classified into three types based on the driving force: hydrostatic, hydromechanic propeller (or pump) and hydrodynamic jet flow driven ALR. M. Moresi (1981) used airlift fermenter (ALR) for continuous production of beer, vinegar, citric acid at different volumetric capacity. Maximum biomass yield (1.20 g mol^{-1}) was achieved under Photon Flux Density (PFD) of 58 µmol/m^2s liter, whereas more than 1200 µmol/m^2s of PFD showed reduced biomass yield (0.272 g mol^{-1}) (Barbosa et al., 2002). Rectangular airlift photobioreactors were used for algal growth to improve mixing for increasing the photosynthesis efficiency (Janssen et al., 2003). Maximum specific growth rate of 0.023 h^{-1} was achieved by cultivating *Phaeodactylum tricornutum* in 12 L concentric tube airlift photobioreactor with a superficial gas velocity of 0.055 m/s (Contreras et al., 1998).

An external loop airlift photobioreactor was designed for swirling motion which enhances mixing (Robert and Legrand, 2009). In comparison with a bubble column reactor, an airlift photobioreactor (ALPBR) with the same volume had achieved higher maximum specific growth rate of 7.41×10^{-2} h^{-1} and a cell concentration of 8.88×10^{6} cells mL^{-1}; the bubble column reactor resulted in a maximum specific growth rate of 3.80×10^{-2} h^{-1} and a maximum cell concentration of 5.8×10^{6} cells mL^{-1}.

Diameter of ALPBR decides the photon loss or even photoinhibition in narrow ALPBR, larger diameter with high turbulent flow inside the system lowers the photoinhibition which tends to favour the biomass. Overheating, photo inhibition or photon loss, biofouling and high initial investment costs are major drawbacks of ALPBR for microalgae cultivation. A multiphysics

was used based on Eulerian approach to predict the effect of shear stress, fluid mixing, radiant transport and algal growth kinetics in ALPBR to simulate the biomass growth (Gao et al.,2018).

2.6.4 Flat panel photobioreactor

Flat panel photobioreactors have short light path and offers good surface area to volume ratio for utilizing maximum light energy. Introduced in 1953 by Burlew, a flat plate photobioreactor (FPPBR), is divided into two types based on the use of a recirculation pump (pump driven) or compressed air (airlift) drive. Issarapayup et al. (2009) used an airlift flat panel photobioreactor for cultivating *Haematococcus pluvialis* NIES-144 by varying the parameters such as area of down comer and riser cross section, size and superficial gas velocity. Maximum cell density of 4.1×10^5 cell mL^{-1} and specific growth rate of 0.52 day^{-1} were achieved using a 17 L flat panel airlift photobioreactor, whereas 90 L reactor capacity yielded a low cell density (40×10^4 cell mL^{-1}) and specific growth rate (0.39 day^{-1}). Performance of 3 L and 17 L cylindrical flat panel airlift photo bioreactors were compared, which revealed a high growth rate and cell density with the 3 L reactor (Issarapayup et al., 2009). Between 17 and 90 L flat panel air lift reactors, the 90 L FP-ALPBR is capable of producing 1g of biomass for 1.16 US dollar which is cost effective as compared with the other reactors systems.

In order to overcome the construction cost and problem due to flow control, Tredici et al (1992) used an alveolar panel reactor with 16 mm thick plexiglas sheets (95% light transparency), surface-to-volume ratio of 80 m^2/m^3 and a thickness of 12.5 mm. Outdoor cultivation of *Anabaena azollae* in a flat panel reactor of surface area 5 m^2 yielded biomass of 28 g/m^2 with a productivity of about 16 $g/m^2 \cdot d$. Thickness of FB-PBR decides the surface/volume ratio and path length of the light; smaller the thickness better is the light transfer which enhances the biomass production (Hu et al., 1996; Zou and Richmond, 1999).

Sudden variation in temperature, inhibition due to light, biofilm formation and expensive materials for construction are considered the main drawbacks of this reactor for large scale application. Tilt angle is defined as the angle at which a reactor is placed to the ground to attain maximum light in order to achieve high biomass. Position of the sun plays an important role in deciding the tilt angle. During summer, a small tilt angle (10° to 30°) is suitable for biomass, whereas in winter need a larger tilt angle around 60° is required. Geographic latitude also influences the tilt angle, direction of the reactor such as west-east or south- north is very important as it can affect the biomass production (Hu et al. 1998, Zhang et al. 2002).

2.6.5 Tubular photobioreactor

Tubular reactors are either straight, bent, or spiral in shape with different arrangements such as horizontal, inclined and vertical; array of tubes in a tubular reactor offers a large surface area to volume ratio for effective utilization of light energy. This reactor has an external unit integrated to the main unit for the exchange of gas and heat that maintains the CO_2 and O_2 concentration and temperature for an optimal algal growth. Recirculation pump is used for mixing and circulation and the cells are harvested using an external unit.

Horizontal tubular photobioreactors (HTPBRs) tend to provide a large surface area to volume ratio than their vertical counterparts because of the ability to decrease the diameter of the tubes, without affecting the structural integrity. Vertical columns require a minimum diameter to support the weight above, whereas horizontal columns are free from this requirement. Horizontal reactors can also overcome the disadvantage with vertical reactor in terms of the requirement of a strong material with minimum diameter to support the media and internal pressure. Angle of incident of light is better in horizontal reactor than in vertical reactor which for raising the temperature very quickly in the horizontal units, which is otherwise difficult in large scale without an external temperature control system (Watanabe and Hall, 1995).

Near horizontal tubular bioreactor are placed at 45° and the construction cost increases to maintain above 45º. The array of transparent pipes are connected with the bottom pipe in order to supply gas which can increase the fluid velocity, gas hold up and nutrient mass transfer between liquid and gas (Ugwu et al. 2002).

Helical tubular bioreactor is a combination of both horizontal and vertical reactors, and it consist of a fluorescent lamp in the middle as the light source. External cooling and degassing units are used to operate the reactor successfully and air is supplied from the bottom of the reactor with the help of a flow meter. Degassing is achieved at the top of the reactor to remove the excess oxygen (Watanabe and Hall, 1995).

Photo loss and inhibition are the main problems in a tubular reactor of small diameter and with low cell density. Array of tubes in these reactor provide protection from excess light, thereby favouring algal growth. Increasing the diameter can also overcome the problem due to high temperature and light to a certain extent, but it can be problematic for nutrient mass transfer in the system. Ugwu et al. (2005) demonstrated an increase in volumetric productivity of biomass by 63% using a static mixer in a 12.5 cm diameter reactor as compared to the same reactor without any mixing unit. Static mixer are made of glass material in helical shape, fitted inside the tubular reactor to improve mixing capacity of the reactor. Different methods used to control the reactor temperature are presented in Table 2.10.

Table 2.9 Different photobioreactors and their operational conditions followed for microalgal cultivation

S. no	Microalgae species	Reactor Configuration	Substrate	Cultivation Conditions	Biomass/ product Productivity	Operation Conditions	Reference
1.	*Chlorella Vulgaris*	Borosilicate bioreactor	1.Pretreated urban wastewater 2.Anaerobically wastewater 3. Final disposing effluent from WWTP 4.Effluent from primary settler + CO_2 (EPS 1) 5.EPS II +activated sludge+ CO_2 6.EPS III+ activated sludge 7.EPS IV +activated sludge 8.Effulent from concentrate I (N/P 0.7) 9. Effluent from concentrate I (N/P 2.0) 10. Effluent from concentrate I (N/P 8.0) 11. Effluent from concentrate I (N/P 15.0)	Mixotrophic	1. 115.7 mg SS /L days 2. 128.2 mg SS /L days 3. 79.82 mg SS /L days 4. 55.65 mg SS /L days 5. 87.42 mg SS /L days 6. 67.42 mg SS /L days 7. 39.28 mg SS /L days 8. 124.8 mg SS /L days 9. 195.1 mg SS /L days 10. 193.7 mg SS /L days 11. 138.2 mg SS /L days	Light intensity 150 µmol/ m^2 s Photoperiod 14:10 12 days' incubation Temperature 20±1°C	Cabanelas et al. (2013)
2	*Chlorella zofingiensis*	10 L glass column	Mixed biogas slurry and municipal wastewater	Mixotrophic	0.28 g/ L. d biomass, 96.3 mg/L. d lipid	Light intensity 150 µmol/ m^2 s	Zhou et al. (2018)

						Photoperiod 12:12 12 days' incubation Temperature 25±1°C	
3	*Cholorella sorokiniana*	Airlift photobioreactor	Bioindustrial wastewater	Mixotrophic	0.023 g dw/ L day, biomass	Temperature 25±1°C 70 DAYS incubation Sun light	Podevin et al. (2017)
4.	*Chlorella Vulgaris*	Multi-layered pilot scale photobioreactor	Real concentrate with waste glycerol	Heterotrophic	460 mg/L d TVS and maximum lipid content 27%	34 days semi continuous mode	Ren et al. (2017)
5.	*Chlorella Vulgaris*	Membrane photobioreactor	Synthetic wastewater	Photoautotrophic	2 mg/L of biomass	Light intensity 2000 lux HRT 2 days	Praveen et al. (2016)
6.	*Scenedesmus obliquus*	Vertical alveolar flat panel Photobioreactor	Cattle wastewater	Mixotroph	213 to 358 mg/L d biomass	HRT 12 days Photoperiod 24:0 Light intensity 58 µmol/ m^2 s	de Mendonça et al. (2018)
7.	*Chlorella zofingiensis*	Tubular bubble column photobioreactor	Piggery wastewater	Mixotrophic	106.28 mg/L d to 296.16 mg/L d biomass 11.85 to 30.14 mg/L d	Temperature 25±1°C HRT 10 days Light intensity 230±20 µmol/ m^2 s	Zhu et al., (2013)

8.	*Botryococcus braunii*	Submerged membrane photobioreactor	Livestock wastewater	Heterotrophic	3500 mg/L biomass	HRT 3,4 and 5 Days SRT 16 days Temperature 25±1°C Light intensity 220 µmol E/ m^2 s	Lee et al. (2018)
9.	Mixed culture 68.4% of *Acutodesmus obliquus*, 21.4% of *Scenedesmus quadricauda* and 10.2% of *Scenedesmus tenuispina*	Column Photobioreactor	Textile wastewater	Heterotrophic	Biomass concentration 0.7± 0.1 g TSS/L	Light intensity 400±51 µmol / m^2 s Light period 12:12 HRT 10days SRT 11 days	Dhaouefi et al., (2018)
10	*Halochlorella rubescens*	Twin layer photobioreactor	Municipal wastewater 1.wastewater after secondary settlement 2. the wastewater after secondary settlement with addition of 1.5 mg L^{-1} $PO4^{3-}$ as KH_2PO_4 to simulate an	Mixotrophic	6.3 g /m^2 d Average areal microalgal growth	HRT 8 days Light intensity 22 -220 µmol/ m^2 s	Shi et al. (2014)

			inactive Phosphate precipitation process 3. Wastewater from denitrification tank 4. wastewater from Bio-Phosphorus tank				
11	*Euglena sp*	Sequencing batch membrane photobioreactor	Secondary effluent	Photoautotrophic	550 mg/L biomass concentration 10.09% lipid content	Biomass retention time (BRT) 60 days HRT 2,4 and 8 days Light intensity 10000 lux	Sheng et al. (2017)
12	*S. obliquus*	Airlift tubular photobioreactor	Urban wastewater	Mixotrophic	21.76.26±0.3 g SS/m^2 d maximum areal productivity	HRT 5 days	Arbib et al. (2013)
13	*Chorella vulgaris* ESP-31		Coke making wastewater		**Batch cultivation** Biomass concentration 3.98 g/L and lipid productivity of 47.1 mg/L/d **Semi-batch cultivation** Biomass concentration and lipid productivity of 5.18 g/L and 77.3 mg/L/d **Immobilization approach** Maximal biomass growth	Light intensity 150 µmol/ m^2 s Temperature 27 °C	Ubando et al. (2019)

					of 5.17 g/L and lipid productivity of 68.4 mg/L/d		
14	*Chlorella pyrenoidosa*	Airlift circulation Photobioreactor	Anaerobic digested starch processing wastewater	Photoautotrophic	630 mg/L d biomass productivity 69 mg/L d lipid productivity	Two phase strategy cultivation 1. Temperature 15°C Light intensity 60 µmol/ m^2 s HRT 3 to 4 days 2.10Temperature 35°C Light intensity 220 µmol/ m^2 s HRT 6 to8 days	Tan et al. (2014)
15	*Chlorella vulgaris*	Membrane photobioreactor	Secondary effluent	Mixotrophic	1.035 to 1.524 g/L biomass concentration	HRT 2 days Biomass retention time (BRT) 21.1 d Light intensity 10.5 to 112.3 µmol/ m^2 s	Gao et al. (2018)
16	*Scnedesmus sp*	Bubble column photobioreactor	Olive mill wastewater	Photoautrophic	86 mg/L d Biomass production, 0.86 g/L to 1.4 g/L Biomass concentration	HRT 21 days	Di Caprio et al. (2018)

17	*Chlorella vulgaris*	Column photobioreactor	Saline wastewater treatment	Photoautotrophic	Biomass concentration stage 1 0.5929 mg/L Biomass concentration stage 2 0.9070 mg/L Biomass concentration stage 3 1.0380 mg/L Lipid productivity stage 1 20.15 mg/L d Lipid productivity stage2 32.33 mg/L d Lipid productivity stage 3 54.25 mg/L d 40% lipid content	Batch cultivation 20 days	Shen et al., (2015)
18	*Chlorella vulgaris*	Membrane photobioreactor	Sewage	Photoautotrophic	39.93 mg/L d	Light intensity 8000 lux Temperature 25±2°C HRT 2.5 days	Gao et al. (2014)
19	*Chlorella vulgaris*	Biofilm membrane Photobioreactor	Secondary effluent	Photoautotrophic	Volumetric microalgal production 0.072 g/L d	HRT 2 days Temperature 25 to 28 °C Light intensity 8000lux	Gao et al. (2015)

20	*Chlorella sorokiniana*	Photobioreactor column	Alcohol distillery wastewater	Photoautotrophic	Biomass concentration 12 g/L	HRT 4 days Temperature 27 °C Light intensity 180 µmol photons / m^2s	Solovchenko et al. (2014)

Abbreviations: HRT - Hydraulic retention time, SRT – Solids retention time , TVS – Total volatile solids, SS –Suspended solids, VSS – Volatile suspended solids

Table 2.10 Different methods followed to maintain the temperature in tubular reactor system

S. No	Methods to maintain temperature	Advantage	Disadvantage	Reference
1	Shading with dark sheets	Reduces the photo inhibition	May lead to photodeficient condition	Ugwu and Aoyagi, (2008)
2	Sprinkling water on the reactor	No shading effect	Loss of water and energy	Prakash et al., (1997)
3	Immersing the reactor in water	Reflection of lights in water can improve the light supply	Moving parts are needed to operate the reactor	Carlozzi et al. (2006)
4	Heat exchanger	Easy to handle	Energy consumption at large scale	Watanabe and Hall, (1995)

Unlike in airlift and bubble column reactors gas transfer rate is very low in tubular reactor which creates substrate (CO_2) limitation and also oxygen accumulation. Lehr and Posten, (2009) injected CO_2 in multiple locations along the axis of third generation smart controlled (G3) bioreactor. Novel gas stripping units in tubular reactor includes airlift reactor in which carbon dioxide is added in the riser column to remove dissolved oxygen and head space further prevents the accumulation of oxygen in this system (Rubio et al., 1999). Advantage and disadvantages of various photobioreactors are provided in Table 2.11.

Table 2.11 Advantages and disadvantages of different photobioreactors

S. no	Photo-bioreactor configuration	Advantages	Disadvantages
1.	Bubble column photobioreactor	1.Low capital cost 2. High surface area to volume ratio 3. Lack of moving parts 4. Satisfactory heat and mass transfer 5. Relatively homogenous culture environment 6. Efficient release of O_2 and residual gas mixture	1.Cosiderable degree of back mixing in both liquid and gas phase which adversely affects substrate conversion efficiency 2.Short gas phase residence time
2.	Airlift photobioreactor	1.Mixing is done by bubbling the gas through sparger in the riser tube without any physical agitation 2. Creates circular mixing pattern where liquid culture passes continuously through dark and light phase giving flashing light effect to algal cells 3. High photosynthetic efficiency	1.Performance depends upon the design and operating conditions 2. The amount of gas which does not disengage in the disengagement zone gets trapped by liquid moving downward in the downcomer 3. Complexity and difficulty with scale-up
3.	Tubular photobioreactor	1.Large illumination surface area 2. Suitable for outdoor cultures 3. Fairly good biomass productivities 4. Relatively cheap	1.Gradients of pH 2. Dissolved oxygen and CO_2 along the tubes 3. Fouling 4. Some degree of wall growth 5. Requires large land space
4.	Flat-plate photobioreactor	1.Large illumination surface area 2. Suitable for outdoor cultures 3. Good for immobilization of algae 4. Good light path 5. Good biomass productivities 6. Relatively cheap 7. Easy to clean up 8. Readily tempered 9. Low oxygen build-up	1.Scale-up require many compartments and support materials 2. Difficulty in controlling culture temperature 3. Some degree of wall growth 4. Possibility of hydrodynamic stress to some algal strains

2.7 Control system and its strategy in photo-bioreactor (PBR):

The impact on control systems and its strategy in biological processes, particularly in photo-bioreactor system, is gaining importance due to many challenges and opportunities associated with it. The control of microalgae cultivation using a photo-bioreactor is highly desired due to the potential use of microalgae in various applications, including in medicine, chemistry, fuel, food, cosmetics, etc. Microalgae can be cultivated in photo-bioreactors under controlled environment to achieve maximum growth rate and high productivity. Microalgae cultures have been conventionally cultivated in raceway ponds /open ponds due to its easy operation and low cost (Jorquera et al., 2010 and Richardson et la.,2012). Unfortunately, this type of open system is not suitable for high volumetric productivity and high value added products due its low efficiency of light utilization, lack of pH and temperature control, high risk of contamination, poor liquid/gas mass transfer and low yield of algal biomass (Pulz., 2001). Hence, closed loop photo-bioreactor systems such as tubular, airlift, bubble column and flat panel photobioreactors, can be used to achieve controlled conditions of temperature, pH, mixing etc. (Garcia et al., 2003).

2.7.1 pH control in tubular photo-bioreactor

In a photobioreactor system pH control accounts for 30% of the production cost which can be reduced by using automatic control system. pH in a PBR is controlled by using data acquisition system (DAQ), pH probe, automatic pneumatic valves for CO_2 supply and peristaltic pump for supplying alkaline solution. DAQ acts as the interface between pH probe and peristaltic pump which is continuously monitored and controlled through the commercially available Labview^{TM} software. Carbon losses can be reduced by less than 30% through appropriate design and operation of PBR. Highly developed control strategies to reduce carbon losses are

required by considering mixing and mass transfer that take place in PBR systems. The model protective control (MPC) strategy was established to control pH, which minimizes the carbon losses to less than 5%. In this strategy, lower layer is controlled by proportional integral (PI) controller pulse feed forward compensator for tracking the reference. The response of pH to changes in CO_2 supply is modelled by using a first order equation with dead time system. These advanced control strategies are used to reduce CO_2 losses and operating cost at large scale level (Tebbani et al.2014).

2.7.2 Control of biomass production in tubular photo-bioreactor

Biomass production and productivity in PBR are controlled by nonlinear predictive control and proportional-integral derivative (PID) with feedback linearization for operating a PBR with constant density mode. A PID control estimates the difference between desired set point and observed set point in a process and then rectifies the difference using proptional, integral and derivatives which is PID. These control strategies can be used to maintain the algal culture at an optimal biomass density wherein pre estimated feeding profile is used as the reference. In a study by Abdollahi and Dubljevic, (2012) interior point optimization, moving horizon estimator and model predictive control were employed to maximize lipid production by a heterotrophic microalgae in a fed-batch bioreactor. Interior point optimization is an algorithm which solves the linear and non-linear optimization problems, moving horizon estimator considers the measurements obtained along with its noise to calculate the estimated value. Model predictive control uses the current data of the experiments to estimate any change in the dependent variable.

2.8 Light in photobioreactor

Microalgae absorbs light energy in 600-700 nm wavelength for photosynthesis. Outdoor systems can a accumulate lot of heat which tends to reduce the biomass production during sunny days. Control and monitoring of natural light is difficult, therefore indoor reactors with solar and artificial lights are useful for the purpose. Incandescent, halogen, fluorescent, compact fluorescent bulbs and LED are the different sources of artificial light. LED bulbs can save 80-90% of the energy as compared to incandescent bulbs. Moreover, it does not contain toxic metals like mercury or lead unlike fluorescent bulbs. When electrons transition happens in conduction band, light emits through band gap in the semiconductor. High concentration of microalgae can create self-shading effect whereas high light intensity can be photoinhibitory of microalgal growth. Optimization of light intensity is needed to achieve a maximum specific growth rate. Flat panel, raceway pond and horizontal tubular reactors can effectively utilize light energy due to their larger surface area. Intensity of light diminishes from outer layer to the center of PBR under high dense biomass conditions. Tubular PBRs are constructed using glass, plastic, acrylic, polyethylene and perspex materials in order to allow light penetration of light photosynthetic organisms. However, use of glass material can increase the reactor cost in large scale.

Successful PBR design relies on maximization of efficiency to utilize light energy, process control for attaining maximum yield and minimizing operation energy to reduce the production cost. Cellular respiration due to day/night cycle can reduce the total biomass produced in daytime to 58% in night time (Jacob-Lopes et al., 2009; Tredici et al., 1991). Light fraction and cycle time are two important factors which can influence day/night cycle. Higher value of light fraction (ratio of light exposure time to total day/ night cycle and lower value of cycle time) can influence biomass production positively (Equation 2.1) (Barbosa et al. 2003).

$$\text{Light Fraction} = \frac{\text{Amount of time exposed to light}}{\text{Total day/night cycle}} \quad (2.1)$$

2.9 Cytotoxicity Assay

Cell lines are used for evaluating the cytotoxicity of a test material under *in vitro* condition. Cytotoxicity assays depend on various cellular mechanisms, such as cell membrane permeability, enzyme activity, cell adherence and adenosine triphosphate production(LI et al., 2015). There are several types of cytotoxicity assay, and selection of a suitable assay is based on the type of test material, sensitivity, specificity and cell lines used. Cytotoxicity assays help to reduce animal testing in short term and reduce the cost involved by integrating with other tests. Results of cell line based cytotoxicity studies can corroborate up to 80% with that of *in vivo* studies. Cell lines such as L29 and HeLa cell lines are predominantly used in cytotoxicity assays for evaluating the biocompatibility(Agarwal, 2001)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow colour chemical which reacts with the mitochondrial enzyme NADPH of live cells to give purple colour compound formazan. Fig. 2.4 shows chemical reaction involved in MTT assay. This assay is widely used for assessing the cytotoxic effect of several biomedical products. The colour formed are read at 500-600 nm wavelength range using a spectrophotometer. This assay is precise and reproducible for measuring cell viability and to study toxicity effect. The formazan formed from MTT are insoluble in water, but soluble in solvents such as dimethyl sulfoxide and isopropanol. Due to crystal formation and solvent addition, variation in results of experiment carried out using the same test sample is possible, necessitating control experiments for finding pseudo/false positive results. Moreover, colour due to test compounds tend to interfere with the results of MTT assay (Stone et al., 2009).

Pal et al. (2007) prepared a novel hydrogel membrane by esterification of polyvinyl chloride and gelatin for application in wound dressing and healing(Pal et al., 2007). Hydrogel has the capability to protect injured skin and absorb exudates from injured cells. L929 fibroblast and mice splenocytes cell lines were used to assess the cell proliferation capability of the novel hydrogel of 4×4 mm size in petri plates. Results showed that proliferation of the cells relative to that of a control was in the range 2.37- 2.38, which suggested its safe utilization for healing of wound due to spleen surgery.

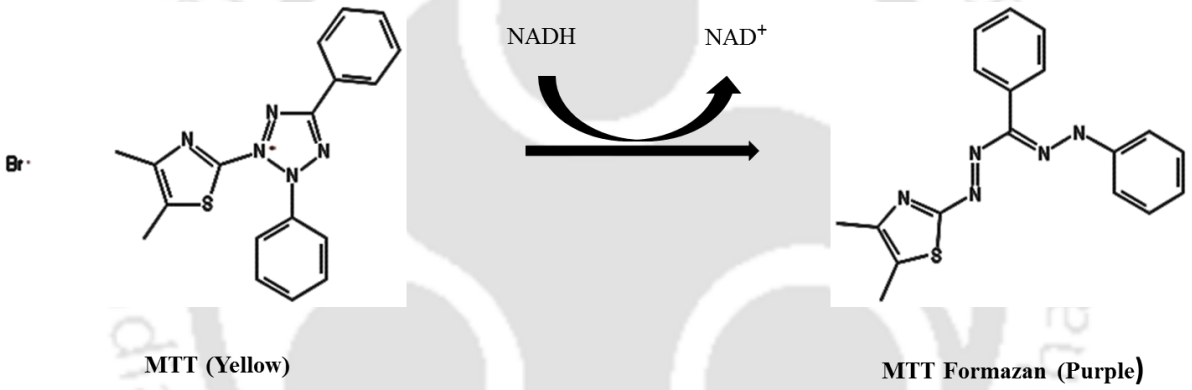


Fig. 2.4 Chemical reaction involved in MTT assay

Kakkar et al. (2014) assessed the biocompatibility of keratin obtained from bovine hooves for its potential application as wound dressing and biomaterial for tissue regeneration. Following pulverisation, defatting, reduction and dialysis, the treated keratin was observed to have a high denaturation temperature of 215°C. Three different concentrations of the processed keratin (10, 25 and 50 µg/well) were tested for its biocompatibility with 3T3-Li fibroblast cells. The results showed aggregation and formation of actin filament with untreated cells, whereas cells treated with 10-25 µg/well were distinct and their internal membranes were segregated. All three concentrations of the biomaterial were found to be biocompatible, but viability of the cells reduced with an increase in keratin concentration.

Balaji et al. (2012) characterized keratin–collagen 3D scaffold and evaluated its biocompatibility with NIH 3T3 fibroblasts and L6 myocytes cells. The results showed that the blend of keratin and collagen with a high thermal denaturation rate reduces fragility and increases strength and flexibility of the scaffold. By MTT assay, the 16 mm diameter scaffold was assessed for its effect on growth and regeneration of the cells in a 24 well culture plate containing cells in the concentration range 2×10^6 - 6×10^6 cells/mL. After 48 h incubation period, the cell density was 3.9–9.7 $\times10^6$ /mL and the cell distribution was uniform due to a high surface area. The scaffold was thus found to be stable and biocompatible.

A through literature survey showed that microalgae are usually cultured using synthetic media for lutein production. There is no report done till date on lutein production using cheaply available waste sources. This study therefore, focused on utilizing cheaply available anaerobic digestate for lutein production from microalgae and development of the bioprocess.

Chapter - 3

MATERIALS AND METHODS

3 Materials and methods

3.1 Microalgal strains and cultivation conditions

Halophilic microalgal strains, namely *Chlorella vulgaris* 92001, *Chlorella vulgaris* 50291, *Chlorella vulgaris* 10241 and *Tetraselmis indica* were procured from National Facility for Marine Cyanobacteria (NFMC), Thiruchirapalli, Tamil Nadu, India. All these strains were cultured in BG11 medium with minerals, vitamin B_{12} (10 µg/L) and NaCl (25 g/L). Prior to inoculation, the initial pH of the medium was set to 7.5 ± 0.2 and after which the cultures were gown at 27 ± 1°C temperature and 125 µmole/m^2s light intensity.

3.2 Selection of anaerobic digestate and microalgae

Synthetic anaerobic digestate of composition similar to those of dairy digestate (DD), municipal digestate (MD) and poultry litter digestate (PD) were prepared based on the literature (Morales-Amaral et al., 2015; Rajagopal et al., 2013; Vasseur et al., 2012; Wang et al., 2010).Composition of these synthetic anaerobic digestate is presented in Table 3.1.

Table 3.1 Composition of synthetic anaerobic digestate used in this study

Composition	**CH_3COO^-** (mg/L)	**NH_4^+** (mg/L)	**PO_4^-** (mg/L)	**K^+** (mg/L)	**NO_3^-** (mg/L)
Dairy digestate	30461.53	2232	249.7	256.210	7463.6
Municipal digestate	3411.53	2276	281	121.7	391
Poultry digestate	1123.07	2473	214	2100	64

All the three anaerobic digestate were diluted with distilled water in 20 and 10 dilution factors and added with 2 g/L of $NaHCO_3$ as the carbon source in case of municipal digestate (MD)and poultry litter digestate (PD). Following dilution, micronutrients and vitamin B_{12} were added in concentrations similar to that in BG11 medium with minerals. Salinity, initial pH, temperature

and light intensity were the same as mentioned in section 3.1 above. All the batch experiments were conducted using 500 ml Erlenmeyer shake flasks (working volume - 200 mL) with agitation at 120 rpm for 10 days and under 12 h light and dark cycle; the initial inoculum concentration was 1.23×10^9 cells/L. Samples were collected every 24 h, whereas biomass and lutein concentrations were analysed on 8th day of the batch experiments.

3.3 Screening and optimization of process parameters

Plackett – Burman design of experiments was used to study the effect of process variables affecting lutein production by *C. vulgaris* 92001 using poultry litter anaerobic digestate based on the previous results. Effect of different parameters such as digestate level (or) dilution factor, pH, $MgCl_2$, NaCl, $NaHCO_3$ and inoculum size were examined in this study. The design consist of 13 experiments and the parameter levels were coded as L, M, H, representing low, middle and high levels. The following first order model equation was used to describe the response: $L = A_0 + \sum A_1 X_i$, where, L is the response, A_0 is model intercept, $A_1$is linear intercept and and X_i is the level of independent variable (Pakshirajan & Mal, 2013; Zininga et al., 2019). All the batch experiments were carried out in shake flasks under constant temperature (27 ± 1°C), agitation speed (120 rpm) and light intensity (125 µmole/m^2 s). Biomass was harvested on 14th day of the experiments to estimate the lutein concentration.

Based on the results of the Plackett – Burman screening design of experiments, the parameters such dilution factor, $NaHCO_3$, NaCl and inoculum size were selected for optimization using response surface methodology (RSM). Central composite design was employed for designing the experimental runs (31), in which five different levels of the variables (+2, +1, 0, -1, -2) were used. The following second order polynomial equation was used for optimization (Equation 3.1).

$$L = \mu_0 + \sum \mu_i Z_i + \sum \mu_j Z_{i2} + \sum \mu_{ij} Z_i Z_j \qquad (3.1)$$

L is the lutein yield, μ_0 is the intercept, μ_i is the coefficient of linear effect due to the variables, μ_j is the squared effect, μ_{ij} is the coefficient of interaction between the parameters and Z_n is the variable (Ravanfar et al., 2016). Minitab version 16 (Penn state, USA) was used for the statistical design and analysis of the results.

3.4 Biokinetics of substrate consumption and biomass formation

Biokinetics of utilization of $NaHCO_3$ and CH_3COONa as the carbon source, *C. vulgaris* 92001 biomass growth and lutein production by the microalgae were studied for different initial concentration of $NaHCO_3$ and CH_3COONa in the range 1000-5000 mg/L and 500 – 2500 mg/L, respectively. In this batch study, synthetic poultry litter digestate was diluted with distilled water in 22.12 dilution factor and added with micronutrients of BG11, 10 µg/L vitamin B_{12} and 27 g/L NaCl. The synthetic poultry litter digestate composition was similar to the natural digestate and same was mentioned in section 3.2. All the experiments were conducted using 500 mL Erlenmeyer shake flasks (working volume - 200 mL) with agitation at 120 rpm for 18 days. The inoculum concentration was 4.82×10^9 cells/L. Samples were collected every 24 h for determining the substrate, biomass and lutein concentration.

The biokinetics of substrate consumption was evaluated by first order, logarithmic, logistic and modified Gompertz models (Table 3.2). The specific substrate utilization rate was described using Monod, Haldane, Edward and Moser models (Table 3.3). Biomass growth kinetics was studied using modified Gompertz and Logistic kinetic models (Table 3.4). Lutein production kinetics was evaluated using modified Gompertz and Luedeking–Piret (Table 3.5). Curve fitting tool in Matlab software was used for fitting the experimental data to the kinetic models.

Table 3.2 Models applied to evaluate substrate utilization kinetics at different initial concentrations

Sl. no.	Kinetic model	Equation	Estimable parameters	References
1.	First order	$S=S_0e^{(-Kt)}$	S_0, K	Sinharoy et al., 2019
2.	Logarithmic	$S=S_0+X_0\left[1-e^{(U_{max}t)}\right]$	S_0, X_0, U_{max}	Sinharoy et al., 2019
3.	Logistic	$S=\frac{S_0+X_0}{1+\frac{X_0}{S_0}\left[e^{\left(\left(\frac{U_{max}}{K_S}\right)(S_0+X_0)t\right)}\right]}$	S_0, X_0, U_{max}, K_S	Sinharoy et al., 2019
4.	Modified Gompertz	$S_0-S=S_{max}e^{\left[-e^{\left[\left(\frac{U_{max}\times 2.71828}{S_{max}}\right)(\lambda-t)+1\right]}\right]}$	S_{max} U_{max}, λ	Mu et al., 2007

In Table 3.2, the model parameters are as follows: S and S_0 - instantaneous and initial $CH_3COONa/NaHCO_3$ concentrations (mg/L), X_0 - initial biomass concentration (g/L), K - first order rate constant (day^{-1}), U_{max} - maximum $CH_3COONa/NaHCO_3$ utilization rate (mg/L/day), K_S - half saturation constant (mg/L), S_{max} - maximum $CH_3COONa/NaHCO_3$ utilization (mg/L) and λ – time of lag phase (day).

Table 3.3 Models applied to evaluate specific substrate consumption rate

Sl. no.	Kinetic model	Equation	Estimable parameters	References
1.	Monod	$U=\frac{q_{max}S}{K_S+S}$	q_{max}, K_S	Mitra et al., 2017
2.	Haldane	$U=\frac{q_{max}S}{K_S+S+\frac{S^2}{K_i}}$	q_{max}, K_S, K_i	Mitra et al., 2017
4.	Edward	$U=\frac{q_{max}S}{K_S+S+\left(\frac{S^2}{K_i}\right)\left(1+\frac{S}{K_s}\right)}$	q_{max}, K_S, K_i	Mitra et al., 2017
5.	Moser	$U=\frac{q_{max}S^n}{K_S+S^n}$	q_{max}, K_S, n	Mitra et al., 2017

In Table 3.3, the model parameter are as follows; U - specific $CH_3COONa/NaHCO_3$ utilization rate (day^{-1}), q_{max} - maximum specific $CH_3COONa/NaHCO_3$ utilization rate (day^{-1}), S - $CH_3COONa/NaHCO_3$ concentration (mg/L), S_m - $CH_3COONa/NaHCO_3$ concentration at which biomass growth ceases (g/L), K_s - half saturation constant (mg/L), K_i - inhibition coefficient (mg/L), K_1 - positive constant and n -empirical constant.

Table 3.4 Modified Gompertz and Logistic models to study *C. vulgaris* biomass growth kinetics

Sl. no.	Kinetic model	Equation	Estimable parameters	References
1.	Modified Gompertz	$X-X_0=X_{max}e^{\left[-e^{\left[\left(\frac{R_{max}\times 2.71828}{X_{max}}\right)(\lambda-t)+1\right]}\right]}$	X_{max} R_{max}, λ	Mu et al., 2007
2.	Logistic	$X-X_0=\frac{X_{max}}{\left[1+e^{\left[\left(\frac{4R_{max}}{X_{max}}\right)(\lambda-t)+2\right]}\right]}$	X_{max} R_{max}, λ	Sinharoy et al., 2019

In Table 3.4, the model parameters are as follows; *Xmax* - maximum biomass concentration (g/L), R_{max} - maximum biomass growth rate (g/L/day) and λ - time of lag phase (day).

Table 3.5 Modified Gompertz and Luedeking–Piret tested to study lutein production kinetics

Sl. no.	Kinetic model	Equation	Estimable parameters	References
1.	Modified Gompertz	$P=P_{max}e^{\left[-e^{\left[\left(\frac{R_{max}\times 2.71828}{P_{max}}\right)(\lambda-t)+1\right]}\right]}$	P_{max} R_{max}, λ	Sinharoy et al., 2019
2.	Luedeking –Piret	$P_L=\left[\frac{\alpha X_m}{1+\exp\left[2+\frac{4v_x}{X_m}(L_x-t)\right]}-\alpha X_0\right]+\left(\frac{\beta X_m^2}{4v_x}\right)\ln\left[\frac{X_0\left(e^{\frac{4v_x t}{X_m}}-1\right)+X_m}{X_m}\right]$	α, β	Ghodke et al., 2018

In Table 3.5 the model parameters are as follows: P is cumulative lutein concentration (mg/L), Pmax is maximum lutein production (mg/L), Rmax is maximum rate of lutein production (mg/L/day) and λ is the time lag phase in lutein production (day). P_L- lutein production, α and β – growth associated (g/g) and non-growth associated constants in Leudeking – Piret model (g/g/h), X_m- Maximum biomass concentration (g/L), X_0 –Initial biomass concentration (g/L), L_x - Log period of the culture (h), V_x – Maximum growth rate (g/L/h)

3.5 Metabolic flux balance analysis of *Chlorella vulgaris* 92001

Metabolic flux balance analysis (FBA) includes three major steps, reconstruction of metabolic pathways and network, creating stoichiometric model and solving them through linear programming by defining the objective function. Metabolic reconstruction involves interconnection of reaction, metabolites, and proteins to form a network. In this study reactions were extracted from Kyoto encyclopedia of genes and genomes (KEGG) data base and from

the literature (Muthuraj et al., 2013), which are detailed in the appendix (Table A1 and A2). The reconstructed metabolic networks were represented in stoichiometric matrix (S) form of size m × n (equation 3.4 - 3.6). Each row of this matrix represents one unique compound (m) and each column represents one reaction (n). The input of the each column represents the coefficients of the reaction. Negative coefficient was used to represent every metabolite consumed, whereas positive coefficient was used for every metabolite produced, a stoichiometric coefficient of zero was used for nonparticipating metabolites. The flux through all of the reactions in a network are represented by the vector v, which has a length of n (Mehaty et al., 2018). The concentrations of all metabolites are represented by the vector x, with length m. The system of mass balance equations was at steady state (dx/dt = 0). efm tool developed by ETH Zurich was used for developing the matrix and optimization of the metabolic model carried out under pseudo steady state approximation by linear programming in Matlab to solve the equation 3.4, 3.5 and 3.6 by setting desired upper and lower bounds.

$$\frac{dx}{dt} = S.v \qquad (3.4)$$

$$S.v = 0 \qquad (3.5)$$

$$\begin{bmatrix} 1 & 0 & 0 \\ 0 & -1 & 0 \\ 0 & 1 & 0 \end{bmatrix} \begin{bmatrix} 0 \\ 0 \\ 1 \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix} \qquad (3.6)$$

$\frac{dx}{dt}$ – Derivative of x with respect to t, S - Stoichiometric matrix, v – Vector, $\begin{bmatrix} 1 & 0 & 0 \\ 0 & -1 & 0 \\ 0 & 1 & 0 \end{bmatrix}$ – Every row represent on compound and every column represent one reaction, $\begin{bmatrix} 0 \\ 0 \\ 1 \end{bmatrix}$ - Flux of all the reactions, $\begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix}$ – Zero matrix

Static flux balance analysis was carried out in autotrophic and heterotrophic mode using sodium bicarbonate (3000 mg/L) and sodium acetate (1500 mg/L), respectively on 7th day. Substrate consumption, biomass production was used for the analysis.

3.6 Lutein production in split column airlift photobioreactor

A laboratory scale split column air lift photobioreactor (SCALPBR) was fabricated using transparent perspex material for this reactor study. The total volume of the reactor was 1.5 L and its working volume was 1.2 L. The reactor was split into two equal half using perspex sheet to create riser and down comer (Table 3.6). The air nozzle with tip made of teflon material was placed at the bottom of the riser. The air flow from a compressor (Tarson - ROCKYVAC™) was controlled using a rotameter with needle valve. The reactor was enclosed in a metal chamber to avoid the influence of external light. LED bulbs were used as the source of light and the intensity of bulbs were adjusted using LED light regulator. Schematic and an image of the reactor are shown in Figure 3.1

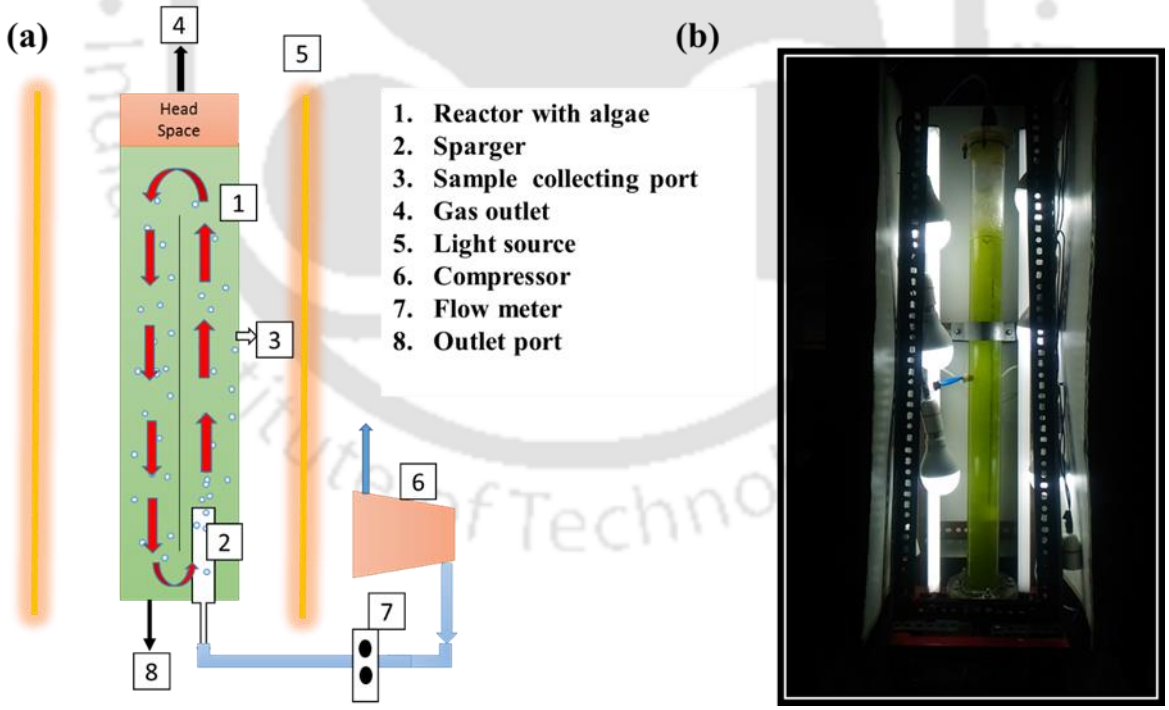


Fig. 3.1 (a) Schematic and (b) image of the split column photobioreactor used in this study

Table 3.6 Dimensions of the split column photobioreactor used in this study

Reactor part	Dimension (cm)
Total height	76.50
Diameter of the reactor	5
Diameter of the riser column	2.375
Diameter of the down comer column	2.375
Thickness of the split sheet	0.25
Height of the liquid	61.11
Head space	15.37
Top clearance	9.1
Bottom clearance	5

3.6.1 Mixing kinetics in split column photobioreactor

Mixing time in the split column photobioreactor was determined by using tracer method, which relies on the time required to attain complete homogeneity of the tracer after its addition. In this alkali method synthetic anaerobic digestate (Initial pH 7) was injected inside the reactor with 5 mL of 0.0241 M NaOH as the tracer at different superficial velocity (4.01×10^{-4} to 1.3×10^{-3} m/s). The tracer was injected from the middle of the reactor and a pH probe was kept at the bottom of the reactor for pH analysis. Endress+Hauser pH probe (model CPF810) was used and the pH values were recorded using a data acquisition system.

Dimensionless concentration of OH^- was estimated using the equation 3.7, liquid phase Bodenstein (B_0) was estimated using the equation 3.9, which is derived from the equation 3.8 of Ficks's second law for diffusion. Closeness of the estimated tracer concentration to the experimental data was evaluated using equation 3.9. Model equation developed by Rice et al. (1981) for Froude number and Bodenstein based on the superficial velocity of the gas was

evaluated (equation 3.10). Correlation between Bodenstein number, cycling time and mixing was evaluated using the equation 3.11. All these equations were solved using Wolfram Mathematica online version and the model fit was assessed using the MATWORKS online version.

Table 3.7 Equations involved in evaluating the mixing kinetics of split column photobioreactor

Equation	Equation No.
$C_{OH^-} = [OH^-] = \frac{[OH^-]_t - [OH^-]_{t_i}}{[OH^-]_{t_f} - [OH^-]_{t_i}}$	3.7
$\frac{\partial M_f}{\partial t} = E_r \frac{\partial M_f}{\partial r^2} + V_i \frac{\partial M_f}{\partial r^2}$	3.8
$\sum_{s=1}^{1}(C_t)_p = (\frac{B_0}{4\pi l})^{\frac{1}{2}} \sum_{p=1}^{1} \exp[-B_0(s-\theta)^2/4\theta]$	3.9
$s = \frac{L_i}{L_o}$, $\theta = \frac{t_i}{t_c}$	
$B_0 < 0.1$(mixing is perfect), $B_0 > 20$ (plug flow)	
$B_o = A\left(\frac{t_m}{t_c}\right)$	3.10
$t_c = \frac{Lo}{V_i}$	
$B_o = \frac{V_i L_o}{E_r}$	

$$Bo_{LG} = \varepsilon(Fr^{1/3})^{\gamma} \qquad 3.11$$

$$Fr = \frac{V_G^2}{gD}$$

$$Bo_{LG} = \frac{V_G D}{E_Z}$$

$[OH^-]$ – Concentration of OH^- ion, t – Instantaneous time, t_i – Initial time, t_f – Final time, B_0 - Bodenstein number, E_r- Axial dispersion coefficient, V_i – Liquid linear velocity, t_c – Circulation time, L_i - Distance covered by liquid at the specific time, L_0 – Length of the circulation loop, t_m – mixing time, A- constant, F_r - Froude number, Bo_{LG} - Bodenstein number based on gas velocity, g – acceleration due to gravity (m/s^2), D – diameter of the vessel, V_G – superficial velocity of the gas based on the diameter of the vessel (m/s), ε – parameter in the equation

3.6.2 Lutein production in splitcolumn photobioreactor

Batch experiment was carried out using the split column airlift photobioreactor with diluted poultry digestate (22.22 dilution factor) along with micronutrients of BG_{11}, 4.5 g/L $NaHCO_3$, 10 µg/L, vitamin B_{12} and 27 g/L NaCl. Initial pH of the medium was 7.5 ± 0.2 and the temperature was maintained at 27 ± 1°C. The effect of different light intensity (150 and 250 150 μ/m^2s) and airflow rate (1, 2 and 3 VVM) was examined to optimize the parameters. Samples were collected every 24 h for measuring biomass, lutein, $NaHCO_3$, K^+, NH_4^- and PO_4. Specific growth rate (µ) of the biomass was assessed using the equation 3.12 (Vaquero et al., 2012).

$$\mu = \ln(\mu_T - \mu_0)/(T_T - T_0) \qquad (3.12)$$

In continuous cultivation mode the split column airlift photo bioreactor was initially run in batch mode till the end of logarithmic growth phase. Subsequently, the reactor was operated in continuous mode at different HRTs (150, 130, 100 and 90 h). The hydraulic residence time

(HRT) was evaluated as per the following equation 3.13 and 3.14 (Tagliaferro et al., 2019) and peristaltic pumps were used to maintain the liquid flow in the continuous mode.

$$HRT = 1/D \qquad (3.13)$$

$$D = F/V \qquad (3.14)$$

where, D is Dilution ratio, F is Flow rate, V is volume of the reactor

3.7 Lutein production in continuous stirred tank photobioreactor

Lutein production in continuous stirred tank photobioreactor (CSTPBR) was carried out using 3L fermenter (Bio Console ADI 1025, Applikon Biotechnology, Holland). Figure 3.2 shows the schematic and image of the CSTPBR used in this study. Anaerobic digestate as mentioned in the previous section was used as the substrate for lutein production. The effect of different light intensity (150, 200, and 250 μ/m^2s), airflow rate (0.5, 1, 1.5, 2, 2.5 VVM) and agitation (100, 150, 200, 250 and 300 RPM) in the batch operated CSTPBR was initially studied for optimizing the process parameters. In continuous mode different HRT (220, 200, 180, 160 h) was assessed for maximum lutein and biomass production using the CSTPBR.

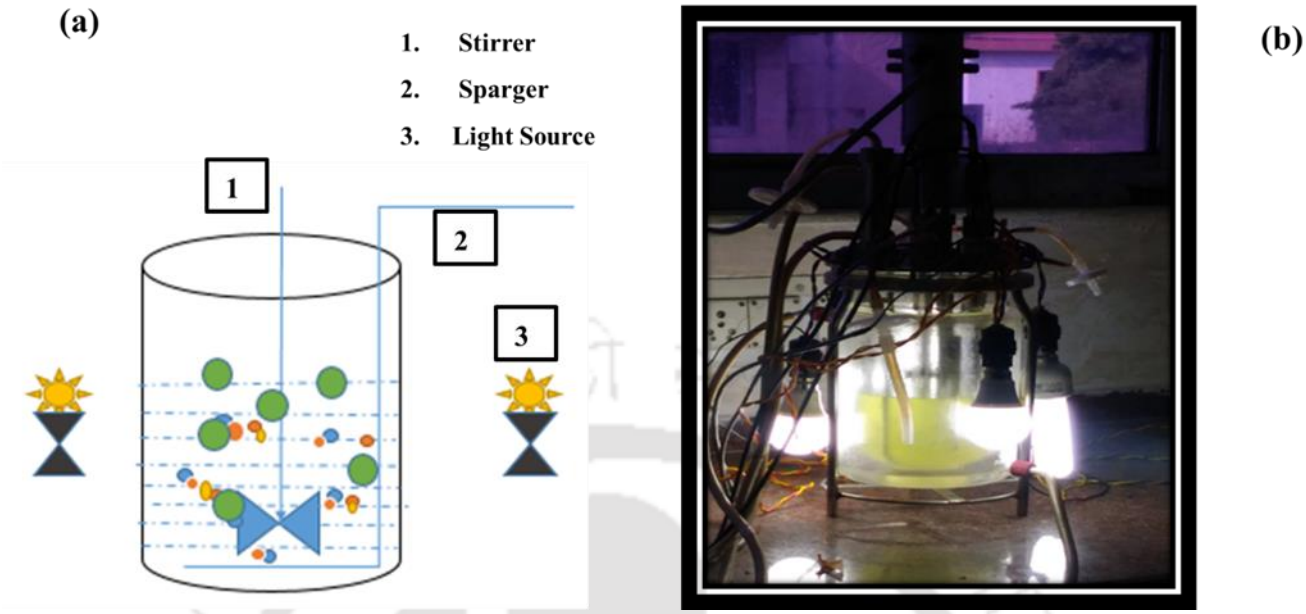


Fig. 3.2 (a) Schematic and (b) Image of the continuously stirred tank photobioreactor used in this study

3.8 Analytical methods used for estimation

Algal biomass in samples were estimated using a hemocytometer and a standard plot was prepared between algal cell number and dry biomass weight. In order to determine lutein, 10 mg of algal biomass was mixed with 3 ml of cellulase enzyme and 7 ml of acetate buffer (pH – 4.5). The enzyme concentration was 6 mg/mL and its activity 0.3 U/mg. The incubation time was 60 min at 40º C in a rotating orbital shaker for enzymatic disruption. Liquid – liquid extraction was then carried out using 10 mL of methanol and dichloromethane (3:1) followed by drying and addition of 1 mL of 4 % KOH to the dried samples. The mixture was left at 40 º C for 45 min for the removal of esters by hydrolysis. Final extraction of lutein was carried out using dichloromethane and its amount was determined by ultra-performance liquid chromatography (Shimadzu, Japan) using C18 column, operated in isocratic mode by passing solvent mixture consist of methanol- 67.5%; dichloromethane-22.5%; acetonitrile-9.5%; and water-0.5% at a flowrate of 1 mL/min, and UV detector set at 445 nm.

Acetate was determined as COD as 1 g of CH_3COONa is equivalent to 0.76 ± 0.03 g of COD. For COD determination in the sample, 2.5 mL of test solution was mixed with 1.5 mL of digestion solution (1.04 % of $K_2Cr_2O_7$ +3.33 % of $HgSO_4$ + 16.8 % (v/v) of conc. H_2SO_4) and 3.5 mL of conc. H_2SO_4 containing 1.012 % of Ag_2SO_4 as a catalyst. Mixture was cooled and incubated in an oven (HACH, DRB 200, USA) maintained at 150º C for 2 h. The mixture was cooled and its optical density was measured at 600 nm. Different concentrations of sodium acetate was used to prepare the COD.

Sodium bicarbonate was determined based on titration method for alkalinity. Test sample (100 mL) was initially added with few drops of phenolphthalein indicator (1% of phenolphthalein in 95 % ethanol) which turned the sample into pink colour due to hydroxyl ions. The sample was then titrated with 0.005 M sulphuric acid till the pink colour disappeared followed by the addition of a mixed indicator (0.1 % bromocresol and 0.02 % methyl red in 95% ethanol) which turned it to blue colour due to the presence of carbonate and bicarbonate. The sample was finally titrated with 0.01 M H_2SO_4 and the volume of acid utilized was noted to estimate the amount of bicarbonate present in the sample as per the following equations.

$$\text{Alkalinity due to } OH^- \text{ ion} = \frac{(\text{Volume of } H_2SO_4)_{PI} \times 0.02\ N \times 50 \times 100}{\text{Total volume of the sample}} \qquad 3.2$$

$$\text{Total alkalinity} = \frac{(\text{Volume of } H_2SO_4)_{MI} \times 0.02\ N \times 50 \times 100}{\text{Total volume of the sample}} \qquad 3.3$$

Alkalinity due to bicarbonate and carbonate = Total alkalinity – Alkalinity due to OH^- ion

In the above equations, $(\text{Volume of } H_2SO_4)_{PI}$ denotes the volume of sulphuric acid consumed after adding phenolphthalein indicator and $(\text{Volume of } H_2SO_4)_{MI}$ is the Volume of sulphuric acid consumed after adding the mixed indicator.

Ammonium estimation was carried out using potassium antimony tartrate solution (Baethgen and Alley, 1989), 1 mL of test solution was added with 5.5 mL of buffer solution (2.3 % Na_2HPO_4 + 8.33 % $C_4H_4KNaO_6.4H_2O$ + 18 % of 50 % NaOH), 4 mL of salicylate/nitroprusside solution (15 % $C_7H_5NaO_3$+ 0.03 %$Na_2[Fe(CN)_5NO].2H_2O$) and 2 mL of hypochlorite solution (6% of 5.25 % sodium hypochloride) in sequential manner with intermediate shaking sequentially. The final solution was incubated at 37ºC for 15 min and then optical density was measured at 650 nm.

Phosphate was estimated using ascorbic acid method (APHA, 1995), in which 3 mL of test solution was mixed with 0.7 mL of reagent (62.5 mL of Con. HNO_3 + 100 mL of 5 % $(NH_4)_6$. Mo_7O_{24}. $4H_2O$ + 100 mL of 0.25 % $(NH_4)_2$ VO_3). The reaction mixture was incubated at 45 °C for 20 min and its optical density was measured at 820 nm. Potassium dihydrogen phosphate was used as the standard phosphate for this analysis.

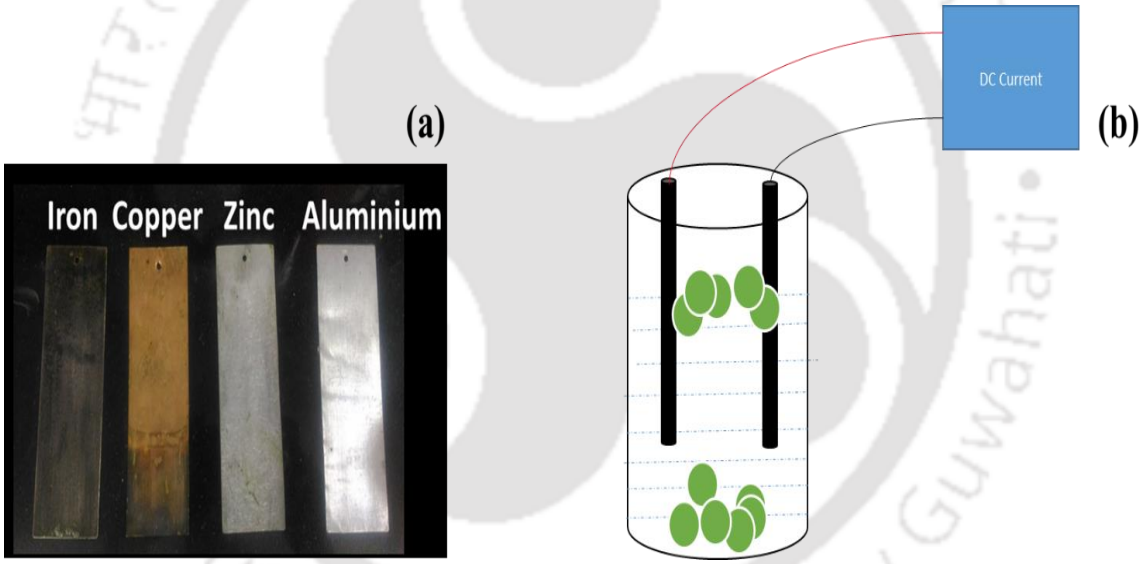
Potassium was estimated by flame spectrometry (Systronics, Model: 128, India), and potassium chloride was used as the standard.

3.9 Downstream processing technology

3.9.1 Electrocoagulation flocculation (ECF) for recovery of *C. vulgaris* biomass

ECF was carried out using a measuring cylinder with a working volume of 400 ml and a Digital DC supplier (Biku, India) as the power source (Figure 3.3 c). Copper, iron, aluminium and zinc electrodes were used for ECF (Figure 3.3 a). Dimensions of the different electrodes were the same with 15 cm height, 0.1 cm breath and 5 cm length. The electrodes were rinsed with 0.01N HCl to eliminate deposits and mounted along the two opposite sides of the cylinder by maintaining a distance of 4 cm. The effect of different current densities (1.5, 2.5, 3.5 mA/cm^2) was evaluated for different ECF duration (10, 20, 30 min). The initial cell and lutein

concentrations used in this study was 0.91 g/L and 4.20 mg/L. The cells were counted using a hemocytometer at the end of each ECF process, followed by recovery of lutein from the separated *C. vulgaris* biomass (Nidal et al., 2017). Algal cell morphology prior to and after ECF was analysed using a field emission scanning electron microscope (FESEM). For FESEM analysis (Zeiss, Sigma, Germany) a small portion of sample containing the microalgae was loaded onto a small aluminium stand supported by double sided carbon tape and then dried overnight. Prior to the FESEM analysis, sample was gold coated using a doping instrument (JEOL JFC-1300).



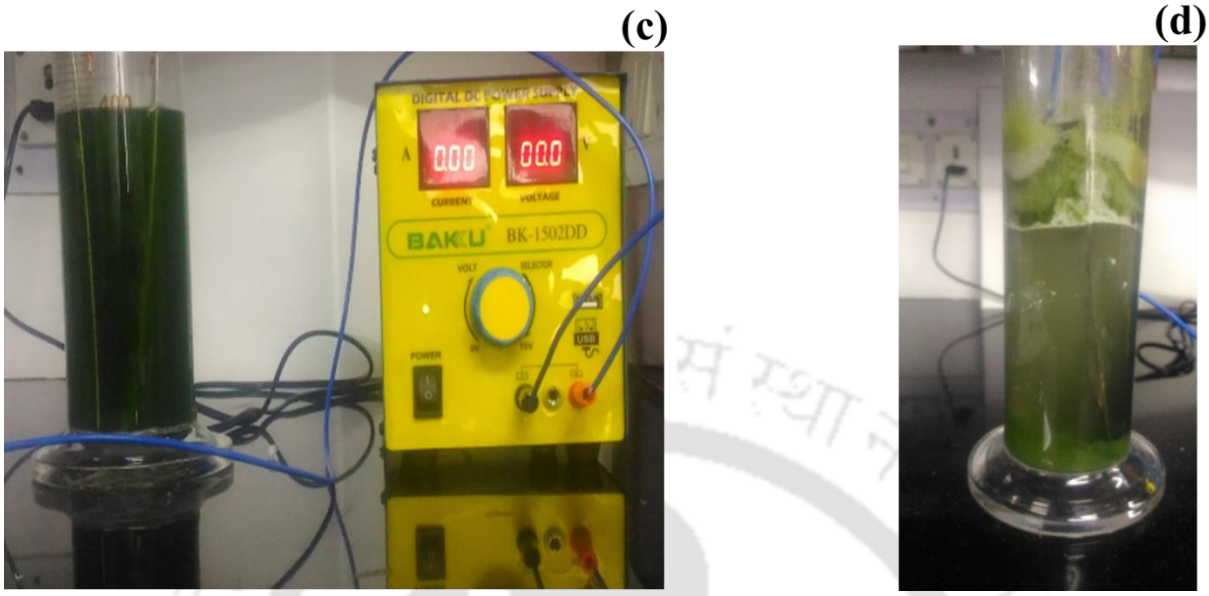


Fig. 3.3 (a) Electrodes used in ECF (b&c) Schematic and image of ECF setup (d) Image showing coagulated and flocculated *C. vulgaris* due to ECF

3.9.1.1 Recovery percentage and power consumption calculation in ECF

To evaluate the cell recovery percentage, 5 ml of liquid samples were collected before and after ECF and analysed for cell count (Equation 3.15). Cells were allowed to settle prior to the cell count after ECF process. Cell voltage during the ECF process was observed and the power consumed was calculated using the equation 3.16. In order to estimate the lutein recovery 10 mg of microalgae was disrupted using enzymatic method as described previously in section 3.8

$$\varepsilon = \frac{\text{Cell numbers}_{\text{initial}} - \text{Cell numbers}_{\text{final}}}{\text{Cell numbers}_{\text{initial}}} \qquad 3.15$$

$$P = \frac{ABt}{1000\ L\ \varepsilon C_0} \qquad 3.16$$

Where, P is power consumption (KWh/kg), A is voltage (V), B is current (A), t is time of ECF treatment (h), L is volume of microalgae biomass (m^3), ε is the microalgae recovery efficiency and C_0 is the initial microalgal concentration (kg/m^3).

3.9.2 Lutein recovery by ultrasonication of *C. vulgaris* biomass

Cell disruption was carried out using a probe type sonicator operated with a 500 Watt ultrasonic processor (Sonics Vibra cells, USA). The diameter of the probe was 1.3 cm and the maximum power output of the sonicator is 500 watts and 20 kHz. The *C. vulgaris 92001* cells were sonicated in a self-standing 50 mL falcon tube, modified to handle 15 mL, biomass denaturation was minimized by keeping the samples in ice bath. Cells were sonicated at 30.4 μm amplitude with 30 secs on and 5 secs off cycle.

Ultrasonication was carried out for different time (2.5, 4.5, 6.5 and 8.5 min) with different cell concentrations of 0.125, 0.25, 0.50, 0.75 g/15 mL. Liquid – liquid extraction using dichloromethane and saponification using KOH was carried out for lutein determination by HPLC as mentioned in the previous section (3.8). Lutein recovered by enzymatic cell disruption as mentioned previously (3.8) was considered as 100% recovery (4.56 mg of lutein/g of *C. vulgaris*). Energy input for the process was recorded at the end of the sonication experiment process. Cell disruption was confirmed by direct observation under light microscope.

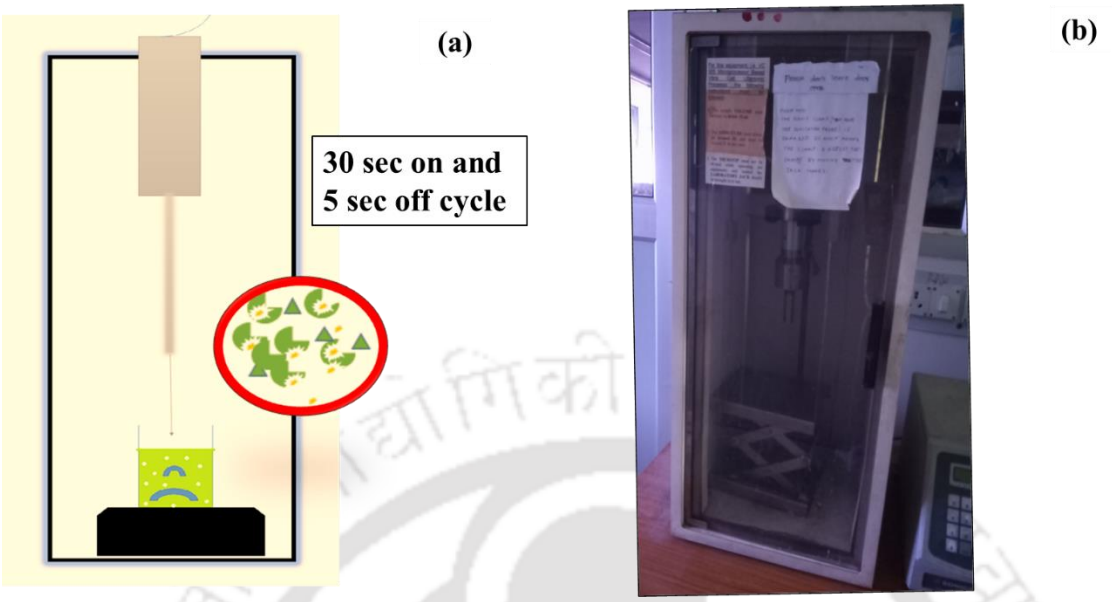


Fig. 3.4 (a) Schematic and (b) Image showing probe type sonicator for cell disruption and lutein recovery

3.9.3 Purification of lutein by nanofiltration

Nanofiltration was carried out using a dead end filtration setup of total volume 400 mL. Hydrophilized polyamide nanomembranes with two different cutoffs were purchased from Permionics membrane Pvt. Ltd, Gujarat, India. The cell effective membrane area was 113.09 cm^2. Filtration experiments were carried out at room temperature and applied pressure using high purity nitrogen (99.996 %), supplied from a cylinder. The membrane conditioning was done by filtering 100 ml ethanol (89 % v/v) and then ethanol flux at 2, 3, 4 and 5 kg/cm^2 applied pressure was calculated with respect to time using a weighing balance (Vinoth et al., 2015; Yaxuan et al., 2016). The membrane permeate was collected in a sealed cylinder and its volume measured. At the end of the filtration process a small amount of sample was taken from the permeate and retentate to analyse lutein content and its purity in the permeate. HPLC was employed to determine the lutein concentration and purity as per the section 3.8.

3.9.3.1 Effect of lutein concentration and membrane cut off

Extraction of lutein prior to the nanofiltration step was carried as outlined in the Figure 3.5. *C. vulgaris* biomass was first centrifuged and then freeze dried, 82.5 mL of ethanol was added to 2.75 g of the lyophilized biomass containing 4.61 mg/g of lutein. The biomass was disrupted by ultrasonication for 4.5 min. The disrupted biomass was vacuum dried by rotavaccum distillation and then added with 8 mL of Tris HCl buffer (pH 7.5) and 3 mL of 7 unit/mL cholesterol esterase enzyme for hydrolysing the esters. The mixture was incubated at 36 ºC with agitation at 25 rpm for 40 min and then 1 mL of stock was subjected to liquid-liquid extraction using dichloromethane for determining the lutein quantity. The stock was diluted using Tris HCl buffer to adjust the volume to 11 mL, followed by addition of 89 mL of ethanol and mixing at 25ºC for 30 min. The extract was filtered using Whatman filter paper and nitrocellulose paper with 0.20 µm pore size filter by vacuum filtration prior to nanofiltration. FETEM and EDX (JOEL,2100F, Japan) analyses of the samples before and after nanofiltration were carried out and for which the samples were loaded onto carbon coated copper grid and dried under nitrogen.

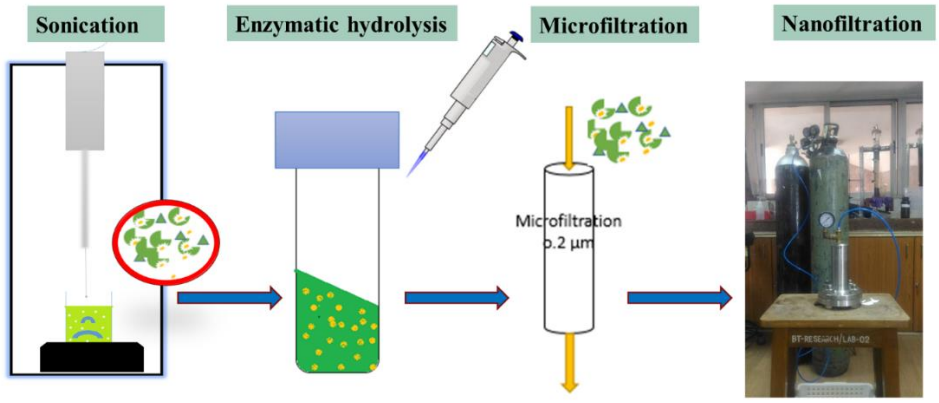


Fig. 3.5 Different steps involved in lutein extraction prior to its purification by nanofiltration

Nanofiltration was evaluated using two different membrane cutoffs (800 -1000 and 600 – 800 Dalton) and different concentrations of lutein (20 – 100 mg/L) at an applied pressure of 4 kg/cm^2 for 30 min. Total lutein content in permeate and its purity were determined using the equation 3.17 and 3.18.

Effect of different applied pressures (2, 3, 4 and 5 kg/cm^2) on the lutein purity and the amount of lutein in permeate was carried out using nanofilter with the cutoff 800 -1000 Dalton. Based on the results of the previous experiments, lutein concentration of 40 mg/L was used for the nanofiltration study.

$$L_p(\%) = \frac{L_A}{T_A} \times 100 \qquad 3.17$$

L_p – Lutein purity (%), L_A - Chromatographic area of lutein, T_A - Total area of all the chromatograms

$$P_L(\%) = \frac{L_{TP}}{L_{TF}} \times 100 \qquad 3.18$$

P_L - Amount of lutein in permeate, L_{TP} - Total amount of lutein in permeate, L_{TS} - Total amount of lutein in the feed

The flux of ethanol and lutein in ethanol was calculated using the following equation

$$J_E = \frac{V}{At} \qquad 3.19$$

J_E – Ethanol flux (m/s), V – permeate ethanol volume (m^3), A – Effective membrane area (m^2), t – time measured (s)

3.10 Cytotoxicity and insecticidal activity of lutein

Lutein extracted from *C. vulgaris* 92001 was dried through vacuum distillation and then subjected to cytotoxicity and insecticidal assay. The Sf9 cell lines and armyworm eggs for these assays were procured from National Center for Cell Science (NCCS), Pune, India. Cell line was derived from immature ovaries of fall armyworm moth (*Spodoptera frugiperda*) and the same was cultured in modified Grace's insect medium (TNM-FH) supplemented with 10% fetal bovine serum (Veeran et al., 2017). Lutein was dissolved in DMSO and then diluted in culture medium such that the final volume of DMSO was 1% (v/v) of the medium. Different lutein concentration ranging from 0.16 to 6 µg/mL were used for this cytotoxicity study. The medium with the test compound was inoculated with 0.1 million cells/mL in the 96 well titter plate with a working volume of 0.2 mL. Cultures in the titter plates were incubated at 27 ºC for 48 h, and after which MTT reagent was added and the mixture was left undisturbed for 4-6 h to obtain insoluble formazan crystals. DMSO was used to solubilize the crystals prior to colorimetric measurement at 590 nm wavelength using a micro plate reader (InfiniteM200pro, Switzerland). SF9 cell lines treated with lutein and untreated cells were centrifuged at 200×g and the pellet containing cells were treated with the fluorescent dyes fluorescein diacetate (FDA) and propidium iodide (P.I) (Jones & Senft, 1985). Cell viability and morphology were observed under Eclipse Ti-S phase contrast fluorescence inverted microscope (Nikon, USA)

For insecticidal activity of lutein extracted from *C. vulgaris* 92001, armyworm moth (*Spodoptera frugiperda*) eggs were maintained at 28 ± 3 ºC temperature and 60 ± 10% relative humidity for 4 days to obtaining the larvae. The hatched larvae were fed with castor plant leaves (*Ricinus communis*) for ten days and then these larvae were used for the insecticidal assay. Lutein was dissolved in 1mL of absolute alcohol in different concentrations of 62.5, 125,

187.5 and 250 µg/mL and then smeared uniformly on castor leaves, followed by air drying in a fume hood. Seven insects were placed in a standard petri dish containing lutein treated and untreated leaves, absolute alcohol without lutein was smeared on the untreated leaves (Hematpoor et al., 2017).

3.11 Lutein enrichment in chicken egg

Poultry experiments were conducted in poultry and agro farm, located in Therampalayam, Coimbatore, India. Twenty-four week old White Hyline chicken breed was employed for this poultry study (Figure 3.6). Twenty-eight chickens were divided into 7 groups. The groups were segregated and maintained at temperature 30±2 ºC. Lutein extracted from *C. vulgaris* 92001 lutein ester and microalgae was separately added to the chicken's basal diet (Table 3.8) so as to maintain the final lutein concentration of 0, 75, 150, 300, 375 and 400 (mg/kg) prior to feeding (Leson et. al., 2004). Each and every chicken was fed separately with 50 g of the respective diet every morning and evening throughout the experimental period. This study was conducted in three phases: in the 1st phase only lutein was added to the diet, in the 2nd phase lutein ester was added in the diet and in the 3rd phase whole microalgae was added in the chicken's basal diet. Each phase lasted for 30 days and a lay off period of 20 days was provided to reduce the lutein content in egg by feeding basal diet without lutein. Eggs were collected every day and the lutein content was measured every second day from three eggs. Roche colour score for egg yolk, total protein, carbohydrate and lipid was estimated on 30th day for all the eggs. The effect of lutein concentration on 30th day and the effect of different forms of lutein in the diet, i.e. lutein, lutein ester and microalgae at 300 mg/kg and 450 mg/kg on the egg lutein content was analysed statistically by one-way ANOVA and Tukey comparison of mean values of the results.

Table 3.8 Ingredients used and their composition in the poultry study

Ingredients	**Composition (w/w %)**
Coconut meal	2.2
Corn	60
Dicalcium phosphate	1.2
Fish meal	1.5
Ground nut meal	3.2
Limestone	8
Rice	10
Rice husk	2.6
Salt	0.3
Vitamin & trace elements	1
Wheat	10
Lutein (mg/kg)	L/LE/MA - 0,75,150,225,300,375,450

L- Lutein, LE – Lutein ester, MA - Microalgae

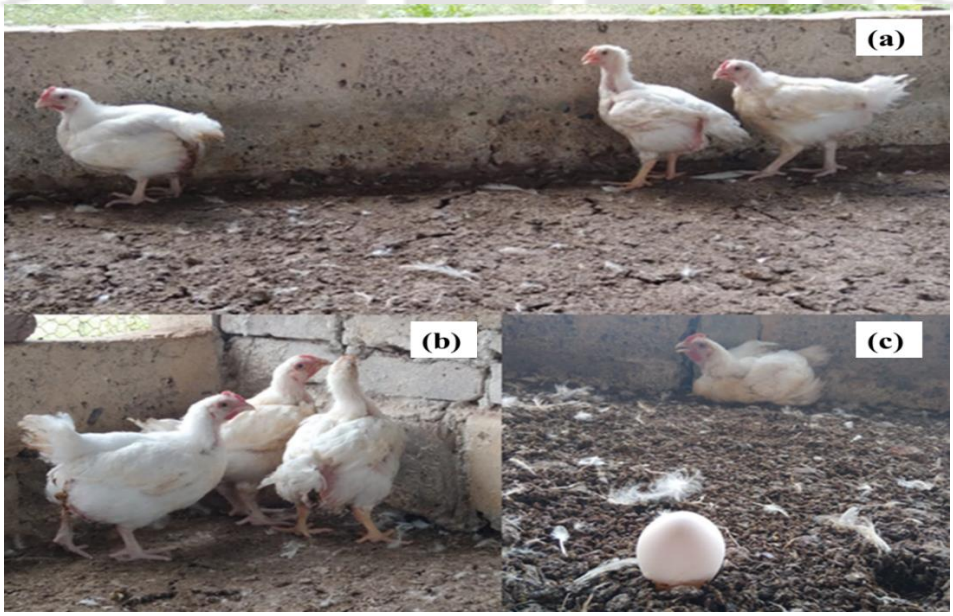


Fig. 3.6 (a&b) Images of white hyline chicken breed used in the poultry experiments (c) Chicken egg

3.11.1 Analytical methods

Weight of the eggs was first analysed with shell or without shell. The egg white and egg yolk were then blended uniformly and freeze dried prior to the determination of total carbohydrate, protein and total fatty acid contents. Total lipid was carried out based on Folch et al. (1957), Bligh and Dyer (1959) method. Uniformly blended egg (3g) was mixed with methanol (15 mL) for 300 s followed by the addition of chloroform (7.5 mL) and mixing. Aqueous saline solution containing 2900 mg/L of NaCl and 200 mg/L of $CaCl_2$ was added and mixed for 600 s. Lipid extraction was carried out using 15 mL of chloroform in two batches followed by centrifugation to remove the supernatant. Saline solution of 7.5 mL was added to the supernatant and then mixed for 15 min prior to centrifugation. Lower part obtained after centrifugation of the mixture was adjusted to the final volume of 25 mL using methanol followed by vacuum drying to obtain the total lipid.

Biuret test was carried out to determine the total protein content of the eggs. Lyophilized egg powder was mixed in water and suitably diluted. Egg sample dissolved in water (1 mL) was mixed with 6 mL of Biuret reagent [$CuSO_4 \cdot 5\ H_2O$ (13.0 mmol/L), potassium sodium tartrate $KNaC_4H_4O_6 \cdot 4\ H_2O$ (32·0 mmol/L), NaOH (0.6 mol/L)] and then incubated at room temperature for 30 min. Optical density of the final solution was measured at 540 nm, bovine serum albumin was used for preparing the standard protein for this analysis.

Carbohydrate was measured by Anthrone and for which 1 mL of the sample was mixed with 3 mL of Anthrone reagent prepared by adding 100 mg of Anthrone's reagent in 100 mL of conc.H_2SO_4. The mixture was initially left at 4º C for 10 min followed by incubation at 100 ºC for 20 min. Optical density of the resultant mixture was analysed at 620 nm, and glucose was used as the standard for this carbohydrate analysis

Roche colour score was measured using Roche scale – 1 to 15 (DSM yolk Fan^{TM}), where a score of 1 represents pale yellow colour, 15 represents dark orange colour. The egg yolk colour score was obtained by comparing the colour of the Roche colour fan and the yolk.

Lutein from the egg yolk was estimated using the following method: 0.5 g of egg yolk was first homogenously mixed and then added with 5 mL of dichloromethane and methanol (3:1). The mixture was vortexed for 10 min and then allowed to settle, after which the solvent containing dichloromethane and methanol was evaporated under nitrogen atmosphere in fume hood. HPLC analysis was carried out as mentioned previously in the section 3.8 for lutein analysis.

Statistical analysis such as one-way ANOVA and Tukey test were carried out using Minitab software (version 16),(Penn state, USA)

Chapter - 4

RESULTS AND DISCUSSION

4 Results and Discussion

Results of screening different microalgae for lutein production using anaerobic digestate as the substrate along with parameter selection and optimization of the process kinetics are discussed in this chapter. Biokinetics involved in biomass formation, lutein production and substrate uptake were analysed and performance of lab scale photobioreactors were evaluated for lutein production from microalgae. Results of potential application of lutein as a biopesticide and coloring agent in the poultry are also presented and discussed in this chapter.

4.1 Screening of digestate and microalgae

Selection of suitable microorganism and media are important for enhanced product yield in bioprocesses. Lutein production varies in different types of microalgal strains due to differences in genetic factor and growth requirements. Figure 4.1 shows the biomass and lutein production by *Chlorella vulgaris* 92001, *Chlorella vulgaris* 52091, *Chlorella vulgaris* 10241 and *Tetraselmis indica* using three different synthetic anaerobic digestate. *Chlorella vulgaris* 92001 produced maximum biomass of 0.46 g/L on 8th day using diluted dairy litter digestate as compared to other digestate. The maximum lutein production by *Chlorella vulgaris* 92001 was 1.85 mg/L in poultry digestate followed by that in municipal digestate with the value of 1.54 mg/L. Biomass and lutein production by all the microalgal strains was higher using 20 times diluted digestate than with 10 times diluted digestate due to low ammonium concentration (Vasseur et al., 2012; Wang et al., 2010). Lutein production was high using poultry and municipal digestate supplemented with sodium bicarbonate as the substrate whereas, it was low with the diary digestate.

Microalgae grown in dairy digestate produced a high amount of biomass but with low lutein content as compared to other digestate. This is due to heterotrophic mode of growth. Plants and microalgae produce lutein to protect the photosynthetic system from scavenging radicals as well as to produce energy using photochemical reaction (Demmig-Adams & Adams, 1992; Miller et al., 2010). Based on the result from this study, *Chlorella vulgaris* 92001 and poultry digestate were selected for further investigation.

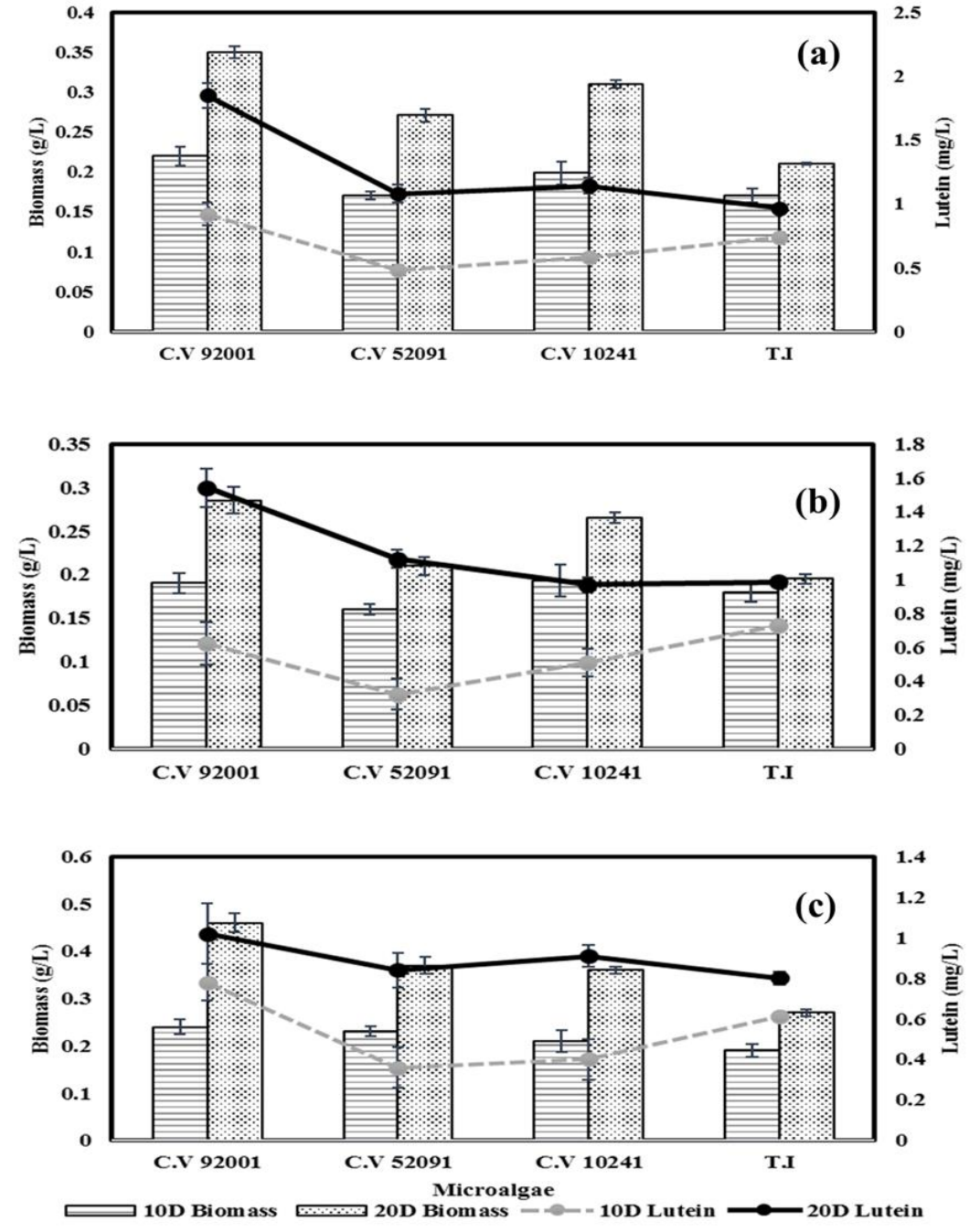


Fig. 4.1 Biomass and lutein concentration on 8th day using different digestate of culture (a) poultry digestate (b) municipal digestate (c) dairy digestate

4.1.1 Effect of different parameters on lutein production

The effect of different factors such as digesatate level (or) dilution factor, pH, $MgCl_2$ (g/L), NaCl (g/L), $NaHCO_3$ (g/L) and inoculum size (cells/L) on lutein production by *C. vulgaris* was examined by performing Plackett-Burman design of experiments and results are presented in table 4.1. Run no. 10 showed the highest amount of lutein (2.86 mg/L) among the thirteen experimental runs. The validity of the results was analysed using analysis of variance (Table. 4.2), where the values of F = 15.97 and P = 0.004 support the significance of the statistical model. The accuracy of the model fit was 95.27 % based on the R^2 value. Student 't' test of the results revealed positive effect of NaCl (g/L), $NaHCO_3$ (g/L) and inoculum size (Cells/L), whereas the effect due to dilution factor was negative (Table 4.3). The effects are found to be significant based on low P value (<0.05) of the different variables. The high lutein concentration in experimental run no. 10 is attributed to lower digestate concentration, high salinity, $NaHCO_3$ and inoculum size. Pareto chart depicted in the Figure 4.2 clearly indicates the effect due to variable; digestate ratio is highly significant as compared to the other.

Dilution concentration of digestate and high inoculum size are reported to be favourable for lutein production by microalgae due to reduced toxicity of the ammonium (Wang et al., 2010). Consumption of CO_2 and NH_3 by microalgae from $NaHCO_3$ and NH_4Cl, respectively, tends to release OH^- ions and H^+ ions in the production medium, which neutralizes the pH effect on lutein production as represented by the following reaction (Scherholz & Curtis, 2013).

$$NaHCO_3 + NH_4Cl \rightarrow NaCl + CO_2 + OH^- + NH_3 + H^+$$

As *Chlorella vulgaris* 92001 was originally isolated from salt pan in which pH tends to vary, the organism is capable of adaptation to a certain range of pH and high salt concentration without affecting biomass production or metabolic activity.

Table 4.1 Plackett Burman design of experiments showing different variables and other levels in each experimental runs along with lutein concentrations

S. No.	Digestate level (Dilution factor)	pH	$MgCl_2$ (g/L)	NaCl (g/L)	$NaHCO_3$ (g/L)	Inoculum (Cells/L)	Lutein (mg/L)
1	Low (20)	7	1	25	6	5.5×10^8	2.53
2	High (10)	7	1	25	2	5.5×10^9	1.63
3	Middle (15)	8	0.55	12.75	4	3.25×10^9	0.56
4	Low (20)	9	0.1	0.5	2	5.5×10^9	0.70
5	Low (20)	9	1	0.5	6	5.5×10^8	0.40
6	High (10)	9	0.1	25	6	5.5×10^8	1.71
7	High (10)	7	0.1	0.5	6	5.5×10^9	0.86
8	High (10)	9	1	0.5	6	5.5×10^9	0.83
9	Low (20)	7	0.1	0.5	2	5.5×10^8	0.44
10	Low (20)	7	0.1	25	6	5.5×10^9	2.86
11	High (20)	9	0.1	25	2	5.5×10^8	0.62
12	High (20)	7	1	0.5	2	5.5×10^8	0.22
13	Low (10)	9	1	25	2	5.5×10^9	2.28

Table 4.2 Analysis of variance of lutein production by *C. vulgaris* in the Plackett Burman screening study

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	6	8.636	8.662	1.443	15.97	0.004
Digestate level (Dilution factor)	1	0.929	0.929	0.929	10.28	0.024
pH	1	0.333	0.333	0.333	3.69	0.113
$MgCl_2$	1	0.0408	0.040	0.040	0.45	0.531
NaCl	1	5.576	5.576	5.576	61.66	0.001
$NaHCO_3$	1	0.907	0.907	0.907	10.04	0.025
Inoculum	1	0.842	0.874	0.874	9.67	0.027
Curvature	1	0.479	0.479	0.479	5.31	0.069
Residual Error	5	0.452	0.452	0.090		
Total	12	9.56				

Table 4.3 Estimated effects and coefficients of the statistical model for the lutein production by *C. vulgaris*

Term	T	P
Constant	-1.97	0.106
Digestate level	-3.21	0.024
pH	-1.92	0.113
$MgCl_2$ (g/L)	0.67	0.531
NaCl (g/L)	7.85	0.001
$NaHCO_3$ (g/L)	3.17	0.025
Inoculum	3.11	0.027
R-Sq = 95.27%	R-Sq(adj) = 88.65%	

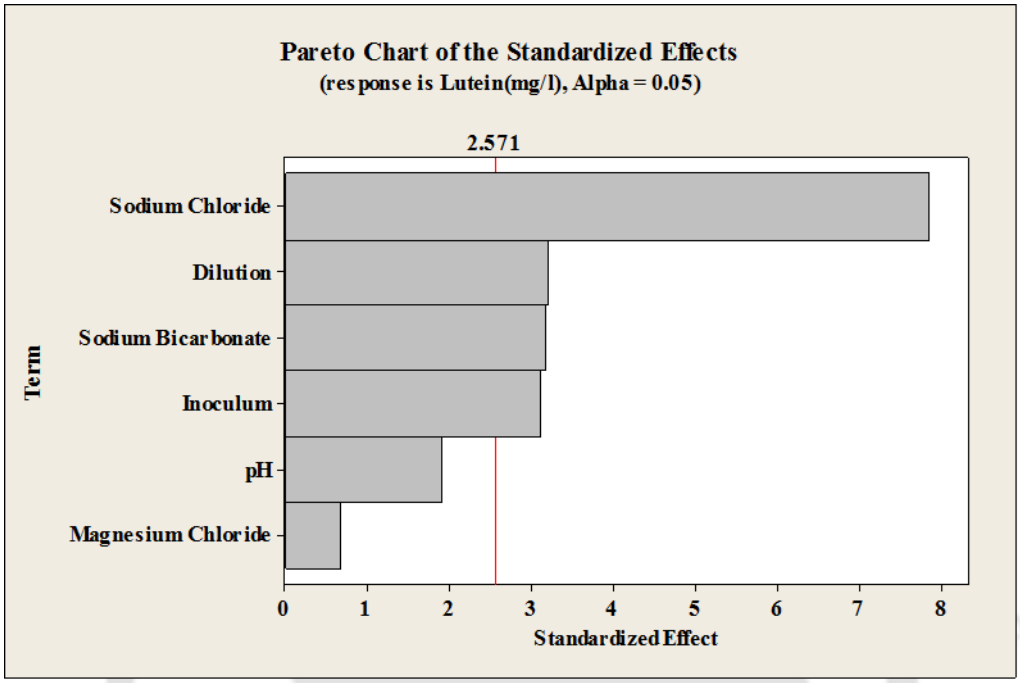


Fig. 4.2 Standardized effect of lutein production for Plackett Burman experimental results

4.1.2 Optimization of parameters affecting lutein production

The significant parameters, NaCl, dilution factor, $NaHCO_3$ and inoculum size on lutein production by *C. vulgaris* were selected based on the Plancket Burman experiment for optimization by central composite design and RSM. As per the design, 31 runs were performed and the maximum lutein production was 3.24 mg/L in the experimental run number 7 (Table 4.4) was achieved. The following polynomial equation 4.1 was developed, and the significance of the regression coefficients were evaluated based on the analysis of variance of lutein production as presented in the table 4.5.

$$\text{Lutein (mg/L)} = 3.10857 + 0.23\ \text{Dilution factor} + 0.46\ \text{NaCl} + 0.13\ \text{Inoculum} + 0.36\ NaHCO_3 - 0.70\ \text{Dilution factor}^2 - 0.36\ \text{NaCl}^2 - 0.26\ \text{Inoculum}^2 - 0.42\ NaHCO_3^2 + 0.23\ (\text{Dilution factor} \times \text{Nacl}) + 0.17\ (\text{Dilution ratio} \times NaHCO_3) + 0.20\ (\text{NaCl} \times NaHCO_3) + 0.144\ (\text{Inoculum} \times NaHCO_3) \quad \textbf{(4.1)}$$

The coefficient of linear, square and interaction effect between dilution factor and NaCl, dilution factor and $NaHCO_3$, NaCl and $NaHCO_3$, Inoculum and $NaHCO_3$ were all significant. The high values of regression coefficient ($R^2 = 0.97$) and adjusted regression coefficient (adj. $R^2 = 0.98$) of regression model indicates its accuracy in predicting the experimental data. Also P = 0 and higher F = 38.40 indicate the significance of the model (Zininga et al., 2019)**.** In order to find the optimal values of these parameters **c**ontour plots between $NaHCO_3$ and dilution factor, NaCl and $NaHCO_3$, NaCl and dilution factor, and $NaHCO_3$ and inoculum on lutein production where obtained (Figure 4.3). The predicted optimal conditions of the parameters for maximum lutein production (3.58 mg/L) are as follows: dilution factor (22.12), NaCl (27 g/L), $NaHCO_3$ (4.5 g/L) and inoculum (4.82×10^9 cells/L) and the experimental lutein value obtained was 3.412 mg/L at these levels

Table 4.4 Central composite design showing the experiments performed and the levels of individual parameters along with lutein production in each experimental run.

S. No	Dilution factor	NaCl (g/L)	Inoculum (g/L)	NaHCO3 (Cells/L)	Lutein (mg/L)
1	20	21	4	4.13×10^9	3.12
2	20	35	4	4.13×10^9	2.874
3	15	14	5	5.5×10^9	1.02
4	25	28	5	2.75×10^9	1.36
5	10	21	4	4.13×10^9	0.117
6	20	21	6	4.13×10^9	2.58

7	25	28	5	5.5×10^9	3.24
8	20	21	4	4.13×10^9	3.07
9	20	21	2	4.13×10^9	1.928
10	20	21	4	1.37×10^9	0.76
11	15	28	5	2.75×10^9	0.73
12	15	28	3	5.5×10^9	1.08
13	25	14	5	2.75×10^9	0.62
14	20	21	4	4.13×10^9	3.19
15	15	14	3	2.75×10^9	0.72
16	25	14	5	5.5×10^9	1.16
17	20	21	4	4.13×10^9	3.08
18	20	21	4	4.13×10^9	3.13
19	25	14	3	5.5×10^9	0.88
20	25	14	3	2.75×10^9	0.56
21	15	14	5	2.75×10^9	0.76
22	20	21	4	4.13×10^9	3.00
23	20	7	4	4.13×10^9	0.84
24	30	21	4	4.13×10^9	0.94
25	15	14	3	5.5×10^9	0.62
26	15	28	3	2.75×10^9	1.08
27	25	28	3	2.75×10^9	1.34
28	15	28	5	5.5×10^9	1.88
29	25	28	3	5.5×10^9	2.64
30	20	21	4	4.13×10^9	3.12
31	20	21	4	6.88×10^9	2.49

Table 4.5 ANOVA of lutein production by *C. vulgaris* in the optimization study by RSM

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	14	32.58	32.58	2.33	39.43	0.000
Linear	4	10.05	10.05	2.51	42.56	0.000
Dilution factor	1	1.29	1.29	1.29	21.79	0.000
NaCl	1	5.11	5.11	5.11	86.64	0.000
Inoculum	1	0.41	0.41	0.41	7.02	0.017
$NaHCO_3$	1	3.23	3.23	3.23	54.79	0.000
Square	4	20.18	20.18	5.04	85.48	0.000
Dilution factor × Dilution factor	1	10.86	14.05	14.05	238.04	0.000
NaCl × NaCl	1	2.66	3.89	3.89	65.90	0.000
Inoculum × Inoculum	1	1.45	2.08	2.08	35.21	0.000
$NaHCO_3$ × $NaHCO_3$	1	5.21	5.21	5.21	88.26	0.000
Interaction	6	2.35	2.35	0.39	6.64	0.001
Dilution factor × NaCl	1	0.86	0.86	0.86	14.58	0.002
Dilution factor× Inoculum	1	0.00	0.00	0.00	0.01	0.943
Dilution factor× $NaHCO_3$	1	0.47	0.47	0.47	7.89	0.013
NaCl × Inoculum	1	0.01	0.01	0.01	0.09	0.769
NaCl × $NaHCO_3$	1	0.68	0.68	0.68	11.60	0.004
Inoculum × $NaHCO_3$	1	0.33	0.33	0.33	5.65	0.030
Total	30	33.52				

S = 0.242942 PRESS = 5.41522

R-Sq = 97.18% R-Sq(adj) = 94.72%

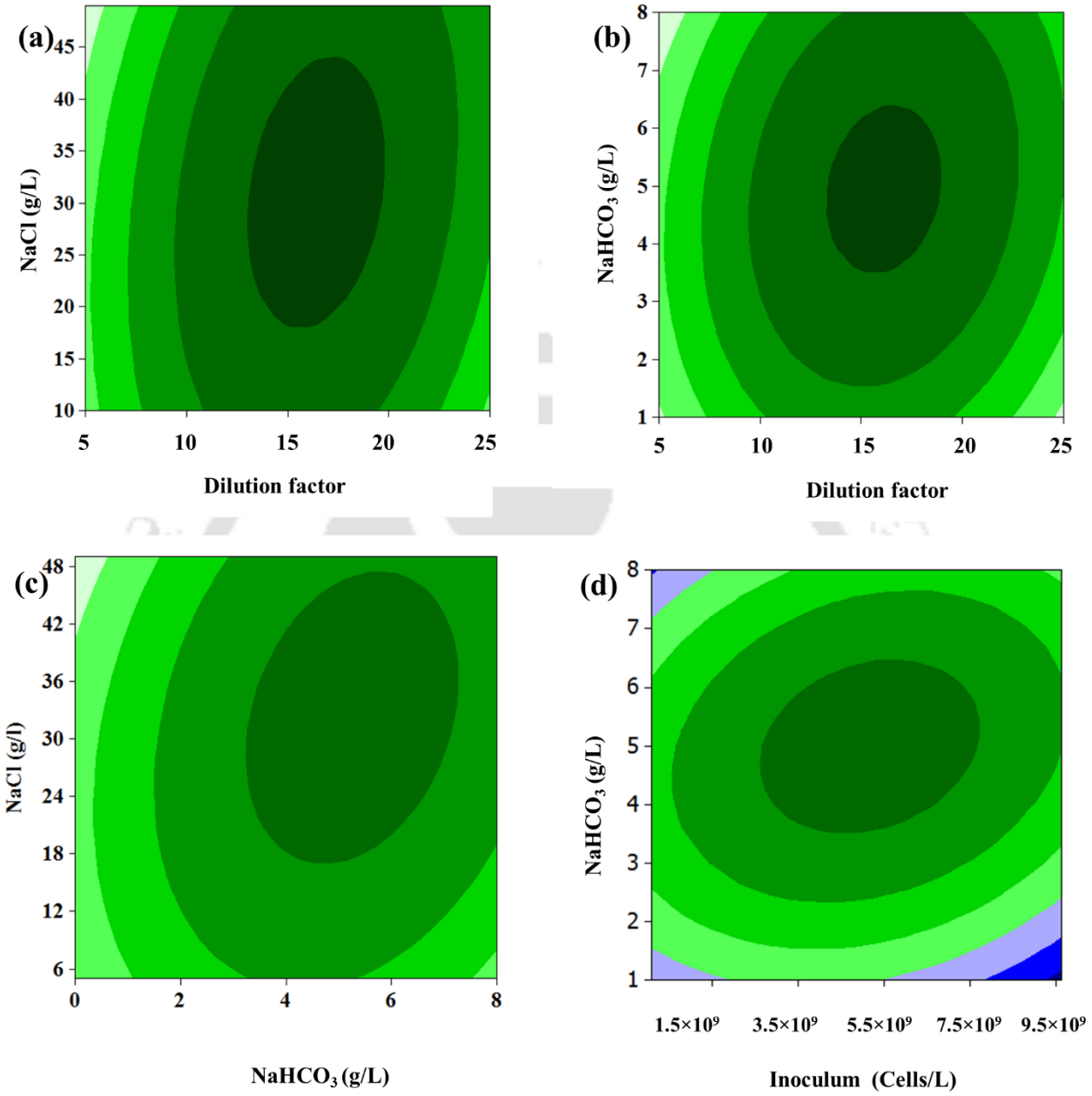


Fig. 4.3 Two dimensional contour plot showing interaction between different parameters on lutein production (a) NaCl (g/L) and Dilution factor (b) $NaHCO_3$ (g/L) and Dilution factor (c) NaCl (g/L) and $NaHCO_3$ (g/L) and (d) $NaHCO_3$ (g/L) and Inoculum (cells/L)

4.2 Biokinetics and flux balance analysis

Biokinetics of lutein production by *C. vulgaris* was analysed using different concentration of substrates in autotrophic and heterotrophic modes. The experimental data on substrate utilization, biomass and lutein production using sodium bicarbonate and sodium acetate as the substrate were fitted to different kinetic models reported in the literature, and the carbon flux balance analysis was carried out for evaluating flux distribution in autotrophic and heterotrophic modes.

4.2.1 Substrate utilization kinetics

Figure 4.4 and 4.6 revealed complete utilization of sodium bicarbonate was up to 3000 mg/L initial concentration and sodium acetate was up to 1500 mg/L initial concentration. Figure 4.5 shows maximum biomass formation (0.737 and 0.736 g/L) at 4000 and 5000 mg/L of initial sodium bicarbonate concentration, which indicates the lack of any substrate inhibition on biomass formation by *C. vulgaris*. In the case of sodium acetate as the substrate maximum biomass formation (0.871 g/l) was observed at 2000 mg/L initial concentration, and above which substrate inhibition was observed (Fig. 4.7). Similar pattern of biomass formation was observed by Chen & Johns (1993) in the case of *Chlamydomonas reinhardtii* on acetate as the substrate at different concentration. Acetate is soluble in lipids of the cell membrane which in turn affects the phosphate channel, thereby inhibiting the microalgal growth (CONWAY & DOWNEY, 1950; Samson et al., 1955). High concentration of sodium in the form of sodium acetate is also detrimental to fresh water microalgal growth (Church et al., 2017), but in this study sodium bicarbonate even at 4000 and 5000 mg/L did not show, inhibition on the biomass growth by *C. vulgaris*.

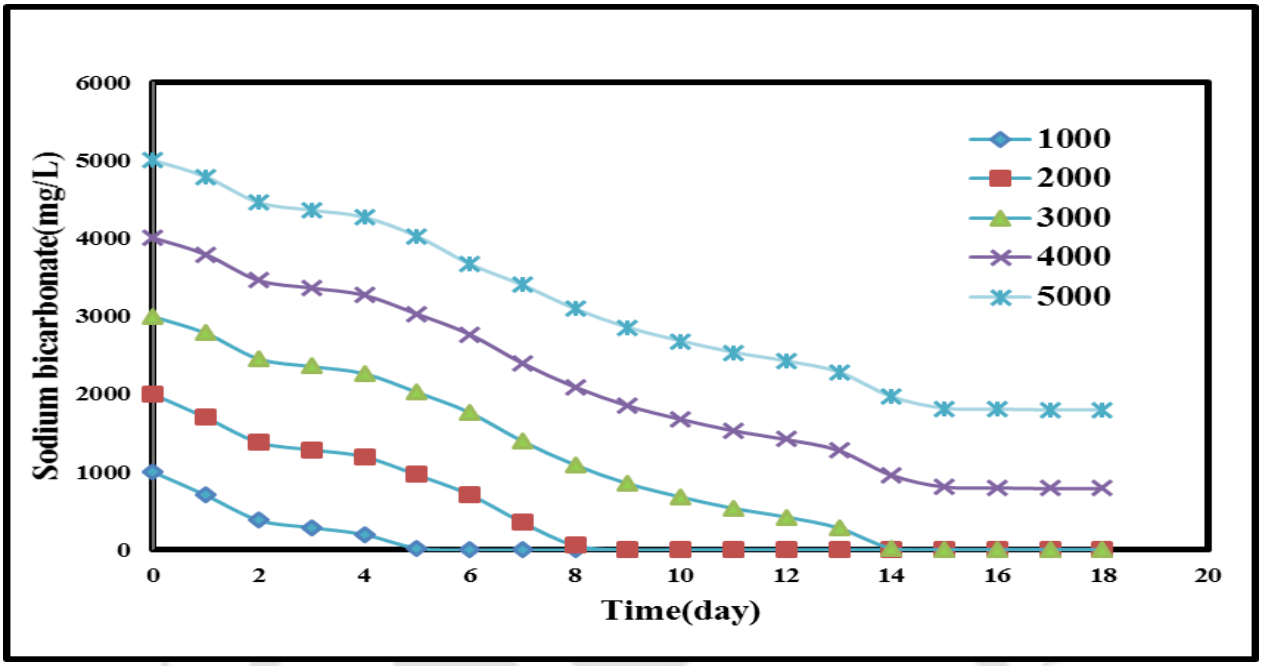


Fig. 4.4 Sodium bicarbonate consumption by *C. vulgaris* at different initial concentrations

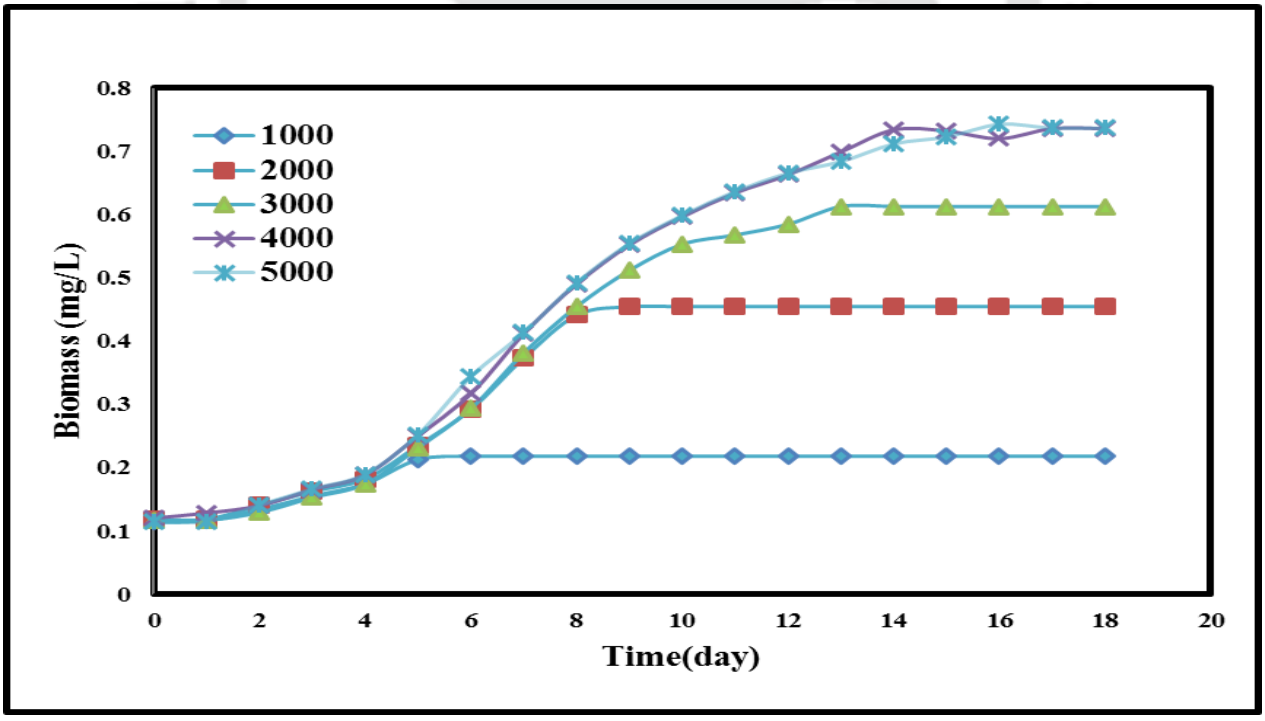


Fig. 4.5 Biomass growth profile at different initial bicarbonate concentrations

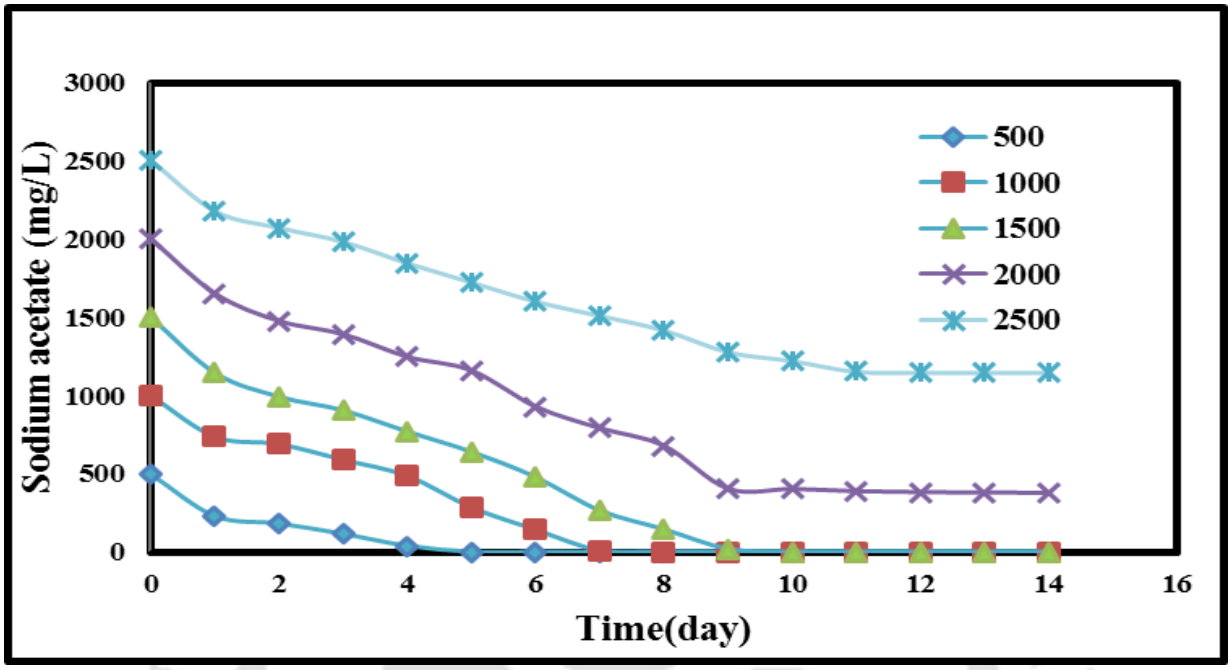


Fig. 4.6 Sodium acetate consumption profile for different initial concentrations

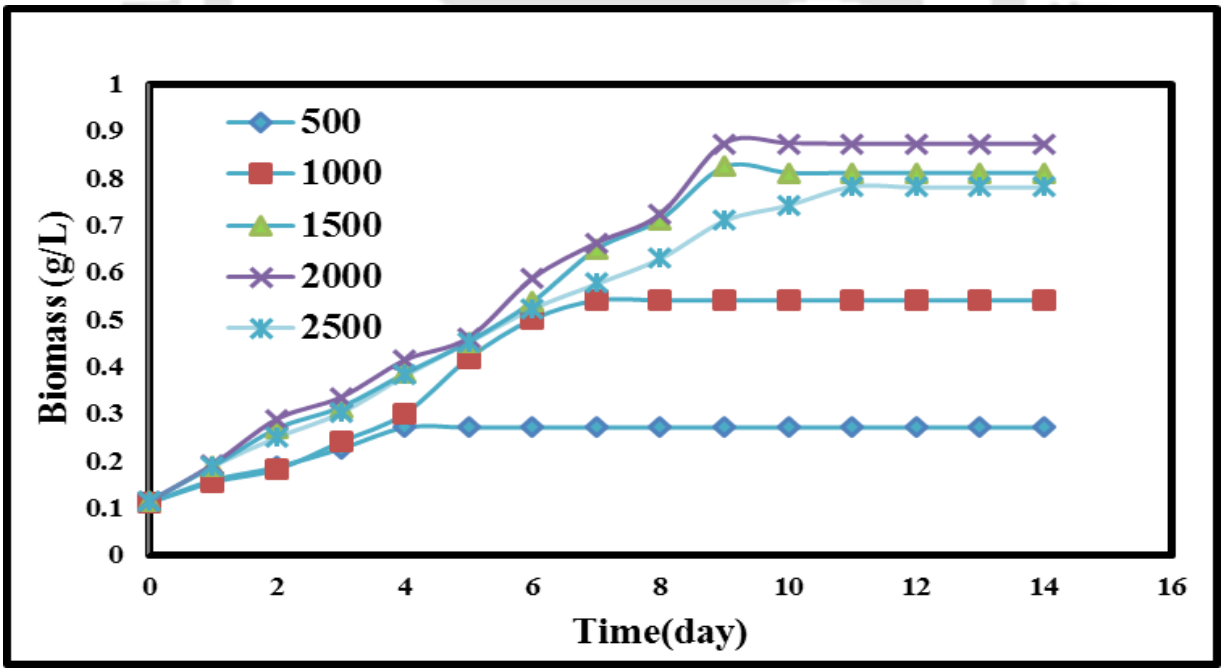


Fig. 4.7 Biomass growth profile for different initial sodium acetate concentrations

Figures 4.8 and 4.9, shows the observed and predicted values of sodium bicarbonate and sodium acetate consumption. Tables 4.6 and 4.7 presents the estimated values of the four models used for assessing substrate consumption by *C. vulgaris*. Logistic and Logarithmic models did not ultimately predict the substrate consumption by *C. vulgaris* as revealed by their low R^2 values, whereas as the prediction due to first order kinetics and modified Gompertz models were accurate with high R^2 values. Both the substrates showed increase in lag phase time (λ) of *C. vulgaris* growth with increase in initial concentration. Çelekli et al. (2008) reported on similar trend in *Scenedesmus obliquus* growth and the substrate consumption kinetics by the microalgae was accurately described using Gompertz model.

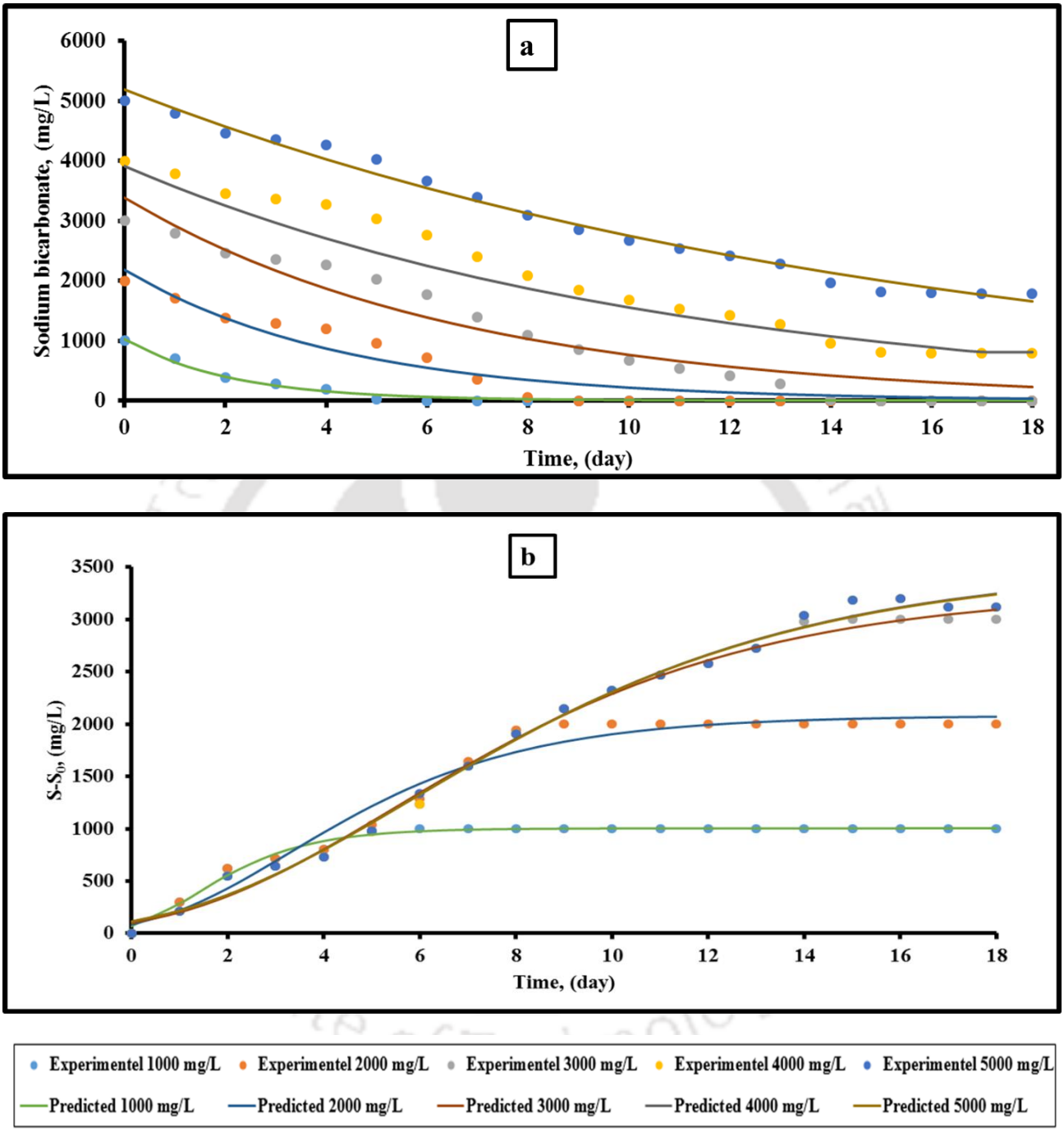


Fig. 4.8 Experimental and predicted values of sodium bicarbonate utilization by *C. vulgaris* (a) First order kinetic model (b) modified Gompertz model

Table 4.6 Estimated model parameters on sodium bicarbonate utilization kinetics by *C. vulgaris*

Model parameters	Initial sodium bicarbonate concentration (mg/L)				
	1000	**2000**	**3000**	**4000**	**5000**
First order model					
K (day^{-1})	0.472	0.229	0.148	0.092	0.063
R^2	0.985	0.932	0.930	0.972	0.985
Logarithmic model					
X_0 (g/L)	247200	< 0.10	< 0.10	< 0.10	< 0.10
U_{max} (mg/L/day)	0.0000281	< 0.10	< 0.10	< 0.10	< 0.10
R^2	< 0.10	0.274	0.177	0.168	0.170
Logistics model					
X_0 (g/L)	30.11	0.731	713.3	< 0.10	2680
U_{max} (mg/L/day)	< 0.10	< 0.10	< 0.10	0.872	< 0.10
K_S (mg/L)	1.171	8.938	30.51	0.935	0.472
R^2	< 0.10	0.1037	0.7468	< 0.10	< 0.10
Modified Gompertz model					
U_{max} (mg/L/day)	103.1	101.4	101.5	99.83	99.8
λ (day)	1.316	3.279	5.5	5.866	5.8
R^2	0.986	0.973	0.993	0.992	0.993

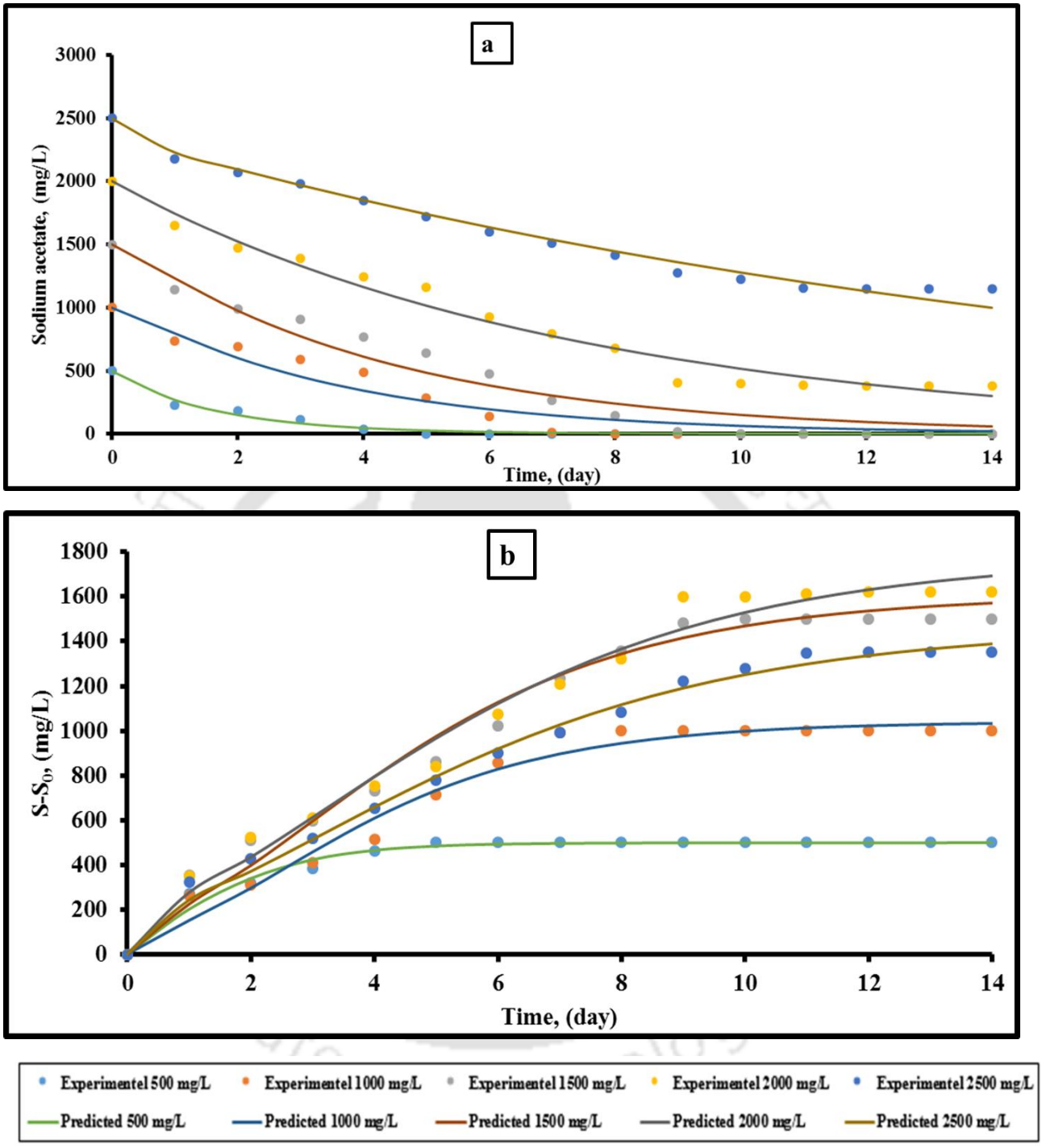


Fig. 4.9 Experimental and predicted values of sodium acetate utilization by *C. vulgaris* (a) First order kinetic model (b) modified Gompertz model

Table 4.7 Estimated model parameters on sodium acetate utilization kinetics by *C. vulgaris*

Model parameters	Initial CH_3COONa concentration (mg/l)				
	500	**1000**	**1500**	**2000**	**2500**
First order model					
K (day^{-1})	0.593	0.281	0.233	0.135	0.062
R^2	0.982	0.937	0.944	0.975	0.975
Logarithmic model					
K (day^{-1})	44520	405600	915800	< 0.10	< 0.10
U_{max} (mg/L/h)	< 0.10	< 0.10	< 0.10	79.51	-4202
R^2	0.431	0.668	0.685	0.190	0.215
Logistics model					
X_0 (g/L)	< 0.10	< 0.10	7.064	< 0.10	< 0.10
U_{max} (mgl/L/h)	< 0.10	< 0.10	4.13	< 0.10	< 0.10
K_S (mg/L)	2.899	1.461	8.216	3.77	4.799
R^2	0.648	< 0.10	< 0.10	0.305	0.327
Modified Gompertz model					
U_{max} (mg/L/h)	57.86	60.19	68.09	67.2	53.6
λ (day)	0.889	2.54	2.983	3.23	3.177
R^2	0.955	0.974	0.979	0.978	0.983

4.2.1.1 Specific substrate utilization rate

Experimental data on specific uptake rate of sodium bicarbonate and sodium acetate as the substrate for *C. vulgaris* was fitted to Monod, Haldane, Edward and Moser models (Figure 4.10 and 4.11). Haldane and Edward models were highly accurate ($R^2 \geq 0.99$) among all the models used in predicting the specific substrate uptake rate of sodium acetate. Increase in sodium

acetate concentration beyond 1000 mg/L reduced its specific uptake rate. Monod, Haldane, and Edward models were highly accurate ($R^2 \geq 0.987$) in predicting the specific uptake rate of sodium bicarbonate. The R^2 value was high at 1000 mg/L of sodium bicarbonate, and the value slightly reduced at a concentration in the range 2000 -5000 mg/L. Monod and Haldane models could predict the experimental data for sodium acetate as the substrate as depicted in Figure 4.11. The estimated model parameters on specific substrate uptake rate of the two substrates are presented in Table 4.8 and 4.9.

Table 4.8 Estimated model parameters on specific uptake rate of sodium acetate by *C. vulgaris*

Model parameters	**q_{max} (day^{-1})**	**K_S (mg/L)**	**K_i (mg/L)**	**n**	**R^2**
Monod	0.542	-138.7	-	-	0.912
Haldane	2.973	1100	522.5	-	0.993
Edward	1.349	334.9	2749	-	0.995
Moser	0.626	640.1	-	7.545	0.86

Table 4.9 Estimated model parameters on specific uptake rate of sodium bicarbonate *by C. vulgaris*

Model parameters	**q_{max} (day^{-1})**	**K_S (mg/L)**	**K_i (mg/L)**	**n**	**R^2**
Monod	2.03	661.5	-	-	0.987
Haldane	2.046	675.1	656800	-	0.987
Edward	2.031	662.1	23840	-	0.987
Moser	1.589	24.32	-	9.416	0.897

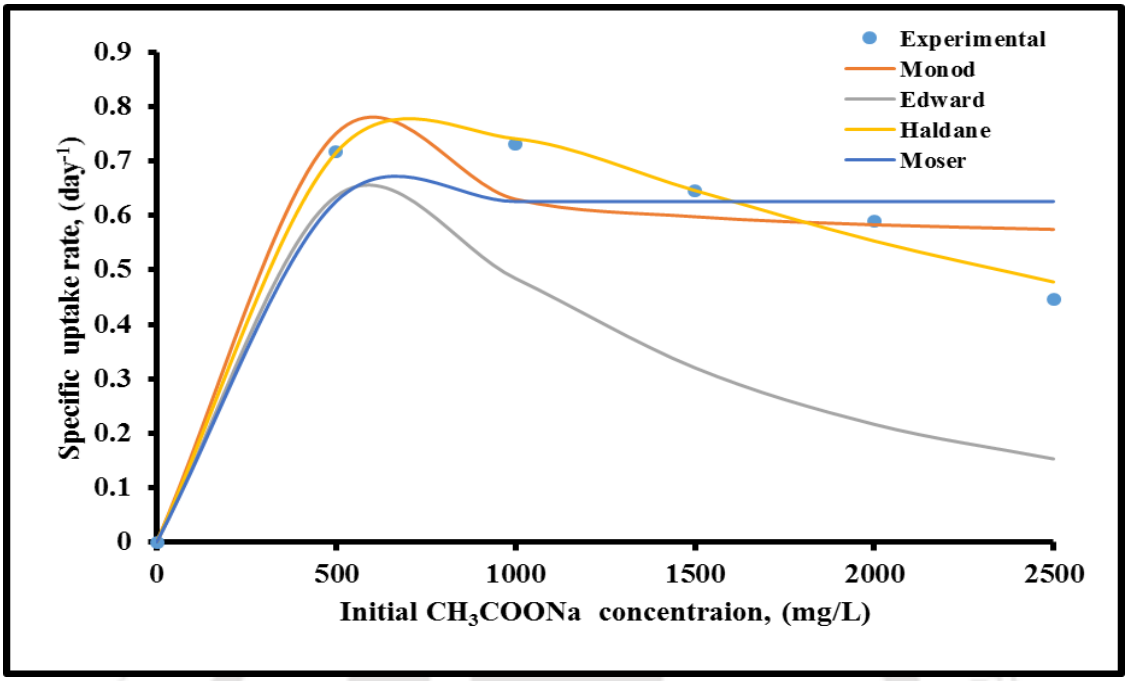


Fig. 4.10 Model predicted and experimental values of specific uptake rate of sodium bicarbonate by *C. vulgaris* for different initial concentrations

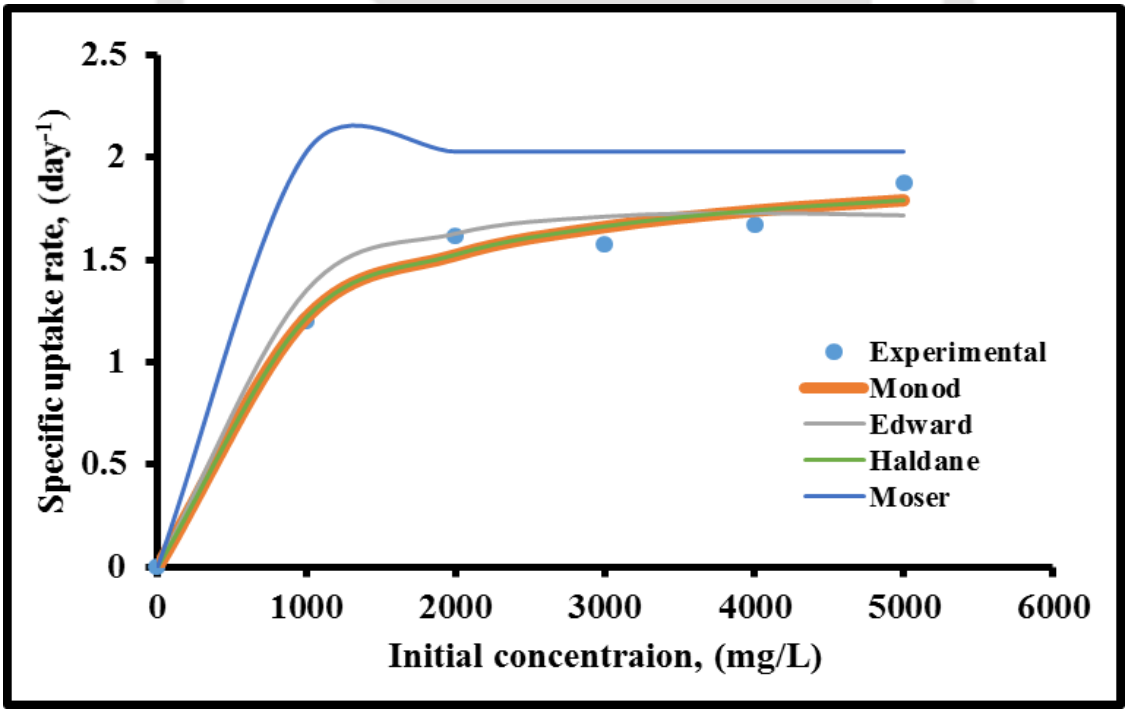
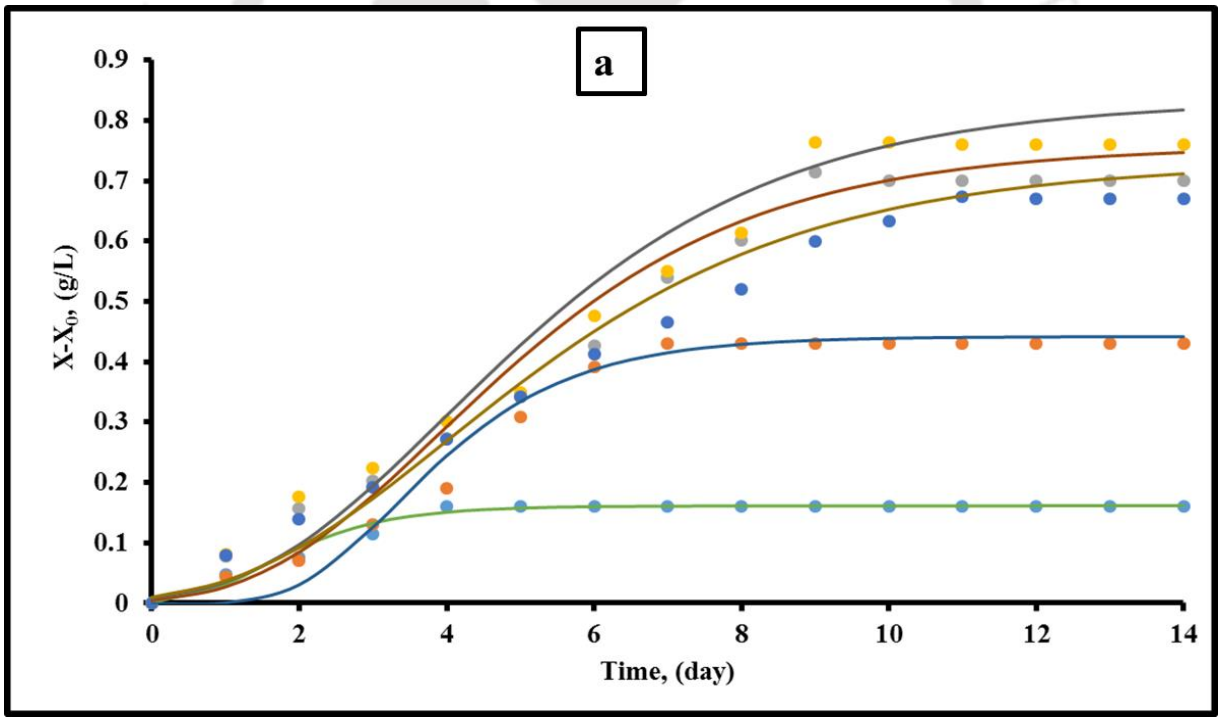


Fig. 4.11 Model predicted and experimental values of specific uptake rate of sodium bicarbonate by *C. vulgaris* for different initial concentrations

4.2.1.2 Biomass growth kinetics

Experimental and model predicted values of biomass growth on the sodium bicarbonate and sodium acetate as the substrate are represented in the Figures 4.12 and 4.13. Both modified Gompertz and Logistic models predicted the biomass formation with high accuracy (R^2= 0.98), for bicarbonate as the substrate at all the initial concentration. The maximum biomass formation was 0.623 g/L on 18th day with sodium bicarbonate as the substrate. The accuracy of the model was also good in case of sodium acetate as the substrate. The maximum biomass formation was 0.78 g/L with sodium acetate as the substrate at 2000 mg/L. The model estimated parameters are presented in Table 4.10 and 4.11 for the two substrates.



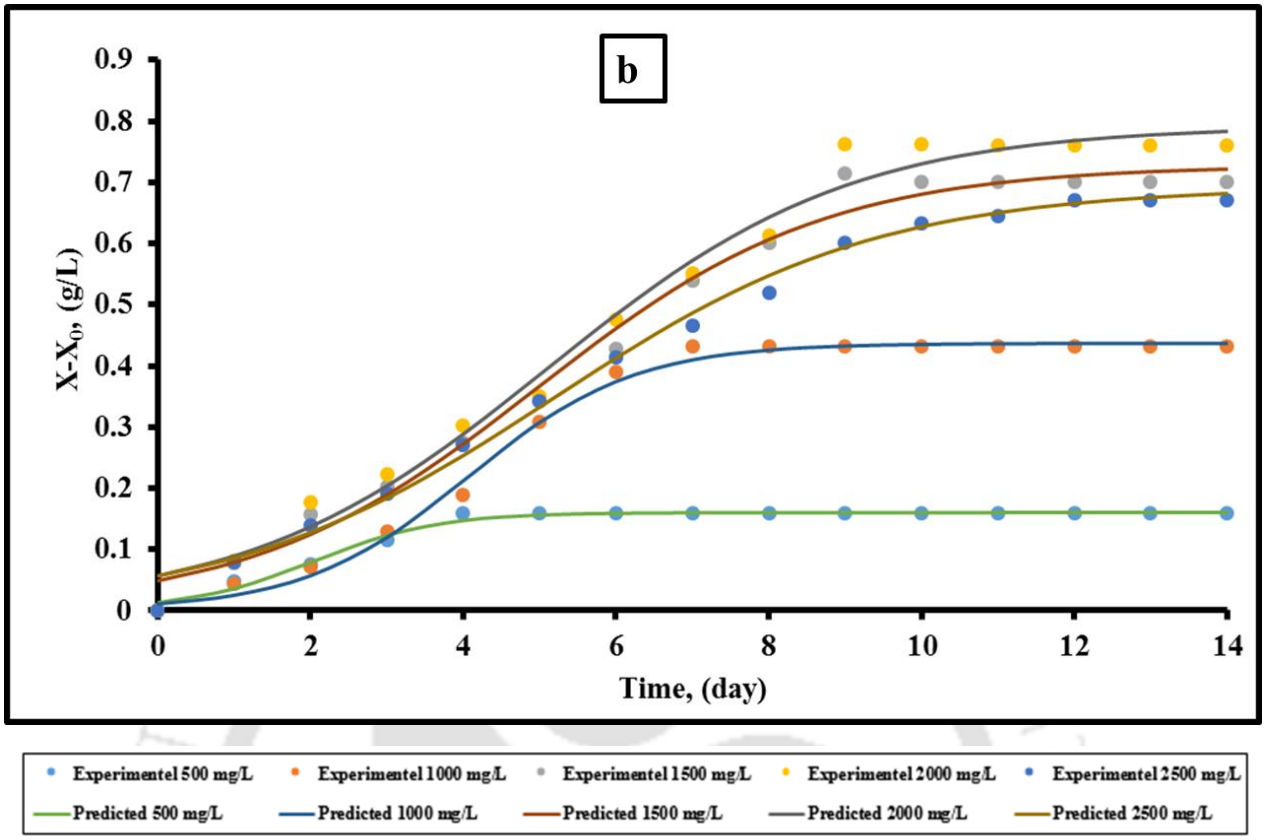
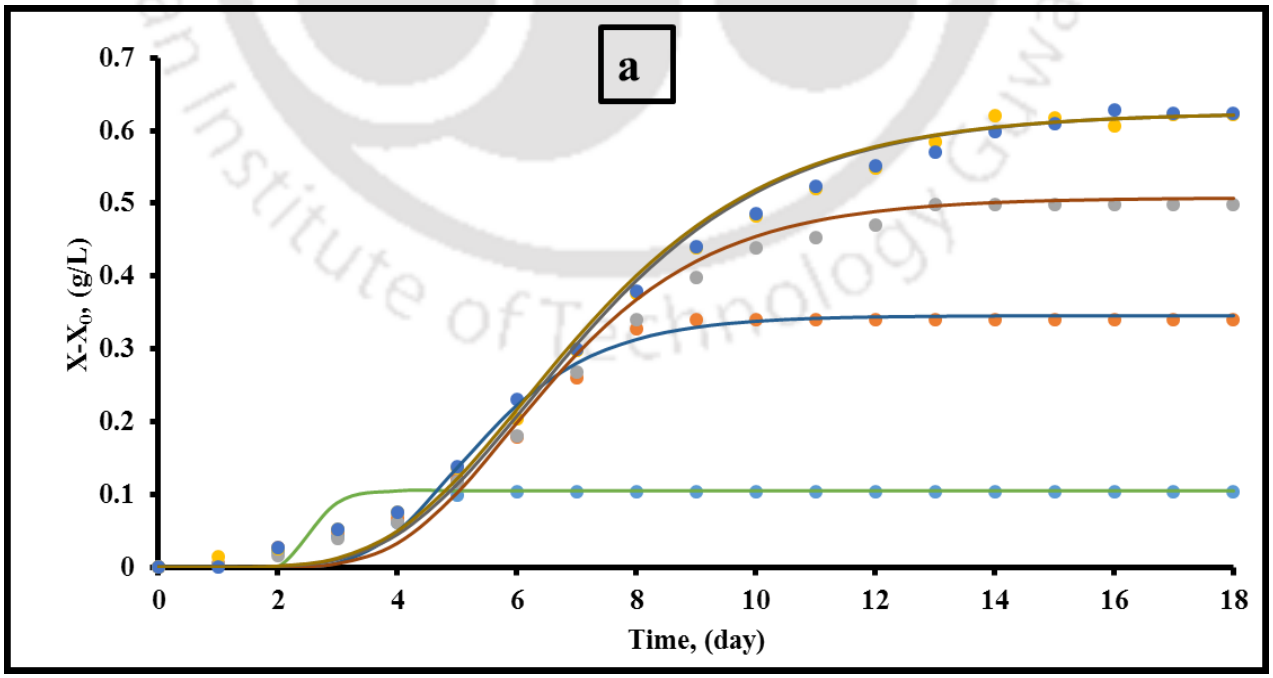


Fig. 4.12 Experimental and model predicted values of biomass growth on sodium acetate at different initial concentrations (a) Modified Gompertz model (b) Logistic model



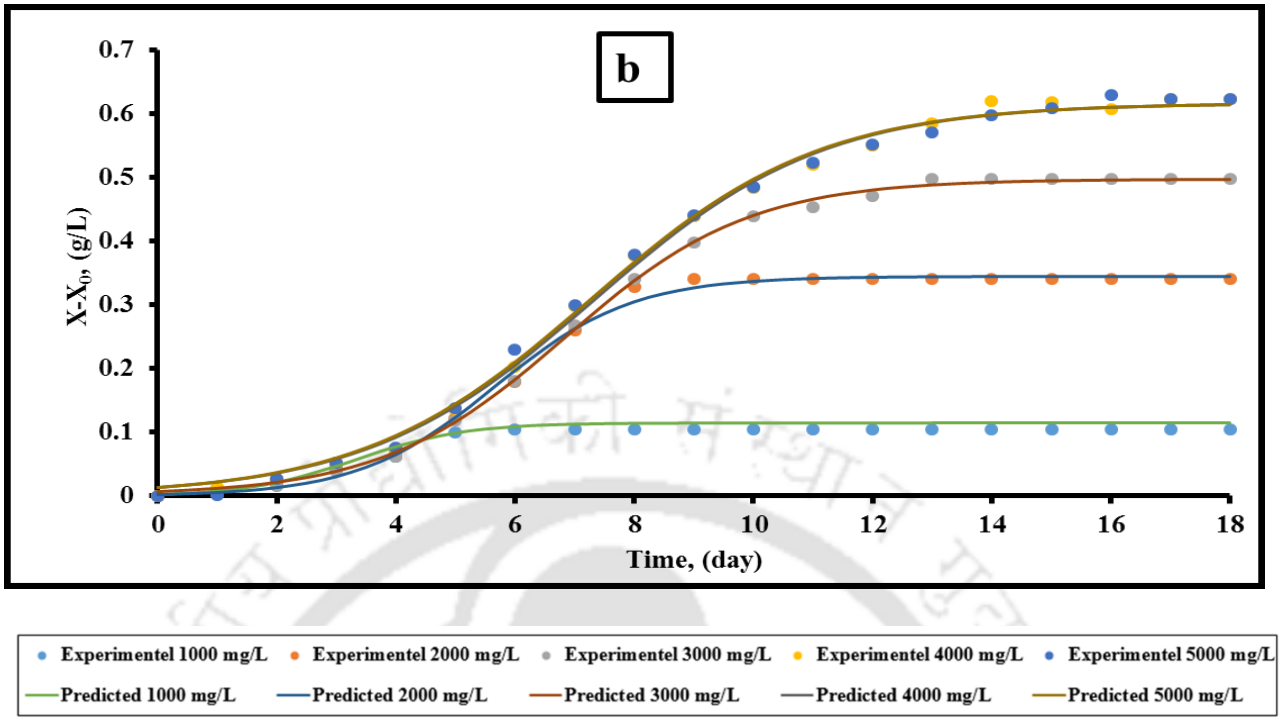


Fig. 4.13 Experimental and model predicted values of biomass growth on sodium bicarbonate at different initial concentrations (a) Modified Gompertz model (b) Logistic model

Table 4.10 Estimated model parameters on biomass growth by *C. vulgaris* with sodium bicarbonate as the substrate

Model parameters	**Initial $NaHCO_3$ concentration (mg/L)**				
	1000	**2000**	**3000**	**4000**	**5000**
Modified Gompertz model					
X_{max}(g/L)	0.038	0.127	0.187	0.236	0.236
R_{max} (mg/L/day)	0.014	0.034	0.036	0.036	0.035
λ (day)	2.825	4.919	5.892	6.239	6.144
R^2	0.9856	0.987	0.998	0.9982	0.999
Logistic model					
X_{max} (g/L)	0.314	1.033	1.493	1.856	1.849
R_{max} (mg/L/day)	0.094	0.225	0.242	0.244	0.241
λ (day)	4.337	6.932	8.544	9.442	9.37
R^2	0.999	0.995	0.999	0.997	0.996

Table 4.11 Estimated model parameters on biomass growth by *C. vulgaris* with sodium acetate as the substrate

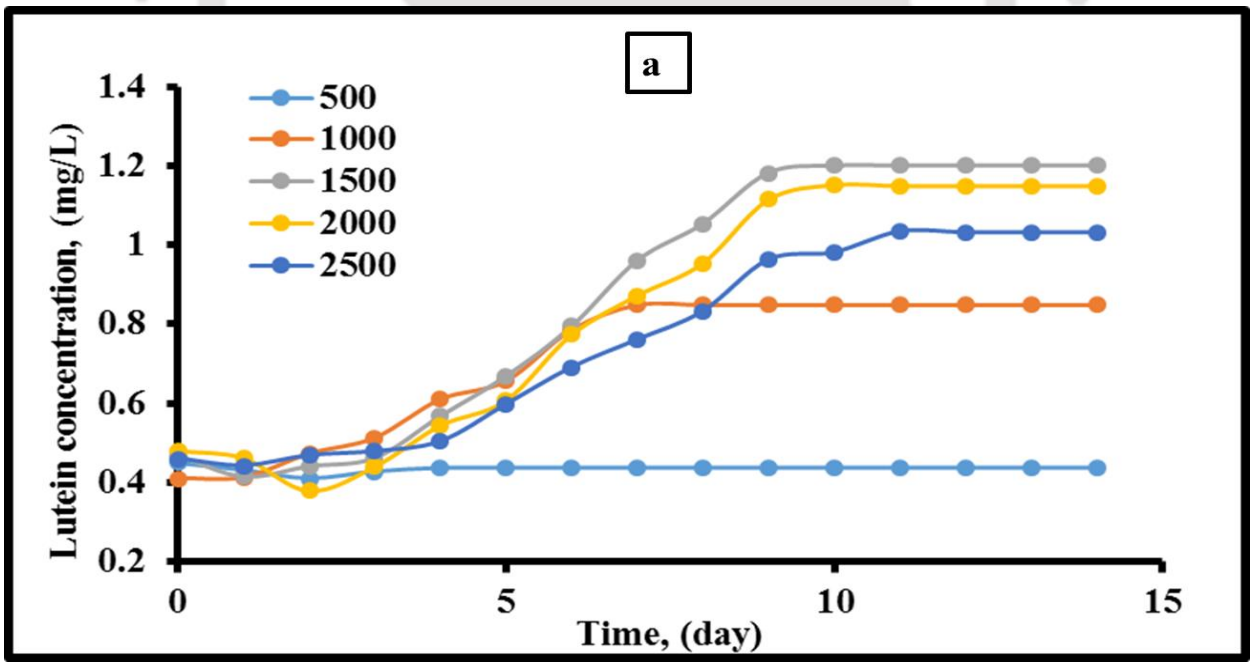
Model parameters	Initial CH_3COONa concentration (mg/L)				
	500	**1000**	**1500**	**2000**	**2500**
Modified Gompertz model					
X_{max}(g/L)	0.059	0.162	0.279	0.307	0.269
R_{max} (mg/L/day)	0.023	0.045	0.043	0.044	0.036
λ (day)	1.458	3.302	3.885	3.971	4.001
R^2	0.979	0.982	0.983	0.980	0.993
Logistic					
X_{max} (g/L)	0.483	1.31	2.181	2.377	2.076
R_{max} (mg/L/day)	0.149	0.302	0.296	0.302	0.244
λ (day)	2.92	5.215	7.026	7.281	7.508
R^2	0.982	0.994	0.989	0.944	0.992

4.2.1.3 Lutein production kinetics

Figure 4.14(a) and 4.15(a) shows the lutein production by *C. vulgaris* with sodium acetate and sodium bicarbonate at different initial concentrations. Maximum lutein concentration of 1.2 and 3.7 mg/L was produced for an initial sodium acetate of 1500 mg/L and sodium bicarbonate of 4000 mg/L, respectively. Increase in sodium acetate concentration from 500 to 1500 mg/L increased the lutein production, but a further increase in initial concentration beyond 1500 mg/L reduced the lutein production value due to substrate inhibition. Microalgae tend to produce high amount of lutein under autotrophic mode ($NaHCO_3$) for protecting its photosystem from free radicals and for photosynthesis, but in heterotrophic/mixotrophic mode (CH_3COONa) microalgae does not utilize the photosystem for its anabolism (Ho et al., 2014).

Leudeking-Piret model and Gompertz model were used for predicting the product formation and cumulative product formation rate for both sodium acetate and sodium bicarbonate (Figure

4.14 b and 4.15 b). Both these models are predominantly used to describe product formation in microalgae. Between the two models, Gompertz model's predicted the experimental data with a high with R^2 value of 0.99 for both the substrates and for all the initial concentrations, whereas accuracy of the Leudeking-Piret model in predicting lutein production was very low for different initial substrate concentrations (R^2 = 0.27 - 0.704). The estimated model parameters values are presented in Tables 4.12 and 4.13. The Leudeking-Piret model parameter values in this study are, $\alpha \leq 0$ and $\beta \neq 0$, which signifies that the lutein production by *C. vulgaris* is non – growth associated (Gaden 2000). Zhang et al. (1999) previously employed Leudeking-Piret model to describe lutein production under heterotrophic mode of cultivation with glucose (35 g/L) as the carbon source. However, this is the first study to describe lutein production kinetics by *C. vulgaris* growth using two different carbon substrates at different initial concentrations.



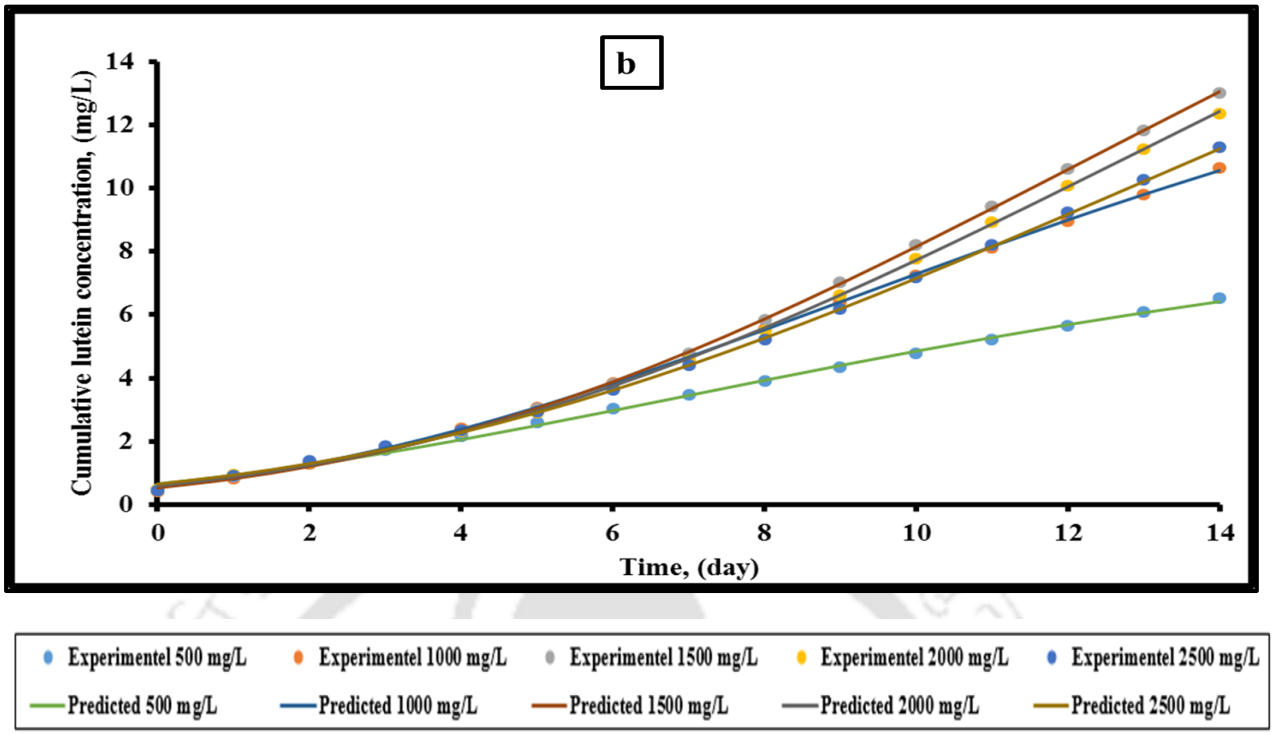


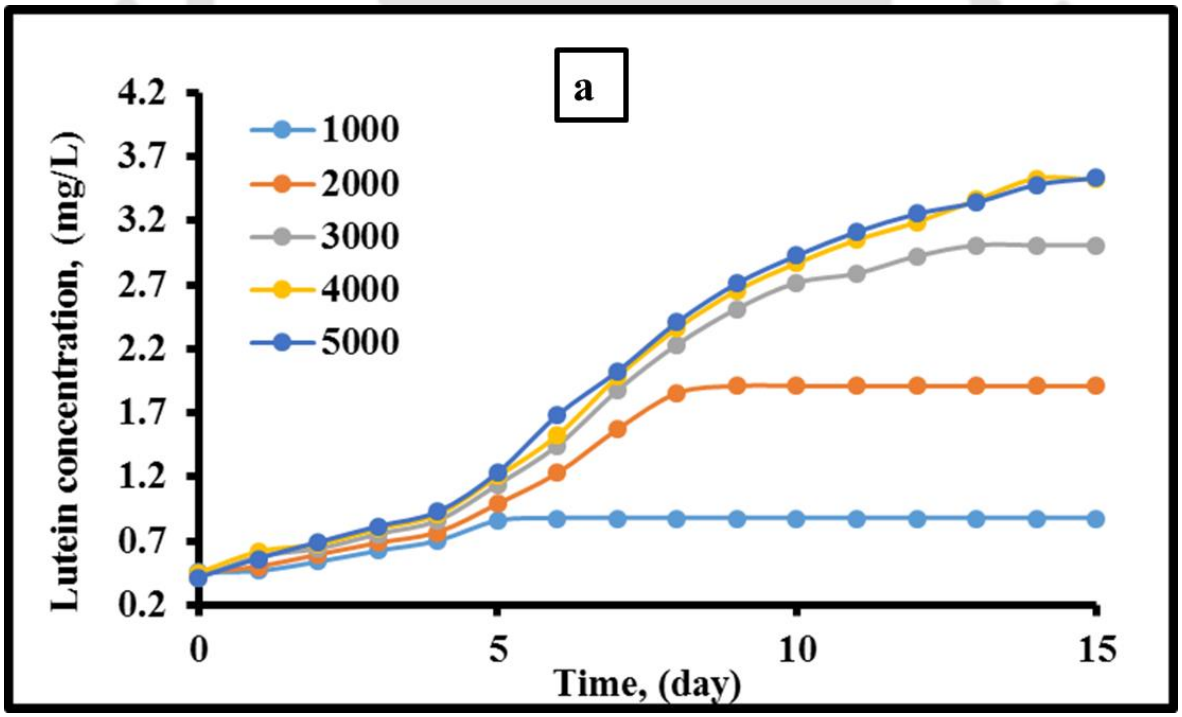
Fig. 4.14 (a) Lutein concentration and (b) cumulative lutein concentration by *C. vulgaris* for different initial sodium acetate concentrations as the substrate. In figure (b) the predicted values are due to Gompertz model

Table 4.12 Estimated model on lutein production kinetics by *C. vulgaris* with sodium acetate as the substrate.

Model parameters	**Initial sodium acetate concentration (mg/L)**				
	500	**1000**	**1500**	**2000**	**2500**
Leudeking-Piret model					
α	0.136	-0.252	0.281	0.435	0.314
β	0.004	0.036	0.001	-0.009	-0.006
R^2	0.535	0.344	0.294	0.278	0.223
Gompertz model					
P_{max} (mg/L)	9.31	17.6	29.63	30.85	27.8
R_{max} (mg/L/day)	0.48	0.885	1.237	1.195	1.04
λ (day)	-0.207	1.762	3.431	3.596	3.16
R^2	0.997	0.999	0.999	0.999	0.999

Table 4.13 Estimated model parameters on lutein production kinetics by *C. vulgaris* with sodium bicarbonate as the substrate

Model parameters	Initial $NaHCO_3$ concentration (mg/L)				
	1000	**2000**	**3000**	**4000**	**5000**
Leudeking-Piret model					
α	-0.466	-0.642	-0.663	-0.570	-0.575
β	0.023	0.058	0.068	0.067	0.068
R^2	0.480	0.704	0.604	0.376	0.524
Gompertz model					
P_{max} (mg/L)	21.38	44.87	73.28	91.35	89.67
R_{max} (mg/L/day)	0.957	2.017	3.075	3.56	3.576
λ (day)	1.631	3.947	5.269	5.818	5.716
R^2	0.998	0.999	0.999	0.999	0.999



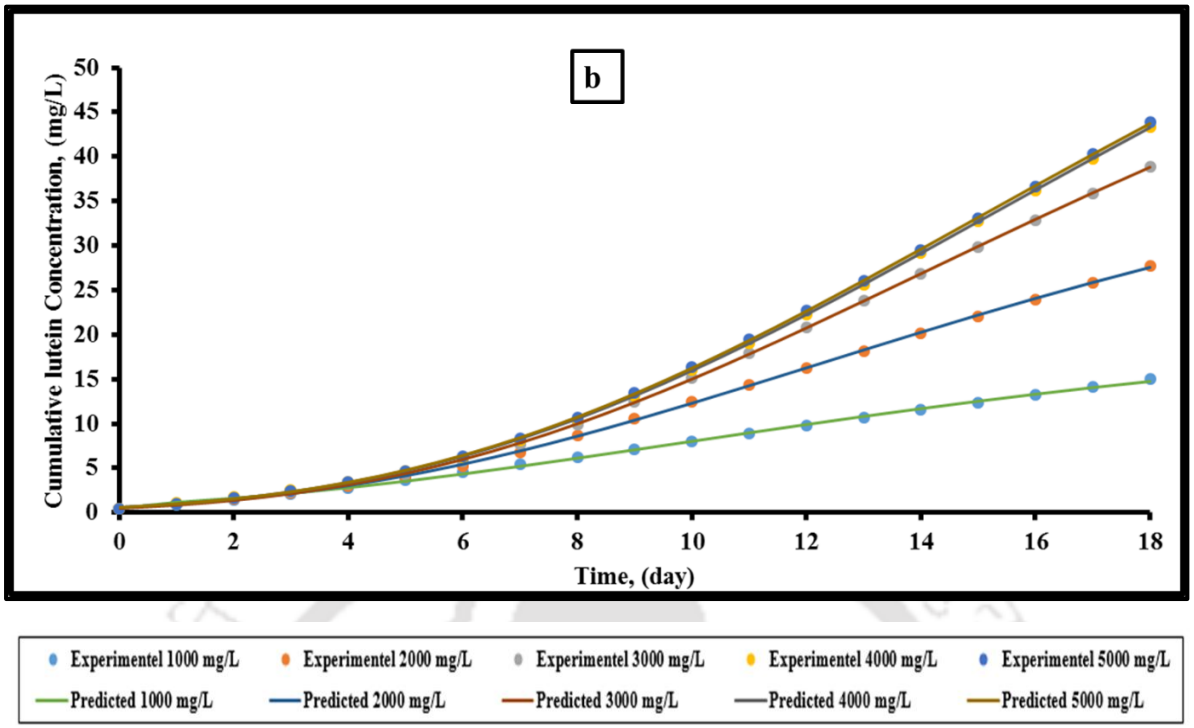


Fig. 4.15 (a) Lutein concentration and (b) Cumulative lutein concentration by *C. vulgaris* for different initial sodium bicarbonate concentrations as the substrate .In figure (b) the predicted values are due to Gompertz model

4.2.2 Static flux balance analysis

Metabolic network was formulated to analyse the carbon flux in *Chlorella vulgaris 92001* on 7th day of culture with synthetic poultry digestate containing sodium bicarbonate (autotrophic mode) and sodium acetate (heterotrophic mode) as the substrate. Equations and abbreviations used in the network are detailed in appendix of this thesis (Table A1 and A2). The metabolic network consists of 159 × 114 and 156 × 111 reactions and metabolites in autotrophic and heterotrophic conditions, as the unknown flux values where higher than the degree of freedom, the linear programing based optimization was selected. Static flux balance analysis is based on pseudo steady state approximation, which excludes the information on metabolite concentration and time (Varma & Palsson, 1994).The elemental composition and

macromolecular constituents of cells were estimated and their composition for different growth conditions are shown in Table 4.14 and 4.15. The biomass equation includes ATP used for polymerization and growth associated maintenance as per the literature (Boyle & Morgan, 2009). The DNA and RNA compositions were kept constant in all the growth conditions as per the literature (Boyle & Morgan, 2009; Muthuraj et al., 2013). The difference between model predicted and experimental values of the specific growth rate where 3 and 8 % for autotrophic and heterotrophic cultivation modes which indicate high accuracy of the model in predicting the carbon flux in the microalgae.

Table 4.14 Percentage of elemental composition of algal biomass

Elemental composition (%w/w)				
	C	**H**	**N**	**O**
Autotrophic	49.5	7.8	3.08	39.62
Heterotrophic	51.2	7.92	3.57	37.31

Table 4.15 Percentage macromolecular composition in algal biomass

Macromolecules	**Composition, (%w/w)**	
	Autotrophic	**Heterotrophic**
DNA	0.002	0.002
RNA	0.0251	0.0251
Proteins	0.48	0.57
Polysaccharides	0.31	0.23
Lipids	0.140	0.167
Chlorophyll	0.042	0.011

In autotrophic mode, sodium bicarbonate equivalent to CO_2 (mM/g DCW/day) was used as the constraint input with ammonium consumption rate. Carbon flux distribution under autotrophic mode is depicted in Figure 4.16, shows that the carbon flux starts through Calvin cycle by

fixing CO_2, in which glyceraldehyde 3 phosphate (GAP) is converted to dihydroxy acetone phosphate (DHAP). One fourth of the DHAP was diverted for producing polysaccharides and the balance was utilized in glycolytic cycle, similar results were reported for flux analysis in autotrophic mode by Yang et al. (2000) and Muthuraj et al., (2013) for *Chlorella* species. Carbon flux was very less towards citric acid cycle (TCA) and pentose pathways (PP) in autotrophic mode and these pathways play an important part in generating energy and anabolic precursors in heterotrophic mode. Energy requirements (ATP and $NAPH^{+}$) for autotrophic mode is from photophosphorylation (PSI I and II) which plays significant role when the flux through TCA and PP are in maintenance mode (Shastri & Morgan, 2005; Yang et al., 2011).

In heterotrophic condition, the flux started with uptake of acetate and then it passed through TCA cycle to produce the energy and malate (MAL), a precursor for producing Phosphoenol pyruvate (PEP) and Phosphatidylglycerol (3 PG) (Figure 4.17). G6P is produced through gluconeogenesis for the production of polysaccharide, whereas carbon flux trough pentose pathway was through 3 PG. Distribution of carbon flux in heterotrophic condition is presented in Figure 4.17.

The autotrophic biomass yield is 13.91 g biomass for every mole of carbon consumed, based on the elemental analysis of biomass, whereas the heterotrophic biomass yield is 19.73 g per mole of carbon. The carbon consumption efficiency of heterotrophic condition is high because the carbon is used only for biomass formation, energy production instead of producing any chlorophyll or carotenoid, which are almost non-essential products in terms of heterotrophic condition. Flux towards chlorophyll biosynthesis was found to be 4 times higher in case of photoautotrophic condition as compared to heterotrophic condition which in turn increases the light harvesting capacity, which is needed for harvesting maximum amount of light to produce

energy (Shastri & Morgan, 2005). The increase in chlorophyll tends to increase the lutein numbers in order to protect the photosystem from oxidative damage and to assist the photosynthesis process in autotrophic condition. From the literature, the biomass yield values with acetate and bicarbonates as the substrate are low as compared with the value for glucose, which is due to the difference in energy content of the substrates. Glucose, with a significantly higher energy content per mole than acetate, has a standard heat of combustion of -2840 kJ/mole as compared to -708.8 kJ/mole for acetate and -948 kJ/mole for bicarbonate.

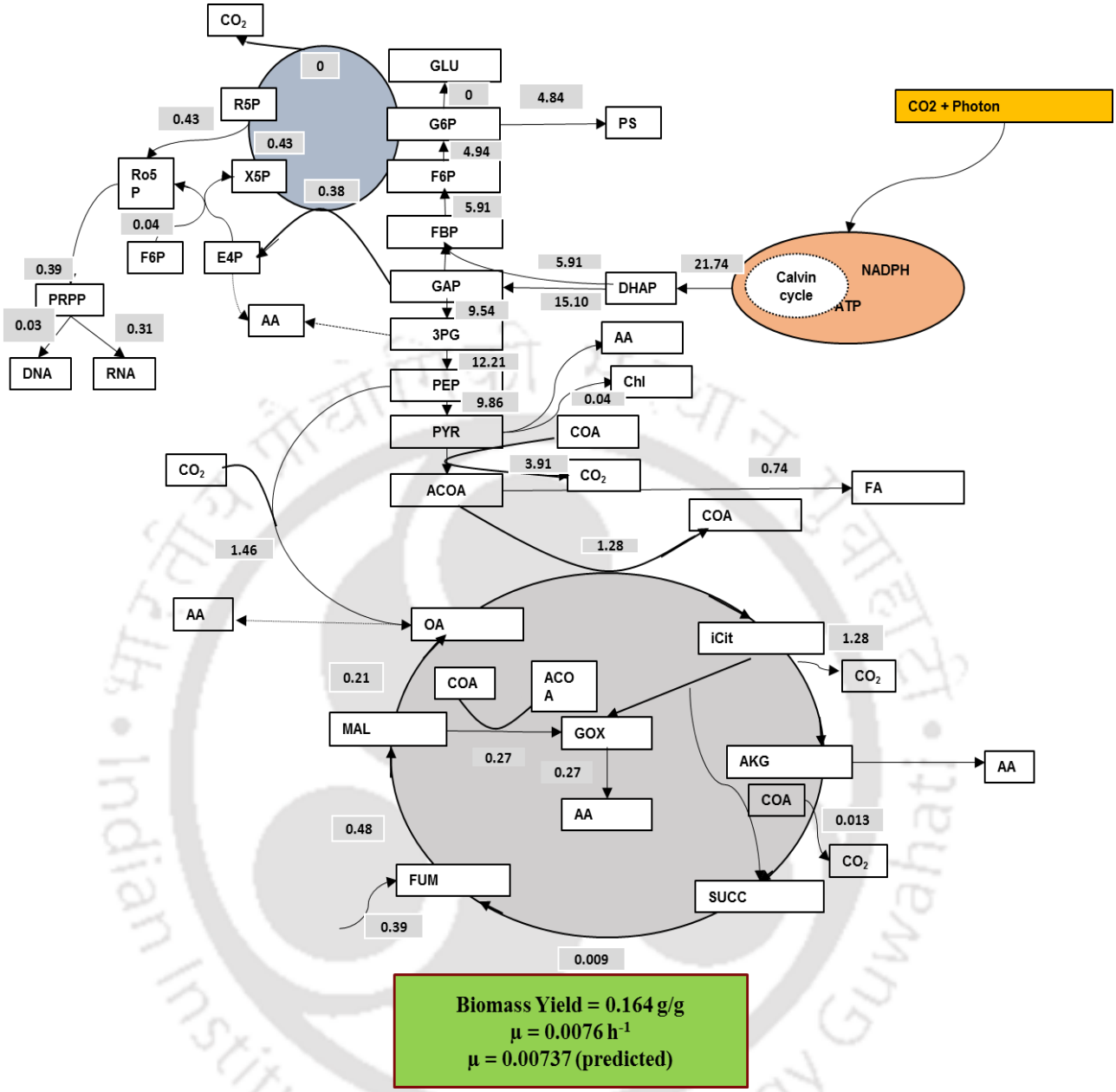


Fig. 4.16 Distribution of carbon flux in *C. vulgaris* on 7th day of cultivation under photoautotrophic mode**.**

were, ace, Acetate; ACOA, Acetyl coenzyme A; AKG, α keto glutarate; Chl, Chlorophyll; Cit, Citricacid; CO_2, Carbon dioxide; COA, Coenzyme A; DHAP, Dihydroxy acetone phosphate; E4P, Erythrose 4 phosphate; F6P, Fructose 6phosphate; FA, Fattyacid; FBP, Fructose1, 6biphosphate; Fum, Fumarate; G6P, Glucose6phosphate; GAP, Glyceraldehyde 3 phosphate; Glu, Glucose; GOX, Glyoxalate; Mal, Malate; OA, Oxaloacetate; PEP, Phosphoenol pyruvate; PG, Phosphatidylglycerol; PRPP, 5phosphoribosyl1pyrophosphate; PS, Polysaccharide; Pyr, Pyruvate; R5P, Ribulose5phosphate; RNA, Ribosenucleicacid; Ro5P, Ribose5phosphate; Succ, Succinate; X5P, Xylulose 5 phosphate.

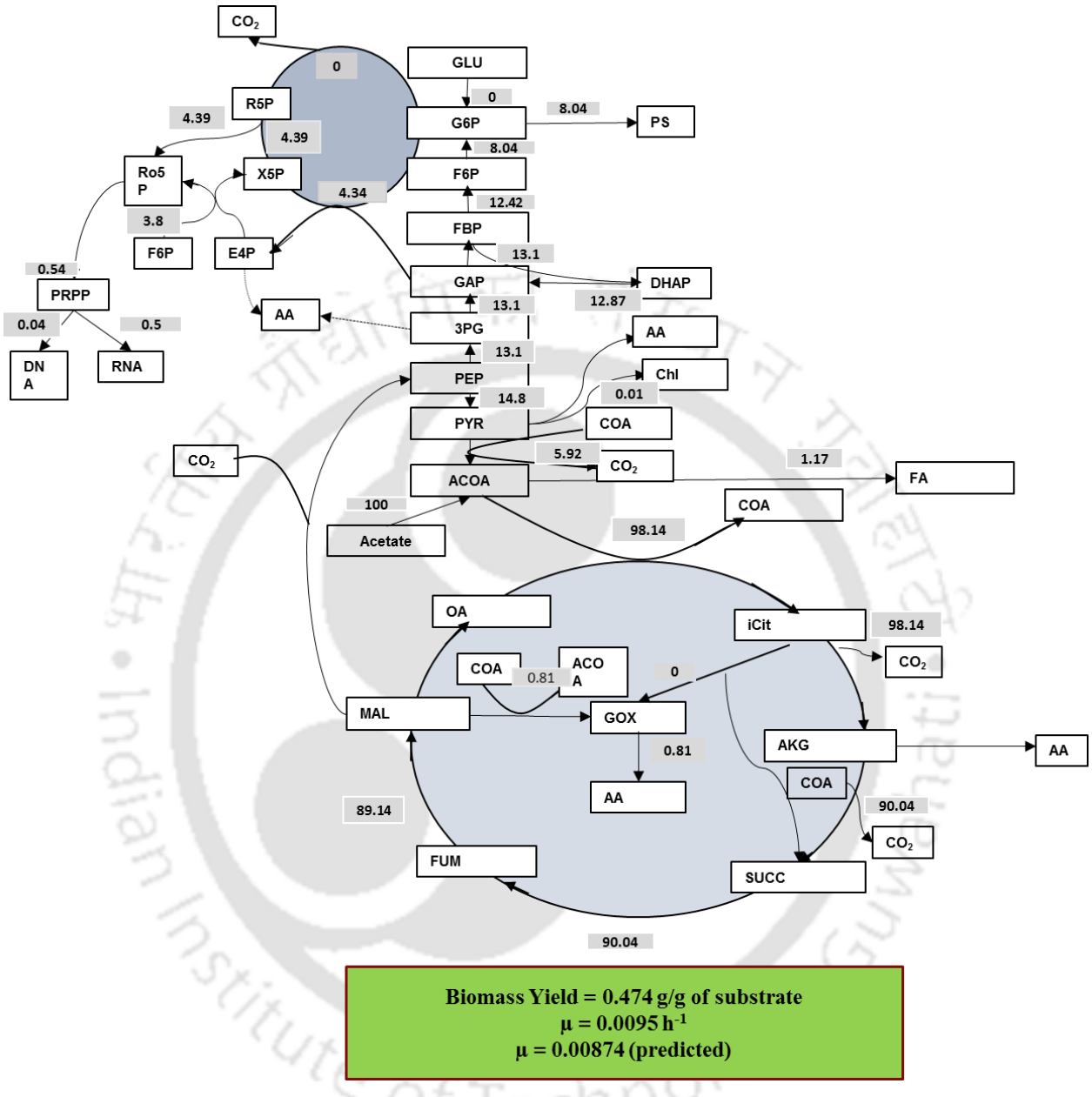


Fig. 4.17 Distribution of carbon flux in *C. vulgaris* on 7th day of culture under heterotrophic mode**.**

were, ace, Acetate; ACOA, Acetyl coenzyme A; AKG, α keto glutarate; Chl, Chlorophyll; Cit, Citricacid; CO_2, Carbon dioxide; COA, Coenzyme A; DHAP, Dihydroxy acetone phosphate; E4P, Erythrose 4 phosphate; F6P, Fructose 6phosphate; FA, Fattyacid; FBP, Fructose1, 6biphosphate; Fum, Fumarate; G6P, Glucose6phosphate; GAP, Glyceraldehyde 3 phosphate; Glu, Glucose; GOX, Glyoxalate; Mal, Malate; OA, Oxaloacetate; PEP, Phosphoenol pyruvate; PG, Phosphatidylglycerol; PRPP, 5phosphoribosyl1pyrophosphate; PS, Polysaccharide; Pyr, Pyruvate; R5P, Ribulose5phosphate; RNA, Ribosenucleicacid; Ro5P, Ribose5phosphate; Succ, Succinate; X5P, Xylulose 5 phosphate.

4.3 Photobioreactor study

Selection of suitable bioreactor is very important for scaling up of lutein production by microalgae. Split column photobioreactor and continuous stirred stank photobioreactors were employed in this study for lutein production by *C. vulgaris* using synthetic poultry digestate in batch and continuous mode of operation.

4.3.1 Split column photobioreactor and mixing kinetics

Mixing performance of an indigenously designed and fabricated split column photobioreactor's performance was first evaluated employing using tracer method at different superficial flow velocity (4.01×10^{-4} to 1.3×10^{-3} m/s). Mixing kinetics of the split column reactor was then evaluated using synthetic digestate as the liquid medium.

4.3.1.1 Mixing time and cycle time

The Blenke's equation was used to determin the Bodenstein number in order to calculate the axial dispersion coefficient (E_z), which indicates the kind of mixing such as plug flow or laminar flow. Figure 4.18 shows the measured and predicted tracer concertation using the Blenke's equation for the superficial flow velocity 4.017×10^{-4} m/s. The mixing time and cycle time were evaluated from the tracer concentration profile for different superficial flow velocity in the photobioreactor. Mixing time and cycle time profiles with respect to different superficial flow velocity are depicted in Figure 4.19 and 4.20, respectively. A decrease in mixing time was observed with increase in velocity, which is due to increase in power input through the fluid. Reduction in cycle time was observed with reduction in mixing time, which is due to increase in mixed head zone of the reactor. Both cycle time and mixing time values saturated above 6.52×10^{-3} m/s. Both these hydrodynamic parameters are dependent on the reactor geometry,

and an increase in dispersion height tends to reduce the mixing time irrespective of the superficial flow velocity (M. Y. Chisti & Moo-Young, 1988; Y. Chisti & Moo-Young, 1993). Several studies have been conducted for estimating mixing time in bubble column and draft tube split column reactors(M. Y. Chisti & Moo-Young, 1988; Petrovit et al., 1995), but there is no study yet on mixing time analysis in split column photobioreactor.

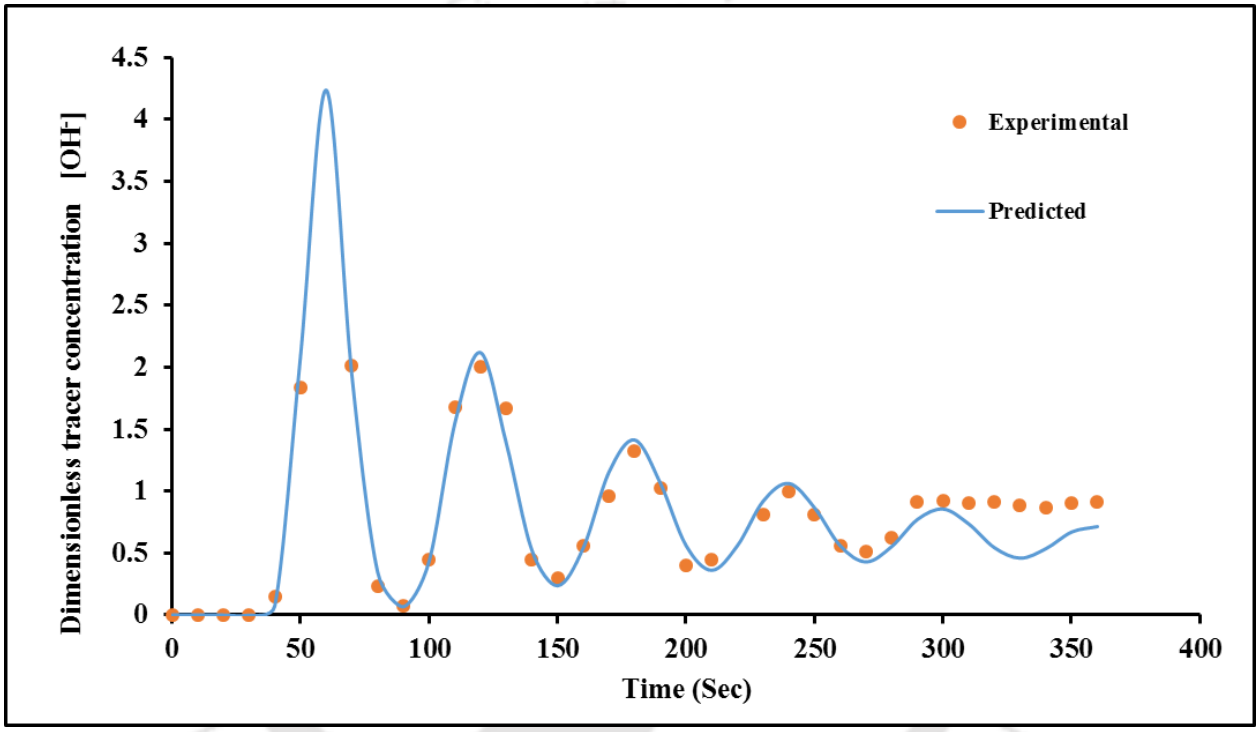


Fig. 4.18 Experimental and predicted values (Blenke's equation) of dimensionless tracer concentration with respect to the time at 4.017×10^{-4} m/s superficial flow velocity in the split column photobioreactor with synthetic poultry digestate as the liquid medium

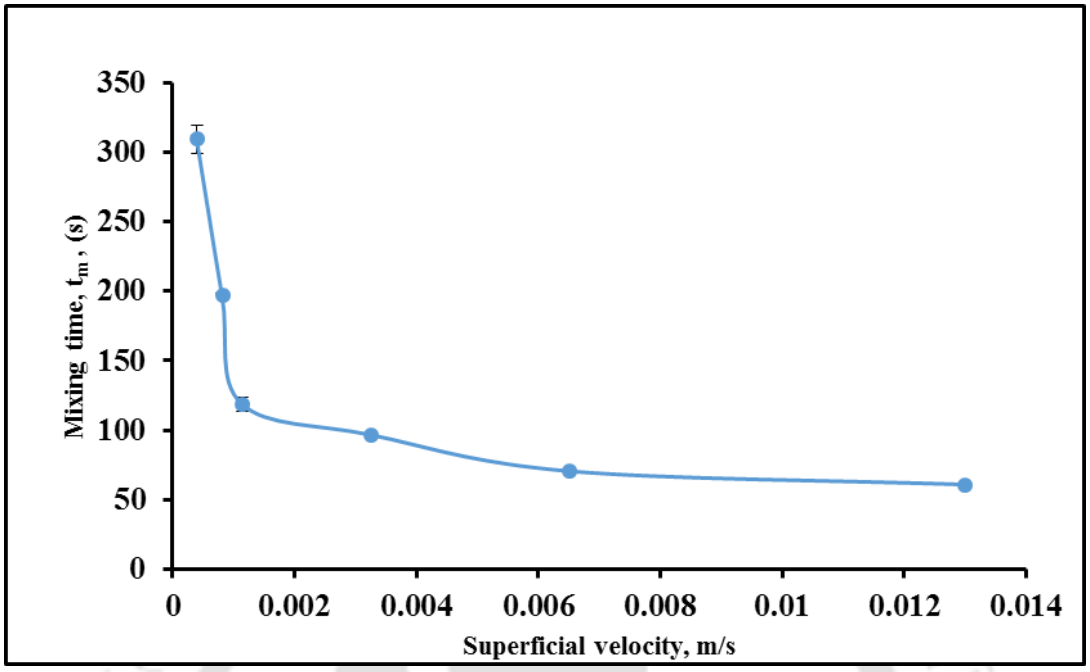


Fig. 4.19 Mixing time in split the column photobioreactor for different superficial flow velocity

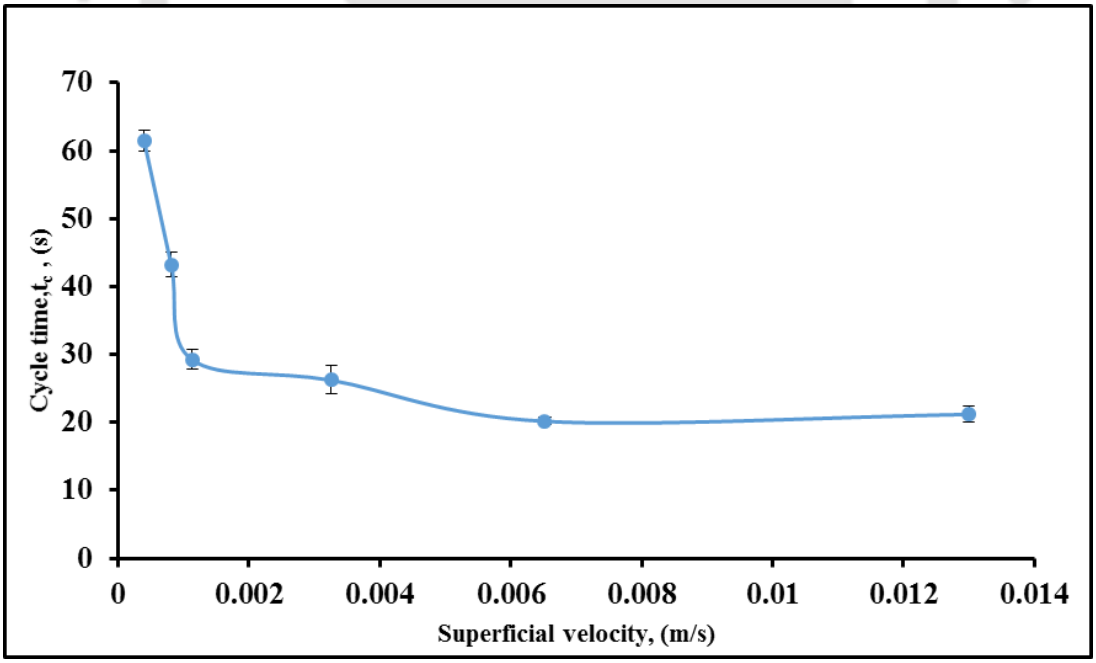


Fig. 4.20 Cycle time in the split column photobioreactor for different superficial flow velocity

4.3.1.2 Axial dispersion coefficient

The axial dispersion coefficient of the split column increased hyperbolically with increase in superficial flow velocity as shown in Figure 4.21. Similar trend was observed by Sanchez et al. (2004) in a draft tube airlift reactor with seawater. Plot shown in Figure 4.23 is based on the equation 3.8 (Bair and Rice, 1975), and it correlates the dispersion coefficient with Froude number. The predicted and the experimental data were in close proximity (R^2=0.958). Estimated value of the exponent to Froude number is 1.042, whereas the pre coefficient is 11.68. These model parameter values are in agreement with the data of Baird and Rice (1975). BO_{LG} values of airlift reactor correlates with its corresponding ratio of mixing time and cycle time as shown in Figure 4.22. The K value of the reactor was 15.75. Bodenstein number and Froude number define the kind of mixing and flow regimes of a fluid inside such reactors. Kinetics analysis of the fluid in the reactors are essential for design and evaluation of the reactors in computational modelling prior to scaling up operations.

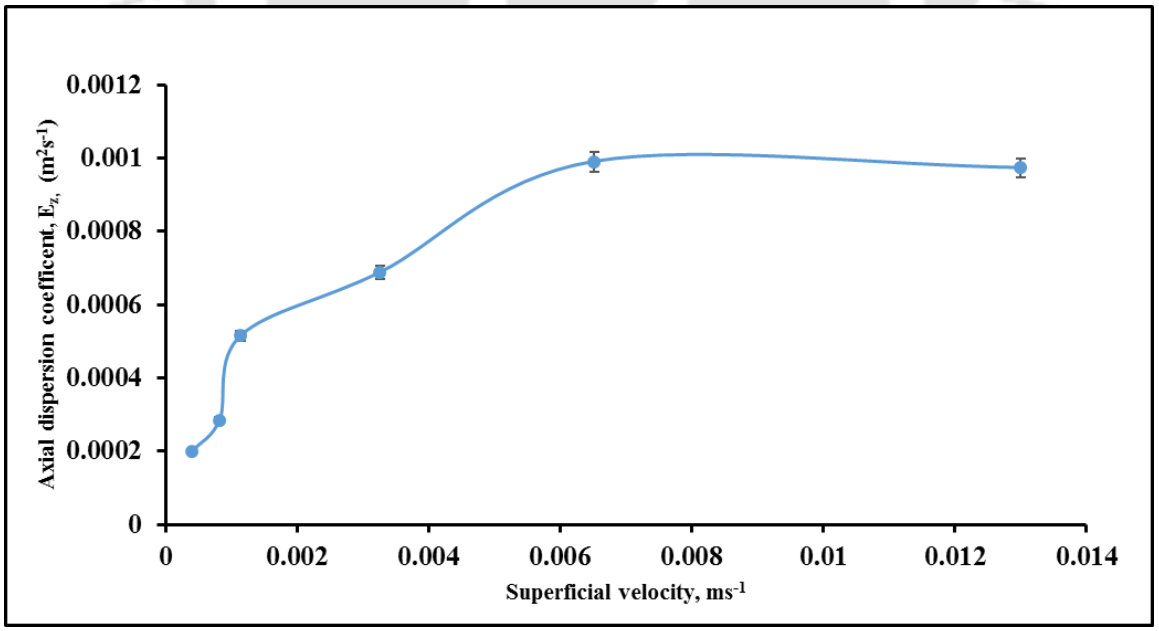


Fig. 4.21 Axial dispersion coefficient in the split column photobioreactor for different superficial flow velocity

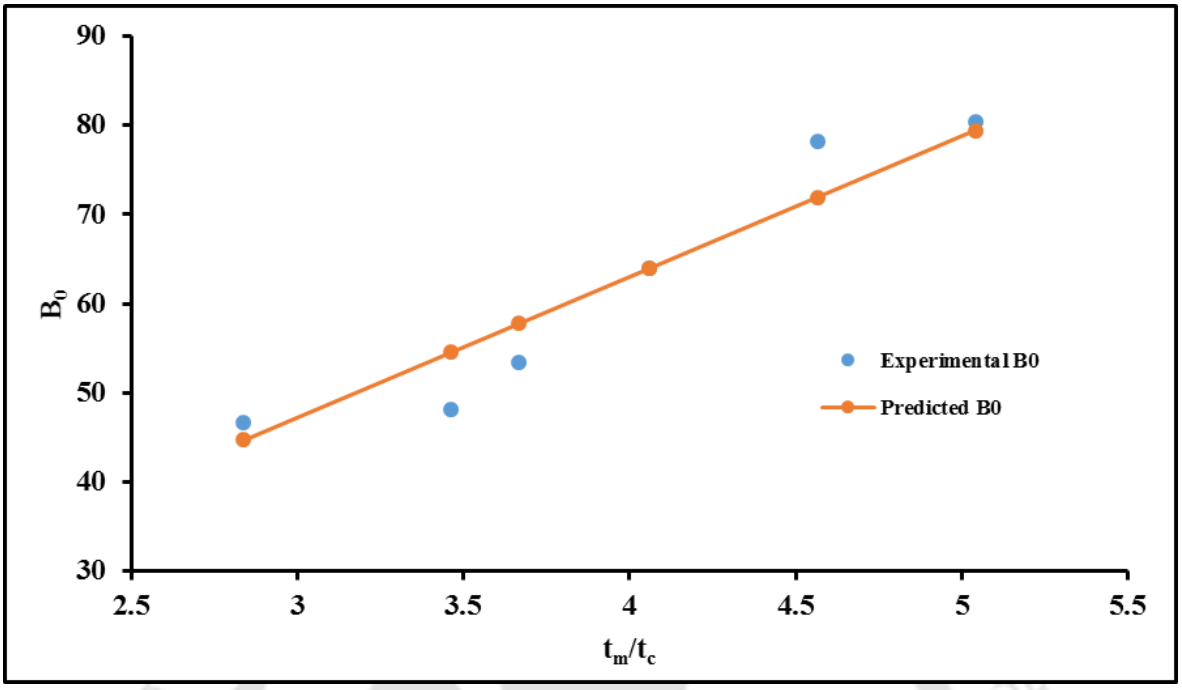


Fig. 4.22 Bodenstein number corresponding to different t_m/t_c ratio

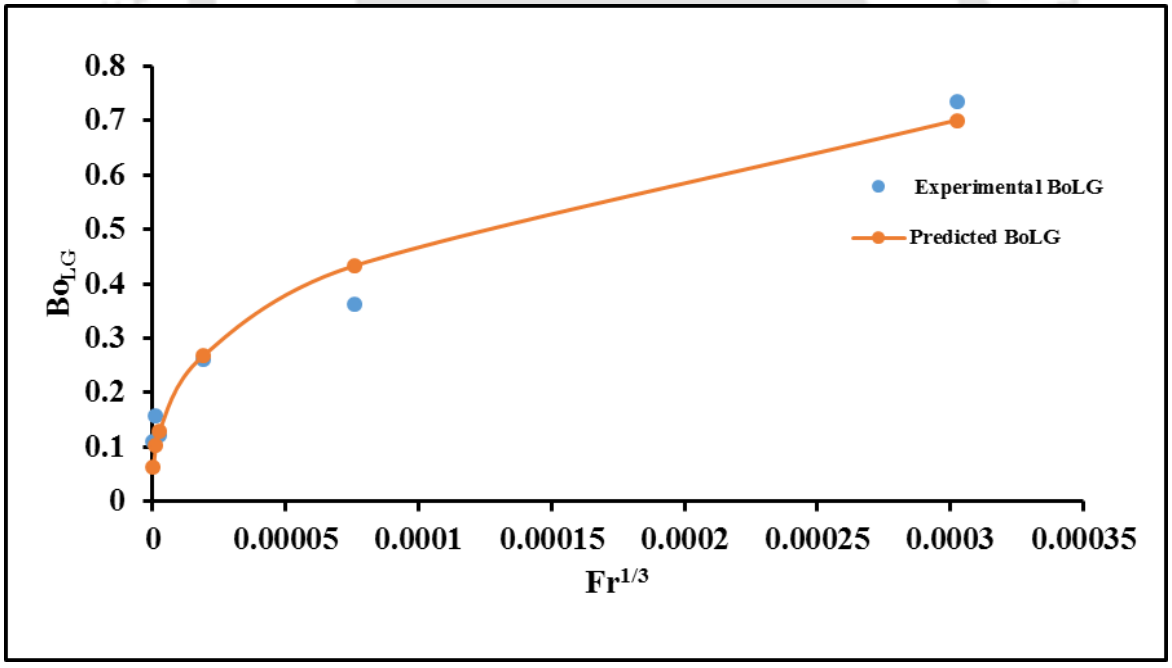


Fig. 4.23 Bodenstein number at different gas velocity Vs the Froude number

4.3.1.3 Lutein production

Table 4.16 presents the effect of airflow rate and light intensity in different combinations on lutein production by *C. vulgaris*, and the highest lutein production was obtained between 14th to 16th day. The highest lutein production (4.31 mg/L) was observed in the experiment number 4 performed at light intensity and airflow rate of 150 μ/m^2s and 1 L/L/min. The lutein production reduced to 2.27 mg/L at 250 μ/m^2s and 1 L/L/min which is due to photo inhibition effect. In experiment number 3 at 250 μ/m^2s and 2 L/L/min, 3.10 mg/L of lutein was produced due to increase in cycle time with respect to light which helped in overcoming the photo inhibition effect.

Light intensity is known to affect the microalgal growth and carotenoid content (Del Campo et al., 2004; Ho et al., 2014). Increase in light intensity beyond 200 μ/m^2s reduced the lutein concentration. Del Campo et al. (2004) and Xie et al. (2013) also reported that increase in light intensity from 150 to 750 μ/m^2s reduced the lutein concentration. Lutein is one of the integral part of light harvesting system, and under high light intensity the size of the light harvesting system tends to reduce which in turn reduces the lutein content in photosystem (Solovchenko and Merzlyak, 2008).

Table 4.16 Effect of light intensity and air flowrate in different combinations on lutein production by *C. vulgaris*

S. no	Light intensity (μ/m^2s)	Air Flow rate (L/L/ min)	Lutein (mg/L)
1	200	3	3.47
2	150	2	2.8
3	250	2	3.10
4	150	1	4.31
5	250	1	2.27

The split column airlift photobioreactor was initially operated under batch condition, and the maximum biomass growth of 0.96 g/L and lutein production of 4.379 mg/L were obtained at 336 h (Figure 4.24). Net biomass growth rate was 0.0102 h^{-1}, whereas the biomass and lutein productivity values were 0.0041 $gL^{-1}h^{-1}$ and 0.0116 $mgL^{-1}h^{-1}$, which are in agreement with previous studies involving marine microalgae (White et al., 2013). The substrate utilization profile at 432 h indicates that 4.29 g/L (95.33 %) of $NaHCO_3$, 82.38 mg/L (73.29 %) of NH_4^+, 8.41 mg/L (86.45 %) of PO_4^{3-}, 11.16 mg/L (11.6 %) of K^+ were utilized from the synthetic anaerobic digestate. The NH_4^+, PO_4^{3-} removal efficiencies are similar to the previous studies involving microalgae for treating anaerobic digestate from dairy manure and municipal waste (Uggetti et al., 2014; Wang et al., 2010).

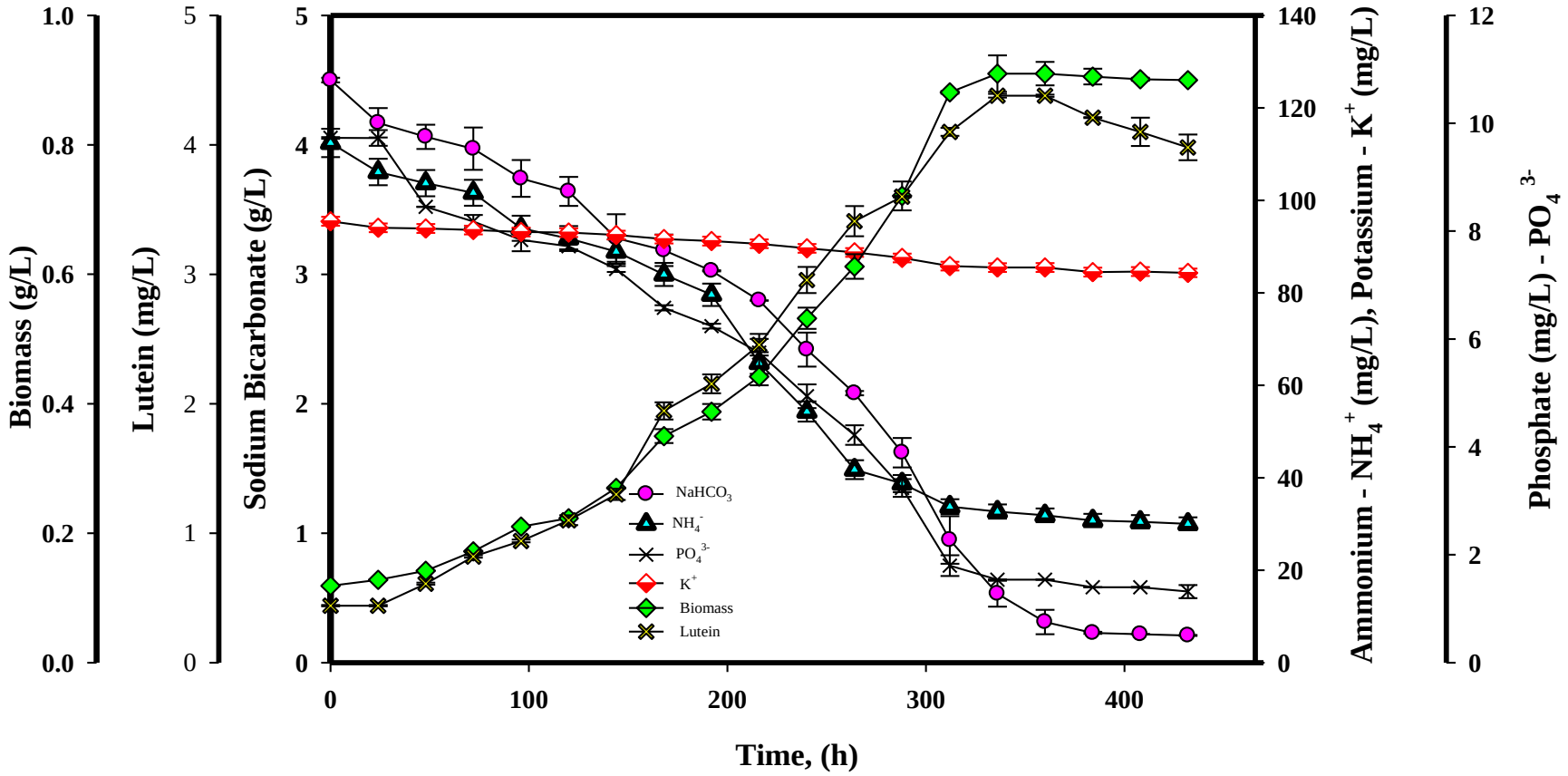


Fig. 4.24 Biomass and lutein production profile along with NH_4^+, K^+, $NaHCO_3$, and PO_4^{3-} utilization profiles in the split column photobioreactor operated in batch mode of operation

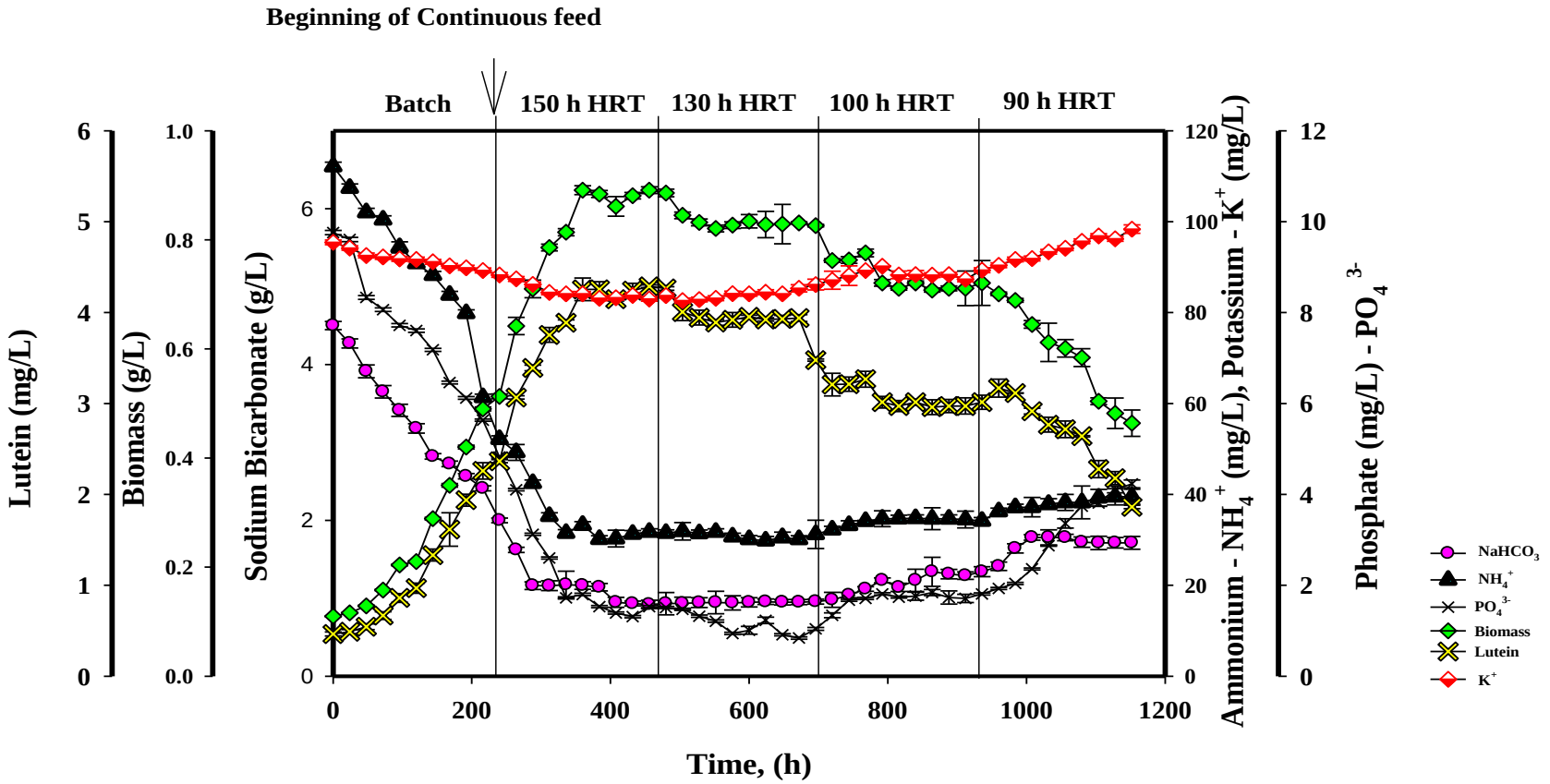


Fig. 4.25 Biomass and lutein production profile along with NH_4^+, K^+, $NaHCO_3$, and PO_4^{3-} utilization profiles in the split column photobioreactor operated in continuous operation mode

Continuous cultivation of the microalgae on the synthetic poultry litter digestate was performed at different HRT (150 -90 h) using split column photobioreactor, and the results are shown in Figure 4.25. Maximum amount of biomass (0.89 g/L) and lutein (4.25 mg/L) were observed at 150 h HRT. Reduction in biomass growth and lutein production was observed when the HRT was lowered but the productivity values were stable, except at 90 h HRT due to biomass washout. At 150 h HRT, the substrate utilization was constant, with the values, 79.53 % of $NaHCO_3$, 71.71 % of NH_4^+, 84.32 % of PO_4^{3-} and 13 % of K^+ from the feed containing synthetic anaerobic digestate (Figure 4.25). The biomass and lutein productivity values were 0.0058 $gL^{-1}h^{-1}$ and 0.026 $mgL^{-1}h^{-1}$. Elemental analysis of the biomass revealed that its CHNO ratio is 49.5:7.8:3.08:39.62, which corresponds well with the nutrient uptake values of 5.010 g of $NaHCO_3$ and 100.6 mg of NH_4 for producing 1 g of the biomass (Gabriel Acien Fernandez et al., 2012). This is the first work study on utilization of synthetic poultry litter anaerobic digestate in a photobioreactor for lutein production by microalgae other previous reports have mainly focused on treating such anaerobic digestate.

As compared to fresh water microalgae the biomass productivity in this study is low, which is common among extremophiles (Cassin, 1974; Tittel et al., 2005). As compared to the extremophile *Coccomyxa onubensis*, reported by Vaquero (2012) the productivity of lutein using *Chlorella vulgaris* 92001 in this study is 1.62 times higher using the split column photobioreactor operated under batch mode.

4.3.2 Lutein production in a continuously stirred tank photobioreactor

Using the continuously stirred tank photobioreactor (CSTPBR), effect of light intensity and airflow rate on lutein production by *C. vulgaris* was studied at a constant agitation speed. At a later stage,

agitation was varied to achieve maximum lutein production. The highest lutein concertation of 3.064 mg/L was obtained at 200 μ/m^{2}s light intensity and 0.5 L/L/min aeration. Increase in airflow rate along with reduction in light intensity decreased the light availability to the microalgae (S.no 2, Table 4.17). Effect of different agitation speed at 0.5 L/L/min and 200 μ/m^{2}s on lutein production by *C. vulgaris* revealed that the maximum lutein production was at 200 rpm (Table 4.18). Light energy requirement by the microalgae was higher in CSTPBR as compared to that in the split column airlift reactor for attaining same lutein concentration, which is attributed to the difference in hydrodynamic and photodynamic conditions of the two reactor systems.

Table 4.17 Effect of light intensity and air flowrate in different combinations on lutein production

S. no	Light intensity (μ/m^2s)	Air flowrate (L/L/min)	Lutein (mg/L)
1	200	0.5	3.064
2	150	1	2.689
3	250	1.5	2.712
4	150	2	2.34
5	250	2.5	2.914

Table 4.18 Effect of different agitation speed on lutein production by *C. vulgaris* at 0.5 L/L/min aeration and 200 μ/m^{2}s light intensity

S. no	AgitationSpeed (RPM)	Lutein (mg/L)
1	100	2.82
2	150	3.128
3	200	3.217
4	250	2.54
5	300	2.01

Under batch operation mode with CSTPBR maximum biomass growth (0.8160 g/L) and lutein production (3.128 mg/L) were obtained at 504 h (Figure 4.26). The net microalgal growth rate was 0.00539 h^{-1}, and the biomass productivity and lutein productivity values are 1.2×10^{-3} g/L.h and 4.9×10^{-3} mg/L.h. These values are lower than that observed previously with the split column photobioreactor. The substrate consumption profile showed that 2.926 g/L (65.02 %) of $NaHCO_3$, 63.26 mg/L (56.22 %) of NH_4^+, 7.695 mg/L (78.92 %) of PO_4^{3-}, 10.443 mg/l (10.91 %) of K^+ were utilized from the synthetic anaerobic digestate. The biomass growth and lutein production in CSTPBR was 15.625% and 28.568% lower than that in the split column airlift reactor used in this study, which might be due to increase in path length of the light in CSTPBR. Moreover, low surface area for illumination and mechanical agitation in the CSTPBR reduced lutein productivity by the microalgae. The change in biomass growth rate due to change in physiochemical parameters is also common in microalgal species.

CSTPBR was operated in continuous mode for 1272 h at four different HRT (220 – 160 h) and the results are shown in Figure 4.27. Maximum amount of biomass (0.7870 g/L) and lutein (2.7860 mg/L) were observed at 200 h HRT than the other HRTs. A drastic reduction in biomass growth and lutein production are observed at 160 h HRT due to biomass washout condition. At 200 h HRT, the steady state substrate utilization values were, 44.44 % of $NaHCO_3$, 50.28 % of NH_4^+, 62.35 % of PO_4^{3-} and 10.79 % of K^+. The biomass and lutein productivity values are 3.93×10^{-3} $gL^{-1}h^{-1}$ and 0.01393 $mgL^{-1}h^{-1}$, respectively.

Lutein content of *C. vulgaris* cultivated in batch modes and continuous modes of operation with the CSTPBR were 1.190 and 1.32 times lower than that using the split column photobioreactor. Ho et al. (2014) reported maximum lutein production of 4.61 mg/g and 1.58 mg/g using

Scenedesmus obliquus FSP-3 in stirred tank photobioreactor fed with Detmer's medium containing 8.0 mM of calcium nitrate and ammonium sulfate as the nitrogen source, in this current study yielded two times higher lutein content using the CSTPBR. Compared with the literature report, ammonium as the nitrogen source the lutein content is 2 times higher in stirred tank photobioreactor. Different species of microalgae utilize different sources of nitrogen for their growth, for example *Neochloris oleoabundans* growth rate is higher on nitrate than ammonium and urea (Y. Li et al., 2008), whereas *Ellipsoidion sp*. growth rate is higher with ammonia (Xu et al., 2001). Redox reaction and low energy requirement for assimilation makes ammonium a preferred nitrogen source for many microalgal species, but the assimilation of ammonium tends to reduce the pH of the medium which is the main factor in reducing the lutein yield (Ramanna et al., 2014). However, the negative effect of ammonium assimilation was avoided in this study by utilizing sodium bicarbonate as the substrate for the halophilic microalgae.

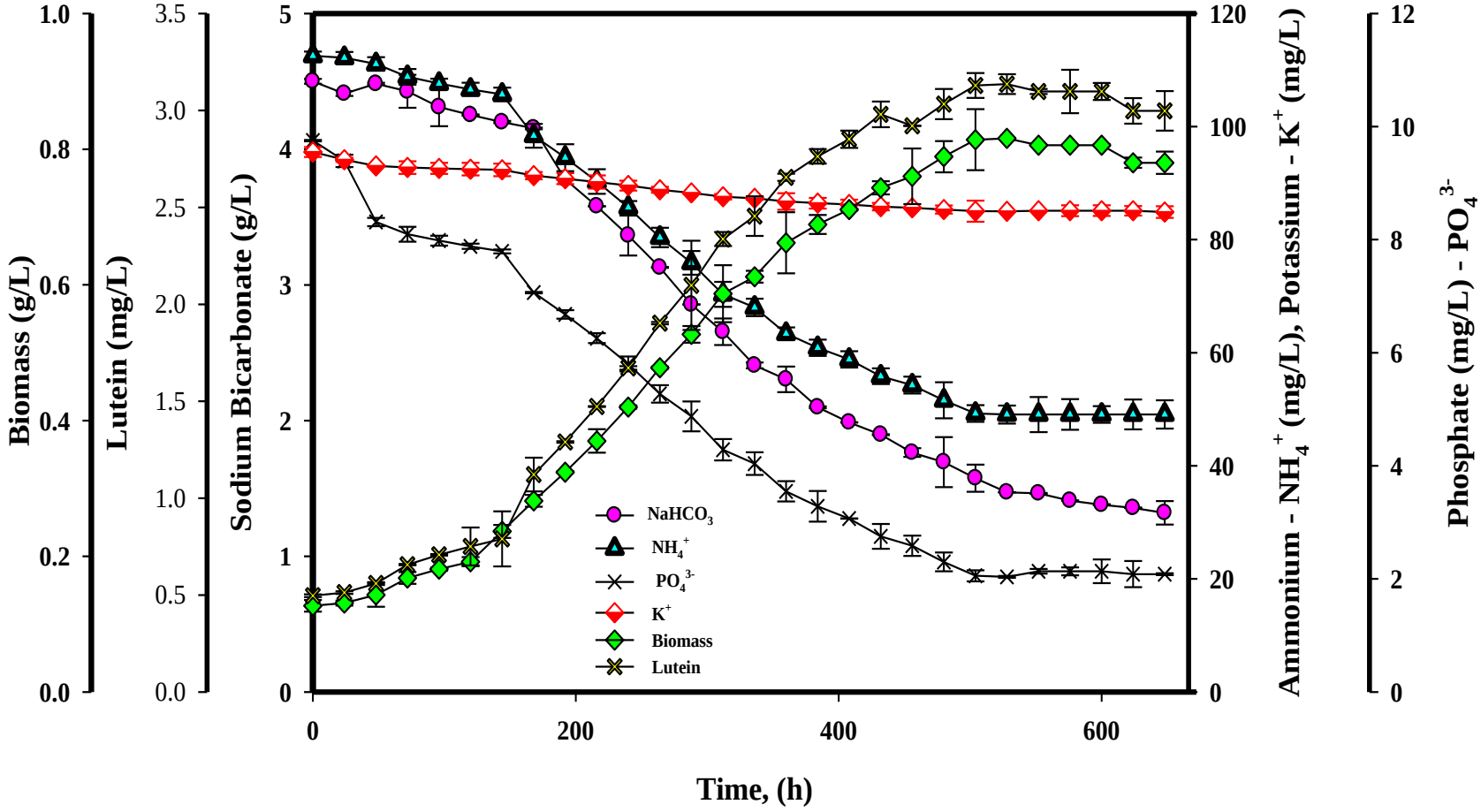


Fig. 4.26 Biomass and lutein production profile along with NH_4^+, K^+, $NaHCO_3$, and PO_4^{3-} utilization profiles in continuously stirred stank photobioreactors under batch mode

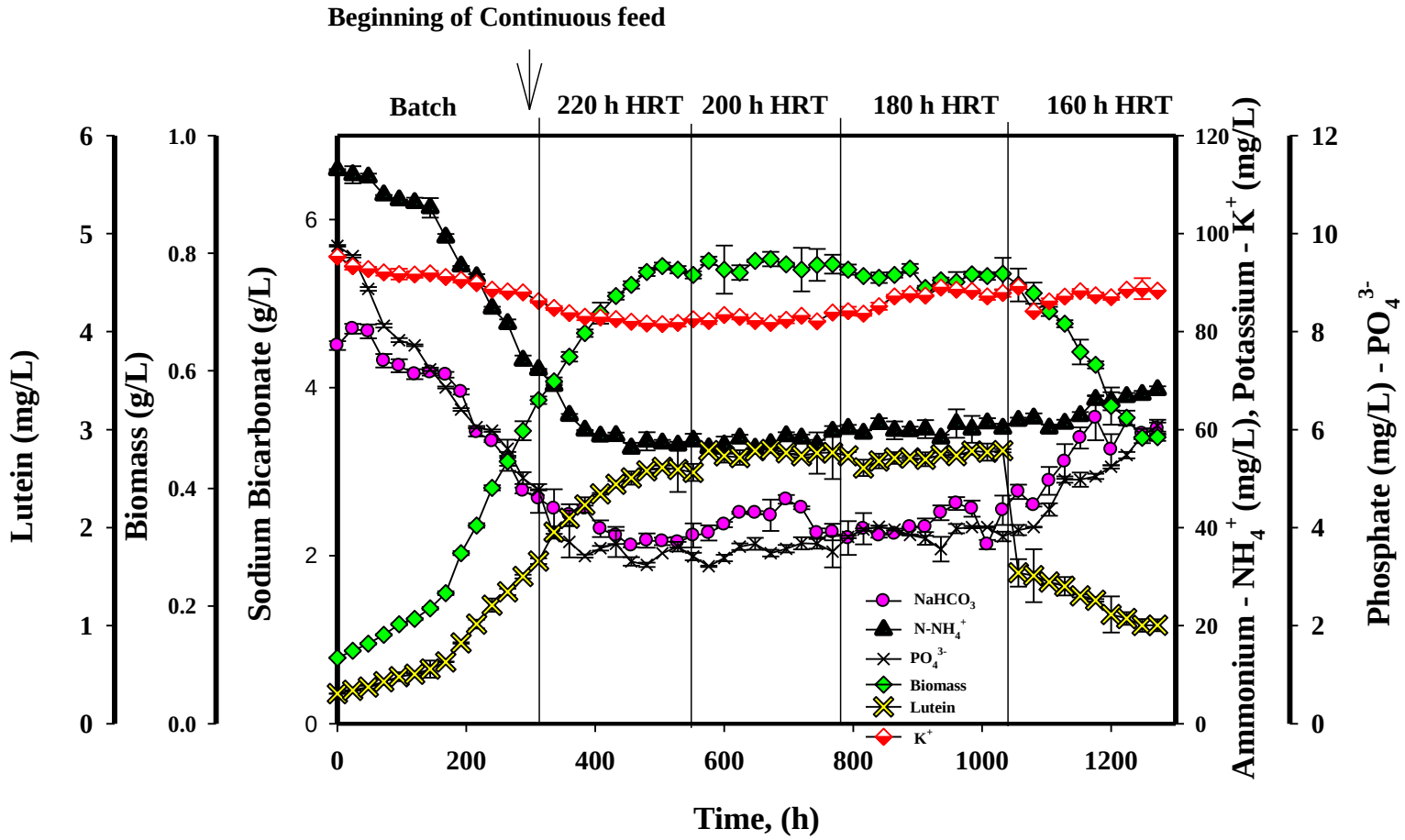


Fig. 4.27 Biomass and lutein production profile along with NH_4^+, K^+, $NaHCO_3$, and PO_4^{3-} utilization profiles in continuously stirred tank photobioreactors under continuous mode

4.4 Downstream processing strategies for lutein extraction and purification from *C. vulgaris*

Extraction and purification of lutein from microalgal biomass involve essential downstream processing steps, such as dewatering, cell disruption and purification (Utomo et al., 2013). In order to increase the recovery percentage and to reduce the energy consumption, selection of suitable downstream processing steps are essential for lutein production. In this study, electro coagulation flocculation (ECF) method for dewatering, ultrasonication for cell disruption and nanofiltration for lutein purification were applied.

4.4.1 Electro coagulation flocculation of *C. vulgaris*

Electronegative charge of the microalgae combined with a dilute concentration and small size of the biomass makes the dewatering process a difficult one in very short term. On the other hand, the use of external energy or chemicals tends to increase the cost of cell separation process (Branyikova et al., 2018; Lee et al., 2013). Cost of cell separation is about 20% of the total biomass production cost and it may increase upto 60% based on the algal species and other factors (Matter et al., 2019; Udom et al., 2013.). Some of the microalgal species can be concentrated by varying the pH and it is one of the cost effective method as compared to all other methods. However, autoflocculation of *C. vulgaris* in this study by varying the pH was not found to be effective. Hence, electrocoagulation flocculation for *C. vulgaris* separation from the culture broth was followed.

The electrode used in the electro flocculation process are sacrificial electrode, in which positively charged metals ions (M^{n+}) are released from the anode, which in turn tends to bind with negatively

charged microalgae to form floc (Figure 4.28). The current used in this process breaks down the s the water by electrolysis to form hydrogen microbubbles which then cause the algal floc containing metal ions to float on the water surface. The reaction involved in the anode and cathode compartment are show in the Table 4.19

Table 4.19 Reactions involved in anode and cathode parts of ECF

Anode	**Cathode**
$M \rightarrow M^{n+} + ne^-$ $2H_2O \rightarrow O_2 + 4H^+ + 4e^-$ $2OH^- \rightarrow O_2 + 2H^+ + 4e^-$	$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$ $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$ $2H^+ + 2e^- \rightarrow H_2 + 2OH^-$ $2H^+ + 2e^- \rightarrow H_2$
Final product from anode - H^+, M^{n+} and O_2 gas	**Final product from cathode - OH^- and H_2**

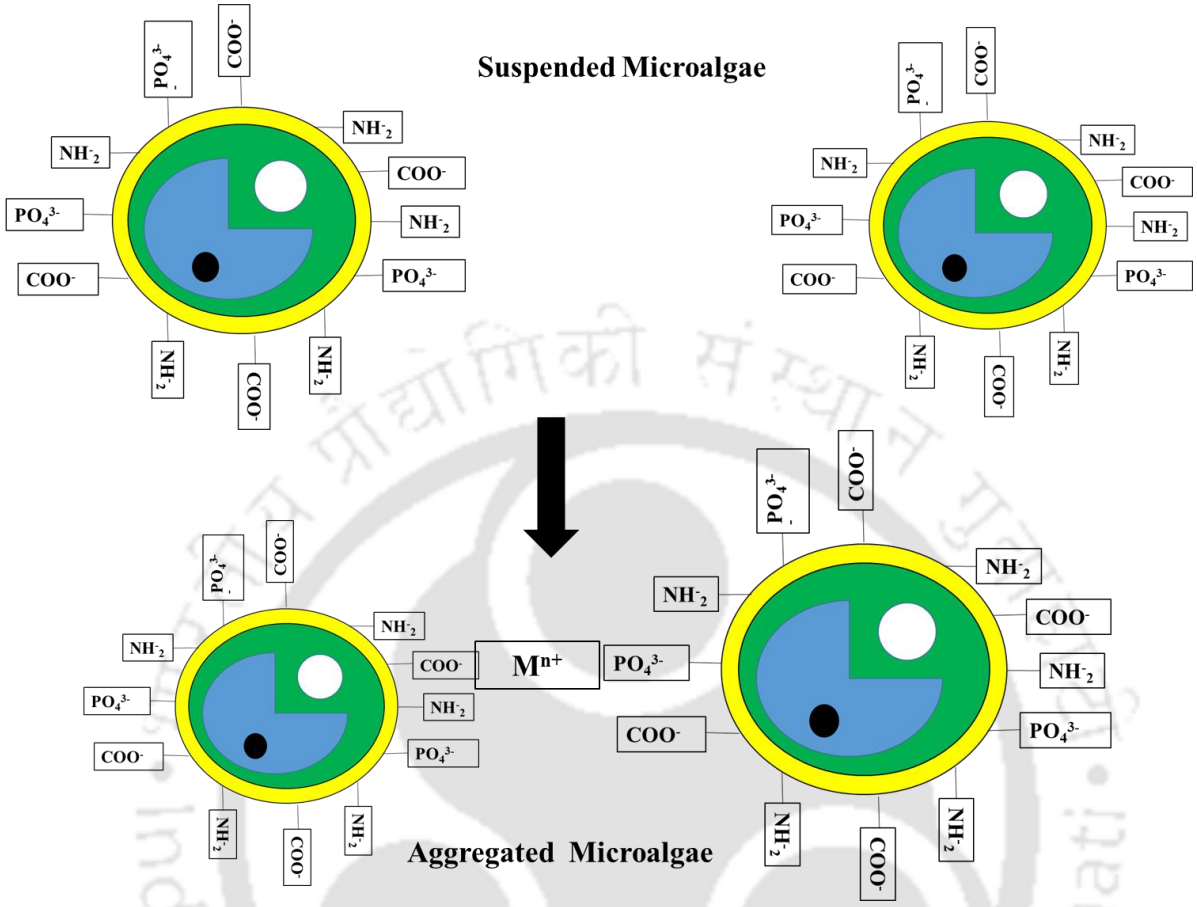
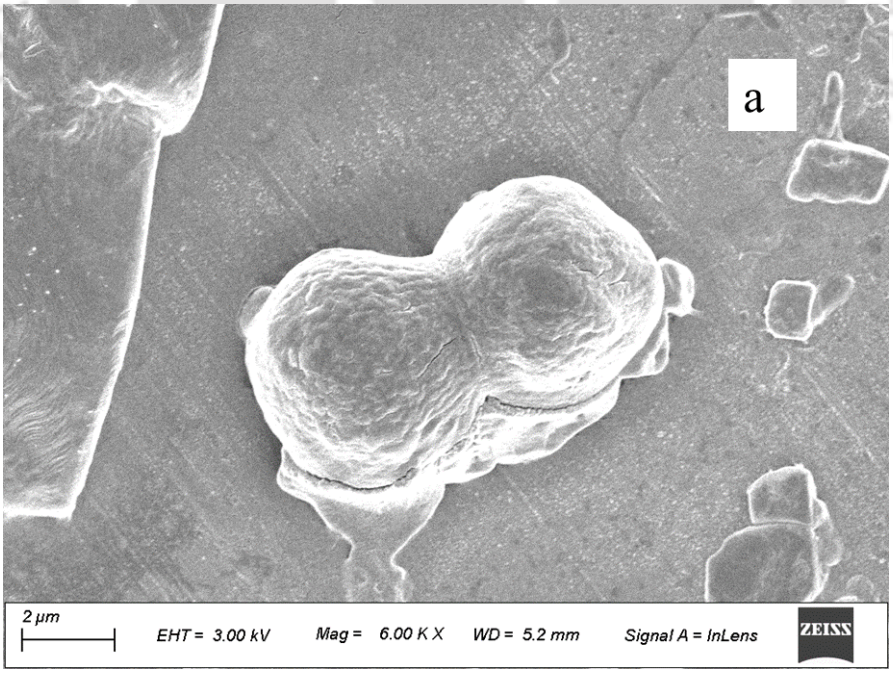


Fig. 4.28 Mechanism of flocculation of microalgal cells due to metal ions

In order to study the dewatering capacity by electro flocculation method, batch experiments were carried out. The effect of different electrodes (aluminium, copper, zinc and iron), current density (1.5 - 3.5 mA/cm^2) and recovery time (10 - 30 min) on the recovery of *C. vulgaris* lutein was studied. *Chlorella vulgaris* 92001 cells were separated from the culture media by using electroflocculation. Table 4.20 presents the value of biomass and lutein recovery by using different electrodes at different current densities for different treatment time.

The effect of electrodes in electroflocculation was significant as the biomass from the liquid media was higher with copper (98 and 98.56 %) and aluminium electrode (98.67 and 99.22 %) at 1.5 and

2.5 mA/cm^2 current density, respectively. The high cell recovery at a low current intensity are based on the conductivity of the electrodes and higher the conductivity of the electrode the recovery was found to be high. Conductivity of the electrodes used in this study are as follows copper (5.96×10^7 S/m) > aluminium (3.5×10^7 S/m) > zinc (1.69×10^7 S/m) > iron (1.00×10^7 S/m). High current intensity (3.5 mA/cm^2) reduced the cell recovery in aluminium and copper electrodes, whereas with zinc and iron the biomass recovery was unaffected due to their lower conductivity. As compared to a low current intensity (1.5 mA/cm^2) high current intensity (3.5 mA/cm^2) showed poor biomass recovery for aluminium and copper electrode (Table 4.20). Increase in treatment time at current intensities of 1.5 and 2.5 mA/cm^2 improved the biomass separation using the different electrodes. Formation of bleach or sodium hypochloride is an electrochemical phenomenon when high current is applied in water containing high sodium chloride. The bleach formation tends to reduce the microalgal floc as reported by Gao et al. (2010).



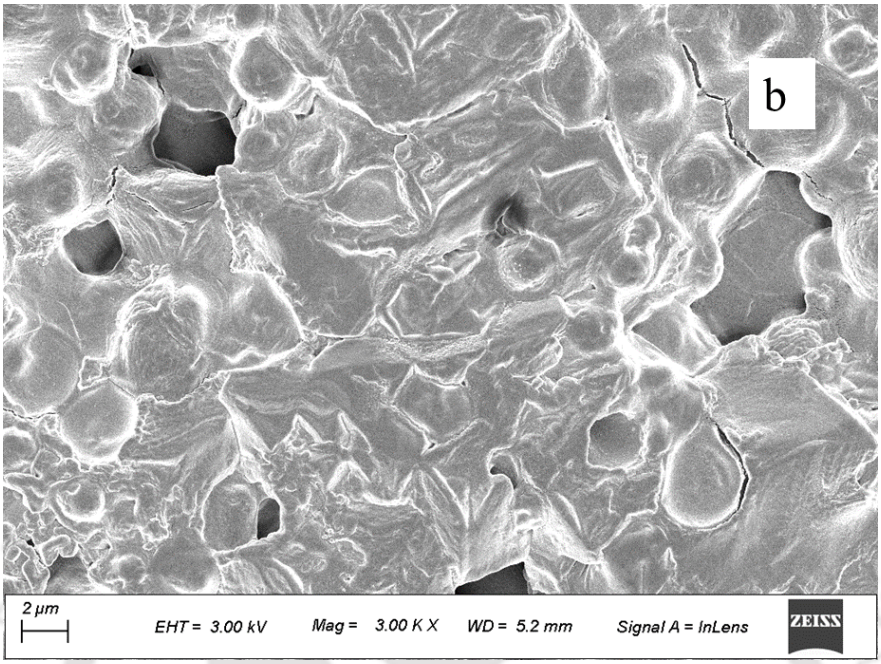


Fig. 4.29 FESEM images of *C. vulgaris* biomass (a) before electrocoagulation and (b) after electrocoagulation using aluminium electrode

Table 4.20 Effect of various process parameters on ECF for recovery of *C. vulgaris* biomass and lutein

Electrode	Time (min)	*C. vulgaris* biomass and lutein recovery at different current densities					
		1.5 mA/Cm^2		2.5 mA/Cm^2		3.5 mA/Cm^2	
		C. vulgaris biomass recovery (%)	Lutein Recovery (%)	*C. vulgaris* biomass recovery (%)	Lutein Recovery (%)	*C. vulgaris* biomass recovery (%)	Lutein Recovery (%)
Aluminium	**10**	95.22	92.05	97.00	90.86	87.00	85.00
	20	97.00	91.83	98.56	87.31	83.00	69.38
	30	98.00	90.81	98.56	83.33	81.00	66.07
Zinc	**10**	86.22	85.45	90.00	84.71	93.00	81.48
	20	90.00	83.48	92.56	83.05	91.56	82.76
	30	95.00	86.55	93.22	82.83	91.22	82.02
Copper	**10**	96.00	87.98	99.22	88.93	92.44	84.86
	20	98.00	87.83	99.11	83.86	90.22	80.86
	30	98.67	82.90	98.89	78.86	89.00	70.86
Iron	**10**	65.00	71.67	70.11	69.19	78.22	64.17
	20	69.00	75.24	74.78	73.83	82.11	68.10
	30	71.44	80.74	77.11	76.10	88.11	70.48

Figure 4.29 shows FESEM images of the microalgae prior to treatment by ECF and after the treatment. In Figure 4.29 (a) shows dispersed biomass in liquid, whereas in figure 4.29 (b) the cells are found in agglomerated form along with aluminium hydroxide to form flocs on the liquid surface. Lutein recovery was also high with aluminium (92.05 %) and copper electrode (88.93 %) at 10 min treatment time and current density of 1.5 mA/cm^2 and 2.5 mA/cm^2, respectively. Increase in the current density or recovery time reduced the lutein recovery, and this is due to heat induced damage from electrochemical reactions. Reduction in lutein recovery was observed with all the electrodes at 10 min and at a high current density. Maintaining high recovery of both biomass and lutein is very important in ECF process as it is evident from the experimental results that 10 min treatment time is found to be the best in this study with copper or aluminium as the electrode. However, due to high cost of copper, aluminium electrode is suitable for the ECF process.

Energy consumption in the ECF process with aluminium electrode was calculated and the results are presented in the Table 4.21. With an increase in the treatment time and current density the power consumption for recovering the biomass also increased. For a current density of 1.5 mA/cm^2 and 10 min treatment time, the energy consumption was 0.27 KW/kg of biomass which was the lowest in this study with the aluminium electrode in the process. The energy consumption values with aluminium electrode closely matches with that reported by Vandamme et al. (2011) for recovering marine algal biomass by ECF. The power consumption for recovering algal biomass from sea water is 10 – 12 times lower than that for fresh water microalgae due to the high salt content, which tends to enhance the electrical conductivity (Lee et al., 2013) for an easy separation. Though increase in current density from 1.5 to 2.5 mA/cm^2 enhanced the biomass recovery, the energy consumption was also high. The power consumption reported for biomass separation by

centrifugation is 16 KW/kg (Danquah et al., 2009; Vandamme et al., 2011). Utilizing the low current densities for short term not only reduces the power consumption in ECF process, but also reduces the formation of aluminium hydroxide and minimize the deposit of aluminium onto the biomass by less than 1% (Fayad et al., 2017; Vandamme et al., 2011)

Table 4.21 Power consumption for biomass recovery by ECF with aluminium electrode for different treatment time

Current density (mA/cm^2)	**Energy consumption for different time (KW/kg of biomass)**		
	10 min	**20 min**	**30 min**
1.5	0.27	0.31	0.40
2.5	0.42	0.67	0.94
3.5	0.56	0.87	1.2

4.4.2 Lutein recovery by ultrasonication

Cell disruption can be achieved through several methods including sonication, bead beating, microwave, osmotic shock, enzymatic and chemical treatment, but these methods have their own advantages and disadvantages (Lee et al., 2017.; Prabakaran and Ravindran, 2011). Sonication does not involve high temperature unlike autoclave and microwave methods, which tend to degrade the lutein. Furthermore, ultrasonication does not need any external agents like beads and NaCl which interfere with the purification process. In addition, ultrasonication can be scaled up for continuous operation.

Effect of different time and biomass concentration on cell disruption for lutein recovery by *C. vulgaris* was studied and the results are shown in Figure 4.30. Biomass concentrations chosen for this study were 0.125, 0.250, 0.50, 0.75 g/15mL that are easily obtained by the electro flocculation process. At a low cell concentration (0.125, 0.250 g/15mL) lutein recovery was higher at a short treatment time (2.5 and 4.5 min), than at a prolonged treatment time (6.5 and 8.5 min) had shown reduced lutein recovery. At a high biomass concentration (0.50, 0.75 g/15mL) lutein recovery was also high than at a low biomass concentration. Formation of free radicals due the sonication process is reported to reduce lutein recovery from such microalgal biomass (Gerde, 2012). The maximum lutein recovery was 95.42% for biomass concentration of 0.5 mg/15 mL at 4.5 min treatment time. Light microscopic images of the microalgae before and after treatment by sonication are shown in Figure 4.32, which reveals the control cells without any treatment and disrupted cells using ultrasonication.

Figure 4.31 represents the energy utilized during sonication of *C. vulgaris* biomass at different set of experiments with different cell concentration and treatment time. For all the four different cell concentrations, the energy consumed was almost similar but the lutein recovery was different. Energy consumed for maximum lutein recovery by sonication was 5351 J/15 ml.

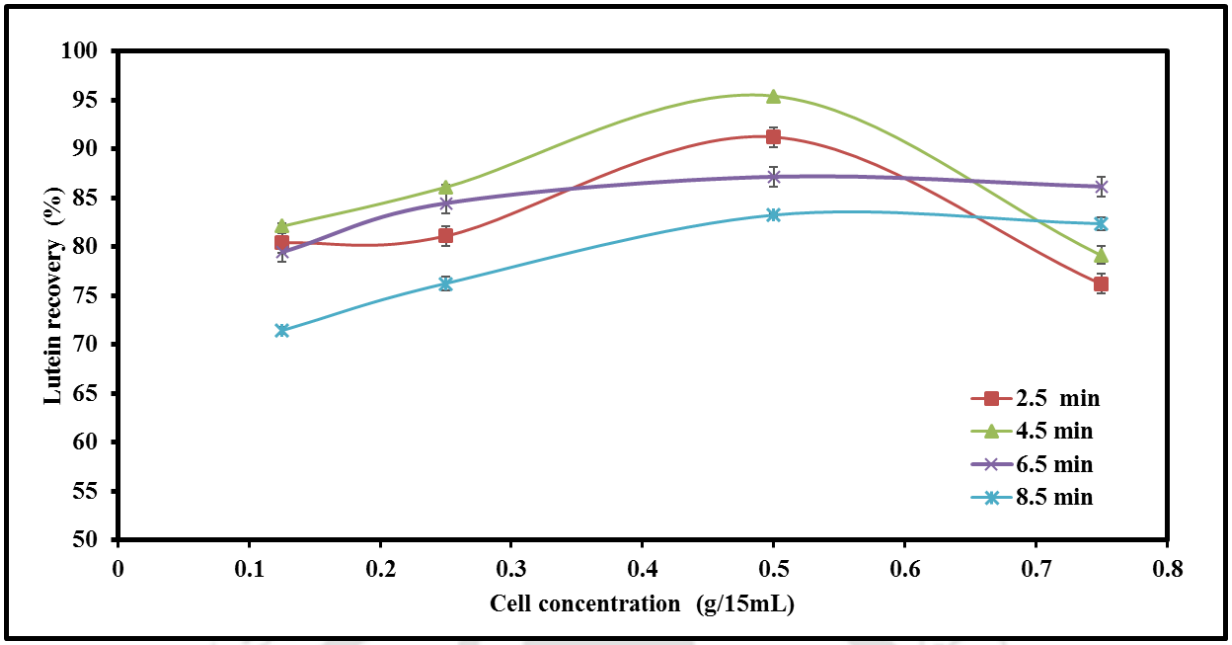


Fig. 4.30 Effect of different biomass concentration on lutein recovery from *C.vulgaris* at different treatment time

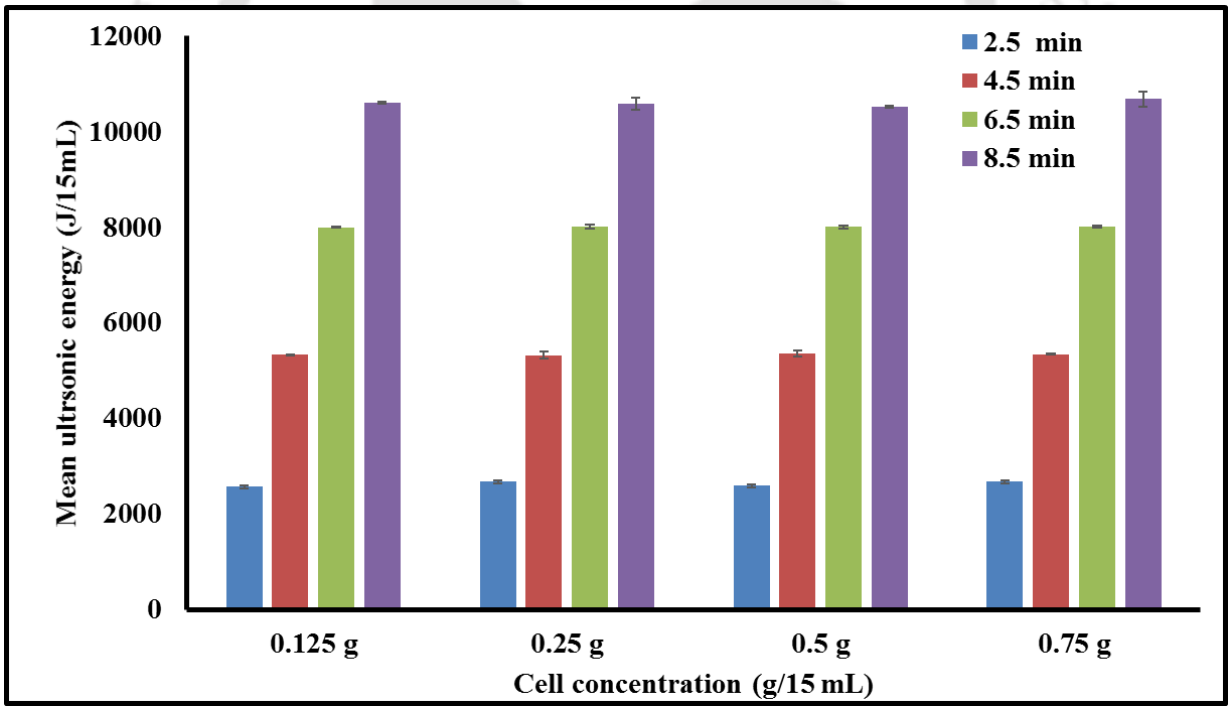


Fig. 4.31 Energy utilized during sonication of *C. vulgaris* at different biomass concentration and for different treatment time

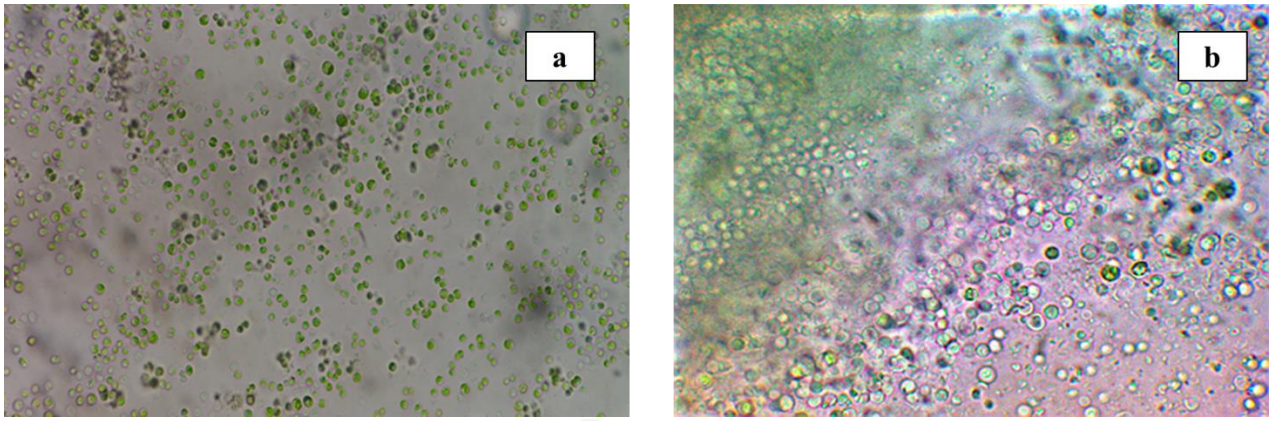


Fig. 4.32 Microscopic image of microalgae (a) Before treatment (b) After treatment using sonication

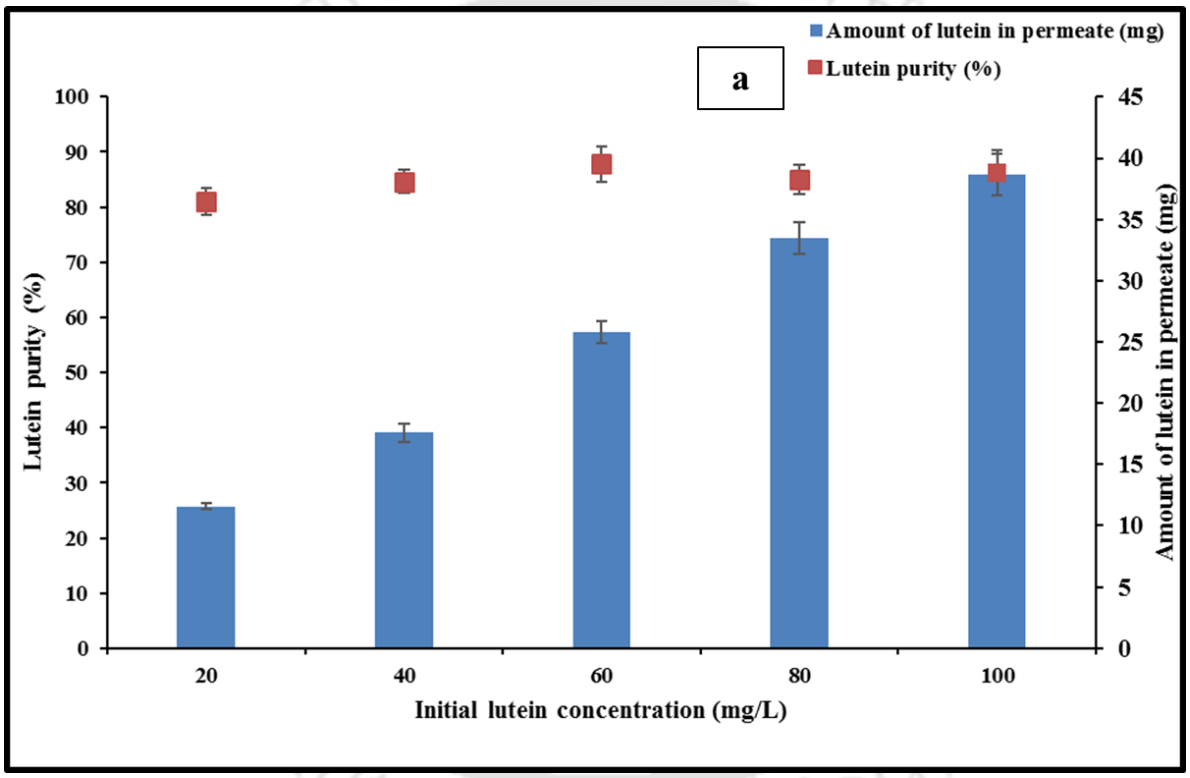
4.4.3 Purification of lutein by nanofiltration

In order purify lutein obtained from *C. vulgaris* biomass by ultrasonication followed by nanofiltration was carried out using dead end filtration. The effect of different lutein concentration in the crude extract and the cut off size of the nanofilters were initially examined, and then the effect of applied pressure on amount of lutein in permeate and purity was studied.

4.4.3.1 Effect of lutein concentration and membrane cut off

Effect of different concentration of lutein in the range 20 -100 mg/L in the ethanol extract and different filter cutoff (600 - 800 and 800 -1000 Da) on amount of lutein in permeate and purity was studied and the results are depicted in Figure 4.33. A high concentration of lutein in the crude extract reduced its separation efficiency with both the filters. Maximum amount of lutein in permeate was observed using 800-1000 Da filter (89.34%), whereas only 57.84 % of lutein was separated with 600 – 800 Da at 20 mg/L. Larger membrane molecular weight cutoff thus enhanced the lutein separation efficiency as the filtration process is mainly based on the principle of sieving. A similar trend in separation efficiency was reported by Pesheva et al. (2011) for treating rosemary by nanofiltration using different membrane cutoff. Use of high cutoff membrane tends to increase the flux as compared to lower cutoff of membrane, which

provides significant advantage in reducing the treatment time (Peshev et al., 2011; Tzibranska et al., 2009). Increase in lutein concentration in the extract reduced the lutein recovery but at a high concentration in the range 40 -100 mg/L, the lutein purity was almost same for all the concentrations. The main impurities while extracting lutein from microalgae are chlorophyll (893.51 Da) and other cell molecules which are larger than 1000 Da. These impurities are effectively retained in the nanofilter used in this study, thereby improving the lutein purification efficiency.



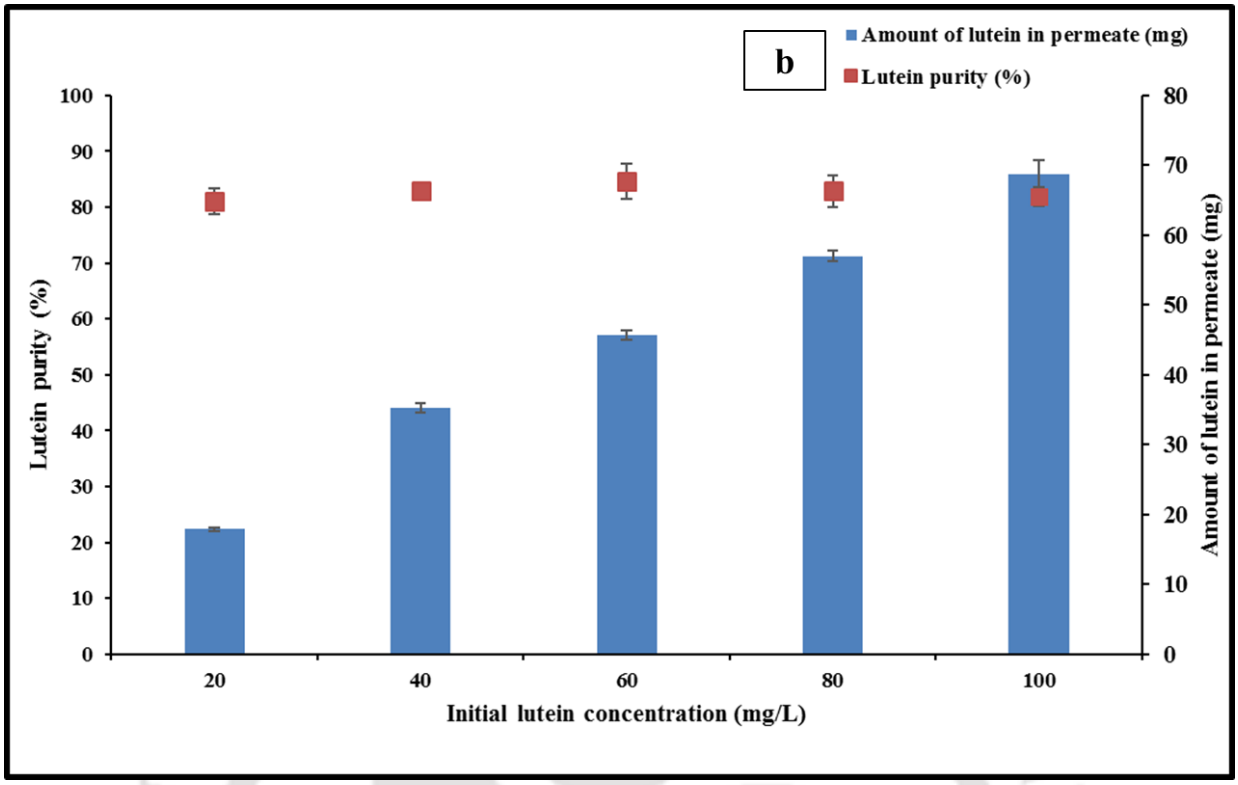


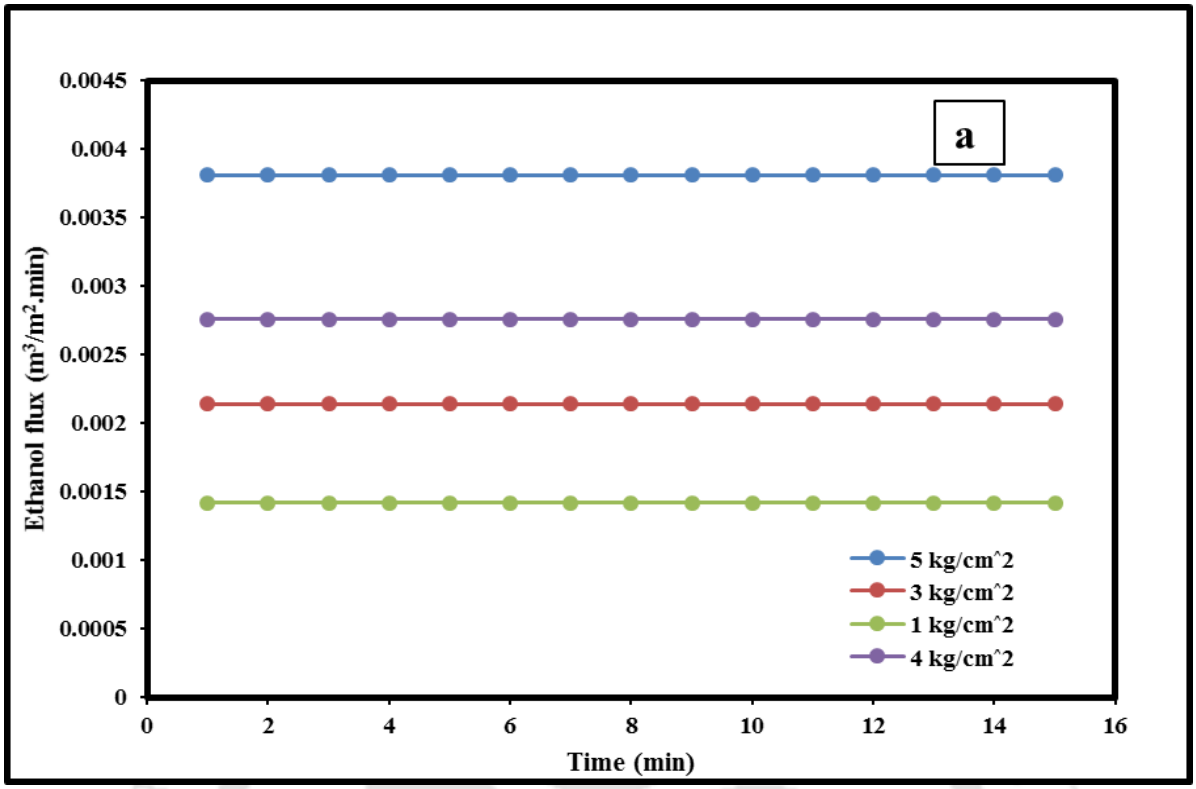
Fig. 4.33 Effect of different molecular weight cutoff membranes on nanofiltration for purification of lutein from ethanol extract at different initial concentrations of lutein (a) 600 - 800 Da (b) 800 -1000 Da

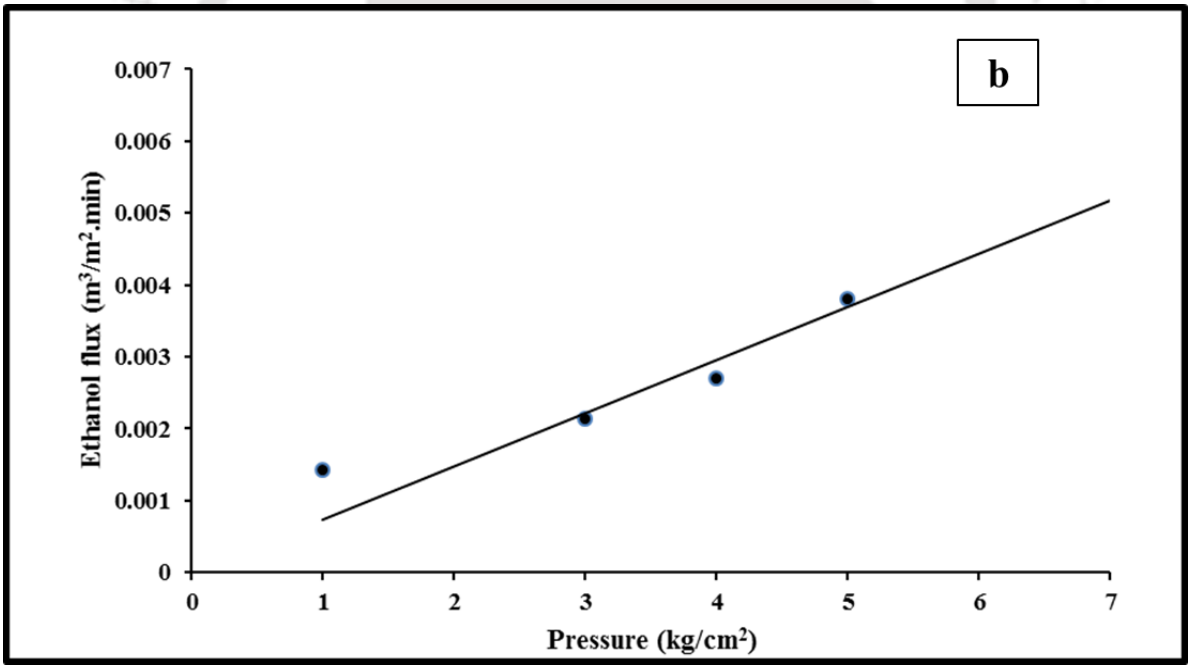
4.4.3.2 Effect of different pressure on nanofiltration

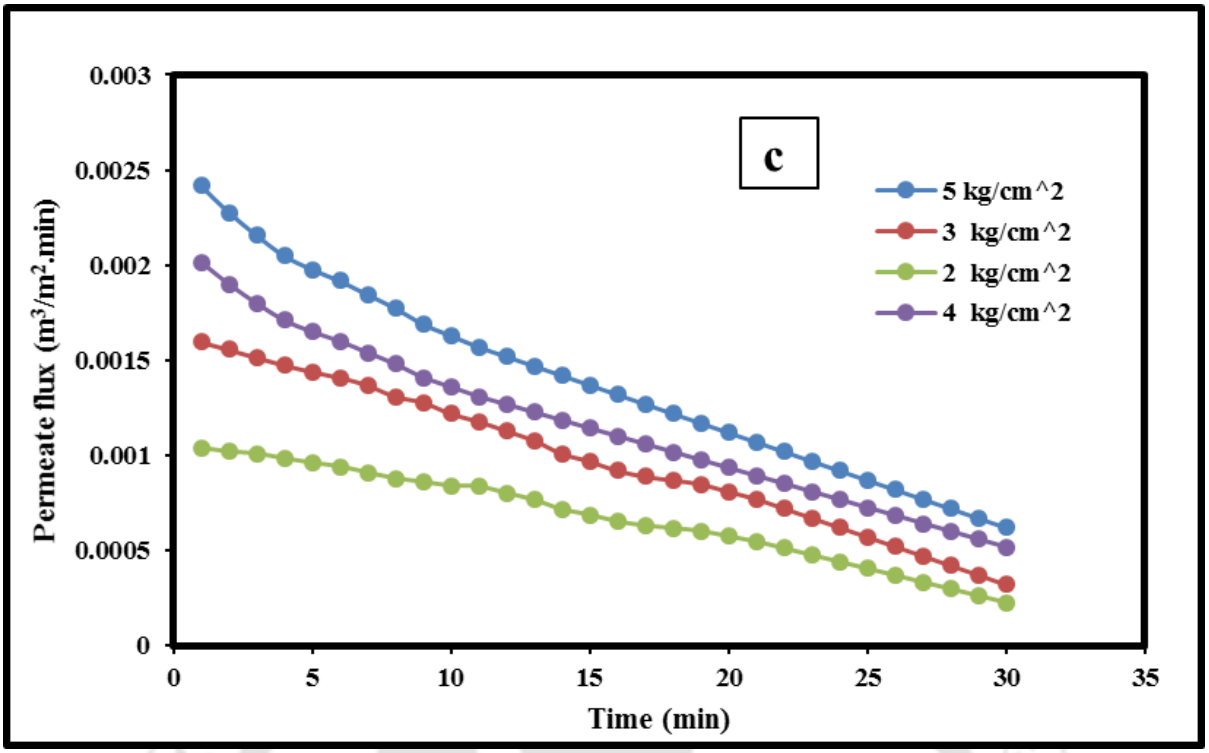
Based on the results from the previous experiments, nano filter with 800 to 1000 Da molecular weight cutoff was selected to study the effect of different applied pressure on the filtration. Nanofilters used in this study was made using polyamide and are resistant to methanol and ethanol. The maximum solubility of lutein in methanol is 200 mg/L, whereas in ethanol its solubility is 300 mg/L (Craft and Soares, 1992). Also, due to toxicity of methanol, ethanol was used in this study. The results of ethanol (89% v/v) flux and permeate flux for ethanol containing lutein (40 mg/L) at the different applied pressure are shown in the Fig. 4.34 (a, b, c). Increase in applied pressure enhanced the flux value, and the values are constant for the ethanol flux, but the flux values of ethanol containing lutein were found to reduce with respect to time. Increase in the applied pressur from 2 to 5 kg/cm^2 showed an increase in lutein

recovery, and the maximum lutein obtained in the permeate was 98.76 % at the applied pressure 5 kg/cm^2 (Figure 4.34d). Lutein purity values were 83.36, 84.84, 84.32 % for the applied pressures 2, 3, and 4 kg/cm^2 respectively. At the applied pressure 5 kg/cm^2 lutein purity was reduced by 12.3 %, which is due to the entry of impurities at a high applied pressure. FETEM image of ethanol extract before and after nanofiltration revealed that the particle size within the range 5 – 20 nm were present prior to the nanofiltration, after filtration particle with uniform size (~5 nm) were present in the permeate (Figure 3.35). EDX of the filtered sample had shown 92 % carbon and 8% oxygen content which is closer to the carbon and oxygen percentage in lutein.

Several studies are available on nanofiltration for extracting phytochemical's from plants in pure form and for removal of specific pollutants from wastewater (Geens et al., 2007; X. Li et al., 2008; Tzibranska et al., 2009; Yao et al., 2016) but there is no reports yet on nanofiltration for purifying lutein. Use of solvents with high lutein solubility such as a dichloromethane, diethyl ether, chloroform for extraction and potassium hydroxide for hydrolysis of lutein ester is known to damage the polyamide membrane. Hence, membrane modification to improve resistance towards such solvents for lutein extraction can further be explored to improve the robustness of the membrane separation process.


a
Ethanol flux (m3/m2.min)
0.0045
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
2
4
6
8
10
12
14
16
Time (min)
5 kg/cm^2
3 kg/cm^2
1 kg/cm^2
4 kg/cm^2


b
Ethanol flux (m3/m2.min)
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
0
1
2
3
4
5
6
7
Pressure (kg/cm2)



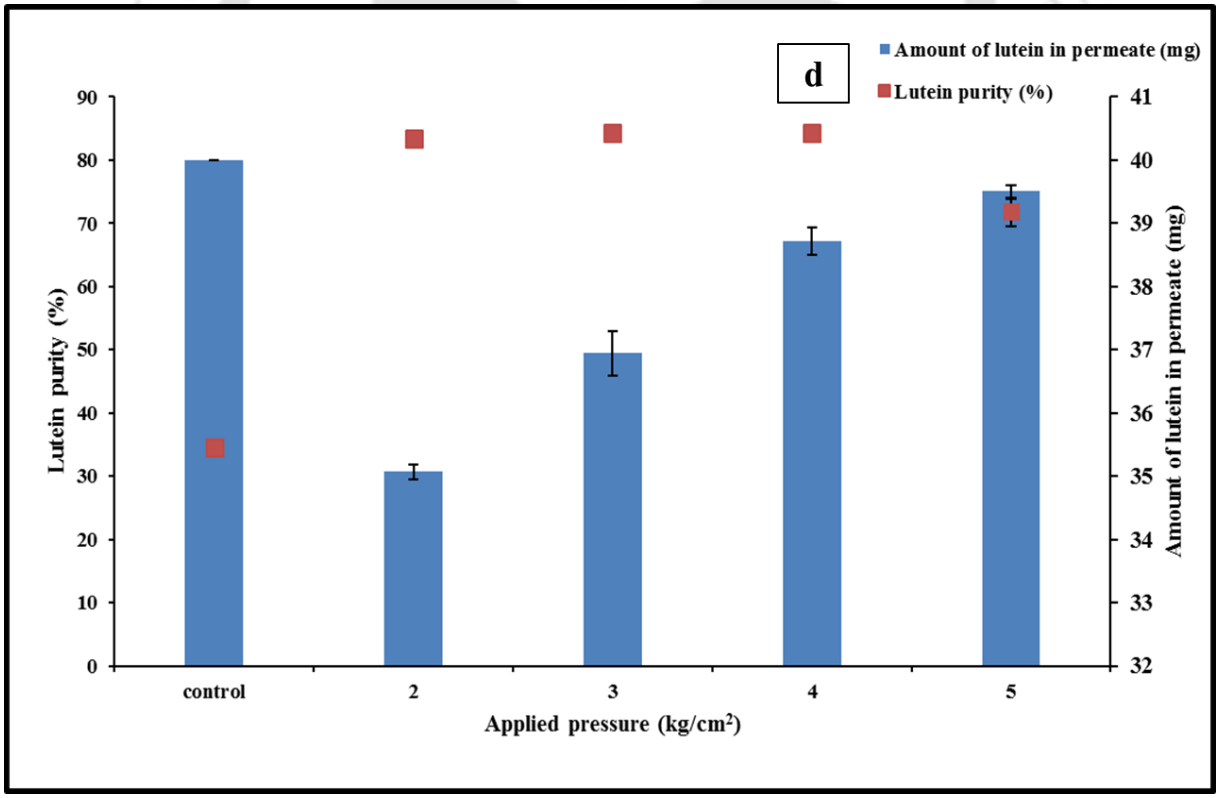


Fig. 4.34 Effect of different applied pressure on (a , b) ethanol flux, (c) permeate flux, and (d) amount of lutein in permeate and purity

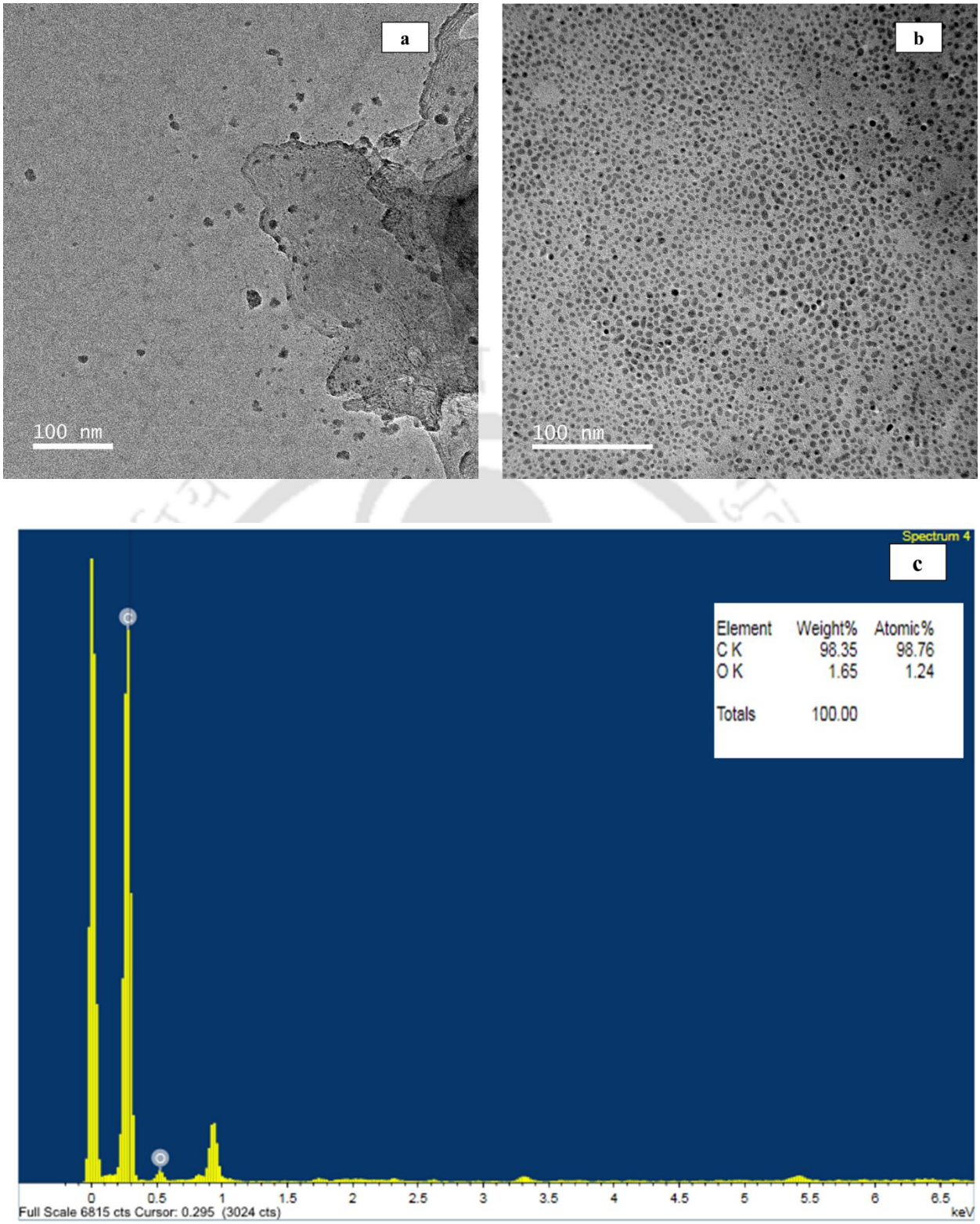


Fig. 4.35 FETEM image of ethanolic extract containing lutein before (a) and after nanofiltation (b) EDX analysis of the ethanolic extract after nanofiltration is shown in the figure (c)

4.5 Cytotoxicity of lutein

Cytotoxicity of the lutein extract obtained from *C. vulgaris* cultivated using synthetic poultry litter anaerobic digestate was assessed by employing MTT assay. Conversion of tetrazolium salts to purple formazan in the assay relates to the viable cell concentration present. Lutein concentration in this study ranged from 0.15µg/mL to 0.9 µg/mL and it did not affect the viability of SF9 cell line. However, an increase in concertation from 1.6 to 6 µg/mL drastically reduced the cell viability (Figure 4.37). Lutein purified by thin layer chromatography (Figure 4.38) exhibited cytotoxicity effect which is close to that the lutein extract which suggests that purification of lutein for use as a biopesticide is not required. Huang et al. (2011) reported that commercially available insecticide (abamectin) reduced cell viability of SF9 cells to 39.27% at 15 µg/mL, whereas in this study viability was reduced upto 94.9% at 6 µg/mL of lutein. The half lethal concentration (LC_{50}) of lutein was 1.6 µg/mL in this study, which is lower than the value LC_{50} (5.28 µg/mL) for human cervical carcinoma cells reported in the literature (Lakshminarayana et al., 2010).

The MTT assay results correlated well with fluorescent microscopic images of the SF9 cells (Fig 4.36). The FDA dye containing non polar ester is hydrolysed by intracellular esterase of living cells to fluorescein which emits green colour in blue light under fluorescent microscope. The PI dye passes through the membrane of the dead cells to bind with nucleic acids present, which in turn emits red light. The fluorescent microscopic images of the cell line are shown in Figure 4.36, control cells and cells treated with lutein at 0.15 µg/mL and 0.9 µg/mL were emitted green fluorescent light. Maximum red fluoresce was observed in cells treated with 6 µg/mL of lutein which indicates the death or damage to the cells (Jones & Senft, 1985). At high lutein concentration, cells were irregular in shape with reduced size, and cell debris were observed due to cellular damage. In the literature, cytotoxicity of xanthophyll at different concentration on cancers cells such as, MDCK, cervical cancer (HeLa) and lung cancer cells

(A549) have been reported is due to apoptosis through biochemical regulatory mechanisms (Lakshminarayana et al., 2010; Saini et al., 2018). Interestingly, literature indicate that normal human cells have the capability to survive at high xanthophyll concentration, even upto 56.80 µg/mL (Kotake-Nara et al., 2001; Krinsky & Johnson, 2005; Niranjana et al., 2015; Saini & Keum, 2018). These reports suggest that lutein can be specifically toxic to insect cells at low concentrations without causing any toxicity to normal human cells.

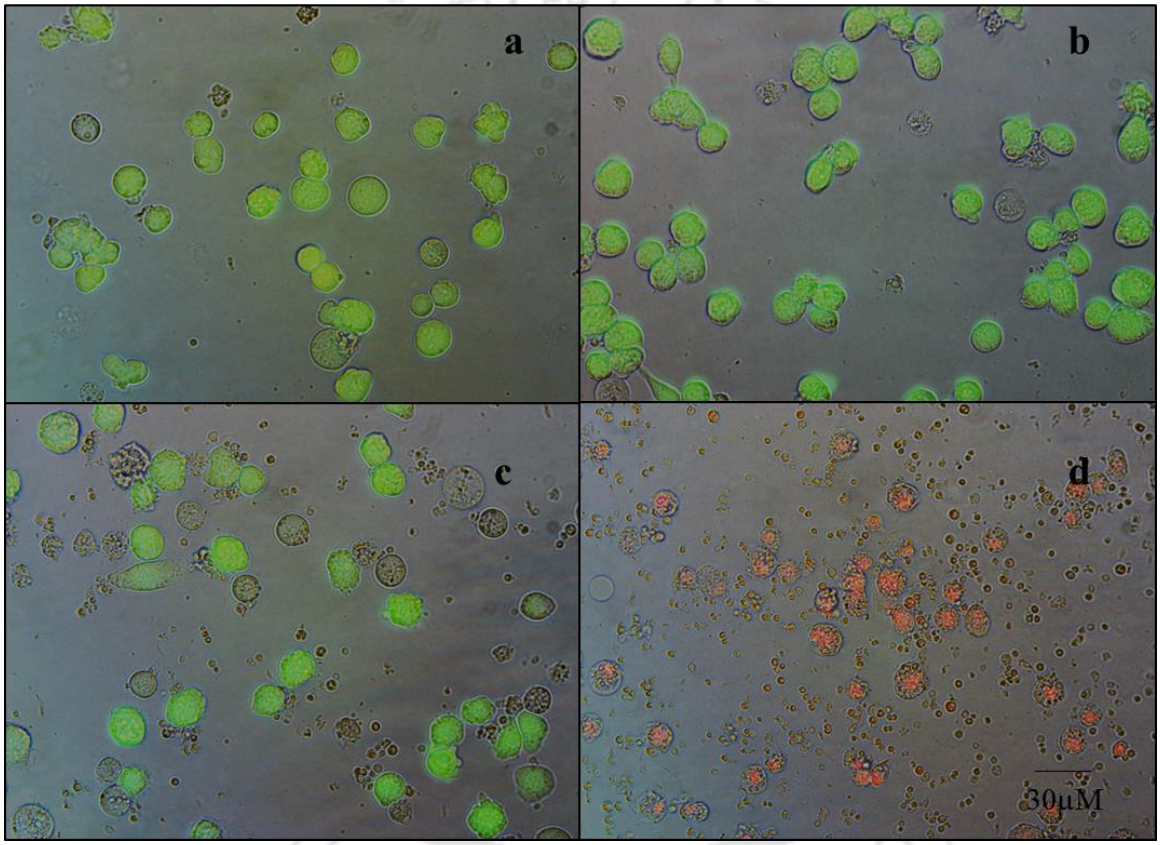


Fig. 4.36 Cellular morphology of SF9 cells under fluorescent microscope after 48 h of treatment with different concentrations of lutein extract (a) Control (b) 0.15 µg/mL (c) 0.9 µg/mL and (d) 6 µg/mL

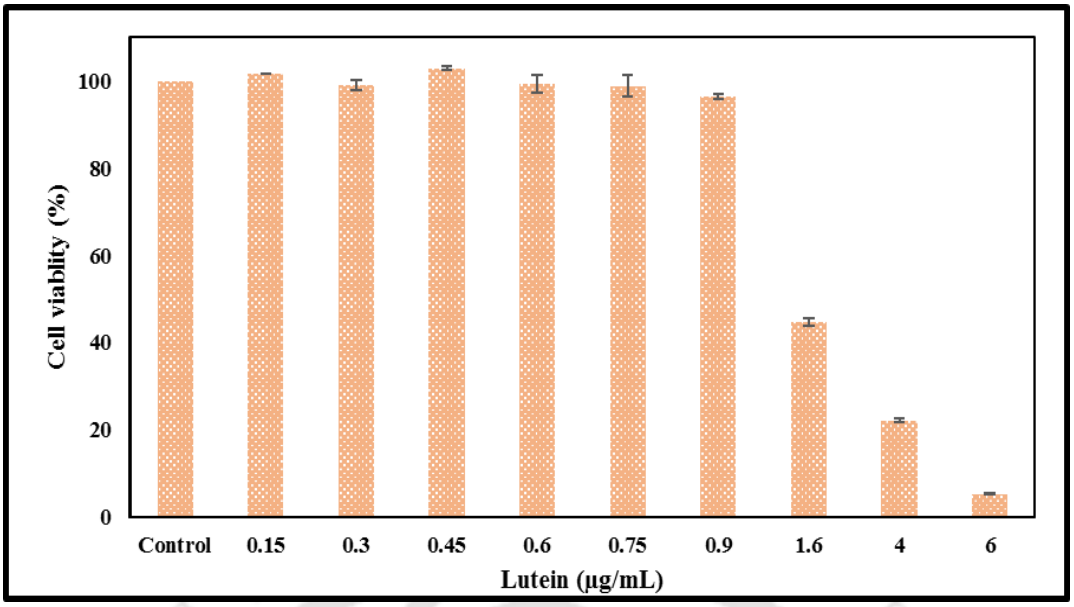


Fig. 4.37 Effect of different concentration of crude lutein extract on the viability of SF9 cell lines in the MTT assay

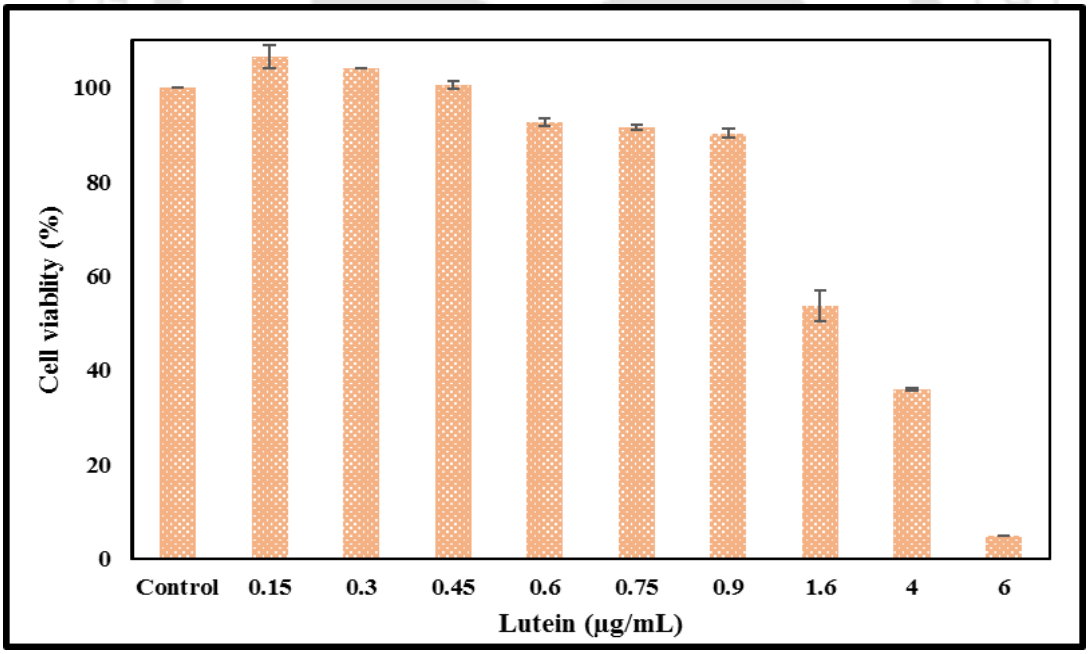


Fig. 4.38 Effect of different concentrations of pure lutein on the viability of SF9 cell lines in the MTT assay

4.5.1 Insecticidal activity of lutein

The effect of different concentration of lutein on the mortality percentage of *Spodoptera litura* is shown in Figure 4.39. Mortality was zero at low concentrations of 62.5 and 125 µg/mL after 72 h of exposure to lutein, whereas mortality was 100% at 187.5 and 250 µg/mL of lutein after 48 h (Figure 4.42 a). Insects that were unexposed or exposed to lower concentration of lutein were able to fully consume the feed (Castor leaves), whereas insects exposed to high lutein concentrations rejected the feed and died after 48h of exposure (Figure 4.40 b and c). It was observed that 26.785 µg/mL of lutein was sufficient to kill one army worm in 48h. In the literature, plant based norditerpenoid alkaloids such as anabasine (60 µg/cm^2) and watropine (50 µg/cm^2) have been shown to induce antifeedance effect on *Spodoptera litura* (González-Coloma et al., 2004). Use of plant extracts as insecticide from *S. pseudocapsicum* (0.625–5 mg/cm^2), *Capsicum annuum L.* (0.5–5 mg/cm^2), *Lycopersicum esculentum Mill* (3.60 mg/cm^2), *Solanum melongena* (0.5–5 mg/cm^2) have also shown antifeed effect, interference in molting and morphological abnormalities in *Spodoptera litura* (Devanand & Rani, 2011; Jeyasankar et al., 2012). A recent study on phenylpropanoid (1′S-1′-acetoxychavicol) extracted from *Alpinia galanga* rhizomes using ethyl acetate showed 50% lethality against single larva at 1.63 and 1.40 µg dose after an exposure time of 24 and 48 h (Poonsri et al., 2019). However, this is the first study to demonstrate insecticidal activity of lutein. Hence, in addition to its antioxidant and coloring properties, lutein also possesses insecticidal property.

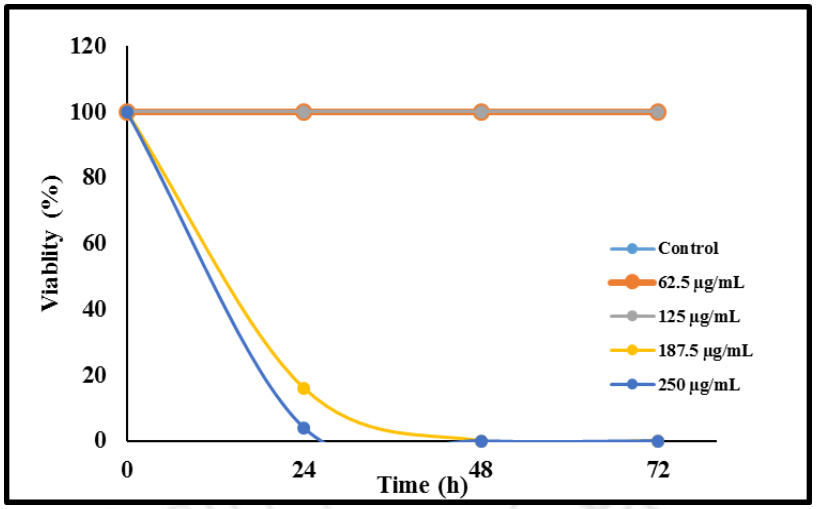


Fig. 4.39 Viability of armyworm treated with different concentrations of lutein at different times of exposure and the viability of control, 62.5 and 125 µg/mL were same.

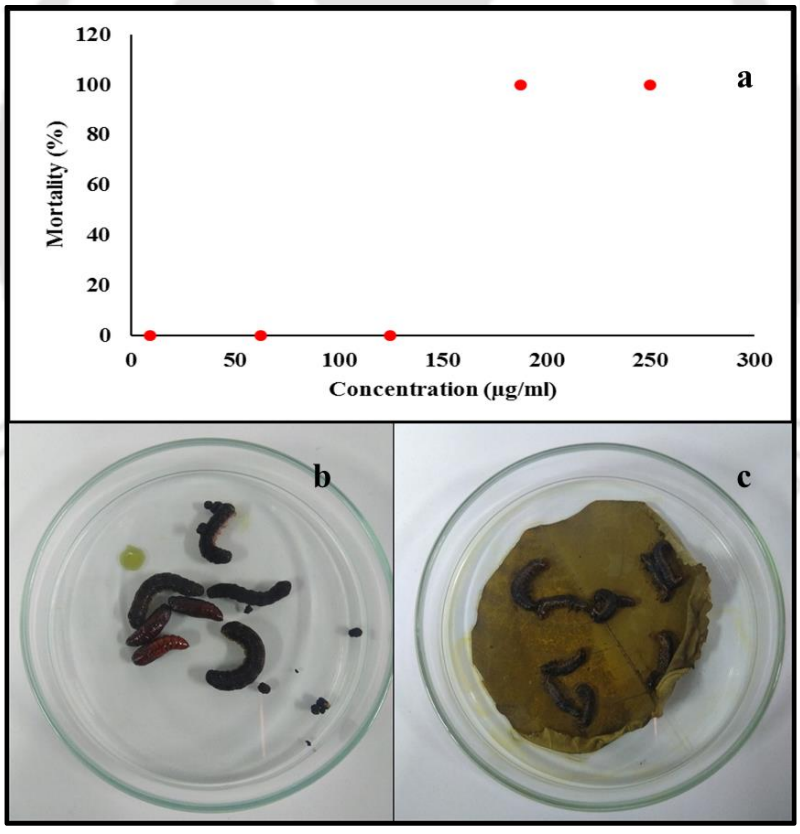


Fig. 4.40 (a) Mortality percentage of armyworm with different concentrations of lutein after 72 h of exposure, and images showing armyworm after 72 h of exposure (b) control (c) leaves treated with 187 µg of lutein

4.6 Lutein enrichment in chicken egg

In order to assess lutein enrichment in chicken egg, chickens were fed with diet containing different concentration of lutein in the form of lutein, lutein ester and *C. vulgaris* biomass. The effect of different lutein concentration and different source on lutein content in egg yolk was examined and the results were statistically analysed based on one way ANOVA and Tukey tests.

Lutein content of chicken eggs increased significantly with increase in lutein concentration ($p<0.001$) through lutein, lutein ester and *C. vulgaris* containing basal diet (Figures 4.41 – 4.43 & Table 4.22). Lutein enrichment was observed in the case of 75 - 375 mg/kg concentration range of lutein in different form. There was no significant difference in the lutein content of egg yolks obtained with feed containing 375 mg/kg and 450 mg/kg of lutein or lutein ester. A similar observation was made by Leeson and Caston (2004) and Steinberg et al. (2000) for enriching lutein in Shaver White SCWL (Single Comb White Leghorn) chicken eggs at a high lutein concentration (375 to 1000 mg/kg) in the feed. Lutein enrichment in the egg was low when *C. vulgaris* was used as the source of lutein in the diet as compared to all other sources of lutein for all different concentrations (Table 4.23). Compared to lutein in the basal diet, lutein ester was found to enhance lutein content in eggs by 5% at 300, 375 and 450 mg/kg concentrations. Adabi et al. (2010) reported increase in egg lutein content from 2.10×10^{-3} mg/g to 2.36×10^{-2} mg/g by supplementing lutein at 250 mg/kg with the basal diet. Addition of 20% w/w of the marine microalgae *Nannochloropsis* for four weeks also increased lutein and zeaxanthin content in eggs upto 0.022 mg/g (Fredriksson et al., 2006; Leeson and Caston, 2004).

Daily recommended lutein content for human is 1 to 2 mg, but its current daily uptake is less than 1 mg. Lutein content of chicken eggs produced in India is only 0.1- 0.2 mg, which can be

improved to 1.123 mg per egg (5.61 times) as demonstrated in this study. Chicken eggs play an important role in importing lutein in the human diet (Englmaierová et al., 2013) and lutein bioavailability through lutein enriched eggs are higher as compared to supplements (lutein, lutein ester) and spinach (Hae-Yun Chung, 2003). Moreover, Handelman et al. (1999) reported 28 % increase in blood serum lutein content when 62 year old men consume $1\frac{1}{3}$ of lutein enriched eggs per day.

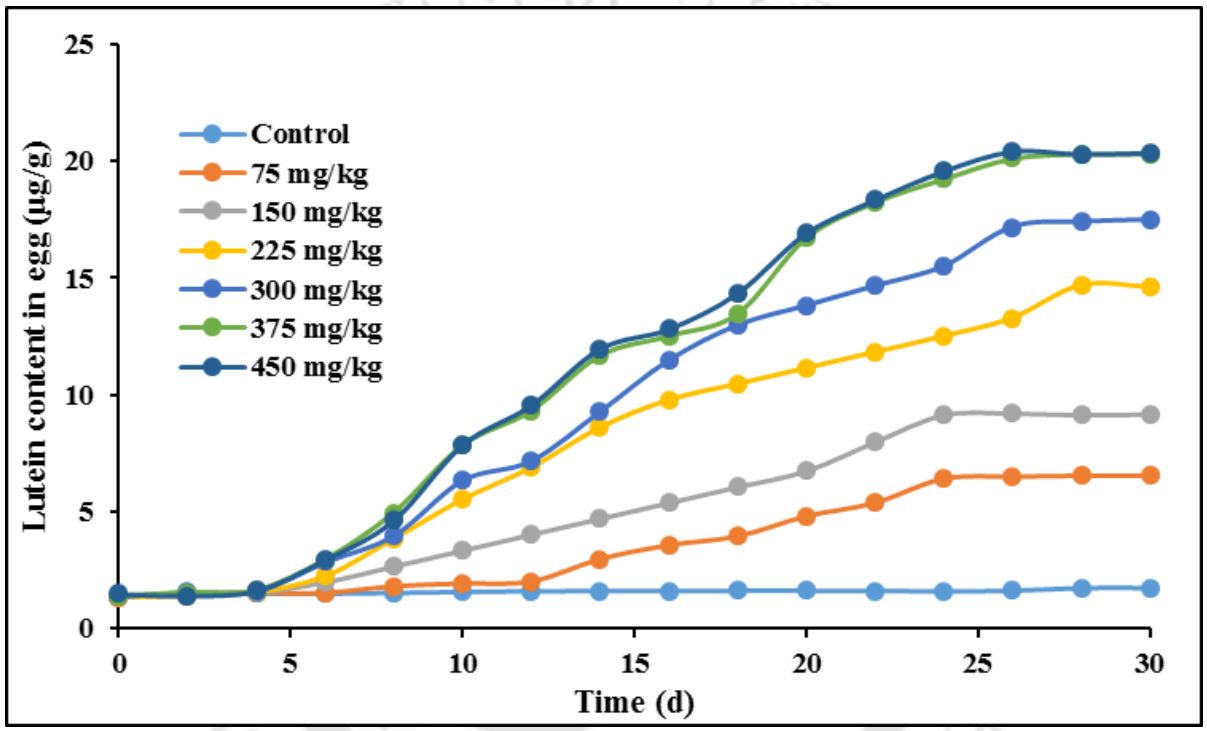


Fig. 4.41 Effect of different lutein concentration in basal diet of chickens on lutein content of the chicken eggs

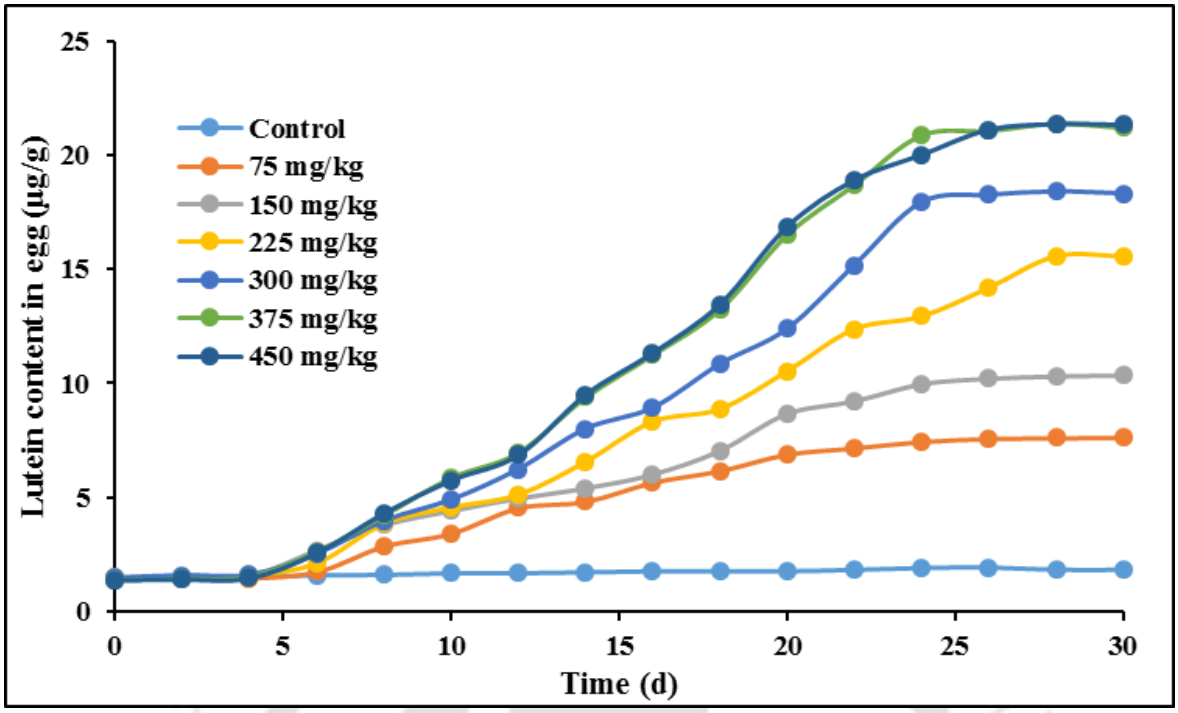


Fig. 4.42 Effect of different lutein ester concentration in basal diet of chickens on lutein content of the chicken eggs

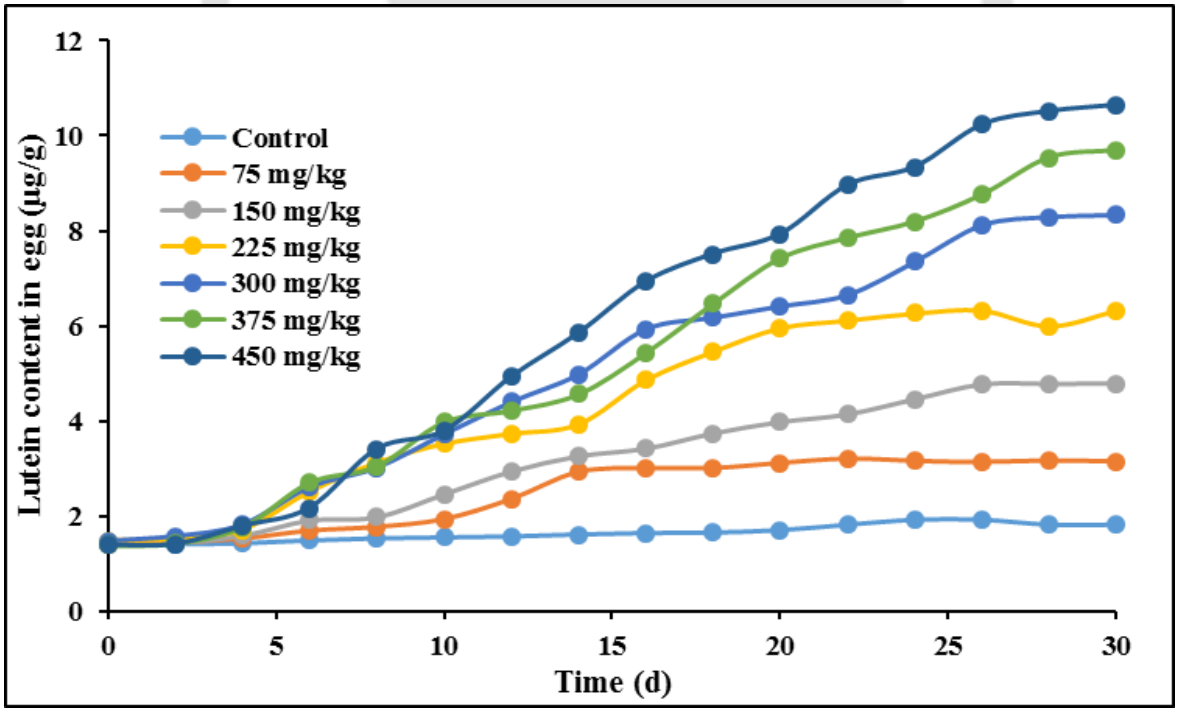


Fig. 4.43 Effect of different microalgae concentration in basal diet of chickens on lutein content of the chicken eggs

Table 4.22 Concentration of lutein in egg on 30th day from chicken fed with different concentrations and various forms of lutein in diet along with their statistical significance

Concentration (mg/kg)	**Lutein**	**Lutein ester**	**Microalgae**
Control	1.69^{f}	1.81^{f}	1.82^{g}
75	6.55^{e}	7.66^{e}	3.15^{f}
150	9.17^{d}	10.35^{d}	4.79^{e}
225	14.60^{c}	15.57^{c}	6.33^{d}
300	17.49^{b}	18.32^{b}	8.30^{c}
375	20.22^{a}	21.2^{a}	9.69^{b}
450	20.37^{a}	21.33^{a}	10.64^{a}
p value	< 0.01	< 0.01	< 0.01
F	2503	5072.000	1907.500

Different superscripts for the mean in the above table indicates the significance between the concentrations

Table 4.23 Concentration of lutein in egg on 30th day from chicken fed with 300 and 450 mg/kg of lutein in various forms of lutein in diet along with their statistical significance

Concentration (mg/kg)	**300**	**450**
Lutein	15.803^{b}	18.67^{b}
Lutein ester	16.513^{a}	19.527^{a}
Microalgae	6.485^{c}	8.817^{c}
p value	< 0.01	< 0.01
F	2070.3	2873.6

Different superscripts for the mean in the above table indicates the significance between the treatment (**Mean values were subtracted with control prior to analysis**)

4.6.1 Egg yolk colour and Biochemical parameters

Increase in egg yolk colour due to feeding of chicken with lutein in various form can be seen from Fig. 4.44. A maximum Roche's colour score of 12 was observed for the eggs obtained by feeding chickens with diet containing 300 mg/kg lutein ester whereas diet with lutein at the same concentration showed a score of 11. Increase in colour score was not observed beyond 300 mg/kg concentration of lutein and lutein ester, whereas diet with microalgae showed increase in colour score beyond 300 mg/kg but the score was lower than that obtained due to the other two form of lutein. Zahroojian et al. (2011) reported that supplementing 2 to 2.5% spirulina in chicken diet increased the adsorption of carotenoids which in turn increased the egg yolk color in layer chicken. In this study, the egg yolk colour was more intense due to lutein ester as compared to the value of free lutein in the diet, which might be due to high bioavailability of the lutein ester. Similarly, Breithaupt et al. (2003) reported increase in lutein content of blood plasma of chickens when lutein ester was used instead of free lutein in the diet.

Diet with lutein and lutein ester did not show any effect on total protein, carbohydrate and lipid content of the eggs (Figure 4.45, 4.46 and 4.47). On the other hand, diet containing microalgae with lutein concentration of 450 mg/kg (~10 % *C. vulgaris* biomass) increased the total protein and lipid content of the eggs by 29.9% and 23% as compared to the control. Thus, addition of 5 to 10% of *C. vulgaris* does not exert any negative effect on the chickens but showed positive effect on the egg's protein and lipid content (Tokuşoglu and Ünal, 2003; Yaakob et al., 2014). Microalgae are rich source of proteins, e.g. spirulina (50 to 70 %) and *Chlorella* (48 %) (Tokuşoglu & Ünal, 2003), which makes them suitable as a feed for poultry (Austic et al., 2013; Spolaore et al., 2006). Microalgae can be a source for other components such as carbohydrates, lipids, vitamins and minerals, omega 3 and 9 fatty acids and phosphorus (El-Sheekh et al., 2020; Yaakob et al., 2014).

Ginzberg et al. (2000) observed reduction in cholesterol and saturated fatty acids and increase content of chicken eggs and increase beneficial fatty acids (PUFA) content by feeding chickens with spirulina. Several studies on inclusion of microalgae in poultry feed have shown to improve the beneficial lipids (PUFA and DHA) content of eggs, which can be recommended for the people with coronary heart disease (Fredriksson et al., 2006; Leeson & Caston, 2004; Lemahieu et al., 2013). Microalgae at very less concentration is sufficient to enhance the lipid content in eggs. Bruneel et al., (2013) reported that the microalgae *Nannochloropsis* in the feed at 5% w/w enhanced the beneficial lipids in eggs by upto 10%. Hence, it is clear that increase in lipid content of eggs due to feeding of chickens with diet containing *C. vulgaris* is due to beneficial lipids added by the microalgae containing lutein.

Concentration of artificial coloruring agents (canthaxanthin and ethyl ester of β-apo-8'-carotenoic) in the poultry are restricted to 8 mg/kg and 80 mg/kg by regulatory bodies as they lead to formation of crystalline deposits in the retina at a high concentration (Geoffrey & Felix, 1991). Use of antibiotics in poultry as a yield promoter is banned in developed countries but not in developing countries. Use of antibiotics tends to increase the microbial resistance to antibiotics and reduces the gut microbial content. Thus, microalgal supplements such as *C. vulgaris* containing lutein can be an interesting alternative for enhancing the colour, lutein, lipid and protein content of chicken eggs in improving the health of individuals.

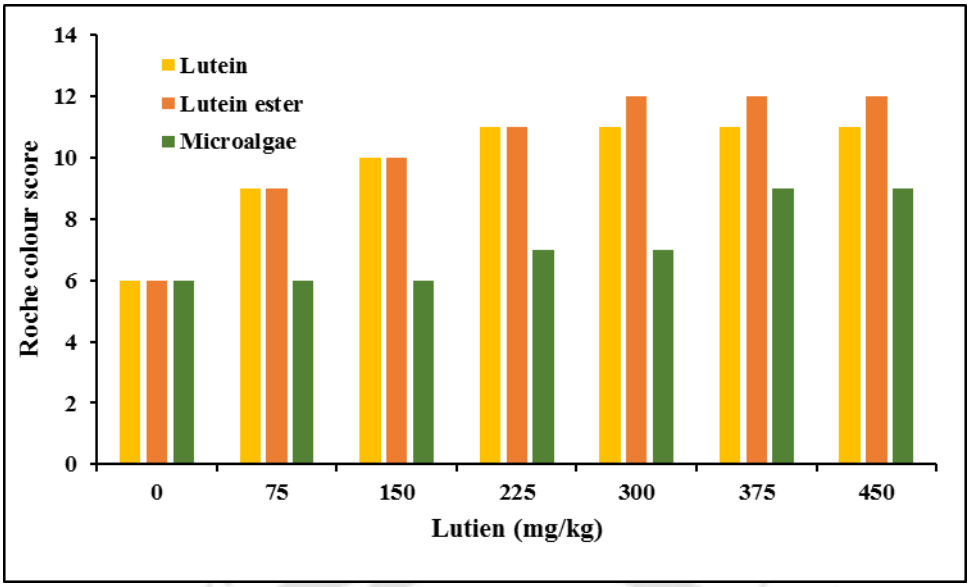


Fig. 4.44 Roche colour score of egg yolk at 30th day from chicken fed with different concentrations and various forms of lutein in the diet

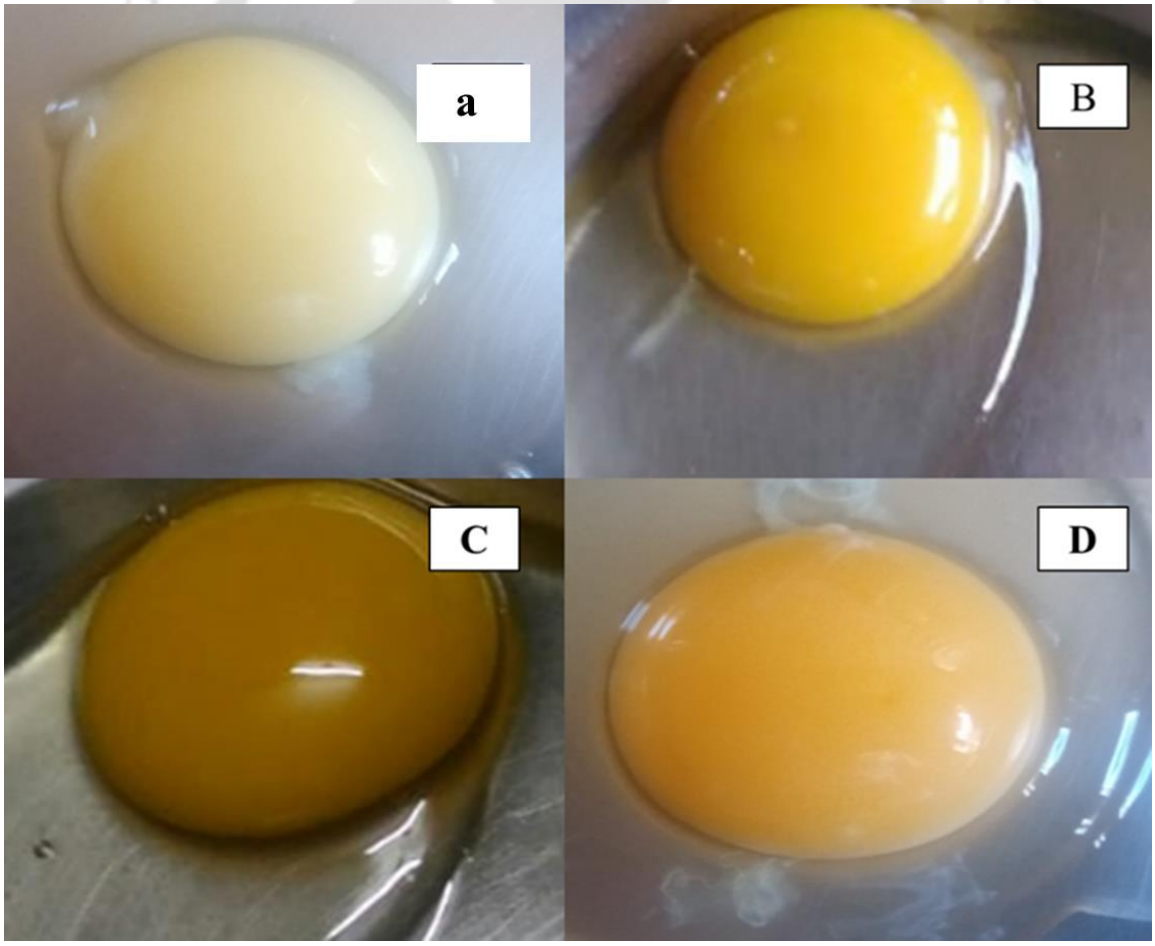


Fig. 4.45 Roche colour score of egg yolk at 30th day from chicken fed with various form of lutein in the diet at 375 mg/kg of lutein (a) Basal diet (b) Lutein (c) Lutein ester (d) microalgae

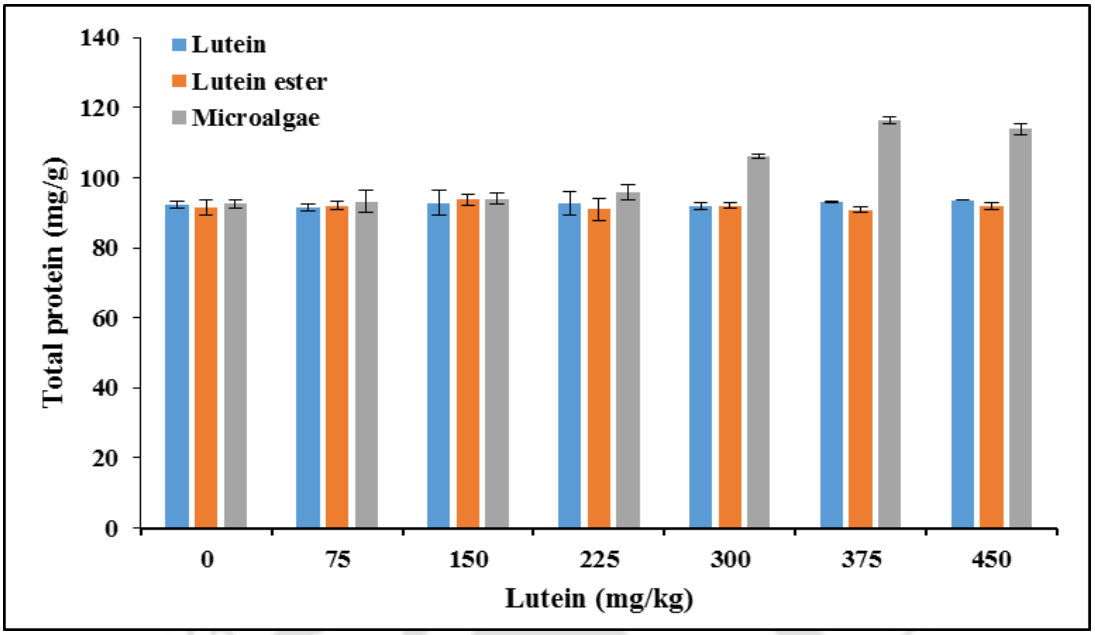


Fig. 4.46 Total protein content of the egg at 30th day from chickens fed with different concentrations of lutein in the diet and various forms

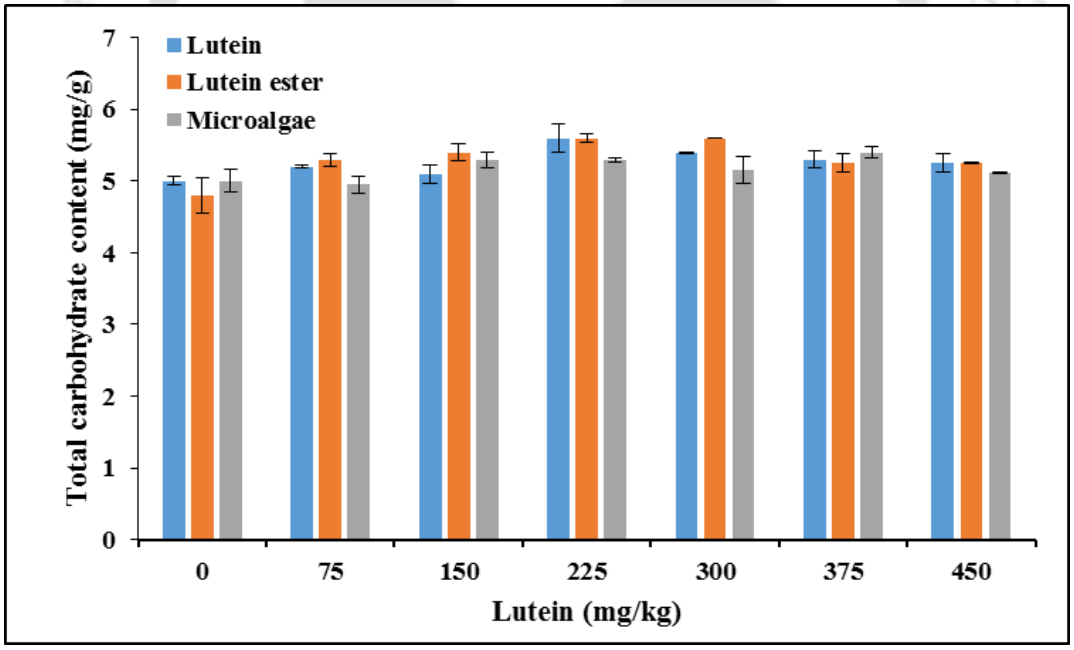


Fig. 4.47 Total carbohydrate content of the eggs at 30th day from chicken fed with different concentrations and various forms of lutein in the diet

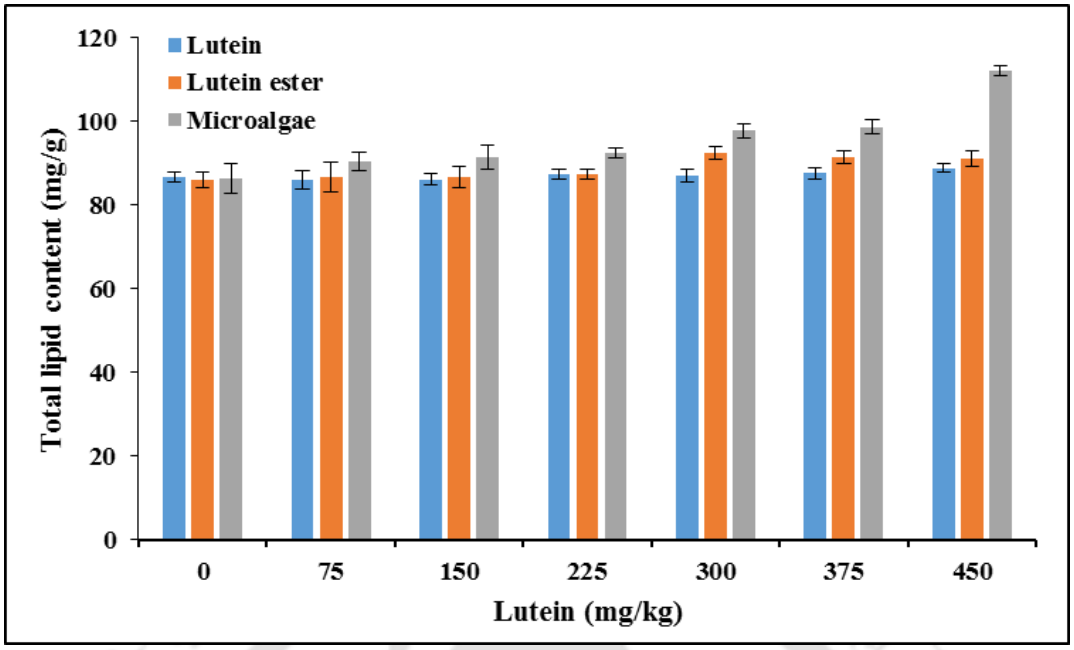


Fig. 4.48 Total lipid content of the egg at 30th day from chicken fed with different concentrations and various forms of lutein in the diet

Chapter - 5

SUMMARY AND CONCLUSIONS

5 Summary and Conclusions

Lutein is currently produced from petals of marigold grown on agricultural land. Production and harvest of marigold flowers involves huge amount of land, man power and fresh water. Besides, utilization of fertilizers, and pesticides for its cultivation are drawbacks of lutein production from marigold flower petals.

This study focus on utilizing anaerobic digestate for lutein production using marine microalgae. Application of lutein as the biopesticide and coloring agents in agriculture and poultry was studied.

Four different marine halophilic microalgae strains such as *Chlorella vulgaris* 92001, *Chlorella vulgaris* 52091, *Chlorella vulgaris* 10241 and *Tetraselmis indica* were cultured in 10 and 20 times diluted synthetic municipal, dairy litter and poultry litter digestates. Among the four strains, *Chlorella vulgaris* 92001 produced maximum biomass (0.46 g/L) and lutein (1.85 mg/L) in 20 times diluted dairy litter and poultry digestates, respectively. Biomass and lutein yield was very low at lower dilution (10 times). *Chlorella vulgaris* 92001 and poultry digestate were selected based on this study for further investigation.

The effect of different parameters such as dilution times, pH, $MgCl_2$ (g/L), NaCl (g/L), $NaHCO_3$ (g/L) and inoculum size (cells/l) on lutein production by *C. vulgaris* was studied by employing Plackett-Burman design at three different levels.

Dilution ratio, high salinity, $NaHCO_3$ and inoculum size significantly influenced the lutein by *C. vulgaris*, and a maximum lutein production of 2.86 mg/L was obtained. The level of the parameters on the lutein production were further optimized by response surface methodology. Under the optimum condition of 22.12 dilution factor, 27 g/L NaCl, 4.5 g/L $NaHCO_3$ and 4.82 $\times 10^9$ cells/L inoculum size, maximum lutein production of 3.412 mg/L was obtained.

Kinetics of substrate utilization ($NaHCO_3$ and CH_3COONa), biomass growth and lutein production by *C. vulgaris* was studied and evaluated using different bio kinetic models. In the

case of $NaHCO_3$ as the substrate, its uptake rate was unaffected even at a high initial concentration, whereas in the case of CH_3COONa (heterotrophic condition) there was a reduction in its specific uptake rate was observed above 1000 mg/L of initial concentration. Static carbon flux distribution in the microalgae was carried out on 7^{th} day of its culture for both heterotrophic and autotropic conditions, which showed that the flux towards chlorophyll biosynthesis in autotrophic mode was 4 times higher than that in the heterotrophic mode.

An indigenously designed and fabricated split column photobioreactor was used for lutein production by *C. vulgaris* 92001 in batch and continuous mode of operation for lutein production. In the batch mode operation, maximum biomass growth of 0.96 g/L and lutein production of 4.379 mg/L was attained at 336 h. The biomass growth rate obtained was 0.0102 h^{-1}, whereas the biomass and lutein productivity where 0.0041 $gL^{-1}h^{-1}$ and 0.0116 $mgL^{-1}h^{-1}$. At the end of the batch operation, the substrate utilization were as follows: 4.29 g/L (95.33 %) of $NaHCO_3$, 82.38 mg/L (73.29 %) of NH_4^+, 8.41 mg/L (86.45 %) of PO_4^{3-}, 11.16 mg/L (11.6 %) of K^+.

In continuous operation mode the effect of different HRT (150, 130, 100, 90 h) on lutein production by *C. vulgaris* 92001 was examined using the split column photobioreactor. Maximum biomass growth of 0.89 g/L and lutein production of 4.25 mg/L were observed at 150 h HRT. The biomass and lutein productivity values were 0.0058 $gL^{-1}h^{-1}$ and 0.026 $mgL^{-1}h^{-1}$. Reduction in biomass growth and lutein production was observed with a decrease in the HRT, but the productivity values were stable, except in the case of 90 h HRT. In the steady state at 150 h HRT, substrate utilization efficiency was 79.53 % for $NaHCO_3$, 71.71 % for NH_4^+, 84.32 % for PO_4^{3-} and 13 % for K^+ from the feed containing synthetic anaerobic digestate.

The performance of a continuously stirred tank laboratory scale continuously stirred tank photobioreactor, operated in batch and continuous mode was evaluated for lutein production

by *C. vulgaris* 92001. In batch mode, maximum biomass growth of 0.8160 g/L and lutein production of 3.128 mg/L was obtained at 504 h. The net highest microalgal growth rate was 0.00539 h^{-1} and the biomass productivity and lutein productivity values where 1.2×10^{-3} $gL^{-1}h^{-1}$ and 4.9×10^{-3} $mgL^{-1}h^{-1}$. These values are lower than that obtained using the split column photobioreactor. The substrate consumption profile showed that 2.926 g/L (65.02 %) of $NaHCO_3$, 63.26 mg/L (56.22 %) of $NH4^+$, 7.695 mg/L (78.92 %) of PO_4^{3-}, 10.443 mg/L (10.91 %) of K^+ were utilized from the wastewater.

In continuous operation mode maximum biomass growth of 0.7870 g/L and lutein production of 2.7860 mg/L was obtained at 200 h HRT. The net highest microalgal growth rate was 0.00539 h^{-1}, whereas the biomass productivity and lutein productivity values where 3.93×10^{-3} $gL^{-1}h^{-1}$ and 0.01393 $mgL^{-1}h^{-1}$. The substrate consumption profile showed that 44.44 % of $NaHCO_3$, 50.28 % of $N\text{-}NH_3$, 62.35 % of $P\text{-}PO_4^{3-}$ and 10.79 % of K^+ were utilized from the feed containing anaerobic digestate.

The effect of different electrodes (aluminium, copper, zinc and iron), current density (1.5 to 3.5 mA/cm^2) and recovery time (10 to 30 min) on the recovery of microalgal biomass and lutein by electroflocculation was studied. Electrodes played a significant role in the recovery of *C. vulgaris* from the culture medium and the recovery was maximum with copper (98.67 and 99.22 %) and aluminium electrode (98 and 98.56 %) at 1.5 and 2.5 mA/cm^2, on the other hand, lutein recovery was higher with aluminium (92.05 %) and copper electrode (87.98 %) at 10 min recovery time and at a current density of 1.5 mA/cm^2. Increase in current density or recovery time reduced the lutein recovery, probably due to heat induced damage from electrochemical reactions. At the current density 1.5 mA/cm^2 and 10 min recovery time, the energy consumption was 0.27 KW/kg of biomass which was the lowest among all the experimental values using aluminium as the electrode.

Effect of different time (2.5, 4.5, 6.5, 8.5 min) and biomass concentration (0.125, 0.250, 0.50, 0.75 g/15mL) on *C. vulgaris* cell disruption for lutein recovery was studied. The maximum lutein recovery was 95.42% for biomass concentration 0.5 g/15 mL and 4.5 min treatment time. Energy consumption for the maximum lutein recovery by cell disruption was 5351 J/15 mL.

Effect of different concentration of lutein in the range (20 -100 mg/L) and different filter cutoff (600 - 800 and 800 -1000 Da) on lutein recovery and purification by nano filtration was studied under different applied pressure. Maximum amount of lutein obtained in the permeate was 89.34% using 800-1000 Da filter, whereas 57.84 % was obtained with 600 – 800 Da at 20 mg/L concentration. Increase in applied pressure from 2 to 5 kg/cm^2 reduced the recovery time, and maximum amount of lutein in the permeate was 98.76 % at an applied pressure of 5 kg/cm^2. Lutein purity was 83.36, 84. 84, 84.32 and 72.02 % for the applied pressure 2, 3, 4 and 5 kg/cm^2 respectively. FETEM image of the lutein sample taken before and after nanofiltration showed that the particle size varied in the range 5 -20 nm prior to the nanofiltration, whereas after filtration particle size were uniform (~5 nm).

Cytotoxicity of the lutein extract obtained from *C. vulgaris* cultivated using synthetic anaerobic poultry litter digestate was assessed employing MTT assay. Lutein concentration ranging from 0.15µg/mL to 0.9 µg/mL showed no effect on the viability of SF9 cell line, whereas an increase in lutein concertation from 1.6 to 6 µg/mL drastically reduced the cell viability. The toxicity effect was found to be dose dependent but not due to presence of any pyrogens.

Insecticidal activity of lutein produced by *C. vulgaris* was investigated against *Spodoptera litura* (army worm) for different concentrations. Mortality was zero at the low concentrations of 62.5 and 125 µg/mL after 72h, whereas the mortality was 100% at 187.5, 250 µg/mL after 48 h. From this study, it was observed that 26.785 µg/mL of lutein was sufficient to kill one army worm in 48h.

Lutein enhancement in egg yolk was carried out by supplementing lutein, lutein ester and microalgae in basal diet of hen in different concentrations (75 to 450 mg/kg). Lutein content of the eggs increased significantly($p<0.001$) with the increase in lutein concentration with lutein, lutein ester and microalgae containing basal diet. However, the lutein enrichment was observed for an initial concentration 75 to 375 mg/kg. There was no significant difference in lutein concentration in the egg yolk for a feed lutein concentration in the range between 375 and 450 mg/kg, which clearly means beyond 375 mg/kg, lutein enhancement is not possible. Moreover, lutein enrichment in egg yolk was reduced when microalgae was used as the source of lutein in the diet as compared to all other sources of lutein. Compared to lutein in the basal diet lutein ester enhanced the egg yolk lutein content by 5% at a concentration above 300 mg/kg.

Furthermore, increase in egg yolk colour was observed in all the diets or different lutein concentrations. Maximum Roche's colour score of 12 was observed for the eggs obtained by feeding the hens with diet containing 300 mg/kg lutein ester, whereas diet with lutein at the same concentration yielded a score of 11. Increase in colour score was not observed beyond 300 mg/kg concentration for lutein and lutein ester on the other hand, diet with microalgae showed increase in colour score beyond 300 mg/kg, but the score was lower than the score obtained using the other two form of lutein in the diet.

This study demonstrated sustainable production of lutein by the marine microalgae *C. vulgaris* using poultry litter anaerobic digestate and its potential applications in agriculture and poultry as a biopesticide and value addition of eggs had increased the closed loop capability of this study.

5.1 Scope for future work

The present work focused on lutein production from *C. vulgaris* using easily available poultry litter digestate and its potential applications. Some suggestions for future work based on this thesis are as follows

- Metabolomics study for evaluating flux towards lutein or carotenoid pathway
- Scale up of split column photobioreactor for lutein production in outdoor conditions using poultry litter digestate
- Non sacrificial electrode for cell separation from the broth and integration of solar panels
- Chitosan/neem coating for lutein to enhance biopesticide activity

BIBLIOGRAPHY

- Abedini Najafabadi, H., Malekzadeh, M., Jalilian, F., Vossoughi, M., and Pazuki, G. (2015). Effect of various carbon sources on biomass and lipid production of Chlorella vulgaris during nutrient sufficient and nitrogen starvation conditions. *Bioresource Technology*, *180*, 311–317. https://doi.org/10.1016/j.biortech.2014.12.076
- Abeliovich, A., and Azov, Y. (1976). Toxicity of ammonia to algae in sewage oxidation ponds. *Applied and Environmental Microbiology*, *31*(6), 801–806. https://doi.org/10.1128/aem.31.6.801-806.1976
- Abubakar, B. S. U. I., and Ismail, N. (2012). Anaerobic digestion of cow dung for biogas production. *ARPN Journal of Engineering and Applied Sciences*, *7*(2), 169–172.
- Acién, F. G., Fernández, J. M., Magán, J. J., and Molina, E. (2012). Production cost of a real microalgae production plant and strategies to reduce it. In *Biotechnology Advances* (Vol. 30, Issue 6, pp. 1344–1353). https://doi.org/10.1016/j.biotechadv.2012.02.005
- Agarwal, A. K. (2001). In vitro cytotoxicity evaluation of plastic biomedical devices. *Human and Experimental Toxicology*, *20*(8), 412–417. https://doi.org/10.1191/096032701682692919
- Ahammad, S. Z., Davenport, R. J., Read, L. F., Gomes, J., Sreekrishnan, T. R., and Dolfing, J. (2013). Rational immobilization of methanogens in high cell density bioreactors. *RSC Advances*, *3*(3), 774–781. https://doi.org/10.1039/c2ra21901h
- Ahmed, F., Fanning, K., Netzel, M., Turner, W., Li, Y., and Schenk, P. M. (2014). Profiling of carotenoids and antioxidant capacity of microalgae from subtropical coastal and brackish waters. *Food Chemistry*, *165*, 300–306. https://doi.org/10.1016/j.foodchem.2014.05.107
- Aikawa, S., Ho, S. H., Nakanishi, A., Chang, J. S., Hasunuma, T., and Kondo, A. (2015). Improving polyglucan production in cyanobacteria and microalgae via cultivation design and metabolic engineering. *Biotechnology Journal*, *10*(6), 886–898. https://doi.org/10.1002/biot.201400344
- Amar, I., Aserin, A., and Garti, N. (2003). Solubilization patterns of lutein and lutein esters in food grade nonionic microemulsions. *Journal of Agricultural and Food Chemistry*, *51*(16), 4775–4781. https://doi.org/10.1021/jf026222t
- Amini, M., Arami, M., Mahmoodi, N. M., and Akbari, A. (2011). Dye removal from colored textile wastewater using acrylic grafted nanomembrane. *Desalination*, *267*(1), 107–113. https://doi.org/10.1016/j.desal.2010.09.014
- Anwar, N., Wang, W., Zhang, J., Li, Y., Chen, C., Liu, G., and Zhang, R. (2016). Effect of sodium salt on anaerobic digestion of kitchen waste. *Water Science and Technology*, *73*(8), 1865–1871. https://doi.org/10.2166/wst.2016.035
- Araya, B., Gouveia, L., Nobre, B., Reis, A., Chamy, R., and Poirrier, P. (2014). Evaluation of the simultaneous production of lutein and lipids using a vertical alveolar panel bioreactor for three Chlorella species. *Algal Research*, *6*(PB), 218–222. https://doi.org/10.1016/j.algal.2014.06.003
- Arenovski, A. L. (1994). The distribution, abundance and ecology of mixotrophic algae in marine and freshwater plankton communities. *The Distribution, Abundance and Ecology of Mixotrophic Algae in Marine and Freshwater Plankton Communities*, *1988*. https://doi.org/10.1575/1912/5582
- Arora, M., Anil, A. C., Leliaert, F., Delany, J., and Mesbahi, E. (2013). Tetraselmis indica (Chlorodendrophyceae, Chlorophyta), a new species isolated from salt pans in Goa, India. *European Journal of Phycology*, *48*(1), 61–78. https://doi.org/10.1080/09670262.2013.768357

- Ascanio, G. (2015). Mixing time in stirred vessels: A review of experimental techniques. *Chinese Journal of Chemical Engineering*, *23*(7), 1065–1076. https://doi.org/10.1016/j.cjche.2014.10.022
- Aslan, S., and Şekerdağ, N. (2016). Salt inhibition on anaerobic treatment of high salinity wastewater by upflow anaerobic sludge blanket (UASB) reactor. *Desalination and Water Treatment*, *57*(28), 12998–13004. https://doi.org/10.1080/19443994.2015.1059369
- Augugliaro, V., Loddo, V., Palmisano, L., and Schiavello, M. (1995). Heterogeneous photocatalytic systems: Influence of some operational variables on actual photons absorbed by aqueous dispersions of TiO2. *Solar Energy Materials and Solar Cells*, *38*(1–4), 411–419. https://doi.org/10.1016/0927-0248(94)00233-9
- Austic, R. E., Mustafa, A., Jung, B., Gatrell, S., and Lei, X. G. (2013). Potential and limitation of a new defatted diatom microalgal biomass in replacing soybean meal and corn in diets for broiler chickens. *Journal of Agricultural and Food Chemistry*, *61*(30), 7341–7348. https://doi.org/10.1021/jf401957z
- Bae, J. H., and Hur, S. B. (2012). Selection of suitable species of chlorella, nannochloris, and nannochloropsis in high- and low-temperature seasons for mass culture of the rotifer brachionus plicatilis. *Fisheries and Aquatic Sciences*, *14*(4), 323–332. https://doi.org/10.5657/FAS.2011.0323
- Banks, C., and Zhang, Y. (2010). Technical Report: Optimising inputs and outputs from anaerobic digestion processes. *Defra Project Code WR0212, March 2009*, 1–213. https://doi.org/WR0212
- Barman, A., and Tamuli, R. (2015). Multiple cellular roles of Neurospora crassa plc-1, splA2, and cpe-1 in regulation of cytosolic free calcium, carotenoid accumulation, stress responses, and acquisition of thermotolerance. *Journal of Microbiology*, *53*(4), 226–235. https://doi.org/10.1007/s12275-015-4465-1
- Basu, S., Khan, A. L., Cano-Odena, A., Liu, C., and Vankelecom, I. F. J. (2010). Membrane-based technologies for biogas separations. *Chemical Society Reviews*, *39*(2), 750–768. https://doi.org/10.1039/b817050a
- Beardsworth, P. M., and Hernandez, J. M. (2014). Yolk colour - an important egg quality attribute. *Internation Poultry Production*, *12*(5), 17, 18. http://www.positiveaction.info/pdfs/articles/pp12.5p17.pdf
- Becker, S. A., Feist, A. M., Mo, M. L., Hannum, G., Palsson, B., and Herrgard, M. J. (2007). Quantitative prediction of cellular metabolism with constraint-based models: The COBRA Toolbox. *Nature Protocols*, *2*(3), 727–738. https://doi.org/10.1038/nprot.2007.99
- Berg, K., Zhai, L., Chen, M., Kharazmi, A., and Owen, T. C. (1994). The use of a water-soluble formazan complex to quantitate the cell number and mitochondrial function of Leishmania major promastigotes. *Parasitology Research*, *80*(3), 235–239. https://doi.org/10.1007/BF00932680
- Bhanushali, D., Kloos, S., Kurth, C., and Bhattacharyya, D. (2001). Performance of solvent-resistant membranes for non-aqueous systems: Solvent permeation results and modeling. *Journal of Membrane Science*, *189*(1), 1–21. https://doi.org/10.1016/S0376-7388(01)00356-8
- Bhatnagar, A., Chinnasamy, S., Singh, M., and Das, K. C. (2011). Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. *Applied Energy*, *88*(10), 3425–3431. https://doi.org/10.1016/j.apenergy.2010.12.064

- Bicas, J. L., Kleinegris, D. M. M., and Barbosa, M. J. (2015). Use of methylene blue uptake for assessing cell viability of colony-forming microalgae. *Algal Research*, *8*, 174–180. https://doi.org/10.1016/j.algal.2015.02.004
- Bickley, R. I., Slater, M. J., and Wang, W. J. (2005). Engineering development of a photocatalytic reactor for waste water treatment. *Process Safety and Environmental Protection*, *83*(3 B), 205–216. https://doi.org/10.1205/psep.04028
- Blenke, H. (1979). Loop Reactors. *Advances in Biochemical Engineering*, *13*, 121–214. https://doi.org/10.1007/3540094687_8
- Boland, M. J. (2002). Aqueous two-phase extraction and purification of animal proteins. *Applied Biochemistry and Biotechnology - Part B Molecular Biotechnology*, *20*(1), 85–93. https://doi.org/10.1385/mb:20:1:085
- Bonora, A., and Mares, D. (1982). A simple colorimetric method for detecting cell viability in cultures of eukaryotic microorganisms. *Current Microbiology*, *7*(4), 217–221. https://doi.org/10.1007/BF01568802
- Boonnoun, P., Opaskonkun, T., Prasitchoke, P., Goto, M., and Shotipruk, A. (2012). Purification of free lutein from marigold flowers by liquid chromatography. *Engineering Journal*, *16*(5), 145–155. https://doi.org/10.4186/ej.2012.16.5.145
- Bopp, S. K., and Lettieri, T. (2008). Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. *BMC Pharmacology*, *8*. https://doi.org/10.1186/1471-2210-8-8
- Bopp, S. K., Bols, N. C., and Schirmer, K. (2006). Development of a solvent-free, solid-phase in vitro bioassay using vertebrate cells. *Environmental Toxicology and Chemistry*, *25*(5), 1390–1398. https://doi.org/10.1897/05-374R.1
- Borowitzka, L. J., and Brown, A. D. (1974). The salt relations of marine and halophilic species of the unicellular green alga, Dunaliella - The role of glycerol as a compatible solute. *Archives of Microbiology*, *96*(1), 37–52. https://doi.org/10.1007/BF00590161
- Borowitzka, M. A. (2013). High-value products from microalgae-their development and commercialisation. *Journal of Applied Phycology*, *25*(3), 743–756. https://doi.org/10.1007/s10811-013-9983-9
- Boyle, N. R., and Morgan, J. A. (2009). Flux balance analysis of primary metabolism in Chlamydomonas reinhardtii. *BMC Systems Biology*, *3*, 1–14. https://doi.org/10.1186/1752-0509-3-4
- Branyikova, I., Prochazkova, G., Potocar, T., Jezkova, Z., and Branyik, T. (2018). Harvesting of microalgae by flocculation. In *Fermentation* (Vol. 4, Issue 4). https://doi.org/10.3390/fermentation4040093
- Breithaupt, D. E., Weller, P., and Grashorn, M. A. (2003). Quantification of carotenoids in chicken plasma after feeding free or esterified lutein and capsanthin using high-performance liquid chromatography and liquid chromatography-mass spectrometry analysis. *Poultry Science*, *82*(3), 395–401. https://doi.org/10.1093/ps/82.3.395
- Bruneel, C., Lemahieu, C., Fraeye, I., Ryckebosch, E., Muylaert, K., Buyse, J., and Foubert, I. (2013). Impact of microalgal feed supplementation on omega-3 fatty acid enrichment of hen eggs. *Journal of Functional Foods*, *5*(2), 897–904. https://doi.org/10.1016/j.jff.2013.01.039
- Buehner, M. R., Young, P. M., Willson, B., Rausen, D., Schoonover, R., Babbitt, G., and Bunch, S. (2009). Microalgae growth modeling and control for a vertical flat panel photobioreactor. *Proceedings of the American Control Conference*, *4*, 2301–2306. https://doi.org/10.1109/ACC.2009.5160260

- Byreddy, A. R., Gupta, A., Barrow, C. J., and Puri, M. (2015). Comparison of cell disruption methods for improving lipid extraction from thraustochytrid strains. *Marine Drugs*, *13*(8), 5111–5127. https://doi.org/10.3390/md13085111
- Camacho, F. G., Rodríguez, J. J. G., Mirón, A. S., Belarbi, E. H., Chisti, Y., and Grima, E. M. (2011). Photobioreactor scale-up for a shear-sensitive dinoflagellate microalga. *Process Biochemistry*, *46*(4), 936–944. https://doi.org/10.1016/j.procbio.2011.01.005
- Cantrill, R. (2004). Lutein from *Tagetes erecta*: Chemical and Technical Assessment. *63rd JECFA in Compendium of Food Additive Specification: Addendum 12*, *1*(127), 1–5.
- Carney, L. T., Reinsch, S. S., Lane, P. D., Solberg, O. D., Jansen, L. S., Williams, K. P., Trent, J. D., and Lane, T. W. (2014). Microbiome analysis of a microalgal mass culture growing in municipal wastewater in a prototype OMEGA photobioreactor. *Algal Research*, *4*(1), 52–61. https://doi.org/10.1016/j.algal.2013.11.006
- Cassano, A., Conidi, C., and Ruby-Figueroa, R. (2014). Recovery of flavonoids from orange press liquor by an integrated membrane process. *Membranes*, *4*(3), 509–524. https://doi.org/10.3390/membranes4030509
- Cassano, A., Conidi, C., Ruby-Figueroa, R., and Castro-Muñoz, R. (2018). Nanofiltration and tight ultrafiltration membranes for the recovery of polyphenols from agro-food by-products. *International Journal of Molecular Sciences*, *19*(2). https://doi.org/10.3390/ijms19020351
- Cassin, P. E. (1974). Isolation, Growth, and Physiology of Acidophilic Chlamydomonads. *Journal of Phycology*, *10*(4), 439–447. https://doi.org/10.1111/j.1529-8817.1974.tb02737.x
- Çelekli, A., Balci, M., and Bozkurt, H. (2008). Modelling of Scenedesmus obliquus; function of nutrients with modified Gompertz model. *Bioresource Technology*, *99*(18), 8742–8747. https://doi.org/10.1016/j.biortech.2008.04.028
- Çelekli, A., Bozkurt, H., and Dönmez, G. (2014). Predictive modeling of β-carotene accumulation by Dunaliella salina as a function of pH, NaCI, and irradiance. *Russian Journal of Plant Physiology*, *61*(2), 215–223. https://doi.org/10.1134/S1021443714010038
- Cerón, M. C., Campos, I., Sánchez, J. F., Acién, F. G., Molina, E., and Fernández-Sevilla, J. M. (2008). Recovery of lutein from microalgae biomass: Development of a process for Scenedesmus almeriensis biomass. *Journal of Agricultural and Food Chemistry*, *56*(24), 11761–11766. https://doi.org/10.1021/jf8025875
- Chae, J. S., Park, S. K., Roh, K. C., and Park, H. S. (2020). Electrode materials for biomedical patchable and implantable energy storage devices. *Energy Storage Materials*, *24*(April 2019), 113–128. https://doi.org/10.1016/j.ensm.2019.04.032
- Chagas, A. L., Rios, A. O., Jarenkow, A., Marcílio, N. R., Ayub, M. A. Z., and Rech, R. (2015). Production of carotenoids and lipids by Dunaliella tertiolecta using CO2 from beer fermentation. *Process Biochemistry*, *50*(6), 981–988. https://doi.org/10.1016/j.procbio.2015.03.012
- Chan, M. C., Ho, S. H., Lee, D. J., Chen, C. Y., Huang, C. C., and Chang, J. S. (2013). Characterization, extraction and purification of lutein produced by an indigenous microalga Scenedesmus obliquus CNW-N. *Biochemical Engineering Journal*, *78*, 24–31. https://doi.org/10.1016/j.bej.2012.11.017
- Chanakya, H. N., and Malayil, S. (2012). Anaerobic digestion for bioenergy from agro-residues and other solid wastes - An overview of science, technology and sustainability. *Journal of the Indian Institute of Science*, *92*(1), 111–143.

- Chatsungnoen, T., and Chisti, Y. (2016). Harvesting microalgae by flocculation-sedimentation. *Algal Research*, *13*, 271–283. https://doi.org/10.1016/j.algal.2015.12.009
- Chen, C. Y., Jesisca, Hsieh, C., Lee, D. J., Chang, C. H., and Chang, J. S. (2016). Production, extraction and stabilization of lutein from microalga Chlorella sorokiniana MB-1. *Bioresource Technology*, *200*, 500–505. https://doi.org/10.1016/j.biortech.2015.10.071
- Chen, F., and Johns, M. R. (1994). Substrate inhibition of Chlamydomonas reinhardtii by acetate in heterotrophic culture. *Process Biochemistry*, *29*(4), 245–252. https://doi.org/10.1016/0032-9592(94)80064-2
- Chen, F., and Johns, M. R. (1996). Heterotrophic growth of Chlamydomonas reinhardtii on acetate in chemostat culture. *Process Biochemistry*, *31*(6), 601–604. https://doi.org/10.1016/s0032-9592(96)00006-4
- Chen, Y., Cheng, J. J., and Creamer, K. S. (2008). Inhibition of anaerobic digestion process: A review. *Bioresource Technology*, *99*(10), 4044–4064. https://doi.org/10.1016/j.biortech.2007.01.057
- Chisti, M. Y., and Moo-Young, M. (1988). Hydrodynamics and oxygen transfer in pneumatic bioreactor devices. *Biotechnology and Bioengineering*, *31*(5), 487–494. https://doi.org/10.1002/bit.260310514
- Chisti, Y., and Moo-Young, M. (1993). Improve the performance of airlift reactors. *Chemical Engineering Progress*, *89*(6), 38–45. http://tur-www1.massey.ac.nz/~ychisti/ImproveAlft.pdf
- Chitchumroonchokchai, C., and Failla, M. L. (2006). Hydrolysis of zeaxanthin esters by carboxyl ester lipase during digestion facilitates micellarization and uptake of the xanthophyll by Caco-2 human intestinal cells. *Journal of Nutrition*, *136*(3), 588–594. https://doi.org/10.1093/jn/136.3.588
- Chung, H. Y., Rasmussen, H. M., and Johnson, E. J. (2004). Lutein bioavailability is higher from lutein-enriched eggs than from supplements and spinach in men. *Journal of Nutrition*, *134*(8), 1887–1893. https://doi.org/10.1093/jn/134.8.1887
- Church, J., Hwang, J. H., Kim, K. T., McLean, R., Oh, Y. K., Nam, B., Joo, J. C., and Lee, W. H. (2017). Effect of salt type and concentration on the growth and lipid content of Chlorella vulgaris in synthetic saline wastewater for biofuel production. *Bioresource Technology*, *243*, 147–153. https://doi.org/10.1016/j.biortech.2017.06.081
- Cisneros, M., Benavides, J., Brenes, C. H., and Rito-Palomares, M. (2004). Recovery in aqueous two-phase systems of lutein produced by the green microalga Chlorella protothecoides. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *807*(1), 105–110. https://doi.org/10.1016/j.jchromb.2004.01.009
- Collos, Y., and Harrison, P. J. (2014). Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Marine Pollution Bulletin*, *80*(1–2), 8–23. https://doi.org/10.1016/j.marpolbul.2014.01.006
- Conway, E. J., and Downey, M. (1950). An outer metabolic region of the yeast cell. *The Biochemical Journal*, *47*(3), 347–355. https://doi.org/10.1042/bj0470347
- Cordero, B. F., Obraztsova, I., Couso, I., Leon, R., Vargas, M. A., and Rodriguez, H. (2011). Enhancement of lutein production in Chlorella sorokiniana (chorophyta) by improvement of culture conditions and random mutagenesis. *Marine Drugs*, *9*(9), 1607–1624. https://doi.org/10.3390/md9091607

- Cory, A. H., Owen, T. C., Barltrop, J. A., and Cory, J. G. (1991). Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Communications*, *3*(7), 207–212. https://doi.org/10.3727/095535491820873191
- Costard, G. S., Machado, R. R., Barbarino, E., Martino, R. C., and Lourenco, S. O. (2012). Chemical composition of five marine microalgae that occur on the Brazilian coast. *Int. J. Fish. Aquacult.*, *4*(October), 191–201. https://doi.org/10.5897/IJFA11.092
- Craft, N. E., and Soares, J. H. (1992). Relative Solubility, Stability, and Absorptivity of Lutein and β-Carotene In Organic Solvents. *Journal of Agricultural and Food Chemistry*, *40*(3), 431–434. https://doi.org/10.1021/jf00015a013
- Csögör, Z., Herrenbauer, M., Perner, I., Schmidt, K., and Posten, C. (1999). Design of a photo-bioreactor for modelling purposes. *Chemical Engineering and Processing: Process Intensification*, *38*(4–6), 517–523. https://doi.org/10.1016/S0255-2701(99)00048-3
- Dalrymple, O. K., Halfhide, T., Udom, I., Gilles, B., Wolan, J., Zhang, Q., and Ergas, S. (2013). Wastewater use in algae production for generation of renewable resources: A review and preliminary results. *Aquatic Biosystems*, *9*(1), 1. https://doi.org/10.1186/2046-9063-9-2
- Dammak, M., Hadrich, B., Barkallah, M., Hentati, F., Ben Hlima, H., Pichon, C., Denis, M., Fendri, I., Michaud, P., and Abdelkafi, S. (2018). Modelling Tetraselmis sp. growth-kinetics and optimizing bioactive-compound production through environmental conditions. *Bioresource Technology*, *249*(October 2017), 510–518. https://doi.org/10.1016/j.biortech.2017.10.028
- Danquah, M. K., Ang, L., Uduman, N., Moheimani, N., and Forde, G. M. (2009). Dewatering of microalgal culture for biodiesel production: Exploring polymer flocculation and tangential flow filtration. *Journal of Chemical Technology and Biotechnology*, *84*(7), 1078–1083. https://doi.org/10.1002/jctb.2137
- Daood, H. G., Bencze, G., Palotás, G., Pék, Z., Sidikov, A., and Helyes, L. (2014). HPLC analysis of carotenoids from tomatoes using cross-linked C18 column and MS detection. *Journal of Chromatographic Science*, *52*(9), 985–991. https://doi.org/10.1093/chromsci/bmt139
- De Jesus, S. S., Ferreira, G. F., Moreira, L. S., Wolf Maciel, M. R., and Maciel Filho, R. (2019). Comparison of several methods for effective lipid extraction from wet microalgae using green solvents. *Renewable Energy*, *143*, 130–141. https://doi.org/10.1016/j.renene.2019.04.168
- Decker, T., and Lohmann-Matthes, M. L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods*, *115*(1), 61–69. https://doi.org/10.1016/0022-1759(88)90310-9
- Deenu, A., Naruenartwongsakul, S., and Kim, S. M. (2013). Optimization and economic evaluation of ultrasound extraction of lutein from Chlorella vulgaris. *Biotechnology and Bioprocess Engineering*, *18*(6), 1151–1162. https://doi.org/10.1007/s12257-013-0213-8
- Del Campo, J. A., García-González, M., and Guerrero, M. G. (2007). Outdoor cultivation of microalgae for carotenoid production: Current state and perspectives. *Applied Microbiology and Biotechnology*, *74*(6), 1163–1174. https://doi.org/10.1007/s00253-007-0844-9
- Del Campo, J. A., Moreno, J., Rodríguez, H., Angeles Vargas, M., Rivas, J., and Guerrero, M. G. (2000). Carotenoid content of chlorophycean microalgae: factors

determining lutein accumulation in Muriellopsis sp. (Chlorophyta). *Journal of Biotechnology*, *76*(1), 51–59. https://doi.org/10.1016/S0168-1656(99)00178-9

- Del Campo, J. A., Rodríguez, H., Moreno, J., Vargas, M. Á., Rivas, J., and Guerrero, M. G. (2004). Accumulation of astaxanthin and lutein in Chlorella zofingiensis (Chlorophyta). *Applied Microbiology and Biotechnology*, *64*(6), 848–854. https://doi.org/10.1007/s00253-003-1510-5
- Del Campo, J. A., Rodríguez, H., Moreno, J., Vargas, M. Á., Rivas, J., and Guerrero, M. G. (2001). Lutein production by Muriellopsis sp. in an outdoor tubular photobioreactor. *Journal of Biotechnology*, *85*(3), 289–295. https://doi.org/10.1016/S0168-1656(00)00380-1
- Demirbas, M. F. (2011). Biofuels from algae for sustainable development. *Applied Energy*, *88*(10), 3473–3480. https://doi.org/10.1016/j.apenergy.2011.01.059
- Demmig-Adams, B., and Adams, W. W. (1992). Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology*, *43*(1), 599–626. https://doi.org/10.1146/annurev.pp.43.060192.003123
- Derrien, M., Badr, A., Gosselin, A., Desjardins, Y., and Angers, P. (2017). Optimization of a green process for the extraction of lutein and chlorophyll from spinach by-products using response surface methodology (RSM). *LWT - Food Science and Technology*, *79*, 170–177. https://doi.org/10.1016/j.lwt.2017.01.010
- Desloover, J., Abate Woldeyohannis, A., Verstraete, W., Boon, N., and Rabaey, K. (2012). Electrochemical resource recovery from digestate to prevent ammonia toxicity during anaerobic digestion. *Environmental Science and Technology*, *46*(21), 12209–12216. https://doi.org/10.1021/es3028154
- Devanand, P., and Rani, P. U. (2011). Insect growth regulatory activity of the crude and purified fractions from Solanum melongena L., Lycopersicum esculentum Mill. and Capsicum annuum L. *Journal of Biopesticides*, *4*(2), 118–130.
- Dineshkumar, R., Dhanarajan, G., Dash, S. K., and Sen, R. (2015). An advanced hybrid medium optimization strategy for the enhanced productivity of lutein in Chlorella minutissima. *Algal Research*, *7*, 24–32. https://doi.org/10.1016/j.algal.2014.11.010
- Dlangamandla, N., Ntwampe, S. K. O., Angadam, J. O., Chidi, B. S., and Mewa-Ngongang, M. (2019). Kinetic parameters of saccharomyces cerevisiae alcohols production using nepenthes mirabilis pod digestive fluids-mixed agro-waste hydrolysates. *Fermentation*, *5*(1). https://doi.org/10.3390/fermentation5010010
- Duellman, S. J., Zhou, W., Meisenheimer, P., Vidugiris, G., Cali, J. J., Gautam, P., Wennerberg, K., and Vidugiriene, J. (2015). Bioluminescent, Nonlytic, Real-Time Cell Viability Assay and Use in Inhibitor Screening. *Assay and Drug Development Technologies*, *13*(8), 456–465. https://doi.org/10.1089/adt.2015.669
- El-Ghany, W. A. A. (2020). Microalgae in poultry field: A comprehensive perspectives. *Advances in Animal and Veterinary Sciences*, *8*(9), 888–897. https://doi.org/10.17582/JOURNAL.AAVS/2020/8.9.888.897
- El-Sheekh, M. M., El-Mohsnawy, E., Mabrouk, M. E. M., and Zoheir, W. F. (2020). Enhancement of biodiesel production from the green microalga Micractinium reisseri via optimization of cultivation regimes . *Journal of Taibah University for Science*, *14*(1), 437–444. https://doi.org/10.1080/16583655.2020.1745505
- Endo, H., Nakajima, K., Chino, R., and Shirota, M. (1974). Growth Characteristics and Cellular Components of Chlorella Regularis, Heterotrophic Fast Growing Strain. *Agricultural and Biological Chemistry*, *38*(1), 9–18. https://doi.org/10.1271/bbb1961.38.9

- Endo, H., Sansawa, H., and Nakajima, K. (1977). Studies on chlorella regularis, heterotrophic fast-growing strain II. Mixotrophic growth in relation to light intensity and acetate concentration. *Plant and Cell Physiology*, *18*(1), 199–205. https://doi.org/10.1093/oxfordjournals.pcp.a075413
- Englmaierová, M., Skřivan, M., and Bubancová, I. (2013). A comparison of lutein, spray-dried Chlorella, and synthetic carotenoids effects on yolk colour, oxidative stability, and reproductive performance of laying hens. *Czech Journal of Animal Science*, *58*(9), 412–419. https://doi.org/10.17221/6941-cjas
- Fan, X. D., Hou, Y., Huang, X. X., Qiu, T. Q., and Jiang, J. G. (2015). Ultrasound-enhanced subcritical CO2 extraction of lutein from Chlorella pyrenoidosa. *Journal of Agricultural and Food Chemistry*, *63*(18), 4597–4605. https://doi.org/10.1021/acs.jafc.5b00461
- Fan, Z., Sun, L., Huang, Y., Wang, Y., and Zhang, M. (2016). Bioinspired fluorescent dipeptide nanoparticles for targeted cancer cell imaging and real-time monitoring of drug release. *Nature Nanotechnology*, *11*(4), 388–394. https://doi.org/10.1038/nnano.2015.312
- Fayad, N., Yehya, T., Audonnet, F., and Vial, C. (2017). Harvesting of microalgae Chlorella vulgaris using electro-coagulation-flocculation in the batch mode. *Algal Research*, *25*, 1–11. https://doi.org/10.1016/j.algal.2017.03.015
- Feng, L., Liu, Z. M., Xu, L., Lv, X., Ning, J., Hou, J., Ge, G. B., Cui, J. N., and Yang, L. (2014). A highly selective long-wavelength fluorescent probe for the detection of human carboxylesterase 2 and its biomedical applications. *Chemical Communications*, *50*(93), 14519–14522. https://doi.org/10.1039/c4cc06642a
- Feoktistova, M., Geserick, P., and Leverkus, M. (2016). Crystal violet assay for determining viability of cultured cells. *Cold Spring Harbor Protocols*, *2016*(4), 343–346. https://doi.org/10.1101/pdb.prot087379
- Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D. P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Häcker, G., and Leverkus, M. (2011). CIAPs Block Ripoptosome Formation, a RIP1/Caspase-8 Containing Intracellular Cell Death Complex Differentially Regulated by cFLIP Isoforms. *Molecular Cell*, *43*(3), 449–463. https://doi.org/10.1016/j.molcel.2011.06.011
- Fernández-Sevilla, J. M., Acién Fernández, F. G., and Molina Grima, E. (2010). Biotechnological production of lutein and its applications. *Applied Microbiology and Biotechnology*, *86*(1), 27–40. https://doi.org/10.1007/s00253-009-2420-y
- Flórez-Miranda, L., Cañizares-Villanueva, R. O., Melchy-Antonio, O., Martínez-Jerónimo, F., and Flores-Ortíz, C. M. (2017). Two stage heterotrophy/photoinduction culture of Scenedesmus incrassatulus: potential for lutein production. *Journal of Biotechnology*, *262*, 67–74. https://doi.org/10.1016/j.jbiotec.2017.09.002
- Franssen, M. C. R., Steunenberg, P., Scott, E. L., Zuilhof, H., and Sanders, J. P. M. (2013). Immobilised enzymes in biorenewables production. *Chemical Society Reviews*, *42*(15), 6491–6533. https://doi.org/10.1039/c3cs00004d
- Fredriksson, S., Elwinger, K., and Pickova, J. (2006). Fatty acid and carotenoid composition of egg yolk as an effect of microalgae addition to feed formula for laying hens. *Food Chemistry*, *99*(3), 530–537. https://doi.org/10.1016/j.foodchem.2005.08.018
- Fu, W., Paglia, G., Magnúsdóttir, M., Steinarsdóttir, E. A., Gudmundsson, S., Palsson, B. T., Andrésson, Ó. S., and Brynjólfsson, S. (2014). Effects of abiotic stressors on lutein production in the green microalga Dunaliella salina. *Microbial Cell Factories*, *13*(1), 1–9. https://doi.org/10.1186/1475-2859-13-3

- Fuente, D., Keller, J., Conejero, J. A., Rögner, M., Rexroth, S., and Urchueguía, J. F. (2017). Light distribution and spectral composition within cultures of micro-algae: Quantitative modelling of the light field in photobioreactors. *Algal Research*, *23*, 166–177. https://doi.org/10.1016/j.algal.2017.01.004
- Gabriel Acien Fernandez, F., González-López, C. V., Fernández Sevilla, J. M., and Molina Grima, E. (2012). Conversion of CO 2 into biomass by microalgae: How realistic a contribution may it be to significant CO 2 removal? *Applied Microbiology and Biotechnology*, *96*(3), 577–586. https://doi.org/10.1007/s00253-012-4362-z
- Gansukh, E., Mya, K. K., Jung, M., Keum, Y. S., Kim, D. H., and Saini, R. K. (2019). Lutein derived from marigold (Tagetes erecta) petals triggers ROS generation and activates Bax and caspase-3 mediated apoptosis of human cervical carcinoma (HeLa) cells. *Food and Chemical Toxicology*, *127*(February), 11–18. https://doi.org/10.1016/j.fct.2019.02.037
- Gao, S., Yang, J., Tian, J., Ma, F., Tu, G., and Du, M. (2010). Electro-coagulation-flotation process for algae removal. *Journal of Hazardous Materials*, *177*(1–3), 336–343. https://doi.org/10.1016/j.jhazmat.2009.12.037
- Garnier, A., and Gaillet, B. (2015). Analytical solution of Luedeking-Piret equation for a batch fermentation obeying Monod growth kinetics. *Biotechnology and Bioengineering*, *112*(12), 2468–2474. https://doi.org/10.1002/bit.25669
- Geens, J., De Witte, B., and Van Der Bruggen, B. (2007). Removal of API's (active pharmaceutical ingredients) from organic solvents by nanofiltration. *Separation Science and Technology*, *42*(11), 2435–2449. https://doi.org/10.1080/01496390701477063
- Gentili, F. G. (2014). Microalgal biomass and lipid production in mixed municipal, dairy, pulp and paper wastewater together with added flue gases. *Bioresource Technology*, *169*, 27–32. https://doi.org/10.1016/j.biortech.2014.06.061
- Geoffrey, B. A., and Felix, M. B. (1991). Canthaxanthin and the Eye: A critical ocular toxicologic assessment. *Cutaneous and Ocular Toxicology*, *10*(1–2), 115–155. https://doi.org/10.3109/15569529109057908
- Gerde, J. A., Montalbo-Lomboy, M., Yao, L., Grewell, D., and Wang, T. (2012). Evaluation of microalgae cell disruption by ultrasonic treatment. *Bioresource Technology*, *125*, 175–181. https://doi.org/10.1016/j.biortech.2012.08.110
- Gherardi, S. R. M., Santos, B. M., Silva, F. A., Stringhini, J. H., and Café, M. B. (2015). Physical and Chemical Changes and Functional Properties of White Eggs as a Function of Time and Conditions of Storage. *XXII European Symposium on the Quality of Poultry Meat XVI European Symposium*, *May*. https://doi.org/10.13140/RG.2.2.21110.09282
- Ghirardini, A., Grillini, V., and Verlicchi, P. (2020). A review of the occurrence of selected micropollutants and microorganisms in different raw and treated manure – Environmental risk due to antibiotics after application to soil. In *Science of the Total Environment* (Vol. 707). https://doi.org/10.1016/j.scitotenv.2019.136118
- Gholami, M., Nasseri, S., Mesdaghinia, A., Vaezi, F., Mahvi, A., and Naddaffi, K. (2001). Dye Removal from Effluents of Textile Industries by ISO9888 Method and Mebrane Technology. *Iranian J. Publ. Health*, *30*(1–2), 73–80. http://ijph.tums.ac.ir/index.php/ijph/article/view/1680/1661
- Ginzberg, A., Cohen, M., Sod-Moriah, U. A., Shany, S., Rosenshtrauch, A., and Arad, S. (2000). Chickens fed with biomass of the red microalga Porphyridium sp. have reduced blood cholesterol level and modified fatty acid composition in egg yolk.

Journal of Applied Phycology, *12*(3–5), 325–330. https://doi.org/10.1023/a:1008102622276

- Giuliano, G., Giliberto, L., and Rosati, C. (2002). Carotenoid isomerase: A tale of light and isomers. *Trends in Plant Science*, *7*(10), 427–429. https://doi.org/10.1016/S1360-1385(02)02329-4
- Golzar Adabi, S. H., Kamali, M. A., Davoudi, J., Cooper, R. G., and Hajbabaei, A. (2010). Quantification of lutein in egg following feeding hens with a lutein supplement and quantification of lutein in human plasma after consumption of lutein enriched eggs. *Archiv Fur Geflugelkunde*, *74*(3), 158–163. https://www.researchgate.net/profile/Jaber-Davoodi-2/publication/285942806_Quantification_of_lutein_in_egg_following_feeding_hens_with_a_lutein_supplement_and_quantification_of_lutein_in_human_plasma_after_consumption_of_lutein_enriched_eggs/links/595735f
- González-Coloma, A., Reina, M., Medinaveitia, A., Guadaño, A., Santana, O., Martínez-Díaz, R., Ruiz-Mesía, L., Alva, A., Grandez, M., Díaz, R., Gavín, J. A., and De La Fuente, G. (2004). Structural diversity and defensive properties of norditerpenoid alkaloids. *Journal of Chemical Ecology*, *30*(7), 1393–1408. https://doi.org/10.1023/B:JOEC.0000037747.74665.0a
- González-González, L. M., Astals, S., Pratt, S., Jensen, P. D., and Schenk, P. M. (2019). Impact of osmotic shock pre-treatment on microalgae lipid extraction and subsequent methane production. *Bioresource Technology Reports*, *7*(February), 100214. https://doi.org/10.1016/j.biteb.2019.100214
- Grimi, N., Dubois, A., Marchal, L., Jubeau, S., Lebovka, N. I., and Vorobiev, E. (2014). Selective extraction from microalgae Nannochloropsis sp. using different methods of cell disruption. *Bioresource Technology*, *153*, 254–259. https://doi.org/10.1016/j.biortech.2013.12.011
- Grudzinski, W., Krzeminska, I., Luchowski, R., Nosalewicz, A., and Gruszecki, W. I. (2016). Strong-light-induced yellowing of green microalgae Chlorella: A study on molecular mechanisms of the acclimation response. *Algal Research*, *16*(April), 245–254. https://doi.org/10.1016/j.algal.2016.03.021
- Gu, N., Lin, Q., Li, G., Tan, Y., Huang, L., and Lin, J. (2012). Effect of salinity on growth, biochemical composition, and lipid productivity of Nannochloropsis oculata CS 179. *Engineering in Life Sciences*, *12*(6), 631–637. https://doi.org/10.1002/elsc.201100204
- Guedes, A. C., Amaro, H. M., and Malcata, F. X. (2011). Microalgae as sources of carotenoids. *Marine Drugs*, *9*(4), 625–644. https://doi.org/10.3390/md9040625
- Guerrero-Cabrera, L., Rueda, J. A., García-Lozano, H., and Navarro, A. K. (2014). Cultivation of Monoraphidium sp., Chlorella sp. and Scenedesmus sp. algae in Batch culture using Nile tilapia effluent. *Bioresource Technology*, *161*, 455–460. https://doi.org/10.1016/j.biortech.2014.03.127
- Guida, V., Niro, E., Landi, N., Chambery, A., Parente, A., Cantarella, L., Cantarella, M., and Di Maro, A. (2014). Immobilised peroxidases from Asparagus acutifolius L. seeds for olive mill waste water treatment. *RSC Advances*, *4*(106), 61482–61490. https://doi.org/10.1039/c4ra11310a
- Guo, J., Zhang, L., Lu, X., Zeng, S., Zhang, Y., Xu, H., and Zheng, B. (2016). Medium optimization and fermentation kinetics for κ-carrageenase production by Thalassospira sp. Fjfst-332. *Molecules*, *21*(11). https://doi.org/10.3390/molecules21111479

- Guo, X., and Mei, N. (2014). Assessment of the toxic potential of graphene family nanomaterials. *Journal of Food and Drug Analysis*, *22*(1), 105–115. https://doi.org/10.1016/j.jfda.2014.01.009
- Guo, X., Yao, L., and Huang, Q. (2015). Aeration and mass transfer optimization in a rectangular airlift loop photobioreactor for the production of microalgae. *Bioresource Technology*, *190*, 189–195. https://doi.org/10.1016/j.biortech.2015.04.077
- Gustavs, L., Eggert, A., Michalik, D., and Karsten, U. (2010). Physiological and biochemical responses of green microalgae from different habitats to osmotic and matric stress. *Protoplasma*, *243*(1), 3–14. https://doi.org/10.1007/s00709-009-0060-9
- Halim, R., Papachristou, I., Kubisch, C., Nazarova, N., Wüstner, R., Steinbach, D., Chen, G. Q., Deng, H., Frey, W., Posten, C., and Silve, A. (2021). Hypotonic osmotic shock treatment to enhance lipid and protein recoveries from concentrated saltwater Nannochloropsis slurries. *Fuel*, *287*(August 2020), 119442. https://doi.org/10.1016/j.fuel.2020.119442
- Halle, I., Janczyk, P., Freyer, G., and Souffrant, W. B. (2009). Effect of microalgae Chlorella vulgaris on laying hen performance. *Archiva Zootechnica*, *122*, 5–13.
- HAMULKA, J., KOCZARA, J., and GRONEK, M. (2005). Lutein content of selected polish foods and estimation of its intake. *Polish Journal of Food and Nutrition Sciences*, *14*(2), 201–206.
- Handelman, G. J., Nightingale, Z. D., Lichtenstein, A. H., Schaefer, E. J., and Blumberg, J. B. (1999). Lutein and zeaxanthin concentrations in plasma after dietary supplementation with egg yolk. *American Journal of Clinical Nutrition*, *70*(2), 247–251. https://doi.org/10.1093/ajcn.70.2.247
- Harun, R., Singh, M., Forde, G. M., and Danquah, M. K. (2010). Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable and Sustainable Energy Reviews*, *14*(3), 1037–1047. https://doi.org/10.1016/j.rser.2009.11.004
- Hematpoor, A., Liew, S. Y., Azirun, M. S., and Awang, K. (2017). Insecticidal activity and the mechanism of action of three phenylpropanoids isolated from the roots of Piper sarmentosum Roxb. *Scientific Reports*, *7*(1), 1–13. https://doi.org/10.1038/s41598-017-12898-z
- Heo, J. Y., Kim, S., Kang, J. H., and Moon, B. (2014). Determination of lutein from green tea and green tea by-products using accelerated solvent extraction and UPLC. *Journal of Food Science*, *79*(5), 816–821. https://doi.org/10.1111/1750-3841.12438
- Hermassi, M., Valderrama, C., Gibert, O., Moreno, N., Querol, X., Batis, N. H., and Cortina, J. L. (2017). Recovery of nutrients (N-P-K) from potassium-rich sludge anaerobic digestion side-streams by integration of a hybrid sorption-membrane ultrafiltration process: Use of powder reactive sorbents as nutrient carriers. *Science of the Total Environment*, *599–600*, 422–430. https://doi.org/10.1016/j.scitotenv.2017.04.140
- Ho, S. H., Chan, M. C., Liu, C. C., Chen, C. Y., Lee, W. L., Lee, D. J., and Chang, J. S. (2014). Enhancing lutein productivity of an indigenous microalga Scenedesmus obliquus FSP-3 using light-related strategies. *Bioresource Technology*, *152*, 275–282. https://doi.org/10.1016/j.biortech.2013.11.031
- Ho, S. H., Xie, Y., Chan, M. C., Liu, C. C., Chen, C. Y., Lee, D. J., Huang, C. C., and Chang, J. S. (2015). Effects of nitrogen source availability and bioreactor operating strategies on lutein production with Scenedesmus obliquus FSP-3. *Bioresource Technology*, *184*, 131–138. https://doi.org/10.1016/j.biortech.2014.10.062

- Ho, S. H., Ye, X., Hasunuma, T., Chang, J. S., and Kondo, A. (2014). Perspectives on engineering strategies for improving biofuel production from microalgae - A critical review. *Biotechnology Advances*, *32*(8), 1448–1459. https://doi.org/10.1016/j.biotechadv.2014.09.002
- Hosseini Tafreshi, A., and Shariati, M. (2009). Dunaliella biotechnology: Methods and applications. *Journal of Applied Microbiology*, *107*(1), 14–35. https://doi.org/10.1111/j.1365-2672.2009.04153.x
- Huang, J. F., Tian, M., Lv, C. J., Li, H. Y., Muhammad, R. ul H., and Zhong, G. H. (2011). Preliminary studies on induction of apoptosis by abamectin in Spodoptera frugiperda (Sf9) cell line. *Pesticide Biochemistry and Physiology*, *100*(3), 256–263. https://doi.org/10.1016/j.pestbp.2011.04.010
- Huang, J., Xia, J., Jiang, W., Li, Y., and Li, J. (2015). Biodiesel production from microalgae oil catalyzed by a recombinant lipase. *Bioresource Technology*, *180*, 47–53. https://doi.org/10.1016/j.biortech.2014.12.072
- Humphries, J. M., Graham, R. D., and Mares, D. J. (2004). Application of reflectance colour measurement to the estimation of carotene and lutein content in wheat and triticale. *Journal of Cereal Science*, *40*(2), 151–159. https://doi.org/10.1016/j.jcs.2004.07.005
- Jalal, K. C. a., Md Zahangir, A., Matin, W. a., Kamaruzzaman, B. Y., Akbar, J., and Toffazel, H. (2011). Removal of Nitrate and Phosphate From Municipal Wastewater Sludge By *Chlorella Vulgaris*, Spirulina Platensis and. *IIUM Engineering Journal*, *12*(4), 125–132.
- Jeon, J. Y., Kwon, J. S., Kang, S. T., Kim, B. R., Jung, Y., Han, J. G., Park, J. H., and Hwang, J. K. (2014). Optimization of culture media for large-scale lutein production by heterotrophic Chlorella vulgaris. *Biotechnology Progress*, *30*(3), 736–743. https://doi.org/10.1002/btpr.1889
- Jeyasankar, A., Premalatha, S., and Elumalai, K. (2012). Biological activities of Solanum pseudocapsicum (Solanaceae) against cotton bollworm, Helicoverpa armigera Hübner and armyworm, Spodoptera litura Fabricius (Lepidotera: Noctuidae). *Asian Pacific Journal of Tropical Biomedicine*, *2*(12), 981–986. https://doi.org/10.1016/S2221-1691(13)60010-6
- Jimenez, B., Noyola, A., and Capdeville, B. (1988). Selected dyes for residence time distribution evaluation in bioreactors. *Biotechnology Techniques*, *2*(2), 77–82. https://doi.org/10.1007/BF01876154
- Jin, E. S., and Melis, A. (2003). Microalgal biotechnology: Carotenoid production by the green algae Dunaliella salina. *Biotechnology and Bioprocess Engineering*, *8*(6), 331–337. https://doi.org/10.1007/BF02949276
- Jones, K. H., and Senft, J. A. (1985). An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *Journal of Histochemistry and Cytochemistry*, *33*(1), 77–79. https://doi.org/10.1177/33.1.2578146
- Kale, S., Bhandare, S., Gaikwad, M., Urunkar, V., and Rajmane, A. (2011). Formulation and in vitro evaluation for sun protection factor of lutein ester extracted from Tagetes erecta Linn flower (Family-Asteraceae) sunscreen creams. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *2*(3), 947–955.
- Karnjanawipagul.P, W. Nittayanuntawech, P. R. and L. S. (2010). Analysis of β-Carotene in Carrot by Spectrophotometry. *Thailand: Mahol University. Department of Pharmaceutical Chemistry, Faculty of Pharmacy*, 37 (1-2), 8–16.

- Kawase, Y., and Moo-Young, M. (1989). Mixing time in bioreactors. *Journal of Chemical Technology and Biotechnology*, *44*(1), 63–75. https://doi.org/10.1002/jctb.280440107
- Kendall, L. (1996). The Osmotic Adjustment of Marine Microalgae and the Complex Role Osmolytes Play in This Process. *Department of Marine Biology, The University of Groningen, April*, 34.
- Khachik, F., Bernstein, P. S., and Garland, D. L. (1997). Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. *Investigative Ophthalmology and Visual Science*, *38*(9), 1802–1811.
- Khin, M. M., Nair, A. S., Babu, V. J., Murugan, R., and Ramakrishna, S. (2012). A review on nanomaterials for environmental remediation. *Energy and Environmental Science*, *5*(8), 8075–8109. https://doi.org/10.1039/c2ee21818f
- Kim, S. H., Liu, K. H., Lee, S. Y., Hong, S. J., Cho, B. K., Lee, H., Lee, C. G., and Choi, H. K. (2013). Effects of Light Intensity and Nitrogen Starvation on Glycerolipid, Glycerophospholipid, and Carotenoid Composition in Dunaliella tertiolecta Culture. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0072415
- Kim, S. I., Kim, H. J., Lee, H. J., Lee, K., Hong, D., Lim, H., Cho, K., Jung, N., and Yi, Y. W. (2016). Application of a non-hazardous vital dye for cell counting with automated cell counters. *Analytical Biochemistry*, *492*, 8–12. https://doi.org/10.1016/j.ab.2015.09.010
- Kliphuis, A. M. J., Klok, A. J., Martens, D. E., Lamers, P. P., Janssen, M., and Wijffels, R. H. (2012). Metabolic modeling of Chlamydomonas reinhardtii: Energy requirements for photoautotrophic growth and maintenance. *Journal of Applied Phycology*, *24*(2), 253–266. https://doi.org/10.1007/s10811-011-9674-3
- Kommareddy, A., and Anderson, G. (2004). Analysis of currents and mixing in a modified bubble column reactor. *ASAE Annual International Meeting 2004, January 2004*, 4105–4114. https://doi.org/10.13031/2013.17681
- Kotake-Nara, E., Kushiro, M., Zhang, H., Sugawara, T., Miyashita, K., and Nagao, A. (2001). Carotenoids affect proliferation of human prostate cancer cells. *Journal of Nutrition*, *131*(12), 3303–3306. https://doi.org/10.1093/jn/131.12.3303
- Kotrbáček, V., Doubek, J., and Doucha, J. (2015). The chlorococcalean alga Chlorella in animal nutrition: a review. *Journal of Applied Phycology*, *27*(6), 2173–2180. https://doi.org/10.1007/s10811-014-0516-y
- Krehbiel, J. D., Schideman, L. C., King, D. A., and Freund, J. B. (2014). Algal cell disruption using microbubbles to localize ultrasonic energy. *Bioresource Technology*, *173*, 448–451. https://doi.org/10.1016/j.biortech.2014.09.072
- Krinsky, N. I., and Johnson, E. J. (2005). Carotenoid actions and their relation to health and disease. In *Molecular Aspects of Medicine* (Vol. 26, Issue 6, pp. 459–516). https://doi.org/10.1016/j.mam.2005.10.001
- Krishna Koyande, A., Tanzil, V., Murraly Dharan, H., Subramaniam, M., Robert, R. N., Lau, P. L., Khoiroh, I., and Show, P. L. (2020). Integration of osmotic shock assisted liquid biphasic system for protein extraction from microalgae Chlorella vulgaris. *Biochemical Engineering Journal*, *157*(September 2019), 107532. https://doi.org/10.1016/j.bej.2020.107532
- Krömer, J. O., Wittmann, C., Schröder, H., and Heinzle, E. (2006). Metabolic pathway analysis for rational design of L-methionine production by Escherichia coli and Corynebacterium glutamicum. *Metabolic Engineering*, *8*(4), 353–369. https://doi.org/10.1016/j.ymben.2006.02.001

- Kumar, K., and Das, D. (2012). Growth characteristics of Chlorella sorokiniana in airlift and bubble column photobioreactors. *Bioresource Technology*, *116*, 307–313. https://doi.org/10.1016/j.biortech.2012.03.074
- Kumar, R. R., Rao, P. H., and Arumugam, M. (2015). Lipid extraction methods from microalgae: A comprehensive review. *Frontiers in Energy Research*, *3*(JAN), 1–9. https://doi.org/10.3389/fenrg.2014.00061
- Kunga, L., Vanags, J., and Centrs, B. (2015). *Cultivation of microalgae in shake flasks and laboratory scale photobioreactor Microalgae*. http://bioreactors.net/wp-content/uploads/2015/10/Photobioreactor_applicationENG.pdf
- Lakshminarayana, R., Sathish, U. V., Dharmesh, S. M., and Baskaran, V. (2010). Antioxidant and cytotoxic effect of oxidized lutein in human cervical carcinoma cells (HeLa). *Food and Chemical Toxicology*, *48*(7), 1811–1816. https://doi.org/10.1016/j.fct.2010.04.011
- Lam, M. K., and Lee, K. T. (2014). Cultivation of Chlorella vulgaris in a pilot-scale sequential-baffled column photobioreactor for biomass and biodiesel production. *Energy Conversion and Management*, *88*, 399–410. https://doi.org/10.1016/j.enconman.2014.08.063
- Lamers, P. P., Janssen, M., De Vos, R. C. H., Bino, R. J., and Wijffels, R. H. (2008). Exploring and exploiting carotenoid accumulation in Dunaliella salina for cell-factory applications. *Trends in Biotechnology*, *26*(11), 631–638. https://doi.org/10.1016/j.tibtech.2008.07.002
- Laurens, L. M. L., Van Wychen, S., McAllister, J. P., Arrowsmith, S., Dempster, T. A., McGowen, J., and Pienkos, P. T. (2014). Strain, biochemistry, and cultivation-dependent measurement variability of algal biomass composition. *Analytical Biochemistry*, *452*(1), 86–95. https://doi.org/10.1016/j.ab.2014.02.009
- Lee, A. K., Lewis, D. M., and Ashman, P. J. (2013). Force and energy requirement for microalgal cell disruption: An atomic force microscope evaluation. *Bioresource Technology*, *128*, 199–206. https://doi.org/10.1016/j.biortech.2012.10.032
- Lee, A. K., Lewis, D. M., and Ashman, P. J. (2013). Harvesting of marine microalgae by electroflocculation: The energetics, plant design, and economics. *Applied Energy*, *108*, 45–53. https://doi.org/10.1016/j.apenergy.2013.03.003
- Leeson, S., and Caston, L. (2004). Enrichment of eggs with lutein. *Poultry Science*, *83*(10), 1709–1712. https://doi.org/10.1093/ps/83.10.1709
- Leeson, S., Gaston, L., and Namkung, H. (2007). Effect of dietary lutein and flax on performance, egg composition and liver status of laying hens. *Canadian Journal of Animal Science*, *87*(3), 365–372. https://doi.org/10.4141/A06-043
- Lemahieu, C., Bruneel, C., Termote-Verhalle, R., Muylaert, K., Buyse, J., and Foubert, I. (2013). Impact of feed supplementation with different omega-3 rich microalgae species on enrichment of eggs of laying hens. *Food Chemistry*, *141*(4), 4051–4059. https://doi.org/10.1016/j.foodchem.2013.06.078
- Li, H. Bin, Chen, F., Zhang, T. Y., Yang, F. Q., and Xu, G. Q. (2001). Preparative isolation and purification of lutein from the microalga Chlorella vulgaris by high-speed counter-current chromatography. *Journal of Chromatography A*, *905*(1–2), 151–155. https://doi.org/10.1016/S0021-9673(00)00987-0
- Li, T., Xu, J., Gao, B., Xiang, W., Li, A., and Zhang, C. (2016). Morphology, growth, biochemical composition and photosynthetic performance of Chlorella vulgaris (Trebouxiophyceae) under low and high nitrogen supplies. *Algal Research*, *16*, 481–491. https://doi.org/10.1016/j.algal.2016.04.008

- Li, W. W., Yu, H. Q., and He, Z. (2014). Towards sustainable wastewater treatment by using microbial fuel cells-centered technologies. *Energy and Environmental Science*, *7*(3), 911–924. https://doi.org/10.1039/c3ee43106a
- Li, X., De Feyter, S., Chen, D., Aldea, S., Vandezande, P., Prez, F. Du, and Vankelecom, I. F. J. (2008). Solvent-resistant nanofiltration membranes based on multilayered polyelectrolyte complexes. *Chemistry of Materials*, *20*(12), 3876–3883. https://doi.org/10.1021/cm703072k
- Li, Y., Chen, Y. F., Chen, P., Min, M., Zhou, W., Martinez, B., Zhu, J., and Ruan, R. (2011). Characterization of a microalga Chlorella sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. *Bioresource Technology*, *102*(8), 5138–5144. https://doi.org/10.1016/j.biortech.2011.01.091
- Li, Y., Horsman, M., Wang, B., Wu, N., and Lan, C. Q. (2008). Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. *Applied Microbiology and Biotechnology*, *81*(4), 629–636. https://doi.org/10.1007/s00253-008-1681-1
- Lichtenthaler, H. K. (1987). Chlorophylls and Carotenoids: Pigments of Photosynthetic Biomembranes. *Methods in Enzymology*, *148*(C), 350–382. https://doi.org/10.1016/0076-6879(87)48036-1
- Lichtenthaler, H. K., and Buschmann, C. (2001). Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. *Current Protocols in Food Analytical Chemistry*, *1*(1), F4.3.1-F4.3.8. https://doi.org/10.1002/0471142913.faf0403s01
- Liliek Nurhidayati, M. I. (2012). Lutein Contente Analysis in Green and Red Spinach (Amaranthus tricolor L.) by visible spectrophotometry. *T He International Seminar on Natural Product s Medicines in School of Pharmacy Bandung Institute of Technology, November 22 Nd - 23 Rd , 2012*, *Rukmana 1994*.
- Lim, D. K. Y., Garg, S., Timmins, M., Zhang, E. S. B., Thomas-Hall, S. R., Schuhmann, H., Li, Y., and Schenk, P. M. (2012). Isolation and evaluation of oil-producing microalgae from subtropical coastal and Brackish waters. *PLoS ONE*, *7*(7). https://doi.org/10.1371/journal.pone.0040751
- Lin, J. H., Lee, D. J., and Chang, J. S. (2015). Lutein in specific marigold flowers and microalgae. *Journal of the Taiwan Institute of Chemical Engineers*, *49*, 90–94. https://doi.org/10.1016/j.jtice.2014.11.031
- Lin, J. H., Lee, D. J., and Chang, J. S. (2015). Lutein production from biomass: Marigold flowers versus microalgae. *Bioresource Technology*, *184*, 421–428. https://doi.org/10.1016/j.biortech.2014.09.099
- Liu, J., Sun, Z., Gerken, H., Liu, Z., Jiang, Y., and Chen, F. (2014). Chlorella zofingiensis as an alternative microalgal producer of astaxanthin: Biology and industrial potential. *Marine Drugs*, *12*(6), 3487–3515. https://doi.org/10.3390/md12063487
- Liu, Z. Y., Wang, G. C., and Zhou, B. C. (2008). Effect of iron on growth and lipid accumulation in Chlorella vulgaris. *Bioresource Technology*, *99*(11), 4717–4722. https://doi.org/10.1016/j.biortech.2007.09.073
- Lizzul, A. M., Hellier, P., Purton, S., Baganz, F., Ladommatos, N., and Campos, L. (2014). Combined remediation and lipid production using Chlorella sorokiniana grown on wastewater and exhaust gases. *Bioresource Technology*, *151*, 12–18. https://doi.org/10.1016/j.biortech.2013.10.040

- Lokaewmanee, K., Yamauchi, K. E., Komori, T., and Saito, K. (2009). Effects on egg yolk colour of paprika or paprika combined with marigold flower extracts. *Italian Journal of Animal Science*, *9*(4), 356–359. https://doi.org/10.4081/ijas.2010.e67
- Lu, S., Wang, J., Ma, Q., Yang, J., Li, X., and Yuan, Y. J. (2013). Phospholipid Metabolism in an Industry Microalga Chlorella sorokiniana: The Impact of Inoculum Sizes. *PLoS ONE*, *8*(8). https://doi.org/10.1371/journal.pone.0070827
- Lu, S., Wang, J., Niu, Y., Yang, J., Zhou, J., and Yuan, Y. (2012). Metabolic profiling reveals growth related FAME productivity and quality of Chlorella sorokiniana with different inoculum sizes. *Biotechnology and Bioengineering*, *109*(7), 1651–1662. https://doi.org/10.1002/bit.24447
- Luengo, E., Martínez, J. M., Bordetas, A., Álvarez, I., and Raso, J. (2015). Influence of the treatment medium temperature on lutein extraction assisted by pulsed electric fields from Chlorella vulgaris. *Innovative Food Science and Emerging Technologies*, *29*, 15–22. https://doi.org/10.1016/j.ifset.2015.02.012
- Luterotti, S., and Kljak, K. (2010). Spectrophotometric estimation of total carotenoids in cereal grain products. *Acta Chimica Slovenica*, *57*(4), 781–787.
- Lynch, F., Santana-Sánchez, A., Jämsä, M., Sivonen, K., Aro, E. M., and Allahverdiyeva, Y. (2015). Screening native isolates of cyanobacteria and a green alga for integrated wastewater treatment, biomass accumulation and neutral lipid production. *Algal Research*, *11*, 411–420. https://doi.org/10.1016/j.algal.2015.05.015
- Ma, Q., Wang, J., Lu, S., Lv, Y., and Yuan, Y. (2013). Quantitative proteomic profiling reveals photosynthesis responsible for inoculum size dependent variation in Chlorella sorokiniana. *Biotechnology and Bioengineering*, *110*(3), 773–784. https://doi.org/10.1002/bit.24762
- Maehara, Y., Anai, H., Tamada, R., and Sugimachi, K. (1987). The ATP assay is more sensitive than the succinate dehydrogenase inhibition test for predicting cell viability. *European Journal of Cancer and Clinical Oncology*, *23*(3), 273–276. https://doi.org/10.1016/0277-5379(87)90070-8
- Magnuson, A. D., Sun, T., Yin, R., Liu, G., Tolba, S., Shinde, S., and Lei, X. G. (2018). Supplemental microalgal astaxanthin produced coordinated changes in intrinsic antioxidant systems of layer hens exposed to heat stress. *Algal Research*, *33*(May), 84–90. https://doi.org/10.1016/j.algal.2018.04.031
- Mahapatra, D. M., Chanakya, H. N., and Ramachandra, T. V. (2014). Bioremediation and lipid synthesis through mixotrophic algal consortia in municipal wastewater. *Bioresource Technology*, *168*, 142–150. https://doi.org/10.1016/j.biortech.2014.03.130
- Mahdy, A., Mendez, L., Ballesteros, M., and González-Fernández, C. (2015). Algaculture integration in conventional wastewater treatment plants: Anaerobic digestion comparison of primary and secondary sludge with microalgae biomass. *Bioresource Technology*, *184*, 236–244. https://doi.org/10.1016/j.biortech.2014.09.145
- Makdi, M., Tomcsik, A., and Orosz, V. (2012). Digestate: A New Nutrient Source - Review. *Biogas*, *14*, 295–312. https://doi.org/10.5772/31355
- Maktedar, S. S., Mehetre, S. S., Avashthi, G., and Singh, M. (2017). In situ sonochemical reduction and direct functionalization of graphene oxide: A robust approach with thermal and biomedical applications. *Ultrasonics Sonochemistry*, *34*, 67–77. https://doi.org/10.1016/j.ultsonch.2016.05.015
- Markou, G., Iconomou, D., and Muylaert, K. (2016). Applying raw poultry litter leachate for the cultivation of Arthrospira platensis and Chlorella vulgaris. *Algal Research*, *13*, 79–84. https://doi.org/10.1016/j.algal.2015.11.018

- Martínez-Huitle, C. A., and Ferro, S. (2006). Electrochemical oxidation of organic pollutants for the wastewater treatment: Direct and indirect processes. *Chemical Society Reviews*, *35*(12), 1324–1340. https://doi.org/10.1039/b517632h
- Mary Leema, J. T., Kirubagaran, R., Vinithkumar, N. V., Dheenan, P. S., and Karthikayulu, S. (2010). High value pigment production from Arthrospira (Spirulina) platensis cultured in seawater. *Bioresource Technology*, *101*(23), 9221–9227. https://doi.org/10.1016/j.biortech.2010.06.120
- Mata-Gómez, L. C., Montañez, J. C., Méndez-Zavala, A., and Aguilar, C. N. (2014). Biotechnological production of carotenoids by yeasts: An overview. *Microbial Cell Factories*, *13*(1), 1–11. https://doi.org/10.1186/1475-2859-13-12
- Matter, I. A., Hoang Bui, V. K., Jung, M., Seo, J. Y., Kim, Y. E., Lee, Y. C., and Oh, Y. K. (2019). Flocculation harvesting techniques for microalgae: A review. *Applied Sciences (Switzerland)*, *9*(15). https://doi.org/10.3390/app9153069
- Mbanga, L., Mulenga, M., Mpiana, P. T., Bokolo, K., Mumbwa, M., and Mvingu, K. (2014). Determination of Sun Protection Factor (SPF) of Some Body Creams and Lotions Marketed in Kinshasa by Ultraviolet Spectrophotometry. *International Journal of Advanced Research in Chemical Science (IJARCS)*, *1*(8), 7–13. www.arcjournals.org
- McMillan, J. R., Watson, I. A., Ali, M., and Jaafar, W. (2013). Evaluation and comparison of algal cell disruption methods: Microwave, waterbath, blender, ultrasonic and laser treatment. *Applied Energy*, *103*, 128–134. https://doi.org/10.1016/j.apenergy.2012.09.020
- Merchuk, J. C., Contreras, A., García, F., and Molina, E. (1998). Studies of mixing in a concentric tube airlift bioreactor with different spargers. *Chemical Engineering Science*, *53*(4), 709–719. https://doi.org/10.1016/S0009-2509(97)00340-0
- Meurer, M. C., Mees, M., Mariano, L. N. B., Boeing, T., Somensi, L. B., Mariott, M., da Silva, R. de C. M. V. de A. F., dos Santos, A. C., Longo, B., Santos França, T. C., Klein-Júnior, L. C., de Souza, P., de Andrade, S. F., and da Silva, L. M. (2019). Hydroalcoholic extract of Tagetes erecta L. flowers, rich in the carotenoid lutein, attenuates inflammatory cytokine secretion and improves the oxidative stress in an animal model of ulcerative colitis. *Nutrition Research*, *66*, 95–106. https://doi.org/10.1016/j.nutres.2019.03.005
- Miller, G., Suzuki, N., Ciftci-Yilmaz, S., and Mittler, R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell and Environment*, *33*(4), 453–467. https://doi.org/10.1111/j.1365-3040.2009.02041.x
- Minhas, A. K., Hodgson, P., Barrow, C. J., Sashidhar, B., and Adholeya, A. (2016). The isolation and identification of new microalgal strains producing oil and carotenoid simultaneously with biofuel potential. *Bioresource Technology*, *211*, 556–565. https://doi.org/10.1016/j.biortech.2016.03.121
- Miyazawa, T., Nakagawa, K., Kimura, F., Nakashima, Y., Maruyama, I., Higuchi, O., and Miyazawa, T. (2013). Chlorella is an effective dietary source of lutein for human erythrocytes. *Journal of Oleo Science*, *62*(10), 773–779. https://doi.org/10.5650/jos.62.773
- Mohammad Mirzaie, M. A., Kalbasi, M., Mousavi, S. M., and Ghobadian, B. (2016). Investigation of mixotrophic, heterotrophic, and autotrophic growth of Chlorella vulgaris under agricultural waste medium. *Preparative Biochemistry and Biotechnology*, *46*(2), 150–156. https://doi.org/10.1080/10826068.2014.995812
- Möller, K., and Müller, T. (2012). Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. *Engineering in Life Sciences*, *12*(3), 242–257. https://doi.org/10.1002/elsc.201100085

- Morales-Amaral, M. del M., Gómez-Serrano, C., Acién, F. G., Fernández-Sevilla, J. M., and Molina-Grima, E. (2015). Production of microalgae using centrate from anaerobic digestion as the nutrient source. *Algal Research*, *9*(6), 297–305. https://doi.org/10.1016/j.algal.2015.03.018
- Morales-Sánchez, D., Martinez-Rodriguez, O. A., Kyndt, J., and Martinez, A. (2015). Heterotrophic growth of microalgae: metabolic aspects. *World Journal of Microbiology and Biotechnology*, *31*(1), 1–9. https://doi.org/10.1007/s11274-014-1773-2
- Moreira, S., Silva, N. B., Almeida-Lima, J., Rocha, H. A. O., Medeiros, S. R. B., Alves, C., and Gama, F. M. (2009). BC nanofibres: In vitro study of genotoxicity and cell proliferation. *Toxicology Letters*, *189*(3), 235–241. https://doi.org/10.1016/j.toxlet.2009.06.849
- Moreno-Garcia, L., Adjallé, K., Barnabé, S., and Raghavan, G. S. V. (2017). Microalgae biomass production for a biorefinery system: Recent advances and the way towards sustainability. *Renewable and Sustainable Energy Reviews*, *76*(January), 493–506. https://doi.org/10.1016/j.rser.2017.03.024
- MOSS, B. (1968). STUDIES ON THE DEGRADATION OF CHLOROPHYLL a AND CAROTENOIDS IN FRESHWATERS. *New Phytologist*, *67*(1), 49–59. https://doi.org/10.1111/j.1469-8137.1968.tb05453.x
- Mottet, A., Habouzit, F., and Steyer, J. P. (2014). Anaerobic digestion of marine microalgae in different salinity levels. *Bioresource Technology*, *158*, 300–306. https://doi.org/10.1016/j.biortech.2014.02.055
- Mu, D., Min, M., Krohn, B., Mullins, K. A., Ruan, R., and Hill, J. (2014). Life cycle environmental impacts of wastewater-based algal biofuels. *Environmental Science and Technology*, *48*(19), 11696–11704. https://doi.org/10.1021/es5027689
- Mu, Y., Yang, H. Y., Wang, Y. Z., He, C. S., Zhao, Q. B., Wang, Y., and Yu, H. Q. (2014). The maximum specific hydrogen-producing activity of anaerobic mixed cultures: Definition and determination. *Scientific Reports*, *4*, 1–7. https://doi.org/10.1038/srep05239
- Mulders, K. J. M., Lamers, P. P., Martens, D. E., and Wijffels, R. H. (2014). Phototrophic pigment production with microalgae: Biological constraints and opportunities. *Journal of Phycology*, *50*(2), 229–242. https://doi.org/10.1111/jpy.12173
- Murugan, D., and Saini, G. K. (2019). Cytotoxic and lethal effects of recombinant β-BUTX-Lqq1a peptide against Lepidopteran insects and cell lines. *Toxicology in Vitro*, *60*, 44–50. https://doi.org/10.1016/j.tiv.2019.05.005
- Muthuraj, M., Palabhanvi, B., Misra, S., Kumar, V., Sivalingavasu, K., and Das, D. (2013). Flux balance analysis of Chlorella sp. FC2 IITG under photoautotrophic and heterotrophic growth conditions. *Photosynthesis Research*, *118*(1–2), 167–179. https://doi.org/10.1007/s11120-013-9943-x
- Nayak, M., Karemore, A., and Sen, R. (2016). Sustainable valorization of flue gas CO2 and wastewater for the production of microalgal biomass as a biofuel feedstock in closed and open reactor systems. *RSC Advances*, *6*(94), 9111–91120. https://doi.org/10.1039/c6ra17899e
- Nielsen, H. (1998). Hen age and fatty acid composition of egg yolk lipid. *British Poultry Science*, *39*(1), 53–56. https://doi.org/10.1080/00071669889394
- Niranjana, R., Gayathri, R., Nimish Mol, S., Sugawara, T., Hirata, T., Miyashita, K., and Ganesan, P. (2015). Carotenoids modulate the hallmarks of cancer cells. *Journal of Functional Foods*, *18*, 968–985. https://doi.org/10.1016/j.jff.2014.10.017

- Nkoa, R. (2014). Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: A review. *Agronomy for Sustainable Development*, *34*(2), 473–492. https://doi.org/10.1007/s13593-013-0196-z
- O'Brien, J., Wilson, I., Orton, T., and Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry*, *267*(17), 5421–5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x
- Ogbonna, J. C., Yoshizawa, H., and Tanaka, H. (2000). Treatment of high strength organic wastewater by a mixed culture of photosynthetic microorganisms. *Journal of Applied Phycology*, *12*(3–5), 277–284. https://doi.org/10.1023/a:1008188311681
- Orth, J. D., Thiele, I., and Palsson, B. O. (2010). What is flux balance analysis? *Nature Biotechnology*, *28*(3), 245–248. https://doi.org/10.1038/nbt.1614
- Özogul, F., Taylor, K. D. A., Quantick, P. C., and Özogul, Y. (2000). A rapid HPLC-determination of ATP-related compounds and its application to herring stored under modified atmosphere. *International Journal of Food Science and Technology*, *35*(6), 549–554. https://doi.org/10.1111/j.1365-2621.2000.00405.x
- Pahl, S. L., Lee, A. K., Kalaitzidis, T., Ashman, P. J., Sathe, S., and Lewis, D. M. (2013). Harvesting, thickening and dewatering microalgae biomass. *Algae for Biofuels and Energy*, 165–185. https://doi.org/10.1007/978-94-007-5479-9_10
- Pakshirajan, K., and Mal, J. (2013). Biohydrogen production using native carbon monoxide converting anaerobic microbial consortium predominantly Petrobacter sp. *International Journal of Hydrogen Energy*, *38*(36), 16020–16028. https://doi.org/10.1016/j.ijhydene.2013.09.129
- Panaite, T. D., Nour, V., Vlaicu, P. A., Ropota, M., Corbu, A. R., and Saracila, M. (2019). Flaxseed and dried tomato waste used together in laying hens diet. *Archives of Animal Nutrition*, *73*(3), 222–238. https://doi.org/10.1080/1745039X.2019.1586500
- Pant, D., Singh, A., Van Bogaert, G., Irving Olsen, S., Singh Nigam, P., Diels, L., and Vanbroekhoven, K. (2012). Bioelectrochemical systems (BES) for sustainable energy production and product recovery from organic wastes and industrial wastewaters. *RSC Advances*, *2*(4), 1248–1263. https://doi.org/10.1039/c1ra00839k
- Parekh, S., Srinivasan, V., and Horn, M. (2008). Bioprocessing Using Novel Cell Culture Systems. *Advances in Applied Microbiology*, *63*(07), 105–143. https://doi.org/10.1016/S0065-2164(07)00003-2
- Park, J., Jin, H. F., Lim, B. R., Park, K. Y., and Lee, K. (2010). Ammonia removal from anaerobic digestion effluent of livestock waste using green alga Scenedesmus sp. *Bioresource Technology*, *101*(22), 8649–8657. https://doi.org/10.1016/j.biortech.2010.06.142
- Park, Y. J., Park, S. Y., Arasu, M. V., Al-Dhabi, N. A., Ahn, H. G., Kim, J. K., Park, S. U., and Iriti, M. (2017). Accumulation of carotenoids and metabolic profiling in different cultivars of tagetes flowers. *Molecules*, *22*(2), 1–14. https://doi.org/10.3390/molecules22020313
- Passos, F., Gutiérrez, R., Brockmann, D., Steyer, J. P., García, J., and Ferrer, I. (2015). Microalgae production in wastewater treatment systems, anaerobic digestion and modelling using ADM1. *Algal Research*, *10*, 55–63. https://doi.org/10.1016/j.algal.2015.04.008
- Pawlett, M., and Tibbett, M. (2015). Is sodium in anaerobically digested food waste a potential risk to soils? *Sustainable Environment Research*, *25*(4), 235–239.
- Peev, G., Penchev, P., Peshev, D., and Angelov, G. (2011). Solvent extraction of rosmarinic acid from lemon balm and concentration of extracts by nanofiltration: Effect

of plant pre-treatment by supercritical carbon dioxide. *Chemical Engineering Research and Design*, *89*(11), 2236–2243. https://doi.org/10.1016/j.cherd.2011.04.014

- Peeva, L. G., Gibbins, E., Luthra, S. S., White, L. S., Stateva, R. P., and Livingston, A. G. (2004). Effect of concentration polarisation and osmotic pressure on flux in organic solvent nanofiltration. *Journal of Membrane Science*, *236*(1–2), 121–136. https://doi.org/10.1016/j.memsci.2004.03.004
- Pei, D., Xu, J., Zhuang, Q., Tse, H. F., and Esteban, M. A. (2010). Induced pluripotent stem cell technology in regenerative medicine and biology. *Advances in Biochemical Engineering/Biotechnology*, *123*(July 2015), 127–141. https://doi.org/10.1007/10_2010_72
- Peng, H., Wei, D., Chen, F., and Chen, G. (2016). Regulation of carbon metabolic fluxes in response to CO2 supplementation in phototrophic Chlorella vulgaris: a cytomic and biochemical study. *Journal of Applied Phycology*, *28*(2), 737–745. https://doi.org/10.1007/s10811-015-0542-4
- Pereira, M. V., Dassoler, A. F., Antunes, P. W., Gonçalves, R. F., and Cassini, S. T. (2020). Indigenous microalgae biomass cultivation in continuous reactor with anaerobic effluent: effect of dilution rate on productivity, nutrient removal and bioindicators. *Environmental Technology (United Kingdom)*, *41*(14), 1780–1792. https://doi.org/10.1080/09593330.2018.1549105
- Perez-Garcia, O., Escalante, F. M. E., de-Bashan, L. E., and Bashan, Y. (2011). Heterotrophic cultures of microalgae: Metabolism and potential products. *Water Research*, *45*(1), 11–36. https://doi.org/10.1016/j.watres.2010.08.037
- Perrine, Z., Negi, S., and Sayre, R. T. (2012). Optimization of photosynthetic light energy utilization by microalgae. *Algal Research*, *1*(2), 134–142. https://doi.org/10.1016/j.algal.2012.07.002
- Peshev, D., Peeva, L. G., Peev, G., Baptista, I. I. R., and Boam, A. T. (2011). Application of organic solvent nanofiltration for concentration of antioxidant extracts of rosemary (Rosmarinus officiallis L.). *Chemical Engineering Research and Design*, *89*(3), 318–327. https://doi.org/10.1016/j.cherd.2010.07.002
- Peter Amala Sujith, A., Hymavathi, T. V., and Yasoda Devi, P. (2010). Supercritical fluid extraction of lutein esters from marigold flowers and their hydrolysis by improved saponification and enzyme biocatalysis. *World Academy of Science, Engineering and Technology*, *37*(March), 1133–1142. https://doi.org/10.5281/zenodo.1331475
- Petrovit, D. L., Posarao, D., and Dudukovlc, A. (1995). Prediction of Mixing Time in Airlift Reactors. *Chemical Engineering Communications*, *133*(1), 1–9. https://doi.org/10.1080/00986449508936308
- Pignolet, O., Jubeau, S., Vaca-Garcia, C., and Michaud, P. (2013). Highly valuable microalgae: Biochemical and topological aspects. *Journal of Industrial Microbiology and Biotechnology*, *40*(8), 781–796. https://doi.org/10.1007/s10295-013-1281-7
- Pishgar, Z., Samimi, A., Mohebbi-Kalhori, D., and Shokrollahzadeh, S. (2020). Comparative Study on the Harvesting of Marine Chlorella vulgaris Microalgae from a Dilute Slurry Using Autoflocculation-Sedimentation and Electrocoagulation-Flotation Methods. *International Journal of Environmental Research*, *14*(6), 615–628. https://doi.org/10.1007/s41742-020-00277-y
- Pohlmann, E. S., Patel, K., Guo, S., Dukes, M. J., Sheng, Z., and Kelly, D. F. (2015). Real-Time Visualization of Nanoparticles Interacting with Glioblastoma Stem Cells. *Nano Letters*, *15*(4), 2329–2335. https://doi.org/10.1021/nl504481k

- Pompelli, M. F., França, S. C., Tigre, R. C., de Oliveira, M. T., Sacilot, M., and Pereira, E. C. (2012). Spectrophotometric determinations of chloroplastidic pigments in acetone , ethanol and dimethylsulphoxide. *Brazilian Journal of Bioscience*, *11*(1), 52–58.
- Prabakaran, P., and Ravindran, A. D. (2011). A comparative study on effective cell disruption methods for lipid extraction from microalgae. *Letters in Applied Microbiology*, *53*(2), 150–154. https://doi.org/10.1111/j.1472-765X.2011.03082.x
- Präbst, K., Engelhardt, H., Ringgeler, S., and Hübner, H. (2017). Basic colorimetric proliferation assays: MTT, WST, and resazurin. In *Methods in Molecular Biology* (Vol. 1601, pp. 1–17). https://doi.org/10.1007/978-1-4939-6960-9_1
- Prado-Cabrero, A., Beatty, S., Stack, J., Howard, A., and Nolan, J. M. (2016). Quantification of zeaxanthin stereoisomers and lutein in trout flesh using chiral high-performance liquid chromatography-diode array detection. *Journal of Food Composition and Analysis*, *50*, 19–22. https://doi.org/10.1016/j.jfca.2016.05.004
- Prajapati, S. K., Kumar, P., Malik, A., and Vijay, V. K. (2014). Bioconversion of algae to methane and subsequent utilization of digestate for algae cultivation: A closed loop bioenergy generation process. *Bioresource Technology*, *158*, 174–180. https://doi.org/10.1016/j.biortech.2014.02.023
- Prommuak, C., Pavasant, P., Quitain, A. T., Goto, M., and Shotipruk, A. (2013). Simultaneous Production of Biodiesel and Free Lutein from Chlorella vulgaris. *Chemical Engineering and Technology*, *36*(5), 733–739. https://doi.org/10.1002/ceat.201200668
- Purnima, M., Arul Manikandan, N., Pakshirajan, K., and Pugazhenthi, G. (2020). Recovery of microalgae from its broth solution using kaolin based tubular ceramic membranes prepared with different binders. *Separation and Purification Technology*, *250*(May), 117212. https://doi.org/10.1016/j.seppur.2020.117212
- Puspasari, T., and Peinemann, K. V. (2016). Application of thin film cellulose composite membrane for dye wastewater reuse. *Journal of Water Process Engineering*, *13*, 176–182. https://doi.org/10.1016/j.jwpe.2016.08.008
- Qiao, Y. Q., Jiang, P. F., and Gao, Y. Z. (2018). Lutein prevents osteoarthritis through Nrf2 activation and downregulation of inflammation. *Archives of Medical Science*, *14*(3), 617–624. https://doi.org/10.5114/aoms.2016.59871
- Qiao, Z., Wang, Z., Zhang, C., Yuan, S., Zhu, Y., and Wang, J. (2012). PVAm–PIP/PS composite membrane with high performance for CO_2/N_2 separation. *AIChE Journal*, *59*(4), 215–228. https://doi.org/10.1002/aic
- R. Todd Lorenz and Gerald R. Cysewsk. (2000). Commercial potential for Haematococcus. . . *Trends in Biotechnology*, *18(4)*, ((4)), 160-167.
- Rajagopal, R., Massé, D. I., and Singh, G. (2013). A critical review on inhibition of anaerobic digestion process by excess ammonia. In *Bioresource Technology* (Vol. 143, pp. 632–641). https://doi.org/10.1016/j.biortech.2013.06.030
- Rakesh, S., Dhar, D. W., Prasanna, R., Saxena, A. K., Saha, S., Shukla, M., and Sharma, K. (2015). Cell disruption methods for improving lipid extraction efficiency in unicellular microalgae. *Engineering in Life Sciences*, *15*(4), 443–447. https://doi.org/10.1002/elsc.201400222
- Rakonjac, S., Bogosavljević-Bošković, S., Škrbić, Z., Perić, L., Dosković, V., Petrović, M., and Petričević, V. (2017). The effect of the rearing system, genotype and laying hens age on the egg weight and share of main parts of eggs. *Acta Agriculturae Serbica*, *22*(44), 185–192. https://doi.org/10.5937/aaser1744185r
- Ramanna, L., Guldhe, A., Rawat, I., and Bux, F. (2014). The optimization of biomass and lipid yields of *Chlorella sorokiniana* when using wastewater supplemented with

different nitrogen sources. *Bioresource Technology*, *168*, 127–135. https://doi.org/10.1016/j.biortech.2014.03.064

- Rampure, M. R., Kulkarni, A. A., and Ranade, V. V. (2007). Hydrodynamics of bubble column reactors at high gas velocity: Experiments and computational fluid dynamics CFD simulations. *Industrial and Engineering Chemistry Research*, *46*(25), 8431–8447. https://doi.org/10.1021/ie070079h
- Ranganathan, A., Hindupur, R., and Vallikannan, B. (2016). Biocompatible lutein-polymer-lipid nanocapsules: Acute and subacute toxicity and bioavailability in mice. *Materials Science and Engineering C*, *69*, 1318–1327. https://doi.org/10.1016/j.msec.2016.08.029
- Ravanfar, R., Tamaddon, A. M., Niakousari, M., and Moein, M. R. (2016). Preservation of anthocyanins in solid lipid nanoparticles: Optimization of a microemulsion dilution method using the Placket-Burman and Box-Behnken designs. *Food Chemistry*, *199*, 573–580. https://doi.org/10.1016/j.foodchem.2015.12.061
- Read, A., Wright, A., and Abdel-Aal, E. S. M. (2015). In vitro bioaccessibility and monolayer uptake of lutein from wholegrain baked foods. *Food Chemistry*, *174*, 263–269. https://doi.org/10.1016/j.foodchem.2014.11.074
- Réhault-Godbert, S., Guyot, N., and Nys, Y. (2019). The golden egg: Nutritional value, bioactivities, and emerging benefits for human health. *Nutrients*, *11*(3), 1–26. https://doi.org/10.3390/nu11030684
- Ringwood, A. H., Conners, D. E., and Hoguet, J. (1998). Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters Crassostrea virginica. *Marine Ecology Progress Series*, *166*, 163–171. https://doi.org/10.3354/meps166163
- Risberg, K. (2015). Quality and function of anaerobic digestion residues. In *Sciences-New York*.
- Risbud, M. V., and Bhonde, R. R. (2001). Suitability of cellulose molecular dialysis membrane for bioartificial pancreas: In vitro biocompatibility studies. *Journal of Biomedical Materials Research*, *54*(3), 436–444. https://doi.org/10.1002/1097-4636(20010305)54:3<436::AID-JBM180>3.0.CO;2-8
- Rise, M., Cohen, E., Vishkautsan, M., Cojocaru, M., Gottlieb, H. E., and Arad, S. (Malis). (1994). Accumulation of Secondary Carotenoids in Chlorella zofingiensis. *Journal of Plant Physiology*, *144*(3), 287–292. https://doi.org/10.1016/S0176-1617(11)81189-2
- Riss, T. L., and Moravec, R. A. (2004). Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay and Drug Development Technologies*, *2*(1), 51–62. https://doi.org/10.1089/154065804322966315
- Rivera, S., and Canela, R. (2012). Influence of sample processing on the analysis of carotenoids in Maize. *Molecules*, *17*(9), 11255–11268. https://doi.org/10.3390/molecules170911255
- Road, P., and Kong, H. (2002). Isolation and Purification of Lutein from the Microalga. *Journal of Agricultural and Food Chemistry*, *14*, 1070–1072.
- Roleira, F. M. F., Tavares-Da-Silva, E. J., Varela, C. L., Costa, S. C., Silva, T., Garrido, J., and Borges, F. (2015). Plant derived and dietary phenolic antioxidants: Anticancer properties. *Food Chemistry*, *183*, 235–258. https://doi.org/10.1016/j.foodchem.2015.03.039
- Rose, G. G. (1974). Tissue Culture Tissue Culture: Methods and Applications Paul F. Kruse, Jr. M. K. Patterson, Jr. *BioScience*, *24*(11), 664–664. https://doi.org/10.2307/1296682

- Rotter, B. A., Thompson, B. K., Clarkin, S., and Owen, T. C. (1993). Rapid colorimetric bioassay for screening of fusarium mycotoxins. *Natural Toxins*, *1*(5), 303–307. https://doi.org/10.1002/nt.2620010509
- Saini, R. K., and Keum, Y. S. (2018). Carotenoid extraction methods: A review of recent developments. In *Food Chemistry* (Vol. 240, pp. 90–103). https://doi.org/10.1016/j.foodchem.2017.07.099
- Saini, R. K., Moon, S. H., Gansukh, E., and Keum, Y. S. (2018). An efficient one-step scheme for the purification of major xanthophyll carotenoids from lettuce, and assessment of their comparative anticancer potential. *Food Chemistry*, *266*, 56–65. https://doi.org/10.1016/j.foodchem.2018.05.104
- Samson, F. E., Katz, A. M., and Harris, D. L. (1955). Effects of acetate and other short-chain fatty acids on yeast metabolism. *Archives of Biochemistry and Biophysics*, *54*(2), 406–423. https://doi.org/10.1016/0003-9861(55)90054-0
- Sánchez Mirón, A., Cerón García, M. C., García Camacho, F., Molina Grima, E., and Chisti, Y. (2004). Mixing in bubble column and airlift reactors. *Chemical Engineering Research and Design*, *82*(10),1367–1374.
- Sandeski, L. M., Ponsano, E. H. G., and Neto, M. G. (2014). Optimizing xanthophyll concentrations in diets to obtain well-pigmented yolks. *Journal of Applied Poultry Research*, *23*(3), 409–417. https://doi.org/10.3382/japr.2013-00912
- Sanna, A., Uibu, M., Caramanna, G., Kuusik, R., and Maroto-Valer, M. M. (2014). A review of mineral carbonation technologies to sequester CO2. *Chemical Society Reviews*, *43*(23), 8049–8080. https://doi.org/10.1039/c4cs00035h
- Sanscartier, D., MacLean, H. L., and Saville, B. (2012). Electricity production from anaerobic digestion of household organic waste in Ontario: Techno-economic and GHG emission analyses. *Environmental Science and Technology*, *46*(2), 1233–1242. https://doi.org/10.1021/es2016268
- Scherholz, M. L., and Curtis, W. R. (2013). Achieving pH control in microalgal cultures through fed-batch addition of stoichiometrically-balanced growth media. *BMC Biotechnology*, *13*(1), 1–17. https://doi.org/10.1186/1472-6750-13-39
- Schmalzer, P. N., Thompson, T. R., and Simpson, A. L. (2008). Trends in deflection with application of repeated loads: Impact on deflection data averaging. *Transportation Research Record*, *2068*(2068), 71–77. https://doi.org/10.3141/2068-08
- Scott, K. J. (2001). Detection and Measurement of Carotenoids by UV / VIS Spectrophotometry. *Current Protocols in Food Analytical Chemistry*, *00*(1), 1–10. https://doi.org/10.1002/0471142913.faf0202s00
- Scudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M. J., Seniff, D., and Boyd, M. R. (1988). Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines. *Cancer Research*, *48*(17), 4827–4833.
- Selvaratnam, T., Pegallapati, A. K., Montelya, F., Rodriguez, G., Nirmalakhandan, N., Van Voorhies, W., and Lammers, P. J. (2014). Evaluation of a thermo-tolerant acidophilic alga, Galdieria sulphuraria, for nutrient removal from urban wastewaters. *Bioresource Technology*, *156*, 395–399. https://doi.org/10.1016/j.biortech.2014.01.075
- Sharma, K. K., Schuhmann, H., and Schenk, P. M. (2012). High lipid induction in microalgae for biodiesel production. *Energies*, *5*(5), 1532–1553. https://doi.org/10.3390/en5051532
- Sharma, O. P., and Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry*, *113*(4), 1202–1205. https://doi.org/10.1016/j.foodchem.2008.08.008

- Shastri, A. A., and Morgan, J. A. (2005). Flux balance analysis of photoautotrophic metabolism. *Biotechnology Progress*, *21*(6), 1617–1626. https://doi.org/10.1021/bp050246d
- Shen, Y. (2014). Carbon dioxide bio-fixation and wastewater treatment via algae photochemical synthesis for biofuels production. *RSC Advances*, *4*(91), 49672–49722. https://doi.org/10.1039/c4ra06441k
- Shen, Y., Fan, Z., Chen, C., and Xu, X. (2015). An auto-flocculation strategy for Chlorella vulgaris. *Biotechnology Letters*, *37*(1), 75–80. https://doi.org/10.1007/s10529-014-1655-6
- Shetty, P. K., Venuvanka, V., Jagani, H. V., Chethan, G. H., Ligade, V. S., Musmade, P. B., Nayak, U. Y., Reddy, M. S., Kalthur, G., Udupa, N., Rao, C. M., and Mutalik, S. (2015). Development and evaluation of sunscreen creams containing morin-encapsulated nanoparticles for enhanced UV radiation protection and antioxidant activity. *International Journal of Nanomedicine*, *10*, 6477–6491. https://doi.org/10.2147/IJN.S90964
- Shi, X. M., and Chen, F. (1997). Stability of lutein under various storage conditions. *Nahrung - Food*, *41*(1), 38–41. https://doi.org/10.1002/food.19970410110
- Shi, X. M., Chen, F., Yuan, J. P., and Chen, H. (1997). Heterotrophic production of lutein by selected Chlorella strains. *Journal of Applied Phycology*, *9*(5), 445–450. https://doi.org/10.1023/A:1007938215655
- Shi, X. M., Jiang, Y., and Chen, F. (2002). High-yield production of lutein by the green microalga Chlorella protothecoides in heterotrophic fed-batch culture. *Biotechnology Progress*, *18*(4), 723–727. https://doi.org/10.1021/bp0101987
- Shi, X. M., Liu, H. J., Zhang, X. W., and Chen, F. (1999). Production of biomass and lutein by Chlorella protothecoides at various glucose concentrations in heterotrophic cultures. *Process Biochemistry*, *34*(4), 341–347. https://doi.org/10.1016/S0032-9592(98)00101-0
- Shinde, S., and Lele, S. (2010). Statistical media optimization for lutein production from microalgae Auxenochlorella protothecoides SAG 211-7A. *International Journal of Advanced Biotechnology and Research*, *1*(2), 104–114. http://www.pdoaj.com/pdf-files/104-114 Vol1, Issue2;2010.pdf
- Shmulewitz, A., Langer, R., and Patton, J. (2006). Convergence in biomedical technology. *Nature Biotechnology*, *24*(3), 277–281. https://doi.org/10.1038/nbt0306-277a
- Shoener, B. D., Bradley, I. M., Cusick, R. D., and Guest, J. S. (2014). Energy positive domestic wastewater treatment: The roles of anaerobic and phototrophic technologies. *Environmental Sciences: Processes and Impacts*, *16*(6), 1204–1222. https://doi.org/10.1039/c3em00711a
- Sierra, L. S., Dixon, C. K., and Wilken, L. R. (2017). Enzymatic cell disruption of the microalgae Chlamydomonas reinhardtii for lipid and protein extraction. *Algal Research*, *25*(May), 149–159. https://doi.org/10.1016/j.algal.2017.04.004
- Sighinolfi, G. L., Artoni, E., Gatti, A. M., and Corsi, L. (2016). Carcinogenic potential of metal nanoparticles in BALB/3T3 cell transformation assay. *Environmental Toxicology*, *31*(5), 509–519. https://doi.org/10.1002/tox.22063
- Singh, D., Barrow, C. J., Mathur, A. S., Tuli, D. K., and Puri, M. (2015). Optimization of zeaxanthin and β-carotene extraction from Chlorella saccharophila isolated from New Zealand marine waters. *Biocatalysis and Agricultural Biotechnology*, *4*(2), 166–173. https://doi.org/10.1016/j.bcab.2015.02.001

- Sirri, F., Iaffaldano, N., Minelli, G., Meluzzi, A., Rosato, M. P., and Franchini, A. (2007). Comparative pigmentation efficiency of high dietary levels of apo-ester and marigold extract on quality traits of whole liquid egg of two strains of laying hens. *Journal of Applied Poultry Research*, *16*(3), 429–437. https://doi.org/10.1093/japr/16.3.429
- Skehan, P., Storeng, R., Scudiero, D., Monks, A., Mcmahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *Journal of the National Cancer Institute*, *82*(13), 1107–1112. https://doi.org/10.1093/jnci/82.13.1107
- Skřivan, M., Englmaierová, M., Skřivanová, E., and Bubancová, I. (2015). Increase in lutein and zeaxanthin content in the eggs of hens fed marigold flower extract. *Czech Journal of Animal Science*, *60*(3), 89–96. https://doi.org/10.17221/8073-CJAS
- Slocombe, S. P., Ross, M., Thomas, N., McNeill, S., and Stanley, M. S. (2013). A rapid and general method for measurement of protein in micro-algal biomass. *Bioresource Technology*, *129*, 51–57. https://doi.org/10.1016/j.biortech.2012.10.163
- Smith, R. G., Vanlerberghe, G. C., Stitt, M., and Turpin, D. H. (1989). Short-Term Metabolite Changes during Transient Ammonium Assimilation by the N -Limited Green Alga Selenastrum minutum . *Plant Physiology*, *91*(2), 749–755. https://doi.org/10.1104/pp.91.2.749
- Sobral, D., Bueno Costa, R. G., Machado, G. M., Jacinto de Paula, J. C., Martins Teodoro, V. A., Nunes, N. M., dos Santos Pires, A. C., and Pinto, M. S. (2016). Can lutein replace annatto in the manufacture of Prato cheese? *LWT - Food Science and Technology*, *68*, 349–355. https://doi.org/10.1016/j.lwt.2015.12.051
- Solovchenko, A. E., and Merzlyak, M. N. (2008). Screening of visible and UV radiation as a photoprotective mechanism in plants. *Russian Journal of Plant Physiology*, *55*(6), 719–737. https://doi.org/10.1134/S1021443708060010
- Sowmya, P. R., Arathi, B. P., Vijay, K., Baskaran, V., and Lakshminarayana, R. (2014). Optimization of LC/MS (APCI)+ Methods for the Determination of Possible Lutein Oxidation Products in Plasma and Tissues of Adult Rats. *Chromatographia*, *77*(23–24), 1633–1642. https://doi.org/10.1007/s10337-014-2765-y
- Sparrow, J. (2008). Commercializing Successful Biomedical Technologies. In *Journal of Commercial Biotechnology* (Vol. 14, Issue 4). https://doi.org/10.1057/jcb.2008.20
- Spolaore, P., Joannis-Cassan, C., Duran, E., and Isambert, A. (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering*, *101*(2), 87–96. https://doi.org/10.1263/jbb.101.87
- Stefels, J. (2000). Physiological aspects of the production and conversion of DMSP in marine algae and higher plants. *Journal of Sea Research*, *43*(3–4), 183–197. https://doi.org/10.1016/S1385-1101(00)00030-7
- Steinberg, W., Grashorn, M. A., Klunter, A. M., and Schierle, J. (2000). Comparative pigmentation efficiency of two products containing either apo-ester or tagetes extracts in egg yolks and liquid eggs. *Archiv Fur Geflugelkunde*, *64*(4), 180–187. https://www.cabdirect.org/cabdirect/abstract/20001418915
- Stone, V., Johnston, H., and Schins, R. P. F. (2009). Development of in vitro systems for nanotoxicology: Methodological considerations in vitro methods for nanotoxicology Vicki Stone et al. In *Critical Reviews in Toxicology* (Vol. 39, Issue 7, pp. 613–626). https://doi.org/10.1080/10408440903120975
- Strober, W. (2001). Trypan blue exclusion test of cell viability. *Current Protocols in Immunology / Edited by John E. Coligan ... [et Al.]*, *Appendix 3*, 2–3. https://doi.org/10.1002/0471142735.ima03bs21

- Sujith A, P. A., and TV, H. (2012). A Study on the Different Methods of Preparation of Lutein from Supercritical Fluid Processed Lutein Esters. *Journal of Nutrition and Food Sciences*, *02*(07). https://doi.org/10.4172/2155-9600.1000154
- Sumanta, N., Haque, C. I., Nishika, J., and Suprakash, R. (2014). Spectrophotometric Analysis of Chlorophylls and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents. *Research Journal of Chemical Sciences Res. J. Chem. Sci*, *4*(9), 2231–2606.
- Sun, X., Zhong, Y., Huang, Z., and Yang, Y. (2014). Selenium accumulation in unicellular green alga Chlorella vulgaris and its effects on antioxidant enzymes and content of photosynthetic pigments. *PLoS ONE*, *9*(11), 1–8. https://doi.org/10.1371/journal.pone.0112270
- Sun, Z., Liu, J., Zeng, X., Huangfu, J., Jiang, Y., Wang, M., and Chen, F. (2011). Protective actions of microalgae against endogenous and exogenous advanced glycation endproducts (AGEs) in human retinal pigment epithelial cells. *Food and Function*, *2*(5), 251–258. https://doi.org/10.1039/c1fo10021a
- Sundström, J. F., Albihn, A., Boqvist, S., Ljungvall, K., Marstorp, H., Martiin, C., Nyberg, K., Vågsholm, I., Yuen, J., and Magnusson, U. (2014). Future threats to agricultural food production posed by environmental degradation, climate change, and animal and plant diseases - a risk analysis in three economic and climate settings. *Food Security*, *6*(2), 201–215. https://doi.org/10.1007/s12571-014-0331-y
- Surendhiran, D., Vijay, M., Sivaprakash, B., and Sirajunnisa, A. (2015). Kinetic modeling of microalgal growth and lipid synthesis for biodiesel production. *3 Biotech*, *5*(5), 663–669. https://doi.org/10.1007/s13205-014-0264-3
- Tagliaferro, G. V., Izário Filho, H. J., Chandel, A. K., da Silva, S. S., Silva, M. B., and Santos, J. C. dos. (2019). Continuous cultivation of Chlorella minutissima 26a in a tube-cylinder internal-loop airlift photobioreactor to support 3G biorefineries. *Renewable Energy*, *130*, 439–445. https://doi.org/10.1016/j.renene.2018.06.041
- Taher, H., Al-Zuhair, S., Al-Marzouqi, A. H., Haik, Y., and Farid, M. (2014). Effective extraction of microalgae lipids from wet biomass for biodiesel production. *Biomass and Bioenergy*, *66*, 159–167. https://doi.org/10.1016/j.biombioe.2014.02.034
- Takaichi, S. (2000). Characterization of carotenes in a combination of a C18 HPLC column with isocratic elution and absorption spectra with a photodiode-array detector. *Photosynthesis Research*, *65*(1), 93–99. https://doi.org/10.1023/A:1006445503030
- Takaichi, S. (2011). Carotenoids in algae: Distributions, biosyntheses and functions. *Marine Drugs*, *9*(6), 1101–1118. https://doi.org/10.3390/md9061101
- Takeshita, T., Ota, S., Yamazaki, T., Hirata, A., Zachleder, V., and Kawano, S. (2014). Starch and lipid accumulation in eight strains of six Chlorella species under comparatively high light intensity and aeration culture conditions. *Bioresource Technology*, *158*, 127–134. https://doi.org/10.1016/j.biortech.2014.01.135
- Tan, C., Xia, S., Xue, J., Xie, J., Feng, B., and Zhang, X. (2013). Liposomes as vehicles for lutein: Preparation, stability, liposomal membrane dynamics, and structure. *Journal of Agricultural and Food Chemistry*, *61*(34), 8175–8184. https://doi.org/10.1021/jf402085f
- Tan, R. K., Eberhard, W., and Büchs, J. (2011). Measurement and characterization of mixing time in shake flasks. *Chemical Engineering Science*, *66*(3), 440–447. https://doi.org/10.1016/j.ces.2010.11.001
- Tao, Q., Gao, F., Qian, C. Y., Guo, X. Z., Zheng, Z., and Yang, Z. H. (2017). Enhanced biomass/biofuel production and nutrient removal in an algal biofilm airlift photobioreactor. *Algal Research*, *21*, 9–15. https://doi.org/10.1016/j.algal.2016.11.004

- Teo, C. L., Jamaluddin, H., Zain, N. A. M., and Idris, A. (2014). Biodiesel production via lipase catalysed transesterification of microalgae lipids from Tetraselmis sp. *Renewable Energy*, *68*, 1–5. https://doi.org/10.1016/j.renene.2014.01.027
- Thrane, J. E., Kyle, M., Striebel, M., Haande, S., Grung, M., Rohrlack, T., and Andersen, T. (2015). Spectrophotometric analysis of pigments: A critical assessment of a high-throughput method for analysis of algal pigment mixtures by spectral deconvolution. *PLoS ONE*, *10*(9), 1–24. https://doi.org/10.1371/journal.pone.0137645
- Tittel, J., Bissinger, V., Gaedke, U., and Kamjunke, N. (2005). Inorganic carbon limitation and mixotrophic growth in Chlamydomonas from an acidic mining lake. *Protist*, *156*(1), 63–75. https://doi.org/10.1016/j.protis.2004.09.001
- Tokuşoglu, Ö., and Ünal, M. K. (2003). Biomass nutrient profiles of three microalgae: Spirulina platensis, Chlorella vulgaris, and Isochrisis galbana. *Journal of Food Science*, *68*(4), 1144–1148. https://doi.org/10.1111/j.1365-2621.2003.tb09615.x
- Tominaga, H., Ishiyama, M., Ohseto, F., Sasamoto, K., Hamamoto, T., Suzuki, K., and Watanabe, M. (1999). A water-soluble tetrazolium salt useful for colorimetric cell viability assay. *Analytical Communications*, *36*(2), 47–50. https://doi.org/10.1039/a809656b
- Tran, K. C., Mendoza Martin, J. L., Heaven, S., Banks, C. J., Acien Fernandez, F. G., and Molina Grima, E. (2014). Cultivation and anaerobic digestion of Scenedesmus spp. grown in a pilot-scale open raceway. *Algal Research*, *5*(1), 95–102. https://doi.org/10.1016/j.algal.2014.06.001
- Tran, T. B., Nguyen, P. D., Um, S. H., Son, S. J., and Min, J. (2013). Real-time monitoring in vitro cellular cytotoxicity of silica nanotubes using electric cell-substrate impedance sensing (ECIS). *Journal of Biomedical Nanotechnology*, *9*(2), 286–290. https://doi.org/10.1166/jbn.2013.1500
- Triantaphylidès, C., and Havaux, M. (2009). Singlet oxygen in plants: production, detoxification and signaling. *Trends in Plant Science*, *14*(4), 219–228. https://doi.org/10.1016/j.tplants.2009.01.008
- Tsai, H. P., Chuang, L. Te, and Chen, C. N. N. (2016). Production of long chain omega-3 fatty acids and carotenoids in tropical areas by a new heat-tolerant microalga Tetraselmis sp. DS3. *Food Chemistry*, *192*, 682–690. https://doi.org/10.1016/j.foodchem.2015.07.071
- Tzibranska, I., Seikova, I., Kochanov, R., Kancheva, I., and Peev, G. (2009). *Perspectives for Integration of Nanofiltration with Solid-Liquid Extraction from Plant Materials*. Nanoscienc and Nanotechnology.
- Udom, I., Halfhide, T., Gillie, B., Dalrymple, O., Zaribaf, B. H., Zhang, Q., and Ergas, S. J. (2012). Harvesting microalgae grown on wastewater. *WEFTEC 2012 - 85th Annual Technical Exhibition and Conference*, *6*, 3646–3658. https://doi.org/10.2175/193864712811726905
- Uggetti, E., Sialve, B., Latrille, E., and Steyer, J. P. (2014). Anaerobic digestate as substrate for microalgae culture: The role of ammonium concentration on the microalgae productivity. *Bioresource Technology*, *152*, 437–443. https://doi.org/10.1016/j.biortech.2013.11.036
- Utomo, R. P., Chang, Y. R., Lee, D. J., and Chang, J. S. (2013). Lutein recovery from Chlorella sp. ESP-6 with coagulants. *Bioresource Technology*, *139*, 176–180. https://doi.org/10.1016/j.biortech.2013.04.025
- Vandamme, D., Pontes, S. C. V., Goiris, K., Foubert, I., Pinoy, L. J. J., and Muylaert, K. (2011). Evaluation of electro-coagulation-flocculation for harvesting marine and

freshwater microalgae. *Biotechnology and Bioengineering*, *108*(10), 2320–2329. https://doi.org/10.1002/bit.23199

- Vaquero, I., Ruiz-Domínguez, M. C., Márquez, M., and Vílchez, C. (2012). Cu-mediated biomass productivity enhancement and lutein enrichment of the novel microalga Coccomyxa onubensis. *Process Biochemistry*, *47*(5), 694–700. https://doi.org/10.1016/j.procbio.2012.01.016
- Varma, A., and Palsson, B. O. (1994). Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type Escherichia coli W3110. *Applied and Environmental Microbiology*, *60*(10), 3724–3731. https://doi.org/10.1128/aem.60.10.3724-3731.1994
- Vasseur, C., Bougaran, G., Garnier, M., Hamelin, J., Leboulanger, C., Chevanton, M. Le, Mostajir, B., Sialve, B., Steyer, J. P., and Fouilland, E. (2012). Carbon conversion efficiency and population dynamics of a marine algae-bacteria consortium growing on simplified synthetic digestate: First step in a bioprocess coupling algal production and anaerobic digestion. *Bioresource Technology*, *119*, 79–87. https://doi.org/10.1016/j.biortech.2012.05.128
- Veeran, S., Shu, B., Cui, G., Fu, S., and Zhong, G. (2017). Curcumin induces autophagic cell death in Spodoptera frugiperda cells. *Pesticide Biochemistry and Physiology*, *139*, 79–86. https://doi.org/10.1016/j.pestbp.2017.05.004
- Vidussi, F., Claustre, H., Bustillos-Guzmàn, J., Cailliau, C., and Marty, J. C. (1996). Short communication: Determination of chlorophylls and carotenoids of marine phytoplankton: Separation of chlorophyll a from divinylchlorophyll a and zeaxanthin from lutein. *Journal of Plankton Research*, *18*(12), 2377–2382. https://doi.org/10.1093/plankt/18.12.2377
- Vijayakumar, J., Anderson, G., Gent, S., and Rajendran, A. (2013). Calculation of biomass capacity of Algae based on their elemental composition. *American Society of Agricultural and Biological Engineers Annual International Meeting 2013, ASABE 2013*, *6*(January 2013), 4709–4719. https://doi.org/10.13031/aim.20131620717
- Vinoth Kumar, R., Kumar Ghoshal, A., and Pugazhenthi, G. (2015). Elaboration of novel tubular ceramic membrane from inexpensive raw materials by extrusion method and its performance in microfiltration of synthetic oily wastewater treatment. *Journal of Membrane Science*, *490*, 92–102. https://doi.org/10.1016/j.memsci.2015.04.066
- Wadaugsorn, K., Limtrakul, S., Vatanatham, T., and Ramachandran, P. A. (2016). Hydrodynamic behaviors and mixing characteristics in an internal loop airlift reactor based on CFD simulation. *Chemical Engineering Research and Design*, *113*, 125–139. https://doi.org/10.1016/j.cherd.2016.07.017
- Wang, D., Li, Y., Hu, X., Su, W., and Zhong, M. (2015). Combined enzymatic and mechanical cell disruption and lipid extraction of green alga Neochloris oleoabundans. *International Journal of Molecular Sciences*, *16*(4), 7707–7722. https://doi.org/10.3390/ijms16047707
- Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., and Ruan, R. R. (2010). Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae Chlorella sp. *Bioresource Technology*, *101*(8), 2623–2628. https://doi.org/10.1016/j.biortech.2009.10.062
- Wang, M., and Yuan, W. (2014). Bacterial Lysis of Microalgal Cells. *Journal of Sustainable Bioenergy Systems*, *04*(04), 243–248. https://doi.org/10.4236/jsbs.2014.44022

- Wang, M., Sahu, A. K., Rusten, B., and Park, C. (2013). Anaerobic co-digestion of microalgae Chlorella sp. and waste activated sludge. *Bioresource Technology*, *142*, 585–590. https://doi.org/10.1016/j.biortech.2013.05.096
- Wang, S., Meng, Y., Liu, J., Cao, X., and Xue, S. (2018). Accurate quantification of astaxanthin from Haematococcus pluvialis using DMSO extraction and lipase-catalyzed hydrolysis pretreatment. *Algal Research*, *35*(August), 427–431. https://doi.org/10.1016/j.algal.2018.08.029
- Wang, Y. Z., Wang, Y. K., He, C. S., Yang, H. Y., Sheng, G. P., Shen, J. Y., Mu, Y., and Yu, H. Q. (2015). Hydrodynamics of an Electrochemical Membrane Bioreactor. *Scientific Reports*, *5*, 1–12. https://doi.org/10.1038/srep10387
- Wensel, P., Helms, G., Hiscox, B., Davis, W. C., Kirchhoff, H., Bule, M., Yu, L., and Chen, S. (2014). Isolation, characterization, and validation of oleaginous, multi-trophic, and haloalkaline-tolerant microalgae for two-stage cultivation. *Algal Research*, *4*(1), 2–11. https://doi.org/10.1016/j.algal.2013.12.005
- White, D. A., Pagarette, A., Rooks, P., and Ali, S. T. (2013). The effect of sodium bicarbonate supplementation on growth and biochemical composition of marine microalgae cultures. *Journal of Applied Phycology*, *25*(1), 153–165. https://doi.org/10.1007/s10811-012-9849-6
- wiaętkiewicz, S., Arczewska-Włosek, A., and Józefiak, D. (2015). Application of microalgae biomass in poultry nutrition. *World's Poultry Science Journal*, *71*(4), 663–672. https://doi.org/10.1017/S0043933915002457
- Wiles, C., and Watts, P. (2012). Continuous flow reactors: A perspective. *Green Chemistry*, *14*(1), 38–54. https://doi.org/10.1039/c1gc16022b
- Wilkie, A. C., and Mulbry, W. W. (2002). Recovery of dairy manure nutrients by benthic freshwater algae. *Bioresource Technology*, *84*(1), 81–91. https://doi.org/10.1016/S0960-8524(02)00003-2
- Williams, J., and Esteves, S. (2011). Digestates: Characteristics, Processing and Utilisation. *Digestates: Characteristics, Processing and Utilisation*, *May*, 33. http://biomethaneregions.cra.wallonie.be/img/download/TrainingTheTrainers_UK_Digestate_Characteristics_and_Processing.pdf
- Wörle-Knirsch, J. M., Pulskamp, K., and Krug, H. F. (2006). Oops they did it again! Carbon nanotubes hoax scientists in viability assays. *Nano Letters*, *6*(6), 1261–1268. https://doi.org/10.1021/nl060177c
- Wu, Z. Y., Shi, C. L., and Shi, X. M. (2007). Modeling of lutein production by heterotrophic Chlorella in batch and fed-batch cultures. *World Journal of Microbiology and Biotechnology*, *23*(9), 1233–1238. https://doi.org/10.1007/s11274-007-9354-2
- Xavier, A. A. O., Mercadante, A. Z., Garrido-Fernández, J., and Pérez-Gálvez, A. (2014). Fat content affects bioaccessibility and efficiency of enzymatic hydrolysis of lutein esters added to milk and yogurt. *Food Research International*, *65*(PB), 171–176. https://doi.org/10.1016/j.foodres.2014.06.016
- Xia, A., and Murphy, J. D. (2016). Microalgal Cultivation in Treating Liquid Digestate from Biogas Systems. *Trends in Biotechnology*, *34*(4), 264–275. https://doi.org/10.1016/j.tibtech.2015.12.010
- Xia, F., Hu, D., Jin, H., Zhao, Y., and Liang, J. (2012). Preparation of lutein proliposomes by supercritical anti-solvent technique. *Food Hydrocolloids*, *26*(2), 456–463. https://doi.org/10.1016/j.foodhyd.2010.11.014
- Xia, Y., Fang, H. H. P., and Zhang, T. (2013). Recent studies on thermophilic anaerobic bioconversion of lignocellulosic biomass. *RSC Advances*, *3*(36), 15528–15542. https://doi.org/10.1039/c3ra40866c

- Xie, Y., Ho, S. H., Chen, C. N. N., Chen, C. Y., Ng, I. S., Jing, K. J., Chang, J. S., and Lu, Y. (2013). Phototrophic cultivation of a thermo-tolerant Desmodesmus sp. for lutein production: Effects of nitrate concentration, light intensity and fed-batch operation. *Bioresource Technology*, *144*, 435–444. https://doi.org/10.1016/j.biortech.2013.06.064
- Xie, Y., Jin, Y., Zeng, X., Chen, J., Lu, Y., and Jing, K. (2015). Fed-batch strategy for enhancing cell growth and C-phycocyanin production of Arthrospira (Spirulina) platensis under phototrophic cultivation. *Bioresource Technology*, *180*, 281–287. https://doi.org/10.1016/j.biortech.2014.12.073
- Xin, L., Hong-ying, H., Ke, G., and Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. *Bioresource Technology*, *101*(14), 5494–5500. https://doi.org/10.1016/j.biortech.2010.02.016
- Xu, N., Zhang, X., Fan, X., Han, L., and Zeng, C. (2001). Effects of nitrogen source and concentration on growth rate and fatty acid composition of Ellipsoidion sp. (Eustigmatophyta). *Journal of Applied Phycology*, *13*(6), 463–469. https://doi.org/10.1023/A:1012537219198
- Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., and Takriff, M. S. (2014). An overview: Biomolecules from microalgae for animal feed and aquaculture. In *Journal of Biological Research (Greece)* (Vol. 21, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/2241-5793-21-6
- Yang, D., Li, L., Cao, L., Chang, Z., Mei, Q., Yan, R., Ge, M., Jiang, C., and Dong, W. F. (2020). Green synthesis of lutein-based carbon dots applied for free-radical scavenging within cells. *Materials*, *13*(18), 1–10. https://doi.org/10.3390/ma13184146
- Yang, H., Roth, C. M., and Ierapetritou, M. G. (2011). Analysis of amino acid supplementation effects on hepatocyte cultures using flux balance analysis. *OMICS A Journal of Integrative Biology*, *15*(7–8), 449–460. https://doi.org/10.1089/omi.2010.0070
- Yang, Y., Zhang, C., and Hu, Z. (2013). Impact of metallic and metal oxide nanoparticles on wastewater treatment and anaerobic digestion. *Environmental Sciences: Processes and Impacts*, *15*(1), 39–48. https://doi.org/10.1039/c2em30655g
- Yao, S., Lu, J., Sárossy, Z., Baggesen, C., Brandt, A., and An, Y. (2016). Neutral lipid production in Dunaliella salina during osmotic stress and adaptation. *Journal of Applied Phycology*, *28*(4), 2167–2175. https://doi.org/10.1007/s10811-016-0794-7
- Yao, Y., Ba, C., Zhao, S., Zheng, W., and Economy, J. (2016). Development of a positively charged nanofiltration membrane for use in organic solvents. *Journal of Membrane Science*, *520*, 832–839. https://doi.org/10.1016/j.memsci.2016.08.041
- Yap, B. H. J., Crawford, S. A., Dumsday, G. J., Scales, P. J., and Martin, G. J. O. (2014). A mechanistic study of algal cell disruption and its effect on lipid recovery by solvent extraction. *Algal Research*, *5*(1), 112–120. https://doi.org/10.1016/j.algal.2014.07.001
- Yeh, T. J., Tseng, Y. F., Chen, Y. C., Hsiao, Y., Lee, P. C., Chen, T. J., Chen, C. Y., Kao, C. Y., Chang, J. S., Chen, J. C., and Lee, T. M. (2017). Transcriptome and physiological analysis of a lutein-producing alga Desmodesmus sp. reveals the molecular mechanisms for high lutein productivity. *Algal Research*, *21*, 103–119. https://doi.org/10.1016/j.algal.2016.11.013
- Yen, H. W., Ho, S. H., Chen, C. Y., and Chang, J. S. (2015). CO2, NOx and SOx removal from flue gas via microalgae cultivation: A critical review. *Biotechnology Journal*, *10*(6), 829–839. https://doi.org/10.1002/biot.201400707

- Yip, D. K., and Auersperg, N. (1972). The dye-exclusion test for cell viability: Persistence of differential staining following fixation. *In Vitro: Journal of the Tissue Culture Association*, *7*(6), 323–329. https://doi.org/10.1007/BF02618887
- Yuhas, R., Mccormick, M., Yachetti, S., Burgher, A. M., Kong, K., and Walsh, J. (2011). A Method for the Measurement of Lutein in Infant Formula. *Food and Nutrition Sciences*, *02*(02), 145–149. https://doi.org/10.4236/fns.2011.22020
- Zadghaffari, R., Moghaddas, J. S., and Revstedt, J. (2009). A mixing study in a double-Rushton stirred tank. *Computers and Chemical Engineering*, *33*(7), 1240–1246. https://doi.org/10.1016/j.compchemeng.2009.01.017
- Zahroojian, N., Moravej, H., and Shivazad, M. (2011). Comparison of marine algae (Spirulina platensis) and synthetic pigment in enhancing egg yolk colour of laying hens. *British Poultry Science*, *52*(5), 584–588. https://doi.org/10.1080/00071668.2011.610779
- Zapata, M., Rodríguez, F., and Garrido, J. L. (2000). Separation of chlorophylls and carotenoids from marine phytoplankton: A new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. *Marine Ecology Progress Series*, *195*, 29–45. https://doi.org/10.3354/meps195029
- Zeng, X., Danquah, M. K., Jing, K., Woo, M. W., Chen, X. D., Xie, Y., and Lu, Y. (2013). Solubility properties and diffusional extraction behavior of natamycin from streptomyces gilvosporeus biomass. *Biotechnology Progress*, *29*(1), 109–115. https://doi.org/10.1002/btpr.1659
- Zhang, D., Dechatiwongse, P., del Rio-Chanona, E. A., Maitland, G. C., Hellgardt, K., and Vassiliadis, V. S. (2015). Modelling of light and temperature influences on cyanobacterial growth and biohydrogen production. *Algal Research*, *9*, 263–274. https://doi.org/10.1016/j.algal.2015.03.015
- Zhang, Q., Lu, Y., Zheng, H., Liu, H., and Li, S. (2016). Differential immune response of vitellogenin gene to Vibrio anguillarum in noble scallop Chlamys nobilis and its correlation with total carotenoid content. *Fish and Shellfish Immunology*, *50*, 11–15. https://doi.org/10.1016/j.fsi.2016.01.001
- Zhang, X.-W., Shi, X.-M., and Chen, F. (1999). Chlorella protothecoides. *Journal of Industrial Microbiology and Biotechnology*, *23*, 503–507.
- Zhang, Y. M., Chen, H., He, C. L., and Wang, Q. (2013). Nitrogen Starvation Induced Oxidative Stress in an Oil-Producing Green Alga Chlorella sorokiniana C3. *PLoS ONE*, *8*(7), 1–12. https://doi.org/10.1371/journal.pone.0069225
- Zheng, Y., Xiao, R., and Roberts, M. (2016). Polymer-enhanced enzymatic microalgal cell disruption for lipid and sugar recovery. *Algal Research*, *14*, 100–108. https://doi.org/10.1016/j.algal.2016.01.010
- Zhou, Y., Schideman, L., Yu, G., and Zhang, Y. (2013). A synergistic combination of algal wastewater treatment and hydrothermal biofuel production maximized by nutrient and carbon recycling. *Energy and Environmental Science*, *6*(12), 3765–3779. https://doi.org/10.1039/c3ee24241b
- Zhu, Y., and Dunford, N. T. (2013). Growth and biomass characteristics of picochlorum oklahomensis and nannochloropsis oculata. *JAOCS, Journal of the American Oil Chemists' Society*, *90*(6), 841–849. https://doi.org/10.1007/s11746-013-2225-0
- Ziegler, J. U., Wahl, S., Würschum, T., Longin, C. F. H., Carle, R., and Schweiggert, R. M. (2015). Lutein and Lutein Esters in Whole Grain Flours Made from 75 Genotypes of 5 Triticum Species Grown at Multiple Sites. *Journal of Agricultural and Food Chemistry*, *63*(20), 5061–5071. https://doi.org/10.1021/acs.jafc.5b01477

- Zininga, J. T., Puri, A. K., Govender, A., Singh, S., and Permaul, K. (2019). Concomitant production of chitosan and lipids from a newly isolated Mucor circinelloides ZSKP for biodiesel production. *Bioresource Technology*, *272*, 545–551. https://doi.org/10.1016/j.biortech.2018.10.035

LIST OF PUBLICATIONS

List of publications

Book chapter

1. Surjith, R., & Pakshirajan K. (2021). Product evaluation: Cytotoxicity assays. Biomedical Product and Materials evaluation: Standards and ethics
2. Surjith, R., Arun, S., & Pakshirajan, K. (2020). An overview of algal photobioreactors for resource recovery from waste. Bioreactors (pp. 215-248). Elsevier.

Presentations in international and national conferences

1. Surjith Ramasamy and Kannan Pakshirajan. 'Lutein production from halophilic microalgae utilizing waste anaerobic digestate as a cheap substrate' RAER 2017, 5th June, IIT Guwahati.
2. Surjith Ramasamy and Kannan Pakshirajan. 'Lutein production in split column airlift photobioreactor from halophilic microalgae using waste anaerobic digestate as a cheap substrate' RACEEE-2019, 14th – 15th February 2019, SSN college of Engineering, Chennai, India
3. Surjith Ramasamy and Kannan Pakshirajan. 'Algae based lutein production from anaerobic digestate: a strategical approach' RAER 2016, 4-5th June IIT Guwahati.
4. 'Carotenoid production from marine algae using cheaply available Anaerobic digestate' MACB 2016, 30th August to 1st September Bharathidasan University, Tamil Nadu.

Manuscripts under preparation

1. Surjith, R., Pakshirajan K., Dhanasing M., & Gurvinder K. S. Process development for lutein production using anaerobic digestate as substrate in splitcolumn photobioreactor and its application as biopesticide
2. Surjith Ramasamy and Kannan Pakshirajan. Biokinetics of biomass growth, substrate utilization and lutein production from $NaHCO_3$ and CH_3COONa by *Chlorella vulgaris* 92001
3. Surjith Ramasamy and Kannan Pakshirajan. Lutein recovery and purification from *Chlorella vulgaris* and its application in poultry
4. Surjith Ramasamy and Kannan Pakshirajan. Evaluation of mixing kinetics in splitcolumn photobioreactor
5. Surjith Ramasamy and Kannan Pakshirajan. Lutein, its source and its applications: a review

Manuscript from collaborative work

1. Arun, S., Ramasamy, S., Pakshirajan, K., & Pugazhenthi, G. (2022). Bioelectricity production and shortcut nitrogen removal by microalgal-bacterial consortia using membrane photosynthetic microbial fuel cell. Journal of Environmental Management, 301, 113871.
2. Arun, S., Ramasamy, S., & Pakshirajan, K. (2021). Mechanistic insights into nitrification by microalgae-bacterial consortia in a photo-sequencing batch reactor under different light intensities. Journal of Cleaner Production, 321, 128752.
3. Manoj, K. M., Ramasamy, S., Parashar, A., Gideon, D. A., Soman, V., Jacob, V. D., & Pakshirajan, K. (2020). Acute toxicity of cyanide in aerobic respiration: Theoretical and experimental support for murburn explanation. Biomolecular Concepts, 11(1), 32-56.

4. Manoj, K. M., Soman, V., Jacob, V. D., Parashar, A., Gideon, D. A., Kumar, M., Surjith, R, Pakshirajan, K. & Bazhin, N. M. (2019). Chemiosmotic and murburn explanations for aerobic respiration: predictive capabilities, structure-function correlations and chemico-physical logic. Archives of biochemistry and biophysics, 676, 108128.

APPENDIX

Appendix

Table (A1) Stoichiometric equations used for flux balance analysis

S. No	Stoichiometric Equations
1	Gluc + ATP --> G6P + ADP
2	G6P --> F6P
3	F6P --> G6P
4	F6P + ATP --> FBP + ADP
5	FBP --> GAP + DHAP
6	DHAP + GAP --> FBP
7	DHAP --> GAP
8	GAP --> DHAP
9	GAP + Pi + NAD --> BPG + NADH
10	NADH + BPG --> GAP + Pi + NAD
11	BPG + ADP --> 3PG + ATP
12	3PG + ATP --> BPG + ADP
13	3PG --> PEP
14	PEP --> 3PG
15	PEP + ADP --> Pyr + ATP
16	Pyr + COA + NAD --> ACOA + CO2 + NADH
17	ACOA + OA --> Cit + COA
18	Cit --> ICit
19	ICit --> Cit
20	ICit + NAD --> AKG + NADH + CO2
21	AKG + COA + NAD --> SCOA + CO2 + NADH
22	SCOA + Pi + ADP --> Succ + ATP + COA
23	Succ + ATP + COA --> SCOA + Pi + ADP
24	Succ + FAD --> Fum + FADH2
25	Fum + FADH2 --> Succ + FAD
26	Fum --> Mal
27	Mal --> Fum
28	Mal + NAD --> OA + NADH
29	'OA + NADH --> Mal + NAD'
30	'OA + ATP --> PEP + ADP + CO2'
31	'PEP + ADP + CO2 --> OA + ATP'
32	'FBP --> F6P + Pi'
33	'G6P --> Gluc + Pi'
34	'ICit --> Succ + GOX'
35	'GOX + ACOA --> Mal + COA'
36	'Mal + COA --> GOX + ACOA'
37	'G6P + NADP --> PGL + NADPH'
38	'PGL + NADP --> R5P + CO2 + NADPH'
39	'R5P --> Ro5P'
40	'Ro5P --> R5P'
41	'R5P --> X5P'

42 'X5P --> R5P'
43 'Ro5P + X5P --> SH7P + GAP'
44 'SH7P + GAP --> Ro5P + X5P'
45 'SH7P + GAP --> E4P + F6P'
46 'E4P + F6P --> SH7P + GAP'
47 'E4P + X5P --> F6P + GAP'
48 'F6P + GAP --> E4P + X5P'
49 3 CO2 + 9 ATP + 6 NADPH --> DHAP + 9 ADP + 8 Pi + 6 NADP'
50 8.57 ACOA + 7.57 ATP + 15.96 NADPH + 0.82 O2 --> FA + 7.57 ADP + 7.57 Pi + 15.96 NADP + 8.57 COA'
51 'UTP + G1P --> UDP_gluc + 2 Pi'
52 'UDP_gluc --> UDP_gal'
53 'DHAP + NADH + 2 FA + 2 ATP + UDP_gal --> MGDG + UDP + NAD + 2 AMP + 5 Pi'
54 'DHAP + NADH + 2 FA + 2 ATP + 2 UDP_gal --> DGDG + 2 UDP + NAD + 2 AMP + 5 Pi'
55 'SO4 + UDP_gluc + DHAP + NADH + 2 FA + 2 ATP --> SQDG + 2 AMP + UDP + NAD + 0.5 O2 + 5 Pi'
56 '2 DHAP + 2 NADH + 2 FA + 2 ATP + CTP --> PG + CMP + 2 AMP + 2 NAD + 7 Pi'
57 'DHAP + NADH + 2 FA + 2 ATP + CTP + Ser --> PE + CMP + NAD + 2 AMP + CO2 + 6 Pi'
58 'DHAP + NADH + 2 FA + 2 ATP + CTP + G6P --> PI + CMP + NAD + 2 AMP + 7 Pi'
59 '0.5 MGDG + 0.2 DGDG + 0.1 SQDG + 0.1 PG + 0.05 PE + 0.05 PI --> PL'
60 'Glu + NH4 + ATP --> Gln + ADP + Pi'
61 'AKG + Gln + NADPH --> 2 Glu + NADP'
62 Glu + 2 NADH --> Pro + 2 NAD'
63 'OA + Glu --> Asp + AKG'
64 'Asp + AKG --> OA + Glu'
65 '2 Glu + 2 ATP + NADH + CAP + Asp + ACOA --> Arg + Fum + ace + AKG + NAD + 4 Pi + ADP + AMP + COA'
66 '2 Glu + ATP + NADH + CAP + Asp --> Arg + Fum + AKG + NAD + 3 Pi + AMP'
67 '3PG + NAD + Glu --> Ser + NADH + AKG + Pi'
68 'Pyr + NH4 --> Ser'
69 'Ser --> Pyr + NH4'
70 'Ser + THF --> Gly + MnTHF'
71 'Gly + MnTHF --> Ser + THF'
72 'ATP + SO4 + FADH2 + 3 NADH --> H2S + 3 NAD + AMP + FAD + 2 Pi'
73 'Ser + ACOA + H2S --> Cys + ace + COA'
74 'Pyr + Glu --> Ala + AKG'
75 'Ala + AKG --> Pyr + Glu'
76 'Ser + Pyr --> Ala + 3HPyr'
77 'Ala + 3HPyr --> Ser + Pyr'
78 'Asp + Gln + ATP --> Asn + Glu + AMP + 2 Pi'
79 'Asp + Pyr + NADH + Glu + ATP + NADPH --> Lys + AKG + NADP + ADP + CO2 + Pi + NAD'
80 'Asp + ATP + 2 NADPH --> HSer + ADP + 2 NADP + Pi'
81 'HSer + ACOA + Cys + MTHF --> Met + THF + Pyr + COA + ace + NH4'
82 'HSer + ATP --> Thr + ADP + Pi'
83 'Pyr + Thr + NADPH + Glu --> Ile + AKG + NADP + NH4 + CO2'
84 '2 Pyr + Glu + NADPH --> Val + AKG + NADP + CO2'
85 '2 Pyr + ACOA + Glu + NADPH + NAD --> Leu + AKG + NADP + NADH + COA + 2 CO2'

86 '2 PEP + E4P + NADPH + ATP --> Chr + NADP + ADP + 4 Pi'
87 'Chr + Gln + PRPP + Ser --> Trp + Glu + GAP + Pyr + CO2 + 2 Pi'
88 'Chr + NADP + Asp --> Tyr + OA + NADPH + CO2'
89 'Chr + Asp --> Phe + OA + CO2'
90 'PRPP + ATP + Gln --> IGo3P + AICAR + Glu + 4 Pi'
91 'IGo3P + Glu + 2 NAD --> His + 2 NADH + AKG + Pi'
92 'Ro5P + ATP --> PRPP + AMP'
93 'CO2 + 2 ATP + Gln --> CAP + 2 ADP + Pi + Glu'
94 'ace + ATP + COA --> ACOA + AMP + 2 Pi'
95 'ACOA + AMP + 2 Pi --> ace + ATP + COA'
96 'Ala + GOX --> Pyr + Gly'
97 'Pyr + Gly --> Ala + GOX'
98 'Ser + GOX --> 3HPyr + Gly'
99 '3HPyr + Gly --> Ser + GOX'
100 'Gly + AKG --> Glu + GOX'
101 'Glu + GOX --> Gly + AKG'
102 '3HPyr + NADH + ATP --> 3PG + ADP + NAD'
103 'THF + NADP --> DHF + NADPH'
104 'DHF + NADPH --> THF + NADP'
105 'MnTHF + NADPH --> MTHF + NADP'
106 '2 NADH + 5 ADP + 5 Pi + O2 --> 2 NAD + 5 ATP'
107 '2 FADH2 + 3 ADP + 3 Pi + O2 --> 2 FAD + 3 ATP'
108 '4 Photon + NADP + 1.285 ADP + 1.285 Pi --> 0.5 O2 + NADPH + 1.285 ATP'
109 'Photon + 0.4283 ADP + 0.4283 Pi --> 0.4283 ATP'
110 'PRPP + 2 Gln + Gly + 5 ATP + Pyr + COA + CO2 + Asp --> AICAR + Fum + 2 Glu + ACOA + 5 ADP + 7 Pi'
111 AICAR + Pyr + COA + Asp + 2 ATP --> AMP + 2 ADP + Fum + ACOA + 2 Pi'
112 'AICAR + Pyr + COA + NAD + Gln + 2 ATP --> GMP + AMP + Glu + 3 Pi + NADH + ACOA + ADP'
113 'Asp + CAP + NADP + PRPP --> UMP + CO2 + NADPH + 3 Pi'
114 'UTP + ATP + Gln --> CTP + ADP + Glu + Pi'
115 'ATP + AMP --> 2 ADP'
116 '2 ADP --> ATP + AMP'
117 'GMP + 2 ATP --> GTP + 2 ADP'
118 'GTP + 2 ADP --> GMP + 2 ATP'
119 'UMP + ATP --> UDP + ADP'
120 'UDP + ADP --> UMP + ATP'
121 'UDP + ATP --> UTP + ADP'
122 'UTP + ADP --> UDP + ATP'
123 'CTP + ADP --> CDP + ATP'
124 'CDP + ATP --> CTP + ADP'
125 'CDP + ADP --> CMP + ATP'
126 'CMP + ATP --> CDP + ADP'
127 'CTP --> CDP + Pi'
128 'AMP + 2 ATP + NADPH --> dATP + 2 ADP + NADP'
129 'GMP + 2 ATP + NADPH --> dGTP + 2 ADP + NADP'
130 'CDP + NADPH --> dCDP + NADP'

131 'dCDP + ATP --> dCTP + ADP'
132 dCDP + ADP --> dCMP + ATP'
133 'dCMP + MnTHF --> dTMP + DHF + NH4'
134 'dTMP + 2 ATP --> dTTP + 2 ADP'
135 '8 Glu + 9 ATP + 7 O2 + SAM + 13 NADPH + PPP --> Chl + 19 Pi + 13 NADP + SAHC + ADP + 4 NH4 + 8 AMP + 6 CO2 + 3 H2O2'
136 'Met + ATP --> SAM + 3 Pi'
137 'SAHC + MTHF --> Met + ade + THF'
138 'ade + ATP --> AMP + ADP'
139 '4 GAP + 4 Pyr + 11 NADPH + 8 NADH + 8 ATP --> PPP + 11 NADP + 14 Pi + 4 CO2 + 4 ADP + 4 AMP + 8 NAD'
140 '2 H2O2 --> O2'
141 'G6P --> G1P'
142 'G1P --> G6P'
143 'G1P + ATP --> PS + ADP + 2 Pi'
144 'PS + Pi --> G1P'
145 '0.226 Ala + 0.124 Arg + 0.056 Asp + 0.056 Asn + 0.0020 Cys + 0.067 Gln + 0.067 Glu + 0.085 Gly + 0.0010 His + 0.027 Ile + 0.068 Leu + 0.015 Lys + 0.0020 Met + 0.028 Phe + 0.039 Pro + 0.017 Ser + 0.068 Thr + 0.0010 Trp + 0.0010 Tyr + 0.049 Val + ATP --> Prt + AMP + 2 Pi'
146 '0.19 dATP + 0.19 dTTP + 0.31 dGTP + 0.31 dCTP + 0.25 ATP --> DNA + 0.25 ADP + 2.25 Pi'
147 '0.19 ATP + 0.19 UTP + 0.31 GTP + 0.31 CTP --> RNA + 2 Pi'
148 'NAD + ATP --> NADP + ADP'
149 'NADH + NADP --> NAD + NADPH'
150 'NADPH + NAD --> NADH + NADP'
151 'ATP --> ADP + Pi'
152 0.002 DNA + 0.0251 RNA + 0.48 Prt + 0.31 PS + 0.140PL + 0.042 Chl + 35.1 ATP --> BM + 35.1 ADP + 35 Pi'
153 '# --> Pi'
154 '# --> Photon'
155 # --> NH4'
156 '# --> CO2'
157 '# --> SO4'
158 'O2 --> #'
159 'BM --> #'

Table (A2) Abbreviations of the substrates and products of the equations

S. no		Abbreviations
1.	3HPyr	3 hydroxy pyruvate
2.	3PG	3-phospho glycerate
3.	ace	Acetate
4.	ACOA	Acetyl coenzyme A
5.	ade	Adenosine
6.	ADP	Adenosine diphosphate
7.	AICAR	5 Aminoimidazole 4 carboxamide ribonucleotide
8.	AKG	α keto glutarate
9.	Ala	Alanine

10.	AMP	Adenosine monophosphate
11.	Arg	Arginine
12.	Asn	Aspargine
13.	Asp	Asparate
14.	ATP	Adenosine triphosphate
15.	BM	Biomass
16.	BPG	1,3 biphospho glycerate
17.	CAP	Carbamoyl phosphate
18.	CDP	Guanosine diphosphate
19.	Chl	Chlorophyll
20.	Chr	Chorismate
21.	Cit	Citric acid
22.	CMP	Cytidine diphosphate
23.	CO2	Carbon dioxide
24.	COA	Coenzyme A
25.	CTP	Cytidine triphosphate
26.	Cys	Cysteine
27.	dATP	Deoxy adenosine triphosphate
28.	dCDP	Deoxy cytidine diphosphate
29.	dCMP	Deoxy cytidine 5' monophosphate
30.	dCTP	Deoxy cytidine triphosphate
31.	DGDG	Digalactosyldiacylglycerol
32.	dGTP	Deoxy guanosine triphosphate
33.	DHAP	Dihydroxy acetone phosphate
34.	DHF	Dihydro folate
35.	DNA	Deoxy ribose nucleic acid
36.	dTMP	Deoxy thymidine 5' monophosphate
37.	dTTP	Deoxy thymidine triphosphate
38.	E4P	Erythrose 4 phosphate
39.	F6P	Fructose 6 phosphate
40.	FA	Fatty acid
41.	FAD	Flavin adenine dinucleotide (Oxidized form)
42.	FADH2	Flavin adenine dinucleotide (reduced form)
43.	FBP	Fructose 1,6 biphosphate
44.	Fum	Fumarate
45.	G1P	Glucose 1 phosphate
46.	G6P	Glucose 6 phosphate
47.	GAP	Glyceraldehyde 3 phosphate
48.	Gln	Glutamine
49.	Glu	Glutamate
50.	Gluc	Glucose
51.	Gly	Glycine
52.	GMP	Guanosine 5' monophosphate
53.	GOX	Glyoxalate
54.	GTP	Guanosine triphosphate
55.	H2O2	Hydrogen peroxide

56.	H2S	Hydrogen sulfide
57.	His	Histidine
58.	HSer	Homo serine
59.	ICit	Isocitrate
60.	IGo3P	Imidazole glycerol 3 phosphate
61.	Ile	Isoleucine
62.	Leu	Leucine
63.	Lys	Lysine
64.	Mal	Malate
65.	Met	Methionine
66.	MGDG	Monogalactosyldiacylglycerol
67.	MnTHF	N5,N10 Methylene tetra hydrofolate
68.	MTHF	N5 Methyl tetrahydrofolate
69.	NAD	Nicotinamide adenine dinucleotide (oxidized form)
70.	NADH	Nicotinamide adenine dinucleotide (reduced form)
71.	NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
72.	NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
73.	NH4	Ammonium
74.	NO3	Nitrate
75.	O2	Oxygen
76.	OA	Oxaloacetate
77.	PE	Phosphatidylethanolamine
78.	PEP	Phosphoenol pyruvate
79.	PG	Phosphatidylglycerol
80.	PGL	6 phospho glucono δ-lactone
81.	Phe	Phenyl alanine
82.	Photon	Photon (light)
83.	Pi	Inorganic phosphate
84.	PI	Phosphatidylinositol
85.	PPP	Phytyl pyrophosphate
86.	Pro	Proline
87.	PRPP	5 phosphoribosyl 1 pyrophosphate
88.	Prt	Protein
89.	PS	Polysaccharide
90.	Pyr	Pyruvate
91.	R5P	Ribulose 5 phosphate
92.	RNA	Ribose nucleic acid
93.	Ro5P	Ribose 5 phosphate
94.	SAHC	S- Adenosyl homocysteine
95.	SAM	S Adenosyl methionine
96.	SCOA	Succinyl coenzyme A
97.	Ser	Serine
98.	SH7P	Sedoheptulose 7 phosphate

99.	SO4	Sulfate
100.	SQDG	Sulfoquinovosyldiacylglycerol
101.	Succ	Succinate
102.	THF	Tetra hydrofolate
103.	Thr	Threonine
104.	Trp	Tryptophan
105.	Tyr	Tyrosine
106.	UDP	Uridine diphosphate
107.	UDP_gal	UDP-galactose
108.	UDP_gluc	UDP-glucose
109.	UMP	uridine 5' monophosphate
110.	UTP	Uridine triphosphate
111.	Val	Valine
112.	X5P	Xylulose 5 phosphate