Process and Environmental Research Division

Faculty of Engineering

Lagooning and Bio-consortium Optimisation for Secondary Level Remediation of Simulated Sugar Factory Wastewater

By

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ABSTRACT

The generation of organic wastewater such as from sugar factories is a source of water pollution particularly in developing countries like Pakistan, where sugar industry is the second largest after tanneries and a major source of nuisance because of the way the wastewater is disposed of mostly as untreated in the outside environment. Since mechanical wastewater treatment processes are capital-intensive, energy-demanding and complex in operation, the common approach is to use waste stabilization ponds or lagoons mostly operated on complete retention basis, howsoever, in a wasteful manner.

This work is an attempt to highlight the possibility of effectively applying wastewater lagooning process utilizing the inherent organic contents of sugar factory wastewater with the aid of the concerned organisms to investigate its capacity as a utilizable resource rather than a useless liability to be disposed of. Thus, lagoon photo tank (LPT) resembling a prototype raceway lagoon was designed and used to carry out mass cell cultivation during prepared sugar-oriented medium for the assessment and inter-optimization of the process conditions such as temperature, incident light (IL), pH, dissolved oxygen (DO). The evaluation of the process performance was observed via the analyses of parameters such as chemical oxygen demand (COD), total organic carbon and cell mass growth.

The first part of this study was related with the baseline assessment of LPT process conditions using distilled water as well as sucrose solution or sugar water to establish basis for a priori analysis of simulated sugar factory wastewater (SFW) carried out in the second part. The suitability of the dosing of copolymer

Polyacrylate polyalcohol was optimised through a series of trial runs to aid in the immobilization of mono or mixotrophic cultures of green algae *Chlorella Vulgaris* and bacteria *Pseudomonas Putida* at the surface of LPT in order to influence reduction in the organic concentration of SFW.

This research study has contributed to the knowledge base of the concerned area of study with respect to hitherto unknown application of copolymer Polyacrylate polyalcohol, which showed viable characteristics in the cultivation medium in terms of cell immobilization at the surface of LPT resulting in the formation of growth-conducive copolymer-algae matrices leading to the rapid growth of the cell mass with increased process efficiency. This process optimisation resulted in SFW depollution by around 89% along with higher energetic biomass growth, suggesting towards the potential of copolymer addition in the system to enhance the efficiency of the organisms inducing optimum substrate utilization.

AFFIRMATION

The work presented in this thesis is original in its contents, thus it can be said that barring the referenced knowledge given in the thesis, the unreferenced part of this thesis is my own work, which has not previously been submitted for any other degree.

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

Abbreviation	Description
ACU	Algae culturing unit
ADP	Adenosine diphosphate
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCU	Bacteria culturing unit
BG	Blue green
BOD_5	Biochemical oxygen demand in 5 days
CAL	Calibration
Cal	Calories
CCAP	Culture collection for algae and protozoa
COD	Chemical oxygen demand
CV	Calorific value
DO	Dissolved oxygen
DW	Distilled water
EDTA	Ethylene diamine tetra acetic acid
GY	Growth yield
HRAP	High rate algal ponds
IL	Incident light
LB	Luria Bertani
LPT	Lagoon photo tank

NADP	Nicotinamide adenine dinucleotide phosphate
$NADP^+$	charged/saturated NADP
NADPH	Strengthened NADP
NBSF	Newark beet sugar factory
NCIMB	National collection of industrial, food and marine bacteria
NDIR	Non dispersive infrared
NEQS	National environmental quality standards
OD	Optical density
OM	Organic matter
OUR	Oxygen uptake rate
PBRs	Photobioreactors
pH	Hydrogen ion concentration
PP	Polyacrylate polyalcohol
PS	Photosystem
RPM	Revolutions per minute
SEM	Scanning electron microscope
SFW	Sugar factory wastewater
SSFW	Simulated sugar factory wastewater
SW	Sugar water
s/v	surface to volume ratio
TN	Total Nitrogen
TP	Total phosphorous
TOC	Total organic carbon
TSS	Total suspended solids

UNEP	United nations environment program
UNESCO	United nations educational scientific and cultural organization
UV VIS	Ultraviolet visible
VP	Volumetric productivity
AP	Areal productivity
VSS	Volatile suspended solids
WSP	Waste stabilization ponds
WHO	World health organisation

SYMBOLS

^0C	degrees centigrade
C	Concentration, Carbon
cm	centimetre
CO_3^-	Carbonates
d	days
e^-	electron
ft	foot/ feet
h	Plank's constant
HCO_3^-	Bicarbonates
hr	hours
I_0	Light intensity
J	Joule
k	Biodegradation constant

$K_L\ a$	Overall volumetric mass transfer coefficient
lb	pounds
Lx	Lux
M	Molarity
mg l^{-1}	milligrams per litre
min	minute
ml	millilitres
nm	nanometre (10^{-9})
OH	Hydroxide
r	specific growth rate
s	seconds
S	Sulphur, Organic substrate or COD
T	Temperature
t	Time
Δ t	Time difference
T_2 / (t_d)	Doubling time
μ	Specific growth rate

CHAPTER I

INTRODUCTION

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INTRODUCTION

I.1 PROBLEM DIAGNOSTIC

Wastewater generation the world over has increased manifold in the last two decades resulting in the corresponding increase in its volume and pollution concentration (Tchobanoglous *et al*., 2003). Inadequate handling of industrial wastewater has serious consequences for human health, the environment and social and economic development. When discharged untreated in the outside environment without any further application, as is the case in developing countries (Khan, 1999), wastewater disposal is a major source of: pollution in receiving water bodies, increased risk to waterborne diseases such as Typhoid, Diarrhoea, gastroenteritis, etc, contamination to groundwater quality and unpalatability to other local ecosystems besides affecting the aesthetic value of the environment.

Organic matter constitutes a large part of much of the wastewater generated all around the world mostly from chemical and food industries including sugar mills (Tchobanoglous *et al*. 2003). Their presence in liquid discharges may result in various biochemical reactions taking place due to the presence of variety of chemicals and materials of organic origin. The organic matter present in wastewater is the most readily degradable material and therefore is one of the major causes for water pollution problem, as under septic or demanding conditions the decomposition of organic matter may lead to nuisance all around in the form of undesirable gases and sludges (Phong, 2008).

In the backdrop of huge costs incurred on the mechanical treatment of wastewater, different disposal modes are practised in developing countries to get rid of such wastewaters, which include drying and evaporation in lagoons or ponds, utilisation by agriculturists for irrigation purposes or direct disposal to receiving water bodies (Tebbutt, 1998). However, organic wastewaters can be utilized for further application to harness the energy contents contained in them to explore more avenues for further application (Cohen, 1989).

I.2 RESEARCH AIM

The aim of this research is to optimize the utilization of algae and bacterial cultures in the surrogate sugar factory wastewater to study the correlation between biomass growth and corresponding reduction in the organic loading of the wastewater. The commonly employed method of wastewater lagooning by most of the industries in developing countries, however, can be effectively carried out by wastewater attenuation using specific allochthonous or inoculated organisms like algae and bacteria. Thus, this research study was aimed at looking at this possibility of reducing the pollution strength in terms of specified organic concentration of wastewater such as generated from sugar factories. Some researchers have pointed out in their investigations that in organic wastewater remediation by algal-bacterial consortium, mechanical aeration may not be required and that the process can be carried out based on efficient algal photosynthesis. The efficiency of the process is boosted by bacterial mineralization of the organic matter releasing carbon dioxide, which can also be consumed by

mixotrophic algae cells for the synergistic cell growth inducing reduction in the organic concentration of the medium (Munoz and Guieysse, 2006). To investigate this hypothesis, sugar water (SW), prepared as a priori cultivation medium to simulated sugar factory wastewater (SSFW), was used in lagoon photo tank (LPT) with or without the aid of organisms to establish the baseline data before using SSFW for its remediation with the optimised inocula volume ratio of the organisms to investigate their propagation as well as corresponding effect on the organic concentration of SSFW. For the suspension of algae cells at the surface of water in LPT, suitable proportion of copolymer Polyacrylate polyalcohol (PP) particles was also investigated to facilitate maximum growth of the cells.

I.3 RESEARCH OBJECTIVES

The impending objectives of this work in the light of the research aim may be characterised as under:

- Baseline assessment of LPT with respect to recirculation flow rate, temperature, pH, dissolved oxygen (DO), incident light (IL) and PP characteristics with respect to its appropriate amount and effect on the water properties such as pH and DO.
- SW lagooning in LPT with and without PP addition to determine the characteristic change in the process parameters.
- SW lagooning in LPT with algae cells with and without PP addition to evaluate the corresponding change in chemical oxygen demand (COD) of SW along with the monitoring of algae cell mass growth.

- SSFW lagooning with introduced bacterial cells to study the impact of cell growth on the medium characteristics.
- SSFW lagooning with free and immobilized cells of algae and bacteria to study the influence of process inputs on SSFW remediation.

I.4 THESIS STRUCTURE

This thesis is comprised of nine chapters including this introduction chapter. Chapter II reviews the wastewater pollution problem with respect to its background followed by the option of feeding algae cells with organic wastewater for influencing reduction in its organic loading. Flocculation of algae cells is discussed next along with wastewater remediation methods. Then wastewater remediation using algae and bacterial consortium is revisited including separate discussion on bacteria and their growth pattern, microalgae and the associated photosynthesis process. The chapter ends with discussing important wastewater remediation parameters such as pH, DO, temperature and COD. Chapter III describes the materials utilised during the experimental work along with the equipment used for the analysis of the parameters concerned and the procedures followed. Chapter IV gives an account of the baseline assessment of LPT for the optimized values of parameters such as recirculation flow rate, pH, DO, IL, temperature. The characteristic behaviour of copolymer Polyacrylate polyalcohol particles in water is also investigated. Chapter V covers the analysis of SW lagooning in LPT without and with copolymer particles to observe the characteristic change in the process variables without the inoculation of the

organisms. Chapter VI deals with the free and suspended cultivation of algae cells via copolymer immobilization in SW to observe the algae growth and corresponding influence on SW characteristics. Chapter VII is about SSFW lagooning with the cultured cells of inoculated bacteria in LPT to study the influence of bacterial growth on the medium characteristics.

Chapter VIII is concerned with the final part of the experimental regime for SSFW remediation using mixed culture of algae and bacteria optimized with different copolymer concentrations for cell immobilization to study the cumulative influence of the mixed culture on the biomass growth as well as on SSFW depollution. Chapter IX presents the summary of observations and conclusions made during the experimental work based on the results achieved.

CHAPTER II

LITERATURE REVIEW

CHAPTER II

LITERATURE REVIEW

II.1 BACKGROUND TO WASTEWATER DISPOSAL AND POLLUTION PROBLEM IN DEVELOPING COUNTRIES

Wastewaters are the unwanted materials originating as domestic sewage or industrial effluents, which for reasons of public health and for recreational, economic, and aesthetic considerations, cannot be disposed of by discarding them untreated in the outside environment including receiving water bodies (Madigan *et al.,* 1997). According to a cautious estimate 90% of wastewater in the developing countries is disposed of untreated. About a sixth or 17% (1.13 bill) of the Earth's population (6.79 bill) have no access to drinking water, while according to World Health Organization five million people die each year from poor quality drinking water contaminated most likely by discharged wastewater contents (Mantzavinos, 2008). For centuries, low population densities in prevailing rural economies meant that water consumption levels were modest and pollution from wastewater was localised. Besides, the natural environment could absorb these modest pollution loads, and thus coastal zones were not really polluted. Nowadays, nature can often no longer cope with the pressing increase in the water consumption levels that has resulted in unmanageable volumes of wastewater generation, threatening the environment as well as coastal zones, which have been turned as virtual industrial receptacles especially in developing countries.

To collect and treat a cubic meter of wastewater is probably more expensive than the treatment and distribution of a cubic meter of drinking water. At present in developing countries, only about a tenth of wastewater, largely domestic in nature, is collected and treated in underperformed wastewater treatment plants (Khan, 1999). For instance, in Pakistan only 7 wastewater treatments plants are installed for 170 million people, out of which 4 are in operation for only the basic or physico-chemical treatment of wastewater via filtration or chlorination, while the remaining lie non-functional for technical reasons (Khan, 1999).

In ultimate analysis, the overall progress in wastewater remediation in developing countries is almost non-existent rather than insignificant, as the major bottlenecks involved in the operation of conventional mechanical methods have proved to be prohibitive in their instalment (Shilton *et al.*, 2008). These include high capital and energy investment, the sophisticated nature of operation along with shortage of required expertise and well-trained operators. For these reasons and coupled with limited technical and financial resources available to their disposal, the need is to use suitable low-cost, energy-efficient and renewable type of method for wastewater remediation, which would likely be tailored to the local conditions as well. For example, high ambient temperatures in arid and semi arid areas of most of these countries make it possible to use simple and economical wastewater treatment processes such as waste stabilization ponds (WSPs). Although, WSPs may be the most commonly employed method of wastewater remediation in most developing countries including Pakistan (Oswald, 1963), but these are operated mostly on a complete retention basis for wastewater impounding, evaporation and drying, often unregulated and non-assayed without further possible application of

the wastewater contents. Thus, it explains the point that WSPs in Pakistan are generally operated without optimisation of the inflows or and monitoring of the inputs such as wastewater organic loading characteristics, influent flow rate, temperature, pH, DO and incident light variations as well as their resulting impacts on the wastewater characteristics (Rehman *et al*., 2006). The unwillingness or non-awareness in technical terms, of the industrialists for wastewater remediation in Pakistan stems from the fact that it is deemed as a capital-intensive issue with no benefits accrued as a result. Therefore, they prefer to discharge untreated effluents in the outside environment and pay the nominal pollution charges based on polluter pay principle ranging between Rs. 5,000 (£40) as a common penalty and Rs. 15,100 (£121) per day in serious violative circumstances (Pakistan Environmental Protection Act, 1997). This is in contrast to investing around one billion pounds as a total estimated capital cost on the installation of a wastewater treatment plant (Oilgae, 2009).

Considering wastewater as an undesirable commodity to be disposed of rather than a possible resource to be recovered, most of the industries in developing countries discharge their wastewaters outside the plants in different ways. For instance in Pakistan, 400 million gallons of industrial wastewater is daily discharged untreated into the sea and roughly around 1% or less of generated wastewater is treated before its disposal (Dawn, 2010). Over there, most of the chemical and food industries have built number of medium-sized to large ditches outside their plants to impound the wastewater discharges in those ponds for natural drying and evaporation, considering that the water temperature in these ponds during the summer remains in the range of 25-35^{0}C for almost eight months from March until

October. Figure II-1 is a photograph taken during a study of wastewater discharge methods adopted by sugar factories. The picture shows the disposal of untreated wastewater generated from Fauji sugar mills Pakistan, as seen in the background, via a dug-in earthen channel to the outside environment.

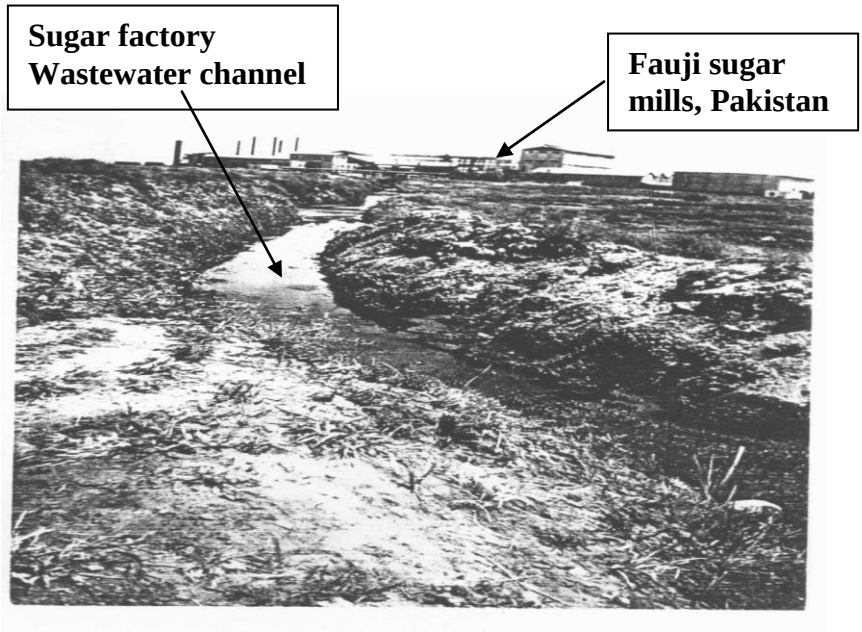


Figure II-1: Wastewater disposal by Fauji Sugar mills Pakistan (Rehman *et al*., 2006).

Table II.1 highlights the cumulative pollution level for the major parameters contained by the wastewaters from chemical and food industries of different countries including Pakistan, the wastewater quality of which is compared with the national environmental quality standards (NEQS). The data in the table suggests that BOD/COD values are higher for Pakistan, which could be associated with the difference in the manufacturing process and variation in the qualitative and quantitative nature of characteristic impurities present in wastewater.

Table II.1: Wastewater pollution strength for different countries (Khan, 1999).

Parameter ($mg\ l^{-1}$)	**Puerto Rico**	**India**	**Pakistan**	**Pakistan Environmental Quality Standards $mg\ l^{-1}$**
pH	5.3 – 8.8	6.8 – 8.4	4.7 – 6.5	6 – 8
BOD_5	112 – 225	267 – 660	600 – 4853	80
COD	385 – 978	890 – 2236	1037 – 19234	150

Considering the wastewater disposal modes and their pollution strength as given in the above table, wastewater remediation in developing countries can be assumed as a lingering problem given the limited resources available with the developing countries. However, the energy contents contained in these liquid discharges, particularly those which are of organic nature such as from sugar mills, could be put to further use employing photobioreactors (PBRs). The PBRs use a lighting source for the propagation of inoculated algae culture and may serve as a major driving force in the utilization of organic contents of wastewater by the allochthonous bacteria thereby boosting algae growth resulting at the same time in the corresponding wastewater remediation.

II.2 SUGAR FACTORY WASTEWATER AS A FEED FOR ALGAE GROWTH

Sugar factories, which are a potential source of organic pollution (Ingaramo *et al*., 2009) use sugar beet or cane containing sucrose contents in the range of 15-17% as a raw material for the manufacture of sugar utilizing large amounts of water mainly for washing and processing the input raw material and cooling purposes. Approximately 15 m^3 of water is consumed per metric ton of beet (or 15 l water per kg beet) processed (Zver and Glavic, 2005). Beet sugar factories produce wastewater of different characteristics from multiple sources mostly of organic origin that are quite homogenous in their characteristics (Rittmann, 2008). Among these include flume discharges that are used for washing beets soaked with dirt as well as conveying beets between processes. The process wastewater originates from the flushing of exhausted sliced beet known as cossettes and then the partial dewatering of the exhausted beet pulp. Steffen wastewater, which is generated during the process of sugar extraction from molasses, results in the discharge of Steffen wastewater. The organic load of process and Steffen wastewaters is comparatively higher than the flume wastewaters (Dilek *et al.*, 2003). Thus, the wastewater generated by sugar factories mostly contains organic matter, the energy recovery of which has been acknowledged as a source of renewable bioenergy (Vorkamp *et al.*, 2001), since the chief component of organic matter is carbon, which is an essential source of food for the growth and development of organisms (Russell, 2006).

This organic colloidal material ranges in size from 1 nm to 1 µm in diameter (Racz and Goel, 2010) thus it is directly taken up by the microorganisms. The considerable level of phosphates, ammonium nitrogen and COD present in sugar factory wastewater (SFW) can be a good feed for the cultivation of algae cells, as the major nutrients for algae growth are also the same (Bojcevska and Tonderski, 2007). Colosi (2009) hypothesizes that feeding algae with rich sources of both organic and inorganic carbon could boost the oil yield in grown algae mass to as much as 40 percent by weight. Zhou *et al.*, (2011) stated that algae that are able to grow mixotrophically or photoheterotrophically grow faster in the light when both inorganic and organic carbon sources are available in the wastewater medium. The soluble, biodegradable organic matter in wastewater is composed of carbohydrates, which can be either readily degradable starches and sugars or cellulose. The microorganisms easily metabolize the starches and sugars, while the cellulose compounds are degraded at a much slower rate probably because of being complex and larger in structure and size (McKinney, 1962).

Therefore, SFW can be a good feed for the growth of microalgae cells as salinity, which suppresses algae growth is not an issue with such type of wastewater; it also does not contain heavy or toxic metals which can prove deterrent to algae growth (Zhou *et al*., 2011). Besides, the review of literature as to the relevant research reveals that SFW has not been investigated for its remediation using algae-bacterial consortium. However, previous research has focussed on SFW remediation by means of methods such as adsorption (Parande *et al*., 2009), electrochemical (Guven *et al*., 2009), anaerobic (Farhadian *et al*., 2007) and end-of-pipe stabilization (Dilek *et al*., 2003).

II.3 WASTEWATER REMEDIATION USING ALGAE CELLS

Wastewater remediation is preferably cost-effective and beneficial conversion of wastewater ingredients or residuals by physical, chemical or biological means under optimally controlled and conducive conditions to produce minimum adverse effects on the environment and public health (Eckenfelder *et al*., 2009 and King *et al*., 1998). Wastewater remediation by means of physical and chemical methods such as evaporation and drying, ultrafiltration, reverse osmosis, chemical coagulation and anaerobic digestion, etc is generally technically or economically unfeasible (Olivieri *et al*., 2006). Besides, the conventional technologies, applied to remove the pollutant nutrients are impractical for developing countries, as these are generally costly to operate and often lead to secondary pollution due to incomplete destruction of the primary pollutants (Paniagua-Michael and Garcia 2003). Contrary to this, the overall objective of biological remediation of wastewater is to transform or oxidize dissolved and particulate biodegradable constituents into acceptable and preferably useful products by removing or reducing the concentration of organic and inorganic compounds present in wastewater. However, wastewater remediation although important from public health, ecological, and aesthetic viewpoint, is generally given low priority, especially in developing countries where there are many competing demands on the limited funds available for environmental development (Shiny *et al*., 2005).

Alternatively, microorganisms such as algae and bacteria are used to oxidise or convert the dissolved carbonaceous organic matter into simple products with additional biomass, which in case of algae mass is a potential source of proteins,

carbohydrates, pigments, lipids and hydrocarbons (Hodaifa *et al*., 2008). The process used during biological wastewater treatment can be suspended or attached-growth via the use of an inert material such as flocculants or polymer particles or the combination of both as in a suspended-attached growth. By the end of the treatment process, biomass with a slightly higher specific gravity than that of water can be removed from the treated liquid by gravity settling (Tchobanoglous *et al*., 2003). The biodegradable characteristics of organic matter present in wastewaters is the most common cause of pollution altering the equilibrium or constituent balance of the receiving environment; thus, the primary aim of organic wastewater remediation is to remove the wastewater organics which would otherwise exert an oxygen demand on water characteristics (Sidwick, 1985).

Wastewater remediation using microalgae, which is also termed as phycoremediation, may be an effective method in removing the nutrients and organic matter contained by organic wastewaters generating added oxygen, which is required during decomposition of the organic matter by other aerobic organisms (Pant and Adholeya, 2006). Munoz *et al*. (2009) concluded that biomass retention in algal-based wastewater remediation processes showed a remarkable potential to maintain an optimum bacterial activity in addition to improving the final quality of the effluent. Roughly one kg of COD removed in an activated sludge process requires one kWh of electricity for aeration. By contrast, one kg of COD removed by photosynthetic oxygenation requires no energy inputs and produces enough algal biomass to generate renewable source of bioenergy that can produce one kWh of electric power (Oilgae, 2009). Tarlan *et al*. (2002) also found utilization of

algae cells as a monoculture effective in the treatment of wood-based pulp and paper industry wastewater.

Different methods involving in the cultivation of algae cells are outlined as under:

II.3.1 Photobioreactors

Photobioreactors (PBRs) essentially using a light source, are used for cultivation of photosynthetic microorganisms such as microalgae, cyanobacteria, plant cells and photosynthetic bacteria for various biotechnological applications (Li *et al*., 2003). PBRs for the treatment of pollutant-laden effluents and mass cultivation of microalgae function with the basic design criteria of high light utilization efficiency, good scalability, control over the reaction conditions, and low hydrodynamic stress on photosynthetic cells (Munoz and Guieysse, 2006).

As compared to algae ponds or raceways, which can be low-cost but their operation mostly entails poor control over prevailing conditions along with low biomass productivity; PBRs permit higher biomass cultivation, productivity and better maintenance of algae cells, leading to certain degree of organic wastewater remediation as well (Richmond, 2004). Successful design and operation of PBRs requires inter-optimization of parameters such as pH, temperature, light incidence, substrate concentration, DO stabilization, and growth yield of algae cells along with the establishment of quantitative relationships between kinetics of concerned parameters and gaseous transport processes (Ahmann *et al*., 2009). Because of the controlled and maintained conditions in PBRs, there is less likelihood of direct exchange between culture species and surrounding contaminants such as dust or aerial microorganisms (Richmond, 2004).

Table II-2 compares the operational characteristics of both PBRs and waste stabilization ponds (WSPs) with respect to different functional parameters.

Table II.2: Comparison between WSPs and PBRs (Oilgae, 2009).

PARAMETER	RELATIVITY	COMPARISON NOTE
Contamination risk	WSPs > PBRs	Much reduced for PBRs
Biomass concentration	WSPs < PBRs	3-5 times higher in PBRs
O_2 inhibition	WSPs < PBRs	O_2 greater problem in PBRs
CO_2 losses	WSPs > PBRs	Much reduced in PBRs
Process control	WSPs < PBRs	Much easier in PBRs

PBRs can be classified on the basis of both design and mode of operation. In design terms, they can be: (1) flat or tubular; (2) horizontal, inclined, vertical or spiral; and (3) manifold or serpentine (snakelike). An operational classification of PBRs may include (4) air pump mixed, (5) single-phase reactors – with gas exchange taking place in a separate gas exchanger, and (6) two-phase reactors in which both gas and liquid are present and continuous gas mass transfer takes place in the reactor itself. On the basis of construction material, PBRs can be: (7) glass or plastic made and (8) rigid or flexible. Besides, the PBR construction material must lack toxicity, have chemical stability along with high transparency, mechanical strength and durability or resistance to weathering and offers ease of cleaning as well.

Productivity of PBRs is evaluated by three ways: (1) as volumetric productivity (VP), which is productivity as mass in grams per unit volume in g l^{-1} d^{-1}; (2) as areal productivity, that is productivity per unit of ground area occupied by the reactor expressed in g m^{-2} d^{-1}; and (3) illuminated surface productivity, which is productivity per unit of reactor illuminated surface area expressed in g m^{-2} d^{-1}. Oxygen production by algae cells is directly correlated with VP in PBRs, and DO concentrations of four to five times higher than the saturation DO values with respect to standard ambient conditions when DO is 8.3 mg l^{-1} at 25^0C, have been found toxic to many phototrophs (Weissman *et al*., 1988). The DO saturation of the cultivation medium may lead to consistent decline in the pH value due to the algal dark respiration (the period when there was no light), which is associated with the adverse effect of oxygen super saturation on algae photosynthesis in the given culture medium (Cheng *et al*., 2006).

II.3.2 Waste stabilization ponds or lagoons

As compared to energy-and-cost-intensive conventional or mechanical wastewater remediation processes, the simpler option of using waste stabilization ponds (WSPs) or lagoons is in extensive use particularly in developing countries (Shilton *et al*., 2008). WSP technology offers some important advantages and interesting possibilities when viewed in the light of renewable sustainable energy and neutral carbon footprint. These include their simple and economical construction, little or no operating and electrical energy requirements for aerobic wastewater remediation and potential for bioenergy generation (Shilton *et al*., 2008).

The WSPs, which may or may not be lined or matted from the bottom with either plastic or linen sheets or cement and clay material, are shallow rectangular lakes in which raw or screened wastewater is treated by natural processes such as sorption, infiltration or leaching, evaporation and drying, based on the activities of autochthonous (indigenous) microorganisms. These are least expensive to operate as well as considerably more efficient in destroying pathogenic bacteria given that the effluents are exposed to relatively higher ambient temperatures for most of the year. Thus, wastewater remediation in WSPs may be carried out for variety of functions including, sedimentation (settling), equalization or neutralization of wastewater ingredients via microbial treatment, and wastewater holding on either partial or complete retention basis and drying (Asadi, 2007).

The procurement of land on comparatively lower prices along with simple maintenance requirements such as its edges, water level and overall safety make WSPs an ideal form of wastewater treatment in developing countries (Martin, 1991). WSPs or lagoons are operated in a variety of ways and therefore are named accordingly. The major types include: Facultative ponds or Aerobic lagoons, Aerated lagoons, and High rate algal ponds. Each type is briefly described here to know and understand their characteristics.

II.3.2.1 Facultative ponds or Aerobic lagoons

These type of ponds with a depth of 3-6 ft, in most cases, are divided into aerobic surface and an anaerobic bottom causing thermal stratification or induction of heating and cooling layers induced by warming at the surface due to the surrounding temperature as well as from biological activity (Eckenfelder *et al*., 2009). Autochthonous or otherwise algae cells and plant life in the pond grow

during the daylight depending upon the depth of the pond resulting in the corresponding oxygen production required for the decomposition of organic matter contained in the wastewater by the indigenous bacteria. With higher pond depth the light penetration decreases resulting in lesser growth of algae cells and hence reduced production availability of oxygen concentration for bacterial activity. Thus, it can be said that the shallower the depth of a given pond, the greater will be the surface area for the activity by photosynthetic organisms yielding in higher growth of algae cells. In this case, the algal rate of oxygen production is frequently so rapid that it is produced faster than it can diffuse in the medium surroundings and thus supersaturated oxygen concentrations are attained (Horan, 1991).

II.3.2.2 Aerated lagoons

An aerated lagoon is comparatively larger in depth than the aerobic lagoon varying between 6 and 15 ft and receives continuous flow of wastewater. However, it differs from aerobic lagoons in that the oxygen required by the bacteria for organics removal is provided not by algal photosynthesis but by using mechanical aerators or diffused aeration devices. Therefore, the resultant turbidity, turbulence, shear stress caused by intensive aeration ceases or greatly reduces the algae growth; hence the primary focus is on promoting bacterial decomposition of wastewater contents rather than the growth of algae mass. The wastewater detention time in these systems ranges from a few days to 2 weeks depending on the organics removal efficiency (Benefield *et al*., 1980).

II.3.2.3 High Rate Algal Ponds

High rate algal ponds (HRAPs) are designed primarily as a unit for maximum carbon conversion for optimal algal productivity in a given medium of water.

Algae biomass thus produced is harvested either by flocculation or filtration on regular basis in order to offset the operational costs. To ensure maximum light penetration, these ponds are built with shallower depths of around 20-45 cm with retention times of 1-3 days. Continuous optimised gentle mixing in HRAPs is carried out by paddle-wheeling to prevent the formation of sludge layer, thus the operation of such a pond is also energy-intensive as both mechanical aeration and mixing make an important part for the functioning of HRAPs. The biological reactions occurring in HRAPs reduce the organic content to a limited extent and the nutrients and organic matter present in wastewater are utilized by algae cells to the maximum resulting in the increased level of photosynthesis as well as higher generation of algal biomass (Oilgae, 2009).

Figure II-2 shows the sketch of a typical HRAP.

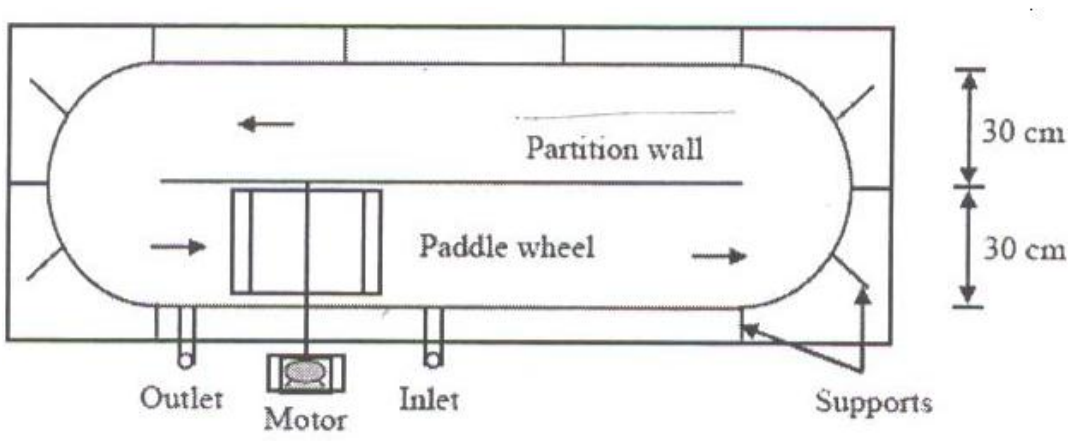


Figure II-2: Schematic of High Rate Algal Pond (Oilgae, 2009).

II.4 IMMOBILIZATION OF ALGAE CELLS

Photosynthetically oxygenated processes are often limited by the slow growth of microalgae and the risk of microalgal inhibition due to settling of algae cells usually near the bottom of the reactor during free cell cultivation, receiving reduced amount of incident light for energy and growth of the cells. Hence algae cells are immobilized by different ways for suspension in the medium near the surface of water (Munoz *et al*., 2009). Cell immobilization can be defined as any technique that limits the free movement of cells. The use of entrapped cells in biological wastewater remediation is drawing attention as it offers several advantages over free cells, including relatively easy cell separation, reuse of the same cells for a prolonged period of time due to continuous cell regeneration. However, the major advantage of cell entrapment is that immobilized cells do not suffer from physical or chemical changes occurring in the bulk medium during the immobilization process. Thus, permeability, null toxicity and transparency of the immobilized matrices create a very growth-conducive environment for immobilized cells (Morino-Garrido, 2008). The green algae such as *Chlorella Vulgaris* are mostly non-motile and known to behave in water in a static way particularly during the initial period of the cell growth as they lack flagella, a part of the cell structure responsible to make the cells moving in the medium. Therefore, mobilizing agents, either chemical or solid substances, are used for algae flocculation in water to keep the cells in suspension near the surface of water for maximising the photosynthetic activity of the cells (Richmond, 2004).

II.4.1 Algae attachment using chemicals

Chemical attachment of algae cells is carried out by using chemical products such as aluminium sulphate, sodium and calcium hydroxides, lime and polyferric sulfate to effect pH adjustment of the medium resulting in the aggregation of cells with the precipitates of the chemicals added (Munoz and Guieysse, 2006). Sandbank (1978) cultivated microalgae *Cyprinus Carpia* cells in wastewater and flocculated it with aluminium sulphate to prepare an algae diet of high pH for aquaculture species. However, many drawbacks have been observed during the use of these products such as use of high dosages for optimum interaction of chemicals with algae cells, the frequent need for pH correction of the culture medium. In addition, chemical flocculation process for algae immobilization is expensive and prone to causing salinity as well (Munoz and Guieysse, 2006).

II.4.2 Algae immobilization using solid substances

Use of various polymer or copolymer compounds and resins for algal cell immobilization is the most widely used technique, which is also termed as gel entrapment (Moreno-Garrido, 2008). Variety of solid substances are used for algae suspension at the surface of water in a reactor such as chitin, a homopolymer that is similar in structure to cellulose (Meanwell and Shama, 2007); chitosan, a linear amino polysaccharide (Moreno-Garrido, 2008); alginate, a natural polysaccharide and potato or starch derivatives, etc. The polymer substances generally have a very high molecular weight in excess of a million and exhibit relatively high flocculation efficiency (Pushparaj *et al*., 1993). Aggregated microalgal cells via

these substances offer advantages of growing faster with enhanced rates of photosynthesis along with ease of cell-broth separation as well.

Flocculation of algae cells is also necessary for biomass recovery, before algae cells are harvested by centrifugation, filtration, or in some cases gravity sedimentation (Grima *et al*., 2003). Flocculation is the coalescence of finely divided solid particles such as algae cells into larger loosely packed conglomerates. In general, the first stage of flocculation is the aggregation of suspended solids in the medium to form larger aggregates resulting from the interaction of the flocculant with the surface charge of these solids. The second stage involves the coalescing of these aggregates into large flocs that settle out of suspension (Knuckey *et al*., 2006). Tenney *et al*. (1969) quoted the model postulation proposed by LaMer and Healy (1963) stating that polymer molecules will attach themselves either by electrostatic or chemical forces, or both, to the surface of the algae cells at one or more sites creating bridges and forming algae-polymer matrix. The optimum flocculant concentration for polymers corresponds to a situation where the cell surface is partially covered by patches of polymers. This agglomeration of cationic polymers with the negatively charged algae cells reverses the charge and thus reduces the force of repulsion between cells. The polymers, because of their size, are able to form a bridge between the cells, bind them together, and bring about flocculation. Organic cationic polymers can induce efficient flocculation of freshwater microalgae at dosages between 100-1000 mg l^{-1} (Asadi, 2007).

Tilton *et al*. (1972) studied the flocculation of *Chlorella Ellipsoidia* at different algal concentrations (50–3000 mg l^{-1}) polymer concentrations (10–1000 mg l^{-1})

and at pH (4–9). They found that no flocculation at polymer concentrations from 10 to 200 mg l^{-1} took place except for cationic polyethyleneamine, which was effective at lower concentration and that there was no effect of pH variation on the flocculation matrix.

Cohen *et al*. (1989) studied the comparative flocculation behaviour of algae cells and showed that a cationic polymer flocculated the algae cells of *Chlorella Vulgaris* but an anionic polymer did not, suggesting the applicability of difference in the surface charges during polymer flocculation of algae cells.

Knuckey *et al*. (2006) observed that the polymer flocculation of shear-sensitive algae cells appeared to be less damaging to the cells than centrifugation, which induces constant shear force on to the cells. They concluded that this form of algae flocculation may prove to be a cost-effective alternative for the propagation of microalgal concentrates. Meanwell and Shama (2006) also observed in their experiments that mechanical stress influenced not only the morphology of microorganisms but also induced the cell lysis.

II.5 ORGANIC WASTEWATER REMEDIATION USING ALGAE-BACTERIA CONSORTIUM

Zhu *et al*. (1999) have referred to organic wastewater as one of the most abundantly obtainable biomass. Organic wastewaters contain energy in the form of bound carbon, which may be utilized by the organisms, as stressed by Polprasert (1996), that the treatment and recycling of organic wastewaters can be most effectively accomplished by biological processes, employing the activities of

microorganism. The suggested pathway for biodegradation of organics may be written as:

Organics → simple sugars → organic acids → CO_2 → microbial protoplasm.

Wastewater organics in a biological treatment process are removed by one or more mechanisms, namely sorption, stripping, or biodegradation, which is the primary step of bioremediation process. When organic matter is removed or taken up from water by aerobic microorganisms, two basic phenomena occur: oxygen is consumed by the organisms for energy, and new cell mass is synthesized. The related resultant reaction can be illustrated by the following generic equation:

$$\text{Organic matter} + a' O_2 + N + P \quad \text{cells (k)} \rightarrow a \text{ new cells} + CO_2 + H_2O \qquad (2.1)$$

Where, k is the rate coefficient and is a function of biodegradability of organic matter in wastewater. The coefficient *a'* is the fraction of the organics removed and oxidized to products for energy and coefficient *a* is the fraction of organics removed that is synthesized to cell mass (Manahan, 2010 and Martin, 1991).

Buzzi (1992) states that most of the microorganisms are quite useful to wastewater bioremediation process; whereas Rittmann (2008) stated that the microorganisms themselves are not the source of energy output but the means to channel electrons to desired products provided the optimum microbial function in a bioreactor is facilitated by the provision and maintenance of favourable conditions of pH, temperature, mixing or recirculation flow rate, DO and nutrients.

Arranz *et al*. (2008) note that photosynthetically oxygenated biodegradation processes offer an attractive and reliable alternative to conventional energy-demanding and greenhouse gases-emitting wastewater treatment processes. The oxygenation mode observed in the consortium is cheaper requiring no further

energy for aeration and the functioning of the consortium has less environmental impacts with low release of greenhouse gases and volatile organic contaminants than the traditional methods.

Guieysse *et al*. (2002) investigated the influence of algal-bacterial microcosm for the degradation of salicylate in a fed-batch culture. They first optimized the inoculation strategy before using the biomass inocula of algae *Chlorella Sorokiniana* and bacteria *Ralstonia basilensis* with algal-bacterial ratio of 5:1 and 1:1, with the former proving viable as compared to latter for salicylate degradation. Borde *et al*. (2003) write about the synergistic nature of microalgae and state that most of the microalgae are found in association with other aerobic microorganisms and the mixed culture of algae and bacteria offer beneficial advantages. First, the biological aeration is economically comparable with the mechanical one; secondly, conversion of light energy to chemical energy phenomenon in ultimate analysis is both renewable and carbon-neutral and finally, wastewater nutrients such as nitrogen and phosphorous are taken up to the maximum for the growth of the cells. Chavan and Mukherji (2008) have referred to the successful investigations made in bioreactor technology utilizing algae-bacterial consortia yielding higher treatment efficiency than the systems run with monocultures. From their own experiments, they concluded that association of phototrophic green algae and bacteria could result in better removal efficiency of the polluting substances as compared to the systems employing either of them alone. Their observation was in agreement with Hammouda *et al.* (1995) when they quoted that wastewater treatment utilizing algal-bacterial system was capable of removing pollutants as 83% COD removal efficiency was obtained.

Munoz and Guieysse (2006) have reported that algae and bacterial consortium was able to remove sodium salicylate at a maximum rate of 87 mg l^{-1} hr^{-1} in a continuous enclosed PBR, which corresponded to an oxygenation capacity of 77 mg O_2 l^{-1} hr^{-1} close to that generated by large-scale mechanical surface aerators. They also concluded that periodical absence of light or dark period caused a halt or sever reduction in the process of photosynthesis, which led to the occurrence of anaerobic conditions in the reactor. They also dilated upon the symbiotic microalgal-bacterial relationship stating that microalgae provided the oxygen necessary for aerobic bacteria to decompose organic matter, consuming in turn the carbon dioxide released from bacterial respiration.

Drapcho and Brune (2000) shared somewhat similar observation when they found out in their work that the external supply of inorganic carbon source for the growth of algae cells in a mixed culture with bacteria was not needed. They further observed that both the organisms do not limit their interactions to a simple CO_2/O_2 exchange; as microalgae can also enhance bacterial activity by releasing extracellular compounds, whereas bacterial growth can enhance microalgal metabolism by releasing growth-promoting factors or by reducing oxygen concentration in the medium.

II.5.1 *Pseudomonas Putida* as a model bacteria in the consortium

Bacteria are single-celled prokaryotes that are generally shaped as rods (bacillus), spheres (coccus), or spirals (vibrios, spirilla, spirochetes) (Srivastava, 2008). Their optimum growth temperature range is between 20–35^{0}C and they can make use of sugars as well (Kucerova, 2006). Given the optimum growth conditions, they can

multiply very rapidly after every 20 min by simple division or binary fission; like all other organisms, they generate specific enzymes naturally for the breakdown and metabolism of organics (Manahan, 2010). Almost all bacteria are 80% water and 20% dry matter. The dry matter is 90% organic and 10% inorganic; the organic fraction consists of 53% carbon, 29% oxygen, 12% nitrogen and 6% hydrogen (Sayler, 1991). The breakup of bacteria into their recognised forms, based on their carbon and energy needs, is shown in Figure II-3.

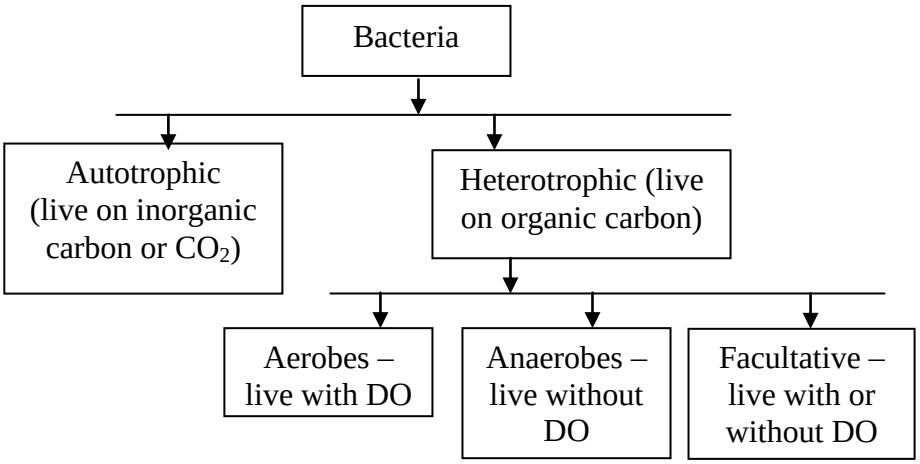


Figure II-3: Types of bacteria based on their energy and carbon needs (Sayler, 1991).

The above figure suggests that bacteria obtain their food and energy either from organic or inorganic carbon sources needed for their metabolic processes and reproduction by mediating chemical reactions via enzymes, which are biochemical catalysts endogenous to living organisms. The two major divisions of bacterial metabolism are catabolism, energy-yielding degradative metabolism that breaks macromolecules down to their small monomeric constituents, and anabolism,

which is synthetic metabolism in which small molecules are assembled into large ones. Oxygen is the terminal electron acceptor in the electron transport chain involved in the process by which bacteria gain energy by oxidizing food materials (McKinney, 1962).

Obligate aerobe *Pseudomonas Putida* (*P. Putida*) is from *Pseudomonad* family, which are ubiquitous in nature. These bacteria engage in important metabolic activities in the environment and have considerable potential for biotechnological applications, particularly in the area of bioremediation or biocatalysis. *P. Putida* strain KT2440 is the best-characterized saprophytic *Pseudomonad* that has retained its ability to survive and function in the environment. In addition to the metabolic potential of this bacterium, the ability of *P. Putida KT2440* to colonize with the plants may facilitate in their growth promotion as well. *P. Putida* cells' dimension ranges between 0.5–4.0 μm and they can move in the culture medium by one or more polar-flagella that they possess.

El-Masry *et al*. (2004) observed in their experiments over 90% removal of COD from polluted wastewater using a biofilm system consisting of eight bacterial strains including *Pseudomonas sp*. Kalyani *et al*. (2009) used *Pseudomonas sp* strain for biodegradation of textile dye effluent and their results showed that within 24 hr 52% COD reduction was achieved. Satyawali and Balakrishnan (2008) used *P. Putida* cells in a bioreactor for distillery wastewater treatment, which resulted in a COD removal of 44% in 24 hr.

Costura and Alvarez (2000) have stated that laboratory studies have shown that some *Pseudomonads* can degrade some specific hydrocarbons such as benzene

toluene and xylene under hypoxic conditions of DO between 0 and 2 mg l^{-1} suggesting the psychrophilic nature of *Pseudomonas* bacteria.

Alagappan and Cowan (2004) studied the growth behaviour of *P. Putida* strain on benzene and toluene correlating the growth with temperature and dissolved oxygen of the growth medium. They observed that when *P. Putida* cells were grown at 35^0C, oxygen uptake rate was between 2 and 15 mg l^{-1} d^{-1}; however, when they were grown at reduced temperature of 25^0C, the oxygen uptake rate was between 1 and 9 mg l^{-1} d^{-1}. In addition, at DO 8.3 mg l^{-1} and temperature 20^0C, the maximum growth rate per day achieved for *P. Putida* was 0.3, showing lesser consumption of oxygen resulting in the reduced growth of the cells.

II.5.2 *Chlorella Vulgaris* as green microalgae in the consortium

Microalgae have an average diameter of 2-3 µm, are photosynthetic microorganisms of prokaryotic (without membrane-bound nuclei) such as blue green algae or Cyanobacteria and eukaryotic nature (with a nucleus) such as green algae *Chlorella Vulgaris* (*C. Vulgaris*). Microalgae have played a significant role in earth's biogeochemistry for billions of years producing about half of the atmospheric oxygen and powerfully influence the cycling of carbon, nitrogen and phosphorous (Nelsen, 2002). Therefore, algae has the potential to be used in engineered systems to produce renewable sources of energy as well as aid in the removal of pollutants from water and wastewater (Andresen, 2005 and Richmond, 2004). So far approximately 30,000 algae species have been discovered and described (Graham *et al*., 2009); whereas Um and Kim (2009) have referred to more than 100,000 known strains of microalgae in the world. With respect to

diversity, according to Smith's freshwater algae of the US, microalgae have been classified into seven divisions as shown in Table II-3.

Out of the seven divisions as mentioned in the Table, the eukaryotic Chlorophyta division consisting of green algae alone comprises an estimated 17,000 species and is relatively widely recognised in the application of water and wastewater remediation (Graham *et al*., 2009).

Table II.3: Classification of microalgae in various divisions (McKinney, 1962).

Algae by colour	Division
Green	Chlorophyta
Motile green	Euglenophyta
Yellow-green to golden green	Chrysophyta
Motile tan to golden brown	Pyrrophyta
Blue-green	Cynophyta
Marine brown	Phaeophyta
Marine red	Rhodophyta

II.5.2.1 Chlorophyta (green algae)

The green algae cells both have nucleus and cell wall. This type of algae appears green due to the presence of chlorophyll and other pigments that are found in membrane bound organelles known as chloroplasts, located in a dense region called pyrenoids. The chloroplast contains a series of flattened vesicles or thylakoids, containing the chlorophylls, and a surrounding matrix called stroma

(Richmond, 2004). Chlorophyll is the principal photoreceptor, which absorbs both blue and red light. The lipid-rich green algae *C. Vulgaris* are one such example, which can be a useful source of modern renewable fuels, like biodiesel (Graham *et al.*, 2009 and Pratt *et al*., 2004).

Chinnasamy *et al*. (2009) concluded from their experiments that microalgae could be grown in organic wastewaters with even better growth rates than if grown in a normal water body.

Weissman *et al*. (1988) in his experimental studies focused on the development of pond system containing suspended microalgae for wastewater remediation, and their results showed that the growth of algae cells was proportional to the level of wastewater remediation achieved.

Oswald (1963) referred to treatment facilities such as stabilization ponds that utilize algae to remove nutrients. He further stated that algae increase oxygen in water by 100% to 400% and that oxygenation systems employing attached algae have a number of advantages as these are odourless and no energy is required for suspending cells, which makes harvesting of algal biomass easier.

Wijanarko *et al*. (2008) observed constancy or slight decrease in the biomass production of *C. Vulgaris* in bubble column PBR during the dark reaction period of photosynthesis. Martinez and Orus (1991) referred to green algae strain of *C. Vulgaris* being isolated from the effluent of sugar refinery, which suggested the tendency of this particular alga towards glucose consumption for its maximal growth. This proposition was verified when they found out in their experiments that the growth rate of *C. Vulgaris* was strongly influenced by the presence of

glucose, as the cells were not growing enough without being supplemented with sugar even after the increase in the light intensity from 30 to 150 $\mu E.m^{-2}.s^{-1}$.

Sayed and El-Shahed (2000) found out during their experiments that above 25^0C, increased growth temperature resulted in decreased growth rate of *C. Vulgaris* and that maximum activity was observed at midday in the cells grown at 25^0C.

Meng *et al*. (2009) have quoted the findings of Papanicolaou *et al*. (2004) regarding the unexpected growth return of algae *Mortierella isabellina*, cultivated in nitrogen-limited media presenting remarkable cell growth up to 35.9 g l^{-1} and high glucose uptake when initial sugar concentration of 100 g l^{-1} was added in the media. Borde *et al*. (2003) observed that *Chlorella* cells commonly colonized in wastewater treatment systems due to their high tolerance towards dissolved and particulate organic compounds. Safonova *et al*. (2004) used nine algae strains for biotreatment of wastewater along with bacteria at the pilot scale and found out that only two of them namely *Chlorella* and *Scenedesmus* proved to be very suitable for use with bacteria in terms of wastewater bioremediation efficiency achieved. Kalin *et al*. (2005) noted that every algae cell has a plausible number of high affinity surface binding sites numbering about 10^7 or 10^8. When the cells are growing, this number of available surface sites was observed to increase, indicating the rationale behind increased amount of photosynthetic oxygenation of the medium particularly during log phase of algae growth.

II.5.2.2 Oxygenic photosynthesis by microalgae

Photosynthesis is an endothermic or energy-requiring process, which is driven by the light source. The electromagnetic radiations of light reach the algae cells in distinct units of energy called quanta. Absorption of light quanta by chlorophyll

pigments begins the process of photosynthetic energy conversion (Madigan *et al*., 2009). Figure II-4 depicts the overall light profile including the photosynthetically utilisable visible light matrix, also known as photosynthetically active radiation (PAR), which constitutes about 43% of the total incident light radiation.

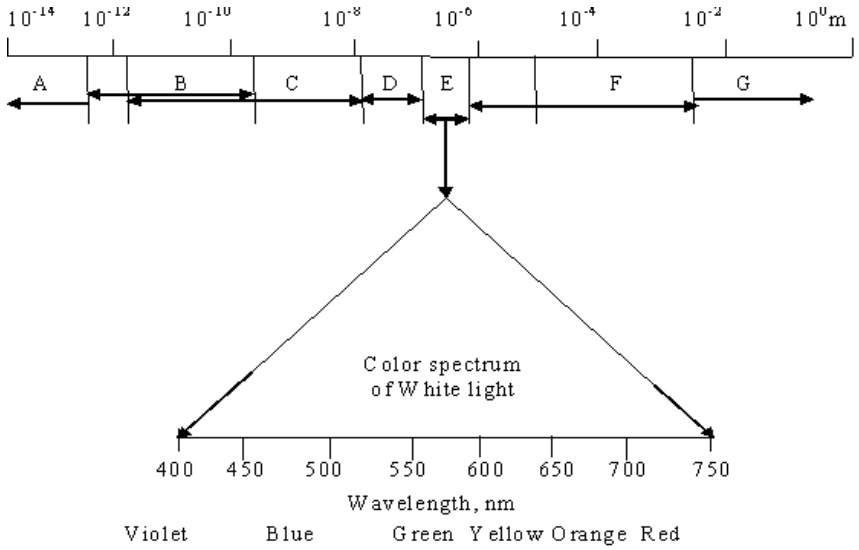


Figure II-4: Spectra of electromagnetic radiation and spectral pattern of visible light. Rays: A= Cosmic, B= Gamma, C= X, D= Ultraviolet, E= Visible, F= Infrared and G = Radio waves (Richmond, 2004).

Assuming the wavelength of light remaining constant, the intensity of light will influence the rate of photosynthesis, but higher light intensity and higher temperature slow down the photosynthetic rate (Nag, 2008). The location of photosynthetic apparatus in microalgae is found within chloroplasts, which contains many light-harvesting pigments such as chlorophylls, carotenoids and phycobilins which are grouped into photosynthetic units each consisting of 300-

400 molecules of pigment. The pigments are capable of absorbing light over a broad range of wavelengths and referred to collectively as antenna pigments or light harvesting complexes (Graham *et al*., 2009). Figure II-5 shows a roughly sketched image of photosynthetic apparatus in microalgae cells to depict the capturing of light by antenna pigments in the reaction centre, with a closer view of inside in a thylakoid.

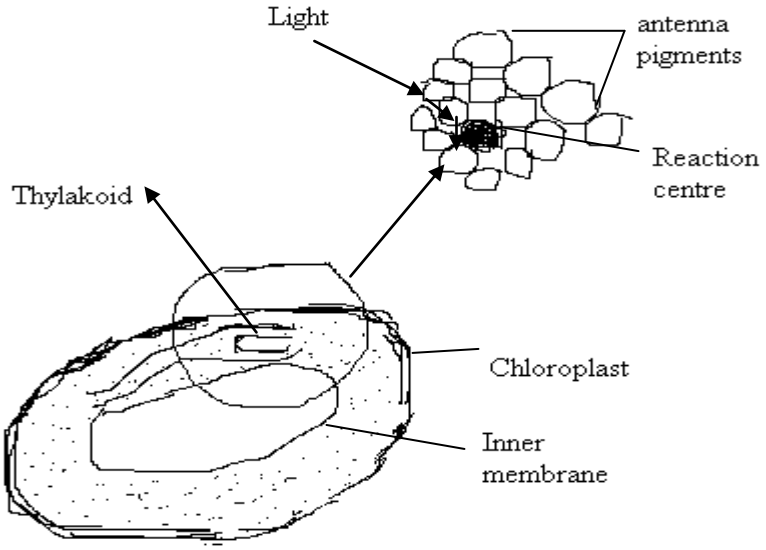


Figure II-5: Schematic of microalgae chloroplast along with inset view of thylakoid (Horan, 1991).

In the first phase of oxygenic photosynthesis also known as light-dependent or light reaction, light energy reaches the reaction centre and transfer of energy takes place resulting in the conversion of elementary energy molecules like adenosine diphosphate (ADP) and nicotinamide adenine dinucleotide phosphate (NADP) into high-energy molecules as adenosine triphosphate (ATP) and strengthened nicotinamide adenine dinucleotide phosphate (NADPH) (Graham *et al*., 2009).

During this process, water is splitted to yield in free molecular oxygen, a waste product that mostly diffuses out of cells into the bulk medium. The first phase of algae photosynthesis also provides the reducing power needed in the second phase, known as light-independent/ dark reactions or Calvin cycle. In these reactions, ATP and NADPH are used to reduce carbon compounds, thereby forming a new protoplasm in a process known as carbon fixation (Graham *et al*., 2009). There are two ways to measure photosynthesis either by carbon uptake or by oxygen evolution. It should be stressed that the dark reaction does not occur during the hours of darkness, but it is so named because it does not require light energy as such but rather utilizes the products of the light reaction (Benefield *et al*., 1980 and Madigan *et al*., 1997).

Figure II-6 highlights the reactions involved in algae photosynthesis process

(a) Light-dependent or Hill reaction:

$$H_2O + Light + Algae + NADP^+ + ADP + P \longrightarrow O_2 + ATP + NADPH + H^+$$

(b) Light-independent or Blackman's reaction:

$$C + NADPH + H^+ + ATP + Algae \text{ ----- } CH_2O + NADP^+ + H_2O + ADP + P$$

Figure II-6: Light and dark reactions during algae photosynthesis (Graham *et al*., 2009).

II.5.3 Microbial growth kinetics

In a given culture medium, microorganisms consume substrate and carry out oxidation-reduction reactions for the growth of cells to occur resulting in the production of new or additional cells (Madigan *et al*., 1997). The initial food-microorganism (F: M) ratio stimulates the growth which results in rapid removal of oxygen and reduction of organic matter in the medium, thus the rate of metabolism decreases as the F: M ratio reduces. Microbial growth is determined by changes in the weight of biomass, and changes in substrate concentration, which is characterized by identifiable phases. With glucose as a model substrate, four distinct growth phases involved during the course of its consumption are shown in the classical growth curve as illustrated in Figure II-7.

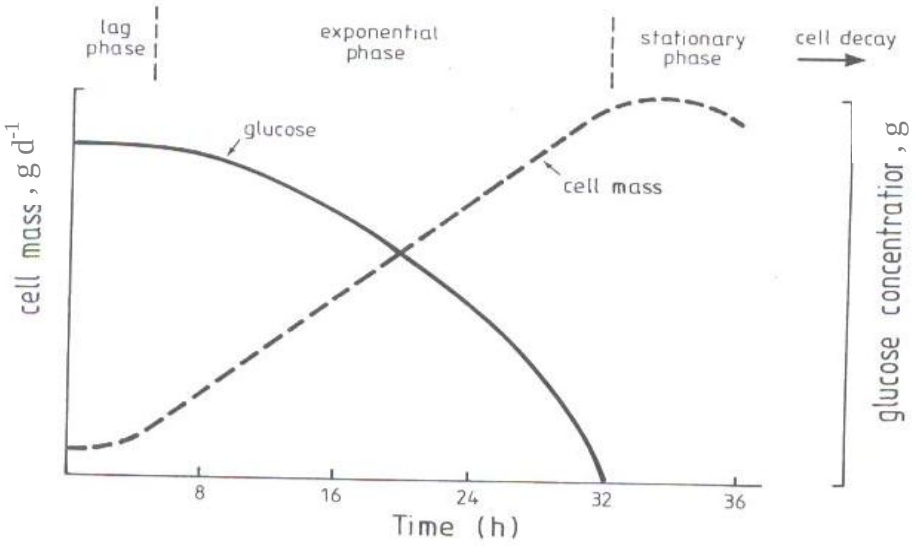


Figure II-7: Typical microbial growth curve for glucose degradation in a batch reactor (Horan, 1991).

a. The lag phase

It represents the time required for the organisms to acclimate to their new environment before significant cell division and biomass production occur. During the lag phase enzyme induction may be occurring and the cells may be acclimating to changes in pH or temperature. Although the biomass is not changing during this period, sufficient metabolic activity is taking place within the organism, and substrate is being assimilated and utilised for the synthesis of new enzymes and for growth of the cell prior to division.

b. The exponential or log growth phase

During this phase microbial cells are multiplying at their maximum rate, as there is no limitation due to substrate or nutrients saturation. The biomass growth curve increases exponentially or rapidly during this period and continues dividing at a rapid speed until such time as the medium is no longer able to support the growth, thus entering into stationary phase. The stagnation in the growth may be accounted for involving certain factors such as depletion of a nutrient essential for growth; depletion or saturation of DO supply and changes in medium pH and temperature.

c. The stationary phase

During this phase, the biomass concentration remains relatively constant with time and the growth is no longer exponential and the amount of growth is offset by the start of cells' death.

d. The death phase.

In the death phase, the decay in biomass concentration is due to cell death, which is observed with an exponential decline in the biomass concentration per day. Finally, death takes over the entire culture to complete the growth cycle

(Tchobanoglous *et al*., 2003). Eckenfelder *et al*., (2009) mentioned that when insufficient nitrogen is present in the culture medium, the amount of cellular material synthesized per unit of organic matter removed increases due to accumulation of polysaccharides, which implies towards increased synthesis of cellular material under nutrient-limiting conditions.

II.6 OXYGEN REQUIREMENT IN A MIXED CULTURE

When a heterotrophic bacteria degrades an organic substrate to fulfil its requirement for carbon and energy, electrons are removed from the substrate and passed on to a suitable electron acceptor, usually oxygen in aerobic biodegradation. This simply means that reduced carbon atoms are oxidized, releasing energy by giving up electrons through the action of biodegradation by bacteria (King *et al*., 1998). This particular reaction occurs for two purposes one for microbial respiration and two for synthesis of new cells (Reynolds and Richards, 1996). In an aerobic biodegradation process, if the demand for oxygen is greater than the supply, anaerobic conditions will set in and problems such as cell decay and generation of malodorous compounds will take place in the system. Munoz *et al*. (2006) have referred to energy savings in oxygen supply by *Chlorella* cells with an equivalent of 12 kg O_2 m^{-3} d^{-1} (8.3 mg l^{-1} d^{-1}), making photosynthetic oxygenation a very attractive process economically.

Drapcho and Brune (2000) investigated the photosynthetic oxygen production by both blue green and green algae strains in mixed cultures cultivated at 20^0C and found out that O_2 generation rate by blue green algae cells was 2.3 mg/ mg TSS

per day in contrast to 3.4 mg /mg TSS per day by green algae cells, which also influenced upon their respective specific growth rates, indicating higher photosynthetic oxygen return by green algae than blue green algae.

II.6.1 Mass transfer phenomenon

The mass transfer in terms of oxygen from gaseous phase to liquid phase is a process in which interphase diffusion occurs when a driving force is created by departure from equilibrium. In the gas phase, the driving force is partial pressure gradient; in the liquid phase, it is concentration gradient. For sparingly soluble gases such as oxygen and CO_2, the liquid-film resistance controls the rate of mass transfer. The solubility of oxygen is influenced by the decreasing partial pressure in the bubble as oxygen is absorbed (Eckenfelder *et al*., 2009).

According to Hilton (1999), as quoted by Kilani and Lebeault (2007), in liquid batch processes the critical parameter is gas exchange or balance between oxygen demand of the culture or oxygen uptake rate (OUR) and oxygen transfer to the culture or oxygen transfer rate (OTR).

McKinney (1962) mentioned that the OTR is a function of oxygen gradient existing between the gas and the liquid, the surface area of contact between the liquid and the gas, the time of contact, temperature, and the characteristics of the liquid

Two-film theory concept

The most widely used and accepted theory to describe the absorption of a gas by liquid medium is known as the two-film theory developed by Lewis and Whitman in 1924 (Lekang, 2007). This envisages that oxygen transfer involves physical

mass transport across a two-film layer that consists of a gas film i.e. oxygen and a liquid film in bulk water, as highlighted in Figure II-8.

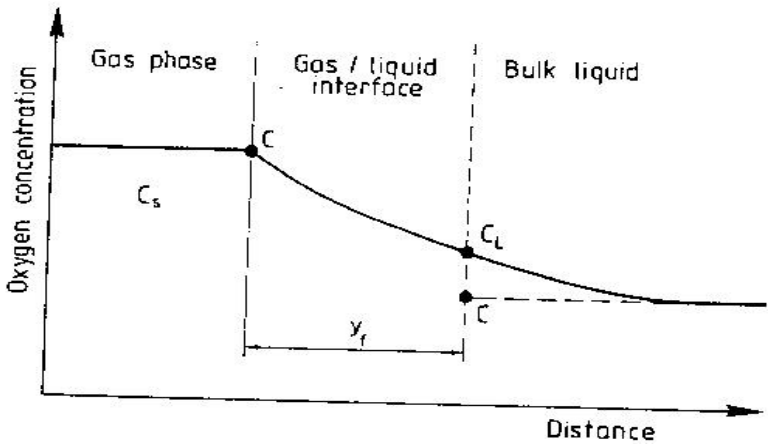


Figure II-8: Two-film theory for gas-liquid transfer (Collins *et al.*, 2004).

Legends: C_s = oxygen saturation concentration; C = actual concentration of oxygen; C_L = concentration of oxygen in the liquid.

Oxygen molecules are initially transferred across the gas film of the bubble to the liquid film and then by diffusion and convection across the liquid film into the bulk liquid. The dissolved oxygen (DO) then diffuses across the microbial cell wall and is utilised in cell metabolism (Madigan *et al*., 1997).

To transfer the large quantities of oxygen in the bulk liquid, additional interfaces can be created via maximum concentration of oxygen bubbles introduced to the water to increase the OTR. Doble *et al*. (2004) noted that for a typical mass transfer across liquid phase film, molecular diffusivity is of the order of 10^{-9} m^2 s^{-1}, while the liquid film thickness is of the order of 10^{-5} m, giving a typical value of liquid side mass transfer coefficient of ~10^{-4} m^2 s^{-1}. On the gas-phase side, the

diffusivity is of the order 10^{-6} m^2 s^{-1} giving two orders of magnitude higher gas-phase mass transfer coefficient.

The aeration of cell culture in bioreactors is usually carried out by bubble aeration, as it delivers large amounts of oxygen into the medium resulting in the high oxygen mass transfer in water. When a gas is dispersed in a liquid in the form of bubbles, the interfacial area of gas-liquid contact is the total surface area of the bubbles. For a specified volume of gas, the interfacial area increases as the number of bubbles into which it is dispersed increase (Czermak *et al*., 2005).

Bubbles smaller than about 1.5 mm dia maintain a spherical shape and behave almost like rigid spheres. The bubble diameter will affect the velocity of rise, the mass transfer coefficient and the excess internal pressure due to surface tension. Smaller bubbles of size between 0.5 to 1 mm in diameter have velocities of rise of about 0.12 m s^{-1} compared to 0.3 m s^{-1} for bubbles of size about 1.5 mm and higher usually produced in conventional aeration, which increases the length of contact time between bubbles and the liquid by 25 times. On the other hand, the smaller bubbles have total interfacial area 20 to 30 times greater than that of coarse bubbles, but due to the lower velocity, the mass transfer coefficient is as much smaller, so that these two effects balance each other out (Winkler, 1981).

Germain and Stephenson (2005) elaborated that the rate of mass transfer is proportional to the contact area between the liquid and oxygen phases, therefore small bubbles have a higher contact area/volume ratio compared to coarse bubbles at equal airflow rates, making them more efficient in terms of overall mass transfer coefficient ($K_L a$). Figure II-9 shows rough schematic of the mass transfer in terms of oxygen via a bubble system.

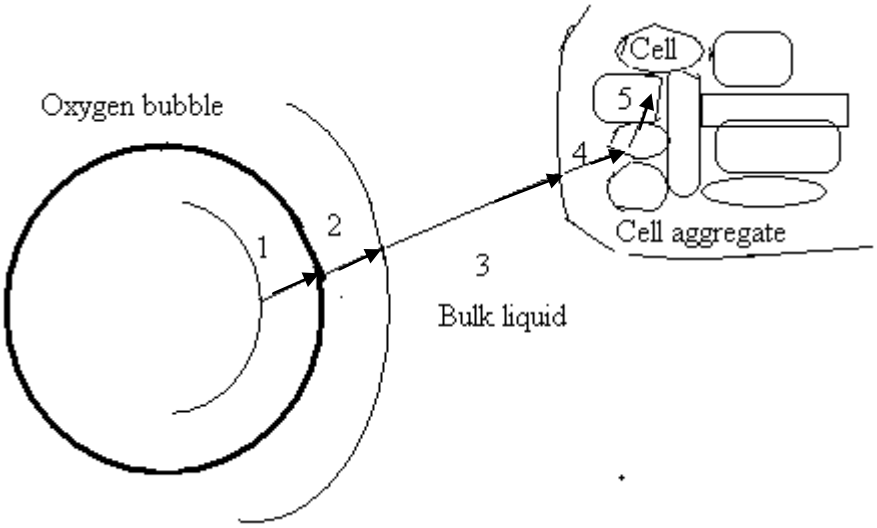


Figure II-9: Chain of mass transfer steps from inside of an oxygen bubble towards the site of reaction inside a cell (Winkler, 1981).

The description of the steps involved in the mass transfer via bubble aeration is outlined as under:

1. Oxygen transfer mainly by diffusion from gas to the interface of liquid phase
2. Transport usually by a combination of diffusion and convection across a thin boundary layer of water phase that surrounds the gas bubble
3. Transport usually by convection or advection through the bulk liquid phase to a thin boundary layer surrounding a microorganism
4. Diffusive transport across this boundary layer to the cell surface; and
5. Diffusive or enzyme-induced transfer over the cell envelope to a reaction site inside the cell.

II.7 WASTEWATER REMEDIATION DETECTORS

To monitor the magnitude of bioremediation function in a given culture medium, following parameters are investigated through their analyses, which are briefly reviewed hereunder:

II.7.1 Dissolved Oxygen

The critical wastewater bioremediation level for dissolved oxygen (DO) has been suggested between 0.7 and 2 mg l^{-1} for aerobic processes (Winkler, 1981 and Phong, 2008). The most important aspect of water pollution is the rate at which DO is depleted, which is a direct function of microbial activity. The depletion of oxygen from the medium results in a deficit at the gas-liquid interface, causing oxygen from the gas to enter the liquid medium, indicating the rate of oxygen transfer being dependent on the concentration gradient of DO in the medium. On the other hand accumulation of excess DO in the medium leads to photoxidative damage resulting in the loss of electrons for excited algae cells causing damage to the algae cell pigment due to photorespiration, which is a reverse phenomena of algal photosynthesis and eventually proving detrimental to overall productivity (Andresen, 2005 and Richmond, 2004). Oxygen solubility may be expressed on the basis of oxygen tension, which may be taken as the necessary partial pressure in the atmosphere to keep certain concentration of the gas in water. If the atmosphere is air, at normal pressure the oxygen tension is 159.6 mmHg, which creates 100% oxygen saturation in the water. If the pressure is less than this, the concentration in the water will also be reduced (Lekang, 2007).

In algal ponds, the net accumulation of DO may vary from about 2 to 5 mg l^{-1} during the night to 20 mg l^{-1} or above during the day due to algae photosynthesis activity during the day time as shown in Figure II-10.

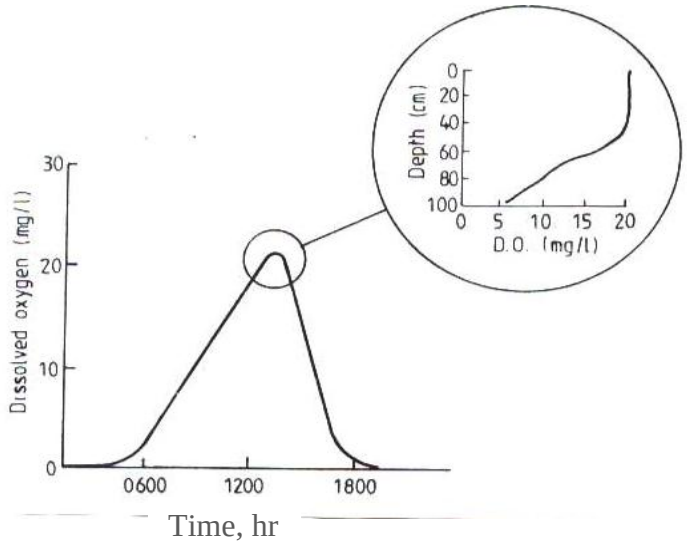


Figure II-10: Diurnal DO profile in an algal pond (Reynolds and Richard, 1996).

From practical standpoint, oxygen uptake and oxygen transfer rates can be measured by determining the residual DO values at various time intervals during the bioremediation process (Sawyer *et al*., 2003).

II.7.2 pH

pH influences cell growth rates at its optimum values and affects the solubility and or bioavailability of substrate materials or compounds at the lower values, which can reduce the biological activity (Burgess *et al*., 1999). pH measurements can be taken as an indicator for the production of acidic products in a given water medium particularly when biodegradation is underway. Lower pH values (< 4) can stop a

biochemical reaction or reduce its rate to almost zero, as hydrogen ion concentration causes denaturation of the key enzyme proteins. Therefore, most microorganisms may not survive below pH 4.0, as microbial growth occur between pH 4.5 and 7.5 (Isaac *et al*., 1995). Similarly, high pH is inhibitory to the biological activity, as accumulation of hydroxyl ions begins to exert a toxic effect when the pH is above 10 (McKinney, 1962). Active microbial growth in culture may well result in pH changes as acidic or alkaline products are released into or depleted from the growth medium. In addition, at pH 8 or 9, the concentration of bicarbonates is 200 times greater than that of dissolved carbon dioxide, which becomes more abundant only at pH around 6 (Andresen, 2005).

II.7.3 Temperature

Temperature of the cultivation media controls the activities and growth rates of algae and bacteria. It affects the dissolution of gases in water and the biochemical reactions and their kinetics (Mayo and Noike, 1995). Wastewater bioremediation is traditionally carried out at ambient temperatures with low temperatures below 20^0C may prove to be a limiting factor in the biodegradation process due to slower pace of biological activity. Whereas higher temperatures of more than 35^0C are known to increase reaction rates, degree of metabolism but may reduce log phase growth period for the biomass production and may result in the foaming problems as well (Cappuccino *et al*., 2005). Thus temperature of intermediate range i.e. between 20 to 30^0C, which is also the optimal temperature range for growing many microalgae (Chisti, 2008) may influence the microorganisms adopt productive metabolic pathways (Burgess *et al*., 1999). The temperature within a microbial cell

is virtually equal to the temperature of its surroundings, thus variation in the temperature of more than ± 2^0C from the optimum range increases the microbial activity up to a point where the cells are killed (Reynolds and Richards, 1996). Chinnasamy *et al*. (2009) found in their experiments that increase in temperature from the optimum range enhanced the process of photorespiration more markedly thus causing depletion of intracellular carbon reserves.

II.7.4 Chemical Oxygen Demand

The efficiency of wastewater treatment is normally expressed in terms of COD removal equivalent to oxygen consumed during the process measured as a percentage of organic matter stabilized during the biodegradation cycle (Phong, 2008). Chemical oxygen demand (COD) is the oxygen-equivalent of the dichromate consumed in the chemical oxidation of organic matter to carbon dioxide during its analysis. Thus, COD is an indirect measure of the concentration of chemically oxidizable organic matter present in water (Casey, 1997).

The COD monitoring is of great interest to measure the growth of the microorganisms, in addition to allowing the study of the evolution of biodegradation process (Jemenez *et al*., 2003). During biodegradation process, one-third of the ultimate COD of a substrate is used for energy and two-thirds result in cell synthesis (Tchobanoglous *et al*., 2003 and McKinney, 1962). Using an oxygen-to-cellular-volatile-solids conversion of 0.7 g VSS/g O_2, 0.47 g VSS may be synthesized for each gram of COD removed. Theoretically, a COD-nitrogen-phosphorus ratio of 100:5:1 is adequate for aerobic treatment, with small variations depending on the type of system and mode of operation (Hammer *et al*.,

2004). Acuner and Dilek (2002) observed that maximum COD removal from wastewater occurred during the exponential growth phase of *C. Vulgaris* indicating growth-associated treatment of wastewater using algae cells.

CHAPTER III

EXPERIMENTAL MATERIALS, EQUIPMENT AND ANALYTICAL PROCEDURES

CHAPTER III

EXPERIMENTAL MATERIALS, EQUIPMENT AND ANALYTICAL PROCEDURES

III.1 INTRODUCTION

This chapter presents the description of materials concerned with the experiments, experimental equipment and relevant analytical procedures. In the materials description, the reaction tank characteristics are elaborated along with the independent input variables of the tank such as microorganisms used and copolymer beads added. The next section provides the information about the equipment utilised for the analysis of the parameters of interest, which are also discussed.

III.2 EXPERIMENTAL MATERIALS

The materials used in the experimental work are outlined hereunder, which are dilated separately:

i. Lagoon photo tank
ii. Simulated sugar factory wastewater
iii. Bacteria strain
iv. Algae strain
v. Copolymer

III.2.1 Lagoon photo tank

Lagoon photo tank (LPT) resembles a small prototype photobioreactor (PBR) or a raceway lagoon of rectangular shape with flat bottom. The rectangular shape of the tank allows its design to include the option of common wall construction, which is the most satisfactory shape for ponds or PBRs as it affords sufficient surface to volume (s/v) ratio for the culture activity (Tebbutt, 1998). The LPT is made from Perspex material, which is transparent enough to facilitate the viewing of culture growth and for visual observations. The LPT body thickness of 1.1 cm offers durability and strength to its structure. The LPT length of 120 cm ensures maximum dilution and saturation of sample water in the tank with respect to organic loading and dissolved gases. The LPT has almost the same altitude and width or inside space in the tank of 22 cm to facilitate the standardized light path of around one foot (Richmond, 2004). The tank is partitioned from inside across the middle in line with the standard PBRs and HRAPs to induce wall turbulence for homogeneity of the tank contents (Farhadian *et al*., 2007 and Busca, 2004). This partition divides the tank into two compartments of equal space creating a gradient environment in LPT with respect to culture growth. The lighting unit is mounted over LPT with the support of bracket stands at the right and left edges of the tank. The lighting field consists of two fluorescent tubes of 38 wattage purchased from Boss Lighting, UK, one emitting blue spectrum (400-500 nm), which efficiently excites fluorescence in algae cells (Gregor *et al*., 2008) and the other cool white luminance (400-700 nm). The schematic of LPT is shown in Figure III-1.

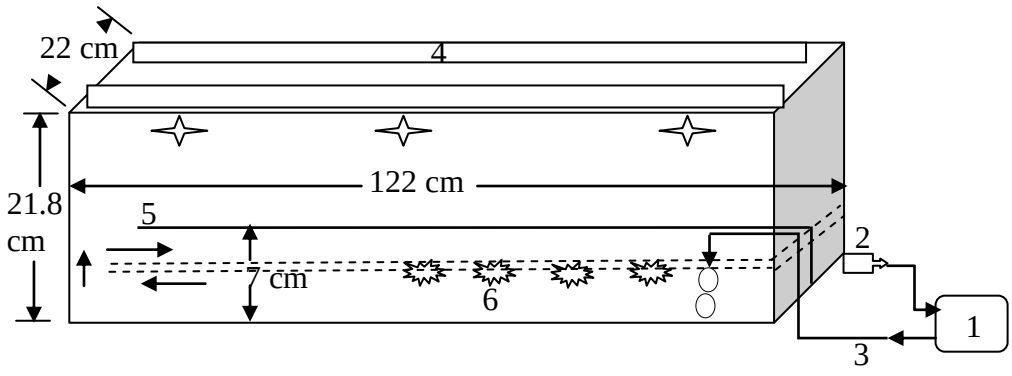


Figure III-1: Schematic of lagoon photo tank.

Legends: 1- Peristaltic pump, 2- Outlet point, 3- Inlet point, 4- Light source, 5- Partition bar, 6- immobilized algae cells.

The working volume of the tank was set as 13 litres to: 1) maintain food (carbon) to microorganism contents below this partition bar level, 2) ensure bioavailability of nutrients and their removal by the organisms within specified points in the tank. Bioavailability is the accessibility of both nutrients and substrate to the culture cells in the cultivation medium. The degree of bioavailability is increased when the nutrients and substrate are present in a dissolved state in the medium, which are more accessible to the suspended culture cells.

Table III-1 shows different levels of water at their corresponding depth of water in the tank within the bar height.

Table III.1: Volume of water at different depths in lagoon photo tank.

Partition depth in LPT, cm	Volume of water in L
6	15.6
5	13
4	10.4
3	7.8
2	5.2

The open space between the overhead lighting unit and LPT top at the back, front and at the right side was covered with black resin sheet to prevent the loss of heat contents in the tank. The gap at the left side was used for the removal of any aerosol particles generated via the contiguous extractor. The front side of the tank was used for sampling and analysis of the tank contents. The camera photograph of LPT is shown in Figure III-2.

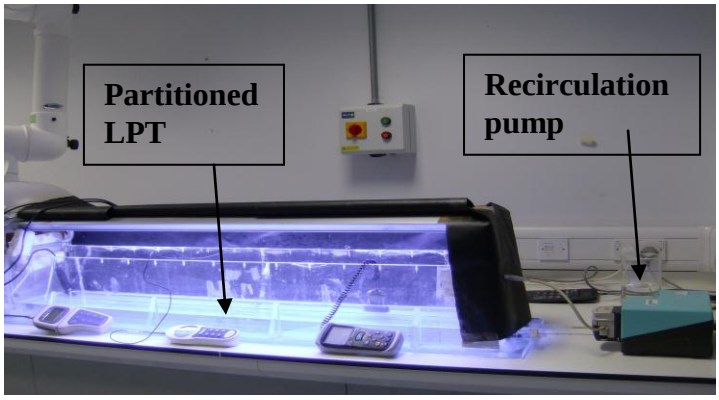


Figure III-2: Camera photo of lagoon photo tank.

The design characteristics of LPT are mentioned in Table III-2. The data in the Table shows that LPT has short light path of 25 cm along with s/v ratio of 89 m^{-1}, which suggests that for 0.013 m^3 or 13 l water in the tank, 1.15 m^2 of surface area is available for the culture growth and corresponding bioreactions to occur in LPT.

Table III-2: Design characteristics of lagoon photo tank.

Parameter Description	Definition
Construction material	Perspex
Body thickness, cm	1.1
Length, cm	120
Height, cm	21.8
Width, cm	22
Partition height, cm	7
Compartment width, cm	10
Light path to the bottom, cm	25
Working volume, L	13
Surface to volume ratio, m^{-1}	89

For mixing of the tank contents including uniform distribution of organic matter or COD, nutrients and algae and bacterial cells leading to their homogeneity in the medium, the tank contents were recirculated via peristaltic pump, which consisted of a display panel for visual confirmation of controlled recirculation flow rate

(Miyake *et al*., 1999 and Balcioglu *et al*., 2007). Recirculation of LPT contents was necessary in the sense that algae matting or biodegradation process leading to biomass generation could stop the wave action in the medium and block photosynthesis. To offset this condition, continuous circulation or recirculation of culture contents would have ensured culture dilution and reduced the surface encrustation, thus facilitating oxygen transfer as well. The pump was connected with polypropylene tube of 7 mm diameter for transfer of the tank contents. The outlet opening of 5 mm was located at the bottom of the back section of LPT to eject water from the tank for recirculation via the inlet opening situated at the top of the front section to allow induction of waves and current in the water, which might also have caused breaking of the surface and gas bubbles allowing mass transfer to occur.

All the experiments were carried out in LPT in batch mode so as to investigate the impact of input materials with respect to their original concentrations. The lagooning time during the experiments in LPT was a variable factor depending on the nature of cell cultivation. For analyses of the samples collected from LPT, diel cycle was taken into account implying that sampling for analyses was done at about the same time after every 24 hr during each run (Liu *et al*., 2000).

III.2.2 Simulated sugar factory wastewater

The simulated sugar factory wastewater (SSFW) is a characteristic replica of organic wastewater discharged by beet sugar factories. SSFW was selected as a case study because sugar factories produce more waste including solid (mud and ash) and liquid (wastewater) than the main product (sugar), by-products (pulp or

bagasse and molasses) combined (Asadi, 2007). Hence, the effects of sugar factory wastes particularly the disposal and handling of wastewater can be significant (Rehman *et al*., 2006). The reason to synthesise SSFW had to do with the factory operation, which being a seasonal plant runs the campaign for only 3 to 4 months every year (November to February), thus representative wastewater collection for continuous analysis during the off-season time of eight months would not be possible. Therefore, for consistency of the results, simulation of SFW was planned and for this, Newark beet sugar factory (NBSF) Nottingham, UK was visited for wastewater sampling to determine the *in-situ* wastewater composition. Based on the analysed contents of NBSF wastewater, SSFW was prepared accordingly. Fine size sugar crystals with mean aperture size of about 200-300 µm was used in the preparation of SSFW. Figure III-3 is the camera photograph taken during the wastewater collection from a sampling point inside NBSF Nottingham.

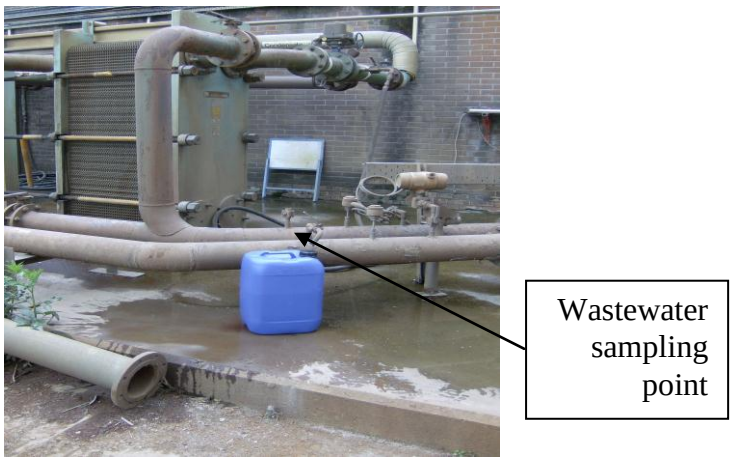


Figure III-3: Wastewater collection from Newark beet sugar factory, Nottingham, UK.

The components of SSFW with respect to the composition of NBSF wastewater are given in Table III-3.

Table III.3: Simulation of real sugar factory wastewater

Parameter in real sugar factory wastewater	**Surrogate component in simulated SFW**	**Chemical formula**
Chemical oxygen demand, as a bound form of carbon	White sugar crystals or Sucrose	$C_{12}H_{22}O_{11}$
Total nitrogen	Ammonium bicarbonate	NH_4HCO_3
Total phosphorous	Potassium dihydrogen phosphate	KH_2PO_4
Calcium	Calcium hydroxide	$Ca(OH)_2$

III.2.2.1 Sugar (Sucrose)

The word sugar comes from Indian sarkara, while its scientific name is Saccharides and the chemical name is sucrose. The ose suffix in sucrose or glucose identifies the sugars. All sugars belong to a largest group known as carbohydrates. Sucrose (sugar), glucose (dextrose) and fructose (levulose) are examples of sweet-tasting sugars caused by the presence of hydroxyl groups (OH) in molecules of sugars, which contribute to their sweetness. Sucrose is a non-polar, non-ionic or non-electrolyte substance and is soluble in polar solvents such as water due to the fact that it contains eight free OH groups, of which five can attach to other molecules depending on the molecule reactivity, as shown in Figure III-4. The sucrose molecule, which is a disaccharide made from glucose and fructose,

contains 51.5% oxygen, 42% carbon, and 6.5% hydrogen. Sugar, a basic ingredient of SFW, as a source of organic matter was used in the preparation of SSFW, since in most cases it is the best organic substrate (Mayo and Noike, 1995).

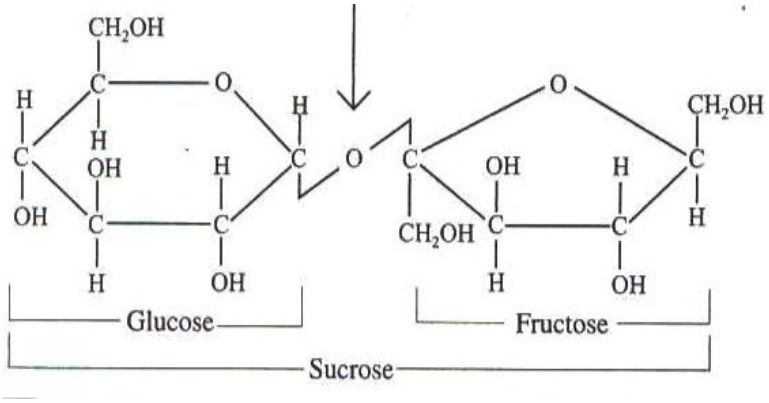


Figure III-4: Structural diagram of sucrose (Asadi, 2007).

III.2.3 Bacteria strain

For biodegradation of SSFW organics, *Pseudomonas Putida* (*P. Putida*) KT2440 (ATCC No. 47054) was selected based on its organic degradation ability as well as being a good biofilm former (Perni *et al*., 2006). *P. Putida* is a harmless NCIMB Group 1 aerobic bacterium (Walker and Weatherley, 1999). The average size of *P. Putida* ranges between 1.0 and 4.0 µm and they may be of straight, curved or rod shape. *P. Putida* is a member of *Pseudomonad* family, which consists of 178 or more different types of strains (Bakker *et al*., 2004). The main characteristics of *P. Putida* include being gram-negative, non-spore forming, typically motile with its polar flagella and non-pathogenic as well. *P. Putida* is non-pathogenic due to the absence of virulence factors in its system like certain exotoxin genes and type III secretion systems, which are found in other members of *Pseudomonad* family such

as pathogenic bacteria *P. Aeruginosa* containing these genes (Nelson *et al*., 2002). *P. Putida* strain is most popular and versatile in aerobic metabolism, which is able to grow on a wide variety of organic substrates and has been found as a free-living saprophytic or capable of feeding on decaying organic matter (Horan, 1991). Aerobic *P. Putida* use low-molecular-weight organic compounds that are always motile and metabolize glucose via the Entner-Doudoroff pathway and produce no toxic acids or gases (Collins *et al*., 2004 and Madigan *et al*., 1997). *P. Putida* strain was purchased from LGC standards Middlesex, UK, which was received as a lyophilized cell line in a tube of around 5 ml as shown in Figure III-5.

Figure III-5: *Pseudomonas Putida* cell line vial

P. Putida lyophilized cell-line after its delivery was immediately put in a freezer for cryogenic preservation at around -80^0C until its use later. The culture vial was taken out of the freezer after few months and 24 hr prior to its culturing was put into refrigerator at around 4^0C for overnight in order to start the revitalization process. Before the strain was transferred into culturing bottle, the refrigerated cell line was thawed at 30^0C for 4 hr using hotplate (ATCC, 2008).

Luria Bertani (LB) broth medium, which was composed of Tryptone (10 g l^{-1}), Sodium chloride (5 g l^{-1}) and Yeast Extract (5 g l^{-1}) was used for *P. Putida* culturing. The LB medium was prepared in the following way:

- 20 g of LB powder was measured and dissolved in a sterilized Duran glass bottle containing 1L distilled water (DW).
- The culturing bottle containing LB solution was covered with aluminium foil for maintaining purity of the medium and was kept in oven for 2 hr at 160^0C for sterilization.
- After 2 hr the sterilized LB solution was taken out of the oven and cooled down to room temperature before use.

III.2.3.1 Culturing *P. Putida*

P. Putida culturing was carried out in bacteria culturing unit (BCU) set up in the laboratory. The first phase of *P. Putida* culturing was carried in the following way: Three sterile tubes of 20 ml size were taken, two of them labelled as *P. Putida* broth 1 and 2 each containing 2 ml of the initial *P. Putida* culture along with 5 ml of prepared LB medium transferred via sterilized pipettes. The third one was retained as a control tube, which contained only LB medium but was not inoculated with *P. Putida* initial culture. A medium-sized beaker containing little water was put into a larger-size beaker also containing some water and was kept on a hot plate, the temperature of which was fixed at 37^0C for the incubation of the culture tubes. All the labelled tubes were placed in an inclined position in the medium size beaker to avoid the cell encrustation inside the beaker. The water in the beaker served the purpose of transferring the heat contents from beaker to the tubes. The temperature of both the surface of the hotplate and the water in large

size beaker was under constant check with the help of thermometer. The BCU was fully covered all around including its top, which was enveloped by an extractor to squeeze out the aerosol particles and other aerial fuming substances. Figure III-6 is a camera photograph of BCU from inside after the start of *P. Putida* culturing.

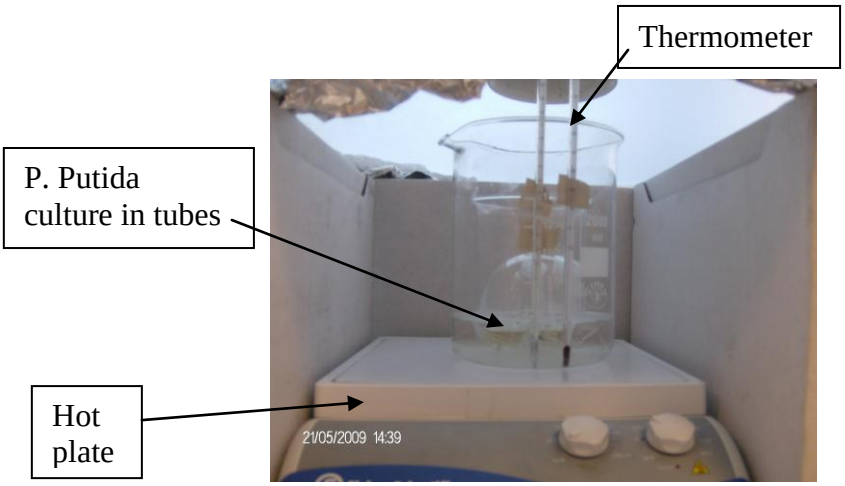


Figure III-6: *P. Putida* pre-culturing using hotplate

III.2.4 Algae

Both blue green and green algae strains were purchased and used for pre-culturing. The basic difference between the two is that green algae are grouped as eukaryotic because they have a well-defined nucleus in their chloroplasts as against blue green algae, which do not possess nucleus in their cell structures and hence they are grouped as prokaryotic, but both are termed as algae for their photosynthetic characteristics. Blue-green algae strain namely *Leptolyngbya sp.* ATCC No. 27894, which is in the family of *Cyanobacteria* was purchased from LGC Standards Teddington Middlesex, UK. The freeze-dried cell-line of *Leptolyngbya* was taken out of the freezer and kept in the fridge at 4^0C for overnight and then the

next morning was thawed using the hot plate at 25^0C for 2 hr to remove the coolness of the culture. The blue green culture vial of *Leptolyngbya* is shown in Figure III-7.

Figure III-7: Lyophilized *Leptolyngbya sp*. vial being thawed on a hot plate

Green algae *C. Vulgaris* cells were also used for culturing, based on their recognised rapid growth pattern, mixotrophic characteristics and biodegradation capability in organic rich conditions (Ono and Cuello, 2006). *C. Vulgaris* is one of the dominant green algae species, which are also used in oxidation pond systems (Sahinkaya and Dilek, 2009; Oilgae, 2009). Two *C. Vulgaris* strains CCAP No. 211/79 were purchased from Culture Collection for Algae and Protozoa (CCAP), Scotland, UK and received the same in 10 ml liquid solution of the medium carrying 2-3 ml of *C. Vulgaris* cells in each tube, as shown in Figure III-8. Upon delivery from the supplier, the vials were preserved and revitalized the same way as stated earlier (III.2.3).

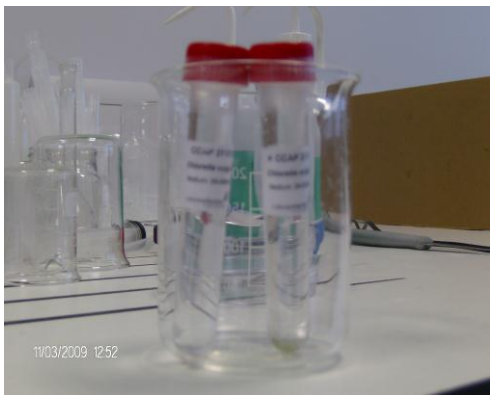


Figure III-8: Cell-line tubes for *C. Vulgaris*

III.2.4.1 Algae culturing unit

To setup the rig for algae culturing unit (ACU), two 30-W cool white fluorescent tubes, purchased from BLT Direct, UK, with wavelength of 400-700 nm, were placed in horizontal position slightly above the lab bench-top. The culturing bottles were evenly placed in front of the lights in culturing cabin, which was hooded from the top by the contiguous extractor to emit out the possible generation of micron size aerosol particles to keep them from being airborne (Kumar *et al*., 2008). The sides of the cabin were wrapped with aluminium foil to enclose the surroundings to retain the temperature. The fluorescent lighting was also covered from both sides with cardboard material for safety reasons leaving only the central part as exposed for the culturing bottles. The distance between the lights and the bottles was dependent on the amount of heat being transferred to the bottles by the light source as well as the light flux in Lux being irradiated towards the bottles. The distance between the bottles and the light source was maintained at 11" to satisfy the required culturing conditions of temperature (28^0C) and incident light (2800 Lux).

For mixing and as a CO_2 feed the culture contents were supplied with air via Algarde 1000 Aquarium air pump, filtered with Pall Acro 0.2 µm in-line air filter, through peristaltic santoprene tubing. The air supply was regulated by the attached stopcock valve to supply the air at low to moderate flow rates in the range of 0.5 to 1 l min^{-1} to avoid the buildup of shear force causing damage to algae cells. The camera image of ACU is shown in Figure III-9.

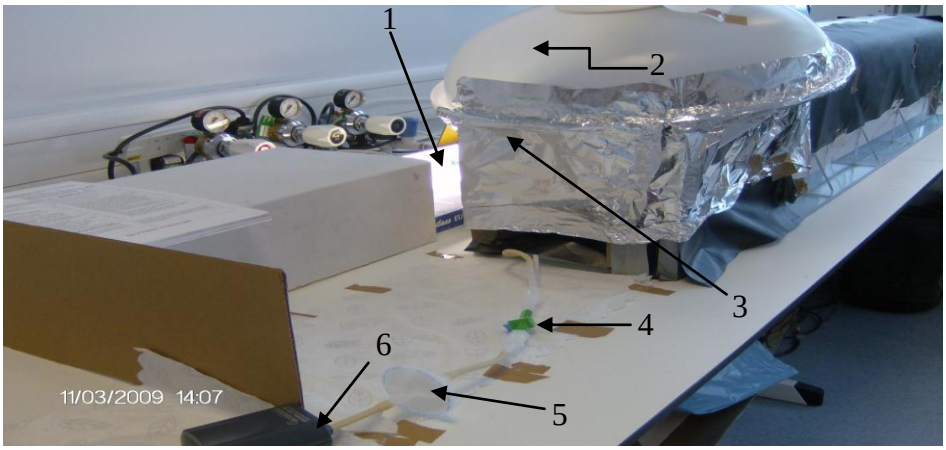


Figure III-9: Algae culturing setup

1- Two 30-W fluorescent tubes, **2-** Extractor, **3-** Hooded culturing cabin,

4- Stopcock, **5-** 0.2 µm in-line air filter, **6-** Air pump.

III.2.4.2 Preparation of BG-11 medium

BG (blue green)-11 was used as the standard broth medium for culturing green or blue green algae (Chinnasamy *et al*., 2009). To prepare one litre solution of BG-11 medium, 1.64 g of BG-11 powder were measured using micro range electronic balance and dissolved in one litre DW in a sterilized glass bottle. 1 mL of separately prepared solution of Trace metal mix was then added in the prepared BG-11 solution, which caused drop in its pH up to 3.8, which was adjusted to 7.1

as per the culturing procedure (ATCC, 2008). To raise the pH, a solution of 1M NaOH was prepared by dissolving 40 g of NaOH in 1L DW. The amount of 1M NaOH required to raise the pH up to 7.1 was determined by adding the prepared alkali solution in a progressive way (millilitre wise) and in all 1.6 ml of the prepared alkali were added to bring the pH of the medium solution to 7.1. The pH-adjusted BG-11 solution was then kept in oven for 2 hr at 160^0C for sterilization (Ball, 1997). Tables III-4 shows the composition of BG-11 medium, which was prepared according to the composition as given by Stanier *et al*. (1971).

Table III-4: Composition of BG-11 medium in 1l DW.

Ingredient	**Chemical name**	**Quantity (mg)**
$NaNO_3$	Sodium nitrate	1500
K_2HPO_4	Potassium biphosphate	40
$MgSO_4 .7H_2O$	Magnesium sulphate	75
$CaCl_2 .2H_2O$	Calcium chloride	36
$C_6H_8O_7$	Citric acid	6
$_6H_{5+4y}Fe_xN_yO_7$	Ferric ammonium citrate	6
$C_{10}H_{16}N_2O_8$	Ethylene diamine tetra acetic acid	1
Na_2CO_3	Sodium carbonate	20
Trace metal mix solution	Various chemicals	1 ml

Table III-5 shows the composition of trace metal mix solution. Only Boric acid (H_3BO_3) was required to be heated at 60^0C for 1 hr to dissolve it in the solution.

Table III-5: Composition of Trace metal mix in 1 l DW.

Ingredient	**Chemical name**	**Quantity (g)**
H_3BO_3	Boric acid	2.86
$MnCl_2$. $4H_2O$	Manganese chloride	1.81
$ZnSO_4$. $7H_2O$	Zinc sulphate	0.22
Na_2MoO_4. $2H_2O$	Sodium Molybdate oxide	0.4
$CuSO_4$. $5H_2O$	Copper sulphate	0.8
Co $(NO_3)_2$. $6H_2O$	Cobalt nitrate	0.05

III.2.5 Copolymer

When an inoculum of algae cells is introduced in a liquid volume in excess of 5 l, the cells normally take to the bottom and settle there until the start of log phase of their growth (Andresen, 2005). Since the volume of sample analyte used in the experiments was 13 l, therefore to aid in the suspension of algae cells at the surface of water in LPT, porous-insoluble as well as inexpensive (10 pence per 1 g) copolymer Polyacrylate polyalcohol (PP) in the form of small particles of transparent white colour with a size range of 100–850 µm, was used for algae immobilization. Polymers are macromolecules which are composed of thousands

of similar or repeating units or building blocks namely monomers and have a high molecular mass in millions (Chertin *et al*., 2010; Asadi, 2007 and Madigan *et al*., 1997). PP with product number P7588, also known as Dialyzorb, was purchased from Sigma Aldrich. The copolymer was also found as biocompatible, meaning that it can be used in contact with the organisms without negative effects.

III.3 EQUIPMENT AND ANALYTICAL PROCEDURES

The equipment utilized to carry out monitoring and analyses during the experimental work are mentioned as under. These are divided into inline (first three), which are portable and use no external reagents in their analyses and offline (last six), for which the samples were collected from the tank before their analyses using the concerned equipment. These instruments are described separately along with their significance, parameter theory followed by relevant hands-on procedures.

i. pH meter
ii. DO meter
iii. Light meter
iv. Hach DR-2800 Spectrophotometer
v. UV-VIS Spectrophotometer
vi. Viscometer
vii. Scanning electron microscope
viii. Bomb Calorimeter

III.3.1 pH meter

pH stands for power of Hydrogen and in chemical terms pH is the negative logarithm of the hydrogen-ion concentration, which can be written as,

$$pH = -\log_{10} [H^+] \qquad (3.1)$$

where negative sign my indicate towards the exponential relationship of the solution and the decreasing trend of the value or the acidic profile of the solution.

pH is important in biological wastewater treatment because most microorganisms grow best at pH values around 6 (Tchobanoglous *et al.*, 2003). This means any continuous variation in pH value may affect the microbial growth in terms of its cell structure and function.

Photo-description of pH meter

The hand held Jenway 370 pH meter, as shown in Figure III-10, was purchased from Fisher Scientific UK and has a measurement range between -2 to 16.00 pH. The meter also contains a probe connected to it from the top.

Figure III-10: Camera image of Jenway 370 pH meter with the attached probe.

pH measurement

The pH measurement is based on the technique known as potentiometry which is a temperature-related hydrolytic process that speeds up at higher temperatures, causing the pH to decrease. The technique is based on the potential difference between a pair of electrodes i.e. responsive and reference, which are located in a solution inside the probe. The responsive electrode measures the concentration of hydrogen ions (H^+) and the other electrode functions as a reference or standard electrode. The pH probe also consists of a membrane, which is permeable to H^+. Between the electrodes a current passes that depends on the concentration of H^+ resulting in the transmission of data on the display of the meter. A unit difference in the pH value represents a 10-fold increase in H^+ if pH value gets smaller by that margin and vice versa.

The concentration of H^+ as expressed by the measurement of pH also suggests the corresponding decrease in the concentration of hydroxide ions OH^- existing in the same sample analyzed for pH. $[H^+]$ and $[OH^-]$ of a solution can never be reduced to zero, no matter how acidic or basic the solution is. However, pH itself can reach zero or even less in highly acidic solutions (Sawyer *et al*., 2003).

The pH meter data is usually expressed in between 0 (most acidic) and 14 (most alkaline), with pH 7 at 25^0C representing absolute neutrality.

Calibration of pH meter

Prior to using the instrument for the first time the meter was calibrated or standardised. In case of continuous use of the meter, periodic calibration regime of the meter was observed. Asadi (2007) also stressed the point of pH meter calibration when he referred to its importance in terms of variation occurring in the

electrical potential of electrodes over time resulting in below par values. The variation in the performance of pH probe after its continuous use for certain time may be caused by the encrustation or layering of the body of pH probe as well as the membrane with the impurities in the samples, fine particles in the ambient surroundings or the dissolved solids in water. In this experimental work, a time period of around one month after its continuous use was found to be reasonable to calibrate the pH meter, after checking its sample readability when compared with pH of normal tap water and distilled water to ensure the results were within the standard limits. For calibration, three capsules of pH 4.0, 7.0 and 10.0 were provided by the supplier. Mostly, pH buffer solution prepared with capsule of pH 4.0 was used for calibration of the meter. Between all the measurements pH probe was rinsed in DW to avoid filming of the bulb and the body of the probe with the sample impurities. The steps involved in pH calibration were as under:

- The pH meter was switched on by pressing down the I/O key for 1-2 s.
- The tip of the probe was immersed into the prepared buffer solution to ensure that the bulb and reference junction of the electrode were inside the solution.
- CAL key was pressed to start the calibration; a reading was displayed close to the pH value of buffer solution in about 5 min.
- CAL key was pressed again to confirm the buffer value followed by the third press of CAL key to complete the calibration and return to measurement mode.

Operational procedure of pH meter

1. The pH meter was turned on by holding down the I/O key for 1-2 s to ensure the meter was in normal operating mode.
2. The pH probe was immersed in the sample water to ensure the bulb of the probe was at least 1 cm under the water.
3. pH value was shown on the display of the meter, which was registered after one minute of its stabilization.

III.3.2 Dissolved oxygen meter

Oxygen that is in a dissolved state in water functions as a terminal electron acceptor in aerobic wastewater biological remediation (Hu *et al*., 2005). Adequate levels of dissolved oxygen (DO) are essential for maintaining optimal biological activity (Chang and Ouyang, 1988). Regular monitoring of DO during the process, thus, is an important requirement to check the progress of the process.

Photo-description of the meter

The portable waterproof Accumet AP74 DO meter, as shown in Figure III-11, was purchased from Fisher Scientific UK. The meter also measures the temperature and the measurement ranges for both DO and temperature are specified as 0–19 mg l^{-1} and 0–50^0C respectively. The meter is connected from the top with a galvanic probe for both DO and temperature analyses, the bottom end of which is covered by a probe guard to protect the membrane. The probe guard is fitted with holes to allow sample water to pass through the holes and make contact with the

probe sensor. The membrane is practically impermeable to water and ionic dissolved matter, but is permeable to oxygen dissolved in water.

Figure III-11: Camera shot of Accumet waterproof AP74 DO meter along with the galvanic probe.

DO Measurement

The cathode in the probe consumes oxygen that is dissolved in the water when the sample water flows past the sensor, and produces an electric current in the probe, which is proportional to the partial pressure of oxygen in the sample, transferring signal which is indicated in the form of DO value in mg/L on display of the meter. Simultaneously, the DO meter also reads the temperature of the sample, which is also highlighted in the bottom field of the display.

Calibration of DO meter

The DO meter can be calibrated either in percent saturation mode or in mg l^{-1} mode depending on the barometric pressure value in air, which in normal circumstances is taken as 760 mm Hg, resulting in a calibration value of 100% saturation in air. Deviation in DO measurement values was observed to occur after approximately every three months, when the probe was checked through the

analysis of tap water or DW, necessitating its calibration. The steps involved in the calibration of DO meter were as under:

- The DO meter was turned on by pressing the on/off key.
- The probe was rinsed with DW, then its bottom end or its probe guard was covered in full with a damp piece of clean cloth to ward off any air contamination.
- The mode key was pressed to select the % saturation mode
- The CAL key was pressed, which made the CAL indicator appear on the display. The current value of measurement as a percent saturation in air was shown on the display, mostly above 100%.
- The probe was held in air until the reading was stabilized taking 20-30 min. The appearance of Ready indicator on the display meant the value was stabilized.
- The Enter key was pressed, the meter automatically calibrated to 100% air saturation and then returned to measurement mode.

Temperature calibration

Temperature values were measured by built-in temperature sensor included in the DO meter probe. The steps involved in the temperature calibration were as under:

- The meter was turned on. The mode key was pressed to select mg/L measurement mode.
- The CAL key was pressed to enter mg/L calibration mode and to make the CAL indicator appear on the display.

- While in mg/L calibration mode, the MODE key was pressed to enter temperature calibration mode. The display showed the last set temperature reading or the value that was recorded in the last measurement.
- The room temperature reading was taken by using a working thermometer and compared with the one on the display of the meter.
- **▲or ▼** keys were used to adjust the reading on the display to agree with the value obtained by the thermometer.
- ENTER key was pressed to confirm temperature calibration and return to measurement mode.

Operational procedure

The operation of DO meter was carried out in the following way:

- DO meter was turned on and rinsed well with DW before taking sample measurement
- Measurement mode was selected in mg/L by pressing the MODE key
- The DO probe was dipped into the sample so as to maintain its vertical position in the water (Kilani and Lebeault, 2007). The probe was gently stirred as well to homogenize the sample and it was ensured that the sample was continuously flowing past the membrane sensor via holes in the probe guard. The corresponding values for both DO and temperature appeared on the display.
- To obtain the stabilized DO values, at least 2 min were required for the READY indicator to stop flashing on the display indicating the value was stabilized to be registered.

III.3.3 Light meter

The Light meter (LX-319), as shown in Figure III-12, was purchased from Farnell, UK. The meter was used for taking the incident light (IL) values irradiated by the lighting field. Light readings were taken by using the water proof probe attached with the meter from the top. The light readings could be obtained in units of either foot candle or lux (lumen m^{-2}) with maximum values of 40,000 and 400,000 respectively, or 3053.4 µmole photon $m^{-2}s^{-1}$ or $\mu Em^{-2}s^{-1}$ (since 1µmole photon $m^{-2}s^{-1}$ is equal to 131 lux). No calibration of the meter was required, as only 9V battery was needed to be changed after being exhausted by four months of its continuous use.

Figure III-12: Camera photo showing LX-319 Light meter and the connected light probe.

The simple steps involved in the light meter operation were as under:

- The meter was turned on by pressing the green power key
- The probe was held in horizontal way so that the white sensor area of the probe was not touched and was exposed to light in full. The variation in the

IL values displayed would occur if the probe was not held at the same spot and in the same position. The horizontal position of the probe was maintained with a flat wooden piece of around 5 mm thickness of the same length as the probe and attached below to it. The difference in the values with and without the attached object to the probe was recorded in advance for adjustment in the sample results.

- The key for desired unit of measurement i.e. lux was pressed
- For stabilization of the value, MAX key was pressed
- The stabilized value was noted and MAX key was pressed again to enable the next measurement.

III.3.4 Hach DR-2800 Spectrophotometer

Hach DR-2800 is a visible spectrum spectrophotometer using Tungsten lamp with a wavelength range of 340 to 900 nm that can be used for the determination of more than 240 analytical methods involved in water and wastewater analyses. The instrument was purchased from Hach Lange, UK. The mode of readout by the instrument is in direct concentration reading of the samples or in absorbance, transmittance percentages.

The DR 2800 spectrophotometer automatically reads the bar codes on the sample cuvettes to detect the appropriate test procedure via laser identification. As the cuvette rotates, it is measured 10 times within 5 seconds for an average value with any readings that are distorted by soiling on the cuvette are automatically eliminated. Figure III-13 shows the camera image of DR 2800 Spectrophotometer.

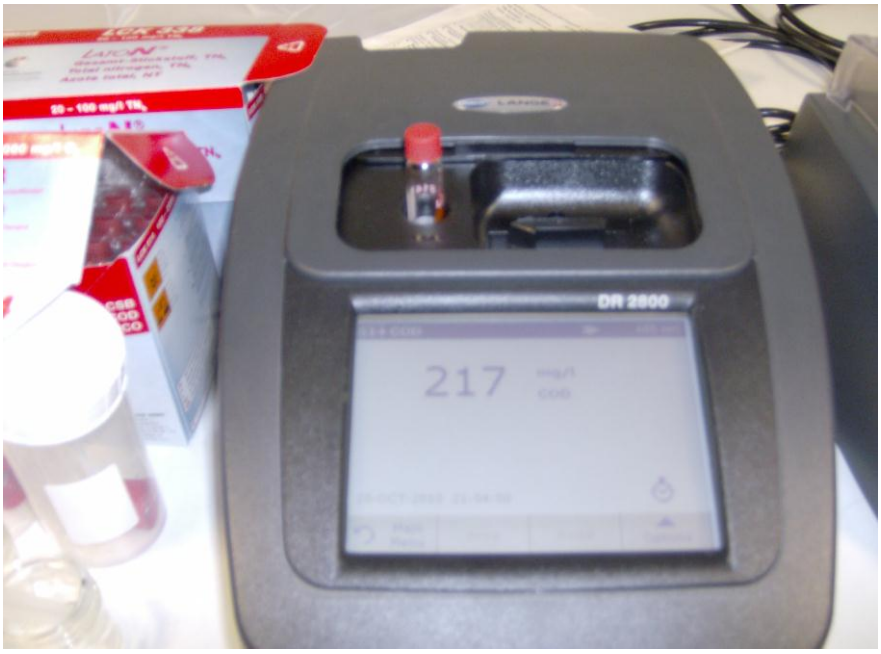


Figure III-13: Camera grab of Hach Lange DR 2800 Spectrophotometer reading the sample cuvette by automatic bar code identification.

The parameters covered in this work using this instrument included chemical oxygen demand (COD) and total nitrogen.

III.3.4.1 COD

Importance of COD test

The biodegradation of organic matter was monitored in terms of COD test considering that this test reflects the remaining concentration of the organic matter which could lead to the determination of mineralization rate of the pollutants as a result of bacterial activity (Kalyani *et al*., 2009 and de-Souza *et al*., 2006). The test is also widely used in the operation of wastewater treatment facilities because of the speed with which results can be obtained in only 2.5 hr (Sawyer, 2003).

Principle and measurement of COD test

COD test measures the oxygen equivalent of the amount of organic matter oxidizable by potassium dichromate in a 50% sulphuric acid solution in the presence of silver sulphate as catalyst, which accelerates the oxidation process, which are all present in the sample cuvettes used for COD measurement. End products are carbon dioxide, water, and various states of the chromium ion. For digestion of the sample in COD cuvettes, an accessory unit of spectrophotometer, LT 200 COD reactor was used, as shown in Figure III-14.

Figure III-14: Photograph showing COD reactor and COD cuvettes being kept for digestion.

Procedure of COD analysis

COD test was carried out by first digesting the prepared sample in COD cuvette in the COD reactor followed by the colorimetric analysis of the digested sample in DR-2800 spectrophotometer using Method No. LCK 514. The COD cuvettes used have a determination range of 100–2000 mg l^{-1}. Considering that the prepared

SSFW COD ranges over 6000 mg l^{-1}, which is above the COD determination range contained by the cuvettes, the samples for COD analysis were diluted with DW with a ratio of 1:100 and the prepared diluted samples were used for COD analysis. The COD value was calculated after adjustment with the dilution factor using the following formula:

$$\text{COD of diluted sample (DS) (mg } l^{-1}\text{)} = \frac{\text{DS test result x total volume of prepared sample}}{\text{Volume of original sample added}}$$

The following procedure was used for COD determination.

- COD cuvette containing 1 ml solution of standard reagents for COD test, was taken and the sediments present in it were brought into suspension form for homogeneity by inverting it few times.
- 1 ml of the sample was collected from the LPT via a digital pipette, which was diluted up to 100 ml with DW in a round flask. 2 ml of the diluted sample were then pipetted into the COD cuvette, which already contained 1 ml of the COD reagent solution.
- The cuvette was closed, thoroughly cleaned from outside and inverted.
- The cuvette was placed in the thermostat of COD reactor for 2 h at 148^0C for digestion of the prepared sample.
- After the digestion, the hot cuvette was removed from the reactor and inverted few times.
- The cuvette was allowed to cool to room temperature in a cooling rack

- After 20 min of cooling, the outside of the cuvette was cleaned before placing it in the cuvette holder of DR-2800 Spectrophotometer for COD analysis.
- After the automatic detection of the cuvette contents by the equipment, the COD value was displayed on the screen of the spectrophotometer.

III.3.4.2 Total nitrogen

Total nitrogen (TN) is defined as the sum of total kjeldahl nitrogen, ammonia, and nitrate-nitrite nitrogen. Wastewater Nitrogen is removed by various means including biological uptake by algae and bacteria. The USA Environmental protection agency classifies an effluent or treated wastewater as low strength having a TN concentration of less than 50 mg l^{-1} (Patterson, 2003).

Principle of TN test

The organic and inorganic bonded nitrogen content present in the prepared sample in the cuvette was oxidized to nitrate by digestion in the reactor with peroxodisulphate. The nitrate ions react with 2,6–dimethylphenol in a solution of sulphuric and phosphoric acid to form nitrophenol (Hach, 2008).

Procedure of TN test

Like COD determination, the digestion of TN cuvette was also carried out in COD reactor while post digestion analysis was done in DR-2800 spectrophotometer. The range of the cuvette for TN is 20–100 mg l^{-1}, while the method of the test is LCK 338. The following steps were involved in the procedure of TN test (Hach, 2008).

- One pre-cleaned and dried reaction tube was taken for TN analysis; 0.2 ml of the sample, 2.3 ml of solution coded as A, and one tablet coded as B

were added in quick succession to the tube. The reaction tube was immediately closed but not inverted.

- The tube was immediately placed in the reactor for heating at 100^0C for 1 hr.
- After digestion, the tube was cooled down to room temperature
- One MicroCap reagent coded as C was added in the tube, then the tube was closed and inverted for a few times until the contents were without any sediments and that all streaks were vanished.
- 0.5 ml of digested sample was slowly pipetted into the cuvette for TN analysis along with 0.2 ml solution coded as D. The cuvette was immediately closed and inverted a few times until no streaks could be seen.
- The outside of the cuvette was thoroughly cleaned and after 15 min the cuvette was placed in the cuvette holder in DR-2800 Spectrophotometer for TN analysis. After rotation of the cuvette, the TN value appeared on the display.

III.3.5 Ultraviolet Visible Spectrophotometer

1240 mini Ultraviolet Visible (UV Vis) Spectrophotometer, Shimadzu, as shown in Figure III-16, was used for the determination of optical density (OD) test to express the concentration of grown cells.

Figure III-15: Photograph showing 1240 mini UV Vis Spectrophotometer.

Principle of operation

UV-Vis absorption spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample with partial absorptions in the ultraviolet and visible light range wavelengths. When an ultraviolet (200 to 320 nm) or visible (400 to 750 nm) light beam hits the sample atoms or molecules, they can either be scattered or absorbed. The absorption of light radiation results in the excitation of electrons to higher energy levels, leading to spectroscopic signal associated with the electron energy levels of the specimen. The technique is routinely carried out to determine the concentration of certain compounds in the solution by a single beam of light based on the concept of Beer's law, which states that absorbance of light (A) is directly proportional to light path length (b) and concentration of the absorbing species (c); or A = ebc, where e is proportionality constant or absorptivity. In the OD test, the sample is subjected to photometric determination of the turbidity level in the sample caused by the concentration of bio cells present in it at a wavelength of 660 nm.

Procedure of OD test

The OD test by UV-Vis spectrophotometer was carried out as under:

- The instrument was switched on along with the connected PC and allowed to complete initialization process.
- Before any measurement, AUTO ZERO key was pressed to zero the machine followed by the selection of SPECTRUM mode.
- 1 ml DW was put in the plastic cuvette as a blank for baseline correction and the wavelength was set at 660 nm
- 1 ml of sample solution was then injected into the cuvette, which was placed into the machine for scanning and the result was noted.

III.3.6 Brookfield Viscometer

Viscosity of culture media

Viscosity is a measure of stickiness or adhesive property of a fluid. It is the tendency of water to resist flow and impose drag on organisms moving through it. If water was significantly less viscous than it is, algae cells would have difficulty remaining in suspension (Graham *et al*., 2009), indicating that certain amount of viscosity may be necessary for the organisms to suspend in the medium. The longer the residence time of bubbles in water, the greater the DO concentration will be in the culture medium and this residence time is increased with increased viscosity, indicating the existence of certain drag force in water necessary for oxygen saturation in the medium (Andresen, 2005).

Viscosity was measured using viscometer as shown in Figure III-17.

Figure III-16: Picture showing Brookfield Viscometer for viscosity measurement.

Procedure for viscosity measurement

Viscosity measurement using Brookfield viscometer entailed the following procedure:

- Before the start of the test, routine checking was done as regards ensuring that the viscometer laid flat and not inclined on the bench top. Then the desired speed, which was 30 (with factor 2) in this case, was selected from the spindle chart for spindle functioning.
- The 600 ml sample was collected in 1 l beaker from LPT for viscosity analysis.
- The spindle was lowered carefully at the centre in the beaker containing 600 ml of the sample until the meniscus of the fluid was at the centre of the immersion groove on the spindle's shaft.
- The instrument was turned on to start the measurement. For better performance, the viscometer was run with the speed starting from the

lowest before increasing the speed up to level 30. The reading at the end was allowed to stabilize.

- The dial reading was noted and viscosity was calculated by multiplying the dial reading with the factor corresponding to the speed of spindle.

III.3.7 Scanning Electron Microscope

For a micro-range view of microbial culture cell growth, certain samples were investigated using scanning electron microscope (SEM). The SEM uses electrons instead of light to form an image of the sample. The combination of higher image magnification coupled with greater resolution, larger depth of focus on the sample areas and ease of observation makes SEM one of the essential tools in the relevant research today. The camera image of Philips XL30 SEM, which was used for the required images is shown in Figure III-18.

Figure III-17: Photograph of Philips XL30 Scanning Electron Microscope.

Principle of SEM operation

In SEM operation, when a sample is introduced into the instrument, a beam of electrons is deflected in the electron optical column and scans over a very fine focal spot progressively in a rectangular area of given sample, producing detailed surface topography of the sample region. When the electron beam finally reaches the sample surface, the interactions between the electrons and sample, such as scattering and absorption, lead to energy transference from electrons to the sample. The energy exchange produces different signals, which result in the formation of corresponding image.

Sample preparation for analysis in SEM

For imaging via SEM, the samples were required to be retained on the surface of the stubs of the size of around 5 mm. An aliquot of 10 ml of representative sample from the tank was collected during the experiment. Then the collected sample was gently mixed for its homogeneity and two drops were taken via a dropper to transfer them onto the stubs. Three stubs were used for each analysis. Then the stubs containing the sample were kept in the covered holder to protect from air contamination and left at room temperature for drying for 24 hr. The dried stubs were then used for SEM analysis.

III.3.8 Bomb Calorimeter

The calorific value (CV) of the cultured cells of grown algae was determined by using C5000 IKA Bomb Calorimeter. CV is the heating value of a substance when its known amount is completely burned and the amount of heat released during the combustion is taken as the CV of the substance. The results for CV are expressed in cal g^{-1} or J g^{-1}. The instrument photograph is shown in Figure III-19.

Figure III-18: Camera image of C5000 IKA Bomb Calorimeter

Procedure for CV test

- O_2 cylinder was turned on for its supply to the equipment and set at 30 bar
- The machine was switched on and it was checked whether the bomb was clean and dry before use.
- The burning cup was filled with approximately 1 g of sample
- Ignition string was attached to bomb to aid in combustion
- 5 ml of tap water were put inside the bomb for cooling effect in the bomb
- The bomb was assembled carefully to ensure it was properly attached with the hook.
- The input data such as sample weight and bomb number was entered on the display screen of the instrument
- The bomb was inserted into stirrup holder
- The test was started by pressing the Start key and it took at least 15 min for combustion process to complete

- At the end of combustion the bomb came out itself and the cup was checked for any sample residue left in it.
- The instrument was switched off following the shut down instructions along the oxygen cylinder by completely closing main cylinder valve and opening regulator valve to relieve the pressure.

III.4 CLEANING AND SAFETY REGIME

At the end of each experiment, the experimental material including LPT, plastic tubing and glassware was washed and cleaned by first soaking these materials in the tank with 5 l of prepared solution of 10% industrial Haychlor for 24 hr. After 24 hr the Haychlor solution was discarded and all the materials were rinsed twice with DW before the materials were left for overnight soaking in the tank with 3 l of 1M HCl prepared solution. The next morning the HCl solution was removed from the tank and the materials were rinsed twice with DW before being used for the next run. This sterilization was carried out to ensure homogeneous conditions of growth in all sections of the tank and to prevent growth of contaminating organisms and light obstruction by unwanted growth all around the tank.

The bench top surface, where the experiments were carried out was swabbed with 70% industrial Methylated spirit before and after the use of the surface (Plymouth, 2007). For disposal of biological materials like discarded microbial solids and used gloves, the wastes were collected in biohazard bags before being disposed of outside properly. Notices for caution with respect to lighting and bacterial culturing were put inside and outside of the laboratory for outsiders.

III.5 REAGENT PREPARATION PROTOCOL

Analytical balance with a readability of 0.0001 g and a capacity up to 200 g was used for the measurement of chemicals. Most of the reagent solutions were prepared slightly over-strength by 5% of the original weight of the chemicals to be added with more DW for standardization at a later time. DW with a conductance of 1 μS cm^{-1}, which corresponds to about 0.5 ppm of dissolved solids, was used in the preparation of the reagents. Polyethylene plastic bottles were used for sampling from the industry as they are less likely to cause organic contamination.

The following protocol for preparation of the reagents was observed:

- Identification of chemicals for reagent preparation from literature
- Calculation of the quantity of chemicals required
- Measurement of the calculated amount of a chemical using the balance
- Dissolution of the chemicals in DW. The chemicals were added in DW only after one added chemical was completely dissolved in the solution and then the next chemical was added and so on.

Each prepared solution was labelled with solution name, solution number, date of preparation and user name for stocking (Connon, 2007).

III.6 QUALITY ASSURANCE AND DATA GENERATION

To ensure the maintenance of overall quality it was necessary to integrate a framework of measures to back up the quality of work at each stage of the process (Wise and Riddington, 2000). Effectiveness of the process was monitored

regularly via the measurement of parameters such as DO, pH, temperature, Incident light, COD and biomass growth, which allowed to assess the performance of the process and the health and efficiency of the input materials such as organisms, nutrients, substrate, copolymer dosing.

To provide the readers and critics, who might come across this research work in furtherance of the relevant research, with the assurance of the quality of this research work, it was imperative to carry out the laboratory research work in the recommended way in order to achieve that desired quality. To maintain the quality of the research work, certain factors needed to be taken care of in terms of their consistency to influence the reproducibility of the results along with their true reflection. For instance, maintaining representativeness of the samples for genuine data generation to afford its meaningful extrapolation and parallelism with relevant literature. This was essentially done by preparing the samples with the same composition to ensure the accuracy of the intended data. Similarly, working samples were collected from a specific point in the bioreactor at the same time for relative point comparison of the data. The input materials used during the conduct of experiments were of analytical grade and applied to the process at their respective standard operating conditions of pH and temperature. The protocol adopted for the preparation of chemicals and their blanks, involved in this work, is discussed just above in section III-5.

Process integrity was maintained by following the specific guidelines as regards standard operating procedure for carrying out the work in the laboratory, which included necessary preparation of process risk assessment before the conduct of the work. This included all the safety and hazard information related with the

chemicals used along with the procedures involved for the conduct of the work. The observance of cleaning and safety regime (section III-4) before and after every cultivation run was also a factor attached with the process integrity. Bio-materials and other process-related wastes in the form of by-products or finished products as well as expired materials were disposed of form the lab using bio-hazard plastic bags for their safe and pointed discharge outside.

The experiments were conducted under controlled conditions of temperature inside the lab to avoid fluctuation in process performance due to ambient conditions. For each case study as a part of experimental plan, control runs were held before the conduct of actual experiments for the sake of comparison between the results obtained from both type of experiments. To register the experimental data during the conduct of experiments specimen data procurement form was made, which was retrofitted subject to the nature of the cultivation run. The data obtained from the experiments was registered minimum of three times before taking out their mean value for the purpose of data presentation.

III.7 STUDY LIMITATIONS

This study was carried out not without experiencing limitations whether inherent or otherwise. To begin with, the concept of the study, though not new, was not developed at the time of start of this study in terms of best-fit criteria involving both qualitative and quantitative parts of the methodology. Hence, micro-level experiments were held to develop a priori baseline before the conduct of experiments with actual representative samples.

The input materials used in this study included those such as copolymer Polyacrylate polyalcohol, which was being used for the first time in this type of study, and for others such as sucrose solution and lagooning, little relevant information was available in the literature for comparison of the results in terms of their reproducibility and validity. Hence, optimization of the process was imperative to minimise the uncertainty level attached with these materials before their use for the actual experiments. Finally, the practical limitation of this study might involve the non-assay of real wastewater from sugar factory, which could not be used for valid reasons of its timely and frequent availability as well as for the sake of process optimization on samples of controlled nature such as its replica simulated sugar factory wastewater (SSFW). Hence, its surrogate version or SSFW was used for this study.

CHAPTER IV

BASELINE ASSESSMENT OF

LAGOON PHOTO TANK

CHAPTER IV

BASELINE ASSESSMENT OF LAGOON PHOTO TANK

IV.1 INTRODUCTION

Distilled water (DW) was used for its lagooning profile in lagoon photo tank (LPT) to establish baseline data without the use of any biomass so that the consumption factor leading to variation in the water properties such as pH and DO with regard to biological activity could be disregarded (Lopez *et al*., 2006). During these baseline experiments, the effect of independent variables such as incident light (IL) and fluid flow rate was studied with respect to dependent process variables like pH, temperature and dissolved oxygen (DO). This was necessary to know since a change in the concentration of these water properties would otherwise have a direct influence on global parameters such as COD and TOC. The working volume of water used for each experiment in LPT was 13 l, which creates a depth of 5 cm in the tank and is 2 cm below the partition cut-off height (7cm). The partition bar divides the tank into two compartments setting the flow path for water movement. The two sections in the tank are taken as front and back, with each section has three specified monitoring points set at right, middle and left spots in LPT. The schematic of the designated points is illustrated in Figure IV-1.

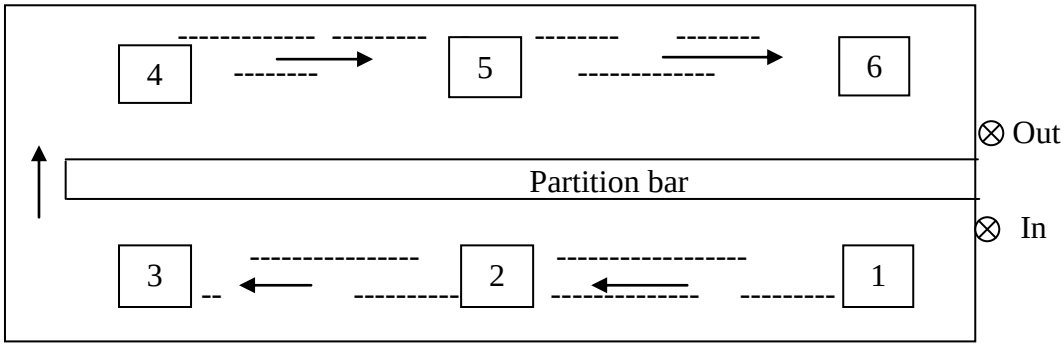


Figure IV-1: Illustration of designated points in LPT.

Legends: 1 = Front Right, 2 = Front Middle, 3 = Front Left, 4 = Back Left, 5= Back Middle, 6 = Back Right.

For comparison of the data, two representative sample points 2 and 5, which were comparatively high-intensity points, were selected as target points. The results were taken both at the bottom and surface layers of each point thrice before taking the mean value with corresponding standard deviation.

IV.2 RECIRCULATION FLOW RATE DETERMINATION IN LPT

For continuous circulation or recirculation of water, peristaltic pump was used, which could be operated with maximum flow rate capacity of 400 ml min^{-1}. To determine the minimum recirculation flow rate, the pump was used at different flow rates beginning from the initial level. The LPT was filled with DW up to 5 cm depth and the pump was turned on at 10 ml min^{-1}. However, not a single drop came out of the inlet tube even after at least 10 minutes. Similar was the case with flow rates from 15 to 25 ml min^{-1}; however, with 30 ml min^{-1} water recirculation in

the tank was possible at the rate of one drop after every four seconds. To decrease the time period from four seconds to the minimum, the flow rate was raised gradually from 30 to 40 ml min^{-1} (50 rpm), when the recirculation was possible at one drop per second, which was also observed to induce water current in the tank. The recirculation flow rate of gave a liquid velocity of 8 cm s^{-1} with Reynolds number of 2000.

EXPERIMENT NO. IV-1

IV.3 DO AND TEMPERATURE PROFILE IN LPT AT DIFFERENT DEPTHS

The LPT was filled with 13 l DW up to 5 cm depth. The temperature and DO readings were recorded at different depths from surface to bottom in LPT with and without water recirculation under continuous incident light. Figures IV-2 and IV-3 show the DO and temperature profiles obtained after DW lagooning in LPT for 24 hr without and with water recirculation at 40 ml min^{-1} respectively. In the Figures, surface level of water is at 5 cm from the bottom in LPT while the bottom layer is taken at the depth of 2 cm, since the bottom values could only be taken 2 cm above the bottom due to the structure of the probes used. The DO graph in Figure IV-2 suggests that with no circulation of water it decreased by 12% near the surface probably due to 3% increase in the temperature caused by the continuous light incidence for 24 hr.

This was in contrast to 5% reduction in DO observed near the bottom of LPT due to relatively lower temperature increase near the bottom of LPT.

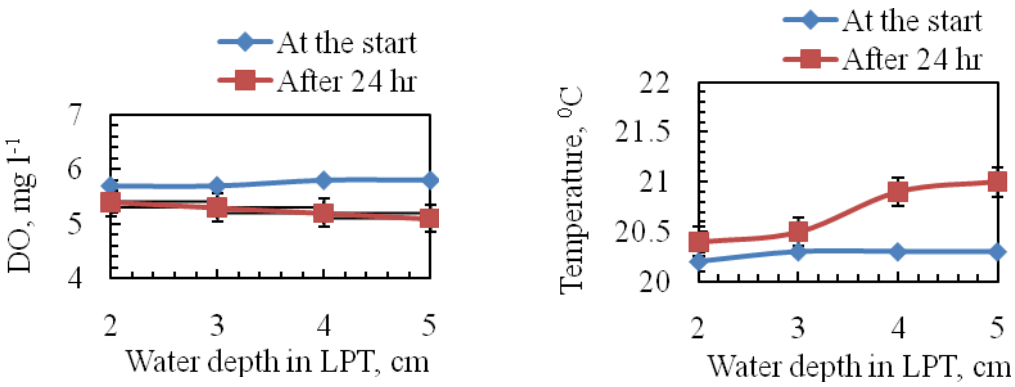


Figure IV-2: DO and Temperature profile at middle point 2 in LPT at different depths after 24 hr of DW lagooning without recirculation.

The results are presented as: mean ± SD; n=3.

Figure IV-3 shows DO and temperature results at different depths in LPT after DW lagooning for 24 hr with water recirculation at 40 ml min^{-1} or 50 rpm. The DO graph suggests that the value was increased by 10~% after 24 hr of DW lagooning with continuous water circulation at 40 ml min^{-1} as compared to the values obtained without water recirculation. Besides, with water recirculation on, visible water current or waves were observed up to the extreme left side of the tank, which could induce slight mixing in the tank as compared to stagnant water profile without recirculation. This was in agreement with the observation made by Phong (2008), who in his preliminary experiments found out that a velocity gradient of 21 rpm was the minimum shear needed to generate a wave motion and keep the solids in suspension in the medium.

The temperature data in LPT suggested that it was increased by around 4% after 24 hr of lagooning particularly at lower depths probably due to continuous lighting, which caused consistent heat buildup at the surface and parallel heat transfer to the bottom of LPT.

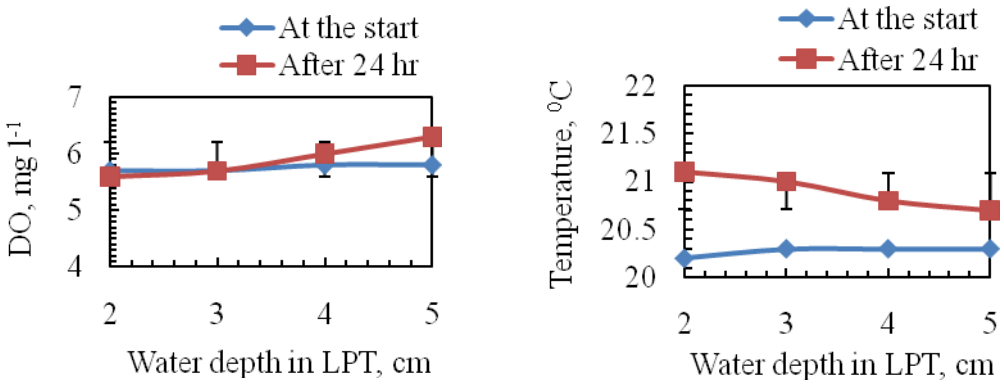


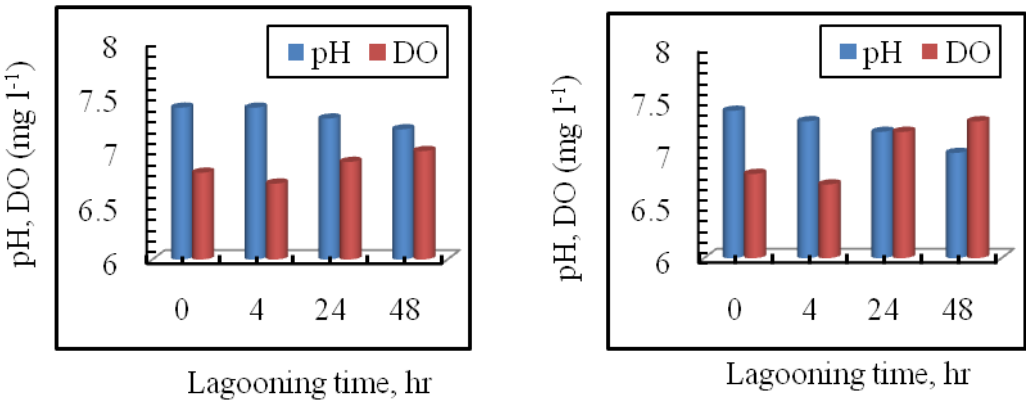
Figure IV-3: DO and Temperature profile at middle point 2 at different depths after 24 hr of DW lagooning in LPT with water recirculation at 40 ml min^{-1}. (Presentation of results: mean ± SD; n=3).

EXPERIMENT NO. IV-2

IV.4 48-hr LAGOONING OF DW IN LPT

In this experiment, DW lagooning for longer time i.e. 48 hr was carried out in LPT with water recirculation at 40 mL min^{-1} with continuous lighting to monitor the variation in parameters such as pH, DO, temperature and incident light. Figure IV-4 shows the results for pH and DO obtained during DW lagooning for 48 hr at the middle point 2 both at the bottom and surface.

The pH data in the graphs suggested that during 48 hr of lagooning it decreased by 3% and 5% at the bottom and surface respectively. The DO registered an increase by 7% at the surface, which can be associated with the water recirculation.



(a) **(b)**

Figure IV-4: pH and DO at the bottom (a) and surface (b) at the middle point 2 after 48-hr lagooning of DW in LPT with water recirculation at 40 ml min^{-1}.

Figure IV-5 presents the temperature and incident light (IL) profile during 48-hr lagooning of DW in LPT both at the bottom and surface. The overall increase of 11% in the temperature was recorded during 48 hr of DW lagooning, of which 3% was influenced by the continuous luminance in LPT. The IL profile suggested that on average around 4% increase in the incidence of light was caused by the light intensity due to constant luminance during 48 hr of DW lagooning. During the course of DW lagooning, 520 ml of water was lost per day likely due to the effect of evaporation under continuous insolation decreasing the depth of water in LPT by 0.2 cm per day.

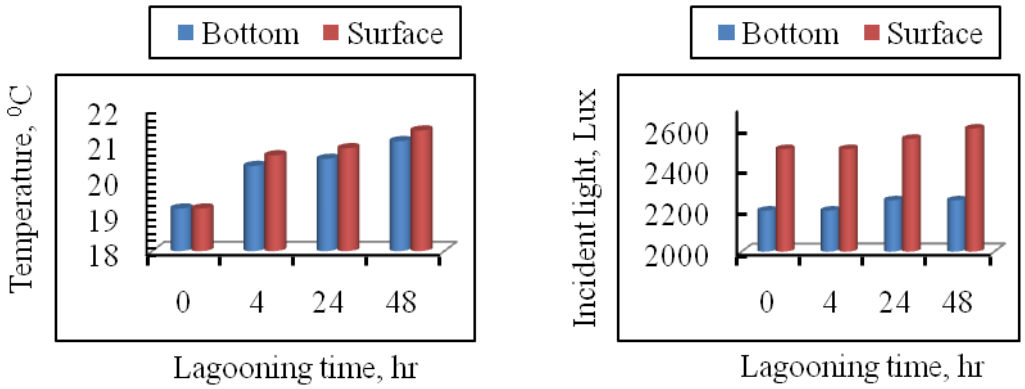


Figure IV-5: Temperature and incident light profiles at the bottom and surface at the middle point 2 after 48-hr lagooning of DW in LPT with water recirculation of 40 ml min^{-1}.

IV.5 POLYACRYLATE POLYALCOHOL

Copolymer Polyacrylate polyalcohol (PP) was used particularly for the immobilization of algae cells for their suspension at the surface of water in LPT, which is discussed in the later chapters. The copolymer was investigated as to its physical characteristics with respect to its expansion or swelling in water leading to the variation in size and its influence on water pH and DO. To investigate its physical characteristics a visual experiment in a beaker, containing 100 ml tap water, was carried out. It was estimated by counting that there are about 100 particles of varying sizes in 1 mg PP or 10^5 or more particles in 1g PP. For the study of physical characteristics of PP in water, 1 mg PP consisting of 100 particles was taken and put into a beaker containing 100 ml tap water.

Table IV-1 presents the observations made during the test for PP.

The copolymer particles were introduced into the beaker with varying sizes ranging from 100 to 850 μm. The integration of the particles with each other might have been caused by the gravity phenomenon in water or due to their becoming heavier after the absorption of water and thus inducing particle collisions forming the resultant copolymer agglomerates.

Table IV-1: Polyacrylate polyalcohol characteristics in water.

Time after PP addition in Water, min	Observation description	Explanation
1	Some particles suspend at the surface and some near the bottom	Probably the larger size particles suspended at the surface, while the smaller size ones took to the bottom
5	Surface particles start coalescing	After water absorption, the particles due to their heavier weight collided with each other
10	Particles below the surface start rising to the surface in colonies	The particles showed the tendency to move upwards while forming associations after water absorption and collision with each other
20	All the particles suspend at the surface coalesced and fully expanded (800 - 1000 μm)	

EXPERIMENT NO. IV-3

IV.6 DW LAGOONING WITH COPOLYMER DOSE OF 700 mg l^{-1} AT RECIRCULATION FLOW RATE OF 40 ML MIN^{-1}.

This experiment was carried out for lagooning 13 l DW with copolymer Polyacrylate polyalcohol (PP) added in LPT at 700 mg l^{-1}, which meant 1500 mg of copolymer was added at each of the six points in LPT for a total copolymer addition of 9000 mg. Figures IV-6 and IV-7 show the pH, DO and temperature results at the bottom and surface middle points respectively during DW lagooning with PP.

The pH of DW was increased by 34% all over the tank due to copolymer addition in LPT, which almost remained unchanged after 24 hr of DW lagooning. The graphs also show that there was no negative effect of copolymer addition on DW DO as the value showed increased by around 10% due to the effect of water recirculation. However, DO at the surface was slightly higher than at the bottom likely due to heat transfer and retention at the bottom than its neutralization at the surface, where the temperature might have been equilibrated by the water movement. However, the surface temperature at the surface was slightly higher by 2% than at the bottom during the course of lagooning due to direct exposure to light.

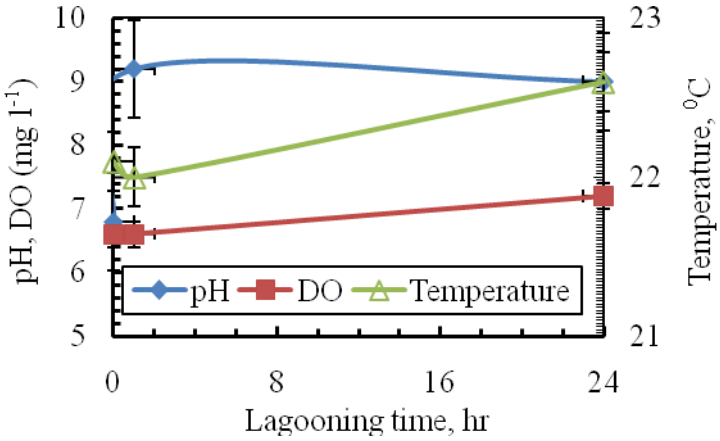


Figure IV-6: pH, DO and temperature results at the middle point at the bottom of LPT during DW lagooning with copolymer Polyacrylate polyalcohol dose of 700 mg l^{-1}. All the results are represented by their mean ± SD; n=3.

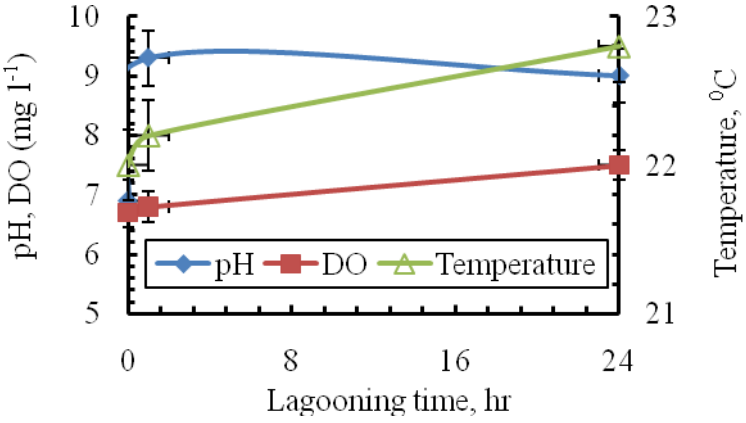
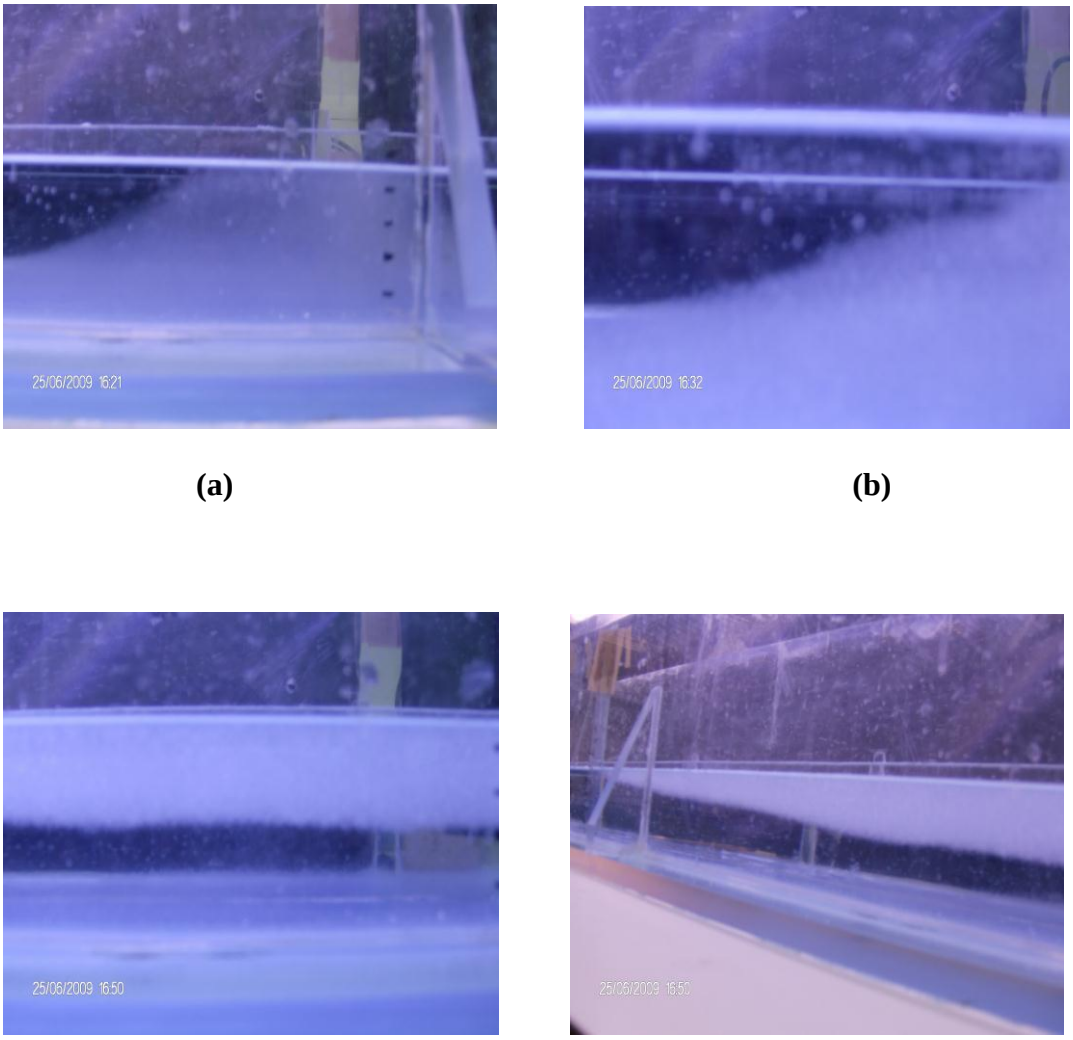


Figure IV-7: pH, DO and temperature results at the middle point at the surface of LPT during DW lagooning with copolymer Polyacrylate polyalcohol added at 700 mg l^{-1}. All the results are represented by their mean ± SD; n=3.

Similar copolymer characteristics during this experiment were observed when it was added with a higher dose in a large volume of 13 l in LPT, as compared to copolymer behaviour with a smaller dose during the test in a beaker (Section IV.5). Figure IV-8 is a combo camera picture containing four grabs (a, b, c and d) exhibiting copolymer expansion process. The copolymer particles after their introduction in LPT initially took to the bottom and started colonising until forming copolymer associations of roughly 1 to 3 mm dia to influence their upward movement (grabs a and b). The copolymer agglomeration took 20 min to complete, when most of the particles were observed suspending at the surface in colonies (grabs c and d).



(a) **(b)**

(c) **(d)**

Figure IV-8: Combination of camera images showing copolymer Polyacrylate polyalcohol (PP) characteristics in DW: (a) PP particles at the bottom of LPT after their addition into the tank; (b) agglomeration of PP particles and corresponding rise to the surface; (c and d) PP associations in colonies suspending at the surface within 20 to 30 min of hydrolysis reaction.

EXPERIMENT NO. IV-4

IV.7 DW LAGOONING WITH COPOLYMER POLYACRYLATE POLYALCOHOL AT HIGHER RECIRCULATION FLOW RATES

This experiment was carried out to study the impact of higher recirculation flow rate on the copolymer characteristics. 9g of copolymer with 700 mg l^{-1} or 1500 mg at each of the six points were added in 13 l DW in LPT for 24 hr lagooning at recirculation flow rate of 100 ml $minl^{-1}$. The pH and DO results obtained from this run are highlighted in Figure IV-9 at middle points 2 (a and b) and 5 (c and d) at the bottom and surface of LPT. The pH and DO results obtained at recirculation flow rate of 100 ml min^{-1} for pH and DO showed similarity with those obtained at the lower recirculation flow rate of 40 ml min^{-1}. The graphs suggested that there was little variation in the surface results, which gave almost an identical pattern as was the case with the results obtained at the bottom of LPT.

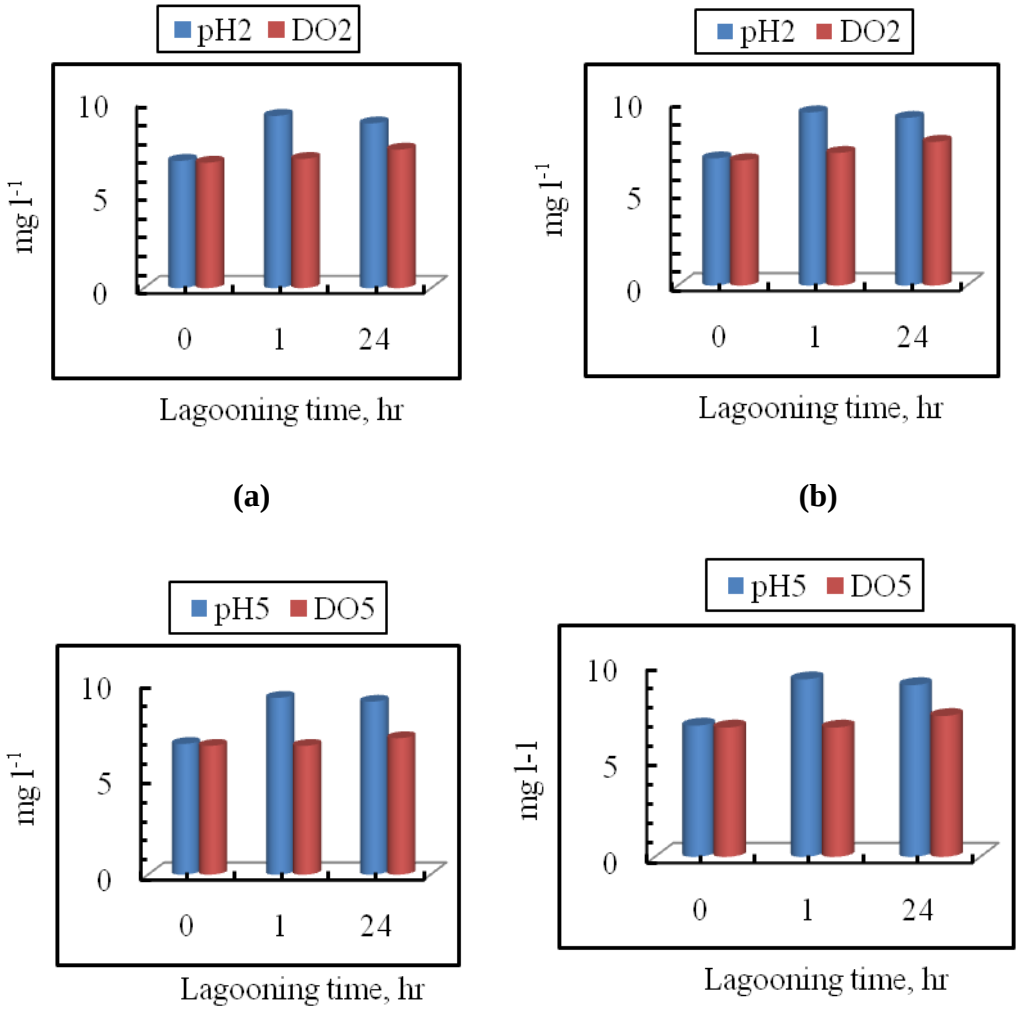


Figure IV-9: pH and DO results during DW lagooning with copolymer dose of 700 mg l^{-1} with water recirculation at 100 ml min^{-1}. Results at middle points 2 (a and b) and 5 (c and d) at both bottom and surface respectively.

Recirculation of water at the higher flow rate resulted in enough turbulence to cause continuous disintegration of copolymer particles, which were mostly found in scattered form particularly near the inlet area. This likely resulted in a condition where proper coalescence of copolymer particles could not take place during 24 hr

of DW lagooning. Figure IV-10 depicts this situation, whereby the particles are suspending in the scattered form after the break-up of the copolymer colonies.

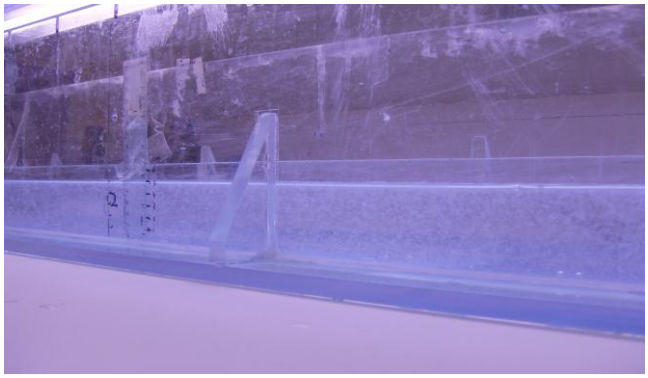


Figure IV-10: Camera image taken after 1 hr of copolymer addition during DW lagooning at 100 ml min^{-1}.

IV.7.1 Incident light profile in LPT

The incident light (IL) profile in LPT observed during DW lagooning with copolymer Polyacrylate polyalcohol is presented in Figure IV-11 to study the influence of copolymer addition on the light incidence. The data in the graph suggested that the middle points 2 and 5 were the high intensity points, which were 18% higher with respect to the incidence of light than the side points 1, 3, 4 and 6. After the addition of copolymer, instant decrease in the IL was registered by 11% at the side points and 8% at the middle points, which remained same after 24 hr.

This variation in IL between the middle and side points was probably due to the fact that in case of the middle points the striking impact of the light photons was direct and maximum; besides, they could also receive the light photons travelling from left and right regions of the tank. In contrast, the side points located at the left and right areas of the tank receive the insolation only within their respective

regions and often in a scattered and diluted form, hence, keeping the striking impact likely indirect and minimum.

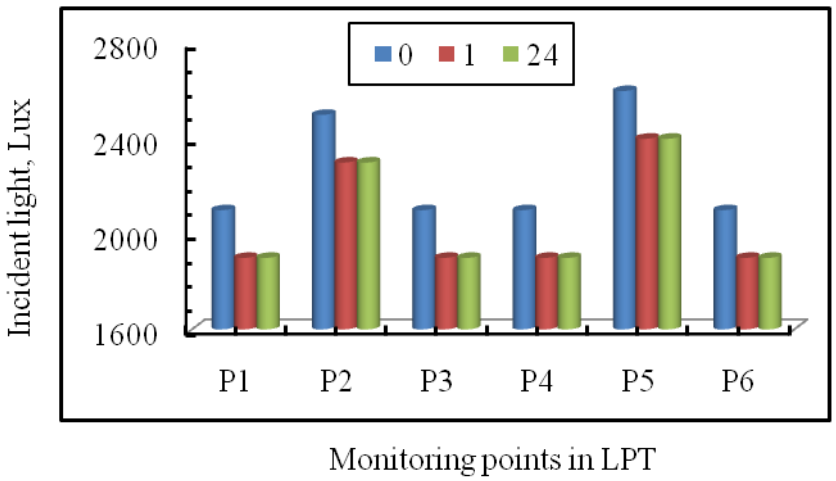


Figure IV-11: Incident light profile at all the points in LPT during 24 hr of DW lagooning with copolymer Polyacrylate polyalcohol.

EXPERIMENT NO. IV-5

IV.8 DW LAGOONING WITH COPOLYMER DOSE OF 160 mg l^{-1} AT 40 ml min^{-1}

In this experiment, DW lagooning in LPT was held at water recirculation flow rate of 40 ml min^{-1} with copolymer dose reduced by 78% than used in the previous run, meaning the copolymer was added in LPT at 160 mg l^{-1} or 333 mg of copolymer added at each of the six points in LPT. pH and DO results at the middle points 2 and 5 at both bottom and surface are presented in Figures IV-12 and IV-13 respectively. The pH graph shows that pH increased by 34% due to copolymer

addition at both points 2 and 5, which was reduced by 27% and 45% at the bottom and surface respectively after 48 hr. DO results implied that the value increased by 12% due to recirculation effect, which suggested that copolymer presence in the water for the longer period did not have negative impact on the DO. It was observed that towards the end of 48 hr of lagooning most of the copolymer associations had dissipated, hence the particles were leaving the surface to suspend or settle near or at the bottom. This dissociation of the particles probably could be a factor in the induction of pH decrease.

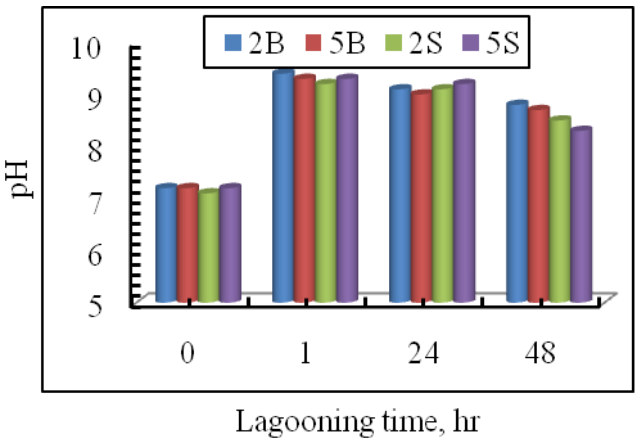


Figure IV-12: pH at middle points 2 and 5 at the bottom and surface of LPT during 48 hr of DW lagooning with copolymer Polyacrylate polyalcohol.

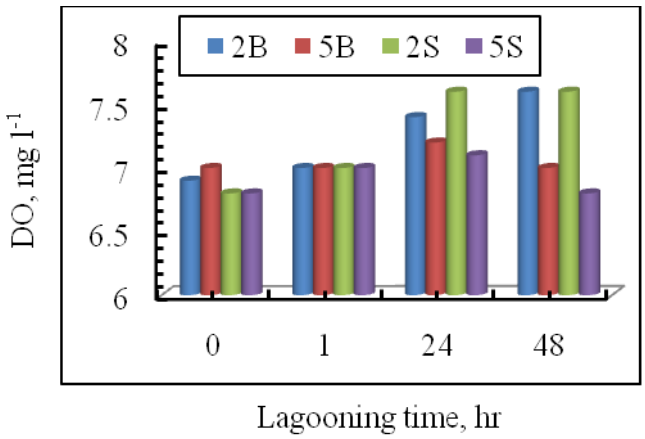


Figure IV-13: DO at middle points 2 and 5 at the bottom and surface of LPT during 48 hr of DW lagooning with copolymer Polyacrylate polyalcohol.

IV.9 SUMMARY

The baseline experiments of DW lagooning were carried out in LPT to study the effects of independent variables such as incident light (IL) and recirculation flow rate (RFR) on dependent variables such as pH, DO and temperature. This was necessary to know as the change in these water properties would otherwise have a direct influence on global parameters such as COD/TOC. It was known that the monitoring points in LPT had different IL profiles likely due to the factors of light photon's travel path and their surface-striking pattern causing variations in the IL. Thus, IL at the middle points (2 and 5) would be higher by 18% as compared to IL at the side or corner points (1, 3, 4 and 6). It was observed that RFR of 40 ml min^{-1} (50 rpm) was suitable in that it did not hinder the coalescence and expansion phenomenon of copolymer particles as compared to that observed with higher RFR. In addition, the conducive RFR caused sufficient visible stirring effect from point 1 to 3 in LPT to influence forward movement of the tank contents. Hence,

the later experiments were planned to be carried out with the optimized RFR. Copolymer addition in water was found to raise the water pH by around 34%, and that it had no negative effect on the water DO. Besides, the use of higher amount of copolymer particles during lagooning experiments in LPT exhibited similar characteristics as was observed in a beaker test with small amount of copolymer. The copolymer particles showed their physical properties such as expansion, coalescence, colonisation in the initial passage of hydrolysis reaction, which was followed by the process of particle dissipation possibly due to rocking phenomenon. This likely caused the breaking up of the integration bonds between the particles with a possible simultaneous decrease in pH. The lagooning experiments in LPT also resulted in the loss of water of around 500 ml d^{-1} due to evaporation caused by the continuous luminance.

CHAPTER V

SUGAR WATER LAGOONING

CHAPTER V

SUGAR WATER LAGOONING

V.1 INTRODUCTION

Prior to study lagooning of simulated version of sugar factory wastewater (SFW) using microorganisms for the optimization of biodegradation process based on several factors such as biomass growth, nutrient consumption and substrate removal, baseline evaluation of environmental parameters such as pH, temperature, IL, DO was carried out via sugar water lagooning (Mrayyan and Battikhi, 2005). For this, sucrose solution or sugar dissolved in water or sugar water (SW) was taken as a baseline component for SFW in that it contained the same amount of COD as SFW. Hence, SW was prepared by adding specific amount of table sugar in DW to reflect the empirical amount of energy contained by SFW COD (Pennington and Baker, 1990). The SW was thus used for lagooning experiments in LPT to monitor the variation in pH, DO and COD without the input of the organisms. To know the COD of SFW beforehand, Newark Beet Sugar Factory (NBSF), Nottinghamshire, UK was visited twice to collect the wastewater samples for their representative characteristics, as outlined in section III.2.2 (p 86). The factory disposes of around 40% of the wastewater generated in 100-120-day campaign each year from November to February, in six wastewater ponds in its vicinity and the rest of the effluent is subjected to anaerobic treatment for biogas production in the wastewater Digestor inside the factory, as shown in Figure V-1.

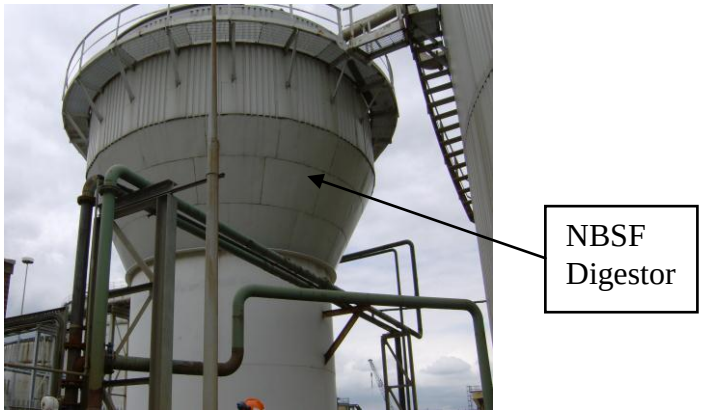


Figure V-1: Wastewater Digestor of Newark beet sugar factory Nottinghamshire, UK.

V.2 NEWARK BEET SUGAR FACTORY WASTEWATER ANALYSIS

Wastewater samples from NBSF were collected into two 25-litre pre-washed plastic containers from inside the factory. For representativeness of the wastewater samples, the bottles were rinsed with the wastewater to reduce the level of internal or external contamination before being filled up by three quarters of the bottle or with 18 l of the sample. The colour of wastewater was greenish indicating either the nature of process or level of organic strength and amount of biological activity. The odour of the sample wastewater was sugarcane juice like but not putrescible or foul suggesting the freshness of samples, with an *in-situ* temperature reading of 22^0C. The samples were then transported to the laboratory within little more than an hour of collection. One sample container was preserved at 4^0C in the fridge and the other was kept in the open in the laboratory at room temperature (23^0C). Both samples were analyzed for DO, pH, temperature and COD parameters for their initial and stabilized values.

Table V-1 presents NBSF wastewater characteristics for parameters of interest such as DO, pH and COD. All the values were determined three times before taking the mean. The analysis of both preserved and non-preserved samples was done immediately after 24 hr and after one and two weeks. The data in the Table indicates that the factory's wastewater contained higher pH in the alkaline range, which had to do with the freshness of the sample as well as the probable dilution of wastewater via combination of different streams within the plant premises. The wastewater had DO value on the lower side, which suggested towards the presence of organic matter in it causing DO depletion. The wastewater COD ranged around 12,000 to 15,000 mg l^{-1} with almost zero oxygen left within one week of biological stabilization at room temperature, which probably caused the COD value to increase owing to possible generation of soluble microbial products (SMPs) or metabolites such as organic acids due to decomposition of sugar by the inherent bacteria (Asadi, 2007).

Table V-1: Newark beet sugar factory wastewater analysis (mean, n=3).

Parameter (immediate values)	Wastewater incubation at 4^0C in the fridge			Wastewater incubation at room temperature (23^0C)		
	Measured after 24 hr	Measured after weeks		Measured after 24 hr	Measured after weeks	
		1	2		1	2
pH (7.7)	7.6	7.4	7.2	7.5	6.3	5.9
DO, mg l^{-1} (1.9)	1.7	1.0	0.0	1.4	0.0	0.0
COD, mg l^{-1} (13,200)	12,700	11,900	9,600	13,100	14,200	14,900

V.3 AMOUNT OF SUGAR AND COD RANGE DETERMINATION

The amount of sugar for addition in water to simulate the organic loading contained by sugar factory wastewater COD was determined as under:

Since 1 g sugar contains 17 kJ energy, and 1 mg COD per l of an organic wastewater is roughly equal to 8 J energy (Pennington and Baker, 1990).

Or, 10000 mg COD per l are equivalent to energy value of 80 kJ.

Using this relationship between COD and energy contents, the following can be deduced:

17 kJ energy in 1g of sugar will roughly contain 2125 mg of COD per l; or 13 g sugar added per l water may yield a theoretical COD range of around 27,000 mg l^{-1}. The COD of prepared SW with 13 g of sugar per litre DW was between 14,500 and 20,000 mg l^{-1} and that of sugar process wastewater it varied between 9,600 and 14,900 mg l^{-1}.

Hence, to determine the empirical value of COD, SW was prepared by adding different amounts of sugar per litre DW such as 1, 5, 10 and 13 mg l^{-1} DW to investigate the proper ratio between amount of sugar and the desired COD range. The results as obtained in this regard are shown in Table V-2, which reflects the analyses data of three prepared samples of SW carried out with a mean of three analyses for each parameter. All four samples were analysed for their initial and final values for pH, DO and COD. The data in the Table explains the point that increasing amount of sugar in the water causes increased level of biological activity resulting in the decreased values of pH and DO and increased values for COD due to probable generation of organic acids by the inherent biomass (Perez-Garcia *et al*., 2011). The COD results relevant with respective amounts of sugar indicated that addition of 13 g sugar in the preparation of SW would be suitable amount to represent COD in the respective range as compared to lower amounts of sugar, which yielded in lower COD ranges.

Table V-2: Analysis of prepared SW for the determination of ratio between amount of sugar and COD range (mean, n=3).

Amount of sugar mixed in 1 l DW (g)	pH at/after time, hr			DO, mg l^{-1} at/after time, hr			COD, mg l^{-1} at/after time, hr		
	0	24	48	0	24	48	0	24	48
1	7.7	7.5	7.3	7.1	6.9	6.3	1,700	1,600	23,00
5	7.5	7.3	7.1	7.3	6.9	6.4	6,400	6,600	7,200
10	7.4	7.1	6.9	7.2	6.8	6.0	11,200	11,900	13,000
13	7.8	7.4	6.8	7.1	6.7	5.7	14,500	15,200	16,900

EXPERIMENT NO. V-1

V.4 SW LAGOONING IN LPT

Taking Table V-2 data into consideration, SW was prepared by dissolving 13g of sugar (0.5 moles/l M) per l DW to prepare 13 l SW, which was poured into LPT directly for lagooning under continuous lighting and without water circulation initially for 48 hr. Figure V-2 shows SW pH, DO and temperature results at point 2 both at the start and after 48 hr at different depths in LPT with 5 cm being the

surface level and 2 cm the bottom level in LPT. The graph shows that after 48 hr of lagooning, pH and DO have decreased by 11% and 13% respectively with maximum reduction occurring at the bottom (2 cm depth); however, the surface values at 5cm depth have comparatively decreased in lesser proportion by 4% and 8% respectivley. Temperature value is showing an increase of 8% at the surface and 10% at the bottom from the initial value due to raise in ambient LPT temperture by 2.5^0C owing to partly by continuous lighting by around 0.5^0C, which was also observed in the baseline with DW. These results suggested that SW degradation by indigenous bacteria began to occur just before 48 hr inducing decrease in pH and DO and partially increasing temperature as well. The biochemical reactions occurring during SW lagooning implied that glucose and fructose promoted the growth of inherent bactial community, with the former resulting in the production of gluconic acid inducting decrease in pH and DO (Asadi, 2007).

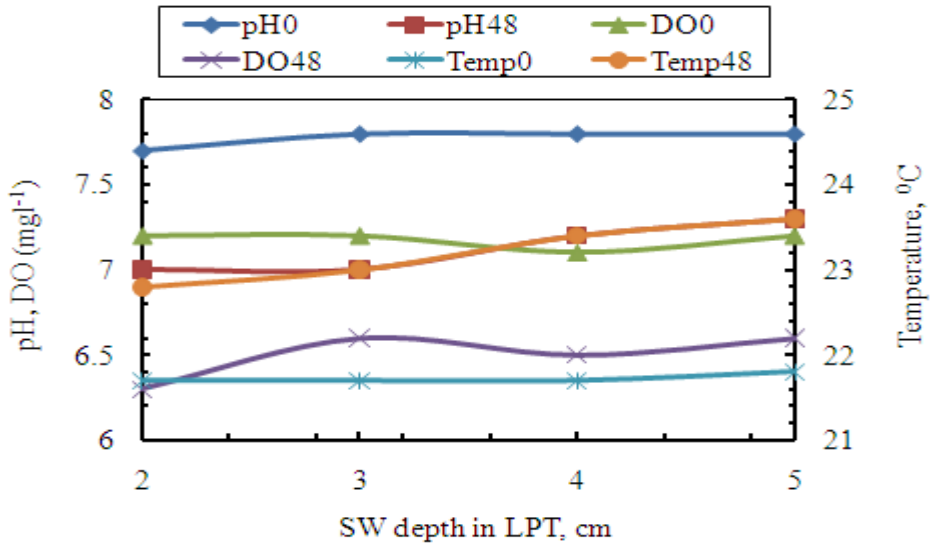


Figure V-2: pH, DO and temperature results at point 2 during SW lagooning for 48 hr without water circulation.

The above run was extended using different recirculation flow rates (RFR) for every 24 hr to study the influence of difference in the RFR on SW pH and DO. Thus continuing from the previous run SW recirculation was started from day 5 until day 9 for every 24 hr at RFR of 100, 200, 300 and 400 ml min^{-1} respectively. Figure V-3 depicts SW pH, DO and temperature values at the surface at point 2 with SW recirculation for every 24 hours at different RFRs. The results for the extended time of SW lagooning showed that before water recirculation was started, pH and DO values were observed to be on a declining path and that DO was completely consumed from SW within five days of lagooning indicating the occurrence of biological activity due to the decomposition of sucrose derivatives (glucose and fructose) by inherent microbial mass. This suggested that DO was more susceptible and responsive to fluctuating environmental conditions influenced by biological activity than pH in a given medium (Singh et al., 2008). This observation was in agreement with the comments made by Joyce et al. (1985), who in their statement made a direct correlation between DO unavailability in the medium and the aerobic bacterial activity. As compared to DW lagooning results, which showed no or little variation in pH (section IV.4), SW pH results in this experiment showed a marked pattern of decrease over a period of five days, thus creating acidic conditions caused by the degradation of sugar derivative compounds by the indigenous biota probably due to nitrogen uptake present in the sugar compounds (Yun et al., 1997).

The consistent decrease in SW pH after day 6 suggested that SW recirculation at higher flow rates might have prompted the biological activity, however, the DO profile in the graph implied that it showed an increase after every 24 hr due to rise

in the RFR. The gradual increase in SW temperature in LPT above the ambient termperature in the lab probably would be due to heat transfer from surface to bottom under the influence of continuous illumination and water recirculation.

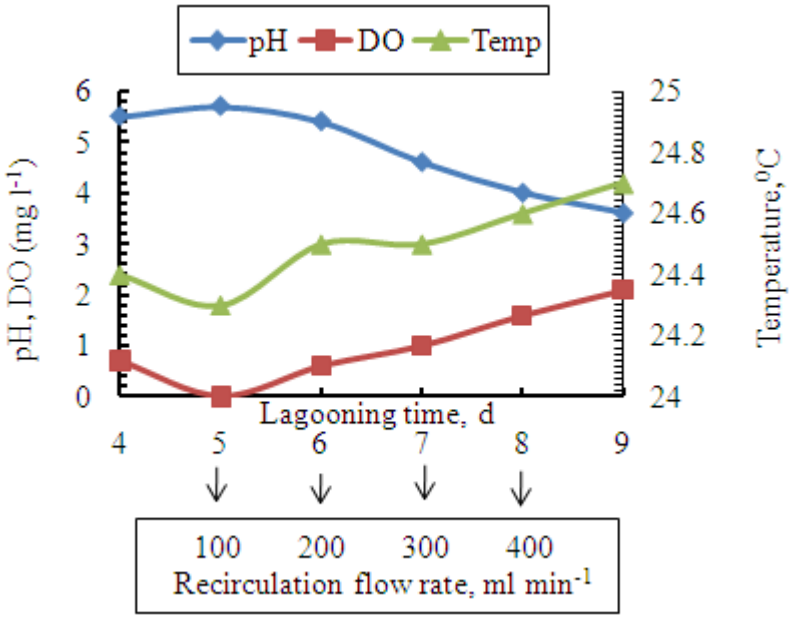


Figure V-3: pH, DO and temperature at middle point 2 at the surface of LPT during SW lagooning for 9 days at different recirculation flow rates.

In addition, water recirculation under higher flow rates caused choking of the attached tubes due to pressure pumping of SW with the micorbial growth produced as a result of sugar inversion followed by its degradation (Asadi, 2007). The displacement of SW caused by recirculation probably neutralized its composition in both compartments of the tank causing interchange of the constituents after every 130 min (2 hr 10 min) with 100 ml min^{-1}, 65 min (1 hr 5 min) with 200 ml min^{-1}, 43 min with 300 ml min^{-1} and 33 min with 400 ml min^{-1}. In contrast, SW recirculation at 40 ml min^{-1} would take 325 min (5.4 hr) to complete one renewal

cycle of the medium. This meant that SW recirculation at 40 ml min^{-1} would have completed 4 renewal cycels per day as compared to 11, 22, 33 and 44 renewal cycles per day with RFR of 100, 200, 300 and 400 ml min^{-1} respectively. Besides, recirculation at 40 ml min^{-1} did not result in choking of the connecting tubes with the biomass due to lower pressure, allowing longer retention time for the majority of input contents to remain in LPT for treatment with lesser displacement cycles. Sugar being an unstable compound tends to divide into two simpler sugars, glucose and fructose, immediately after its dissolution in water due to hydrolysis of sugar molecule, with glucose consumption resulting in the production of gluconic acid due to microbial activity prompting acidic conditions in the medium within 2-3 days of biochemical reaction (Asadi, 2007). However, time factor involved in sugar biodegradation and its effect on water pH and DO were the factors that were investigated in this experiment under different recirculation flow rates.

V.4.1 Comparison of IL profile

Figure V-4 shows the comparison of incident light (IL) values at the surface obtained during this experiment for SW lagooning, which are compared with the results obtained during DW lagooning at the middle points 2 and 5. The graph shows that the IL values remained higher during DW lagooning as compared to the values obtained during SW lagooning, which was probably caused by sucrose degradation and subsequent suspension of microbial mass in the tank. This condition resulted in the decrease of light incidence at the surface by 4% during SW lagooning for 4 days without water recirculation in contrast to none during DW lagooning.

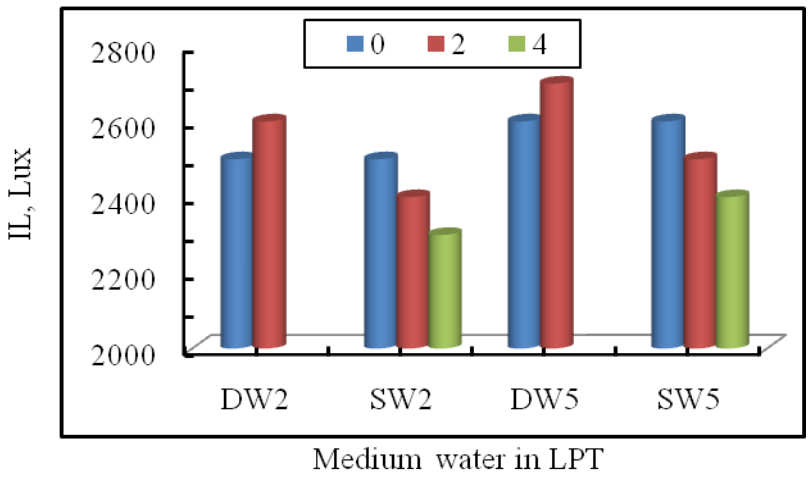


Figure V-4: Incident light profile comparison at middle points 2 and 5 during DW and SW lagooning at the surface of LPT without water recirculation.

Figure V-5 presents the cumulative view of IL at points 1, 2, 5 and 6 during 4 days of SW lagooning without recirculation. The graph shows the higher cumulative IL values at points 5 (9800 Lx) and 2 (9400 Lx) as compared to points 1 (8100 Lx) and 6 (8500 Lx). The cumulative IL data is also inclusive of 4% decrease occurred during 4 days of SW lagooning.

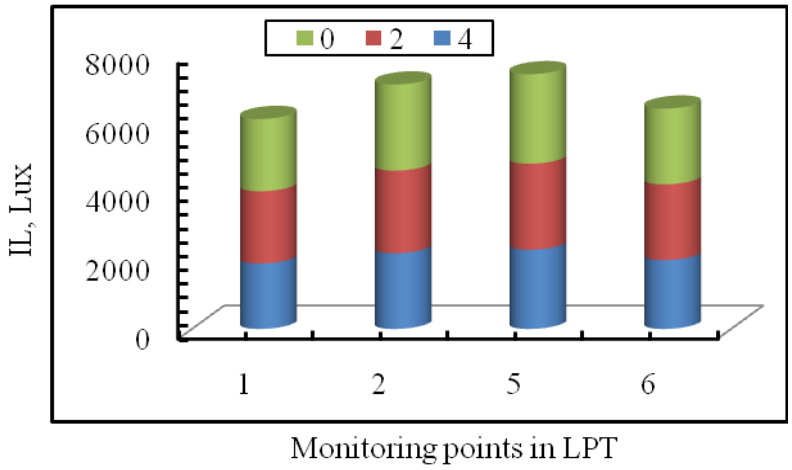


Figure V-5: Cumulative IL profile at the surface of LPT at monitoring points 1, 2, 5 and 6 during SW lagooning without water recirculation (mean, n=3).

V.4.2 SW COD analysis

Figure V-6 shows the COD values at the middle points 2 and 5 during SW lagooning with different recirculation flow rates for every 24 hr. The data in the graph suggested that on the whole in nine days of lagooning SW COD increased by 7% with most of the increase was registered during SW lagooning with recirculation regimes. The steady increase in the COD values could be attributed to the generation of indigenous soluble microbial mass and their metabolites including the production of organic acids during the degradation of sugar compounds (Kim et al., 2010).

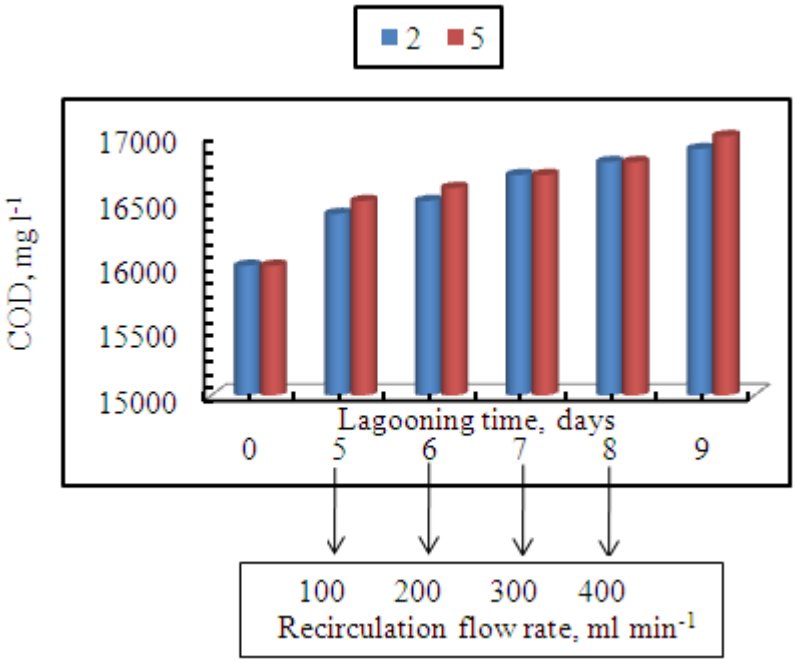


Figure V-6: COD at middle points 2 and 5 during SW lagooning at different recirculation flow rates (mean, n=3).

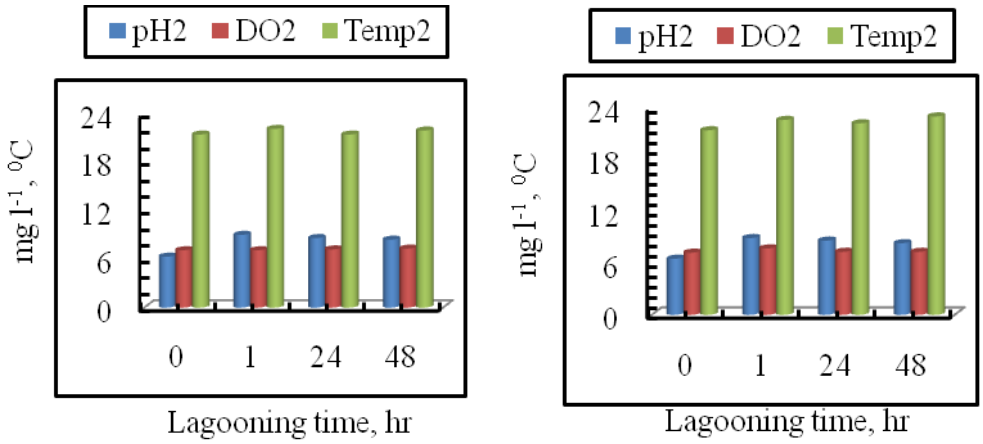
V.5 SW LAGOONING WITH POLYACRYLATE POLYALCOHOL

SW lagooning was carried out with copolymer Polyacrylate polyalcohol, which was also used during DW lagooning, to study the deviation or otherwise of the typical copolymer characteristics when added in SW. Hence, in the ensuing experiments the integration and disintegration of the copolymer particles and its influence on the parameters like DO, pH and temperature.

EXPERIMENT NO. V-2

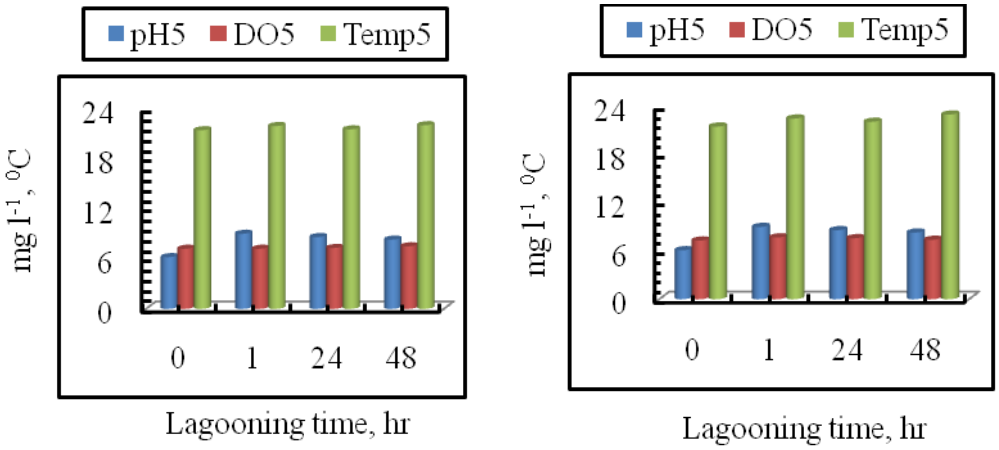
V.5.1 SW lagooning with copolymer dose of 615 mg l^{-1} with recirculation at 40 ml min^{-1}

Figures V-7 and V-8 show pH, DO and temperature results during SW lagooning for 48 hr with water recirculation at 40 ml min^{-1} and continuous lighting for the middle points 2 and 5 at both bottom and surface levels in LPT. The total amount of copolymer added was 8000 mg (615 mg l^{-1}) with 4000 mg being added each at points 2 and 5. The average temperature at the bottom was 22^0C, whereas it was slightly higher at the surface i.e. 22.6^0C. The addition of copolymer in SW increased the pH by 36%, which was slightly higher as compared to that observed during DW lagooning with copolymer (Section IV-6), which could be associated with the presence of hydroxyl ions in SW. The pH was decreased by 8% during 48 hr of SW lagooning, likely due to sugar degradation by the inherent bacteria. The DO more or less remained the same suggesting that there was no negative effect of copolymer addition in SW on the DO concentration.



(a) **(b)**

Figure V-7: pH, DO and temperature results at the bottom (a) and surface (b) at point 2 in LPT during SW lagooning with copolymer dose of 615 mg l^{-1} with recirculation flow rate of 40 ml min^{-1} (mean, n=3).



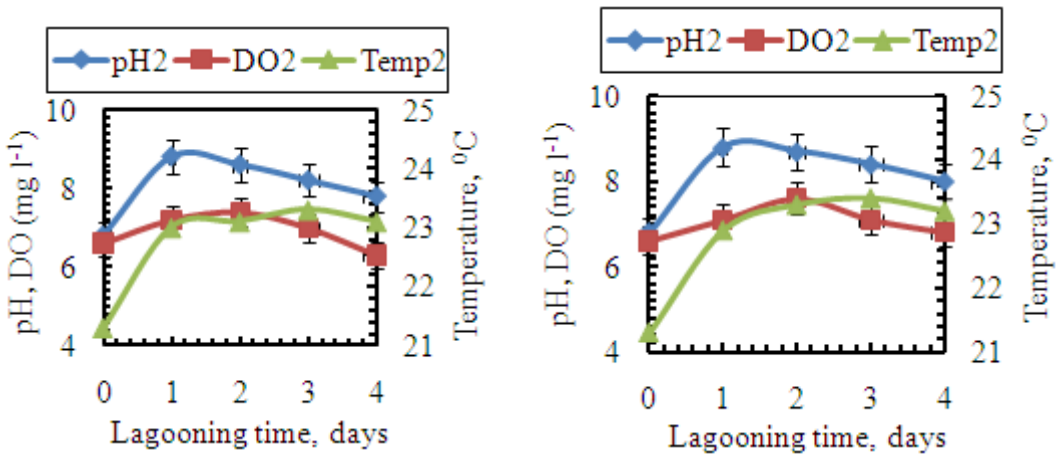
(a) **(b)**

Figure V-8: pH, DO and temperature results at the bottom (a) and surface (b) at point 5 in LPT during SW lagooning with copolymer dose of 615 mg l^{-1} with recirculation flow rate of 40 ml min^{-1} (mean, n=3).

EXPERIMENT NO. V-3

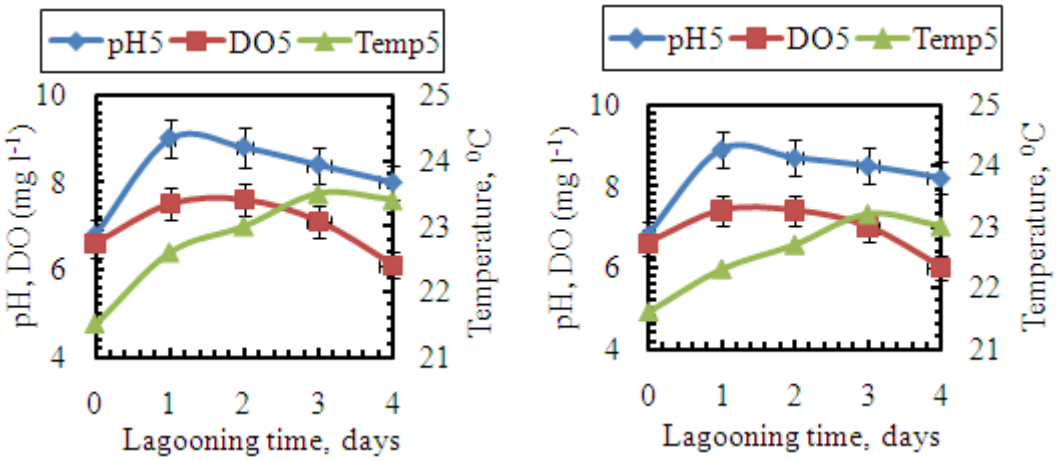
V.5.2 SW lagooning with copolymer dose of 310 mg l^{-1} with recirculation at 40 ml min^{-1}

SW lagooning in this experiment was carried out with 50% lower dosing of copolymer to observe the influence of reduction in copolymer addition on the pH and DO profiles. The total amount of copolymer added in this experiment was 4000 mg (310 mg l^{-1}) with 2000 mg being added each at points 2 and 5. Figures V-9 and V-10 are the results for pH, DO and temperature at the bottom and surface of LPT for the middle points 2 and 5 separately during SW lagooning with 4g copolymer at recirculation flow rate of 40 ml min^{-1}. The average temperature during this run was recorded as 22.5^0C at both surface points 2 and 5, while at the bottom of these points, the temperature was slightly higher (22.7^0C) possibly due to retention of heat at the bottom level by the suspension of polymer particles at the surface. The data in the graphs suggested that after initial increase in the pH by the same margin, consistent decrease in the pH was observed until the end of the run during 96 hr of SW lagooning when overall it was reduced by 12%, which was higher than it was observed in the previous experiment. This could be indicative of the impact of reduced amount of copolymer dosing in SW in terms of biological activity. This was also reflected by 20% decrease in DO, which was not the case in the previous experiment.



(a) **(b)**

Figure V-9: pH, DO and temperature results at the bottom (a) and surface (b) at point 2 in LPT during SW lagooning with copolymer dose of 310 mg l^{-1} with recirculation flow rate of 40 ml min^{-1} (mean, n=3).



(a) **(b)**

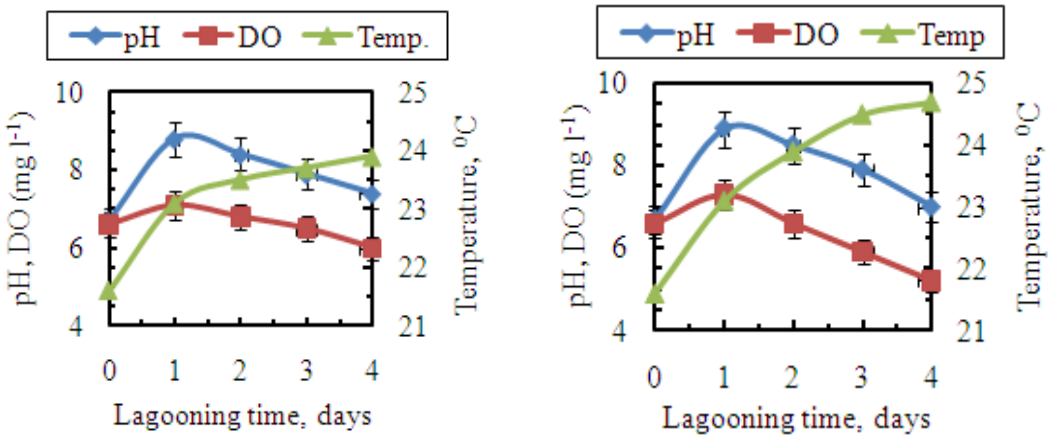
Figure V-10: pH, DO and temperature results at the bottom (a) and surface (b) at point 5 in LPT during SW lagooning with copolymer dose of 310 mg l^{-1} with recirculation flow rate of 40 ml min^{-1} (mean, n=3).

EXPERIMENT NO. V-4

V.5.3 SW lagooning with copolymer dose of 160 mg l^{-1} with recirculation at 40 ml min^{-1}

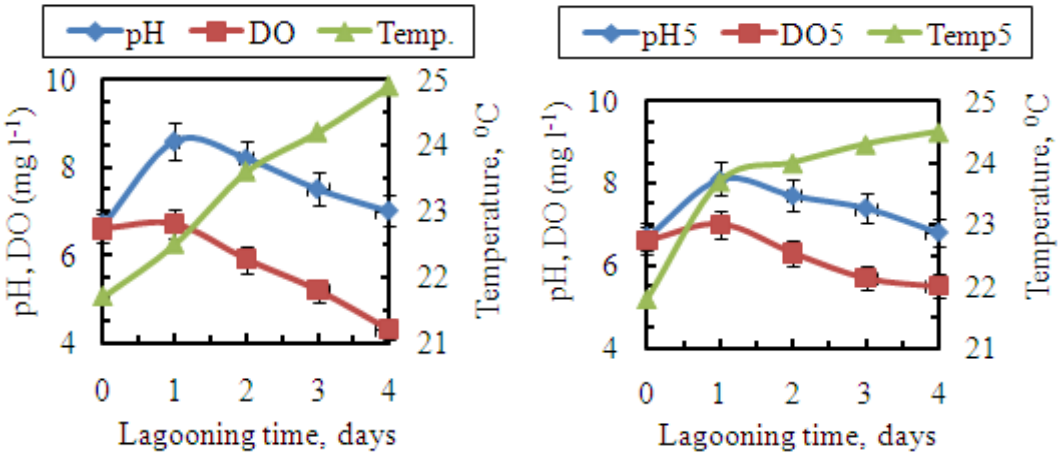
In this experiment, copolymer dosing was further lowered by 50% for SW lagooning when 2065 mg (160 mg l^{-1}) of copolymer were added at the middle points 2 and 5 in LPT. The medium was recirculated at 40 ml min^{-1} for 4 days under continuous insolation. Figures V-11 and V-12 present the pH, DO and temperature results obtained during this run at the given points.

The average temperature during this run was 23.6^{0}C with a maximum temperature of around 25^{0}C was observed, which was higher by at least 1^{0}C as compared to previous experiment (section V.5.2). This suggested that higher concentration of copolymer was resulting in process heat losses from LPT by 8% during the earlier experiment (section V.5.2). The pH value decreased by 22% with reduction occurring more at the bottom than at the surface probably due to accumulation of acidic compounds at the bottom. Similar trend was observed in the DO concentration as there seemed to be a steady decrease of DO after 48 hr until the end of lagooning at 0.6 mg l^{-1} d^{-1}, which was 33% higher reduction in DO than in the previous experiment (section V.5.2). During SW lagooning experiments, the total loss of water in LPT due to evaporation (Andresen, 2005) and taking samples for analyses was estimated to be around 720 ml per day decreasing the depth of water in LPT by 0.3 cm per day.



(a) **(b)**

Figure V-11: pH, DO and temperature results at the bottom (a) and surface (b) at point 2 in LPT during SW lagooning with copolymer dose of 160 mg l^{-1} with recirculation flow rate of 40 ml min^{-1} (mean, n=3).



(a) **(b)**

Figure V-12: pH, DO and temperature results at the bottom (a) and surface (b) at point 5 in LPT during SW lagooning with copolymer dose of 160 mg l^{-1} with recirculation flow rate of 40 ml min^{-1} (mean, n=3).

V.6 SUMMARY

This chapter covered the baseline experiments carried out in LPT using prepared sugar water (SW) without and with SW recirculation at different flow rates as well as using variant dosing of copolymer Polyacrylate polyalcohol to study the variable nature of SW properties such as pH, DO and temperature. SW was prepared with the relevant amount of COD as contained by UK beet sugar factory, which was in the range of around 9,000 to 15,000 mg l^{-1}. It was observed that recirculation flow rate (RFR) of 40 ml min^{-1} was suitable in that it did not hinder the expansion or suspension of copolymer particles in contrast to the higher flow rates, when the particles were mostly found in scattered form. In addition, induction of higher RFRs were contributing towards slightly higher COD values as compared to the COD profile observed with lower RFRs. SW lagooning results transpired that sugar degradation probably started to occur after 40 hr resulting in the subsequent decrease in DO and pH values and corresponding little increase in the COD values probably due to increase in the soluble carbonaceous matter and degradation metabolites. The results also showed that DO was more susceptible and responsive than pH to fluctuating medium characteristics induced by the bacterial activity. The addition of copolymer Polyacrylate polyalcohol during SW lagooning raised the pH slightly higher than that observed during DW lagooning with the copolymer. The baseline study helped optimize the operational parameters during DW and SW lagooning such as RFR suitability, IL-intensive points in LPT, pH, DO and temperature variations before running the actual process with organisms.

CHAPTER VI

PHOTOHETEROTROPHIC CULTIVATION OF ALGAE IN SUGAR WATER

CHAPTER VI

PHOTOHETEROTROPHIC CULTIVATION OF ALGAE IN SUGAR WATER

VI.1 INTRODUCTION

Photoheterotrophic mass cultivation of algae in sugar water (SW) was planned in LPT to assess the growth potential of algae cell mass solely based on the organic carbon present in SW along with the corresponding influence on the organic strength of SW. The propensity of algae cells feeding on the organic carbon was realized during preculturing of algae strain, when the addition of SW in the growth medium spurred the growth of the cells within 24 hr, as discussed in section VI.2.2. However, the growth potential of algae at the mass scale such as in LPT was required to be investigated as a means of organic wastewater remediation using algae culture (Safonova et al., 2004). Algae cultivation in SW was carried out in LPT under the similar conditions as observed during the baseline experiments as to temperature, incident light (IL), optimized SW recirculation flow rate (RFR), copolymer addition and organic loading (COD) specific to sugar factory wastewater. The performance parameters during algae cultivation, like IL, pH, temperature, DO and COD were also investigated by Hohe and Reski (2005), who referred to the importance of optimum profiles of these parameters on the growth and development of algae cells. They observed that algae cultures grew better under continuous illumination with doubling time of 1.7 days as compared to 2.3 days with light and dark cycles.

VI.2 ALGAE PRECULTURING ANALYSIS

The selection and culturing of algae strains is given in Chapter III (Section III.2.4), here, monitoring and analysis of the cultures is discussed.

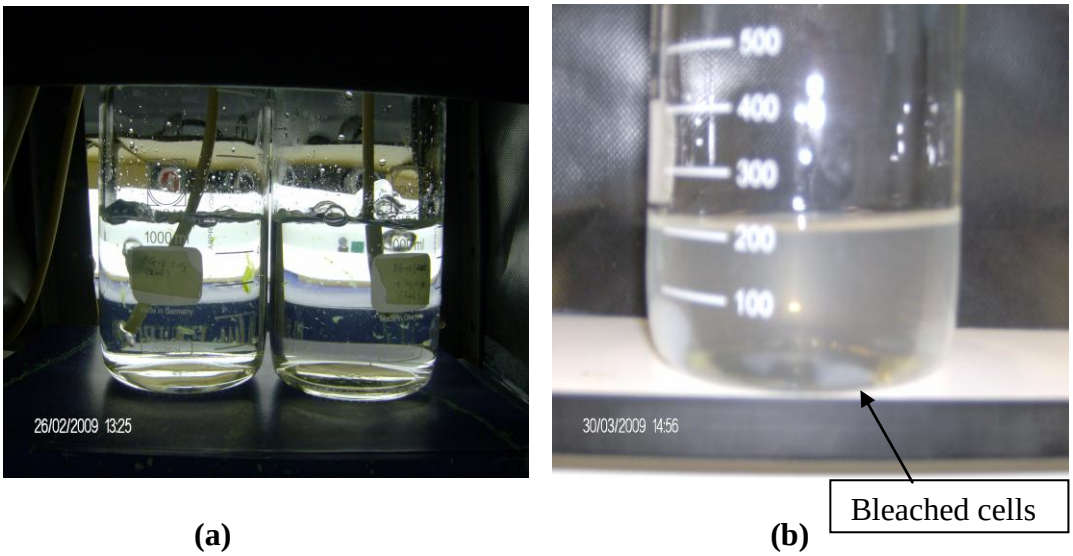
VI.2.1 Blue green algae (*Leptolyngbya*)

Leptolyngbya, a blue green algae strain was cultured in algae culturing unit (ACU). The monitoring of the culture bottles was daily carried out for the maintenance of culture conditions such as IL, temperature and pH for adjustment in case of variation from the values as given in Table VI-1, which shows the standard culturing data as provided by the supplier of the culture, to maintain the culture protocol (ATCC, 2008).

Table VI-1: Specifications for culturing algae cells

Description	Observed/ standard value
Temperature of lighting unit at the source, ^{0}C	35
Culturing bottle temperature, ^{0}C	28
Incident Light at the skin of the bottle, Lux	2800
Distance between light and culturing bottles, cm	24
pH of the culture	7-7.2
Initial culture composition: DW	100
BG-11 medium, ml	125
Starter algae culture, ml	5

During the culturing period, the IL and temperature values were observed as almost the same since there was no variation in the light intensity thus in the temperature as well, as the distance between the culture bottles and the light source was not changed, thus no adjustment was required for the IL and temperature. pH of the culture medium showed decrease from 7.2 and 6.9 during culturing of *Leptolyngbya*, which considering the decrease negligible also did not require to be adjusted. The culture was fed via sterilized pipette with 25 ml of the fresh medium after every 24 hr as nutrient supplement. After seven days of culturing, the culture did not show any growth as regards the visible increased amount of algae cells in the culture. This could have been because of lack of direct supply of pure CO_2 to algae culture or the inadequacy of the same in the indirect supply of CO_2 through aeration of the culture, which was probably the case here. On the other hand, it could be that the algae strain was already contaminated via competition with the foreign and unwanted elements in the culture and hence lost their protoplasm, as in the ACU the culture was preserved from airborne contamination (Chisti, 2008). After six days of culturing, it was noticed that the little green tinge contained by the starter culture had converted into a white particulate matter or bleached probably owing to autolysis (Ozaki et al., 2008), thus turning the water cloudy in the culture bottle with the onset of decaying cells, as shown in Figure VI-1. Abed and Koster (2005) also experienced similar type of problem when out of five strains of cyanobacteria, one strain *Halomicronema exentricum* during culturing lost pigmentation and subsequently died after few days. The *Leptolyngbya* culture was discarded and the ACU setup was cleaned, sanitized and refreshed for culturing of the next strain.



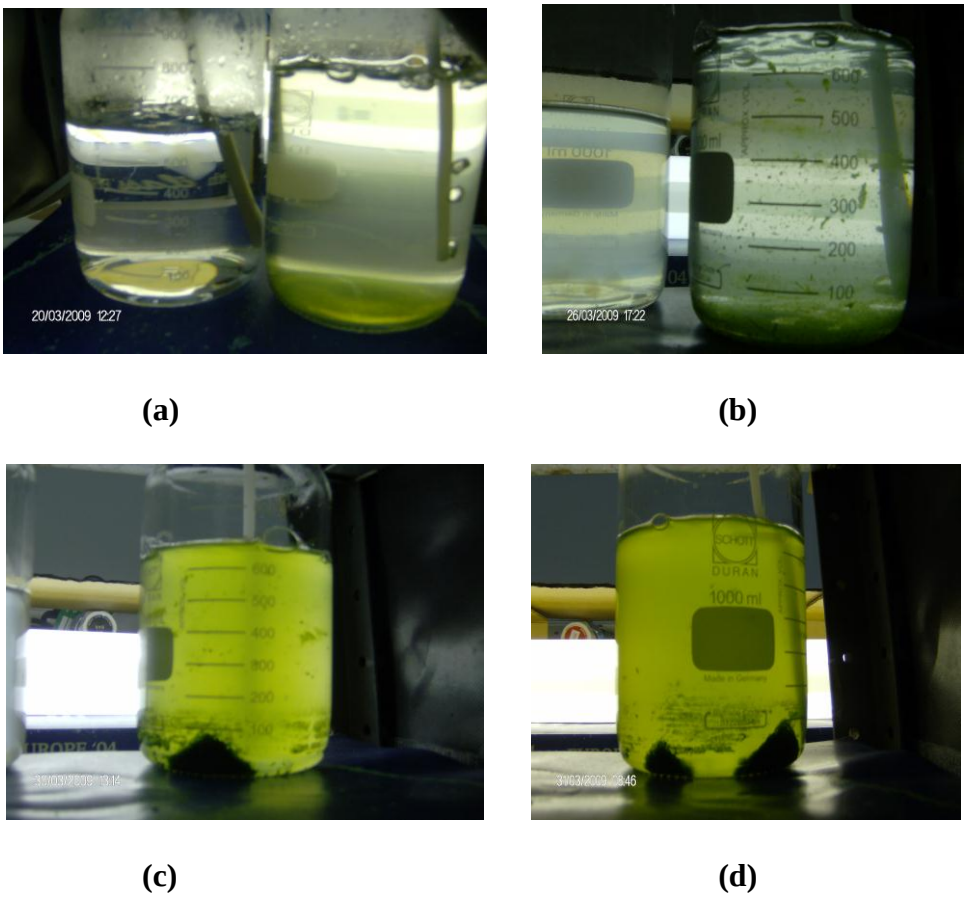
(a) **(b)**

Figure VI-1: *Leptolyngbya* grabs during its pre culturing showing initial growth of the culture after day 1 (a) and whitening of the culture after day 6 (b).

VI.2.2 Green algae (*Chlorella Vulgaris*)

Green algae cells of *Chlorella Vulgaris* (*C. Vulgaris*) were cultured next in the ACU. Two culturing flasks of 100 ml were taken, each containing 3 ml of the starter algae culture along with 50 ml of BG-11 medium and were kept in the ACU. After 48 hr of culture propagation, one of the flasks showed growth, which was visible in the form of increased amount of algae cells. In addition, the pH of the grown culture in the flasks also registered an increase in pH in the range of 0.4-0.7, indicating the consumption of inorganic carbon by the cells supplied via air. After 10 days of preculturing, the contents of both the flasks were transferred for expansion or sub-culturing in a larger volume to check the viability of the cultured cells (Koussemon et al., 2001). The sub-culturing bottles were already added with 500 ml of BG-11 medium each. Figure VI-2 shows the images taken during two

weeks of sub-culturing. The image (a) was taken after 24 hr of the start of subculturing, while image (b) was taken after 7 days of subculturing showing visible growth of algae cells in the culturing bottle, inoculated with the propagated cells. The growth of the cells was measured daily in terms of algae cell concentrate volume by means of either sedimentation or filtration, which transpired that the cells were growing at the rate of 2.1 ml of algae concentrate per day. Image (c) was taken after 11 days when the culture cells showed a colonial growth after the addition of 2 ml of prepared SW the previous day, which increased the growth rate of algae cell volume up to 3.4 ml per day. This meant that the cell volume was increased by 1.3 ml or by 60% per day in comparison to the lower growth rate observed without the addition of SW. Image (d) was taken on day 12 of subculturing, when the cell growth volume rate was increased from 3.4 to 4.2 ml per day yielding total algae concentrate volume of 50.4 ml after 12 days of subculturing. The cell growth rate obtained after day 12 suggested that the cell mass volume was increased by 24% giving a growth reduction of 36% in contrast to previous day growth rate likely due to limiting sugar in the medium. The colonial as well as rapid growth of *C. Vulgaris* after the addition of SW as observed on day 11 of subculturing might indicate towards *C. Vulgaris* tendency for organic carbon as a preferred feed for their growth than CO_2, which did not result in the colonial as well as faster growth of the cells. Rather, with CO_2 as the feed for the growth of algae cells, the cells were always seen in scattered form perhaps suggesting that the cells were under a stress, which either implied towards insufficient carbon availability in the medium or its decreased consumption.



(a) **(b)**

(c) **(d)**

Figure VI-2: Camera images showing *C. Vulgaris* growth pattern during its sub-culturing. Photos taken after days: [a] = 1, [b] = 7, [c] = 11, and [d] = 12.

The growth pattern of *C. Vulgaris* during its sub-culturing suggested that the cell growth was stimulated after the addition of SW into the culture medium, which showed *C. Vulgaris* affinity towards sugar-oriented culture medium and hence its applicability for its cultivation such as in SW (Martinez and Orus, 1991). In addition, it also negated the earlier notion that the growth failure of *Leptolyngbya* strain was the result of either the direct non-supply of pure CO_2 or the inadequacy of the same in the indirect supply of CO_2 through aeration to the culture, which was proved wrong during *C. Vulgaris* culturing.

VI.3 *C. VULGARIS* CULTIVATION IN SW IN LPT

The cultured cells of *C. Vulgaris* were used with SW and nutrient broth (BG-11) in LPT. The sub-culturing of *C. Vulgaris* transpired that the growth of the cells was triggered more by the introduction of SW into the culture medium than by the aeration. Hence, SW was considered as the direct source of organic carbon for the cultivation of *C. Vulgaris* in a larger volume of culture medium in LPT.

EXPERIMENT NO. VI-1

VI.3.1 Free cell cultivation of *C. Vulgaris* in SW with 100% BG-11 broth

SW was prepared for a total working volume of 13 l with a ratio of 13 g sugar per l DW and was directly poured into LPT holding a depth of 5 cm in the tank. *C. Vulgaris* concentrate volume of 40 ml was mixed with a solution of BG-11 broth medium prepared in 50 ml SW containing 22.1 g of BG-11 medium (1.7 g per l SW) along with corresponding trace metal mix solution volume of 13 l (1 ml per l SW). The prepared mix of *C. Vulgaris* and BG-11 broth medium was transferred progressively (millilitre-wise) into LPT so as for the cells to stick to the surface of water in LPT. The inoculation was done at points 1 and 2 in LPT, yielding *C. Vulgaris* cell volume to SW ratio of 1 ml: 260 ml and *C. Vulgaris* cell volume to BG-11 broth ratio of 2.26 ml: 1g or that for every ml of *C. Vulgaris* concentrate, 440 mg of the broth was available in the medium. Before transferring the prepared mix into LPT its pH, which was reading 2.9 was adjusted to 7.2 by adding 1.4 ml of 1M NaOH progressively. 100% BG-11 broth contained nitrogen with a total

concentration of 116 mg l^{-1}, while total phosphorous concentration was 11 mg l^{-1}, giving an N and P ratio of around 10: 1 in the broth. SW recirculation flow rate was set at 40 ml min^{-1} to induce gentle mixing in the medium (OriginOil, 2008). The luminance profile in LPT was set as continuous until the end of the experiment since continuous light to the cultures was preferred in case when the light flux was not variable and of lower intensity around 3000 lux as well (Andresen, 2005). The other reason for continuous mode of light supply to the algae culture had to do with the consistent production of photosynthetic oxygen in the medium, in particular, when the culture was of mixed nature comprising of both algae and bacteria. The results obtained during this experiment were mostly presented in 3-D format and the rotation range while making the graphs for x and y axes along with perspective and depth values were mostly taken as same with respect to each parameter for the sake of relative comparison.

VI.3.1.1 DO, pH and Temperature profile at the bottom of LPT

Figure VI-3 shows the breakup of DO at the bottom of LPT at all monitoring points during 9 days of free cell cultivation of *C. Vulgaris* with 100% BG-11 broth. The data sheet in the graph suggests that SW DO showed a reducing trend towards zero between second and third day of cultivation, giving a reduction rate of 2.6 mg l^{-1} d^{-1}. This higher DO reduction was probably caused by the presence of maximum concentration of nutrients in the cultivation medium, which likely stimulated the growth of inherent bacterial activity thus depleting DO in the medium. However, in the next 24 hr DO was increased up to as high as it was lost during the previous 2 days due to the mass growth of *C. Vulgaris*.

Towards the end of the cultivation run, the DO was again reduced to zero either owing to algae cell lysis, which brought a halt in the growth benthic *C. Vulgaris* or due to the increased oxygen consumption rate by the autochthonous (inherent) bacteria, whose mushroom growth was probably spurred by the presence of saturated nutrients in the medium. This observation appeared to be consistent with the one made by Park et al. (2001).

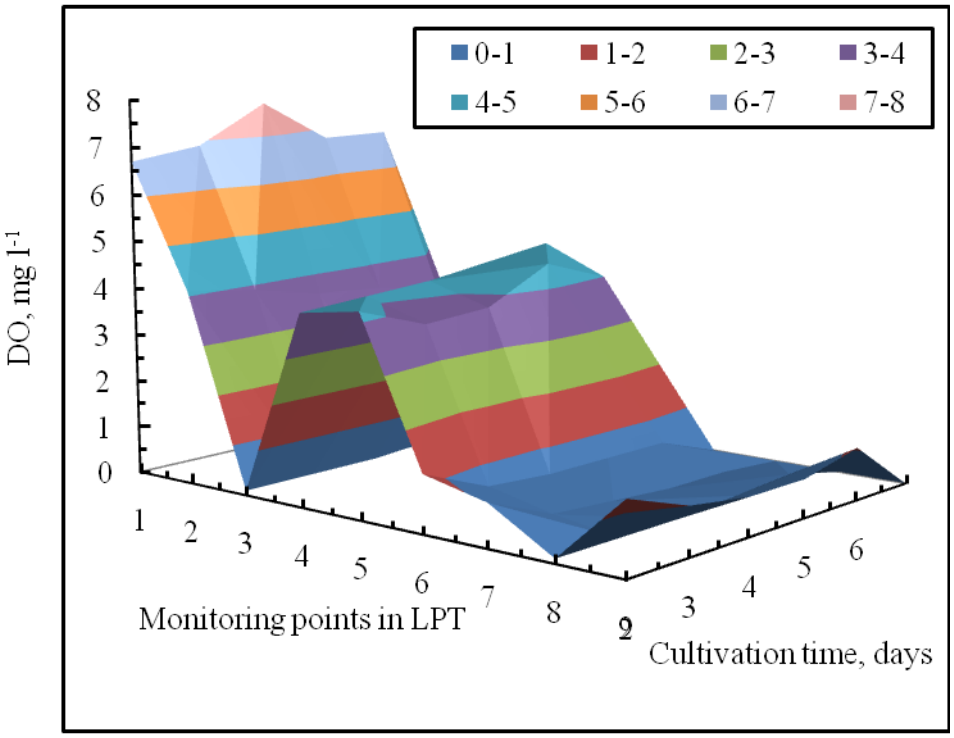


Figure VI-3: DO at all monitoring points at the bottom after 9 days of free cell cultivation of *C. Vulgaris* with 100% BG-11 broth in SW (mean, n=3).

SW pH data as shown in Figure VI-4 represents the values for both the surface and bottom in LPT. The graph shows that pH started to decrease after day 2 by 30%, which further decreased after day six by almost the same margin. This reduction

might have been caused by the production of refractory (resistible to biodegradation) organic acids (Chang et al., 2008). In addition, higher growth rate in algae cultures was also one of the factors in causing a pronounced decrease in the pH value of the cultivation medium (Hohe and Reski, 2005).

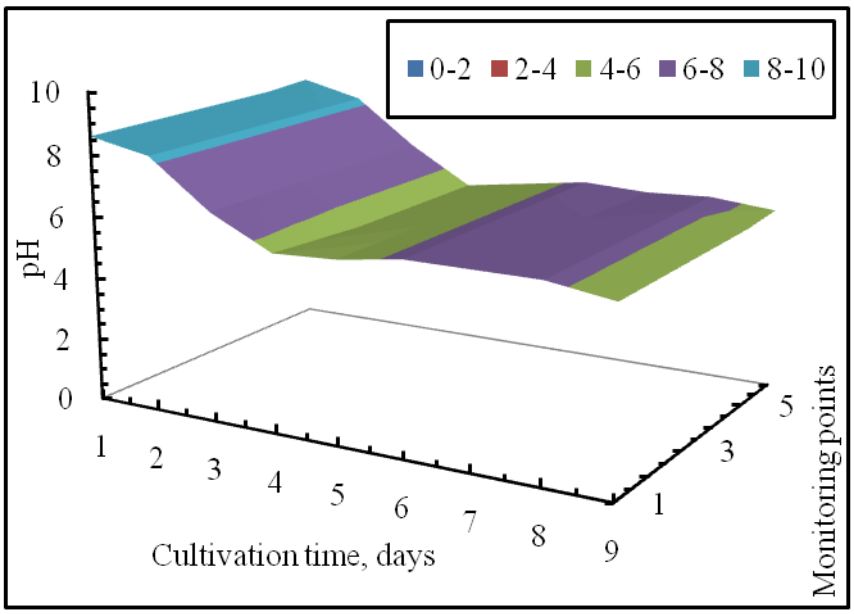


Figure VI-4: pH at all monitoring points at the bottom after 9 days of free cell cultivation of *C. Vulgaris* with 100% BG-11 broth in SW (mean, n=3).

The temperature profile at the bottom of LPT during *C. Vulgaris* cultivation for 9 days is presented in Figure VI-5. The worksheet in the graph shows that temperature at the bottom remained in the range of 20-25^{0}C, with the fluctuation in the temperature during 9 days of cultivation was around 3^{0}C on the lower side and by 1^{0}C on the upper side from the daily average temperature of 24.1^{0}C.

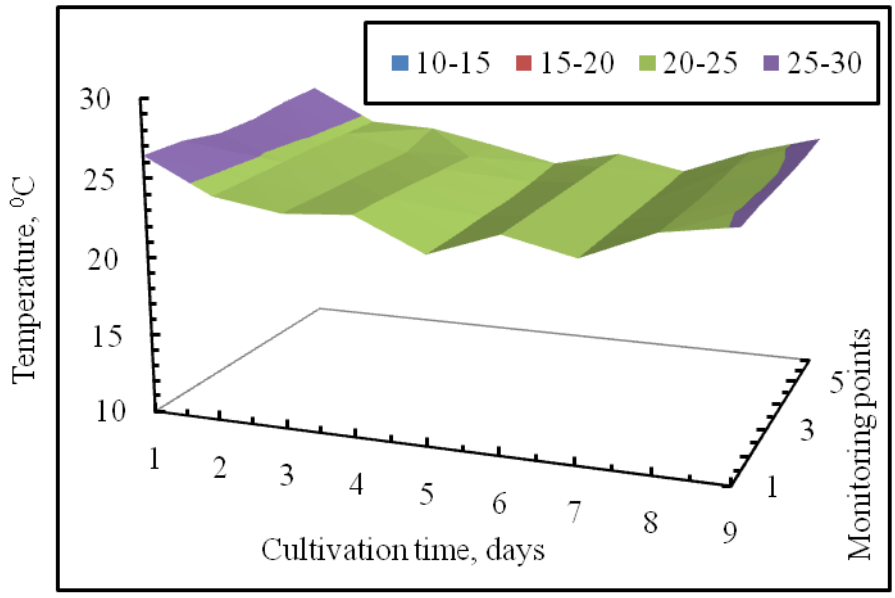


Figure VI-5: Temperature at all monitoring points at the bottom after 9 days of free cell cultivation of *C. Vulgaris* with 100% BG-11 in SW (mean, n=3).

VI.3.1.2 DO, pH and Temperature profile at the surface of LPT

DO values obtained at the surface of LPT during 9 days of *C. Vulgaris* cultivation in SW are charted in Figure VI-6. The graph depicts similar DO decreasing pattern as was observed at the bottom; however, the surface graph for DO also suggested that the lowest range of DO values between 0-2 mg l^{-1}, highlighted by the blue colour in the Figure, occurred after day 5 as compared to after day 6 at the bottom as shown in Figure VI-3. This implied that higher oxygen tension was present at the bottom due to benthic nature of *C. Vulgaris*.

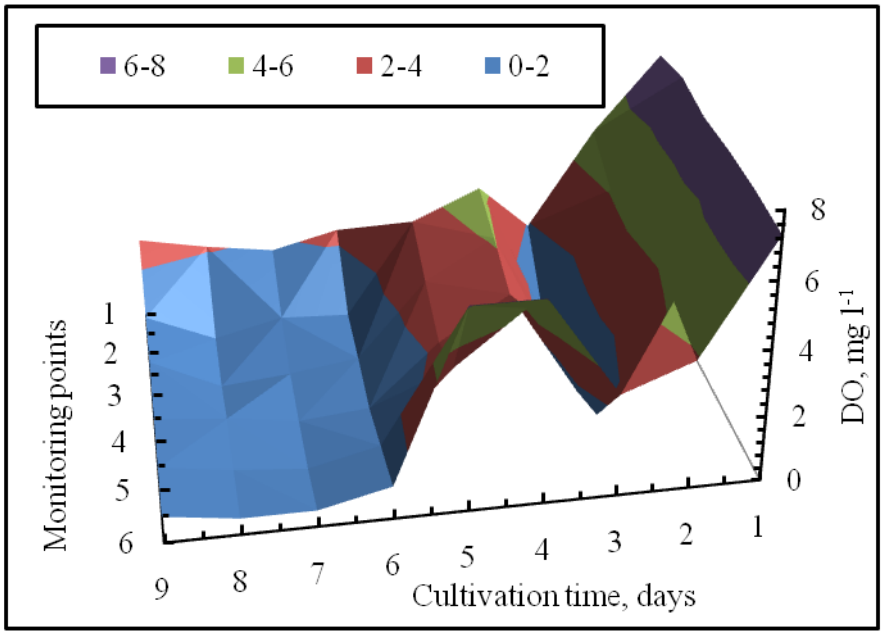


Figure VI-6: DO at all monitoring points at the surface after 9 days of free cell cultivation of *C. Vulgaris* with 100% BG-11 broth in SW (mean, n=3).

The data obtained for SW pH at the surface resembled with that of the bottom values (Figure VI-4) hence not separately shown. The temperature account at the surface of LPT is presented in Figure VI-7. The datasheet in the graph shows that temperature at the surface for the first 4 days was in the range of 25-30^{0}C and from day 5 onwards it was under 25^{0}C. The fluctuation in the temperature caused by the ambient room temperature was in the range of 3^{0}C. The average point temperature at the surface was 24.4^{0}C as compared to 24.1^{0}C at the bottom.

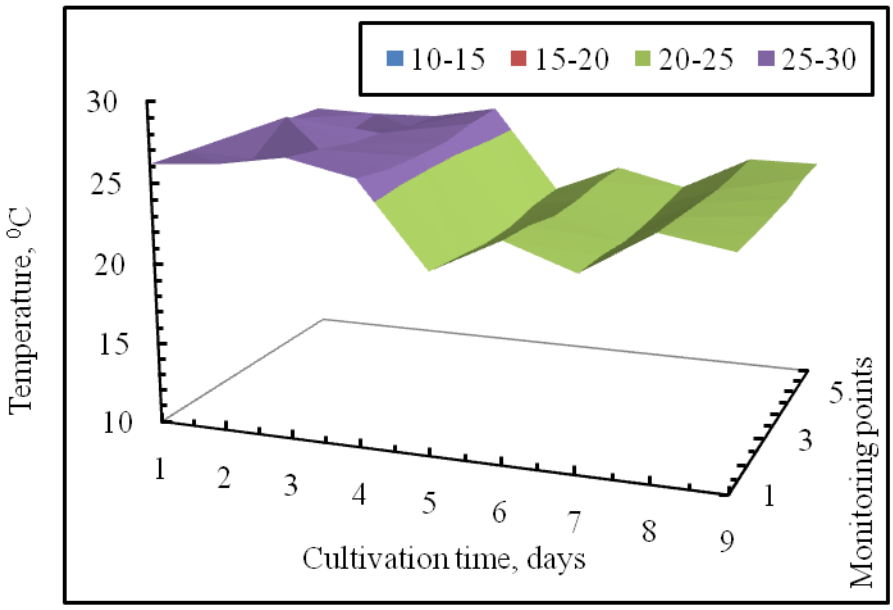


Figure VI-7: Temperature at all monitoring points at the surface after 9 days of free cell cultivation of *C. Vulgaris* with 100% BG-11 in SW (mean, n=3).

VI.3.1.3 Incident light profile

Incident light (IL) values at all the monitoring points during 9 days of *C. Vulgaris* cultivation are highlighted in Figures VI-8 and VI-9 for both bottom and surface layers in LPT respectively. The insolation (light incidence) profile in the bottom graph suggested that maximum decrease in the luminance occurred at points 2 and 5, the high intensity points, as the reduction of around 28% was recorded at these points. The higher IL reduction was likely caused by the presence of higher amount of BG-11 broth in the cultivation medium, which probably led to the turbid conditions all over LPT thus decreasing the incidence of light.

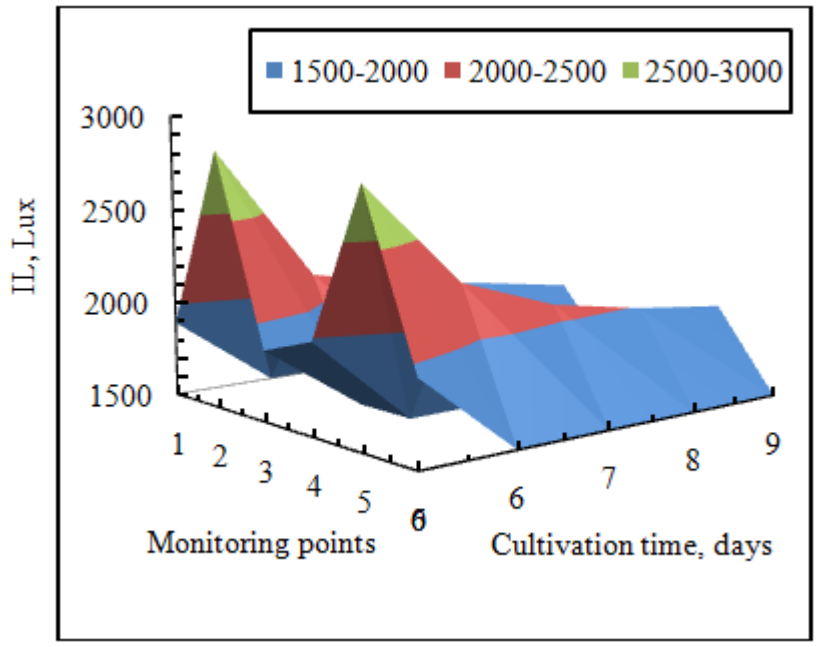


Figure VI-8: Incident light profile at the bottom at all monitoring points during 9 days of *C. Vulgaris* cultivation with 100% BG-11 in SW (mean, n=3).

The reduction in insolation at the surface was lesser as compared to the bottom, as the algae cells were mostly settled at or near the bottom of LPT. The IL at point 2 showed maximum of 15% reduction, whereas it was 19% at point 5 due to higher accumulation of biomass there. As highlighted in Figure VI-9, decrease in IL at the surface started to occur after day 4 of *C. Vulgaris* cultivation, when the algae cells had probably entered into the log phase of their growth. In actuality, the IL attenuation observed at the surface was probably higher than that observed at the bottom, as IL availability at the bottom was already reduced by 18% due to increase in the light path by 5 cm. Thus IL attenuation at the surface was probably higher as compared to the bottom during the log growth phase of *C. Vulgaris*, which could be related with the growth of the biomass in terms of their size and density, which would have allowed the cells to suspend near the surface of LPT.

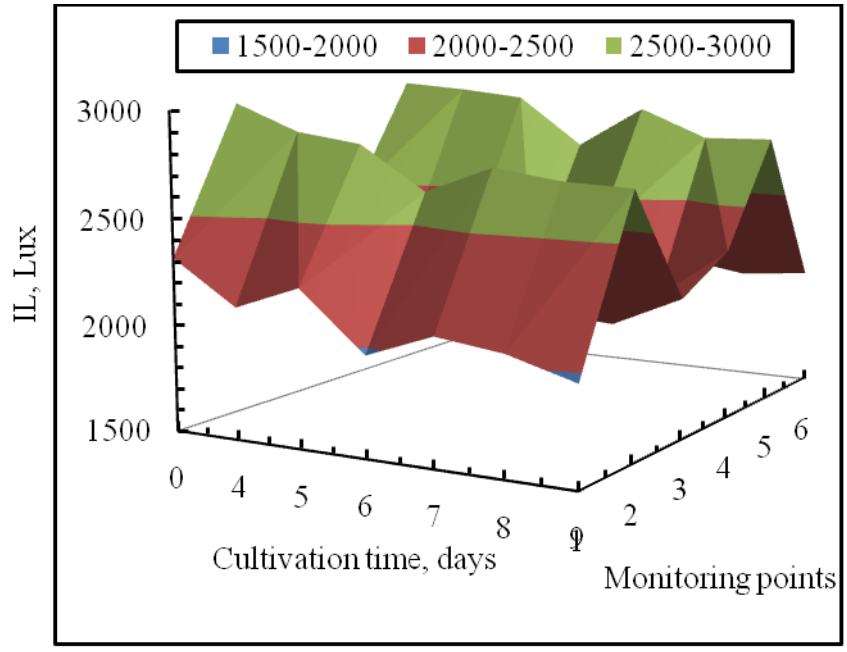


Figure VI-9: Incident light profile at the surface at all monitoring points during 9 days of *C. Vulgaris* cultivation with 100% BG-11 in SW (mean, n=3).

VI.3.2 Experiment Synthesis

Free cell cultivation of *C. Vulgaris* was carried out in SW without external supply of CO_2 via aeration with organic carbon present in SW being the sole source of feed for *C. Vulgaris* growth. After the inoculation of *C. Vulgaris* into the tank, the cells immediately took to the bottom and settled there, as pointed out by A in Figure VI-10. It was observed that the cells were lying in a static condition at the bottom without much growth for the first 3 days suggesting the length of time associated with the lag phase of cell growth since the cells were getting reduced amount of IL at the bottom by 18%. The acclimation period of 3 days for benthic *C. Vulgaris* was perhaps necessary for adaptation of the cells to high concentrations of substrate (sugar) and nutrients present in the cultivation medium.

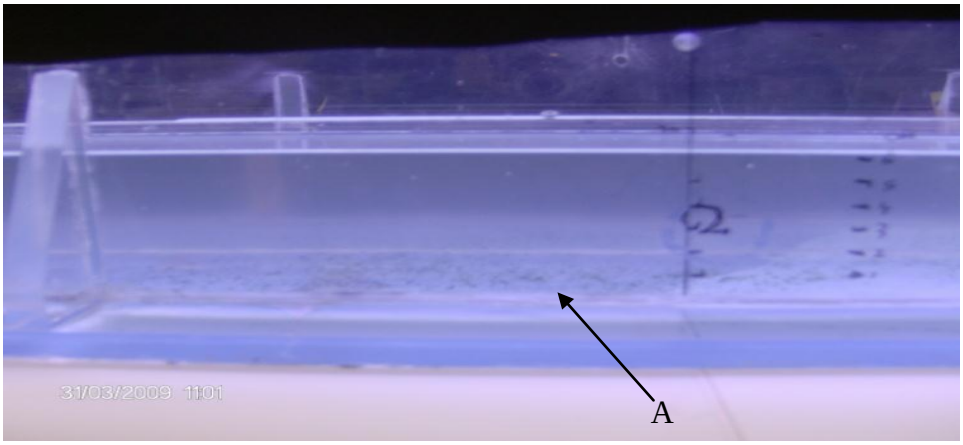


Figure VI-10: Camera photo showing *C. Vulgaris* cells settled at the bottom of LPT immediately after cell inoculation, as indicated by A.

With respect to benthic mode of cultivation of *C. Vulgaris*, two factors can be put forward as an argument. First, the photosynthesis process probably took longer time of 3 days or more to take effect as reflected by the lesser and slower growth of the cells; second and as a resultant effect of the first, the cell-doubling time was perhaps too high (3.3 days) to cause substantial impact on the COD reduction. As observed during the baseline study discussed in the first two chapters, the aerobic degradation of organic carbon in SW started to occur after 40 hr thereby depleting DO in the process. However, during *C. Vulgaris* free cell cultivation in SW, it was observed that DO became a limiting factor within 3 days when it was reduced to zero indicating that until that time or during lag phase of *C. Vulgaris* growth there was actually no substantial evolution of photosynthetic oxygen in the cultivation medium (De Schryver et al., 2008). This drastic decline in DO, which was not the case during the baseline experiments was perhaps triggered by the addition of higher concentration of BG-11 broth exacerbating the oxygen depletion due to probably rapid growth of autochthonous or indigenous bacteria, resulting in the

faster consumption of DO in the medium. The argument concerning the DO replenishment by *C. Vulgaris* until after 3 days of free cell cultivation appeared to be consistent with those made by Martinez and Orus (1991) as well as Irvine (2003) who stated that *C. Vulgaris* respiratory or photosynthetic rates were clearly stimulated by sugar addition in the culture medium and that it took 72 hr for *C. Vulgaris* to enter into their log growth phase. This meant that mixotrophic cultivation of *C. Vulgaris* took the cells 72 hr or higher to enter into log growth phase as observed by the authors, whereas in this study of heterotrophic cultivation of *C. Vulgaris* it took the cells around the same time to enter into exponential growth phase without the traditional supply of inorganic carbon to the culture. Figure VI-11 is the image taken after 72 hr showing the start of *C. Vulgaris* growth as well as the cell suspension little above the bottom of LPT.

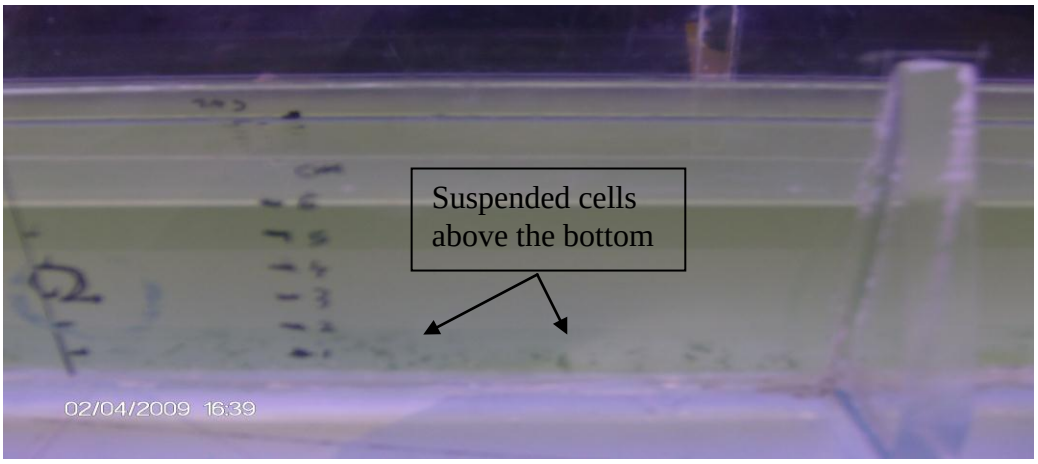
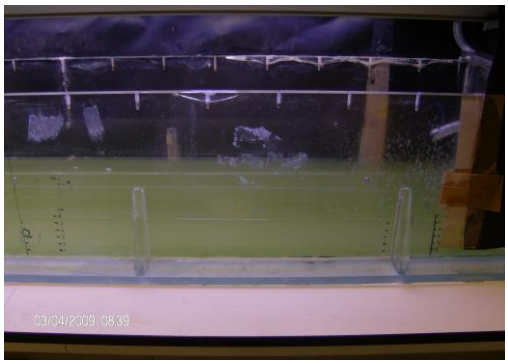


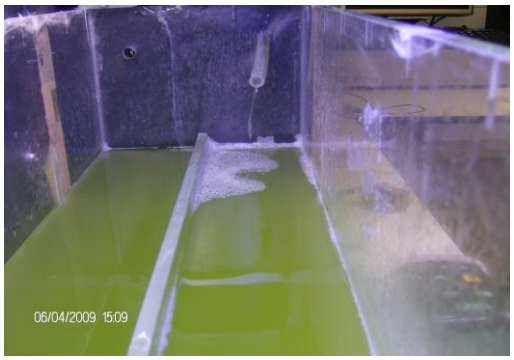
Figure VI-11: Camera photo taken after 72 hr of free cell cultivation of *C. Vulgaris* with 100% BG-11 broth showing the start of the algae cell growth as indicated by the green color of medium and suspension of the cells 1 cm above the bottom.

The pH reduction started to occur after 48 hr of cell cultivation initially due to the breakdown of organic carbon into its derivatives by hydrolysis reaction and later owing to algae cell growth, which ensured saturated DO values in the medium encouraging inherent bacterial activity resulting in the release of acidic compounds thereby reducing the pH (Song et al., 2011). After 96 hrs of cultivation, when pH reduction was around 35% and with pH reading as 5.8 all around the tank, it was buffered progressively with 1.5 ml of 1M NaOH at each of the six points in the tank, which increased the pH above 6 at points 1 to 4. Since SW recirculation in the tank was causing the contents to move ahead through the action of water waves towards point 5 and 6, this resulted in likely accumulation of most of the acidic compounds at these points, hence pH neutralization at points 5 and 6 required more buffer of 0.5 ml each. Every 1 ml of the prepared alkali solution added in 13 l SW in LPT was causing an increase in pH by 0.2-0.3. This meant that pH was raised from 5.8 to 7.4 after the addition of 10 ml NaOH in toto. It was also noticed that during the bacterial activity time period of SW decomposition (after the first 48 hr), SW pH reduced at 1.2 day^{-1}. However, after the addition of alkali buffer solution, it diminished with a faster ratio of 4.8 day^{-1} likely due to the presence of hydroxyl ions in the buffer solution, which probably stimulated the degradation rate causing a sharp decline in pH. This meant that the use of BG-11 broth with higher concentration necessitated buffering of the cultivation medium for pH regulation after triggering its sharp reduction. Figure VI-12 shows multiple grabs of *C. Vulgaris* growth in LPT with image A showing the possible start of exponential growth phase occurring after 3 days of cultivation as depicted by light green color of SW. Image B depicts the log phase growth of *C. Vulgaris* until day

5 of the cultivation as suggested by the bright green color of SW. Image C highlights the stationary or decreasing growth of *C. Vulgaris* after 8 days of cultivation caused by the cell decay probably due to fluctuating environmental parameters such as pH reduction, DO depletion and saturation and COD increase, which is highlighted by the disappearing green colour of the cultivation medium (Danquah et al., 2009).



A



B

C

Figure VI-12: Camera snaps showing different growth phases of *C. Vulgaris* cultivation with 100% BG-11 in SW in LPT after time (days): A = 3, end of lag growth phase and start of log growth phase, B = 5, continuance of log growth phase, and C = 8, decay or death or stationary phase.

Table VI-2 shows growth of *C. Vulgaris* in terms of cell dry mass determined after every 24 hr. Each time 100 ml sample was taken at the same time in the morning from the middle point 2 in LPT, which was first centrifuged at 40 rpm for 30 min followed by drying of separated algae cell mass in oven for 1 hr at 105^0C. The data in Table VI-2 vindicated the argument that *C. Vulgaris* cells started to multiply at faster rate after 3 days of cultivation with a maximum doubling time of almost 2 in 24 hr. This growth pattern continued until the end of day 6 with an overall doubling time of 1.2 for the 6 days. The last 2 days of cultivation showed dwindling growth of *C. Vulgaris* likely due to cell decay, which could have been caused by limiting growth conditions in LPT. This condition was likely caused by the IL attenuation due to mushroom cell growth in the medium as well as possible generation of inherent bacterial mass as well as the non-removal of saturated DO from the cultivation medium (Chisti, 2008).

Table VI-2: *C. Vulgaris* growth analysis during its free cell cultivation in SW with 100% BG-11 broth (mean, n=3).

Cultivation time, days	*C. Vulgaris* cell dry mass, g 100 ml^{-1}	*C. Vulgaris* cell dry mass, g l^{-1}
0	0.02	0.2
1	0.03	0.3
2	0.04	0.4
3	0.08	0.8
4	0.21	2.1
5	0.43	4.3
6	0.78	7.8
7	0.15	1.5
8	0.04	0.4

Table VI-3 presents SW COD profile monitored after every 24 hr of *C. Vulgaris* cultivation. Each time 1 ml of the sample for COD analysis was taken from the middle point 2 at the same time in the morning for comparative analysis. However, as a check with other points in the tank, samples from point 5 were also analysed randomly, which showed insignificant difference in the results as compared to ones shown in the following table. The data in Table VI-3 suggested that maximum reduction in COD occurred after 7 days by 67%, suggesting the continuous consumption of organic carbon by *C. Vulgaris* cells. However, the last

2 days of cultivation yielded in an increase of COD by 49% implying the cell decay and probably resulting in the increase of organic profile or COD of the cultivation medium. From this observed COD profile it can be inferred that free cell cultivation of *C. Vulgaris* in SW had resulted in overall COD reduction 0f around 65%.

Table VI-3: Diurnal SW COD profile during *C. Vulgaris* free cell cultivation in SW with 100% BG-11 broth (mean, n=3).

Cultivation time, days	COD remaining, mg l^{-1}	Cumulative COD removal %
0	16,000	0
1	15,100	+ 6
2	13,900	+ 13
3	12,300	+ 23
4	10,600	+ 34
5	8,400	+ 48
6	6,800	+ 58
7	5,300	+ 67
8	10,400	- 49

Figure VI-13 presents the graphical comparison of COD results obtained during free cell cultivation of *C. Vulgaris* with those obtained without the inoculation of algae cells (Figure V-6). The graph clearly shows the impact of using *C. Vulgaris* cells in SW on its organic loading (COD) during the course of cell cultivation as compared to SW lagooning without using the algae cells, which also resulted in bacteria-induced increase in the COD. The graph suggested that maximum cell growth occurred during days 3 and 7, which resulted in 66% of the COD removal during this time. However, the last 24 hr saw an increase in the COD by around 44% likely due to the release of organic fraction of cell detritus in the medium caused by the cell decay, which was probably induced by IL attenuation in LPT (Chisti, 2007). All the COD values were adjusted with the following formula with the amount of water lost (720 ml day^{-1}) due to water evaporation and sample collection for analysis from the tank.

$$\text{Adj. COD, mg } l^{-1} = \text{COD result } \frac{[\text{working volume of SW} - \text{SW volume lost} * n]}{\text{working volume of SW}}$$

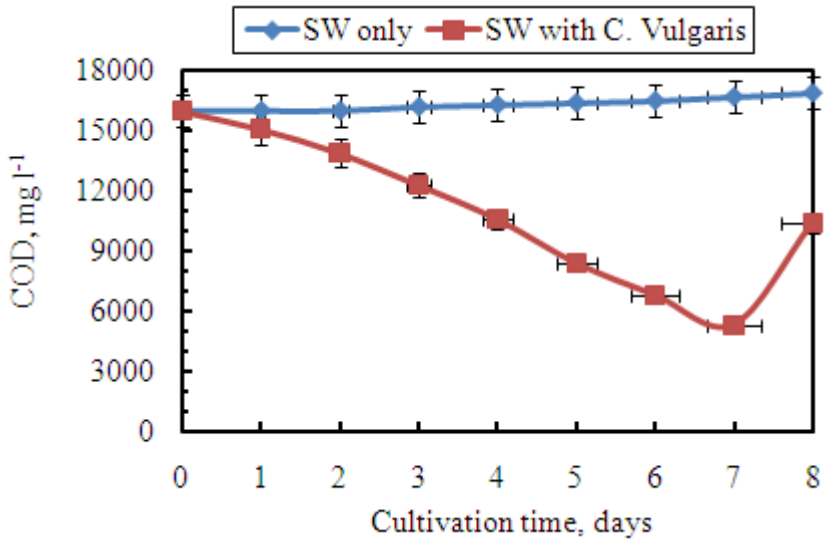


Figure VI-13: COD profile without and with *C. Vulgaris* free cell cultivation in SW with 100% BG-11 broth (mean, n=3).

Figure VI-14 depicts diurnal *C. Vulgaris* growth as against the COD values obtained during free cell cultivation *C. Vulgaris* in SW. The study of the curves in the graph suggested that there is a correlation between the two lines as increase in the growth percentage of *C. Vulgaris* seemed to be proportional to the simultaneous reduction in SW COD. This was fittingly highlighted by the steep rise in *C. Vulgaris* growth after day 4, which coincided with the simultaneous decrease in SW COD until day 6 and similarly by the drop in *C. Vulgaris* growth after day 6 resulting in the corresponding increase in the COD value from the same day. On the whole, it was determined that during the log phase growth of *C. Vulgaris* every 1 g growth in *C. Vulgaris* cell mass caused a COD reduction of 13%. Similarly, the drop in *C. Vulgaris* growth observed after day 6 transpired that every 1g drop in *C. Vulgaris* growth coincided with a COD increase of 730 mg l^{-1}, which was probably caused by the release of soluble organic fraction of the

decaying algae cells along with the suspension of insoluble algae detritus in the cultivation medium.

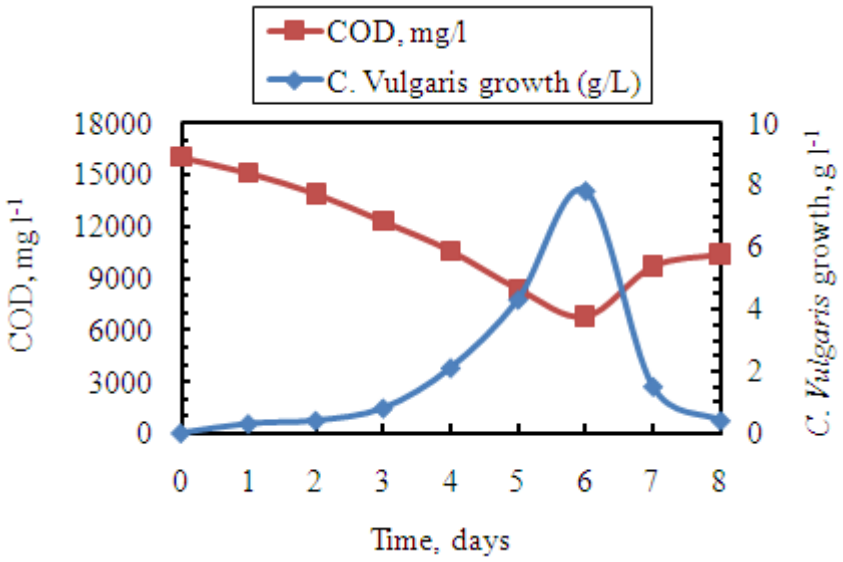


Figure VI-14: Diurnal growth profile of *C. Vulgaris* versus COD removal obtained during *C. Vulgaris* cultivation in SW with 100% BG-11 (mean, n=3).

EXPERIMENT NO. VI-2

VI.3.3 Free cell cultivation of *C. Vulgaris* in SW with 20% BG-11 broth

C. Vulgaris free cell cultivation was carried out with lesser amount or 20% BG-11 broth keeping the algae cell volume inoculum of 40 ml as same. SW was prepared by dissolving 170 g of commercial sugar into 13 l DW (13 g per l) and was poured directly into LPT creating water depth of 5 cm in the tank. *C. Vulgaris* cell concentrate volume of 40 ml, separated from culture medium by centrifugation, were added in a freshly prepared solution of BG-11 broth of 20% purity. The solution was prepared by taking 4.5 g of the broth powder which were dissolved in

1 l DW and added with 2.7 ml of trace metal mix solution. The prepared mixture of *C. Vulgaris* and BG-11 broth gives the cells to broth ratio of 11 ml: 1 g or 1 ml: 0.1 g as compared to 1 ml: 0.4 g used during *C. Vulgaris* free cell cultivation with 100% BG-11. The prepared mix of *C. Vulgaris* and the broth was transferred progressively via sterilized pipette into LPT at points 1 and 2 with equal volumes. The cultivation time for this run was set as maximum of 4 days to investigate the algae growth pattern until the exponential phase of cell growth. SW recirculation was set at 40 ml min^{-1} and the luminance exposure to the cells was kept continuous because of the threshold level of light intensity of around 2600 lux. Figure VI-15 shows the pH data at points 2 and 5 in LPT during *C. Vulgaris* cultivation with 20% BG-11 in SW. The surface values were observed to be identical with the bottom values. The graph shows slight but steady decline in the pH value by 19% until the end of experiment with an overall decreasing rate of 0.38 day^{-1}, which was around 40% lesser decrease in pH than that observed during *C. Vulgaris* free cell cultivation in SW with 100% BG-11 broth.

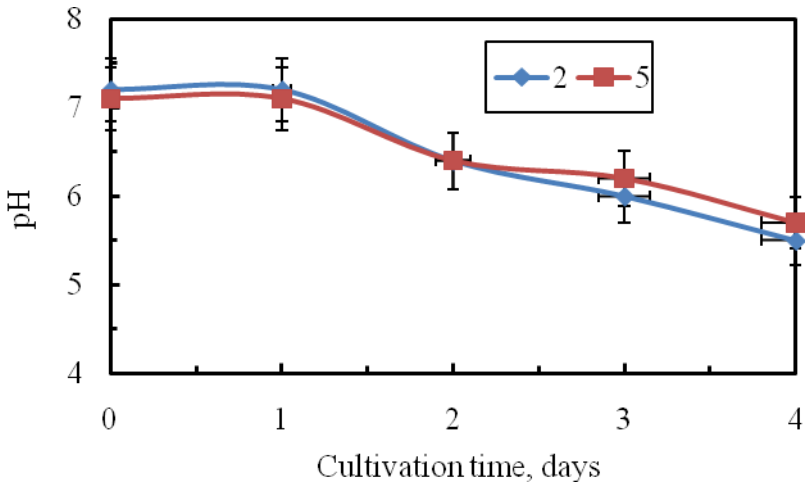


Figure VI-15: pH during C. Vulgaris free cell cultivation in SW with 20% BG-11 broth in LPT (mean, n=3).

SW DO values obtained at points 2 and 5 during *C. Vulgaris* free cell cultivation in SW with 20% BG-11 broth are shown in Figure VI-16. The graph shows reduction pattern in SW DO until day 3 of cultivation, caused by the indigenous microbial activity feeding on the organic matter in SW. However, the decline in DO was relatively lesser by 49% as compared to *C. Vulgaris* cultivation with 100% BG-11 broth in which DO was completely consumed and reduced to zero. This could be attributed to the addition of lesser amount of the nutrient broth during this run, thus resulting in reduced impact on the DO profile leading to lower consumption of DO by the inherent bacterial activity probably due to decrease in the mushroom growth of autochthonous bacteria (Song et al., 2011). The periodic analysis of DO results in the graph suggested that during the first 2 days SW DO decreased by 29% lesser and increased by 12% during the next 2 days of log growth phase as compared to none during *C. Vulgaris* free cell cultivation with the higher broth. This suggested that net photosynthetic oxygen generation due to *C. Vulgaris* growth was around 4-5 mg l^{-1} d^{-1}, considering the daily oxygen consumption by the native bacteria.

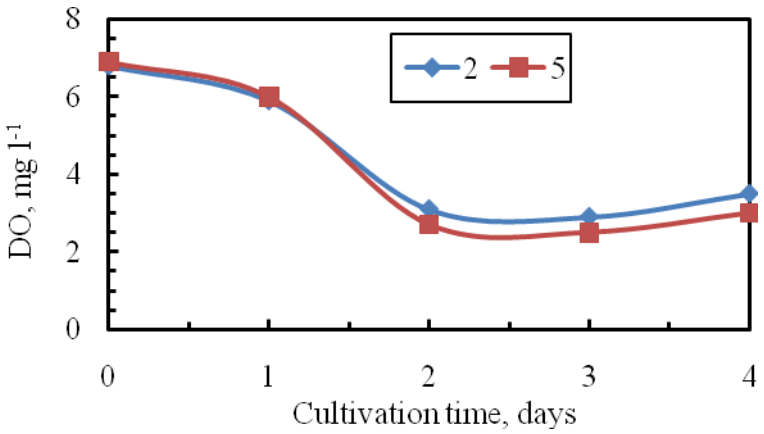


Figure VI-16: DO during C. Vulgaris free cell cultivation in SW with 20% BG-11 broth in LPT (mean, n=3).

Figure VI-17 presents the comparison of average SW temperature at both the surface and bottom of LPT. The daily temperature was 24^0C with fluctuation in the temperature was observed by around 1^0C either side of the average value. The graph also shows that bottom temperature was 10% on average higher than the surface temperature particularly between days 2 to 4. This was likely due to the increase in the algae cell concentration as a result of their growth close to the bottom along with the influence of recirculation inducing heat transfer from surface to the bottom, thus neutralizing the surface temperature and likely causing the retention of heat contents more at the bottom (Sayed and El Shahed, 2000).

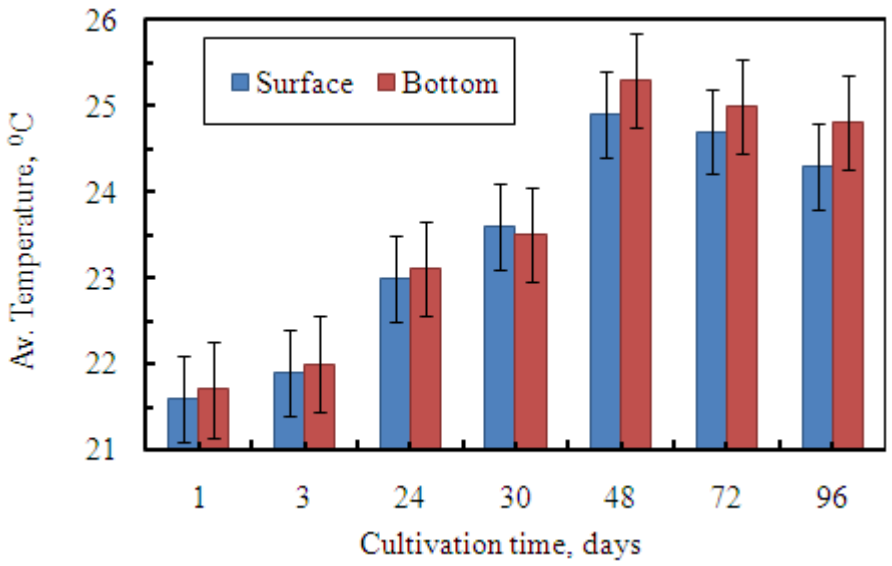


Figure VI-17: Average temperature at the surface and bottom of LPT during C. Vulgaris free cell cultivation in SW with 20% BG-11 broth (mean, n=3).

Figure VI-18 is the comparison graph between the COD values obtained during *C. Vulgaris* free cell cultivation in SW with 100% and 20% BG-11 broth. It is evident from the graph that use of lower concentration of BG-11 broth during *C. Vulgaris* free cell cultivation resulted in the COD values which were comparable with those obtained during *C. Vulgaris* cultivation with higher BG-11 broth. This could mean that with the utilization of lesser concentration of the nutrients in the cultivation medium maximum uptake of the nutrients by *C. Vulgaris* cells was effected under their limiting concentration. This implied that the use of high concentration of nutrients during algae cultivation could be avoided, which resulted in the faster depletion of DO due to mushroom growth of autochthonous bacteria in the cultivation medium spurred by nutrient saturation.

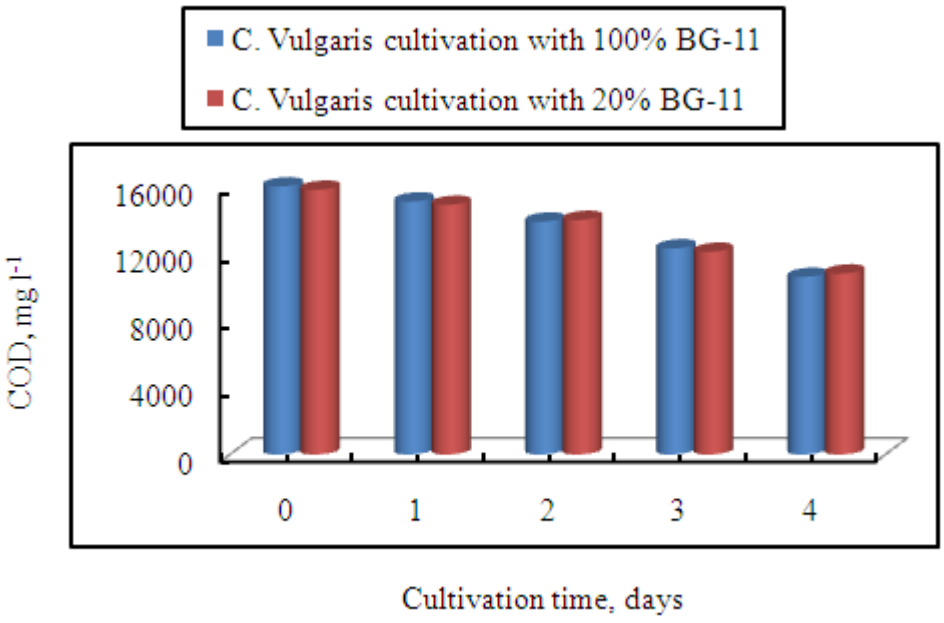


Figure VI-18: COD comparison during *C. Vulgaris* free cell cultivation in SW with 100% and 20% BG-11 broth (mean, n=3).

Figure VI-19 depicts the correlation between *C. Vulgaris* growth and COD values obtained during *C. Vulgaris* free cell cultivation in SW with 20% BG-11 broth. The data in the graph indicated that during the lag phase initial cell growth by 50% may be correlated with 10% COD removal, while an increase in the cell mass by 100% or more during the log growth phase induced 21% decrease in the COD value. By the end of the cultivation run total grown cell mass of *C. Vulgaris*, which was 24% lower than that obtained during *C. Vulgaris* cultivation with 100% BG-11, had resulted in total COD removal of 41% at 1650 mg l^{-1} d^{-1}. It was estimated that every 1 g growth in the cell mass was equivalent to COD removal of around 2900 mg l^{-1}, which was 14% higher than that observed during *C. Vulgaris* free cell cultivation with 100% BG-11 for 4 days. This suggested that *C. Vulgaris* cells were showing more affinity towards substrate utilization under limiting concentration of the nutrients.

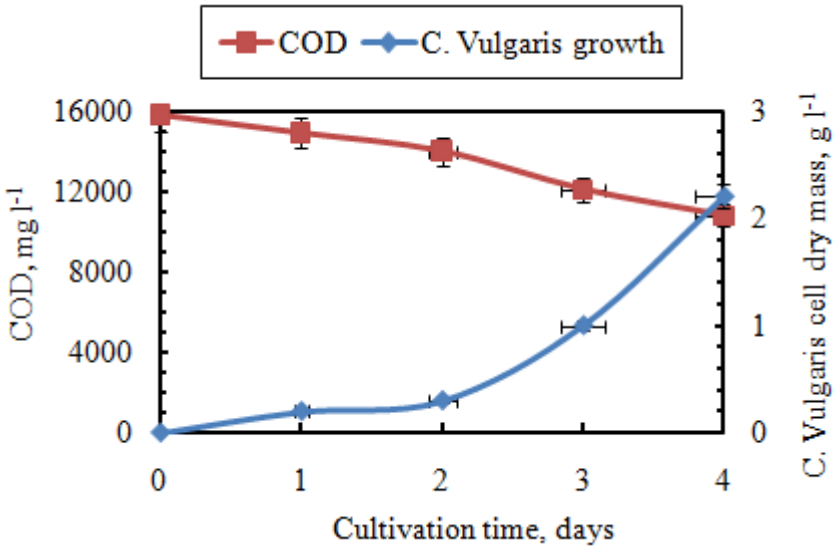


Figure VI-19: *C. Vulgaris* growth versus COD removal during *C. Vulgaris* free cell cultivation in SW with 20% BG-11 broth (mean, n=3).

VI.3.4 Experiment Synthesis

C. Vulgaris free cell cultivation was carried out with BG-11 broth, which was lower in concentration by 80% as compared to that used in the previous run. The results obtained in this experiment indicated that the addition of lower amount of nutrient broth yielded in slightly higher uptake of the substrate as compared to the overall lower substrate utilization observed during *C. Vulgaris* cultivation with higher concentration of the broth up to 4 days. This could be because of the triggering effect of limiting concentration of nutrients present in the medium during *C. Vulgaris* free cell cultivation with 20% BG-11 on the substrate utilization (Scragg et al., 2002). The use of the lower concentration of the broth also resulted in reduced impact on pH and DO values as against the limiting profiles of pH and DO observed during *C. Vulgaris* cultivation with 100% BG-11 broth. Overall COD removal observed during this run was 26% lower than that obtained during *C. Vulgaris* cultivation with 100% BG-11 broth. The lower COD removal observed during *C. Vulgaris* free cell cultivation with 20% BG-11 broth probably reflected maximum substrate consumption mostly by *C. Vulgaris*, whereas during *C. Vulgaris* free cell cultivation with 100% broth the substrate utilization might also have been influenced by the presence of inherent bacterial mass mushroomed by the higher concentration broth. The cells were observed to be in static state at the bottom of the tank for the first 2 days before their rise 1 cm above the bottom during the log phase of their growth likely due to size and mass gain by the grown-up cells. To improve upon the overall growth profile of *C. Vulgaris* via cell immobilization at the surface of the tank copolymer addition with optimum dosing may be a factor towards increased efficiency of the process.

VI.4 IMMOBILIZED CULTIVAITON OF *C. VULGARIS* IN SW WITH COPOLYMER POLYACRYLATE POLYALCOHOL

EXPERIMENT NO. VI-3

VI.4.1 *C. Vulgaris* cultivation in SW with N and P ratio of 5: 1 immobilized with copolymer dose of 160 mg l^{-1}

SW was prepared by the same weight-volume ratio of 13 g sugar per litre DW for 13 l of prepared SW, which was poured directly into LPT already treated with the anti-bacterial chemicals such as Sodium hypochlorite and Hydrochloric acid. The copolymer, Polyacrylate polyalcohol (PP), which, as transpired from the literature, has not been trialled before for algae cell immobilization, was used during the ensuing cultivation runs. For *C. Vulgaris* suspension at the surface various other polymers such as Polystyrene divinylbenzene copolymer (Zhang and Chuang, 2001), Praestol (Pushparaj, 1993) and polyelectrolytes (Tenny, 1969) have been used for the immobilization of algae cells. The copolymer dose of 2 g (160 mg l^{-1}) was added around the middle points 2 and 5 in LPT. After 20 min of copolymer stretching and suspension at the surface, 40 ml of cultured cells of *C. Vulgaris* were progressively inoculated on expanded copolymer colonies at the surface. The medium water was recirculated continuously at 40 ml min^{-1} under continuous luminance. This cultivation run was carried on for 7 days to study the performance parameters such as pH, DO and COD profile for the longer time. For nitrogen and phosphorous supplementation in the cultivation medium relevant chemicals were

added in LPT as a source of these nutrients at the start of the run. Table VI-4 shows the chemicals used along with their quantities.

Table VI-4: Chemicals used with a ratio of 5: 1 as nutrient source during immobilized cultivation of *C. Vulgaris*.

Chemical name	Formula	Quantity used, g
Ammonium bicarbonate for Total Nitrogen	NH_4HCO_3	8.3
Potassium dihydrogen phosphate for Total Phosphorous	KH_2PO_4	1.7

VI.4.1.1 pH

Figure VI-20 presents pH values at points 2 and 5 in LPT during 7 days immobilized cultivation of *C. Vulgaris*. The addition of copolymer in the cultivation medium increased the pH by 34%, which started to decrease after 2 days during the log growth phase of *C. Vulgaris*. By day 4, pH had decreased at 0.4 d^{-1}, compared to the decrease of 0.5 d^{-1} observed during SW lagooning with 2 g PP without the inoculation of algae cells. By the end of the run, pH had further decreased at cumulative rate of 0.3 d^{-1}.

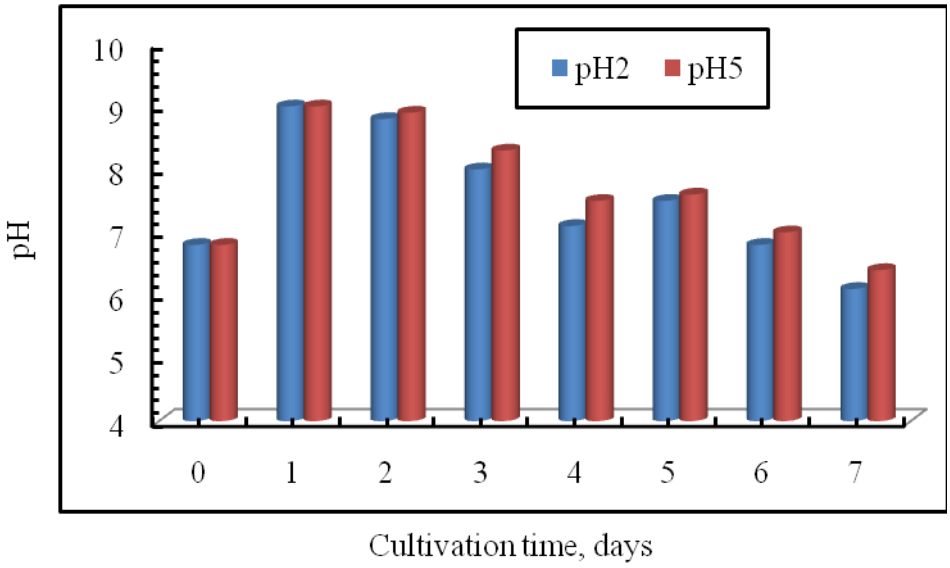


Figure VI-20: pH account at the middle points 2 and 5 during 7 days of *C. Vulgaris* cultivation in SW immobilized with copolymer dose of 160 mg l^{-1} (mean, n=3).

VI.4.1.2 DO and Temperature

The data for DO and temperature at the middle points 2 and 5 is shown in Figure VI-21. The graph shows that DO increased at 0.3 d^{-1} during the first 4 days of cultivation as compared to previous cultivation run during *C. Vulgaris* free cell cultivation when DO tended to decrease during the initial phase of growth. However, afterwards and until the end of the run DO started to decrease for the next 3 days at the higher rate of 0.4 d^{-1}. An increase in the temperature by 1.5^0C was observed until the first 4 days of cultivation before it fluctuated by almost the same margin during the next 3 days.

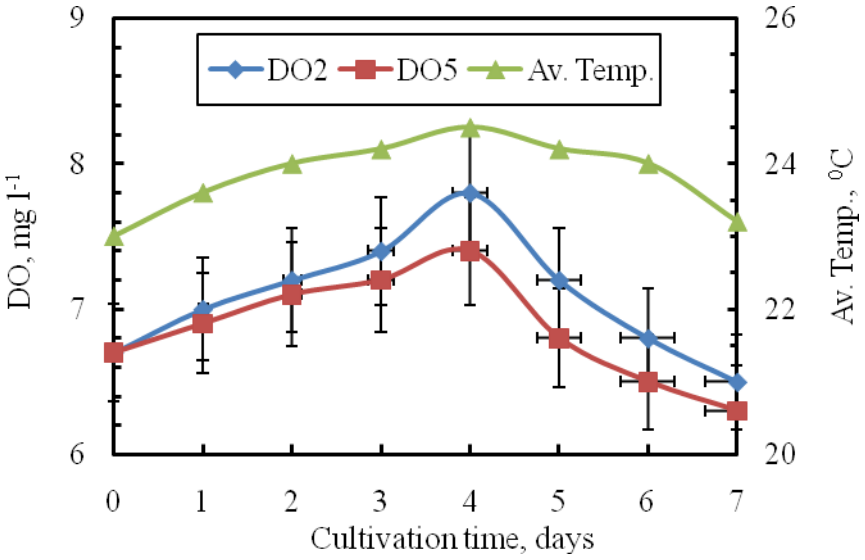


Figure VI-21: DO and average temperature data at the middle points 2 and 5 during 7 days of *C. Vulgaris* cultivation in SW immobilized with copolymer dose of 160 mg l^{-1} (mean, n=3).

VI.4.1.3 COD

SW COD results obtained during C. Vulgaris cultivation immobilized with copolymer dose of 160 mg l^{-1} are presented in Figure VI-22, which are compared with COD values obtained during C. Vulgaris free cell cultivation with 20% BG-11 broth. The initial COD removal after 2 days during immobilized cultivation was 14% as compared to 17% during free cell cultivation. Almost the same trend was observed after 4 days of cultivation when 38% of COD removal was recorded during immobilized cultivation as compared to 42% during free cell cultivation. However, after 4 days and until the end of the run COD was observed to increase by 7% on average after every day with the value increasing by 23% as a whole.

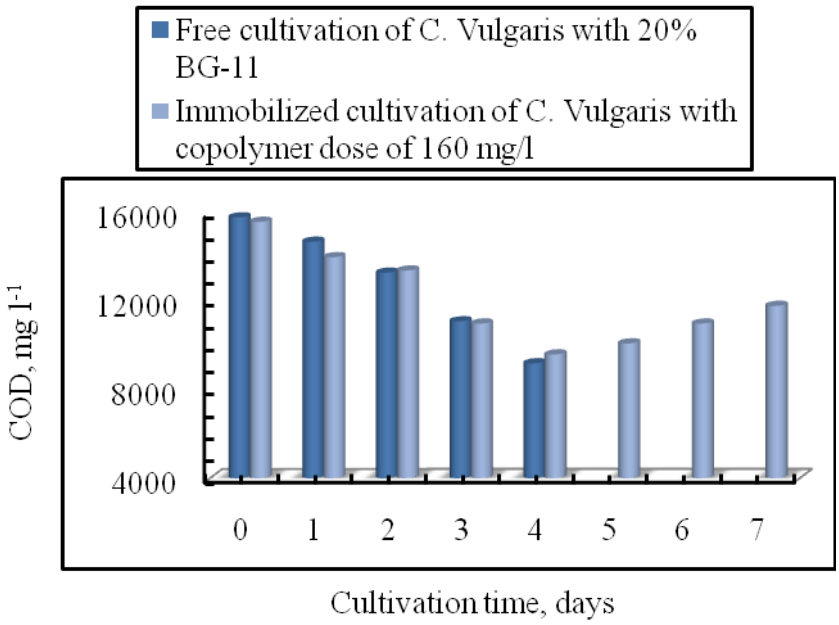


Figure VI-22: COD profile comparison between free cell cultivation of *C. Vulgaris* with 20% BG-11 and immobilized cell cultivation of *C. Vulgaris* in SW with copolymer dose of 160 mg l^{-1} (mean, n=3).

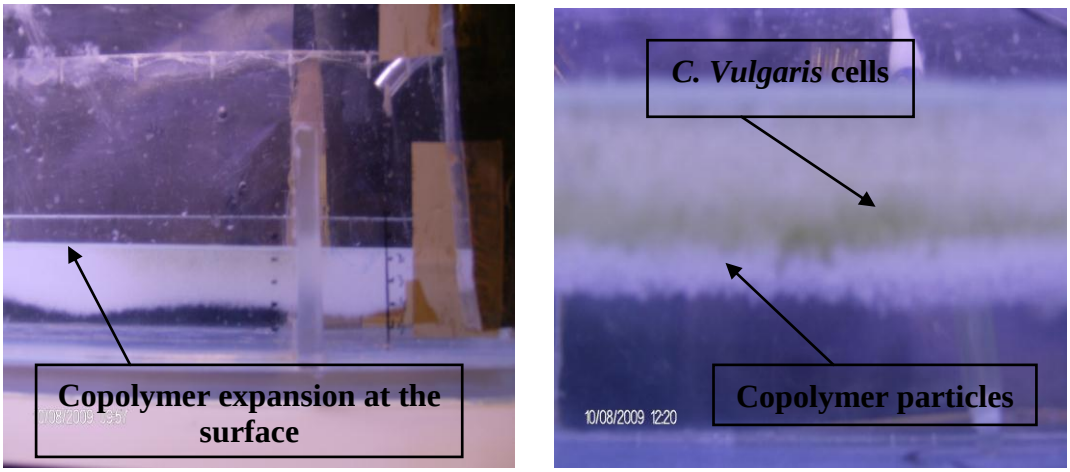
VI.4.1.4 Experiment Synthesis

This experiment was carried out to analyse the influence of using copolymer, Polyacrylate polyalcohol, on the growth of *C. Vulgaris*, which will have a direct bearing on SW COD removal. As was observed previously in SW lagooning with copolymer only, the pH in this experiment was also increased by the same margin of 34%. However, from the experiments held during the baseline study (Chapters IV and V), it was found that increase in the pH value was not dependent on the amount of copolymer used as was observed by using different doses of copolymer during SW lagooning resulting each time in the similar increase in the pH value. This was perhaps because of the specific number of active hydroxyl ions present in

the largely-structured copolymer molecule coming into contact with the hydroxyl ions present in water thus increasing their concentration resulting in the increased pH value of the medium water. The pH decrease of 18% was recorded in this experiment after 4 days of cultivation as compared to 22% during free cell cultivation with almost similar decreasing rate of 0.4 d^{-1}. However, because of copolymer influence pH value in this experiment remained at or near the alkaline range even after cultivation for extended time of 7 days, as opposed to the previous experiment when it reached the acidic range after 4 days of cultivation, which might affect the cell growth. SW DO pattern until 4 days of cultivation was observed as same in both the experiments. However, during this experiment DO was observed to be decreasing after 4 days until the end of experiment, indicating probably the restricted growth of *C. Vulgaris* thus affecting photosynthetic oxygen generation. In addition, the retention of DO value of around 100% observed at the end of immobilized cultivation of *C. Vulgaris* with copolymer dose of 160 mg l^{-1} as compared to the value observed at the end of free cell cultivation of *C. Vulgaris* indicated towards the difference that the use of copolymer during immobilized cultivation of *C. Vulgaris* made. The use of copolymer for the entrapment of algae cells would probably have caused *C. Vulgaris* to grow faster owing to receiving increased IL flux at the surface resulting in the sustained photosynthetic oxygen production as well as reducing the lag phase duration from 72 hr to less than 40 hr. The COD results in this experiment implied that it started to increase after 4 days of *C. Vulgaris* cultivation likely due to halt in the growth of the cells as well as in COD removal, which was probably caused by the higher dosing of copolymer particles affecting the growth cycle of algae cells. *C. Vulgaris* immobilization by

using copolymer colonies at the surface was meant for the cells to receive maximum exposure of fluorescent light at the surface of LPT, which was higher in insolation by 18% than that at the bottom due to decrease in the light path by 5 cm thus influencing the increase in the photosynthetic efficiency by the immobilized cells resulting in a simultaneous impact on the COD removal rate as well.

Figure VI-23 shows the suspension of copolymer dose applied during this experiment in (a) along with *C. Vulgaris* cells being trapped in copolymer colonies in (b).



a **b**

Figure VI-23: Camera images showing in: (a) = Copolymer Polyacrylate polyalcohol particles after 20 min of expansion are suspending at the surface of LPT; and (b) = Admixture of *C. Vulgaris* cells and copolymer particles at the surface after 2 hr of bridging process.

EXPERIMENT NO. VI-4

VI.4.2 *C. Vulgaris* cultivation in SW with N and P ratio of 5: 1 immobilized with copolymer dose of 80 mg l^{-1}

This experiment was held to study the growth pattern of *C. Vulgaris* immobilized with lower copolymer dose of 80 mg l^{-1} while keeping all other inputs of the previous experiment same. The copolymer particles were added around the middle point 2 in LPT before inoculating 40 ml of cultured *C. Vulgaris* after 20 min of copolymer expansion and rise to the surface of LPT.

VI.4.2.1 pH and DO

Figure VI-24 presents the data for SW pH and DO obtained during 7 days of immobilized cultivation of *C. Vulgaris* with copolymer dose of 80 mg l^{-1}. SW lagooning with 50 mL CV cells and 1g polymer Polyacrylate polyalcohol. As observed earlier, reduction in the copolymer dose did not affect the corresponding rise in the pH during this experiment as well. As compared to previous experiment, in which higher copolymer dose of 160 mg l-1 was used, higher pH and DO values were obtained during this cultivation run, which suggested the suitability of the copolymer dose in terms of conducive immobilization of *C. Vulgaris* at the surface of LPT resulting in likely the optimum growth of the algae cells. Overall, pH reduction in this experiment was slightly higher than it was during *C. Vulgaris* cultivation with higher copolymer dose. Similarly, DO results suggested *C.*

Vulgaris cultivation with lower copolymer dose yielded in 19% higher DO retention than that observed during *C. Vulgaris* cultivation with higher copolymer dose.

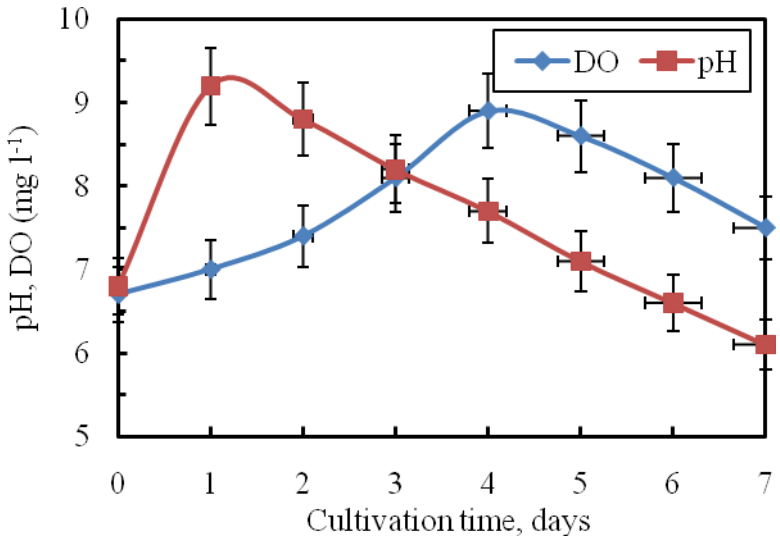


Figure VI-24: pH and DO profile during 7 days of *C. Vulgaris* cultivation in SW immobilized with copolymer dose of 80 mg l^{-1} (mean, n=3).

VI.4.2.2 COD

Figure VI-25 compares the data for SW COD obtained *C. Vulgaris* cultivation immobilized with copolymer dose of 80 mg l^{-1} to that obtained using copolymer dose of 160 mg l^{-1}. The COD results obtained during *C. Vulgaris* cultivation immobilized with lower copolymer dose of 80 mg l^{-1} showed a steady decline until the end of the run as compared to COD removal pattern observed during *C. Vulgaris* cultivation immobilized with the higher copolymer dose of 160 mg l^{-1}, in which COD decrease was halted after 4 days of cultivation.

The halt in COD removal was probably caused by the restricted growth of *C. Vulgaris* due to higher copolymer concentration in the cultivation medium resulting probably in covering of the cell surfaces thereby affecting the photosynthetic process. Overall, 52% COD was removed at 1157 mg l^{-1} d^{-1} during *C. Vulgaris* cultivation immobilized with lower copolymer dose as compared to 24% at 543 mg l^{-1} d^{-1} during *C. Vulgaris* cultivation immobilized with higher copolymer dose.

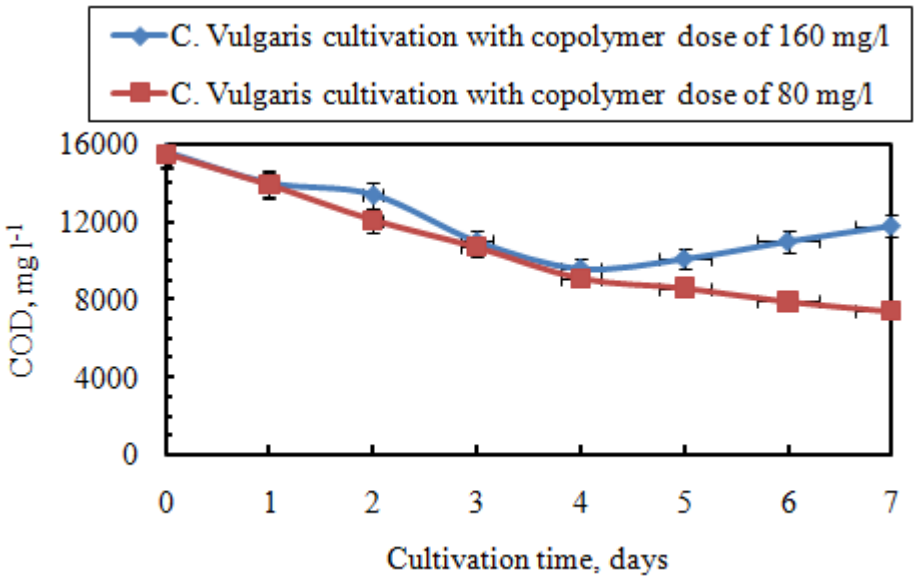


Figure VI-25: COD profile during 7 days of *C. Vulgaris* cultivation in SW immobilized with copolymer dose of 80 mg l^{-1} (mean, n=3).

VI.4.2.3 *C. Vulgaris* growth analysis

C. Vulgaris cell growth was determined by gravimetric analysis in terms of cell dry mass. 50 ml samples were collected after every 24 hr at the same time from the middle point in 100 ml beaker. The collected samples were first centrifuged at 40 rpm for 30 min followed by drying of separated *C. Vulgaris* cells in oven for 1 hr at 105^0C. The combined results for dry mass of *C. Vulgaris* from all the four cultivation runs are presented in Figure VI-26 after adjusting the values obtained in g ml^{-1} to g l^{-1}. It can be inferred from the graph *C. Vulgaris* growth obtained using lower copolymer dose of 80 mg l^{-1} was almost comparable to that observed during *C. Vulgaris* free cell cultivation with 100% BG-11 broth. The overall *C. Vulgaris* growth rate in g l^{-1} d^{-1} obtained with immobilization dose of 80 mg l^{-1} was 0.81 as compared to 0.41 with immobilization dose of 160 mg l^{-1}, 0.52 during free cell cultivation with 20% BG-11 broth and 1.02 during free cell cultivation with 100% BG-11 broth. The structure of the curves in the graph suggests that rapid *C. Vulgaris* growth started to occur from day 2 onwards, with maximum growth occurring between days 3 to 5 of cultivation.

To compare the cell mass growth during the log phase between day 2 to 5, 85% cell mass growth at 1.3 g d^{-1} was obtained during *C. Vulgaris* cultivation immobilized with lower copolymer dose of 80 mg l^{-1}. This was in contrast to 72% and 91% at 0.53 and 1.3 g d^{-1} during *C. Vulgaris* cultivation immobilized with higher copolymer dose of 160 mg l^{-1} and during free cell cultivation of *C. Vulgaris* with 100% BG-11 broth respectively.

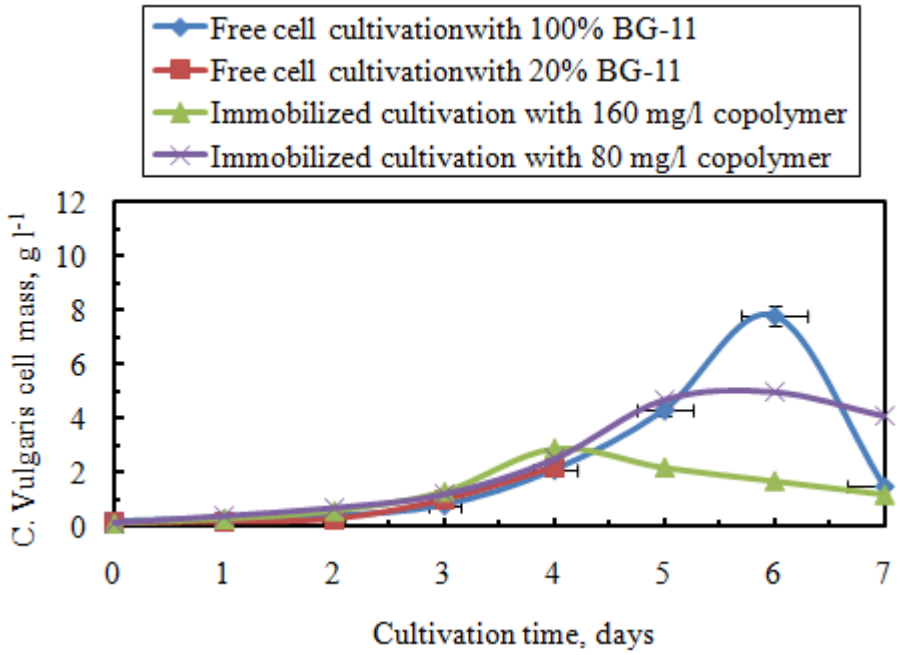


Figure VI-26: *C. Vulgaris* growth profiles during free cell cultivation in SW with 100% and 20% BG-11 broth and immobilized cell cultivation with copolymer doses of 160 and 80 mg l^{-1} (mean, n=3).

VI.4.2.4 Experiment Synthesis

This experiment was held to study the influence of 100% reduction in copolymer concentration on the growth of immobilized *C. Vulgaris* cells. The pH results for this experiment suggested that the use of lower copolymer dose of 80 mg l^{-1} influenced the overall growth of *C. Vulgaris* leading to the increase in SW pH and corresponding reduction in its decrease rate. *C. Vulgaris* cultivation with higher copolymer dose of 160 mg l^{-1} yielded in lower DO profile by the end of cultivation in contrast to that observed during immobilized cultivation with lower copolymer dose. The possible understanding of this could be the likely covering of the cell

surfaces hindering the photosynthetic efficiency of the cells leading to poor substrate and nutrient utilization as well as blocking of the catalytic degradation pathways of the organics by both algae and indigenous bacteria (De Bashan and Bashan, 2010). The initial COD removal of 22% observed during the first 2 days of *C. Vulgaris* cultivation with lower immobilization dose was better than 14% observed during *C. Vulgaris* cultivation immobilized with higher immobilization dose. This was also reflected by the overall COD removal profile with 52% COD removal during *C. Vulgaris* cultivation with lower immobilization dose was observed as against 24% during *C. Vulgaris* cultivation with higher immobilization dose. Use of lower copolymer dose for *C. Vulgaris* immobilization also proved to be a positive multi-factor with respect to conducive *C. Vulgaris* suspension at the surface resulting in enhanced consumption of both the substrate and the nutrients. This likely resulted in the increase in photosynthesis activity by the cells spurred by the reduction in path length by 5 cm as well as increase in the availability of IL by 18% at the surface of LPT.

VI.5 SUMMARY

During pre-culturing of *C. Vulgaris*, it was observed that the cells had showed the tendency towards sugar-oriented culture medium when the cell mass growth rate increased by 60% after the addition of sterile prepared SW into culturing bottles, indicating the ability as well as affinity of *C. Vulgaris* cells to consume organic carbon present in SW. It was observed that the addition of buffer solution of sodium hydroxide to raise the pH of cultivation medium during *C. Vulgaris* cultivation with 100% BG-11 broth, was contributing towards rapid pH reduction, higher by 85% than without alkali addition into LPT, probably stimulating the inherent microbial activity. Most of the DO reduction by over 50% occurred during the log growth phase of *C. Vulgaris* cultivation between day 2 to 4, indicating that the indigenous biological activity started after the initial hydrolysis reaction by day 2. During free cell cultivation of *C. Vulgaris* the cells immediately took to the bottom for the first 3 days and then during the log phase of their growth between day 3 and day 5, the cells rose from the bottom by around 1 cm likely under the influence of larger cell size and density due to their growth. This condition led to the reduced amount of light for the cells to grow at optimum level thus slowing down the cell-doubling time as well.

Points 2 (front middle) and 5 (back middle) in LPT were the high intensity points receiving higher amount of incident light (IL) in the range of 2300 and 2600 Lux at the surface and 2100 and 2300 Lux at the bottom of LPT. The maximum reduction in IL by an average 18% due to the growth of algae cells and SW degradation by the inherent bacteria also occurred at these points.

The COD profile obtained during *C. Vulgaris* free cell cultivation indicated that algae cultivation in SW had direct bearing on SW COD, which was in contrast to COD profile of SW obtained without the inoculation of algae cells. Use of higher broth concentration during *C. Vulgaris* cultivation was resulting in mushroom growth of autochthonous bacteria in the cultivation medium, which were inducing significant variations in pH, DO, IL and temperature of the process leading to declining algae growth during the process as well as increasing the organic profile of the cultivation medium. COD results obtained during *C. Vulgaris* cultivation suggested that increase in the growth percentage of *C. Vulgaris* seemed to be proportional to simultaneous reduction in SW COD on daily basis. However, though it was observed that *C. Vulgaris* was able to be cultivated in SW, yet it was also found out that *C. Vulgaris* cultivation with either lower or higher concentration of the broth nutrients did not result in complete COD removal. The use of lower proportion of nutrient broth for *C. Vulgaris* cultivation yielded in slightly acidic pH values making pH environment conducive for the growth of algae cells. In contrast, use of higher concentration of nutrient broth likely created the condition of nutrient super-saturation resulting in strongly acidic conditions in the cultivation medium needing pH adjustment besides inducting sharp hypoxic DO levels as well as COD increase during the process.

The addition of copolymer Polyacrylate polyalcohol during immobilized cultivation of *C. Vulgaris* resulted in the pH remaining at or near the alkaline range during the process, thus influencing pH-neutral conducive growth environment for the algae cells. Visual observation of the bonding between *Chlorella* cells and the copolymer particles suggested that lower copolymer dose of 80 mg l^{-1} resulted in

an effective *C. Vulgaris* and copolymer agglomeration at the surface of LPT. The higher copolymer dose of 160 mg l^{-1} was observed to factor in the reduced values of DO indicating towards lesser growth of *C. Vulgaris* thus resulting in reduced production of photosynthetic oxygen. This condition probably had occurred due to possible covering of most of the cell surface area with expanded copolymer surface layer thickness of 1-2 cm downwards for the first 2 days during the stretching phenomenon by the particles, causing suppression in the functional activity of the cells. The increased growth rate observed during *C. Vulgaris* cultivation immobilized with lower copolymer dose resulted in the stabilization of COD values as lower COD values with better COD removal rates were accrued as compared to those observed during *C. Vulgaris* immobilization with higher copolymer dose, which yielded in higher COD values mainly due to a halt in *C. Vulgaris* growth. However, this pattern of COD increase did not occur during *C. Vulgaris* cultivation with lower copolymer dose and thus resulting in a consistent decline in COD value, which could be attributed to the non-restricted and continuous growth of *C. Vulgaris*.

CHAPTER VII

SIMULATED SUGAR FACTORY WASTEWATER LAGOONING WITH BACTERIA

CHAPTER VII

SIMULATED SUGAR FACTORY WASTEWATER LAGOONING WITH BACTERIA

VII.1 INTRODUCTION

The results obtained in the previous chapter during *C. Vulgaris* cultivation in SW without and with the addition of copolymer Polyacrylate polyalcohol led to couple of observations come to the fore, which probably necessitate the use of allochthonous or external bacteria in conjunction with the algae cells as a consortium to influence the efficient COD removal rate. Two main factors can be deduced from the results obtained in the previous chapter; One, the minimum DO availability of at least 2 mg l^{-1}, which was in agreement with the observations made by Phong (2008) and Tchobanoglous (2003); Two, stabilization of the COD value by the algae cells through direct consumption of organic carbon present in SW reducing the COD value by around 60%. However, with the inoculation of specific bacteria for the direct breakdown of organic matter, the required maximum COD reduction might be achieved given a conducive cell growth environment comprising of suitable operating conditions of temperature, pH and DO. Given that during cultivation runs with *C. Vulgaris*, a DO profile of around 7-8 mg l^{-1} was retained during immobilized *C. Vulgaris* cultivation with lower copolymer dose, external aeration might not be required for consistent aerobic biodegradation of SW organics by the bacteria (Liu et al., 2007).

VII.2 MONITORING OF BACTERIAL CULTURE

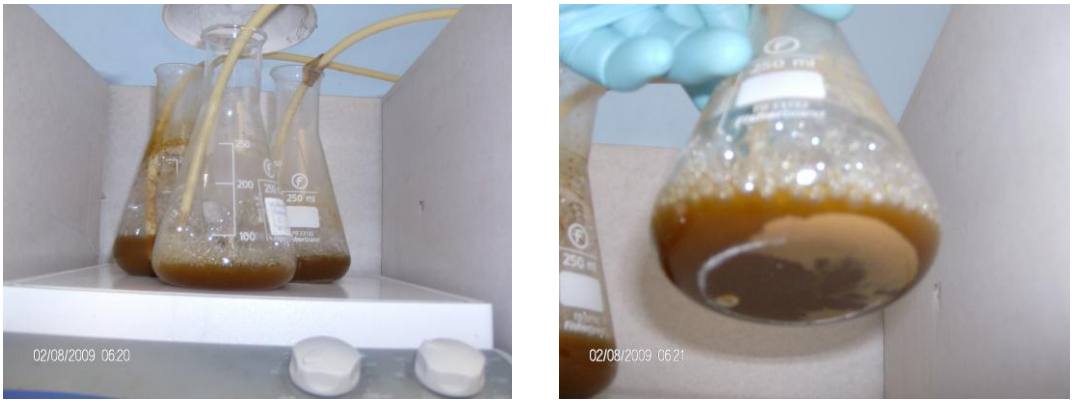
Pseudomonas Putida (*P. Putida*) strain was cultured in the bacteria culturing unit (BCU), which was setup in the lab. Three 20 ml sterilized tubes, two of them each containing 2 ml of the starter culture and the third only 10 ml of the growth medium as a blank to check the emergence of any contaminant bacterial colonies, were kept in BCU for preculturing of the cells for 48 hrs at 37^0C. The periodical monitoring of the culture tubes was held by visually observing the growth of the cells in the form of the development of any suspension in the growth medium. After 40 hr of culture incubation, tubes 1 and 2 had developed certain suspension of off-white color as compared to the blank tube, which was free of any suspension and thus remained as such. For sub-culturing, the contents of tubes 1 and 2 were directly transferred into two 250 ml sterile white glass flasks each already containing 50 ml of the prepared Luria Bertani (LB) medium along with a third flask containing only the same amount of the medium. The flasks were supplied with filtered air at 250 ml min^{-1} continuously via 0.2 µm in-line air filter, through an air pump to maintain the aerobic conditions in the culture medium. Visual observation of the cell growth in culture flasks was done periodically after every 8 hr and suggested that there was no growth visible after the lapse of 24 hr. This was also checked by determining the optical density (OD) test of the cultures using UV-Vis Spectrophotometer. Table VII-1 presents the data related with the OD determination of the culturing flasks. The data in the table shows that until 48 hr of sub-culturing, the cultures had not grown much so as to give optimum OD value of above 0.7 (70% growth), which was obtained after 56 hr of subculturing regime.

Table VII-1: Optical density of the bacterial culturing flasks at different times

Culturing time after, hr	Optical density at 660 nm		
	Flask 1	Flask 2	Flask 3 (blank)
0	0.05	0.04	0.01
24	0.16	0.12	0.01
30	0.31	0.26	0.02
48	0.56	0.49	0.02
56	0.76	0.71	0.03

The grown colonies, which expanded roughly by 0.7 cm in width and 2.4 cm in length and were brownish in colour. For mass culture of grown cells, the cultured contents of the flasks were transferred after every 45 hr of sub-culturing into a fresh sterilized flask by removing the used volume of medium through centrifugation. The retrieved cells were soaked into 5 ml of fresh LB medium and transferred directly into the new sterile flask for sub-culturing, as noted by Andresen (2005), that the transfer of culture into a fresh medium refreshes the performance of the culture. Every day the cultures were supplied with 25 ml of fresh LB medium as nutrient supplement for consistent bacterial growth.

Figure VII-1 shows two images taken during *P. Putida* sub-culturing with the right frame showing the formation of a colony inside the culturing flask. A close look at the colony suggested that it was a large homogenous cluster of the cells with no other colony existing in the flask, hence indicating purity of the culture growth, since a single colony in the bacterial cultures permit to visualize purity of the culture and the presence of more than one colony is indicative of a contaminated culture (Madigan et al., 2009).



(a) **(b)**

Figure VII-1: *P. Putida* sub-culturing showing in: (a) bacteria sub culturing underway on a hot plate at controlled temperature of 37^0C, and (b) growth of *P. Putida* colony.

VII.3 SIMULATED SUGAR FACTORY WASTEWATER LAGOONING WITH *P. PUTIDA*

The last three chapters were focussed mainly on SW lagooning as a priori study for simulated sugar factory wastewater (SSFW) to develop the database for its application in the treatment of SSFW. Thus, the ensuing experiments were aimed at achieving this objective. SSFW was prepared based on the wastewater characteristics of Newark beet sugar factory, Nottingham, UK. The main contaminant sources in the wastewater are COD (sugar), nitrogen and phosphorous. The wastewater sample from Newark beet sugar factory was collected and analyzed for these contaminant parameters to determine the corresponding amounts of the chemicals to be used for the preparation of SSFW.

Table VII-2 shows the chemicals representing the respective contaminants in SSFW together with the contaminant concentration present in the sugar factory wastewater, along with the relative amounts of chemicals added for the preparation of SSFW. The main contaminant components of sugar factory wastewater i.e. carbon, nitrogen and phosphorous were mixed with a C: N: P ratio of 100:5:1 for the preparation of typical SSFW (Kajitvichyanukul and Suntronvipart, 2006). Guven et al., (2009) also used these components to simulate SFW for its electrochemical treatment with the same ratio except that they used only 0.4% of phosphorous instead of 1% and a higher amount of calcium hydroxide of 13%, probably to suit their respective wastewater treatment protocol.

The addition of the chemicals for the preparation of SSFW was optimized by taking the precisely required amounts of the reagents in harmony with the actual

constituent values of N, P, and COD based on the analysis of the real wastewater samples. For example, total nitrogen and total phosphorous analyses of the wastewater sample from the factory ranged in between 43-49 and 7-10 mg l^{-1} respectively. The prepared SSFW was then tested for these parameters until it was in conformity to their specified values of real wastewater. Hence, the optimized respective amounts of chemicals were taken for the preparation of SSFW. Table VII-2 shows the identity of the chemicals used along with their respective amounts in relation to real wastewater concentrations for these parameters for the preparation of SSFW.

Table VII-2: Composition of prepared SSFW

Surrogate substance or chemical used	**Concentration in Sugar factory wastewater, mg l^{-1}**	**Weight in g for 13 l SSFW**
Sugar for COD (100%)	13,000-15,000	13 x 13 = 169
Ammonium bicarbonate for nitrogen (5%)	43-49	0.65 x 13 = 8.5
Potassium dihydrogen phosphate for phosphorous (1%)	7-10	0.13 x 13 = 1.7
Calcium hydroxide for Calcium (4%)	14-19	0.52 x 13 = 6.76

VII.3.1 SSFW lagooning with *P. Putida*

13 l of prepared SFW was poured into LPT and analysed for initial values of DO, pH, temperature, IL and COD. 10 ml volume of *P. Putida* cells (0.76 ml/l SW) separated from the cultured flask by centrifugation was inoculated progressively into LPT via sterilized pipette with 5 ml each at points 1 and 2. After the centrifugation the supernatant medium solution was removed from the centrifuge tubes and 5 ml of fresh Luria Bertani (LB) medium was added into the tubes to make *P. Putida* paste, which was equivalent to total volume of the inoculum of 10 ml. The recirculation flow rate was set at 40 ml min^{-1} and light unit was turned on for the whole duration of the experiment. The room temperature for the experiment was set at 25^0C via heating/cooling unit to make the surrounding temperature constant to check variation in the temperature as a result of the biological activity during the entire run in LPT.

VII.3.1.1 pH analysis

Figure VII-2 shows the pH data at the surface at the middle points 2 and 5 and side points 1 and 6 in LPT during 24 hr of lagooning with *P. Putida*. The pH values at the bottom being identical to the surface data are not presented separately. The graph shows 24 hr pH profile after the inoculation of the bacterial culture into SFW indicating the impact of bacterial culture on SFW pH in a 24-hr cycle. The pH results as depicted in the graph, suggest that there was no reduction in the pH for the first 4 hr of lagooning. However, for the next 16 hr the pH was reduced by 10%, with maximum reduction of 53% taking place in the final 4 hr of lagooning. The overall view of the graph suggests that pH decreased by 26% in 24 hr after the

inoculation of *P. Putida* cells with a decreasing rate of 1.9 d^{-1}. Moreover, the decreasing pattern at all the points, as depicted by the curves, is almost symmetrical implying towards the unanimous growth of the cells followed by the spread of *P. Putida* all over the tank due to the medium recirculation.

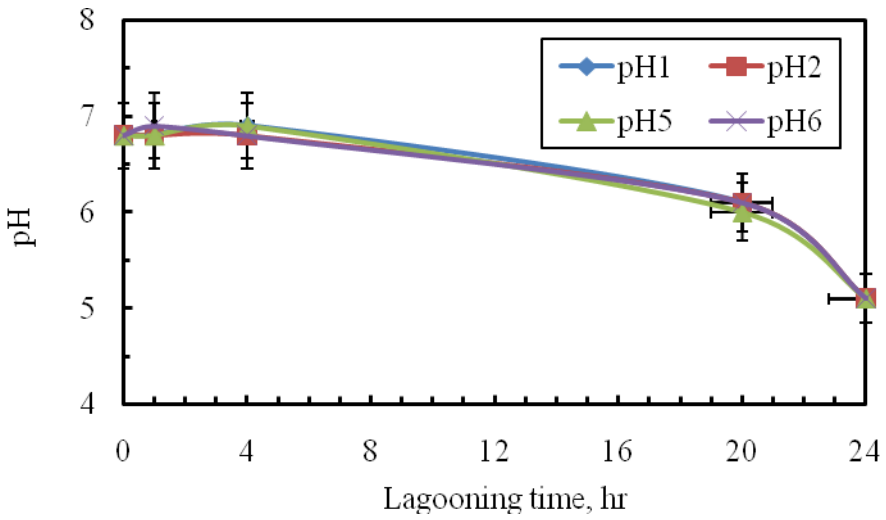


Figure VII-2: pH account at the surface at points 1, 2, 5 and 6 during 24 hr of SSFW lagooning with 10 ml *P. Putida* (mean, n=3).

VII.3.1.2 DO and Temperature

The DO results at points 1, 2, 5 and 6 in LPT after lagooning for 24 hr with the bacterial culture are presented in Figure VII-3 along with the temperature data. The results at the bottom were not different from the surface results thus not shown separately. Like the pH results of this experiment, the impact of inoculated bacterial culture was apparent on the DO results in only 24 hr of lagooning. The data in the graph suggested that DO decreased by 35% in the first 20 hr of lagooning due to the bacterial of SSFW organics while taking in oxygen from the

medium. In addition, it also indicated that the rapid degradation of SSFW organics by the allochthonous *P. Putida* culture started after 20 hr, which may be deemed as the beginning of log phase growth time for the *P. Putida* cells. The overall kinetics of the organic degradation suggested that SSFW DO decreased by 88% at 6 mg l^{-1} in 24 hr (0.25 mg l^{-1} hr^{-1}) of lagooning. The average temperature in LPT was observed as higher by 1^0C after 24 hr than it was at the start.

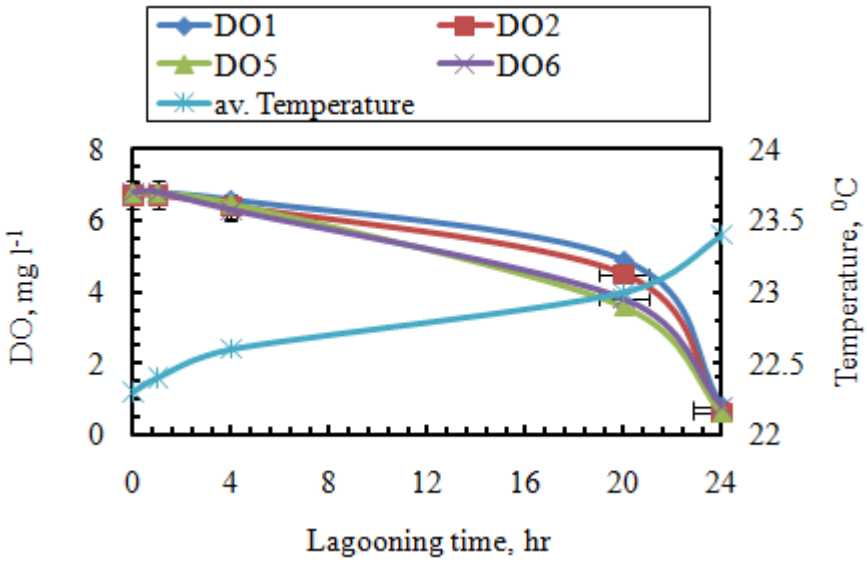
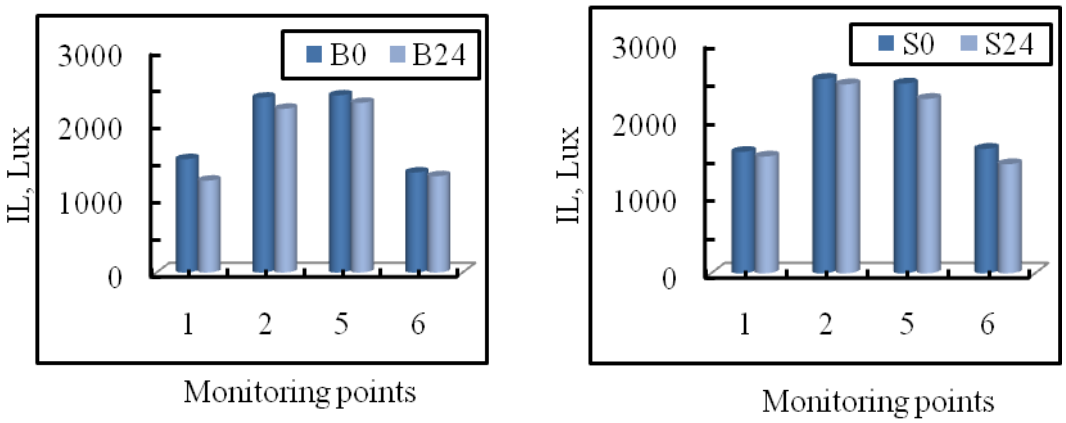


Figure VII-3: DO and average temperature profile at the surface at points 1, 2, 5 and 6 during 24 hr of SSFW lagooning with 10 ml *P. Putida* (mean, n=3).

VII.3.1.3 Incident light profile

Incident Light (IL) profile in the tank at the start and after 24 hr is shown in Figure VII-4. The bacterial breakdown of organic matter in SFW probably produced fine derivatives in SFW turning the color of SFW turbid thus reducing the IL magnitude by the end of experiment. The effect of this change was highlighted by the IL profile in the tank.

The IL values showed reduction at all the given points after 24 hr of lagooning with *P. Putida* cells. The results suggested that the reduction in IL at the bottom (6.4%) was more than at the surface (2.8%) implying towards bacterial activity occurring mostly near the bottom, influencing accumulation of the degradation products and subsequent IL reduction more at the bottom.



(a) **(b)**

Figure VII-4: IL profile in LPT at the start and after 24 hr of SSFW lagooning with 10 ml *P. Putida*: (a) at bottom and (b) at surface (mean, n=3).

VII.3.1.4 COD analysis

Figure VII-5 shows COD data obtained during SSFW lagooning with *P. Putida* for 24 hr, which are referenced with DO values. The graph highlights the influence of bacterial culture on the degradation of SFW organics in terms of variation in the COD values, which is also correlated with the consumption of SFW DO during the process. The study of the curves in the graph suggested that until the DO became limited factor after 20 hr of SSFW lagooning with *P. Putida* cells COD was observed as being stabilized for the first 20 hr with 14% COD reduction occurring

during that time. However, during the last 4 hr with SSFW medium containing depleted DO, COD value increased by 9% implying towards non-consumption of the substrate by the aerobic bacteria probably due to hypoxic conditions prevailing in the medium. In the event of non-availability of DO in the medium, it was likely that the bacterial cells released soluble fraction of synthesis products in the medium, thus increasing the COD level in addition to halt in COD reduction.

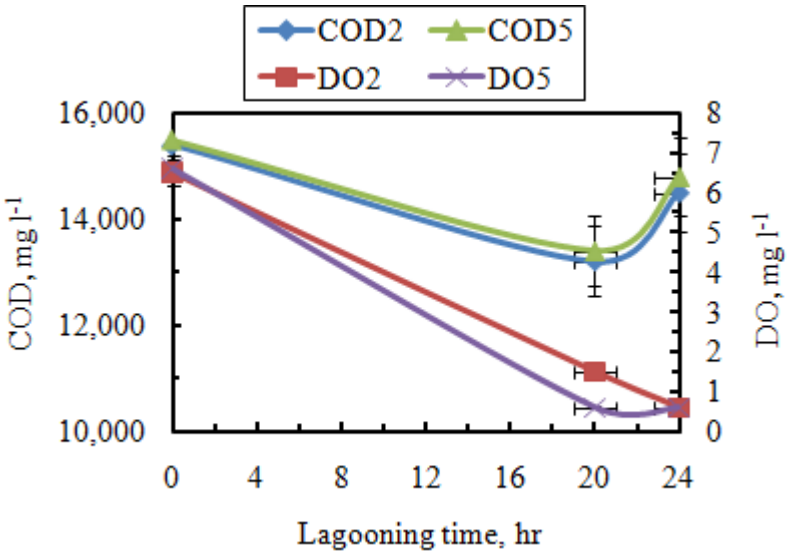


Figure VII-5: COD at points 2 and 5 in reference to DO values after 24 hr of SSFW lagooning with 10 ml *P. Putida* (mean, n=3).

VII.4 SUMMARY

This experiment was held to analyse the influence of bacterial inoculation on SSFW characteristics without the presence of algae cells in the cultivation medium. The results of this experiment transpired that pH decrease of 1.9 d^{-1} in this experiment was double the decrease observed during SW lagooning with *C. Vulgaris*. Similarly, allochthonous *P. Putida* activity during SSFW lagooning yielded in DO reduction of 6 mg l^{-1} d^{-1} along with COD pattern that was observed to be increasing after 20 hr of lagooning, when DO was a limiting factor in the medium. With the inoculation of algae cells along with *P. Putida* culture, the steep decrease in DO value, however, could be avoided resulting probably in the stabilization of COD value by the combined cells of algae and bacteria. Moreover, the direct generation of CO_2 by *P. Putida* cells may also trigger higher oxygen saturation cycles via mixotrophic cultivation of algae cells prompting in return probably the improved rates of organics degradation by the bacterial cells.

CHAPTER VIII

SIMULATED SUGAR FACTORY WASTEWATER REMEDIATION IN LAGOON PHOTO TANK WITH ALGAE AND BACTERIAL CONSORTIUM

CHAPTER VIII

SIMULATED SUGAR FACTORY WASTEWATER REMEDIATION IN LAGOON PHOTO TANK WITH ALGAE AND BACTERIAL CONSORTIUM

VIII.1 INTRODUCTION

In this chapter simulated sugar factory wastewater (SSFW) remediation using combined cells of *C. Vulgaris* and *P. Putida* without and with the addition of copolymer Polyacrylate polyalcohol was aimed at in lagoon photo tank (LPT) to investigate their combined influence on SSFW characteristics such as pH, DO and COD as well as on the growth of algae-bacterial biomass.

VIII.2 FREE CELL CULTIVATION OF COMBINED CELLS OF *C. VULGARIS* AND *P. PUTIDA* IN SSFW

SSFW was prepared with a required working volume of 13 l and with C: N: P ratio of 100:5:1. An inocula protocol comprising of 40 ml of cultured *C. Vulgaris* cells along with 10 ml of cultured *P. Putida* cells were used for their cultivation in SSFW in LPT. The room temperature for the duration of the experiment was set at 25^0C. SSFW recirculation was set at 40 ml min^{-1} and the light supply to the culture was continuous. Prepared SSFW was poured into LPT and immediately its initial analysis was carried out for pH, DO, temperature, IL and COD. After the initial

assaying, 20 ml of *C. Vulgaris* cells were inoculated each at points 1 and 2 through sterilized pipette. The *P. Putida* cells were not added immediately, rather these were inoculated 24 hr after the introduction of algae cells, considering the results obtained during SSFW lagooning with *P. Putida* only, whereby pH and DO limiting conditions prevailed within the first 20 hr of bacterial activity. Hence it was planned to inoculate the bacterial cells after 24 hr to let the algae cells complete the lag phase of their growth under conducive profile of process parameters such as pH and DO. Besides, the bacterial cell inoculation after 24 hr in the cultivation medium meant that by the time the bacteria would start their activity, algae cells would probably enter into their log phase of growth. This would have increased the algae cell mass along with photosynthetic oxygenation of the medium, while the bacterial activity during this particular period of time might instead be beneficial and growth-promoting to algae cells. After 24 hr of *C. Vulgaris* inoculation, 5 ml of cultured cells of *P. Putida* were introduced into LPT via sterilized pipette each at points 1 and 2.

VIII.2.1 pH analysis

Figure VIII-1 presents pH results at the middle points 2 and 5 at the bottom and at point 5 at the surface during combined free cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW for 6 days. The graph shows consistent decrease in the pH value after the introduction of *P. Putida* cells after 24 hr of *C. Vulgaris* cultivation at 0.96 d^{-1} until the end of the experiment. This meant that 50% reduction in the pH decrease rate was observed during this run as compared to the rate observed during SSFW lagooning with *P. Putida* only.

This implied towards the influence of *C. Vulgaris* cell growth, which probably tends to increase the pH of cultivation medium in particular reference to CO_2 uptake by the algae cells, released due to bacterial degradation of sugar. As a result, this neutralizes the net decrease in the pH reduction rate caused by the bacterial activity in medium. All the monitoring points, as highlighted in the graph, exhibited almost similar decreasing pattern for pH, which indicated the homogeneity and scattering of the culture cells in LPT likely due to the effect of recirculation.

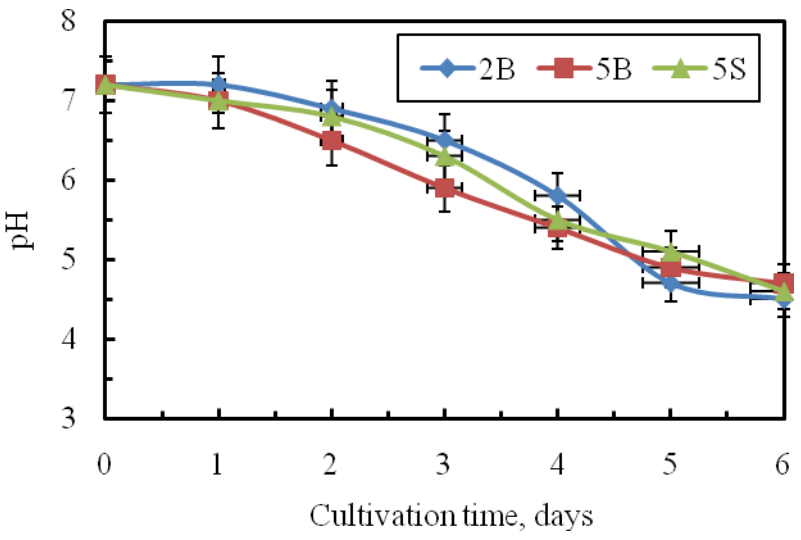


Figure VIII-1: pH account at the bottom points 2 and 5 and surface point 5 during 6 days of combined free cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW in LPT (mean, n=3).

VIII.2.2 DO and Temperature

Figures VIII-2 and VIII-3 depict SFW DO and average temperature results at points 2 and 5 at the bottom and surface respectively. The introduction of *P. Putida* culture after 24 hr of *C. Vulgaris* cultivation caused DO to decrease for the next 24 hr by 5.1 and 3.6 mg l^{-1} at the bottom and surface respectively, which was cumulatively 28% lower than the rate obtained during SSFW lagooning with *P. Putida* only. However, after 48 hr during the log phase of growth, DO value showed an overall increase of 0.4 mg $l^{-1}d^{-1}$ until the end of the run. This meant that daily net DO replenishment of 5.5 mg l^{-1} or higher was in effect due to *C. Vulgaris* growth, which included the DO decrease of around 5 mg $l^{-1}d^{-1}$ caused by the activity of allochthonous *P. Putida*. However, if the decrease in DO of around 3-4 mg $l^{-1}d^{-1}$, influenced by the autochthonous bacterial community, as observed during *C. Vulgaris* cultivation in SW, was also considered then it can be assumed that total net photosynthetic oxygen generation during free cell cultivation of *C. Vulgaris* and *P. Putida* probably was in the range of 10-11 mg $l^{-1}d^{-1}$. The average temperature in LPT during the course of the cultivation run was 23.5^0C at the bottom and 22.9^0C at the surface with bottom temperature was higher by around 1^0C than the temperature at the surface by the end of the experiment. This could be associated with either the heat transfer from surface to bottom or the microbial activity taking place more at the bottom than at the surface.

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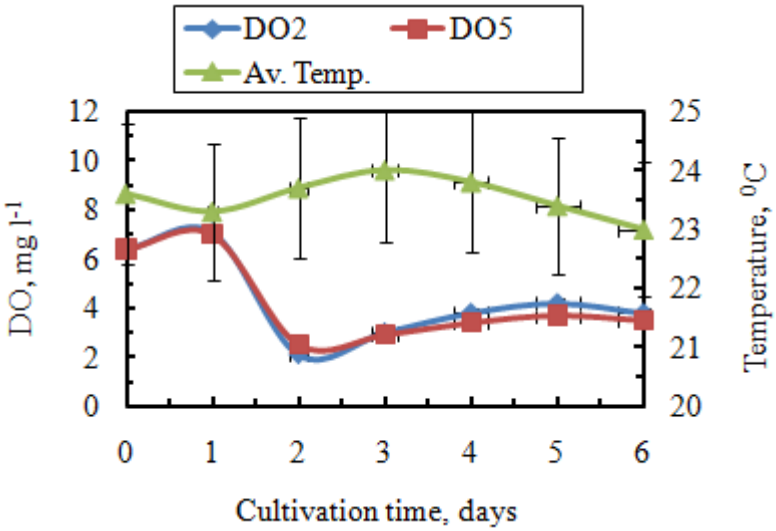


Figure VIII-2: DO and average temperature profiles at the bottom points 2 and 5 during 6 days of combined free cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW in LPT (mean, n=3).

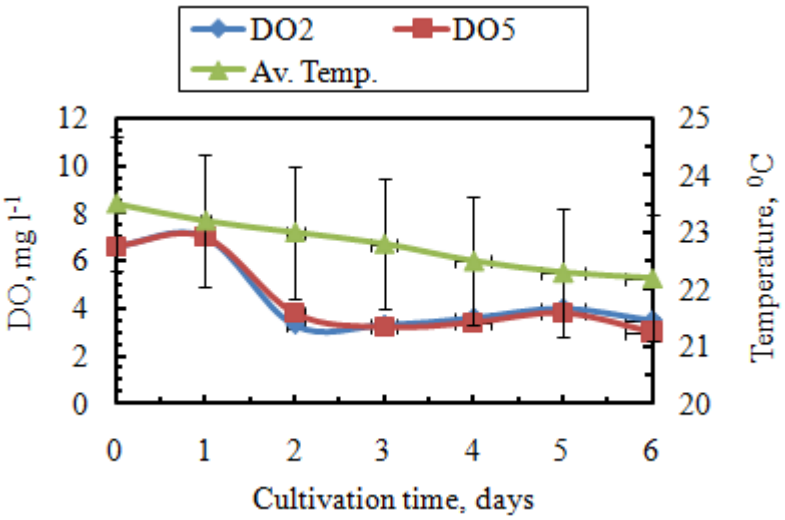


Figure VIII-3: DO and average temperature profiles at the surface points 2 and 5 during 6 days of combined free cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW in LPT (mean, n=3).

VIII.2.3 Incident light profile

Figure VIII-4 shows the incident light (IL) profile at the start and at the end after 6 days of combined free cell cultivation of *C. Vulgaris* and *P. Putida* both at the bottom and surface at points 1, 2, 5 and 6. The data in the graph highlights cumulative reduction in IL with decreasing IL values being observed more at the bottom than at the surface as the culture cells were mostly settled at the bottom. The IL at the bottom points 1 and 6 decreased by 30%, whereas points 2 and 5 showed similar decrease in IL by 18% after the end of the run. The higher reduction in IL could be related with substrate degradation by combined cells of benthic *C. Vulgaris* and *P. Putida*, which was influenced by photosynthetic activity by *C. Vulgaris* and parallel aerobic biodegradation of SSFW organics by *P. Putida*. Resultantly, this synergistic cell activity probably resulted in increased amount of degradation products such as organic acids and their metabolites in the cultivation medium raising its cloudiness as well as the incidence of light to the bottom.

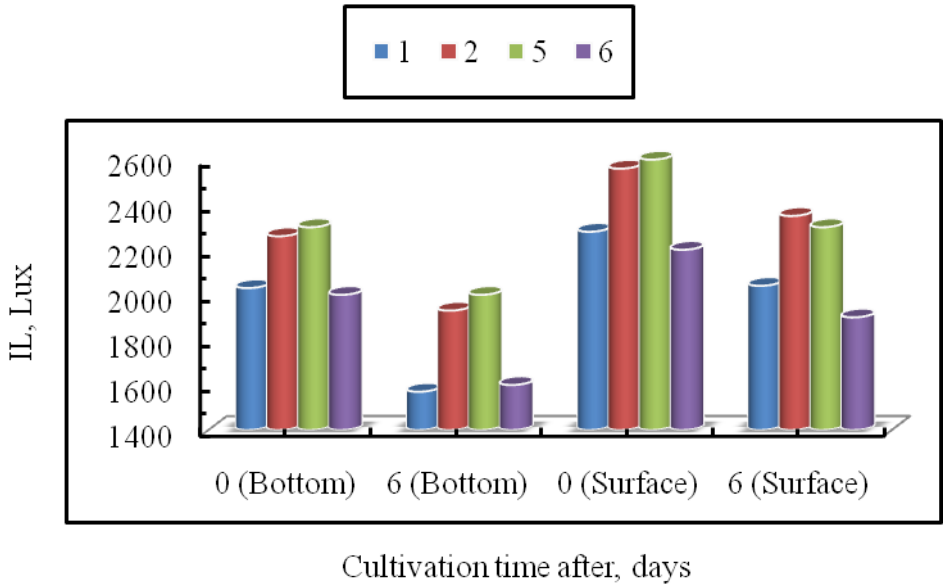


Figure VIII-4: Incident light profile at the start and after 6 days at the bottom and surface points 1, 2, 5 and 6 during combined free cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW in LPT (mean, n=3).

VIII.2.4 COD

SFW COD values obtained at the middle points 2 and 5 in LPT during 6 days of combined free cell cultivation of *C. Vulgaris* and *P. Putida* are presented in Figure VIII-5. The graph shows almost similar pattern of COD removal at the designated points as exhibited by the curves in the graph. The lag phase growth of combined free cell cultivation until the first 2 days yielded in COD removal of 24% at COD removal rate of 1900 mg $l^{-1}d^{-1}$. The log phase growth COD removal from day 3 to 5 was around 51% with a combined COD removal rate of 2100 mg $l^{-1}d^{-1.}$ However, day 6 COD results registered an increase in the COD by around 10% suggesting the onset of stationary or declining cell growth period. Overall, combined free cell

cultivation of *C. Vulgaris* and *P. Putida* in SSFW yielded in COD removal of 62% at 1980 mg $l^{-1}d^{-1}$. The growth cycle of the cells in terms of COD removal suggested that algae photosynthetic activity probably continued until day 5 with corresponding oxygen replenishment of the cultivation medium, thus sustaining the bacterial activity in the process as well. This combined cell uptake of medium COD would have resulted in biomass production, which likely created the condition of IL attenuation in the cultivation medium resulting in a halt in further growth of the cells. Thus, the increase in the COD value was likely related with the declining growth of the cell mass as well as the release of soluble fraction of degradation products including organic acids during the cultivation process in the cultivation medium.

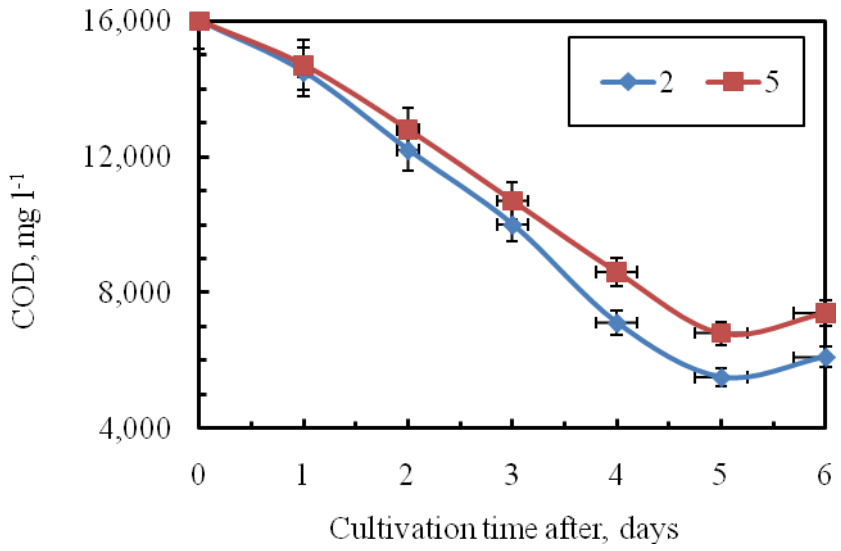


Figure VIII-5: COD profile at the middle points 2 and 5 during 6 days of combined free cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW in LPT (mean, n=3).

VIII.2.5 Biomass growth analysis

VIII.2.5.1 *P. Putida* cell mass growth

Bacterial mineralization of organic matter is assayed either by the disappearance of substrate in the medium (COD reduction) or by the growth of bacterial mass (Whiteley and Lee, 2006). For the analysis of the growth of *P. Putida* during combined free cell cultivation of both *C. Vulgaris* and *P. Putida*, 50 ml sample was collected in a small beaker daily from the middle point 2 in LPT for volatile suspended solids (VSS) determination using APHA method no. 2540E (APHA, 1998). VSS represented indirectly the active mass of *P. Putida* cells present in the given sample (Khelifi et al., 2008). The sample was first filtered via filter paper (analytical grade) to remove the suspended matter present in the collected sample such as larger-sized algae cells. The filtrate containing the dissolved organic matter or the bacterial cell mass was burnt at 550^0C for 30 min in oven for the evaporation of water from the sample leaving the dry cell mass at the bottom of the beaker. The data obtained by gravimetric analysis was used in the following formula to calculate VSS.

$$\text{VSS, mg l}^{-1} = \frac{(A - B) \quad \text{x } 10^6}{C} \qquad (8.1)$$

Where A = combined weight of volatile solids left after evaporation plus beaker, g

B = weight of dried empty beaker used during the test, g

C = sample volume taken for analysis, ml

Table VIII-1 shows the VSS data for this experiment representing the indirect grown mass of Pseudomonas Putida cells inoculated after 24 hrs of SFW

lagooning in LTP. The data in the table shows steady growth of the cells with a growth rate of 248 mg $l^{-1}d^{-1}$ until 5 days of cultivation, while the last 24 hr accrued decrease in the growth of the cells by 7% indicating the stationary or start of the declining phase of the cell growth.

Table VIII-1: *P. Putida* cell mass expressed as VSS produced during combined free cell cultivation in SSFW in LPT (mean, n=3).

Time days	**VSS mg l^{-1}**
2	171
3	301
4	520
5	745
6	695

VIII.2.5.2 Algae-bacterial cell mass growth

Table VIII-2 presents the data related with the growth of algae-bacterial cell mass during combined cell cultivation in SSFW in LPT. Various growth-related factors such as growth yield (Y), specific growth rate (r) and doubling time (T_2) were determined to analyse the cell growth. “Y” is the difference in cell mass between the initial mass and the mass obtained after some time. "r” is the increase in cell mass per unit time. The maximum growth of the biomass occurred during the log growth phase of combined cell cultivation between days 3 to 5, with the cell mass increasing by 67% per day. However, during the last 24 hr the biomass showed decrease in the cell mass by 26% largely due to the attenuation in IL by around 30%. The growth yield (Y) suggested that the biomass was consistently increasing after every 24 hr during the log growth phase. The specific growth rate (r) determined the growth rate of biomass that occurred in 24 hr duration time, which implied that better cell growth occurred between days 3 and 4 of the cultivation, as after that r value showed decrease in the growth rate. This probably occurred due to higher cell density in the cultivation medium influencing decrease in the IL at the bottom and as a result affecting further cell growth. Cell doubling time (T_2) expression was based on the r value, as increase in the r value was decreasing T_2 and vice versa.

Table VIII-2: Growth analysis of algae-bacterial cell mass production during combined free cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW in LPT (mean, n=3).

Cell mass in g l^{-1}

Time interval (d)	**0 – 1**	**1 – 2**	**2 – 3**	**3 – 4**	**4 – 5**	**5 – 6**
Cell mass (X) from day 1 to 6 →	0.3	0.5	0.9	1.5	2.3	1.7
$X_t - X_0$ (GY), g		0.2	0.4	0.6	0.8	-0.7
$\Delta t = (t_t - t_0)$, d		1	1	1	1	1
$r = \frac{\ln X_t - \ln X_0}{\Delta t}$ ($g\ l^{-1}d^{-1}$)		0.51	0.59	0.5	0.43	-0.3
$T_2 = 0.6931/ r$		1.35	1.17	1.38	1.61	-2.31

X_0 , X_t = cell dry mass at the beginning and at the end of log growth phase;

r = specific growth rate; Δt = time difference; T_2 = cell doubling time;

GY = growth yield

VIII.2.6 Experiment Synthesis

In this experiment combined free cell cultivation of *C. Vulgaris* and *P. Putida* was carried out as a consortium to observe symbiosis between the two species in relation to the influence of their combined activity on the growth of the combined cell mass as well as SSFW COD reduction. The experiment yielded in pH

reduction 0.6 d^{-1} as compared to pH reduction of 1.9 d^{-1} observed during free cell cultivation of *P. Putida* only in SSFW. Overall, lower reduction in pH by around 50% was observed during this experiment, which resulted in pH stabilization of the process and that likely occurred due to *C. Vulgaris* uptake of CO_2, produced as a result of bacterial degradation of the substrate, thus increasing the pH of the medium and reducing the overall pH decline. The DO profile during combined free cell cultivation, observed after *P. Putida* inoculation, suggested that 24-hr presence of the allochthonous bacterial cells in the cultivation medium resulted in comparatively lower reduction by 20% than that observed during SSFW lagooning with *P. Putida*. It was also observed that during the log phase of algae growth, SSFW DO increased at 0.4 mg $l^{-1}d^{-1}$ until the end of the experiment. This oxygen profile improved further during log growth phase of *C. Vulgaris* that enhanced SSFW DO concentration in the cultivation medium keeping the net DO value from falling below 2 mg l^{-1}, which was the optimum DO concentration required for biological activity (Phong, 2008 and Tchobanoglous et al., 2003). IL penetration was lesser by at least 12% at point 1 as compared to other points in LPT most likely due to the accumulation or suspension of recirculated mass of substrate degradation products. The reduction in IL penetration at other points was likely the result of the biomass growth and SSFW degradation. COD profile obtained for this experiment transpired that inoculation of *P. Putida* in the cultivation medium alongside the algae cells resulted in overall COD reduction of 62%, which meant that the cultivation medium or SSFW still contained sizable portion of organic matter in the form of remaining COD to afford manoeuvring of the process.

VIII.3 IMMOBILIZED CULTIVAITON OF *C. VULGARIS* AND *P. PUTIDA* IN SSFW WITH COPOLYMER POLYACRYLATE POLYALCOHOL

Free cell cultivation of combined culture of *C. Vulgaris* and *P. Putida* in SSFW transpired that the process efficiency in terms of SSFW remediation could further be improved and that the treatment protocol could be manoeuvred by changing the mode of cell cultivation. This was exactly to this direction that the cell cultivation was planned via the optimized use of copolymer Polyacrylate polyalcohol to afford the growth of algae cells in particular at the surface of LPT to maximise the cell growth as well as influence the efficient COD removal by the synergistic activity of both algae and bacteria. Three separate cultivation runs were held with 4:1 inocula volume ratio of *C. Vulgaris* (40 ml) and *P. Putida* (10 ml) immobilized with different copolymer doses of 40, 80 and 160 mg l^{-1}. The designated time for immobilized cell cultivation was fixed as 96 hr (Ojumu et al., 2005) and the results obtained at the middle point 2 in LPT were presented and discussed for the parameters under investigation. SSFW for each of the run was prepared after dissolving sugar in DW with a ratio of 13 g: 1 l for a total working volume of 13 l. After the introduction of SSFW in LPT followed by its initial analysis, respective copolymer dose was added into LPT at point 2. After 20 min of copolymer stretching or expansion, 20 ml of cultured *C. Vulgaris* cells were inoculated progressively via a sterilized pipette on top of the copolymer colonies in the surface area of point 2. After 24 hr of immobilized cultivation of *C. Vulgaris* at the surface of LPT, 10 ml of cultured cells of *P. Putida* were progressively pipetted

into LPT. The overhead light unit atop LPT was operated continuously. SFW recirculation flow rate was set at 40 ml min^{-1} and room temperature in the laboratory room was fixed at 25^0C using heating unit of the lab in order to keep the ambient temperature constant throughout the run to highlight the difference in temperature incurred via the process.

VIII.3.1 pH

Figure VIII-6 shows pH values at the middle point 2 in LPT during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW immobilized with copolymer doses of 40, 80 and 160 mg l^{-1}. Irrespective of the polymer dosage applied, the pH showed an increase by 34% after the copolymer addition in SSFW. The pH results obtained after 48 hr of *P. Putida* inoculation in the medium suggested that pH decreased by 18%, 15% and 10% during cell cultivation immobilized with copolymer doses of 40, 80 and 160 mg l^{-1} respectively. Whereas, during the last 24 hr pH decrease with immobilization dose of 80 mg l^{-1} was observed as higher by around 50% than that observed with immobilization doses of 40 and 160 mg l^{-1} decreasing at the rate of 0.6, 1.4 and 0.8 d^{-1} with copolymer doses of 40, 80 and 160 mg l^{-1} respectively. The overall pH decrease during combined cell cultivation immobilized with copolymer doses of 40, 80 and 160 mg l^{-1} was 29%, 37% and 22% respectively as compared to 22% observed during 4 days of combined free cell cultivation. The higher pH decrease observed during combined cell cultivation immobilized with lower copolymer doses was probably related with the increased bioconsortium activity by the immobilized cells incurring efficient utilization of both the substrate as well as nutrients.

This was apparently more the case with intermediate copolymer of 80 mg l^{-1}, which likely resulted in better cell entrapment leading to their optimum growth. Another advantage of reduced pH observed during cell immobilization with copolymer dose of 80 mg l^{-1} was that the grown-up flocs of *C. Vulgaris* were seen suspending at the surface after typical copolymer disintegration, which started to occur after 60 hr of cultivation. This condition probably occurred due to the decreased pH the cells, which brought the cells closer to their isoelectric point, whereby the cells induct themselves into a process of autoflocculation (Uduman, et al., 2010).

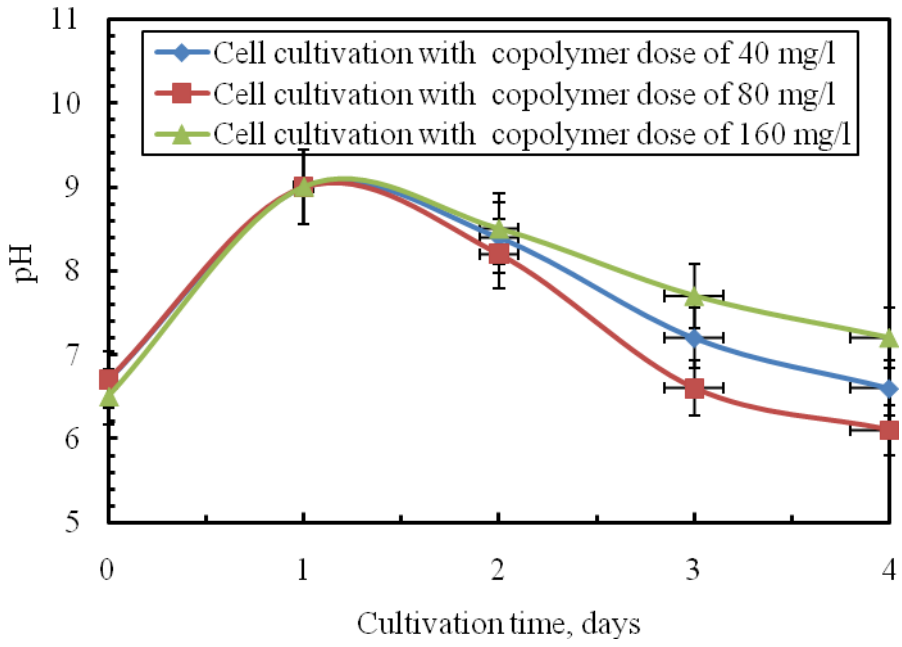


Figure VIII-6: pH profile at the middle point in LPT during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol (mean, n=3).

VIII.3.2 DO

Figure VIII-7 highlights the DO values during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW immobilized with copolymer doses of 40, 80 and 160 mg l^{-1}. In contrast to DO results obtained during combined free cell cultivation of *C. Vulgaris* and *P. Putida* varying DO profiles were observed during the three immobilized cell cultivation runs. During lag growth phase of immobilized cultivation, increase in DO was observed in each of the cultivation run by 34%, 63% and 28% with copolymer doses of 40, 80 and 160 mg l^{-1} respectively. However, during the log growth phase or the last 48 hr of immobilized cell cultivation, decrease in DO was observed by 31%, 35% and 60% at DO reduction rate of 1.4, 1.8 and 2.5 mg $l^{-1}d^{-1}$ with copolymer doses of 40, 80 and 160 mg l^{-1} respectively. This suggested that *P. Putida* degradation of the substrate was efficiently supported by consistent provision of photosynthetic oxygen due to optimum growth of *C. Vulgaris*, which was probably spurred by concomitant bacterial generation of CO_2 and its simultaneous uptake by *C. Vulgaris*.

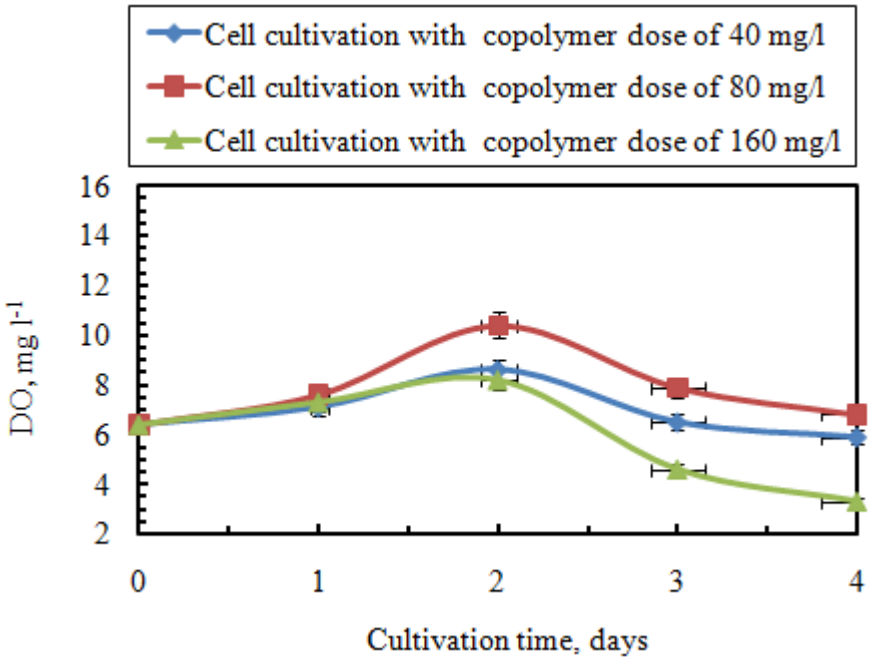


Figure VIII-7: DO profile at the middle point in LPT during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol (mean, n=3).

VIII.3.3 Temperature

SSFW temperature profile at the surface of LPT is shown in Figure VIII-8. The temperature results suggested that the bottom temperature during each run was observed as slightly higher by 0.2^0C than the surface temperature. The overall temperature rise observed during each of the cultivation run was recorded as 0.8^0C, 1.5^0C and 0.6^0C with copolymer doses of 40, 80 and 160 mg l^{-1} respectively. Most of the increase in temperature occurred during the log growth phase of immobilized cultivation, as highlighted by the curves in the graphs. Moreover, 45% increase per day in temperature was observed during log phase growth of the

combined culture immobilized with 80 mg l^{-1} as compared to combined cell cultivation immobilized with lower and higher copolymer doses.

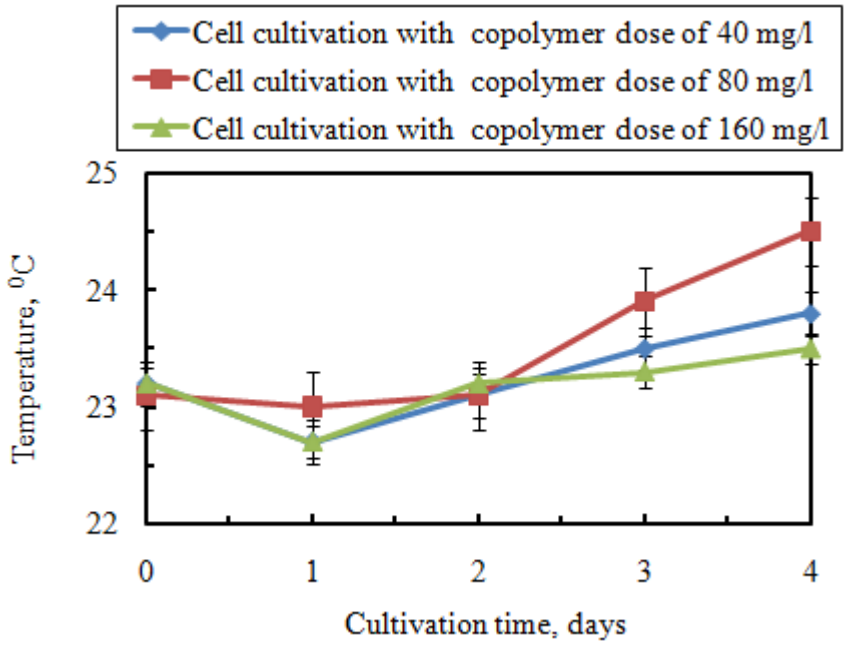


Figure VIII-8: Temperature profile at the surface of LPT during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol (mean, n=3).

VIII.3.4 Incident Light

Figure VIII-9 depicts the incident light (IL) values at the surface during combined cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW immobilized with copolymer doses of 40, 80 and 160 mg l^{-1}. The data in the graphs suggested that the decrease in IL, which was likely caused by *C. Vulgaris* growth and the simultaneous biodegradation activity by *P. Putida* doubled during the log growth phase of the cells as 8% decrease was observed during this period as compared to

4% during the lag phase of immobilized cell cultivation using lower copolymer doses of 40 and 80 mg l^{-1}. The IL results obtained during cultivation run immobilized with higher copolymer dose of 160 mg l^{-1} yielded in IL decrease of 20% at the surface and 22% at the bottom (data not shown separately), which was higher by more than 100% than that observed during cell immobilization with lower copolymer doses.

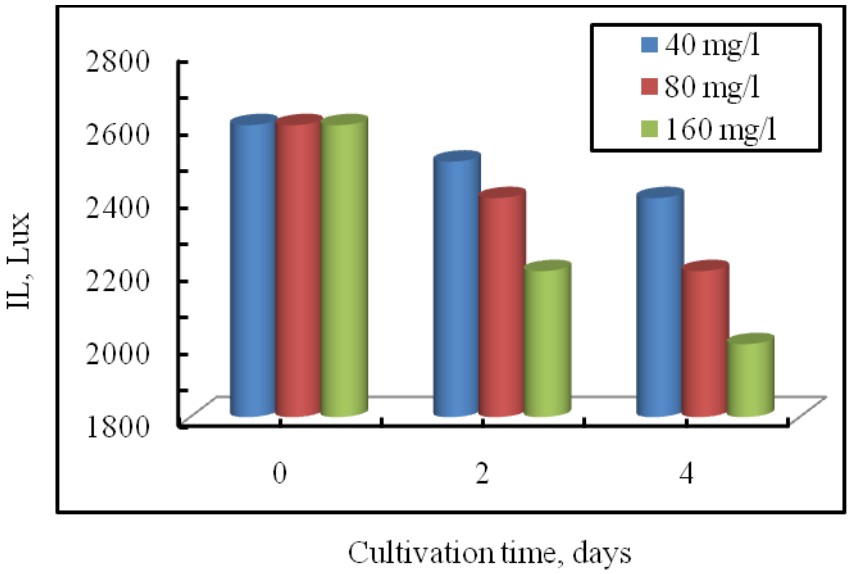


Figure VIII-9: Incident light profile at the surface of LPT during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol (mean, n=3).

VIII.3.5 COD

Figure VIII-10 shows the COD values obtained at the middle point 2 during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol. Before the inoculation of allochthonous bacterial culture in the cultivation medium, immobilized cultivation of *C. Vulgaris* resulted in COD removal of 14%, 17% and 6% with copolymer doses of 40, 80 and 160 mg l^{-1} respectively in contrast to 7% observed during combined free cell cultivation. The introduction of *P. Putida* into the medium yielded in efficient overall COD removal of 75% 89% and 55% during combined cell cultivation immobilized with copolymer doses of 40, 89 and 160 mg l^{-1} respectively as compared to 56% observed during combined free cell cultivation. The COD profile observed during immobilized cell cultivation suggested that *P. Putida* inoculation into the medium was probably more conducive to *C. Vulgaris* during immobilized cultivation as compared to that observed during combined free cell cultivation, when perhaps due to slow and inefficient growth of *C. Vulgaris*, the efficient COD removal could not be realized. The overall COD removal obtained during immobilized combined cell cultivation indicated that the substrate degradation or consumption by the combined culture of *C. Vulgaris* and *P. Putida* occurred to the extent of COD depletion in the cultivation medium, which was likely driven by the formation of continuous DO gradients in the cultivation medium resulting in efficient COD removal via interactive substrate consumption.

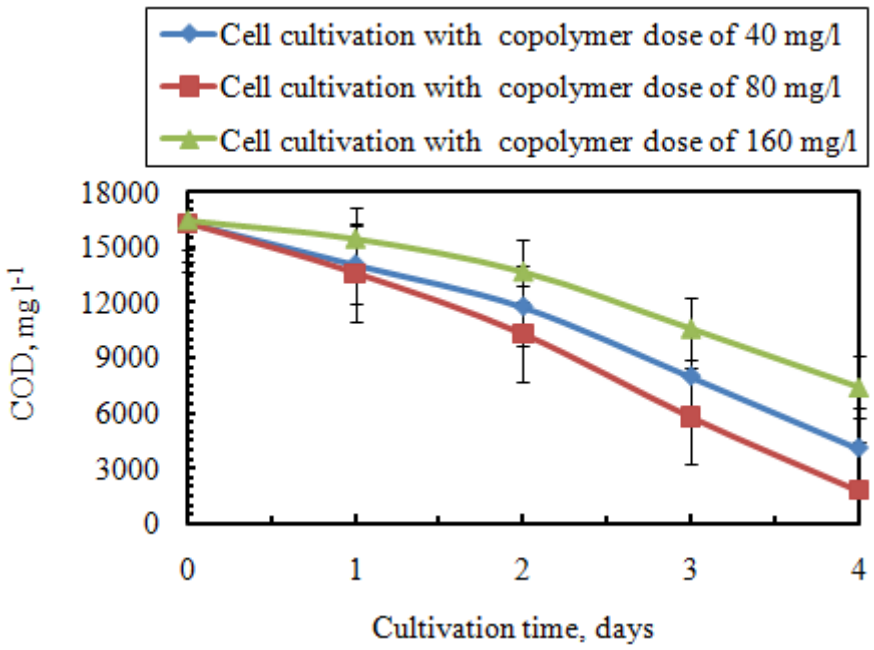


Figure VIII-10: COD profile at the middle point in LPT during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol (mean, n=3).

VIII.3.6 Viscosity

Viscosity measurements during each of the immobilized cultivation run indicated that the use of higher copolymer dose of 160 mg l^{-1} resulted in higher viscosity value of 5.3 cp as compared to 1.3 and 2.4 cp observed during combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with lower copolymer doses of 40 and 80 mg l^{-1} respectively. The immobilization of the culture cells with higher copolymer dose resulted in the medium being more viscous than in the case when copolymer dosing was lower by 100% or less. The viscous medium affected the process of culture renewal cycles as well as the influence of culture recirculation in the cultivation medium by hampering the fluid advection, which

probably resulted in cell encrustation leading to restricted growth of the cells due to multiple factors including IL attenuation along with lower substrate and nutrient utilization.

VIII.3.7 Nutrient uptake

Nutrient check during all the three cultivation runs was carried out by measuring the concentration of total nitrogen (N) and phosphorous (P) at the start and at the end to determine their combined uptake by *C. Vulgaris* and *P. Putida*. The N and P concentration at the start of each cultivation run was measured as 49 and 9 mg l^{-1} respectively. The results obtained after 4 days of combined immobilized cell cultivation for N and P consumption suggested that combined cell cultivation immobilized with higher copolymer dose of 160 mg l^{-1} yielded in higher N and P residual of 36% and 56% severally in contrast to 19% and 33% and 7% and 11% during combined cell cultivation immobilized with lower copolymer doses of 40 and 80 mg l^{-1} separately. This meant that higher N and P consumption of 93% and 89% was recorded during combined cell cultivation immobilized with copolymer dose of 80 mg l^{-1} as compared to 81% and 67% and 71% and 59% with copolymer doses of 40 and 160 mg l^{-1} respectively. The higher consumption of the nutrients during cultivation immobilized with intermediate copolymer does of 80 mg l^{-1} occurred due to faster *C. Vulgaris* growth within the copolymer matrices inducing a recurring cycle including formation of nutrient gradients followed by constant supplementation of nutrients from outside the matrices, thus maintaining sustained nutrient consumption. The consistent occurrence of this probable cycle vindicated the triggering effect on biomass growth with immobilization dose of 80 mg l^{-1}

indicating that higher nutrient consumption was proportional to the optimum growth of the biomass. Whereas, with both lower and higher copolymer doses influencing ineffective immobilization of the cells, relatively lower nutrient consumption was recorded leading to respective lower growths of the biomasses.

VIII.3.8 Biomass growth analysis

The cell mass growth of *P. Putida* and *C. Vulgaris* during the three immobilized cell cultivation runs was monitored via standard method of gravimetric analysis.

VIII.3.8.1 *P. Putida* growth

The growth of *P. Putida* cell mass during the course of cultivation was monitored via volatile suspended solids (VSS) determination, which represents an indirect measure of bacterial cell mass as per the standard method 2540E (APHA, 1998). 50 ml sample collected in 100 ml beaker from the middle point in LPT was filtered via filter paper (analytical grade) to separate the suspended matter such as *C. Vulgaris* and copolymer particles from it. The filtrate containing the dissolved matter was burnt at 550^0C for 30 min in oven for the disappearance of medium water, leaving behind the residue at the bottom, taken as bacterial cell mass. The data obtained during the weight analysis was used to determine VSS using equation 8.1. Table VIII-3 shows the VSS data obtained during combined cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol. The data in the table points towards higher growth of the bacterial cell mass by 27% 24 hr after their inoculation in LPT and by 24% after 48 hr of their introduction, when compared to

that observed during combined free cell cultivation of *C. Vulgaris* and *P. Putida*. It was observed that the bacterial growth realized during cell cultivation immobilized with intermediate copolymer dose of 80 mg l^{-1} was higher by 28% and 48% than that obtained with copolymer doses of 40 and 160 mg l^{-1} respectively. Overall, after 4 days of combined cell cultivation immobilized with intermediate copolymer dose yielded in higher bacterial cell mass by 51% in contrast to 18% and 2% with lower and higher copolymer than that obtained during combined free cell cultivation for the same time period.

Table VIII-3: Growth of *P. Putida* cell mass expressed as VSS during Combined cell cultivation in SSFW immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol.

Cultivation time, days	VSS with immobilization dose of (mg l^{-1})		
	40	80	160
2	179	183	188
3	390	412	341
4	614	786	532

VIII.3.8.2 Algae-bacterial cell mass growth

To determine the growth of *C. Vulgaris* via gravimetric analysis, the samples were collected from the middle point in LPT after every 24 hr at the same time in the morning during the course of combined immobilized cell cultivation in SSFW for 4 days. The results are shown in Figure VIII-11. 100 ml collected samples were first centrifuged at 40 rpm for 30 min to separate the medium water from the cell concentrate. After the centrifugation, the supernatant was poured out from the centrifuge tube and the extracted biomass was dried in oven pre-set at 105^0C for 1 hr. After drying of the biomass, the respective weight of the cellmasses was measured in g 100 ml^{-1}, which was converted into g l^{-1}. The graph suggested that maximum growth of the cell mass was observed during cell cultivation immobilized with copolymer dose of 80 mg l^{-1}, which proved suitable for the conducive suspension of *C. Vulgaris* at the surface of LPT resulting likely in better coalescence of *C. Vulgaris* cells with the stretched copolymer particles. This was in contrast to the cell suspension with lower and higher copolymer doses of 40 and 160 mg l^{-1} when the respective copolymer doses apparently materialized in ineffective immobiliztion of the cells. It was observed that cell immobilization with lower copolymer dose resulted in possible leaking of the cells from the suspension due to diluted nature of the copolymer colonies, whereas saturated immobiliztion of the cells due to higher concentration of copolymer particles resulted in obstructive covering of *C. Vulgaris* cells' surface thus affecting the growth related functions by the cells. This observed condition during cell cultivation immobilized with lower and higher copolymer doses resulted in reduced process efficiency in each case as highlighted in the graph.

Most of the cell mass growth occurred during the last two days during the log phase of growth in each of the cultivation run. Cell cultivation immobilized with intermediate coplymer dose of 80 mg l^{-1} yielded in higher cell mass growth by 160% than 125% and 110% observed during cell cultivation immobilized with lower and higher copolymer doses of 40 and 160 mg l^{-1} respectively. In terms of overall growth, cell cultivation immobilized with intermediate copolymer dose of 80 mg l^{-1} resulted in higher cell growth by 44% and 86% as compared to cell growth observed during cell cultivation immobilized with lower and higher copolymer doses of 40 and 160 mg l^{-1} respectively.

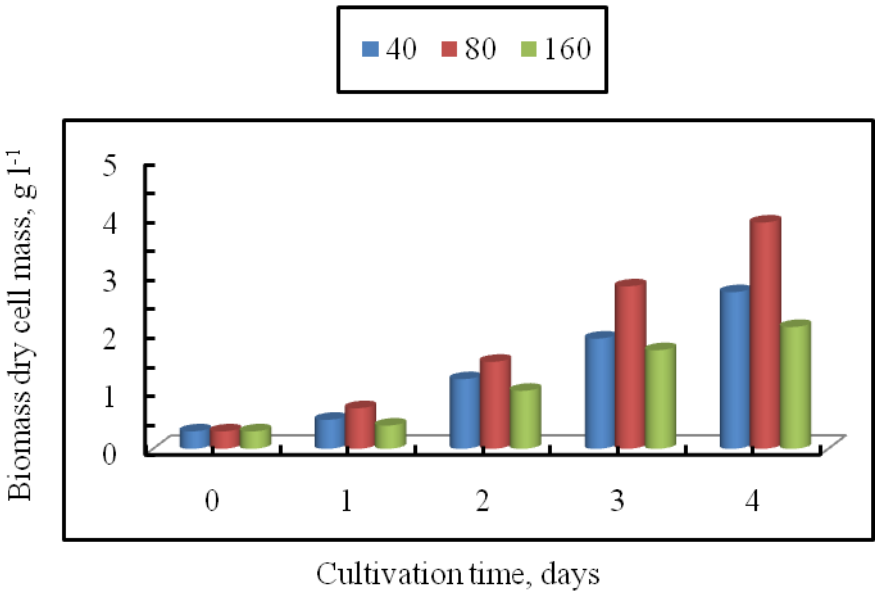


Figure VIII-11: Suspended cell dry mass obtained during 4 days of combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol (mean, n=3).

Table VIII-4 presents the estimated values for different growth-related parameters such as growth yield (GY), specific growth rate (r) and doubling time (T_2). The data in the table suggested that the cell mass increased by 120% per day with immobilization dose of 80 mg l^{-1} as compared to 80%, 60% and 67% observed during combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with 40 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol and during combined free cell cultivation respectively. This was also reflected by GY values, which were higher during immobilized cultivation with intermediate copolymer dose as compared to the values obtained during cell immobilization with lower and higher copolymer doses. This was perhaps indicative of the process being run under optimum environmental conditions of pH, temperature, net DO balance and IL availability during cell cultivation immobilized with intermediate copolymer dose of 80 mg l^{-1}, which proved conducive for the cell mass growth. However, cell cultivation with lower and higher copolymer doses this was not the case as indicated by lower GY values obtained for these cultivation runs. The "r" value showed that better growth occurred between day 2 and 3 of immobilized cultivation as compared to combined free cell cultivation where the growth started to occur after day 3.

Table VIII-4 Analysis of growth parameters for combined cell cultivation immobilized with 40, 80 and 160 mg l^{-1} of copolymer Polyacrylate polyalcohol (mean, n=3).

Time interval (days)	**1 – 2**	**2– 3**	**3 – 4**	**Immobilization dose, mg l^{-1}**
Cell mass (X) **(g l^{-1})**	1.2 1.5 1	1.9 2.8 1.7	2.7 3.9 2.1	40 80 160
GY = $X_t - X_0$ (g)	0.7 0.8 0.6	0.7 1.3 0.7	0.8 1.1 0.4	40 80 160
$\Delta t = t_t - t_0$ (d)	1	1	1	40 80 160
$r = \frac{\ln X_t - \ln X_0}{\Delta t}$ (d^{-1})	0.87 0.76 0.91	0.46 0.62 0.53	0.35 0.34 0.21	40 80 160
$T_2 = 0.6931/ r$ (d)	0.79 0.91 0.76	1.5 1.11 1.3	1.98 2.03 3.3	40 80 160

X_0 , X_t = cell dry mass at beginning and at the end of the log growth phase;

GY = growth yield, r = specific growth rate; Δt = time difference;

T_2 = cell doubling time;

Biomass generation in terms of wet cell mass volume was determined with relatively higher volume sample of 500 ml collected from region of middle point 2 in LPT. The collected sample volume was strained through 0.45 µm sieve to separate the wet biomass from bulk water. Figure VIII-12 shows the wet cell mass production of around 100 ml in a 24 hr cycle during combined cell cultivation immobilized with copolymer dose of 80 mg l^{-1} as compared to 82 and 59 ml obtained during combined cell cultivation immobilized with copolymer doses of 40 and 160 mg l^{-1} respectively. The difference observed in the volume of wet cell mass generation verified the earlier analysis of the biomass in terms of weight, when the cell dry mass obtained during cell cultivation immobilized with intermediate copolymer dose of 80 mg l^{-1} was higher than those obtained during combined cell cultivation immobilized with lower and higher copolymer doses.

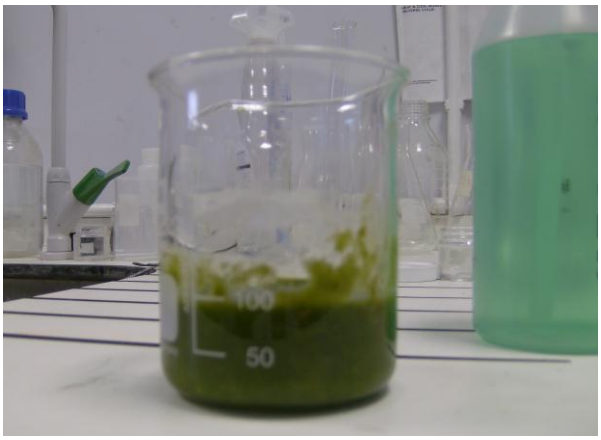


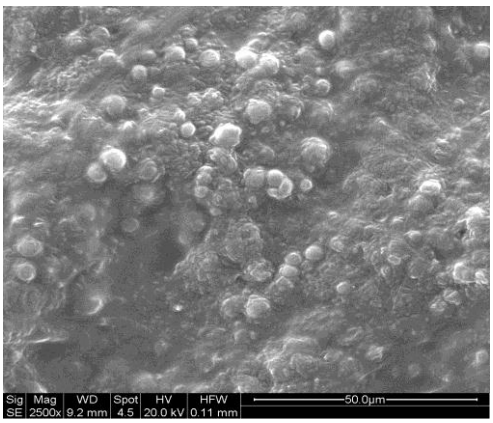
Figure VIII-12: Combined wet cell mass of around 100 ml of *C. Vulgaris* and *P. Putida* collected in a 24-hr cycle during combined cell cultivation immobilized with intermediate copolymer dose of 80 mg l^{-1} during log phase.

VIII.3.9 Calorific value

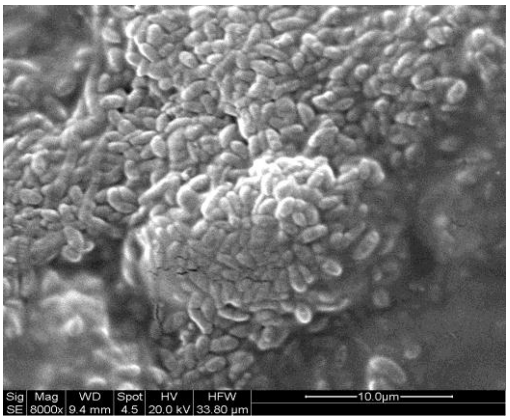
Calorific value (CV) test of the harvested cell mass was carried out after drying of 1 g of the cell mass in oven at 105^0C for 1 hr. The CV test results revealed that higher value of 27 kJ g^{-1} for the cell mass sample was obtained during cell cultivation immobilized with intermediate copolymer dose as compared to CVs of 23 and 20 kJ g^{-1} for cell mass samples obtained from cell cultivation immobilized with lower and higher copolymer doses respectively. The higher CV result accrued from the cell mass obtained from cell cultivation immobilized with copolymer dose of 80 mg l^{-1} was comparable with the CV result for *C. Vulgaris* cell mass as reported by Scragg et al., 2002. The cell mass containing higher CV suggested towards optimum growth of *C. Vulgaris* immobilized at the surface of LPT via efficient entrapment of the cells in association with the copolymer particles resulting in probably the higher chlorophyll concentration of the cells thus increasing their CV. This was in contrast to the cell masses obtained with immobilization doses of 40 and 160 mg l^{-1}, when CV results of 15% and 26% were obtained that were lower than the CV result obtained from the cell mass cultivated with intermediate copolymer dose. The lower CV results indicated that the cells probably were not immobilized properly either with the lower or with higher copolymer doses thus affecting the growth-related functions carried out by the cells resulting in poor growth of the cells, which perhaps led to accumulation of lower level of chlorophyll compounds at the surface of cells and thus induction of lower CV results.

VIII.3.10 Scanning electronic microscopy of biomass growth

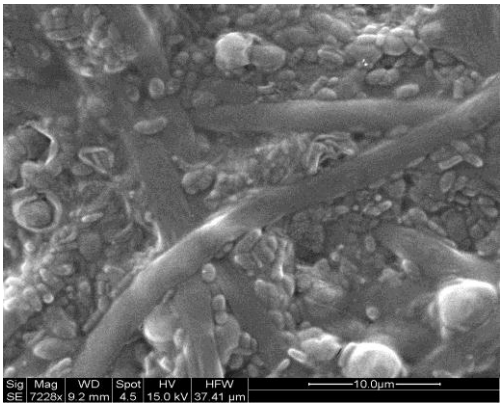
The close range images taken via scanning electronic microscope (SEM) are shown in Figure VIII-13 to highlight the growth pattern of the biomass during immobilized cultivation of combined cell mass of *C. Vulgaris* and *P. Putida* in SSFW with copolymer dose of 80 mg l^{-1}. The samples for SEM analysis were collected after every 24 hr from 48 to 96 hr. Image (a) likely depicts growing cells of *C. Vulgaris*, which were comparatively higher in size than the bacterial cells, due to probably higher cell density. Image (b) taken on a sample collected during cultivation time of 48 to 72 hr and probably exhibits logarithmic growth of *P. Putida* cells, which is reflected by rapid growth of the cells. Image (c) was taken on a sample of cell mass collected at the end of cultivation run or after 96 hr, which probably shows the cell clusters of both algae and bacteria in a sustained case of micro-amalgamation of the cells along with some filamentous growth of the biomass that is a commonplace occurrence during biodegradation of sugars.



(a)



(b)



(c)

Figure VIII-13: SEM images to highlight the growth pattern of biomass obtained during combined cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW immobilized with copolymer Polyacrylate polyalcohol: Images taken after time (hr): (a) 48, (b) 72 and (c) 96.

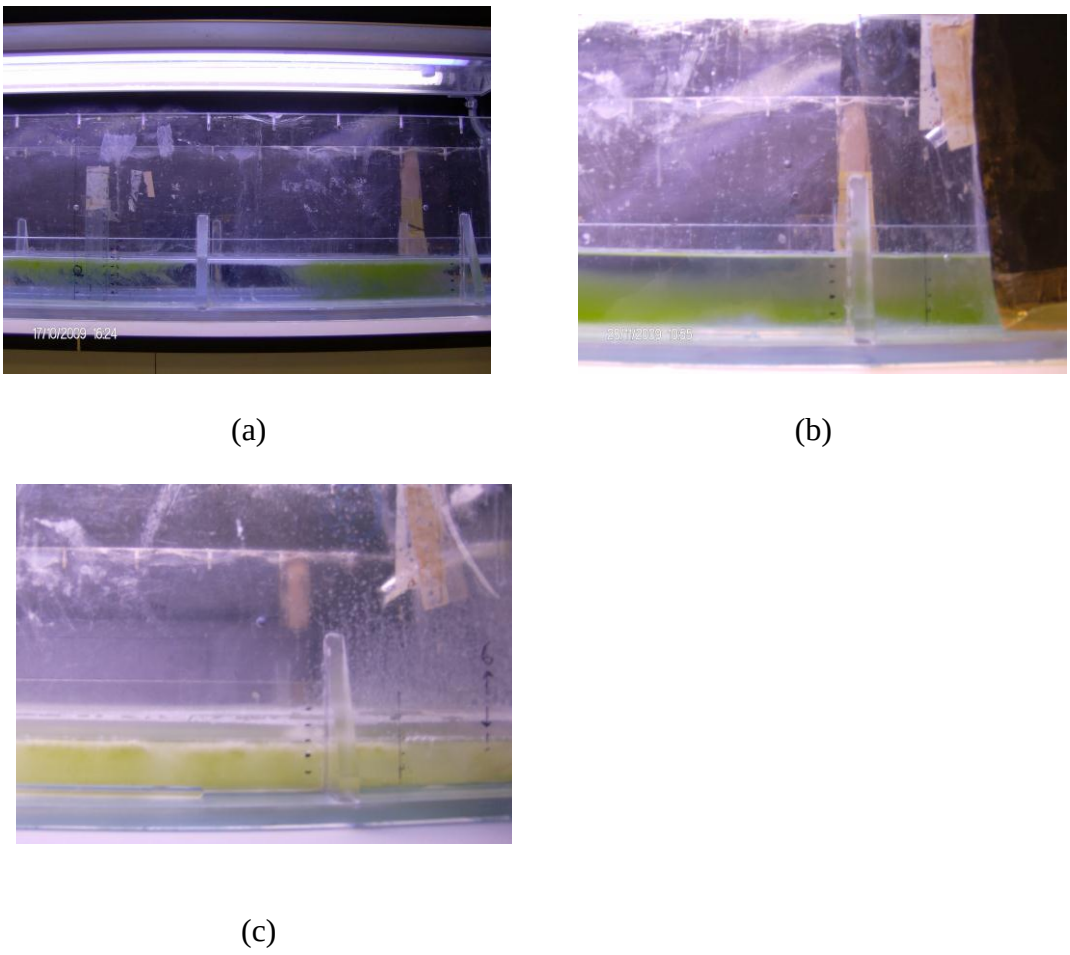
VIII-4 SUMMARY

Combined cell cultivation of *C. Vulgaris* and *P. Putida* both as free and immobilized cell cultivation in SSFW was carried out with different copolymer doses in the light of two findings transpired from *C. Vulgaris* cultivation in sugar water (SW) without and with copolymer immobilization. One, the retention of DO value of above 2 mg l^{-1} remaining after nine days of cultivation and two, the reduced or stabilized values of COD remaining by the end of cultivation, which was not the case during SW lagooning without the addition of both. During combined free cell cultivation in SSFW, *P. Putida* inoculation in LPT was carried out 24 hr after *C. Vulgaris* cultivation. This was done to avoid pH-limiting conditions occurring early in the lag phase growth period of *C. Vulgaris* as was observed during the cultivation run with allochthonous *P. Putida* only. However, pH results during combined free cell cultivation showed stabilization neutralizing the high pH reduction as was observed with *P. Putida* culture in SSFW. The combined free cell cultivation also resulted in lesser depletion of DO by 28% as compared to DO profile observed during SSFW lagooning with *P. Putida* culture. the previous experiment of SFW lagooning with only the bacterial culture. In addition, due to *C. Vulgaris* growth starting after 48 hr of cultivation, DO in the cultivation medium was replenished at 0.6 mg $l^{-1}d^{-1}$, with possible net rate of photosynthetic oxygen production of around 11 mg $l^{-1}d^{-1}$. The breakup of this total DO account included daily oxygen consumption of around 10 mg $l^{-1}d^{-1}$ by both autochthonous and allochthonous bacteria, which was probably sufficient to aid the bacterial activity during culture cultivation for the longer period.

With this observed profile of DO, the COD results did not show the steep rise that occurred during SSFW lagooning with *P. Putida* due to limiting DO condition. However, considering that around 38% of COD was still remaining as observed during combined free cell cultivation of *C. Vulgaris* and *P. Putida*, which indicated that major portion of organic matter still remained non-consumed in SSFW. One contributing factor for this would likely be the benthic nature of *C. Vulgaris* cells that were mostly settled at the bottom of LPT during the free cultivation regime. This observed growth profile of *C. Vulgaris* likely resulted in reduced growth of the cells due to incidence of reduced light flux at the bottom by 18% leading to decreased photosynthetic oxygen generation rate by 35% resulting in inefficient biological activity as well. In addition, this mode of algae growth was found prone to causing an increase in the COD profile particularly towards the end of the run, when the cells probably were in stationary growth phase. However, this particular context of the cell growth could be improved by applying optimum dose of copolymer during immobilized cultivation for *C. Vulgaris* suspension at the surface of LPT for increased rates of photosynthesis resulting in enhanced oxygen generation associated with consistent bacterial degradation of the substrate to influence synergistic COD removal. To address this issue, combined cell cultivation immobilized with different copolymer doses was held with separately. The varying copolymer doses were applied to determine their suitability in relation to cell immobilization at the surface reducing the light path distance by 5 cm and to facilitate maximum photosynthetic activity by enhancing the exposure to the fluorescent light for efficient growth of *C. Vulgaris* along with possible occurrence of gradient microenvironments for nutrient and substrate consumption.

The overall process performance in terms of COD removal of 89% during combined cell cultivation immobilized with optimum intermediate copolymer dose of 80 mg l^{-1} was the likely result of synergistic assimilation of the substrate or COD, which continued until 96 hr of the cultivation regime.

Figure VII-14 highlights the effect of copolymer concentration on the immobilization and growth of *C. Vulgaris* cells in relation to different copolymer doses applied during combined cell cultivation of *C. Vulgaris* and *P. Putida*. The intermediate immobilization dose of 80 mg l^{-1} had resulted in viable suspension of the cells resulting in better cell growth and process efficiency in terms of COD removal, as depicted in Figure VIII-14 (b). This was in contrast to application of lower and higher copolymer doses of 40 and 160 mg l^{-1}, which resulted in consistent leaking of the cells from copolymer and cell suspension at the surface in case of lower copolymer dosing and obstruction to cell activity due to covering of the cell surface in case of higher copolymer dosing.



(a) (b)

(c)

Figure VIII-14: Combination of camera pictures taken during combined cell cultivation immobilized with different copolymer doses showing the influence of respective copolymer doses on the growth of cell mass.

Copolymer dose (mg l^{-1}): (a) 40 (b) 80 and (c) 160.

The initial raise observed in the pH of the cultivation medium after the addition of copolymer was likely caused due to the increase in the –OH ions concentration in SSFW by the OH ions contained in the copolymer molecule. Higher pH reduction rate observed during combined cell cultivation immobilized with copolymer dose of 80 mg l^{-1} indicated towards maximum amount of bacterial activity as well as optimum growth of *C. Vulgaris*, which was likely promote by the suitability of the

copolymer dose. This finding was in agreement with the work of Sayler, 1991 who reported that bacterial decomposition of organics and growth of algae cells would turn the water medium acidic, thus reducing its pH value. The better cell suspension at the surface via optimum intermediate copolymer dose also ensured better DO profile during the course of cell cultivation in terms of DO consumption by the bacterial cells and DO generation by the algae cells than that during cell immobilization with lower and higher copolymer doses. In this regard, the lesser DO decreasing rates incurred during cell cultivation immobilized with intermediate copolymer dose pointed towards maximum growth of *C. Vulgaris* thus producing abundant photosynthetic oxygen, which influenced increased biodegradation activity by the bacteria in oxygen rich environment. In addition, continuous removal of the excess amount of photosynthetic oxygen by the bacterial cells prevented the possible condition of oxygen inhibition for algae cells as observed during baseline study of *C. Vulgaris* cultivation in SW. On the other hand, during combined cell cultivation immobilized with lower and higher copolymer doses, higher DO reduction by the end of cultivation runs was observed implying towards limited *C. Vulgaris* growth generating lesser photosynthetic oxygen likely due to reduced activity of photosynthesis. This condition probably occurred due to leaking and covering of the cells together with the impact on the incidence of light in case of higher copolymer dose, which probably affected the bacterial degradation activity as well. Since the light intensity was kept constant throughout the experiments, it was likely that during the exponential growth phase of algae cells photosynthetic oxygen must have been generated at a constant rate as well particularly during cell cultivation with optimum intermediate copolymer dose.

The optimum and conducive copolymer dosage of 80 mg l^{-1} proved suitable to make viable copolymer colonies, made after stretching phenomenon of the copolymer particles, of such nature that these were observed as neither thin and diluted nor thick and saturated, which was the case during cell immobilization with lower and higher copolymer doses respectively. During these cultivation runs, the cells either leaked or penetrated these colonies to sink to the bottom or were isolated within the denser copolymer colonies probably with covered cell surfaces thus inhibiting the cellular functions as well as photosynthesis process as indicated by lesser growth of the cells in each case. The influence of copolymer dose on *C. Vulgaris* growth was also reflected by COD results, when better COD removal was observed during cell cultivation immobilized with intermediate copolymer dose of 80 mg l^{-1} than reduced COD removal observed during cell cultivation immobilized with lower and higher copolymer doses of 40 and 160 mg l^{-1}. The COD results also showed that cell cultivation period between 48 and 96 hr was the most optimum phase of biological activity in each of the cultivation run, when higher COD removal rates were observed during this period as compared to before it. The low pH and DO values accrued during cell cultivation immobilized with higher copolymer dose of 160 mg l^{-1} implied towards lesser production of photosynthetic oxygen by *C. Vulgaris*, which probably led to poor COD removal rates likely due to the restricted biological activity caused by copolymer shading of the cells. This observation could be paralleled with the one made by Joyce et al. (1985), who stated that low photosynthetic DO production along with high microbial activity induced lower DO values in the medium causing increasing values of COD in the medium.

CHAPTER IX

CONCLUSIONS AND

FURTHER WORK

CHAPTER IX

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This study was undertaken with a view to working on the solution of twin problem of organic wastewater disposal and treatment observed mostly in the developing countries with regard to sugar factories. Since sugar industry is strictly seasonal in nature, and continuous representative sampling would not be possible most of the time its synthetic replica was planned and used to test the hypothesis of wastewater organic reduction, using algae and bacteria as a monoculture or a consortium at the laboratory level. The experiments carried out at the laboratory scale transpired, that even under optimum conditions of temperature, light, pH, DO, and carbon source, the given algae cells may not treat the wastewater up to a desired level, though, the cells may be growing resulting in a bulk of cell mass. However, it could be doubly useful with respect to wastewater remediation with reduced organic load as well as production of algae cell mass with high-energy contents by the end of operation. The general conclusions made from this study may be summarised as under.

Baseline assessment of lagoon photo tank (LPT) transpired that recirculation flow rate (RFR) of 40 ml min^{-1} was the minimum possible flow rate for culture recirculation, which caused an increase in DO by 0.5 mg l^{-1} as compared to DO with non-circulating water. Addition of copolymer, Polyacrylate polyalcohol, in water was found to raise the water pH by 36% and it was also found that this copolymer had no negative effect on the water DO. Besides, copolymer particles were observed to exhibit the flocculating characteristics forming colonies in the initial passage of reaction in water and rising from bottom to suspend at the

surface. The copolymer associations were also found to start disintegrating after almost 48 hr of colonisation process thus descending back to the bottom suggesting that the copolymer was insoluble in water. It was also observed that increasing the flow rate from 40 to 400 ml min^{-1} caused a respective amount of increase in the advective motion in water hindering the flocculation process among the copolymer particles. Sugar water (SW) was used during the initial experiments as a priori for optimization of the experimental protocol before using simulated sugar factory wastewater (SSFW) for actual experiments. SW lagooning in LPT without recirculation transpired that degradation of sugar derivatives in SW likely started to occur from 40 hr onwards resulting in the subsequent decrease in DO and pH values and simultaneous increase in the COD values due to production of indigenous microbial metabolites into the medium thus contributing to an increase in COD. The DO value was observed to reduce to almost zero within 72 hr of SW lagooning suggesting that DO might be more acute and responsive to fluctuating environmental conditions than pH in the given medium.

C. Vulgaris during its pre-culturing showed the tendency towards sugar-oriented culture medium, when the culture showed increased growth after the addition of sterile prepared SW in the culturing bottles, indicating *C. Vulgaris* ability and preference for organic carbon consumption. During mass free cell cultivation of *C. Vulgaris* with 100% BG-11 broth, the addition of buffer solution to neutralize sharp decrease in SW pH was necessary but it was also contributing towards even higher pH reduction, by 85% than without alkali addition possibly stimulating microbial activity as well.

Most of the DO reduction by over 50% during SW lagooning occurred during 40 and 72 hr of lagooning indicating that the indigenous biological activity started after 40 hr of lagooning. During photoheterotrophic free cell cultivation of *C. Vulgaris*, it was observed that immediately after cell inoculation into LPT, the cells took to the bottom lying there in static position, which probably affected the rate of photosynthesis and the growth of the cells due to cell exposure to reduced incident light (IL). It was also found from the COD results that *C. Vulgaris* might be able to grow in an organic medium such as SSFW, yet it might not completely consume the organic matter particularly to the extent of substantial COD removal. Visual observation of the bonding between *Chlorella* cells and copolymer particles suggested that the addition of suitable intermediate copolymer dose of 80 mg l^{-1} resulted in effective *C. Vulgaris* suspension, as most of the cells were seen suspending at the surface of LPT within copolymer matrices, which continued until 60 hr of cultivation. The disintegration of copolymer particles started to occur after 60 hr, leaving behind at the surface the grown-up algae flocs, as the algae cells by that time had entered into autoflocculation under the influence of likely increase in the algae cell size and density.

Combined free cell cultivation of *C. Vulgaris* and *P. Putida* in SSFW did not result in efficient cell growth and concomitant COD removal, which could be due to benthic mode of cell cultivation and allied drawbacks to the overall process such as IL attenuation, limited uptake of nutrients and substrate from the medium along with rapidly fluctuating environmental parameters such as pH, DO and COD. However, combined cell cultivation of *C. Vulgaris* and *P. Putida* immobilized with optimum copolymer dose of 80 mg l^{-1} resulted in efficient biomass growth of 1.2

d^{-1} with corresponding COD removal of 89% and with a higher calorific value of 27 kJ g^{-1} as compared to relatively lower cell growth, COD removal and calorific values obtained during combined cell cultivation immobilized with lower and higher copolymer doses of 40 and 160 mg l^{-1}. These results suggest that reduction in organic concentration of SSFW was associated as much with the growth of algae cells as with the bacterial activity in parallel, to effect a cumulative degradation impact resulting in the increased level of SSFW remediation, which indicated the synergism of the consortium used.

To sum up, this research study concerning SSFW remediation using bioconsortium of algae and bacterial cells promoted via copolymer immobilization was carried out without mechanical aeration at the lab scale in lagoon photo tank in terms of organics removal from SSFW, yielded in the production of algae-rich energy biomass in addition to depollution of SSFW. However, with regard to the application of experimental results obtained in this study at the larger scale may entail utilizing the larger size photobioreactor for real sugar factory wastewater remediation applying similar strategy for the bioconsortium inocula immobilized with optimized dosing of copolymer Polyacrylate polyalcohol after necessary stoichiometric calculations for the input materials relevant with the photobioreactor characteristics.

Further work in this regard should focus on green algae *C. Vulgaris* cultivation with slightly higher intensity of light by 20-30% with the induction of light and dark cycles or with time-varying higher incident light to investigate the growth pattern of the algae cells and corresponding impact on the wastewater remediation efficiency.

Another aspect of interest in wastewater remediation using bioconsortium in a photobioreactor could be the investigation of microbial degradation products in the culture medium for their qualitative and quantitative analysis along with the influence of these metabolite products on the medium characteristics and the biota involved as well.

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